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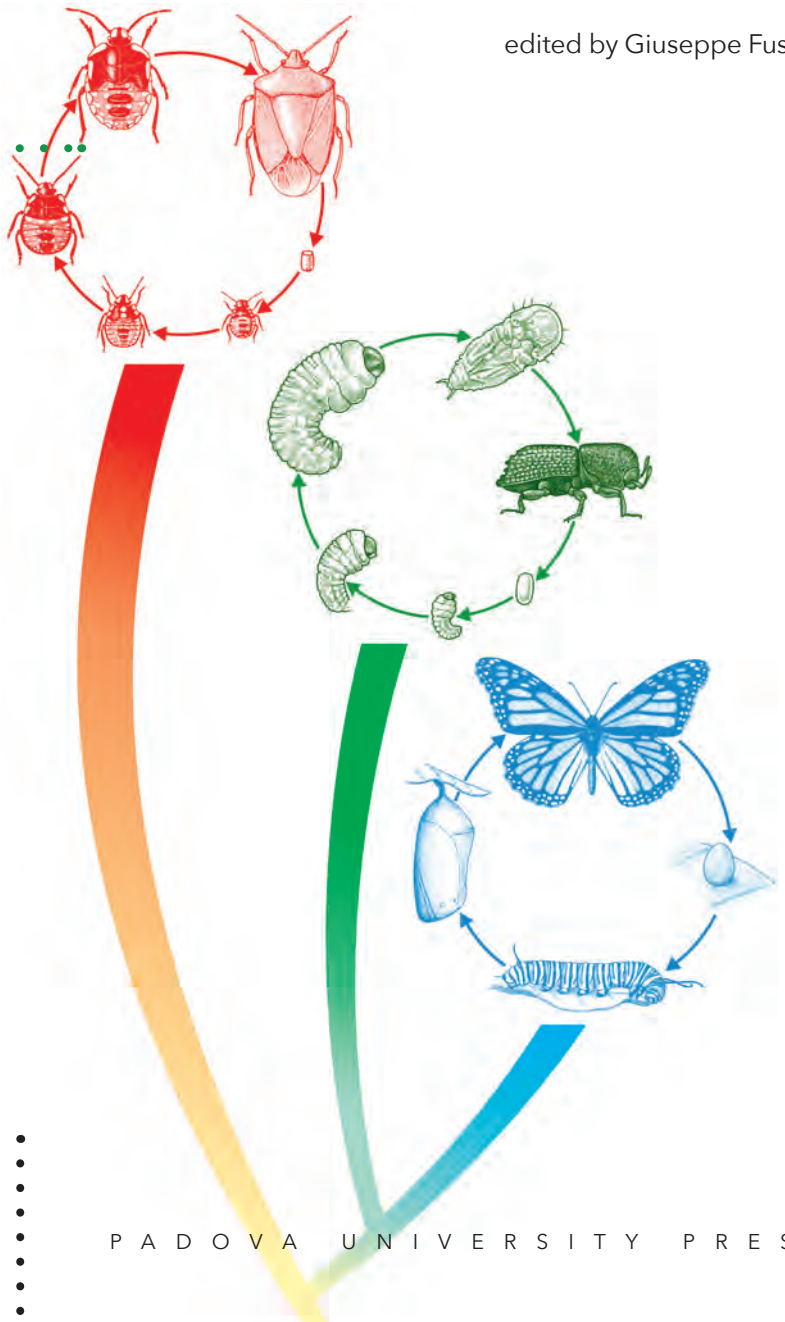
Perspectives on Evolutionary and Developmental Biology

Essays for Alessandro Minelli

edited by Giuseppe Fusco



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First edition 2019, Padova University Press

Università degli Studi di Padova
via 8 Febbraio 2, Padova
www.padovauniversitypress.it

ISBN 978-88-6938-140-9

Printed in January 2019

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Preface

This book is about development, evolution, and their fertile interface, evolutionary developmental biology (evo-devo). It is dedicated to Alessandro Minelli, distinguished zoologist and evolutionary biologist, on the occasion of his 70th birthday.

The collection of short to medium-size essays spans a wide range of approaches to the subject, including: analysis of the development and evolution of specific features in plants and animals, phylogenetic inference, historical and philosophical revision of key aspects of the disciplines, theoretical elaborations on fundamental notions, and conceptual modelling of developmental and evolutionary dynamics. It might seem, and actually it is, a very diverse book. However, the volume covers only a fraction of the approaches and questions that are currently debated in these fields of study, to many of which Alessandro Minelli has significantly contributed. Indeed, evolutionary (and) developmental biology studies are experiencing an enormous expansion, partly driven by the advent of new techniques in experimental investigations, but also by the growing theoretical connections with related fields, such as comparative genomics, systems biology, symbiosis studies, bioinformatics, epigenetics, heredity and ecology. Essays are grouped into five sections of broad topics, but the classification is not intended to be too strict. While being mainly aimed at professional researchers in the field, this essay compilation, as a whole or selected parts of it, could also provide material for discussion groups in undergraduate and graduate university courses.

The book consists of a collection of perspectives, as the title anticipates. One can neither expect to find "one long argument", nor a complete lack of conflict of views among authors. Moreover, the 29 essays put under scrutiny a very diverse array of organismal features, biological processes and theoretical concepts. However, despite its heterogeneity, this ensemble is less disparate than might appear at first sight from simply skimming through the table of contents.

While offering a vivid portrait of what is going on in these diverse areas of research, it reveals a central idea common to all the essays: in order to get a better understanding of the extraordinary variety of phenomena in development and evolution, with their intimate and complex interconnections, it is often helpful, at least at times, to step back from the lab bench or the computer screen, to try to consider our research in a wider, more inclusive context. This can be pursued, for instance, by thinking retrospectively about how we got to where we are, re-evaluating the meaning of the words we use, exploring possible connections among apparently unconnected research areas, considering the possible refining of old concepts (or the devising of new ones) to accommodate new evidence, or even advocating novel theoretical frameworks. For such aims, even an apparently heterogeneous, tumultuous cross-talk between readers in the field can produce fruitful suggestions and provide an opportunity to make significant advances. Participation in the assembly of a book like this one involves seizing exactly this sort of opportunity. The 38 contributors are for the most part Alessandro Minelli's colleagues and collaborators, who over many years have participated in events that he has organized – meetings, summer schools, conferences and symposia – and often contributed to the resulting publications.

The book was generously sponsored by the Istituto Veneto di Scienze Lettere ed Arti and by the Department of Biology of the University of Padova. I am very grateful to Luca Illetterati (Editor in Chief of Padova University Press), who with no hesitation endorsed the project of this volume, and to Cesare Montecucco (Secretary of the Natural Sciences Class of the Istituto Veneto di Scienze, Lettere ed Arti), Gerolamo Lanfranchi (Head of the Department of Biology of the University of Padova) and Telmo Pievani (Delegate for Institutional Communication of the University of Padova), who in different ways and at different stages provided precious support for its realization. I also would like to thank the editorial staff of Padova University Press, who generously complied with the unavoidable strict deadlines of a festschrift. Last but not least, my warmest thanks to the authors, who, in addition to contributing their essay, have served as reviewers, providing insightful comments on other contributors' work. These interactions were the start of a dialogue, of the kind that Alessandro Minelli has always cultivated and vigorously promoted over many years, which I hope will continue and expand through the pages of this book.

Giuseppe Fusco
December 2018

Part I Theoretical investigations

The causal structure of development and its evolution

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Abstract

I focus on the causal structure of development and the ways in which that structure influences evolution, and in turn is modified by the evolutionary process. It is clear that the nature of this structure is a complex issue, and one that can be dealt with in many different ways. Here, I take a conceptual approach that emphasizes the pattern of interconnections among quasi-distinct parts of the overall developmental system. Three ways in which the causal structure of development could interact with evolution are outlined. These can be called: *Panglossianism*, *simple burdenism*, and *realism* (or *complex burdenism*). The realism view is, as its name suggests, correct. However, this is just a launching point from which to consider the much tougher question: which version of realism applies?

The causal structure of development

The overall development of an animal or plant can be thought of as a complex network of causal interactions. To the extent that development is modular, each module can be thought of as a complex network in its own right; and the within-module networks are connected up by a between-module network. Any one causal interaction (from here on called *causal link*) can be dealt with at different levels of abstraction or (conversely) detail. For example, an influence of one tissue on another (for example overlying) tissue can be represented simply by an arrow (A influences B); or it can be represented by a word that indicates the broad nature of the interaction – such as induction. Alternatively, we can describe the causal link at a molecular level, for example by characterizing the morphogen that is responsible for the inductive process and the way in which it acts.

These particular points on the abstract-to-detail spectrum by no means exhaust the range of possible types of description of a single causal link. At a

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formal level, a causal link is rarely a simple arrow from A to B; instead it is likely to involve various types of cross-talk and feedback. At the molecular level, a morphogen (such as retinoic acid or a hedgehog family protein) is just one player in a complex biochemical process involving receptors and multiple other reactants. At a quantitative level, the dynamics of the causal link must involve not just the various molecular players, but also their concentrations, and their rates of interaction.

Starting with any one causal link at any stage of development, we can trace connections backwards and forwards through developmental time. Ultimately, a particular causal link must be connected, directly or indirectly, with most or all other causal links in the development of the organism concerned. Given the number of links, the vast majority of interconnections between two randomly chosen links will be indirect – a chain whose number of links is very large.

Notice the difference between *chain* and *network*. One is a linear series of arrows, end-to-end. The other involves arrows converging and diverging in complex patterns. Perhaps a good analogy can be found in the pattern of traffic flow in a large city. An actual series of roads taken to get from starting point to finishing point (say 10 km apart) is a chain. The pan-city pattern of traffic flow is a network.

Here, I am concerned with the shape of the overall network that underlies the developmental process of a direct-developing animal such as a mammal, or one phase of the development of an indirect-developing animal such as a holometabolous insect. The making of a *Drosophila* larva from a fertilized egg is hugely different in detail (although based on the same cellular and molecular processes) to the making of an adult fly from a series of imaginal discs and histoblast nests scattered through the larval body (Lawrence, 1992). At a formal level, the development of the larva from the egg is probably more equivalent to the development of a mammal, as it has a single-cell starting point. The pattern of causal links making a fly from discs must be somewhat different. In any event, in such a life-cycle, there must be causal links connecting the two developmental phases as well as the more numerous within-phase links.

The shape of the network

Now we come to a key question: what is the shape of the overall network of causal links that underlies the development of a mammal, a fly larva, a land-snail, or a tyrannosaur? Does each of these have some broad shape that we can characterize at least in rough terms? And is this broad shape the same for all the types of animal mentioned, and others?

It's easy to rule out some shapes, so that's a good way to start. The network, for example, does not consist of a series of parallel chains that rarely or never talk to each other. Nor is it so modularized that almost all of development can be thought of as intra-module (e.g., developing heart or kidney), with just a few causal links making contact between one module and another. The dynamics of the network are not bidirectional: although there are many feedback systems at work, the process of development has a certain overall directionality to it. Another way of saying this is that it does not look the same going backward and forward in time. And indeed the 'backward in time' is merely a mental construct, because most developmental systems cannot operate in reverse (with a few possible exceptions: see Piraino *et al.*, 1996). When the result of a developmental process is undone, as when a snail's tentacle or a salamander's leg is excised by an experimenter, the regenerative process is a sort of variant re-run of the original development of these structures, with the same broad temporal directionality.

So, the overall network of development is not disconnected, as in the cases of parallel chains or hyper-modularity, and its dynamics are usually not reversible in time. Or, to give the positive counterpoints to these negative points, development is highly interconnected and essentially unidirectional. But there are still too many possibilities in terms of its shape. We need to be more specific – if possible. This is the point at which we move from the realm of quasi-certainty to the realm of hypotheses.

The most pessimistic hypothesis is that the overall developmental process is too complex, too irregular, and/or too variable between species to be characterizable by a certain shape. But let's ignore that possibility and take a more positive approach, and propose that one particular shape is a key element of the causal structure of development, namely a *radiating hierarchy*. There are many types of hierarchy, and many adjectives to describe them. Among these are: inclusive, exclusive, divergent and aggregational (Panchen, 1992). The taxonomic hierarchy that we use to order our knowledge of nature (several species within a genus, several genera within a family, and so on) is called divergent. The hierarchy that I am proposing to constitute an important element of the causal structure of development also has this feature, in that several downstream processes are initiated by an upstream process. However, since this is an active series of events rather than a passive form of grouping, I think it deserves a different name, and have chosen *radiating hierarchy* as an appropriate one.

Notice that I have not described the radiating hierarchy as constituting the whole of the causal structure of development, but rather as a key element of it. This is important. There are other key elements, including the cross-talk and feedback mentioned earlier. However, it is the hierarchical element whose effect

on evolution I wish to explore here. The other elements may also have important evolutionary consequences, but I leave those to other authors to explore.

Here is one way to think about the hierarchical aspect of development. At the outset, key decisions are made about the body plan. In bilaterians (i.e., the vast majority of the animal kingdom), a decision is made about which end of the early embryo is the head and which the 'tail'; the other body axes, dorsal-ventral and left-right, are likewise established. For these purposes, the tiny early-stage embryo is behaving as a single developmental field. However, as the embryo grows, it becomes divided and subdivided into a progressively larger number of progressively smaller (in proportional terms) sub-fields, such as organ and limb primordia. Eventually, sub-sub fields, such as those for the digits of the human hand, go their own quasi-independent ways, producing quantitative variations on the same qualitative theme – for example finger versus thumb. Thus the radiating hierarchy of developmental decisions leads through various branching events from the first few key decisions to the multitude of 'final' small decisions, always remembering that 'final' should not be interpreted in absolute terms – we should always heed Minelli's (2009) advice not to be too 'adultocentric'.

Approaches to the evolution of animal form

Here is one possible approach to the evolution of animal form: Any developmental process, at any stage of the overall flow of development from zygote to adult, is equally modifiable by natural selection. The range of possibilities is entirely unconstrained by, and in more general terms uninfluenced by, developmental process or stage. The developing animal is like a lump of putty that can be moulded at will by selection in any way that environmental features dictate, in the sense of rendering some variants fitter than others. I call this the *Panglossian* view of morphological evolution, following the 'Panglossian paradigm' of the famous spandrels paper (Gould and Lewontin, 1979).

Here is a very different approach to the evolution of animal form: The earliest stages of development are relatively hard to change, in evolutionary terms, because so much of what follows in the developmental process depends on them; conversely, late stages of development are much easier to change evolutionarily, because then only the finishing touches of the developmental process are affected. Another way of putting this is that a property related to the *difficulty* of making evolutionary changes decreases through developmental time – this property can be called *burden* (Riedl 1978), *constraint* (Arthur, 1984; Thomson, 1988), or *generative entrenchment* (Wimsatt, 1986). Looked at the other way round, a property related to the *ease* of making evolutionary changes – evolv-

ability (Kirschner and Gerhart, 1998) – increases through developmental time. I call this the *simple burden* view of morphological evolution.

Here is how Riedl (1978) defines burden (p. 80): "By burden I mean the responsibility carried by a feature or decision." Later (p. 104), he says that the *degree* of burden is "specified by the number of decisions that depend on a preliminary [=earlier] decision". This can be taken to mean that, other things being equal, the degree of burden increases going backward in developmental time from the adult to the zygote (assuming again a direct developing system). This idea is also behind an important claim made by Thomson (1988, p. 92): "In principle, we should be able to reconstruct for any species or any higher group a sequence of levels of morphological characteristics that define all the higher groups to which the taxon belongs, and to match these up with particular points in the hierarchy of morphogenesis." The idea of burden was also behind my own assertion (Arthur, 1984, p. 217) that "the shape of the mega-evolutionary tree [...] is to some extent determined by the structure of the morphogenetic tree". And Riedl's "ontogenetic burden" is also the "generative entrenchment" of Wimsatt (1986; see also Schank and Wimsatt, 1986). Wimsatt and Schank (2004) define generative entrenchment as "the magnitude of [...] downstream dependence" of a node in the developmental network.

While all the above authors noted many complexities, their major focus was on the property that increases going back in developmental time, and constrains the evolution of early developmental stages, regardless of its name. Thus I use their combined body of work on that property as defining what I call herein the simple burden approach.

By now, it is clear that both the Panglossian view and the simple burden view are wrong. However, I don't think that their errors are symmetric; and indeed the views themselves are not symmetric – in the following sense. Under the Panglossian view (which can be traced back to Wallace, 1897), nothing about development influences the ease of evolution. Selection is the unopposed absolute monarch of the evolutionary process. In my opinion the Panglossian view is fundamentally flawed. Indeed, there are probably few biologists today, even in the most extreme wing of neo-Darwinism, who would support it. Its error – in terms of denying any effect of the developmental process on the action of natural selection – is a fatal one. This complete denial means that there are no variant versions of the Panglossian view – it is a single, clear, and incorrect view of the evolutionary process.

In contrast, the name I've given to the alternative view – simple burdenism – implies that it is one of a series of variant views, with the others being more complex forms of burdenism. One form of complex burdenism is already ap-

parent, and connects with the hourglass or egg-timer pattern of evolutionary variation in developmental stages (Duboule, 1994), and the related concept of a phylotypic stage representing the narrow waist of the hourglass (Sander, 1983; Slack *et al.*, 1993). Examples are the pharyngula stage of vertebrates and the germ band stage of insects. In hourglass burdenism, the patterns of change over developmental time in burden, and in its positive counterpart evolvability, are not monotonic, but rather take the form of an asymmetric hourglass, with the point of constriction much nearer to the start than the end of development.

Hourglass burdenism is closer to the truth than either of the views that we started with; but it is still too simple. The rationale for it emerged from comparing embryonic phenotypes. When we bring genes explicitly into the picture, there are some signs of an hourglass pattern in both animals (Kalinka, *et al.*, 2010; Domazet-Loso and Tautz 2010) and plants (Quint *et al.*, 2012), but another complexity arises, as follows. The mapping of genes to developmental stages is messy. It was once possible to think of early-acting "master genes" that were involved in making key developmental decisions versus late-acting, relatively minor-effect genes involved merely in refining the processes put in place by the master-genes – these minor-effect genes would include the polygenes (or QTL) of quantitative genetics. However, it is now clear that some of the genes that control development have different phases of expression (e.g., certain Hox genes; Salser and Kenyon, 1996), with an early phase and a later phase. Sometimes the later phase is *much* later; sometimes there is a third phase; and so on.

Towards realism

Expanding on the asymmetry

The fact that the Panglossian and simple burden approaches are both flawed does not mean that we are heading for a more realistic approach that is somehow intermediate between the two. Rather, the realistic approach we hope to find that corresponds best to actual developmental systems and their evolution must necessarily be an elaboration of the simple burden approach. In fact, another way to distinguish different approaches here – in contrast to the threefold distinction I have used up to now (Panglossianism, simple burdenism, and realism) – is twofold (Panglossianism and burdenism). In such a two-fold classification, we have one approach that denies a link between a process's location in the overall developmental system and its evolvability, and one approach that accepts such a link.

A realistic model must, in my view, accept such a link, so it belongs in the overall burden genre. Thus the correct way of viewing the relationships among the various approaches can be represented as follows:

1. Panglossianism
2. Burdenism
   a) Simple burdenism
   b) Realism (=complex burdenism)

Complex burdenism

It is possible to imagine a form of burdenism, which I call *complex burdenism*, that is based not on the overall developmental system but on the multiple radiating hierarchies that collectively comprise it. Thus rather than having a monotonic trend in constraint/evolvability through developmental time, or even a slight departure from this (hourglass model), we should recognize trends in constraint/evolvability in terms of different developmental modules, and, where applicable, different phases of the lifecycle. In such a model, the relationship between the degree of ontogenetic burden and the distance through developmental time (in the sense of time elapsed since the zygote stage) might be quite complex, even if the relationship between burden and position within the appropriate radiating hierarchy were simple – which it probably will not be, given the pleiotropic developmental effects of particular genes.

Evolution affecting the developmental hierarchy

At the start of this article, I stated that I would discuss not only the way in which the causal structure of development influences evolution, but also the way that structure "in turn is modified by the evolutionary process". So far, I have focused on the former side of the coin; now it is time to turn to its other side, because a satisfactory complex burdenism must also take this into account.

A simple burdenism approach tends to lead toward a saltational view of the origin of higher taxa for the following reason. The degree of burden of the earliest developmental stages is deemed to be so high that these are doomed to permanent evolutionary stasis – unless something radical happens to temporarily alleviate this stasis. In this context, Goldshmidt's (1940) idea of "hopeful monsters" did not gain general acceptance because all the big-early-effect mutations we know of, such as homeotic mutations, are associated with a severe decrease in fitness. There are other possible solutions to this problem, including my own idea of *n*-selection (Arthur, 1984), in which the important version of fitness is the *n*et reproductive rate rather than a comparative measure such as the cross-product ratio; this idea remains largely untested.

However, if we include in our complex burdenism approach the effect of evolution on the causal structure of development as well as the converse effect,

we may have a solution to the origin of body plans that avoids the need for either hopeful monsters or atypical forms of selection. The *degree of complexity* of the causal structure of development evolves – in both upward and downward directions – over the course of evolutionary time. Perhaps lineages in which this complexity has decreased provide a temporary escape from very high levels of burden of early developmental stages – because such decreases involve a reduction in "downstream dependence". A change in early development might be tolerated in such a situation, but later evolutionary increases in the complexity of the causal structure of development in the lineage concerned might subsequently restore a high degree of downstream dependence and once again lead to a higher degree of constraint, burden, or generative entrenchment.

Envoi

This article has two key take-home messages. The first, which is uncontentious, and could perhaps be regarded as a simple statement of fact, is as follows. *The causal structure of development affects and is in turn affected by, the evolutionary process.* The second, which is more likely to be contested by some evolutionary biologists, takes the following form. *An important part of a future theory of the evolution of development will be a connection between the position of a causal link in a radiating hierarchy of developmental interactions and the property that can on the one hand be called evolvability and on the other hand developmental constraint, ontogenetic burden, or generative entrenchment.* How far away is our future theory of the evolution of development? Although we cannot yet answer this question, the importance of arriving at such a theory is clear. As Minelli (2009) said of the relationship between evolution and phylogeny: "If we are interested in evolution, the tree is not the final target of our investigations, but the branching topology against which we can study a long and not necessarily progressive history of change." That history includes, as a major component, the evolution of development.

Acknowledgements

I thank Giuseppe Fusco and Mark Olson for their helpful comments on the manuscript; and Sandro Minelli for scientific advice and interaction extending over a period of more than 30 years, initially as strangers, subsequently as friends.

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Towards a developmental biology of holobionts

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Abstract

We do not develop as monogenomic organisms, instructed solely from the DNA and cytoplasm of the zygote. Rather, we are *holobionts*, symbiotic consortia containing numerous microbial genomes, whose signals are critically important for our normal development. Microbes play crucial roles in forming and maturing animal guts, immune systems, nervous systems, and reproductive organs. In some species, they regulate such developmental phenomena as the proper orientation of the anterior-posterior axis and metamorphosis. One of the biggest challenges to developmental biology, then, is studying the developmental biology of holobionts, where co-development is the rule, and where the body is seen as a collection of interdependent ecosystems.

What a profession this is – this daily inhalation of wonder.
(Jean Rostand, 1962)

You complete me.
(Dorothy Boyd, in *Jerry Maguire*, by Cameron Crowe, 1996)

Becoming with others

In the past century, the biological world has gone from a Darwin-Wallace paradigm, through a Dawkins-Collins phase, and is now entering the Margulis-Lewontin era. To be sure, the worldviews of each of the earlier eras, like evolving species or religions, are still present while the newer ones arise; but the Margulis-Lewontin view of biology emphasizes cooperative interactions and interpenetrations between individuals, rather than the predominantly competitive

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interactions of the earlier paradigms. As Richard Lewontin (2002) documented, the boundaries of the organism are more porous, interpenetrating and interacting with the environment. The genotype gives us a repertoire of potential phenotypes, and the phenotype is often determined by environmental agents. And as Lynn Margulis (1998) showed, organisms can no longer seen as "monogenomic," bearing only the genes derived from the zygote. Rather, each organism is a *holobiont*, a symbiotic consortium with numerous microbes. Each organism is an ecosystem, and complex organisms, such as ourselves, are biomes, containing numerous ecosystems. Indeed, in this new view of the world, those animals most fit to survive are often those with the best systems of cooperation. As Richard Powers (2018, p. 142) concluded in his analysis of forests and their humans, "Competition is not separable from endless flavors of cooperation."

The Margulis-Lewontin perspective of biology highlights developmental plasticity and symbiosis (Levins and Lewontin, 1985; Margulis and Sagan, 2003; Gilbert and Tauber 2016). Developmental plasticity is most obviously seen in "individuals", where the environment has agency, along with the genome, such that environmental agents generate different phenotypes from the same genotype (West-Eberhardt 2003; Minelli and Fusco 2010; Sultan 2017). Temperature, for instance, can determine the pigment patterns of some butterflies and the sex of many reptiles. Plasticity can also be seen in the "environment". Here, the environment is not a given context. Rather, habitats are formed by interactions between the organisms developing in them and as part of them. This extension of plasticity into the environment is called *niche construction* (Laland *et al.*, 2008).

Symbiosis can be a source of both constraint and flexibility (Bennett and Moran 2016). In the latter mode, it provides the organism with flexibility derived from numerous other genetic systems. Indeed, while we receive some 22,000 pairs of genes from our parents, we get on the order of 8 million different genes from our symbionts (Funkhauser and Bordenstein 2013; McFall-Ngai et al., 2013). Although symbiosis can be parasitic or mutualistic, symbiosis is usually used to describe mutualistic, reciprocally beneficial, interactions between consenting adults. The cow, for instance, is a domesticated female bovid that digests grass. Only, it can't digest grass, as its genome contains no gene encoding cellulose-digesting enzymes. Ditto for wood-eating termites, whose genome contains no lignin-digesting genes. In both cases, their respective abilities to digest cellulose and wood come from the colonies of microbial symbionts located within their guts. The microbes get food and shelter; the animal gets a crucial source of nutrition.

Indeed, symbiosis is the signature of life on earth, whether we are speaking about the nitrogen-fixating symbioses of legumes and rhizobacteria, the mycor-

rhizal interactions with plant roots and seeds, the coral reef and tidal seagrass symbioses that sustain oceanic diversity, or the insect pollinators of plants. And within these grand symbioses are the smaller symbioses we call organisms, cells, and genomes. The "organism" is not an "individual," in the sense of being a solitary organism. Rather, it is a collection of interpenetrating ecosystems. The microbes on our skin and in our guts are essential for our normal physiological, mental, and immune relationships (Gilbert *et al.*, 2012, 2015; McFall-Ngai *et al.*, 2013).

What fascinates me is that symbionts are not only required for normal animal functioning; they are also necessary for normal animal development. This is revolutionary. Throughout the Twentieth Century, it had been assumed that the zygote contained all the genes and proteins needed for normal development under permissive conditions. Development was seen as a read-out of the genes acquired at fertilization (Keller, 1992, 2002). This was our origin story, following the standard Western origin narrative of unity, diversity, and restoration (Haraway, 1985, 2017). Developmental symbiosis – *sympoiesis* – has literally queered the story, adding an important layer of interactive non-heterosexual intercourse – the microbes.

The new story of developmental symbiosis has several points of origin, of which two groups framed much the discussion – Margaret McFall-Ngai and Ned Ruby's studies of the squid light organ and Jefferey Gordon's studies of mouse intestines. The squid isn't born with a light organ. Rather, it binds members of a particular marine bacteria species onto its abdomen (poisoning all others), and the light organ is formed by the interactions of the squid cells and the *Vibrio fischeri* bacteria. The light organ then houses the bacteria, brings them to a critical density, and controls its bioluminescence (McFall-Ngai, 2014; Aschtgen *et al.*, 2016). In Gordon's laboratory, the Paneth cells of the mouse intestine were seen to transcribe different amounts of mRNA depending on whether particular bacteria are present (Hooper *et al.*, 2001; Camp *et al.*, 2014). Certain species of *Bacteroides* are responsible for the "normal" amounts of mRNA that encode enzymes (such as colipase), paracrine factors (such as angiogenin-4), and structural proteins (such as Sprr2a). Germ-free mice (having no gut microbes) have about 10% the amount of angiogenin-4 mRNA as conventionally raised mice; and the normal amount of this message can be regained by adding *Bacteroides* to the gut. The Angiogenin-4 protein helps make gut capillaries, the blood vessels that bring food to the rest of the body. The gut capillary network of germ-free mice is very poor (Stappenbeck *et al.*, 2002). So we mammals get a lot of work from our *Bacteroides* symbionts. They help make us who we are. And *Bacteroides* gets help from the host, the zoon. Not only does the mammalian gut provide

Bacteroides with good food and housing; the host's Angiogenin-4 has a second use – it kills *Listeria*, the major competitor of *Bacteroides* (Hooper *et al.*, 2003; Cash *et al.*, 2006). Development involves some niche construction on the part of the microbes.

Developmental symbiosis has been found throughout the animal kingdom (McFall-Ngai, 2002; Douglas, 2010, 2018; Gilbert and Epel, 2015). In mammals, bacteria are critical for the development of the gut capillaries, the enteric neurons, and the gut-associated lymphoid tissue. In zebrafish, bacteria regulate the division of the gut stem cells as well as the normal proliferation of the insulin-producing beta-cells of the pancreas. Without these particular microbes, there is a paucity of differentiated gut epithelium (Rawls, 2004; Hill *et al.*, 2016). Moreover, some of these developmentally critical bacteria are rather rare members of the microbiome. In zebrafish, for instance, the *Aeromonas* bacteria that stimulates beta cell proliferation are such a very rare component of the gut microbiome that it has no signature in the genomic sequence data (Hill *et al.*, 2016). This leads to the concern that our desire for cleanliness might be wiping out bacteria that are essential for *our* normal development (Blaser, 2014).

We mammals inherit most of our microbes from our mother. Indeed, this is a third pattern of inheritance, following those of nuclear chromosomes and mitochondria (Funkhauser and Bordenstein, 2013; Chiu and Gilbert, 2015; Roughgarden *et al.*, 2017). After our amnion breaks and we pass through the birth canal, we become colonized by microbes. Moreover, the microbes we pick up are not the usual ones. Rather, the microbial populations of the vagina and distal gut are changed during the last trimester of human pregnancy (Koren *et al.*, 2012: Romero *et al.*, 2014). And when the mother feeds the new baby, not all of the food is for the baby. Another part, consisting of oligosaccharides unable to be digested by mammals, are specifically for *Bifidobacteria*, one of the microbes that is helpful for the colonization of the gut by other beneficial microbes (Garrido *et al.*, 2016). The bacteria in mothers' milk appear to be particularly important in inducing the formation of the helper T cells that prevent opportunistic infections (Ardeshir *et al.*, 2014). A specific set of microbes is passed from generation to generation to complete normal development. Birth is the passing from one set of symbiotic relationships to another.

In invertebrates, there are particularly strong associations between bacteria, immune defense, and metamorphosis (Douglas 2010, 2018). Here, the interactions of microbes and development are so strong that many insects develop special cells, *bacteriocytes*, to contain the symbionts. These interactions between invertebrates and microbes can start very early. In the nematode *Brugia malayi*, *Wolbachia* bacteria are responsible for the correct anterior-posterior pattern of

the second mitotic division (Landmann *et al.*, 2014). In pillbugs, *Wolbachia* can transform genetically male pillbugs into females. In several species, symbionts are critical for the development of reproductive organs or general larval growth. Microbes are also critically important for molting and metamorphosis in several species. Many species cannot molt properly without the digestive enzymes produced by symbiotic microbes, and many marine invertebrate need other organisms (bacteria, algae) to provide the signals for settlement and metamorphosis (Hadfield, 2011; Gilbert and Epel, 2015).

The brain and the immune system present their own developmental interactions with microbes. Gut microbes are not only capable of communicating with the adult brain, but they also appear to be critical for normal brain development (Sampson and Mazmanian 2015). In germ-free mice, the brain microglial cells (tissue macrophages that are critical in homeostasis and disease prevention) do not complete their maturation (Erny *et al.*, 2015), and Diaz Heijtz *et al.* (2011, p. 3051) concluded that "during evolution, the colonization of gut microbiota has become integrated into the programming of brain development, affecting motor control and anxiety-like behavior." Indeed, there are two major ways to experimentally generate symptoms of autism in mice by manipulating the microbes of the mother. First, mice born from germ-free mothers and who are themselves without microbes have a syndrome that includes obsessive self-grooming and asocial behavior (Debonnet *et al.*, 2014). Second, one can induce such autism-like features in young mice by giving a large immune insult to the mother while she is pregnant. This causes changes in brain development *in utero*, but these alterations only arise if particular types of bacteria are present to augment the immune challenge (Kim *et al.*, 2017). Moreover, several of these symptoms seem to be cured by adding a different set of microbes into the newborn mice's guts (Hsaio *et al.*, 2013). Thus, there is an entirely new region of developmental neurobiology – how the symbionts interact with the developing brain.

And there is another new science of holobiont immunology (Tauber, 2008, 2017; Pradeu, 2012; Gilbert and Tauber, 2016). If the immune system is supposed to kill all that is not "self", then how do these bacteria even enter our body? Just as developmental biology is changing from seeing development as the readout of the genome, so immunology is changing from the view that the immune system exists to defend the organism against the hostile outside world. Certainly that's a part of it (as development also involves the readout of the genome), but it's far from being the whole picture. The defensive role of the immune system appears to be a subset of a much larger function in mediating our relationships, both positive and negative, with microbes. Just like the immune system of the bobtail squid, the mammalian immune system allows certain microbes

entry, while preventing the penetration of other bacteria and fungi. Not only are microbes needed for the maturation of the gut lymphoid tissue; microbial colonization is also critical for the normal development of T-lymphocytes and B-lymphocytes in the intestinal mucosa (Wesemann *et al.*, 2013) as well as for inducing the specific lymphocyte populations that balance the immune response at mucosal surfaces (Ohnmacht *et al.*, 2015). Lee and Mazmanian (2010, p. 1768) conclude, "Multiple populations of intestinal immune cells require the microbiota for their development and function." Different types of T cells are made depending on which bacteria colonize our guts (Ardeshir *et al.*, 2014). The immune system is a holobiont property; it's not merely the host's immune system. It's the holobiont's immune system. So this means that we should no longer consider ourselves genetically pure. Our immune system facilitates the entry of some microbes and excludes the entry of others.

We complete each other

This has major implications for evolutionary biology (Roughgarden *et al.,* 2017). First, the "tree of life" has become like real trees – full of symbionts. In addition to the genetic lineage provided by our reproductive parents, there are also genetic lineages provided by the symbionts we acquire from our mother and from our environment (Margulis and Fester 1991; Margulis and Sagan 2003). These microbial lineages interact with the eukaryotic lineage in many different ways. Indeed, the microbial lineages can provide selectable genetic traits (Douglas, 2010; Gilbert *et al.*, 2010; Kikuchi *et al.*, 2012; Moran and Yun, 2015), and they are involved with species formation (Brucker and Bordenstein, 2103). Second, if (as evolutionary developmental biology postulates) changes in development are critical for making evolutionary changes in anatomy and physiology, those changes in development could also entail symbionts. Such symbiont-mediated changes in development may even be responsible for such evolutionary transitions as the origins of animal multicellularity (Dayel *et al.*, 2011; Alegado *et al.*, 2012), the mycorrhizal symbiosis that enabled plants to live on land (Heckman *et al.*, 2001), the origin of mammals (Dupressoir *et al.*, 2011; Lynch *et al.*, 2011), and origins of herbivory in insects and vertebrates (Gilbert, in preparation).

We have numerous genomes whose products interact to generate our phenotypes. Monogenomic organisms are in the clade of Cryptid vertebrates whose other members include Nessie, Sasquatch, and the Abominable Snowman. It is dubious that any exist. Therefore, zoology (as well as plant sciences) should deal with this fact. Physiology, developmental biology, immunology, neurobiology, and evolutionary biology each have to concern themselves with this "new imperative for the life sciences" (McFall-Ngai *et al.*, 2013). Developmental biol-

ogy can no longer be seen as the read-out of the zygote genome. Development entails "becoming with" others (Haraway, 2008), generating a body consisting of physiologically connected ecosystems. Developmental biology has also to consider co-development, the body as a constructed niche (Laland *et al.*, 2008; Gilbert *et al.*, 2012). That is the challenge for our field – to study the developmental biology of holobionts.

Acknowledgements

I wish to acknowledge the enjoyment of being with Sandro Minelli around a restaurant table at a Vatican conference and near the statue of Niccolo Tomasseo "El Cagalibri", in Venice. And I wish to thank him and Giuseppe Fusco for their creating the wonderful environment for young scientists at the Summer School for Evolutionary Developmental Biology. I thank G. Fusco and A. Moczek for their very constructive comments on earlier stages of this manuscript.

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An evolutionary biology for the 21st century

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Abstract

Evolutionary developmental biology (*evo-devo*) has revolutionized our understanding of why and how evolution unfolds the way it does. At the same time, much of evo-devo remains steeped in traditional perspectives and established dichotomies; these need to be overcome if evo-devo is to remain relevant in the coming century. In particular our conception of developmental evolution has to embrace the nature and consequences of developmental bias, the self-constructing nature of living systems, and the reciprocal interdependencies of development and environment in evolution.

The shape of things to come

Evolutionary developmental biology (*evo-devo*) has revolutionized our understanding of why and how evolution unfolds the way it does (reviewed in Minelli and Fusco, 2008; Minelli, 2015). We now no longer view organismal diversity in phenotypes as underlain by a corresponding diversity of genes or developmental pathways. Cooption and ubiquitous recombining and repurposing have joined descent with modification as means to conceptualize the evolutionary process. Heterochrony, heretotopy, heterometry, and heterocyberny have emerged as ubiquitous developmental means that delineate evolutionary degrees of freedom, enabling and biasing developmental evolution in the process (Gilbert and Epel, 2009). Collectively, evo-devo now allows us to understand the structure of phenotype space in much greater depth, and to reconstruct the crooked routes of life's evolutionary path with much more clarity.

In the process we have learned to appreciate developmental bias – how the nature of development shapes and directs developmental evolution – as more than just a constraining force. Instead of just describing what is possible and what is not, evo-devo is now providing us with an increasingly deeper un-

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derstanding which among the possible changes are likely, and which changes are most likely to yield convergence, parallelism, perhaps allowing the state of characters to diverge while their identities are maintained (Uller *et al.*, 2018). An evo-devo perspective on evolution thus significantly enhances, expands, and deepens our understanding of the evolutionary process. It has transformed a world of *p*'s and *q*'s, of little *a* and big *A*, into one filled with genes and their products, pathways and their interactions, cells and their cooperative behaviors, and morphogenetic movements and their manifestations in three dimensions.

At the same time, much of evo-devo remains steeped in very traditional perspectives and established dichotomies: phenotypes and phenotypic variation ultimately result from genes and genetic variation, and some map – however complicated – is apparently connecting the two. Similarly, environmental conditions, even though by now abundantly recognized as critical for providing both selective conditions and developmental signals, remain passive, separable from, and external to the developing organism (Moczek, 2012). This of course is fine! It is important *and* productive to fully explore how much better we can make sense of the world around us through the tools added to our conceptual portfolio by evo-devo. It is also important and productive because it allows us to be fully certain when and where this novel explanatory power runs into its own limits, and what evo-devo may need to confront if it is to remain the vibrant and extraordinarily productive discipline it is today. Sandro has always been at the forefront of pushing evo-devo to be more; more holistic, more mechanistic, more theoretical, predictive, etc., and I trust that he has his own opinions on the expansions I am about to argue for. Specifically, I see the most significant challenges in two areas

(a) Developmental bias, facilitated variation, and the self-constructing nature of living systems

Genes and genomes don't make embryos, light organs, limbs, nervous systems, or courtship displays. The metaphor of genes and genomes as blue prints or programs for organisms and their parts have outlived their usefulness long ago (though I admit continue to roam as persistent conceptual zombies in biologists' collective mind space). Instead it is now abundantly clear that complex traits emerge from complex developmental systems characterized by self-constructing and context-dependent behavior (Gerhart and Kirschner, 2010). Throughout ontogeny, and across levels of biological organization, organisms and their bits and pieces to a significant degree take charge of their own development, as cells communicate during the formation of layers and organs, as organ system instruct each others' development and function, and as social group behavior

emerges through the contributions and interactions of individuals. Genes and genomes of course matter in all of this tremendously as they contribute vitally important interactants. But by themselves they usually remain insufficient to explain how biological form and function come into being.

This *agency of living systems* (Walsh, 2015) to direct their own formation and function remains to be embraced, and studied, by evo-devo practitioners. Our focus on too many genes at the expense of too little development will have to change, but I would posit that such a change is likely to be worth it. For starters, by focusing on *agency as a process*, this will deepen our understanding of the developmental, physiological, behavioral, etc., mechanisms by which organisms and their component parts exert control over their own ontogenetic future. But perhaps even more rewarding will be the study of *agency as a product*: once evolved, how does agency in living systems influence subsequent evolution? How does it affect the generation of selectable phenotypic variation, contribute to adaptation, enable resilience in the face of developmental stress or perturbations? More generally, how have organisms influenced and shaped their own evolutionary history? Few questions could hold more consequential answers for our understanding of the evolutionary process.

(b) Environments as cause and effect in development and developmental evolution

Environmental conditions provide, one way or another in all organisms, information critical for the completion of normative development. Nobody seriously argues with that anymore. To develop is to interact with the environment. But if this is correct then developmental evolution becomes possible, in fact becomes the necessary outcome, whenever such interactions are altered in a heritable manner. This should put the interdependencies between environment and development at the forefront of our conceptualization of developmental evolution. Yet evo-devo has been slow to embrace this area, even though there would be so much to learn!

For starters there is the growing realization that development – environment interactions play a critical role in determining which mutational variation becomes phenotypically expressed and thus visible to selection, and which would remain cryptic (Paaby and Rockman, 2014). And accumulate-- until, perhaps, the environment changes, say as a species invades a new habitat or an entire planet starts to warm. Then there are phenotypic and genetic accommodation, two still poorly studied phenomena that posit that in response to environmental perturbations organisms will output altered, even novel, but nevertheless well integrated and functional phenotypes, which have the potential to become sta-

bilized genetically over generations through selection on for instance previously cryptic but now phenotypically visible genetic variation (Pfennig *et al.*, 2010). In the process, environment-development interactions create bridges from the initiation of novel or transitional phenotypes toward their genetic canalization and subsequent elaboration in populations (Moczek *et al.*, 2012).

Yet just as important is the growing realization that environmental conditions are after all not nearly as separable from the organism nor as passive as we generally assume (Moczek, 2015). From cells to tissues and organs, and from individuals to social groups, organisms and their parts create both internal and external circumstances to which they themselves respond in subsequent rounds of phenotype construction. Moreover, environments created or altered in one generation frequently influence development and phenotypic variation in subsequent generations, thereby contributing to heritable variation, albeit often through non-genetic means of transmission (Laland *et al.*, 2014). Finally, lasting environmental modifications may feedback to not just the fitness of the initial modifying individual, or descendent generations, but entire suites of species, as in the case in reef building organisms, or those involved in the creation of wetlands. Known as *niche construction* or *eco-evolutionary feedback* or *ecosystem engineering* this ubiquitous property of organisms to influence their own selective environments has been studied in increasing depth by ecologists, but remains to be integrated into evo-devo research programs (reviewed in Laland *et al.* 2008; Schwab and Moczek, in press).

This has to change if evo-devo want to remain at the forefront of advancing evolutionary biology. It is time that evo-devo re-emphasizes *devo* and embraces *eco*. To develop is to interact and partly create environments. To evolve is to alter these interactions in a heritable manner. And to study all this is to move beyond unproductive and unrealistic dichotomies. There is no better time to put these new perspectives to the test than the present: evo-devo research is now feasible in a more diverse range of organisms than ever before, thanks in large part to the democratizing effects of broadly applicable approaches, from RNAseq to RNAi and CRISPR/Cas9. At the same time an ever-growing range of organisms is experiencing rapidly changing ontogenetic conditions brought about through a changing global climate and the rapid homogenization of the worlds' flora and fauna. The time to assess the importance of agency in development, and the reciprocal interdependencies of development and environment in evolution *is now*. We are bound to learn so much, push evo-devo to its next level in leading the growth of biological thought, and perhaps save a few species along the way. So as we recognize in this *Festschrift* the accomplishments of a generation of evo-devo researchers and celebrate the work of Sandro and all in-

spired by him, lets stand on their shoulders and take measure of the challenges before us. Today we shall celebrate, tomorrow we shall get to work!

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Evo-devo's challenges to the Modern Synthesis

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Abstract

The Modern Synthesis, as a particular instantiation of the neo-Darwinian paradigm, is often believed to represent a definitive version of evolutionary theory. But theories develop, and recently there is intensified debate about whether the significant advancements in many areas of evolutionary biology constitute a challenge to the standard theory. In this chapter, I focus on the theoretical consequences of evo-devo. I argue that its challenges to the Modern Synthesis theory lie not so much in contradictions of its key tenets but in the differences of predictions that result from the evo-devo account. In concert with conceptual innovations in other areas of evolutionary theory, evo-devo contributes to an Extended Synthesis that can address distinct phenomena of phenotypic evolution not covered by the received theory.

Introduction

Evo-devo is a quest for understanding how evolution and development interact to generate the diversity of organismal forms observed in nature. In its theoretical domain, evo-devo is an endeavor for complementing evolutionary theory. It is here that most of the controversies about its implications arise. Some authors see evo-devo's theoretical results in line with the standard theory of evolution (or neo-Darwinian paradigm) and, hence, easily integratable with it. Others see theoretical evo-devo as a challenge to fundamental tenets of the standard theory, prompting its reform. Alessandro Minelli, to whom this volume is dedicated, and who had a major role in the establishment of evo-devo as a research field, prefers to minimize the conceptual challenges that evo-devo offers to the neo-Darwinian paradigm (Minelli, 2009; Minelli, 2015), whereas less civilized commentators, such as Coyne (Whitfield, 2008), display open contempt for the idea that such challenges exist or deny that they have any importance (Futuy-

G. Fusco (ed.) *Perspectives on Evolutionary and Developmental Biology.*

ma, 2017). In this essay, I will argue that the "evo-devo is no challenge" position relies on basic assumptions about evolution that are themselves shaped by the received paradigm. More precisely, they are dictated by the particular instantiation of neo-Darwinian thought that formed almost a century ago, but that is still called the Modern Synthesis.

A common strand in the criticism of evo-devo's theoretical challenges should be briefly mentioned, before we turn to more substantial issues. It is the this-has-been-said-before argument. In a recent meeting on New Trends in Evolutionary Biology at the Royal Society in London, a well-known proponent of population genetics rose after nearly every presentation to declare that the expounded argument was not new but had already been used by so-and-so decades earlier. Others make more subtle points about the nature of previous criticisms of the standard theory (Huneman and Walsh, 2017) or assert that certain representatives of the Synthesis were "keenly interested" in various non-canonical components of evolution such as development. Standard villains of evolutionary theory, such as Haeckel, Goldschmidt, or Waddington, are often evoked in these contexts in an attempt to indicate that any such heretical notions have either been rejected or tacitly assimilated. However, it is clear to anyone with an earnest interest in theory that it is unimportant whether an idea, a perspective, or a theoretical concept has been considered previously or whether someone had mused about it – except if a strong claim had been made that it was completely original and novel. What counts is whether a theoretical framework has formally included that concept, and whether it has become part of the explanatory and predictive canon of the accepted theory. If this is not the case, whereas empirical findings support the concept in question, its repetition (if it actually is one) cannot be held against it. Thus, we may dismiss the accusation of repetitiveness as part of a distraction from the actual conceptual challenges that arise from evo-devo and other areas of evolutionary biology.

The true challenges are manifold. They concern fundamental theoretical questions, such as whether genetic variation is a sufficient explanation for the generation of phenotypic variants, whether natural selection is the decisive determinant of organismal structure, whether inheritance among generations is only via genetic material, and whether populations of organisms are merely passively exposed to natural selection. Such theoretical challenges arise from different fields in evolutionary biology (Laland *et al.*, 2015; Müller, 2017), but here I will deal only with issues related to evo-devo and their controversial aspects addressed by Minelli (2009) and other commentators (Laubichler, 2009; Futuyma, 2015; Moczek *et al.*, 2015). In this context, three issues stand out: the interpretation of the goals of evo-devo, the levels of evolutionary explanation, and the (non)distinction between genotype and phenotype.

Goals of evo-devo

The evo-devo research program has been characterized in multiple ways (Hall, 1992; Arthur, 2002; Laubichler and Maienschein, 2007; Müller, 2005, 2008; Fusco, 2015; Minelli, 2015; Moczek *et al.*, 2015). Usually its conceptual targets are seen in the elucidation of the mechanisms that generate selectable variation, the biases inherent to these processes (constraints, facilitation), the origin of phenotypic complexity (body plans, novelty), the tempo and patterns of phylogenetic change (discontinuity, emergence), and several other issues related to development, such as plasticity or self-organization. Following Hendrikse *et al.* (2007), Minelli (2009) emphasizes "evolvability" as the central target of evo-devo, although the perceived goals were expanded in later writings (Minelli, 2015). If we stay with evolvability for a moment, we need to acknowledge that – in its dominant usage today – it is itself a deeply neo-Darwinian concept. It refers to the evolving rapport between genes and phenotype, the so-called genotype-phenotype (g-p) map, a supposedly representational relationship that is thought to enhance and canalize adaptive variability. The structure of this mapping in turn is taken to be under genetic "control" and therefore to be evolvable in a neo-Darwinian sense, i.e. random variation and selection could modify and "improve" it (Wagner and Altenberg, 1996). Thus, evolvability is taken to evolve itself during the course of selection for plastic but robust developmental processes that are responsible for the generation of complex characters (Kirschner and Gerhart, 1998).

Without doubt, evolvability is a significant factor in organismal evolution, and, in a general way, many of the major evo-devo topics (integration, modularity, constraint, etc.) can be interpreted as contributing to it, especially in the sense of developmental evolvability (Hendrikse *et al.*, 2007; Minelli, 2017). But it is curtailing the goals of evo-devo to designate evolvability its only proper evolutionary target. As pointed out previously (Müller, 2005, 2007, 2008), understanding issues such as emergence and organization of phenotypic complexity represent quite different but equally important goals. These can be understood as addressing another property of the genotype-phenotype relation, namely its capacity to reflect not merely abstract statistical relationships between genes and form, but also to account for discrete structural motifs that arise in organismal architectures. These morphogenetic templates will not be the result of (genetic) evolvability but of given forms of developmental organization, in which a range of different g-p mappings will correspond only to a limited number of phenotypic solutions, whether in the evolution of primary body assemblies at the origin of metazoan life or in later modifications of existing organismal architectures (Newman *et al.*, 2006). Whereas the evolvability interpretation is in

some sense circular, because the structure of the genotype-phenotype map itself is taken to depend on genetic control, the latter view is rooted in physical development, i.e. it shifts the causality of phenotypic solutions to the processes that build biological structures through the reiterated dynamical feedback between genes, cells, and tissue masses, including non-genetic components such as function or environment. Here, the phenotype is not merely an extrapolation of the genotype but gains its specific constitution through the constructive processes of development. At the same time, development is the locus that translates continuous genetic variation and selection into discrete phenotypic states.

In summary, while the concept of evolvability certainly has its merits, I contend that the general goal of evo-devo is not to simply elaborate these neo-Darwinian features but to establish a theory of phenotypic evolution (Callebaut *et al.*, 2007; Laubichler, 2009). If a gene centered version of evolvability is designated the central target of evo-devo, then it is no surprise that what is taken as its results coincides with the standard theory. However, whereas evolvability is not predictive of phenotypic outcomes, evo-devo is.

Levels of explanation

It is common understanding that evolution is a process that takes place at multiple levels of biological organization. The "hardened" version of the Modern Synthesis narrowed the goal of the neo-Darwinian project to explanations at the genetic level. Unfortunately, evo-devo seems to be undergoing a similar hardening. Whereas early evo-devo sought explanations primarily and explicitly in causes above the genetic level (Alberch *et al.*, 1979; Hall, 1992; Müller and Newman, 2003), increasing gene centrism characterizes the field today, producing a steady stream of results in which gene expression patterns are superimposed on body plan sketches. This led to the notion that evo-devo is descriptive and primarily concerned with a "comparative evaluation of developmental genetics" (Minelli, 2009) or "focused on the developmental genetic machinery that lies behind embryological phenotypes (Arthur, 2002), or even more narrowly on "which Hox gene turns on, where does it turn on, and when does it turn on" (Goodman and Coughlin, 2000). This reductionist attitude continues to be upheld (Moczek *et al.*, 2015), even though overwhelming evidence points to the fact that it is not gene expression and regulation that singularly define body structures but the systemic processes of interaction between genes, cells, and tissues as well as the physics and physiologies of the involved entities and their interactions with numerous factors of the environment (Gilbert and Epel, 2009; Newman and Linde-Medina, 2012; Noble, 2017; Müller *et al.*, 2018).

Regarding the different levels at which evolutionary explanation can be sought, we are again faced with a certain circularity: if the project of evo-devo is defined at the genetic level alone, all results and explanations will only be registered at that level. Undoubtedly, many interesting insights into developmental evolution may be gained this way, but we should be aware that it represents a limited perspective, constituting merely a segment of the evo-devo enterprise. The non-standard challenges of evo-devo all follow from approaches that focus on different levels of organization, such as cell behaviors, physics, physiology, development-environment interactions, etc. (e.g., Newman *et al.*, 2006; Badyaev, 2011; Bateson and Gluckman, 2011; Abouheif *et al.*, 2014; Sullivan *et al.*, 2016; Sultan, 2017).

The distinction between genotype and phenotype

Waddington (1975) famously lamented that "in all these paradigms [of genetic evolution], only lip service, if that, is paid to the distinction between genotypes and phenotypes." More than half a century later, his dictum is still true. Whereas the study of the g-p relation has become an overarching goal in evo-devo, it is generally overlooked that genetic evolution and phenotypic evolution also pose questions specific to each of these domains. Even though structuralist strands of evo-devo take the distinction seriously and explore the mechanistic foundations of phenotypic evolution, this approach is often tainted by accusations that it is serving idealistic goals, or it is confused outright with typological attitudes. Yet, evo-devo is not about types but about the causal foundations of internal organization in evolving organisms (Laubichler, 2009). Fears of idealistic reminiscences are unfounded and need to be overcome.

It is difficult to trace the roots of these misunderstandings and to disentangle the intricate web of concepts and ideas, interpretations and controversies, myths and prejudices that ensnares the study of phenotypic organization. One crucial aspect, however, is the composition of the phenotype of recurrent building units, usually called homologues. Whether or not we find the homology concept problematic, there is no way of avoiding it. It deals with the fundamental heritable organization of phenotypes, whether morphological (biological homology) or taxonomical (phylogenetic homology) (DiFrisco, 2018). Since evo-devo rests squarely on the comparative method, it is inevitably concerned with homology.

In taxonomical homology it is correct to say that a body plan (a phylum-specific configuration of homologues) "is nothing but a typological abstraction of a set of diagnostic characters" (Minelli, 2009), but in biological homology, the evolution of body plan organization is the explanandum. As a mechanistic science,

evo-devo is not occupied with diagnostic characters, but with morphological building elements. A radius, for instance, is part of the forelimb skeletal architecture shared by all tetrapods that possess full forelimbs. This is not merely a diagnostic feature, but it is primarily a constructional feature. If we argue that homologues are mere abstractions and, hence, no hierarchical ordering, no body plans, and no novelties exist, then the core explanandum of structural phenotypes and of how these characteristic features of biological evolution originate, is eliminated. The problem of origins may be disliked, but Darwin's theory fundamentally is about "Origins".

The denegation of the genotype-phenotype distinction in evolutionary theory has a self-fulfilling aspect: if the reality of phenotypic organizing features is contested and the study of this phenomenon declared illegitimate, then it is only consequential that the "legitimate" aspects of analysis, the evolution of the genetic underpinnings of the phenotype, are in tune with the Modern Synthesis narrative. Some have criticized extreme positions in these debates, but to take the genotype-phenotype distinction seriously should not be seen as an extreme.

Challenges to the Modern Synthesis paradigm

Theoretical incompatibilities in current evolutionary biology are not a consequence of taking extreme positions. Rather, challenges to a theory arise when its predictions are not met by the empirical findings or when its explanations don't cover significant phenomena in the domain it purports to explain. Today, both kinds of challenges apply in the case of the Modern Synthesis (MS) theory, as numerous authors have noted (e.g., Kutschera and Niklas, 2004; Shapiro, 2005; Jablonka and Raz, 2009; Noble, 2013; Laland *et al.*, 2015). Coined in the 1930s and 40s, the Modern Synthesis was an innovative integration of Darwinian evolutionary factors, such as variation, differential reproduction, and natural selection, with population dynamics and Mendelian inheritance. In its core, the MS consists of a correlational account of how genetic variation spreads in populations and under which conditions variants are modified and maintained. The theory rests on a canon of basic assumptions, such as genes as the sole vehicles of transgenerational inheritance, random and steady genetic variation in populations, and natural selection acting on incremental differences as the exclusive agent driving changes in gene frequencies and adaptation. A characteristic set of predictions derives from the MS theory, primarily regarding allelic distributions in populations but also regarding phenotypic evolution: e.g., the requirement of genetic change always preceding phenotypic change, the unbiased character of phenotypic variation arising in populations, and the exclusively continuous and gradual progression of phenotypic evolution.

Whereas this may have represented an adequate reflection of selected empirical results in the study of evolution at the time of the Synthesis, our understanding of the factors at work has changed considerably since then. The rise of molecular biology (including molecular genetics and genomics), systems biology, and evolutionary developmental biology, amongst others, have dramatically expanded our comprehension of organismal evolution. This has permitted the development of modified and new theoretical assumptions that were not part of the MS framework. In the case of evo-devo, these include the dependence of phenotypic change on non-genetic factors (e.g., developmental, environmental, physical), the non-randomness of variation arising in populations (both genetic and phenotypic), the possibility that phenotypic modification may precede genetic change, and the oftentimes non-gradual nature of evolutionary change. The predictions that result from these theoretical assumptions are substantially different from the MS's predictions. They include that phenotypic variation will be systematically biased by developmental constraint and facilitation; that genetic evolution has a stabilizing role rather than a generative one; that the origin of phenotypic novelties is due to emergent and self-organizing properties of developmental systems; that phenotypic evolution will exhibit discontinuities, and several more. For these and other predictions, substantial empirical support is available today (see references herein or in Laland *et al.*, 2015; Müller, 2017).

In connection with theoretical advances in other areas of evolutionary biology, such as genomics, epigenetics, physiology, ecology, plasticity research, regulatory evolution, behavioral biology, or systems biology, and with further support from the cultural and social sciences as well as philosophy of science, these findings have led to reformulated accounts of evolutionary theory (e.g., Kutschera and Niklas, 2004; Jablonka and Lamb, 2006; Shapiro, 2011; Laland *et al.*, 2015)). One such framework often raised in recent discussions is the Extended Evolutionary Synthesis (EES), which integrates standard evolutionary factors, such as heredity, differential reproduction, variation and selection, with concepts from evo-devo and those of several other areas of evolutionary biology, such as niche construction and plasticity theory. However, the overall predictions of the EES differ substantially from those of the MS (Laland *et al.*, 2015; Müller, 2017). In brief, the theoretical hallmarks of the EES are constructive development and causal reciprocity. The constructional part includes, among others, the physical organizing forces underlying the generation of specific structural motifs that arise in evolving organisms, a principle termed *inherency* (Newman and Müller, 2006; Newman, 2018). Reciprocity between organisms and environment is accounted for by niche construction theory (Odling-Smee *et al.*, 2013). While the EES, besides adding new factors, acknowledges many of

the components of the standard framework, it offers different interpretations of their role in the evolutionary process. Natural selection, for instance, is not seen to function only as the elimination of the unfit but primarily to release the generative potential of development, thus generating biased phenotypic variants and novelty.

Conclusions

Although theory is often avoided by the evo-devo literature (Fusco, 2015), a characteristic suite of theoretical assumptions and predictions derives from modern evo-devo. Together with conceptual innovation in other areas of evolutionary biology, these amount to a significant distinction from some of the classical tenets of the Modern Synthesis, challenging its gene centrism, its gradualistic prerequisites, and its adaptationist proclivity. The challenges are strongly dependent on how evo-devo is defined, how its goals are perceived, and at what level explanation is sought. If, by reducing its conceptual contribution to evolvability, the goals of evo-devo are themselves defined in neo-Darwinian terms, it appears as if the evo-devo challenges constitute merely a "Scheinproblem". But if the neo-Darwinian preconceptions are relinquished, evo-devo's distinct theoretical consequences become very apparent. Evolutionary theory evolves, and it is interesting to note that, in a possibly non-coincidental parallel with biological evolution, theoretical progress can be continuous over time, but the eventual theory structure and resulting domains of explanation are – at least partly – discontinuous, almost in a Kuhnian sense (Kuhn, 2012). This is why some commentators argue that the Extended Synthesis is an expanded version of the standard theory while others regard it as a paradigmatic shift.

In summary, the challenges of evo-devo to the Modern Synthesis arise not so much from theoretical *contradictions* but rather from the differences in testable *predictions* regarding the evolution of phenotypic discreteness. The genotype-phenotype distinction demanded by Waddington needs to be taken seriously and is key for understanding evo-devo's important theoretical contribution. Furthermore evo-devo's research program should not be confused with its theoretical content. Although it places the phenotype at the center of its examinations, this doesn't mean that evo-devo is simply descriptive or typological. Rather, we need to acknowledge that the Modern Synthesis account lacks a theory of the phenotype and, hence, permits no predictions regarding the evolution of phenotypic specificity. Minelli's (2009) criticism of the population genetic predominance in the neo-Darwinian program is a first step towards a reform of the received theory, but we must not shy away from recognizing the crucial theoretical consequences of evo-devo.

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Ever since Darwin: Why plants are important for evo-devo research

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Abstract

In this essay, I provide examples of: (i) the presence of fractal properties and a continuum of forms in living organisms; (ii) the potential contributions of plant evo-devo towards a general theory of development encompassing various multicellular organisms; (iii) the "arrival" of a wealth of forms in plants that cannot be explained by natural selection alone. As elucidated by evo-devo studies, evolutionary diversification is also due to, e.g., (epi)genetics, correlation, phenotypic integration, self-organization, and physical constraints. Four kinds of phyllotaxis patterns in vascular plants – from Fibonacci systems with divergence angles around 137.5° to spiral systems with divergence angles below 80° – are described and illustrated: *Cycas* (gymnosperm), *Huperzia* (clubmoss), *Pandanus* (screw palm), and *Costus* (corkscrew ginger). They serve as examples of morphogenetic variation in plants that call for evo-devo explanations beyond (or prior to) the "survival of the fittest". Charles Darwin was already convinced that natural selection had not been the only driving force in evolution.

Towards a theory of development encompassing various multicellular organisms

Vervoort (2014, pp. 209-210) proposed a theory of development comparing plant versus animal development, especially in respect to the independent origin of multicellularity. He stated that "most developmental biologists working on animals do not feel competent to address and discuss plant developmental data, and vice versa".

Minelli (2018) is clearly a glorious exception. His recently published book *Plant Evolutionary Developmental Biology* proves that he succeeded in doing "the metamorphosis of an evo-devoist trained in zoology to something like a plant evo-devoist" (Minelli, 2018, p. ix). With his comparison of lichens and galls

G. Fusco (ed.) *Perspectives on Evolutionary and Developmental Biology.*

(Minelli, 2017) he had already showed his flair for switching between various kingdoms of life, and for floating unconventional ideas.

Comparisons between animal and plant development were already made in the 18th century. Caspar Friedrich Wolff [1734-1794] and Johann Wolfgang von Goethe [1749-1832] belong to the pioneers capable of doing both (Arber, 1946; Aulie, 1961; Rutishauser and Moline, 2005; Abzhanov, 2018; Rutishauser, 2018). There is an old tradition to look first at plants to better understand the architecture and development of animals. Wolff had begun his inquiries with plant studies in order to get a heuristic Ariadne thread ("Richtschnur") before entering the supposedly much more complex bauplans of animals. Wolff (1759), who was cited several times by Goethe (1790, 1823), belonged to the epigeneticists (Wyder, 1998). Wolff understood the morphogenesis of organisms and their parts as a succession of developmental processes, including tissue differentiation. He first detected the existence of *shoot apical meristems* (*SAMs*) in the buds of vascular plants (Figs. 1-4).

In Darwin's view, plants and animals share a common ancestry and therefore have physiological properties in common. Darwin studied the circular motion (circumnutation) of searching tendrils and climbing shoots. According to him, the climbers belong to the most animal-like groups of plants: How do they find and get in contact with supporting objects? (Costa, 2018). Both animal-like plants and plant-like animals were of interest to Darwin. For example, the modified trichomes of sundews (*Drosera*) with their liquid droplet tips reminded him of the tentacles of marine invertebrates (Rutishauser, 2009).

Today, we are learning more and more about how plants perceive their environment and how they react accordingly. Neurotransmitters as known from animals are also active in plants (Baluška *et al.*, 2006; Baluška and Mancuso, 2007). Thus, we have to accept that there is something like intelligence and learning behaviour in plants. Now it is up to us as researchers to "think like a plant" (Holdrege, 2005), and to get a "feeling for the organism", as experienced by Nobel Prize winner Barbara McClintock [1902–1992] (Keller, 1983; Rutishauser, 2018).

Developmental aspects (including genetics and comparative morphology) of all kinds of multicellular organisms are needed in order to create a theory of development (Minelli and Pradeu, 2014). Thus, not only metazoans (multicellular animals) and land plants (including bryophytes and vascular plants) but also fungi, lichens and various algal clades belonging to other eukaryote lineages need to be studied for the identification of general principles of development. These principles comprise gene regulatory networks through which genes act not as soloists but in concert (Huang 2011; Benitez *et al.,* 2018).

Various multicellular organisms such as land plants as well as brown and red algae show polar growth that may last for a long period. Especially known for indeterminate apical growth are shoots of vascular plants with meristematic tips, the shoot apical meristems. They show a unique morphogenetic potential giving rise to leaves as lateral appendages (Figs. 1-4), and – by lateral branching – also to daughter shoots and flowers. Many biologists and mathematicians are attracted by the regular spiral (helical) leaf arrangement patterns in vascular plants, often coming close to Fibonacci systems with divergence angles between consecutive leaves of ca. 137.5° (Fig. 1a). Thus, plants (especially bryophytes and vascular plants) have distinctive morphogenetic modalities that are rare in, or absent from, other multicellular organisms such as metazoan animals lacking indeterminate apical growth (Benitez *et al.*, 2018).

Developmental geneticists, biophysicists and specialists in computer simulation have already started to better understand the various phyllotactic patterns, especially in model organisms such as *Arabidopsis*, linking molecular (e.g., auxin, cytokinin) drivers with biophysical processes (Cooke, 2006; Smith *et al.,* 2006; Bainbridge *et al.,* 2008; Newell *et al.,* 2008; Besnard *et al.,* 2014; Runions *et al.,* 2014; Rutishauser, 2016b; Minelli, 2018, p. 103]. Most spiral phyllotactic patterns follow Hofmeister's rule: A new leaf primordium tends to form at the shoot apical meristem as far away from the previously initiated leaves, resulting mainly in Fibonacci systems with divergence angles (d) around 137.5° (Fig. 1), and related Fibonacci-type patterns such as Lucas systems (with d = ca. 99.5°) and bijugate systems (with d = 180° + ca. 68.8°). However, phyllotaxis researchers will also have to explain spiral systems violating Hofmeister's rule, such as those found in clubmosses, screw palms and corkscrew gingers (Figs. 2-4).

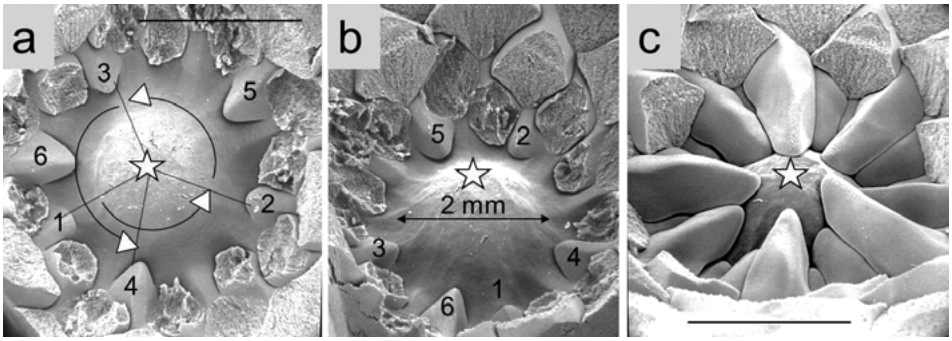

Figure 1. Shoot tip of the gymnosperm *Cycas circinalis*: (a) and (b) show a quite large shoot apical meristem (SAM, diameter 2 mm, stars indicate its centre), seen from above and from a slightly lateral position; (c) shows the same shoot tip prior to the removal of young tightly packed leaves surrounding the SAM. The youngest six leaf primordia reveal typical spiral phyllotaxis (Fibonacci system) with divergence angles between consecutive leaf primordia close to 137.5° (as indicated by crescents with arrowheads). Scale bars = 2 mm [SEM micrographs by RR, UZH]

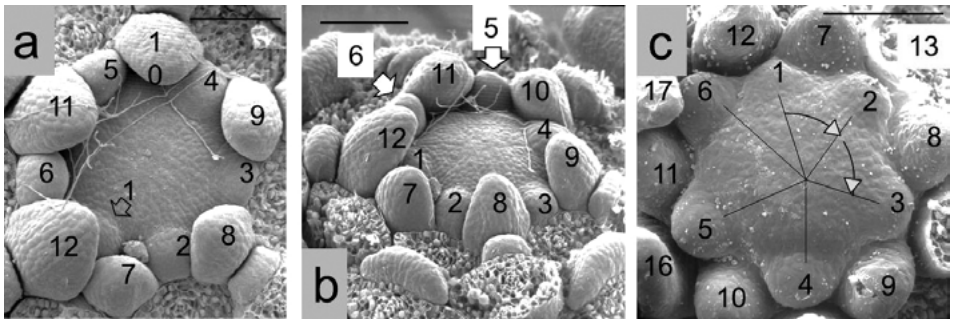

Figure 2. Shoot tips of the clubmoss *Huperzia squarrosa* (Lycopodiaceae): The youngest 12 leaf primordia reveal «Fibonacci-type» spiral phyllotaxis with divergence angles of consecutive leaf primordia close to 65.5°. This (1,5,6) spiral system has contacts between leaves that show age differences of 5, 6 and 11 plastochrones. This aberrant phyllotactic pattern is often found in lycophytes, but very rare in seed-plants including conifers (Fierz, 2014; Gola and Banasiak, 2016). See more on this and related «Fibonacci-type» phyllotaxes in Rutishauser (1998). Scale bars = 150 µm [SEM micrographs by RR, UZH]

The phyllotaxis patterns presented in Figures 1–4 allow some conclusions (for details see figure legends): (i) SAMs are usually 0.1–0.5 mm in diameter (Figs. 2-4). Larger SAMs are rare; they can be found in only a few vascular plants such as cycads (Fig. 1a, b). (ii) Highly regular phyllotaxes probably evolved as optimal solutions of package problems (Fig. 1c), answering the question: How can young leaves be arranged so that they are as compact as possible within a shoot bud? Thus, irregular (seemingly chaotic) phyllotaxis patterns in vascular plants seem to be much rarer than regular ones (Rutishauser, 2016b). (iii) Certain features, such as the shape of leaf primordia and relative frequencies of peculiar spiral patterns, may be genetically fixed as well as the result of developmental correlations because they are restricted to one group of vascular plants, being absent from other closely related taxa. For example, spiral patterns with divergence angles of exactly 120° are favoured in combination with leaf primordia showing triangular shapes, fitting into each other along the corners of an equilateral triangle, as found in screw palms (*Pandanus*) and Cyperaceae (sedge family) among monocots (Fig. 3). (iv) Regular spiral patterns with divergence angles as low as 50-80° are frequent in clubmosses (lycophytes, Fig. 2) and in a subgroup of monocots, the corkscrew gingers (Costaceae) of the banana–ginger alliance (Fig. 4). It seems best to accept that the spiral patterns with divergence angles below 80° resulted from convergent evolution in these two distinct groups of vascular plants that evolved leaves independently: microphylls in lycophytes,

megaphylls in seed plants (Pires and Dolan, 2012; Gola and Banasiak, 2016). The completely different shape of the leaf primordia, being tangentially elliptical in clubmosses and crescent-like in corkscrew gingers, may be another argument in favor of convergent evolution (see Minelli, 2018, pp. 313-327 for additional examples of convergence and parallelism in plants).

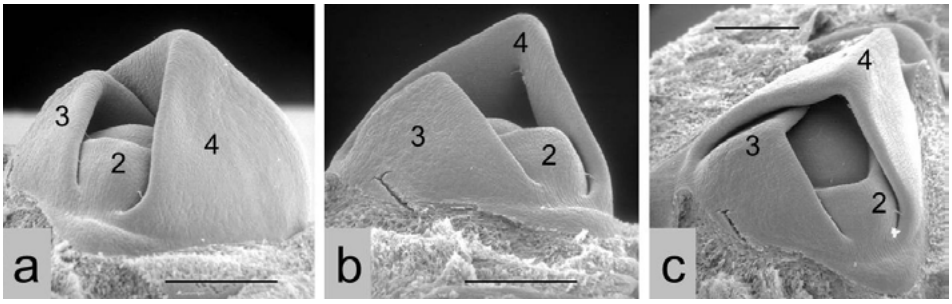

Figure 3. Shoot tip of the screw palm *Pandanus utilis* (Pandanaceae): Three different views of the same tip. The triangular shape of the leaf primordia 2–4 next to the shoot apical meristem is strongly correlated with orthotristichous phyllotaxis (divergence angle 120°). The youngest leaf primordium (1) is hidden. This variant of spiral phyllotaxis is also found in many Cyperaceae. In *Pandanus*, older leaf stages towards the rosette periphery start to twist their position by secondary stem torsion, leading to the leaf arrangement of typical «screw palms» with divergence angles clearly exceeding 120°. Scale bars = 300 μm [SEM micrographs by RR, UZH]

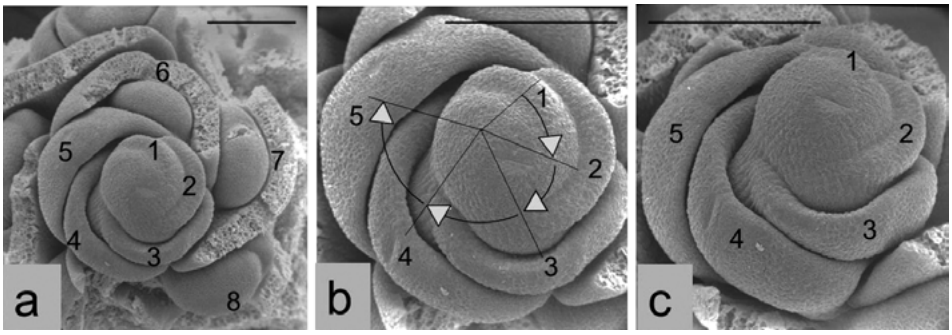

Figure 4. Centre of developing inflorescence in corkscrew ginger *Costus scaber* (Costaceae). Its spiromonostichous phyllotaxis resembles a spiral staircase (divergence angles ca. 60°). Note the crescent-shaped bract primordia 1–7, with floral buds in the older axils. In *C. scaber*, the bracts of the terminal spike-like inflorescence continue with divergence angles as low as ca. 60° (as shown here), whereas other corkscrew gingers such as *Chamaecostus cuspidatus* switch back to Fibonacci systems (with divergence angles of ca. 137.5°) when starting inflorescence development at the tip of aerial shoots (Kirchoff and Rutishauser, 1990). New aerial shoots (starting from the sympodial rhizome) of corkscrew gingers (Costaceae) first show Fibonacci angles (or nearly so), but gradually diminish their divergence angles below 90° along the leafy zone of the upright stems. Scale bars = 200 μm [SEM micrographs by RR, UZH]

Continuum and fractal patterns in plants as compared with animals

Goethe (1823) wrote: "Nature has no system; she has – she is – life and development from an unknown centre towards an unknowable periphery." Thus, Goethe believed that nature's patterns are not fixed – he detected and tried to explain all kinds of transitions both between and within organisms (Abzhanov, 2018; Sattler, 2018).

Furthermore, Goethe (1790) was aware that there are complementary perspectives on vascular plants. Some of the various architectural models designed to conceptually dismantle a growing shoot (e.g. leaves and stems as subunits) were already described by Goethe (1790: see Rutishauser and Sattler, 1985; Rutishauser, 2018; Sattler, 2019 this volume).

Various kinds of homology ("sameness") exist in metazoan animals as well as vascular plants. Comparing iterated parts (e.g., limbs, leaves) in organisms with modular (metameric) construction we tend to speak of "serial homology" (Darwin, 1872; Rutishauser and Moline, 2005; Minelli and Fusco, 2013). Serial homology in arthropods and vertebrates depends to some degree on genetic networks including *Hox* genes. Serial homology in seed plants depends on regulatory networks with e.g. MADS-box genes giving "identity" to the floral organs typically found in four kinds (Vervoort, 2014).

Serial homology may also exist between the whole body of a multicellular organism and its parts. Minelli (2003b, p. 574) provided evidence for fractal patterns in modular animals (arthropods, vertebrates): "It is possibly not by chance that segmented appendages are only present in animals whose main body is also segmented." In vertebrates (e.g., mice) the single limb shares some kind of homology ("sameness") with the main body axis that runs from head to tail. Therefore, Minelli (2003a, 2003b, p. 573) proposed the *paramorphism concept* for modular animals: "It may be justified, instead, to look for correspondences between the appendages and the main body axis of the same animal, as the latter might be the source of the growth and patterning mechanisms which gave rise to the former."

A similar kind of axis paramorphism is present in vascular plants with respect to the iteration of both shoots and leaves, as recently summarized by Minelli (2018, pp. 243–244): "[…] it is sensible to expect that paramorphism is a kind of 'modulated fractal pattern', where the iteration of a small set of rules over different body axes (the longitudinal axis of shoot and leaf) is to some extent constrained by the different context but nevertheless results in recognizable repetitions of a basic regularity." Thus, a single leaf of a vascular plant shares a kind of homology with the whole shoot, which includes both stem and leaves. This process of iteration that continues from the whole shoot with its

leaves to each leaf with its leaflets was observed by botanists well before the times of contemporary evo-devo. In particular, Agnes Arber (1950) and Rolf Sattler (1994, 1996) considered a single leaf of vascular plants (especially when compound) as a partial repetition of the whole shoot to which it belongs. Arber (1950) presented her ideas as the "partial-shoot theory" of the leaf (see Kirchoff, 2001; Rutishauser and Isler, 2001; Flannery, 2003), whereas Sattler used concepts such as homeosis, partial homology and the "continuum model" to explain developmental similarities between compound leaves and shoots with respect to growth modes, architectural complexity and symmetry (arguments further developed by Sattler, 2019 this volume).

Fractal patterns in plants and animals are attractive because growing organisms seem to be able to produce them in an elegant way using simple algorithms (Minelli, 2018, p. 238). Thus, fractal properties according to the holographic paradigm may help to explain modular construction in both metazoan animals and vascular plants: The whole is built up of the parts in such a way that each part bears something of the whole within it (Rutishauser and Isler, 2001; Rutishauser and Moline, 2005). Fractal properties are obvious in phyllotaxis patterns as shown in Figures 1-4: The patterns observable towards the periphery of a shoot bud are repeated by new leaf primordia at the shoot apical meristem (see more on phyllotaxis in Minelli, 2018, pp. 102-106).

Riverweeds (Podostemaceae) as well as bladderworts (Lentibulariaceae, especially genus *Utricularia*) serve to illustrate the continuum and fractal properties in vascular plants (Rutishauser *et al.*, 2008; Rutishauser, 2016a; Minelli, 2018, pp. 254-256). Both of these groups of flowering plants may be called "morphological misfits" because they do not fit the classical root-shoot model of typical seed plants. If, however, for ease of communication, we cling to structural categories such as 'leaf' and 'stem' and 'root' for the description of morphological misfits in vascular plants, we get into trouble with either/or homology ("sameness") of the various plant parts. Then we are forced to accept the existence of structural intermediates such as "stem-leaf mixed organs" in Podostemaceae, as found and genetically analyzed by Katayama *et al.* (2010).

Process philosophical approach in biology: 'leaf', 'stem' and 'root' are usually taken for granted as organs in vascular plants. However, when we realize that these structural categories are arbitrary concepts to some degree, each of them encompassing a certain set of developmental processes, then we are prepared to abandon structural concepts and instead refer to combinations of developmental (morphogenetic) processes that depend – to some degree – on gene regulatory networks. This radical view was proposed by Sattler (1992, 1994, 1996, 2018, 2019 this volume), Sattler and Rutishauser (1997) and Langdale (2008).

According to Sattler (see his "Beyond-Wilber" website): "A structure is not seen as having processes, a structure is seen *as* process(es)." Thus, there is no longer a structure-process dualism. This process philosophical approach was used by Nicholson and Dupré (2017) for all kind organisms: "The living world is a world of process rather than a world of things."

Somewhere between the structural approach and process philosophy are the concepts of dynamic patterning modules and biogeneric materials as proposed by Newman and Bhat (2009), Hernández-Hernández *et al.* (2012) and Benitez *et al.* (2018). They may prove to be heuristically quite fruitful concepts when we want to fully understand the mechanisms responsible for the major evolutionary transitions among eukaryotic lineages that became multicellular.

Natural selection is insufficient to explain the wealth of forms in vascular plants and other multicellular organisms

Minelli (2018) pointed to four aspects of evolutionary developmental biology, while focusing on plants (see also Langdale, 2008; Wagner, 2014; Harrison, 2017; Rutishauser, 2018): (i) During the last 20 years there was a rapid growth of evo-devo as a new approach to understanding the evolution and development of organismal form. (ii) To a considerable extent, evo-devo deals with developmental genes, their evolution and their expression. (iii) Evo-devo explains the *arrival* of the fittest whereas Darwinism explains the *survival* of the fittest. (iv) There is a strong need to focus on the phenotype which is at the same time the product of development and the direct target of selection. Accordingly, Minelli (2018) calls for a renaissance of comparative plant morphology in evo-devo. Such a discipline complementing developmental genetics may be labelled as "MorphoEvoDevo" (Wanninger, 2015).

Darwin (1872) showed in the 6th edition of "Origin of Species" that he was well aware that natural selection is not sufficient to explain the wealth of forms ('bauplans') in the various kingdoms of life. To make sure that the reader of his book received this key message, Darwin wrote not once but twice (in the Introduction as well as in Chapter VII) that "I am convinced that natural selection has been the main, but not the exclusive, means of modification." Darwin (1872, chapter VII) also admitted: "Many characters appear to be of no service whatever to their possessors, and therefore cannot have been influenced through natural selection." With respect to plants he pointed to "an admirable essay", written by the botanist Carl Wilhelm von Nägeli [1817-1891]: "He specifies the arrangement of the cells in the tissues, and of the leaves on the axis, as cases in which natural selection could not have acted." Darwin (1872, chapter VII) continued his objection to natural selection as exclusive means of morphological

change ("modification") in evolution: "It should always be borne in mind that when one part is modified, so will be other parts, through certain dimly seen causes, such as an increased or diminished flow of nutriment to a part, mutual pressure, an early developed part affecting one subsequently developed, and so forth, – as well as through other causes which lead to the many mysterious cases of correlation, which we do not in the least understand. These agencies may be all grouped together, for the sake of brevity, under the expression of the *laws of growth*."

The *'laws of growth'* as proposed by Darwin – and later articulated more formally by D'Arcy Thompson (1917, 1961) – may in a contemporary interpretation also encompass developmental genetics and all interacting ontogenetic processes from the molecular to organismal level, including epigenetics, correlation, self-organization, phenotypic integration (i.e. interdependence of morphological traits, also termed synorganization), morphogenetic fields and gradients, physical constraints such as intrinsic material properties and tissue tension during development, and even neuronal aspects in plants (Baluška *et al.*, 2006; Newman, 2014; Vecchi and Hernández 2014; Wanninger, 2015; Abzhanov, 2017; Cabej, 2018; Bateman and Rudall, 2019 this volume).

Darwin's and Thompson's 'laws of growth' got a refreshing renewal in the 'law-of-form' approach by Newman *et al.* (2006). They are aware that the roots of their approach go back well before the rise of contemporary evo-devo, amalgamating ideas of Goethe, Geoffroy St-Hilaire, Owen, Bateson, D'Arcy Thompson, and also Brian Goodwin (Newman, 2014, p. 107). Newman *et al.* (2006) favour a kind of evolutionary saltationism when they suggest: "[…] once multicellularity had been achieved, the emergence of distinct body plans likely occurred with much less genetic change and at a faster pace than would be predicted by gradualistic models of evolution by natural selection."

As already admitted by Darwin (1872, see underlined words in the quotation above) phyllotaxis patterns as observable in vascular plants (Figs. 1-4) appear as developmental patterns that are not under the control of natural selection. There are developmental constraints ('laws of growth') that force most spiral patterns to approach the famous Fibonacci angle, which is about 137.5° (Cooke, 2006; Mirabet *et al.*, 2012; Swinton *et al.*, 2016). Fierz (2014) examined the phyllotaxes of 6,000 cones of one single European black pine tree (*Pinus nigra*). She counted 5,838 cones (97%) exhibiting the main Fibonacci pattern with 8 and 13 parastichies. Additional nine aberrant spiral patterns with "Fibonacci-type" sequences were quite rare and occurred with different frequencies. Interestingly, all of them have something to do with the golden ratio 0.618. With only one cone observed, the (1, 5, 6) spiral system was the rarest phyllotaxis observed

among the 6000 pine cones, showing divergence angles of d = ca. 65°! This is exactly the pattern that is much more frequent in clubmosses (Fig. 2). It seems that vascular plants with leaf primordia that are much smaller than their shoot apical meristem tend to deviate more easily from the typical Fibonacci phyllotaxis, escaping to other kinds of Fibonacci-type spiral systems or even to irregular ("chaotic") ones. Thus, we have to consider – besides mathematical rules – also physical constraints imposed by the shoot apical geometry (Rutishauser, 1998, 2016b; Cooke, 2006).

Fibonacci systems (with divergence angles approaching 137.5°) and related spiral patterns are – besides land plants such as lycophytes and seed-plants – also known from brown algae (Phaeophyceae, e.g. *Sargassum*) and red algae (Rhodophyceae). These multicellular eucaryotes gained indeterminate apical growth and repeated formation of lateral appendages as a result of convergent evolution. The lineages on the tree of life leading to brown algae, red algae and land plants (as part of *Chara*-like green algae) diverged from unicellular ancestors more than 1,000 millions of years ago (Pires and Dolan, 2012; Peaucelle and Couder, 2016). Fibonacci spirals were even obtained in physics experiments that had no relation to biology (Douady and Couder, 1998). Thus, there are strong arguments in favour of the view that self-organization processes beyond natural selection allowed the emergence of Fibonacci systems and related patterns in living organisms.

Conclusions

Not everything is possible in plant development. There are architectural constraints, favouring some body-plan features while excluding other imaginable patterns in living organisms. This short essay gives emphasis on evo-devo research of land plants. For example, the *paramorphism concept* as proposed for modular animals by Minelli (2003a, b) has its counterpart in land plants when compound leaves repeat the developmental pathways ("programs") of the shoots to which they belong. Thus, there are fractal properties common to both multicellular animals and multicellular plants. Unlike metazoan animals, plants (as well brown and red algae) may form multicellular bodies with indeterminate apical growth and iteration of lateral appendages (Minelli, 2018). The resulting regular Fibonacci-type patterns obey Hofmeister's rule with a new leaf primordium positioned in the least crowded spot around the shoot apical meristem (Fig. 1). Much rarer spiral patterns that violate (at least to some degree) Hofmeister's rule are also found in plants (Figs. 2–4). Thus, the various spiral patterns in plants and other multicellular organisms cannot be explained exclusively by natural selection. They follow 'laws of growth' (e.g. self-orga-

nization, gene regulatory networks, auxin gradients), as already foreseen by Charles Darwin. It is now time to switch in evolutionary biology from the Modern Synthesis to the Extended Synthesis by the inclusion of developmental and evolutionary processes that contribute to non-aptation (Bateman and Rudall, 2019 this volume), giving rise to a wealth of forms in living organisms beyond (or prior to) natural selection (Pigliucci and Müller, 2010; Huang, 2011; Horsthemke, 2012; Wagner, 2014). As concluded by Minelli and Baedke (2014), "Investigating evolvability means shifting the focus from the survival of the fittest to the arrival of the fittest".

Acknowledgements

I am very grateful to Richard Bateman, Paula Rudall, Bruce Kirchoff and Rolf Sattler for valuable comments and suggestions.

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Structural and dynamic approaches to the development and evolution of plant form

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Abstract

Structural approaches are based on morphological categories and frameworks of these categories, such as the root-stem-leaf model (framework) in vascular plants. There are different categories and frameworks that can complement one another and thus provide a more inclusive understanding of plant form. According to factorial or combinatorial homology, features of different categories can be combined in one structure. Two types of dynamic approaches can be distinguished: first, the commonly used approach in which structures *have* processes, and second, process morphology according to which structures *are* processes. When structures are seen as process(es), the structure-process dualism is overcome and the fluidity of plant form in development and evolution can be understood unconstrained by the barriers created by structural categories and frameworks.

Introduction

In this article plant form is understood as the morphology of plants at the organismal level. At this level, plants have usually been described in terms of structural categories such as root, stem, leaf, metamer, telome, enation, and trichome. Some of these categories such as leaf and metamer have been considered mutually exclusive, but they can also be understood as complementary perspectives on the diversity of plant form.

Plant development has been understood as the formation of structures that belong to one or the other category or are intermediate between these categories. And then plant evolution has been seen as the modification of these structures during evolution. Although useful to some extent, this common approach entails a conceptual dismemberment or fragmentation of the wholeness and continuum of individual plants into distinct structures. To overcome this frag-

G. Fusco (ed.) *Perspectives on Evolutionary and Developmental Biology.*

mentation the *theory of anaphytosis* (Schultz, 1843) can be used in conjunction with *process morphology* (Sattler, 1990, 1992, 1994).

The structural approach

The structural approach to the development and evolution of plant form is usually based on structural categories and frameworks of such categories. Different categories and frameworks have been used and often these frameworks have been seen in competition with one another. In terms of Aristotelian either/or logic that is still often taken for granted in our culture and science, it has been assumed that *either* one *or* the other must be correct. However, we know also other kinds of logic that go beyond either/or logic but may include it as a special case. Such kinds of logic have been known since antiquity. According to Yin-Yang thinking, Yin contains Yang and vice versa. Buddhist logic (according to Nagarjuna) accepted either/or, but added both/and as well as neither/nor (Sattler, 2010). According to Jain logic, there are seven perspectives for any proposition (see Sattler, 2010). Applying these more comprehensive kinds of logic to structural frameworks appears liberating because one can recognize then that they need not be antagonistic to one another but complementary. And thus all of these frameworks together provide a richer and more complete understanding of plant development and evolution. It may turn out, however, that in any particular situation one framework may be more appropriate than others.

For algae and the thallose liverworts the thallus category appears generally accepted. For some primitive fossil vascular plants the notions of the telome and enation appear useful. And for the majority of living vascular plants most plant morphologists accept the categories of root and shoot. However, with regard to the shoot different categorical frameworks have been distinguished. Rutishauser and Sattler (1985, Table 1) enumerated the following five frameworks or models:

1. The *stem and leaf model*, the most popular model, also referred to as the classical model.
2. The *fertile leaf model* in which the leaf includes the axillary bud.
3. The *leaf-skin model* in which the leaf descends into the outer part of the stem.
4. The *phytonic model sensu stricto* in which the leaf comprises the axillary bud and descends into the stem below.
5. The *metameric model* in which the leaf comprises the axillary bud and the stem segment below. (The phytonic model sensu lato comprises both 4 and 5)

Depending on which model we use, we get a different view of plants and their evolution. And seeing these views as complementary provides a more compre-

hensive view. I know a mountain near Montreal that on one side has a steep slope and on the other a flatter one. Looking at it only from one side gives us only a one-sided picture of that mountain, whereas including all sides gives us a more comprehensive picture. The same applies to plant morphology (see also Sattler, 2018).

Besides both/and logic and complementarity another important innovation in logic is fuzzy logic (see Kosko, 1993). According to this logic, membership in a set or class is a matter of degree, ranging from 0% to 100%. This contradicts Aristotle's law of the excluded middle according to which membership in a class is either zero or 100%.

There is much evidence that fuzzy logic applies in many instances in plant morphology since many plant structures belong to a category only to some degree. Since fuzzy logic also includes the extremes of Aristotelian logic (0% and 100%), we may characterize plant morphology as fuzzy morphology. Honoring Agnes Arber, Rutishauser and Isler (2001) referred to "Fuzzy Arberian Morphology".

Fuzzy morphology leads to *continuum morphology* that recognizes a continuum between structural categories. Sattler and Jeune (1992), using multivariate analysis, demonstrated a continuum between the categories root, shoot, stem, leaf, and trichome. Thus, classical morphology and typology in which these categories are considered sharply delimited and mutually exclusive seems no longer generally tenable. However, as long as it is applied only to the typical forms and not to the intermediates between them it still works. Timonin (2002) criticized continuum morphology as inconsistent because "it regularly needs hidden applications of concepts that have been formulated in typological morphology". But the concepts he considered hidden such as root, stem, and leaf, are not at all hidden in the multivariate analyses by Sattler and Jeune (1992). In fact, they are explicitly used to show that they are linked through intermediate forms so that a continuum of forms becomes evident. Besides the continuum between morphological categories, continuum morphology also emphasizes the continuum within individual plants that is usually fragmented by the use of structural units such as root, stem, and leaf.

The recognition of a continuum and fuzziness in plant morphology changes the notion of homology from all-or-nothing relationships to homology as a matter of degree. Therefore, Minelli (2016) concluded, "homology should be treated as relative, or partial." I came to the same conclusion (Sattler, 1994). Although most authors still cling to homology as an all-or-nothing concept (that is, a structure is homologous or not), Minelli (2016) noted that since the '80s of the past century several authors have emphasized partial homology. In

line with this kind of thinking, Minelli (1998, 2016, 2018) and Minelli and Fusco (2013) proposed and endorsed the concept of *factorial* or *combinatorial homology*, which means that a structure may combine features of different structures or structural categories. Many examples of this type of homology could be given. Phylloclades (flattened structures in the axils of bracts) such as those of *Ruscus aculeatus* show a combination of shoot and leaf features (Cooney-Sovetts and Sattler, 1987). This combination is also evident at the molecular genetic level: during the development of the phylloclade genes are expressed that normally are expressed at the shoot apex and in leaf primordia (Hirayama *et al.,* 2007). Rutishauser (2016) demonstrated many examples of combinatorial homology in the Podostemaceae and in *Utricularia* (bladderworts). Furthermore, combinatorial homology is also known in common structures such as compound leaves that combine shoot and leaf features, which led Arber to formulate her partial-shoot theory of the leaf (Arber, 1950). Her theory has been confirmed for compound leaves by more recent developmental studies (see, for example, Lacroix *et al*., 2003). On the basis of molecular genetic research, Eckardt and Baum (2010) concluded, "it is now generally accepted that compound leaves express both leaf and shoot properties." In as much as the combination of properties is seen as a combination of processes, combinatorial homology is compatible with process morphology (see below). (See Sattler (2018) for a more detailed discussion of homology, homotopy, homeosis, and related issues).

Dynamic approaches

Two types of dynamic approaches can be distinguished: first, the commonly used approach in which structures *have* processes, and second, *process morphology* (that I will explain below), according to which structures *are* processes. The first type, although considered dynamic, shares much with the structural approach because structures are still basic in this approach. Although the structural approach (with or without processes) appears practical and can provide much insight, it has limitations and disadvantages. It requires a dismemberment or fragmentation of the plant into structural units such as root, stem, and leaf. But the plant represents a continuum. There are no sharp demarcations in the plant between the root, the stem and the leaves and other kinds of structures (that I pointed out above as the 5 frameworks). Demarcations are more or less artificial.

To avoid this artificiality we may resort to the *theory of anaphytosis*, which has been developed long ago by Schultz (1843), who was also known as Schultz-Schultzenstein (1867). This theory has been almost completely forgotten and is only very rarely mentioned in the modern literature (see Rutishauser and

Sattler, 1985; Cusset, 1982; Sattler, 2018). Its physiological aspect is no longer tenable, but its morphology provides a dynamic view of plants in terms of two fundamental processes: branching and articulation. Note that branching here is understood in a broad sense as ramification, the formation of a new growth center or primordium, which may produce a branch, leaf, leaflet, root, telome, or any other structure, that is, a process combination (see below). Branching leads to articulation, which refers to the formation of articles between subsequent branchings. These articles are called anaphytes and therefore the theory of anaphytosis has also been referred to as the theory of anaphytes (Cusset, 1982; Rutishauser and Sattler, 1985; Sattler, 2018). If the anaphytes are understood dynamically (as I shall explain below), then there is no need to demarcate them as structural units and thus the theory of anaphytosis can be seen as a completely dynamic theory of plant morphology.

However, although it seems easy to say that everything flows (changes), to arrive at a completely dynamic view of plant morphology is not an easy task. It requires superseding the structure-process dualism that seems inherent in almost all morphological investigations because, although they may refer to morphogenetic processes, these processes are said to occur in structures such as stems and leaves, and thus structures are implied. As I pointed out elsewhere: "To render morphology and biology more dynamic we have to see structure itself as process. Then there is only process and therefore the structure-process dualism is overcome (Sattler 2018, p. 63). Woodger (1967, p. 330) communicated this insight very well when he wrote: "It seems, then, that what is required is an enlargement of our concept of 'structure' so as to include and recognize that in the living organism it is not merely a question of spatial structure with an 'activity' as something over against it, but that the concrete organism is a spatio-*temporal* structure and that this spatio-temporal structure *is* the activity itself". In short: "structure itself is process" (Sattler, 2018). When we conceive structure in this way, we have to realize that this understanding of structure deviates radically from the traditional definition according to which structure is opposed to process. Claβen-Bockoff (2005, p. 46) criticized the structure-as-process view as illogical because she could not see beyond the traditional definition of structure and therefore she remained caught in the structure-process dualism, which closes the door to a completely dynamic morphology that I also called *process morphology* (Sattler, 1990, 1992, 1994). In this process morphology, instead of accepting structures in the traditional sense such as root, stem, and leaf (that could lead to the structure-process dualism), I started with the following four fundamental processes:

- growth and decay
- differentiation and dedifferentiation

Then I distinguished different parameters (modalities) for these four processes (see Sattler, 1990, 1994). One of the growth parameters is symmetry (symmetrisation). The parameters are fuzzy so that the whole observable range of symmetries is covered. As a result of this approach, structures can be seen completely dynamically as process combinations. Thus, *what changes during development and evolution, ontogeny and phylogeny are process combinations*. It has been shown that they form a *dynamic continuum* (Jeune and Sattler, 1992; Sattler, 1994).

Sattler and Rutishauser (1990) demonstrated how the structures of complex plants such as species of *Utricularia* can be described in terms of process morphology. Sattler (1992) explained how evolutionary changes and innovations can be understood in terms of process morphology. Evolutionary changes such as heterochrony and heterotopy may involve the change of only one or few processes, whereas other changes such as heteromorphy, deviation, and homeosis may entail the change of more processes (for details see Sattler, 1992). It remains a challenge for plant evolutionary developmental biology (evo-devo) to understand developmental changes during evolution in terms of process morphology. So far it seems that development has been described mainly in terms of structures and processes have been seen as belonging to structures, which implies a structure-process dualism. However, it is not always clear to what extent authors actually imply a structure-process dualism (see below).

Returning to the theory of anaphytosis: As I pointed out above, it is based on branching and articulation. If the articles (anaphytes) that are formed as a result of branching are understood as process combinations, then we obtain a completely dynamic theory of anaphytosis in line with process morphology: branching, which is a process, leads to the same or a new process combination. For example, in a telome truss, the new article (anaphyte), which is also a telome, repeats the same process combination, whereas when a leaf is initiated on the shoot apex a different process combination is formed. I am referring here to the structural categories of telome, shoot apex, and leaf only for means of communication. I understand them as process combinations. But the fact that I am referring to structural categories shows that these categories are useful for communication. Hence, even in a dynamic outlook they retain a practical usefulness. And therefore we may say that structural morphology and process morphology complement one another. However, process morphology appears to be closer to reality than structural morphology because reality appears to be fundamentally dynamic. As Minelli (2016) affirmed, "nothing in nature – in the living nature especially – escapes change." And in "A manifesto for a processual philosophy of biology," Dupré and Nicholson (2018, p. 3) concluded, "the world

– at least insofar as living beings are concerned – is made up not of substantial particles or things, […] but of processes. It is dynamic through and through." This has been recognized long ago, when Heraclitus stated that everything flows ("panta rhei"). Daoism also emphasizes dynamics, and Buddhism underlines impermanence (change). However, common interpretations of Plato's and Aristotle's philosophies shifted the emphasis toward essences and essentialism that shaped Western culture and science to a great extent. Essences are static. Troll (1937-1943, 1949), who had an enormous influence on 20th century plant morphology, followed explicitly the essentialist tradition (see also Nickel, 1996). Other morphologists did not endorse essentialism but used the same categories as Troll. Even morphologists who used an evolutionary approach often reduced the diversity of plant form to mutually exclusive categories. I consider this implicit essentialism. It seems more widespread than is generally admitted. Much dynamic morphology also operates within the framework of structures and static structural categories where processes are operative within structures. But processes do not require underlying structures. "Instead of thinking of processes as belonging to things [structures], we should think of things [structures] as being derived from processes" (Dupré and Nicholson, 2018, p. 13). Some of the dynamic morphology may point in this direction and thus may converge more or less with process morphology. For example, when combinatorial homology is seen as a combination of different processes, then it includes process morphology.

The distinction between the process morphological view and the dynamic view according to which processes belong to structures is not always clear but rather fuzzy, especially when structures are defined by processes and then as a shortcut for communication the process combinations are referred to as structures. Thus, authors who refer to structures may have a process morphological view in mind. The danger is that through the use of language structures and the structural approach may become reified even when structures are defined dynamically.

Sometimes I have been told that process morphology is not really different from the dynamic morphology as most modern morphologists practice it. I think this criticism overlooks a fundamental difference: in the commonly practiced dynamic morphology structure is primary and process is secondary. "This pervasive bias towards things [structures] is reflected in our everyday language, and it has a direct effect on how scientific research is conducted and how its results are interpreted" (Dupré and Nicholson, 2018, p. 11). Thus, a statement like "This leaf grows," that we usually take for granted as a factual statement, implies a philosophical bias towards structural thinking and against process philosophy

because it takes structure as more basic than process. This bias seems to be built into the noun-verb structure of our language. Before we can refer to a verb that expresses process we need a noun that implies statics and thus process becomes secondary. In contrast, in process morphology process is primary and structure (if we want to refer to it) is secondary. Therefore, structure can be conceived as an abstraction from the primary dynamics. As I pointed out above, reference to structures is useful for easy communication and it also provides insight. But the insight is limited because process appears to be more fundamental than structure: "processes must be, in some sense, more fundamental than things" (Dupré and Nicholson, 2018, p. 4). "Living organization is dynamic and fluid down to the organism's genome" (Ho and Fox, 1988, p. 15). And these authors added: "Surely, there is a lesson here for us: unless we too are intellectually supple, we shall never really come to grips with nature" (ibid.).

Although the process view of this article deals only with the morphological organismal level of plant development and evolution, it can be extended to other levels of organization such as the molecular genetic level and the ecological level (see, for example, Nicholson and Dupré, 2018).

Process morphology is also relevant to other biological disciplines such as cladistics. Weston (2000) claimed that process morphology does "not impact severely on the ability of cladistics to achieve its primary goal: to reconstruct taxic relationships." However, he added that decomposing continuous variation into characters and character states "may sometimes result in considerable loss or distortion of information." The problem is that the characters and character states used in cladistic analysis may fragment a developmental continuum, whereas process morphology emphasizes the latter (Cronk *et al.*, 2002, p. 513).

Conclusions

Approaches to plant development and evolution in terms of structures and their modification during ontogeny and phylogeny have been practical and useful and will remain important. However, one of their major limitations and disadvantages for a more complete understanding of development and evolution is the fragmentation they entail: fragmentation of the diversity of plant forms into mutually exclusive categories, and fragmentation of individual plants into structural units that usually imply the categories. *Continuum morphology*, supported by much empirical evidence, has counteracted this fragmentation: morphological categories are continuous with one another; and structural units within individual plants are also continuous with one another and therefore they do not exist as separate entities or things – they are no-thing. In general, "there is really no 'thing' in the world" (David Bohm, quoted by Jaeger, 2018). "Things are

abstractions from an ever-changing reality" (Jaeger, 2018). And ever-changing manifest reality arises out of the unmanifest unnamable mystery that, since it remains unnamable, is beyond statics and dynamics. This insight is proclaimed already in the very first sentence of the Daode jing (Tao Te Ching): "Existence is beyond the power of words to define: terms may be used but are none of them absolute" (Bynner 1944/1972). Thus, not even dynamics or process are absolute.

Although not absolute, with regard to manifest reality process that entails impermanence is considered fundamental in Daoism and Buddhism. *Process morphology* and a process-morphological interpretation of the *theory of anaphytosis* resonate well with this ancient Daoist and Buddhist wisdom. In the West Heraclitus also emphasized that everything flows (changes). But subsequently Western culture and science have become dominated by the emphasis of Plato's philosophy of forms (essences) and Aristotle's essentialism and his logic of identity and either/or. However, some recent innovations in biology, including the study of development, evolution, and evolutionary developmental biology (evo-devo), have surmounted these static strictures at least to some extent. Alessandro Minelli has greatly contributed to these innovations.

Acknowledgements

I am very grateful to James Griesemer and Rolf Rutishauser for valuable comments and suggestions.

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Part II Conceptual elaborations

Homology and homoplasy of life cycle traits

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Abstract

Life cycle traits, such as metagenesis and metamorphosis, often take on functionally varied forms in evolution. Like other biological traits, life cycle traits can be classified phylogenetically under a dual aspect, as homologies or homoplasies. Several examples of indeterminate boundaries between metamorphosis, metagenesis, and asexual reproduction demonstrate the utility of classification in terms of homology. Life cycle homologues, as events, processes, or temporal patterns, fit and extend Hennig's semaphoront view of homology.

> The significance for biological systematics that attaches to the variability of the individual in time is that, strictly speaking, one and the same individual assumes a different place in most systems at different times of its life [...] We should not regard the organism or the individual (not to speak of the species) as the ultimate element of the biological system. Rather, it should be the organism or the individual at a particular point of time.
>
> (Hennig, 1966, p. 6)

Introduction: life cycle variation

The aim of this essay is to develop a novel application of the concept of homology for purposes of classifying and comparing life cycles.

Life cycles are the trajectories of generative processes, including both developmental and reproductive processes, through which specific forms of organismic life recur over time. We tend to think of life cycles on the human or vertebrate model as comprising a developing individual followed by a reproductive event leading to another developing individual of the same type. But more complex life cycles are present in many animals, plants, fungi, and protists. In *multigenerational* life cycles, using the terminology of Fusco and Minelli (in

G. Fusco (ed.) *Perspectives on Evolutionary and Developmental Biology.*

press), an individual can reproduce sexually or asexually to give rise to a second individual possessing radically different morphology, physiology, development, ploidy, and behavior. This second type of individual may then reproduce the first type of individual directly, as occurs in land plants and some marine invertebrates, or it may reproduce still other types of individuals (as in trematodes), sometimes with facultative branching paths (as in aphids and rotifers), before eventually closing the cycle back on the first type of individual. These complex life cycles are called "multigenerational" because the cyclical return to the first type of organism requires at least one generation of organisms of a different type separated by reproductive events. In contrast, *monogenerational* life cycles (as in humans) are occupied by a single individual bounded by reproductive events, with differences between phases of development recognized as *stages* rather than generations (see Fusco and Minelli in press, for a more extended treatment of these distinctions).

The existence of multigenerational life cycles suggests that life cycles may be the more general category than organisms or biological individuals *per se* for investigating how forms of individuality get repeated on developmental and inter-generational time scales (see Fusco, 2019 this volume; Jaeger, 2019 this volume). Difficult conceptual questions about biological individuality quickly arise for the study of life cycles, however. When is a given form of life a *stage* and when is it a *generation*? The answer seems to be that stages are temporal parts of an individual's development whereas generations involve reproduction of new individuals. But how do we know when a given generative process is a development of the same individual, and when it is the reproduction of a new individual? (Minelli, 2011; DiFrisco and Mossio, in press) This last question can be specified further: how do we distinguish metamorphic development, a time-heterogeneous series of developmental stages, from metagenesis, a time-heterogeneous series of reproductive generations?

These sorts of questions call for a system of classification for life cycles that is both orderly and inferentially reliable. The trouble is that life cycle evolution presents a bewildering array of variation in the forms and sequences that biological individuality can take. Some of the main traits used to categorize life cycles and their stages include the following: monogenerational versus multigenerational; sexual versus asexual; gonochoric (separate sexes) versus hermaphroditic (or dioecious versus monoecious); nuclear phase or ploidy sequence (i.e., haplontic, diplontic, haplodiplontic); direct versus metamorphic development; and egg, larval, juvenile, and adult stages. For virtually any proposed inferential link between these traits that is not a matter of stipulated definition, there exist major exceptions.

For example, multigenerational life cycles often do not include metamorphic development, but sometimes they do (Cnidaria). Multigenerational life cycles can comprise strictly alternating asexual and sexual generations (metagenesis), but they can also comprise more than two generations with facultative sexuality at specific generations (facultative parthenogenesis), and multiple options of sexual reproduction. Sexual as well as asexual reproduction can occur in both gonochoric and hermaphroditic species as well as at different stages and generations of their life cycles. For example, trematodes can have multigenerational life cycles involving alternating gonochoric and hermaphroditic generations. In haplodiplontic life cycles, haploid generations can be sexual or asexual, as can diploid generations, with multiple asexual generations possible (as in certain ferns). Sexual and/or asexual reproduction tends to occur in adult stages, but it can also occur in earlier stages (pedogenesis), with offspring reproducing parthenogenetically even before they are born in certain aphids, mites, and flatworms.

One of the major hindrances to classification in this disorderly situation is that biologists are frequently imprecise about how the above traits are being defined. Like other biological traits, life cycle traits can be classified phylogenetically as *homologies* or *homoplasies*.[1] Homologies (synapomorphies) are shared derived traits definitive of a monophyletic group, or the same trait in different taxa that was inherited from their most recent common ancestor. Homoplasies, by contrast, are similar traits that are present in different lineages as a result of convergent evolution rather than inheritance from a common ancestor. When zoologists use the term "metamorphosis," are they referring to a homology or a homoplasy? In fact, it can be either one. If theorists are attempting to reach a definition or characterization of metamorphosis that applies equally well to amphibians, fishes, cnidarians, echinoderms, and holometabolous insects (e.g., Bishop *et al.*, 2006), then metamorphosis is being treated as a homoplasy. The most recent common ancestor of these groups almost certainly did not itself have metamorphic development, thus it is extremely unlikely to be homologous. Metamorphosis in Holometabola, on the other hand, *is* a homology: flies, butterflies, beetles, and ants all have this same trait as a result of inheriting it from a common ancestor.

Generally, homology captures structural similarity between traits due to common descent, whereas homoplasy tends to capture functional similarity. In fact, the homology-homoplasy distinction does not always neatly map onto the structure-function distinction. One reason is that convergent evolution can give rise to similarity in the structures that perform similar functions. This is why

[1] Note that not all traits will be either homologous or homoplastic.

theorists typically include among the identifying criteria of homology the presence of shared features that are too needlessly complex to be likely products of independent evolution (Remane, 1956; Riedl, 1978; Patterson, 1982). Another difficulty is that functions or activities of body parts can themselves be considered as homologies (Love, 2007; Brigandt, 2017). Nonetheless, the connection between the homology-homoplasy distinction and the structure-function distinction is biologically robust enough to rely on it as a heuristic in classifying life cycle traits.

Life cycle traits like metamorphosis, metagenesis, and the adult stage of development are typically characterized in functional rather than historical or structural terms. In comparative contexts, this makes them more amenable to being treated as homoplasies, when the phylogenetic background of independent evolution is explicit, or as analogies, when functional similarities are considered without reference to phylogeny. But functional classification of large scale phylogenetic patterns is particularly vulnerable to the vicissitudes of local evolutionary processes. Life cycle traits tend either to be major fitness components or to directly influence major fitness components, affecting generation times, schedules of birth, growth, and mortality, and lifetime reproductive output. Accordingly, these traits can diverge under selection when the same life cycles are placed in new ecological niches (Istock, 1967). It also seems that certain life cycle traits are fairly easily subject to secondary loss, such as the loss of a stage, a generation, or as sometimes happens, the complete loss of sexuality or gonochory in a species. This phenomenon undermines a different line of response to the problem of variation, which is to make all classifications of life cycle traits taxonomically highly specific. Secondary loss also occurs in narrowly-defined taxonomic groups and on short evolutionary time scales. A "taxonomic narrowing" approach only addresses the problem by giving up on the prospect of a comparative biology of life cycles.

Homology is a useful classificatory tool in this situation because it is independent of variations in "form and function," as Owen's (1843) definition originally put it. Traits that are functionally similar but structurally distinct, such as asexual reproduction and metamorphosis, can be distinguished by their non-homology. Conversely, traits that share the same historical origin but that have acquired divergent functional roles, such as the juvenile stage in pedogenetic versus non-pedogenetic aphids, can be grouped together by their homology. The secondary loss of a trait in one branch of a clade need not disrupt comparative generalizations over the members of the clade if the surviving rudiments of the trait permit counting it as a homologue. In many cases homology preserves the inferences and generalizations that we can make about a class of functionally divergent but structurally similar traits.

In the next sections I will examine a few examples that illustrate homology and homoplasy of life cycle traits. The concluding section explores broader questions about how life cycle traits can be integrated into existing work on homology.

Examples: metamorphosis, metagenesis, and asexual reproduction

Developmental processes are classified as *metamorphic* when they comprise multiple stages that differ markedly in physiology, morphology, ecological niche, behavior, and/or reproductive capacity, with transitions between stages being abrupt on developmental time scales (Bishop *et al.*, 2006). Metamorphosis is something that happens to a single individual. It is not itself a multigenerational life cycle, though it may be part of one. *Metagenesis* is nearly the same – comprising differences in physiology, morphology, ecological niche, behavior, and/or reproductive capacity – except that it is a type of multigenerational life cycle. The stages are *generations* that produce each other sexually and then asexually, mostly in alternating fashion. What is the real basis for the distinction between stages and generations, between metamorphosis and metagenesis?

A sensible answer is that generations reproduce whereas stages do not. The transition between generations can be identified as a process of reproduction rather than development because (1) the parental generation typically survives some time after the offspring generation is produced, and (2) a single parent can reproduce more than one offspring. In metamorphosis, by contrast, the earlier stage *becomes* the later stage rather than surviving as a distinct organism, and it can only produce one later stage rather than many. This distinction allows us to explain why the presence of gametes does not make every monogenerational sexual life cycle a metagenetic life cycle with alternating somatic and gamete generations. Gametes cannot reproduce additional organisms in multiplicative fashion but can only fuse, and so they are not a distinct generation.

Metagenesis and metamorphosis in Cubozoa

One clade with metagenesis are the Cnidaria, which alternate between asexual polyp generations and sexual medusa generations. In the typical cnidarian life cycle, free swimming larvae find a benthic site and develop into sessile polyps, which split into discs that become juvenile pelagic medusae. Adult medusae reproduce sexually to form larvae, thereby closing the cycle.

In the cubozoan class of cnidarians, however, the polyps directly transform into medusae (Fig. 1). Although the polyps can produce other polyps asexually, each polyp gives rise to just one medusa and does not survive the process as a

distinct organism. Should this polyp-to-medusa transition be considered a case of metagenesis or metamorphosis? (see Minelli, 2011; Godfrey-Smith, 2015)

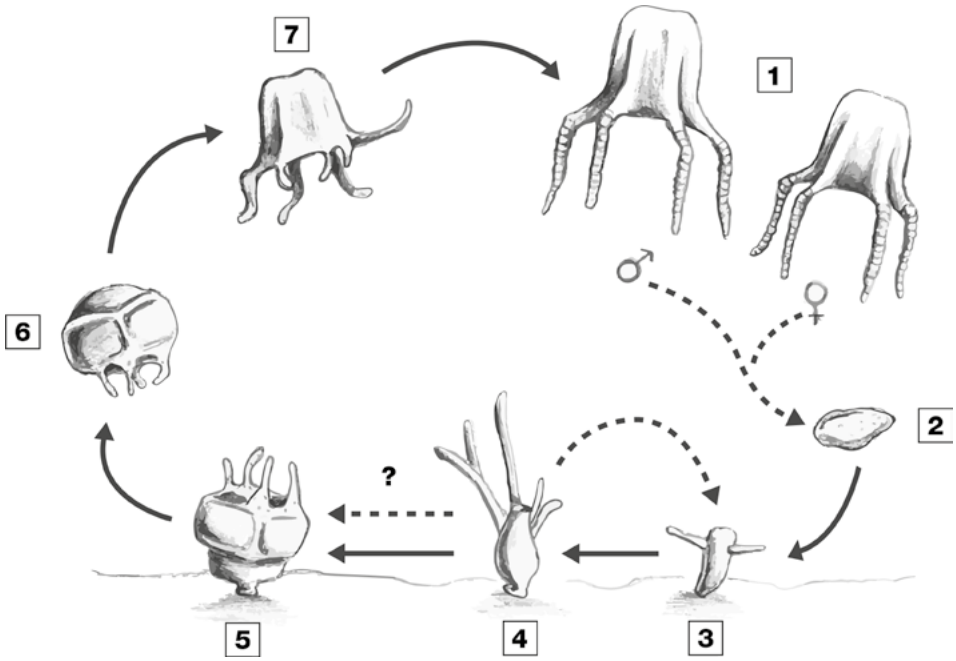

Figure 1. The life cycle of cubozoans. Dashed lines indicate reproduction and solid lines indicate development. Adult medusae (1) sexually reproduce to form larvae (2); larvae develop into sessile polyps (3) which can eventually (4) produce more polyps asexually; polyps give rise to individual juvenile medusae (5) which return to the pelagic zone (6) and develop into sexually mature medusae (7). Drawing by Eva Zaffarini.

According to the two criteria just offered, it should count as metamorphosis. The polyp-to-medusa transition in cubozoans does not involve reproduction because the polyp does not survive the process and it produces only one medusa. Reclassifying this transition as metamorphosis suggests a parallel reclassification of the stages: the polyp becomes a larva and the juvenile medusa becomes the adult, unlike the stages and generations of other cubozoans.

Is this sort of reclassification desirable, however? A major aim for any system of classification is to suggest reliable inferences by grouping things together that are genuinely similar. Is the cubozoan life cycle really more similar to the metamorphic life cycle of frogs and butterflies than it is to the metagenetic life cycle of other cnidarians? (see Minelli, 2011) The former grouping is more

comparable in certain functional respects, but the life cycles within the clade Cnidaria are more comparable *in every other respect*. This is because many of their life cycle traits are *homologous* in spite of some functional divergence.

The cubopolyp and cubomedusa are homologous to the polyp and medusa generations of other cnidarians because they almost certainly derive from the same generations in a shared common ancestor. The cubopolyp is like a larva and the cubomedusa like an adult in metamorphosing organisms, but they are homologous to adult polyps and juvenile medusae, respectively, in other cnidarians. We can even say that the polyp-to-medusa transition is homologous to the same transition in other cnidarians. Even though the former is functionally similar to metamorphosis, it is homologous as a generative process to asexual reproduction in Cnidaria. It sounds less strange to say there is a homology between a process of asexual reproduction and a transformation of a single, spatiotemporally unified organism once we recognize that similar developmental mechanisms are at work in both cases. In one cubozoan species (*Tripedalia cystophora*) the basal portion of the polyp sometimes survives, re-grows, and produces a subsequent medusa, revealing its historical relationship with other cnidarians (Collins 2002).

Metagenesis can be legitimately treated as a synapomorphy of Cnidaria (or at least of medusozoan Cnidaria). Classification based on homology is particularly useful in this clade given that many of the traits are developmentally simple and prone to homoplastic variation (Marques and Collins, 2004). Certain hydrozoan cnidarians have secondarily lost the polyp generation and others the medusae, with compensatory functional changes, but this arguably shouldn't shift the identity of the stages or generations and make them less comparable to other cnidarian polyps and medusae.

Metamorphosis versus asexual reproduction with parental death

We saw that metamorphosis can be functionally distinguished from asexual reproduction (in multicellular organisms) by the fact that asexual reproduction is potentially multiplicative and the parent survives the production of an offspring. But what if the parent generates only one offspring and dies immediately afterwards? From a functional perspective the two processes would be essentially the same. Should asexual reproduction be re-categorized as metamorphosis whenever this happens? The persistence of a parental corpse that can be distinguished from the offspring is not enough to differentiate the two cases. In the metamorphosis of many insects and marine invertebrates, only a small portion of the material constituting the larval stage goes to form the adult stage – the "set-aside cells." The remainder of the larval body that is not set aside is exactly analogous to a parental corpse.

In discussing this example, Godfrey-Smith (2009, p. 104) argues that it doesn't matter much whether we consider it metamorphosis or asexual reproduction because non-multiplicative reproduction will have no significant effect on Darwinian processes anyway. Although the last part seems largely right, having an effect on Darwinian processes is not the only way that life cycle classifications can matter. Classifications also pick out units of comparison that serve as bases for the formation and application of generalizations and theoretical models, such as models from life history theory, developmental evolution, and phylogenetic analysis. This role has been largely missed by biologists and philosophers who have examined problems of biological individuality solely from an evolutionary-functional perspective focused on current evolving populations (see DiFrisco 2018a).

In the present case, the same classification strategy as before can be deployed. Even if non-multiplicative asexual reproduction with immediate parental death is *analogous* to metamorphosis, it is not *homologous* to metamorphosis. It is homologous to asexual reproduction in whatever monophyletic group in which it occurs. In this case, homology of life cycle traits can prevent the misleading comparison with normal metamorphosis.

Metamorphosis and metagenesis in echinoderms

Echinoderm life cycles are typically described as monogenerational and metamorphic, unlike cnidarian life cycles, which are typically multigenerational, metamorphic, and metagenetic. In most cases of echinoderm development, a juvenile develops from stem cells in the larva and proceeds to absorb the larva before growing into an adult. Larva and juvenile echinoderms are therefore considered stages in the same developmental process. In the starfish *Luidia sarsi*, however, the larva is not absorbed into the juvenile that it produces, and can instead swim independently for a further three months (Williamson, 2006; Fusco and Minelli, in press). This example presents the opposite problem from the cubozoan example. Is the life cycle of *L. sarsi* actually metagenetic and multigenerational, with an asexual generation of larvae distinct from the juvenile generation, instead of being metamorphic and monogenerational like other members of its clade?

One reason why this case is strange is that, although metamorphic development often leaves behind detritus, generally what is left behind is not a living, functioning organism. The proposed functional criteria for distinguishing reproduction from development included the presence of an identifiable parent organism that survives the production of offspring, as well as the reproductive process's being potentially multiplicative. From a functional perspective,

then, the life cycle of *L. sarsi* is metagenetic, and in this respect is more similar to cnidarian than to echinoderm life cycles. But metagenesis in *L. sarsi* is not homologous to metagenesis in cnidarians, since there is substantial evidence that there is no unbroken continuity of metagenetic life cycles connecting them phylogenetically. Instead, the larva-to-juvenile transition in *L. sarsi* is homologous as a generative phase to the larva-to-juvenile stages of metamorphosis in echinoderms. Its larval stage is functionally like an adult, but it is homologous as a developmental stage to the larvae of other echinoderms and is more similar to them in every other respect.

Discussion: homology of life cycle traits

The preceding discussion of examples outlines a general classification strategy for dealing with variations that arise from functional divergence in life cycle traits. This strategy centrally depends on a distinction between homologous life cycle traits and analogous or homoplastic life cycle traits. It can be applied to many other developmental phenomena such as the indeterminate boundary between asexual reproduction and regeneration, polyembryony and subsequent fusion, pedogenesis or heterochrony and stage identity, and more. Although theorists tend to think of life cycle traits in terms of function, analogy, and homoplasy, the structural and historical criteria of homology thinking are often more informative for comparative purposes.

That being said, there is no need to choose one classification scheme as the uniquely correct one – i.e., the one that determines what metamorphosis *really* is – while rejecting the other scheme entirely. Biological traits have this dual aspect, and theorists are free to choose the classification scheme that is most conducive to their aims in specific cases. In general, one can expect that functional classification will be more conducive to investigation of current evolution and life history theory, whereas homology classification will be more conducive to comparative developmental biology and phylogenetic analysis. These dual aspects reflect the two main traditions in the evolutionary study of complex life cycles (Moran, 1994).

One reason why homology of life cycle traits is not already a well-established theoretical category is that life cycle traits seem to be quite different from paradigmatic exemplars of homology – i.e., morphological traits like bones and eyes, and biomolecules like genes and proteins. The life cycle traits we have considered are not material objects like body parts, but are instead events, processes, or temporal patterns. Further development of the proposal of this essay will have to explore how these sorts of traits fit into existing thinking about homology. I close by sketching a few points in this direction.

Established criteria of homology include descriptive similarity (Owen, 1843), similarity in topological position (ibid.), congruence (Remane, 1956; Patterson, 1982), and similarity or sameness in developmental mechanisms producing the trait (Roth, 1984; Wagner, 1989). Topological position can plausibly be replaced with the temporal position of the trait within its life cycle. In this case heterochrony would be a problem, just as ectopic expression is problematic for the criterion of spatial position (Minelli 1998). Similarity in developmental mechanisms may be useful for parsing homologies between processes like asexual reproduction, embryogenesis, and regeneration, and may also serve as the basis for distinguishing *parallelism* from homology and homoplasy. Life cycle traits are already used in congruence tests in cladistics (Collins, 2002; Marques and Collins, 2004).

Generally, homology of life cycle traits is well-suited to phylogenetic or cladistic approaches to homology. Unlike developmental and morphological approaches, here there are usually no strong restrictions on what sorts of traits can be homologized (DiFrisco, 2018b). Homology of life cycle traits also fits naturally into Hennig's semaphoront view of homology (Hennig, 1966; Havstad *et al.*, 2015). Hennig thought of semaphoronts, or phases of life cycles, as the primary bearers of homologous traits. Organisms, which are normally assigned this role, were re-conceptualized as a taxonomic category lower than species, comprising *groups* of semaphoronts. But Hennig's view can be pushed one step further: semaphoronts and their temporal patterns can themselves be homologous traits whose bearers are 4-dimensional life cycles.

Acknowledgments

Thanks to Johannes Jaeger and Giuseppe Fusco for insightful comments. Many of the examples discussed in this essay derive from my reading of Fusco and Minelli's (in press) book on the biology of reproduction. I gratefully acknowledge funding support from the Research Fund – Flanders (FWO) and the Konrad Lorenz Institute for Evolution and Cognition Research.

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Objects or processes? Theoretical terms or frame-concepts? Coupled changes in the life sciences and in their epistemology

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Abstract

The awareness of a new congruence between two fields that have so far been regarded as separated, namely ontology and epistemology, is increasing in the life sciences and needs to be explored in the light of the fact that both fields are time-dependent. The pivotal role of historical constraints invests any reasoning on the living world and, in parallel, the epistemological tools for dealing with it. That role consists in highlighting a double link between objects or processes, on the reality side, and the primacy of syntax or semantics, on the epistemic side. Many criteria of knowledge change according to contemporary evolutionary theories, and consequently their feed-back on our self-perception affects our relationship with the explanatory criteria of scientific knowledge.

Stylistic resonances between ontology and epistemology

The separation between the ontological level, i.e. the level of reality, and the epistemological level, i.e. the level of explanation, should not allow, as a rule, any interference. This is a standard criterion needed for the cognitive hygiene in traditional philosophy of science. One should never let that the forms of reality of the world interfere with the systems of knowledge; in other words, the 'state of things', i.e. the *explanandum* of nature, has to be kept separate from the narrative and experimental logics aimed at explaining it, i.e. the *explanans*.

However, in the effort itself of identifying reality, of establishing which functions matter, of deciding whether we look for *entities* (hence the etymology of '*onto*logy') through the regularities of their structures or their different functions, or for *processes* which are more or less unpredictable, some perspectives are implicitly introduced. We 'cut' reality in a way that is already cognitively denoted. The reality as such does not tell anything *to us*. The mere reality cannot

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ever be an *explanandum* as such: reality can only *become* an *explanandum*. And this depends on the perspectives of our questions.

Today, moreover, other and more specific contaminations crack the demarcation rule between reality and *episteme*. The criteria of knowledge, the categories themselves through which we think, change according to the developments of the life sciences and their consequent feed-back on our self-perception: the increase of knowledge produced by evolutionary biology, ecology, epigenetics, neuroscience, affects the understanding of our relationship with the world, the knowledge of the ways in which we know and, as a consequence, the explanatory criteria of scientific knowledge.

Every disciplinary context sets the bounds of its theoretical assumptions, but, well in advance, any knowledge, even the scientific one, depends on another 'hidden depth': the remote cognitive presuppositions of the species to which we belong. These presuppositions have probably been developed starting from our cognitive and perceptive, but also 'enactive'[1], relationship with the world. If we are aware of that, this is due to the research in evolutionary palaeoanthropology, ethology and neuroscience (Kiverstein and Clark, 2009; Berthoz and Debru, 2015). The acquisitions that derive from the whole of the life sciences and concern our knowing and acting in the world, cannot but influence, as a consequence, the epistemic worlds by naturalizing them (Gontier *et al.*, 2006; Sassaroli, 2008; Cellucci, 2013; Sterpetti, 2015).

It is thus at work a *connective* circularity: a given *explanandum*, as for instance the brain-body system, with its interactive processes ingrained in the world, allows us to rethink the abstractness of the instruments of its *explanans* and influence the models of knowledge thanks to the increasing amount of neurobiological information. Our knowledge of the mental functioning, its ontogeny, its phylogeny and the selective role played by active protocultural choices, is growing (Tommasello, 1999; Nöe, 2009; Laland and O'Brien, 2011).

Darwinian evolutionism, which has been furtherly confirmed, corroborated and broadened by current biology and ecology (Pigliucci and Müller, 2010), *recursively* comes into play as a theoretical and explanatory model, to shape

[1] According to 'philosophical enactivism', cognitive structures emerge from the recurrent sensorimotor dynamics of an embodied agent who is embedded in a natural environment, and, beyond the Kantian apriorism, the 'meaningful' mind is regarded as 'extended' and embodied (Huttoand and Myin, 2013). From Maturana and Varela (Varela et al., 1991) to the sensorimotor contingency (O'Regan and Noë, 2001; Noë, 2009), the experience depends on what the subject does or knows to do, in a particular natural environment, and on the interactions that such a world of actions establishes with that environment. Knowledge is embodied in the sensorimotor dynamics and in the environment, and consciousness, between the external constraints and the internal generated activity, emerges from the interaction between brain, body and natural environment. This mutual co-determination, that is embodied and rooted in the context, distinguishes the 'enactive' point of view both from constructivism and biological Neo-Kantianism.

novel interpretations about the relationship between the world and our species, and thus about our knowledge, including scientific knowledge, as in the case of naturalized epistemologies[2].

The awareness of what biology discloses about the functions of ourselves, as biological objects and, at the same time, subjects, determines some transformations in the standard distinction between the tools of the explanation and the phenomena to be explained. Moreover, this indirectly undermines also the standard distinction between the *descriptive metaphysics*, which refers to how we spontaneously represent the world, and the *prescriptive metaphysics*, which is based on the recognition of a structure of the world independent of our representation.

According to this perspective, if we are on a metalevel, or better on the viable boundary between the ontological and the epistemic field, we are better able to grasp resonances or symmetries between the two fields. Stylistic similarities arise between the frames with which the meanings of the living world are pointed out, and the frames of the tools and languages used to explain them: methods and theories. A sort of congruence, i.e. a 'stylistic' coupling (Hacking, 2017), can be grasped and drawn between the portion of reality we selected, i.e. 'where' one goes to look, and 'how', instrumentally and operationally, the research works.

Let us try to keep together these two moments in a well-known scenario. The isolation of the phenomena and the identification of the invariant elements have been identified as the 'substance view' (Waddington, 1977; Fabris, 2016; Nicholson and Dupré, 2018). This perspective belongs to any discourse on the 'entities' of ontology. On the epistemic level we can match this 'atomizing' structure with the scientific explanatory languages of logical neo-positivism, with its worlds of formalization and invariance of meanings. Grasping the real as a sum of *entities*, *objects* and their *functions*, i.e. the 'thingness' of the objects on the ontic level, matches with a methodological view based on an atomized and compositional approach to the language. Moreover, on one side, the isolated and natural datum, which is classified and apt to be manipulated, becomes the experimental 'fact' (Fleck, 1979), according to a line of research which focuses on entities and functions, whose variables can be subjected to a separate control. On the other side, we find, on a formal level, the lexicons that fit with links of theoretical *statements* that are detached and aligned by implicative and

[2] Evolutionist or naturalized epistemologies have been originally developed by Quine in the 1970s (Quine 1995) and have been successively expanded by the ethological researches of Konrad Lorenz, Thomas Campbell and the epistemology of Karl Popper. Now, those epistemologies have spread as a multi-functional model that connects researches on knowledge – as a biological product – with the cognitive dynamics of scientific knowledge.

connective elements, belonging to a set of *formalizations*. That is an algorithmic ideal that focuses on the rigor of a compositional syntax.

Contrary to that, if we change the style of thought, we will observe other congruencies (Buiatti and Longo, 2013). If on the level of reality the attention is addressed to the historical processes, the complex systems and the entanglement of phenomena, then the explanatory method makes use of linguistic, observational and modelling tools, which can account for the developments and flexibility of such phenomena. Thus, a different kind of congruence between the *episteme* and the reality looms.

Where the contingencies and the transformative processes of the living matter come into play, entities become instantaneous fragments of a continuous flux, i.e. a sort of moments of still image. This different focus seems to go hand in hand with a different epistemic style: an attention to different forms of scientific language in which not as much the syntax, but rather the meaning becomes essential. So, the nomological method can be compared with the narrative and abductive method, and with the primacy of the heuristic models that sometimes take the place of the theories (Gayon, 2005; Mayr, 2005; Minelli, 2009).

The unchanging accuracy of the theoretical terms is replaced by the historical depth of 'influential concepts' (Frezza and Gagliasso, 2014) and 'constitutive metaphors' (Black, 1962; Hesse, 1966; Ortony, 1993). At the same time, a *hybridization* between thematic fields in transformation is at work by connecting systemic and historical criteria to each other. Many influential concepts derive from metaphors, that are stratified over time, and their past history gives thickness to their current use. It follows that the scientific and linguistic tools for defining, explaining and demonstrating, show to be pervaded by an intrinsic historicity (Canguilhem, 1955, 1968) that cannot be ignored by us.

Now, let us consider how the perspective of such double bind transforms the landscape of biology, corroborates the evolutionary naturalizations and broaden the networks of connective relationships between the level of the reality and the one of the explanation.

Processes constrained over time

The intrinsic 'thingness' of the invariants, which characterizes classical physics and mathematics, has been both a premise and a goal in biology for a long time: from the phenomenal and morphological invariances in the taxonomic criterion, to the regularity of the organic functioning, to repetition of the phases in the development of organisms, as well as to the identification of invariance as the

fundamental key to the 'code' of DNA[3]. On the other hand, as regards the method, combinations based on invariant formal and syntactic entities have been the basis of the explanations as predictions. The identification of such traits has allowed us for the construction of predictive (or probabilistic) explanations based on the linear repetition of the identical or the similar in the future.

On this view, the time of the transformative processes has been unessential for a long time, and its intrinsic contingency has been ruled out in order to obtain structures, bodies, substances and systems, which have been grasped in their immanence or in their controlled dynamics.

Although this picture has already been questioned by evolutionism for two centuries, there is still room for the contrast between an immanent view and a process view, i.e. between entities and processes.

But entities, structures, and processes are actually less mutually exclusive than they appeared in the past. So, we can develop interesting connective links on the ontic level and then on the epistemic one.

Indeed, if the world of the invariant entities ('substance view') and that of the processes ('process view') (Fabris, 2016; Nicholson and Dupré, 2018) focus on two different characteristics of reality, a sort of bridge between the two can be found in the role of the *structural constraints* (Gagliasso, 2009).

The thresholds of immutability, both morphological and developmental, as well as those of DNA, show the body planes of the various taxa (*Bauplane*). But any organic structure which is repeated over time – since it is no longer explained as an expression of fixism but as an evolutionary product – incorporates and sediments a past of remote biological processes: the history.

Thus, a structural conformation which steadily occurs, is not the expression of an invariant determinism, as in the case of crystals. Even when the phenomenal appearance is as such, a recurring conformation is indeed a historical constraint tightly knotted in the past because of the action of natural selection and other evolutionary drives. Examples of that are the 'frozen genes', the symbiotic origin of mitochondria and chloroplasts, the exchanges of plasmids between bacteria, the morphogenetic *bauplane*, with their bilaterally symmetric or radial structures, and the metabolic functions produced in remote and fixed phases too.

The picture *results* to be determinist. But if by 'determinism' we mean, not an invariant synchrony, but the result of a historical flux (Nicholson and Dupré, 2018), we can see the entanglement of precise constraints in the deep remote time.

[3] From morphological and embryological aberrations, to genetic mutations, all these phenomena have been regarded as 'errors' in code copying for a long time, a sort of deviations from the norm, as background 'noise'.

Those are the constraints of 'phylogenetic inertia' (Gould, 2002), which express the marks of the ancestral organisms in the current one and now are unmodifiable; or developmental constraints, which are immune to further evolutionary mutations and canalize specific phases of embryonic development: ancestral gene networks that are hierarchically integrated (Minelli, 2009; Buiatti, 2013).

Since the 1980s these two types of constraints, phylogenetic and ontogenetic, have been placed in relation with each other (Gould, 1977) and also with their environments, but only today this interdependence has become the driving force of a specific research field that brings together the evolution of species and that of the development of individuals: the *Eco-Evo-Devo Theory* (Maienschein and Laubicher, 2007; Samson and Brandon, 2007; Minelli, 2009; Pigliucci and Müller, 2010; Gilbert, 2010; Minelli and Pradeu, 2014). Before the phylogenetic and the developmental constraints, there are the more fundamental 'architectural constraints'. These constraints have already been pointed out in the 1970s together with their anti-adaptationist function (Gould and Lewontin, 1979). Here we are dealing with the 'necessities' imposed by the state of matter and the energy of the planet, and with the 'materials' of which all living beings are made: constraints of organic compatibility with the static and dynamic conditions of the medium in which the bodies, the cells, the apparatus are immersed (atmospheric pressure, gravity, viscosity, gaseousness, etc.).

There is a style of thinking that allows us to make a link between entities and processes, or between scarcely modifiable structures and their genesis. It consists in connecting and grasping in their conjunction over time the passive constraints of phylogenetic kind (the stratified history of species and their remote and selected structures), those of ontogenetic sort (the 'immutability' of some parts of genome as the frozen genes), those of architectural kind (constructive materials and physical-chemical constants that govern the possibilities of living matter on the planet).

So, while entities and processes become categories that are no longer opposed, even two traditionally antagonistic lines of research, both strictly deterministic, undergo some transformations. On one side, we can observe the structural biology (sensitive to the conformational invariance of morphologies and based on a geometric-topological view; D'Arcy Thompson, 1917; Webster and Goodwin, 1988); on the other side, the informational determinism of first molecular genetics (with its predictive invariance contained in the coded program of DNA).

Structures, entities, as historical micro and macro products have therefore a selected and historical basis, in the light of which the genome is much more than a code (Buiatti and Longo, 2013).

Metaphors, models and the history inside concepts

This explanatory twist at the level of reality transforms *structural invariants* into *products of remote evolutionary constraints*, crystalized in deep time, seems to have an epistemic parallelism of some sort. We have already seen, indeed, that emphasizing invariant formal structures, composed of statements and algorithms, and indifferent to their meanings, represents the epistemology as nomological approach – and an ontology based on 'entities' ('substance view') seems to fit well with this approach.

On this perspective, one cannot but notice that the ontological plan (facts, substances, entities, etc.) and the epistemological one (statements and their connective rules), although distinct, imply each other.

But, if we focus our attention on the *meanings* of the concepts of the biological lexicon, another interpretative scenario opens up. Those meanings, indeed, reveal their role as 'epistemic accesses' to problems and the hidden presence of their historical stratification comes to light.

So, when theoretical concepts and terms are not only understood as invariant statements on the syntactic level, but also as stratified products of the path of scientific thought, even their constitutive process becomes accessible. From different epistemic and historical perspectives, theorists of biology and historians of ideas depicted the role of concepts in biology as a crucial one (Canguilhem, 1955, 1968; Gayon, 2005; Mayr, 2005), whereas epistemologists and semiologists investigated the methodological elasticity of concepts as products of *constitutive metaphors* (Hesse, 1966; Black, 1983; Ortony, 1993).

Constitutive metaphors in science connect different fields and linguistic elements of widespread culture and common sense. Constitutive metaphors by their use, circulation and crystallization, can be transformed into theoretical terms, can contribute to interpretative (or simulative) models, and produce influential concepts, for a privileged access to specific properties and dynamics of the life. So, the primacy of concepts and models in biology is far more heuristic than laws or, at least, it relativizes them (Mayr, 2005).

Moreover, systems of explanation, that better fit with a biology of processes, easily bring together a narrative methodology with a semantics of metaphorical languages and the heuristic of models. Concepts produced by constitutive metaphors, capture in their current meaning the memory of their genealogical course. So, the epistemic access occurs through these cognitive and heuristic tools containing thick cultural apparatus, views of the world and implicit ideologies (Kincaid *et al.*, 2007): their use, on one side, corroborates them, but on the other, often conceals their implicit ideologies (Frezza and Gagliasso, 2014). Thanks to metaphors and heuristic models, we can focus on processes but also

on patterns of mutations, migrations, genetic drifts, levels of selective units, kinds of cell divisions. These explanatory tools are part of a continuum that links influential concepts, models and thematic fields in the light of their methodological flexibility rather than their nomology. Explanatory tools are useful for the reconstruction of the phylogenetic past of the relationship between organisms and environments, and for many description and explanation of morphological and developmental regularities.

Moreover, it seems that adopting different research styles (Hacking, 2017), which are produced by different accesses on the same thematic field and aimed at interrogating the same biological phenomenon, can offer unpredictable cross-covering integrations that work as explanatory corroboration. So, such a 'multilingualism' is increasingly one of the most interesting features of the methodological specificity of the sciences of the living world.

At the end of this comparison we can observe a particular form of double changes. This is the *double bind* in biology between objects or processes, on the reality side, and, between the primacy of syntax (logical statements, algorithms, etc.) or the primacy of semantics (constitutive metaphors, historical frames and models), on the epistemic side. Moreover, the splitting between two classical separate fields, as ontology and epistemology, is changing too. A new congruence needs to be explored in respect to time-dependence, because of the pivotal role of historical constraints in reality and even in conceptual instruments. Finally, from the basis of bio-evolutionary research, an interesting feedback is provided and it affects the forms of human knowledge, with significant consequences on the basic system of epistemology in its broadest sense too.

Acknowledgments

I am honoured to dedicate this article with increasing friendship and admiration to Alessandro Minelli, inspired by his new perspectives on the dynamics of living forms and by his theoretical reflections in contemporary biology.

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Categories of developmental biology: Examples of ambiguities and how to deal with them

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Abstract

Patterns of post-embryonic development are usually roughly categorised into only two different groups. Despite the fact that the literature is full of some rather different phrases to describe post-embryonic patterns of development, many of these are treated as synonyms or at least as roughly equivalent, resulting in the two groups mentioned above. *Group 1* unites direct development, hatching as small adult, absence of larvae, absence of metamorphosis and gradual development. *Group 2* comprises indirect development, presence of larvae, metamorphosis and saltatory development. I outline here that many of these terms are in fact non-synonymous and should be used more carefully to avoid misunderstandings.

Background

We communicate via words. Yet, words can be tricky units of communication as some people may use certain words in a different way than other people do. This phenomenon causes ambiguities when we communicate, or, even worse, it may cause misunderstandings. Therefore, it is worthwhile to take some time and think about the meaning of certain words. Here I want to think about some words frequently used in developmental biology, terms that we find in many textbooks and papers, and what they might mean to some people, but not to others.

This contribution is inspired by a paper by Minelli *et al.* (2006), in which the authors discuss the ambiguities of the "standard" marker points during ontogeny of an animal, the moment of hatching and the reaching of adulthood. As the authors have lain out, both marker points are much more fuzzy than most of us tend to think. Depending on the system in focus they might mean quite different states.

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Here I want to talk about some other general terms that are frequently used to describe and differentiate patterns of post-embryonic development, such as: direct and indirect development, metamorphic development, gradual development, larval development and the like. I do not attempt to provide new and "better" definitions of these terms. In general, I personally do not like the concept of "definition". This may be related to my mother tongue (German) or to my interest in mathematics (e.g. Haug and Haug, 2015, 2016), but my understanding of "defining" is that it can 1) in principle be independent of the real world, and 2) provide the impression to be the "correct" use, not to say "the only correct" use.

Concerning point 1, there are examples in mathematics in which I can define mathematical sets that are empty sets. Such sets may make sense in a mathematical context. Yet, if I define a term so that it has no representation in the real world, it is in my view not immediately appropriate for a scientific discipline such as biology which attempts to describe what occurs in nature in order to entangle the processes behind it. To immediately row back a bit, it can be a benefit if we hypothesise a certain category and recognise that it is not existing in the real world and then start asking why this is the case. Still, in my view the starting point for categorisation should be observation.

Concerning point 2, I do believe there is no "correct use" of a word and also not the "correct word" for something. All I care about is about reducing ambiguities. I do not want to be misunderstood or mislead anyone, but I also do not want to misunderstand anybody. My standard example, of course German-flavoured: if an author insists on addressing a structure (or process) as Günther (a common German first name) I am OK with it as long as he outlines properly what he means by that term, so that I can understand him precisely. Hence I am interested in how others use terms and what they mean with them.

And here comes the tricky part: it is sometimes very complicated to extract the exact meaning of a term used by a certain author. I can only outline what my impression is of how certain terms are used and also what I understand by these terms.

The main problem: pretended synonyms

As shortly outlined above, there is quite a number of different terms to describe different patterns of post-embryonic development of animals. Still, somehow all these words are in my view generally sorted into only two different *boxes*, making many of the different terms seem like synonyms. The two-boxes phenomenon can be seen in many older, typologically driven categorisation schemes.

So if I think that most authors would distinguish only two types of post-embryonic developmental patterns, why are there so many different terms in use?

I think indeed that the terms do have rather different meanings, but it has become a kind of habit to use them as synonyms. This touches another problem I see with the use of synonyms in general. As stated, I am interested in an unambiguous way of expression, but the use of synonyms makes this in fact more difficult. In language classes, at least in German schools, teachers call for the use of synonyms to prevent terms from re-occurring too often. Yet, I believe that urgently trying to use synonyms leads to the effect that terms are treated as synonyms that are effectively not synonymous. This is often not helpful or a sign of good style, but rather a bad habit. I indeed think that most of the seemingly synonymous terms used in developmental biology have in fact quite different meanings. I discuss the details in the following.

Box 1. The first group of terms used as synonymous by some authors includes the following terms: *Direct development* is usually seen as synonymous with 'having no larval stages', but also 'lacking metamorphosis' and sometimes also 'hatching as small adults'. Developmental patterns that lack metamorphosis are furthermore sometimes addressed as gradual development. While not all of the terms are directly used as synonyms, one can easily see the "chain" that connects all these.

Box 2. The second group should include all the opposites, hence: *Indirect development* is usually seen as synonymous with 'having larval stages' and also developing through a metamorphosis. More rarely used is the term 'saltatory development' (as opposing to 'gradual development') and can indeed be found in coupling to metamorphosis.

The case is, of course, much more complicated as many authors will not agree what a larva is or how we should call the immature stages of animals that develop according to Box 1. While the term 'juvenile' is frequently used, the term is also used for animals developing according to Box 2 that already went through metamorphosis but have not yet reached adulthood (e.g., *Glossary* in Martin *et al.*, 2014). Finally, sometimes the term juvenile is used as a kind of umbrella term including larva as a subcategory. I have dealt with the term larva elsewhere (Haug, in press-a) and also with the different evolutionary processes that lead to metamorphosis (Haug, in press-b). So I will concentrate mostly on the other terms.

The approach

I will outline which meaning is in my view implied by certain terms, or better phrases, and how this differs from other terms/phrases that have been used as synonyms. Everybody is welcome to disagree with me, I just outline how I interpret certain terms. This can act as reference point for anybody reading

contributions by me, but hopefully it may also motivate others think about how they understand and use certain terms. For illustrating my interpretations of the terms, I will use a graphical aid, with relative developmental time on the x-axis and the morphology on the y-axis. The y-axis is, in principle, a projection of a multidimensional morphospace that is corrected for body size.

Hatching as small adult

This term describes a pattern where the hatchling occupies the exact same position in morphospace as its corresponding adult. The phrase has been used to address the development of "ametabolous" insects (for ambiguities of this expression, see Haug *et al.*, 2015) or peracaridan crustaceans such as amphipodans (e.g., Wolff, 2014). It seems that the idea of hatchlings being identical to but smaller than the adult is only a matter of the degree of viewed details. Even in species that do indeed not add or reduce certain structures we will very likely see allometric changes. This is not only true for the arthropod examples above (Krapp *et al.*, 2006; Haug *et al.*, 2015), but also for tetrapods including mammals (e.g., Alexander *et al.*, 1979), and even aceolomorphan worms (Semmler *et al.*, 2010). It may turn out that there is no example of a metazoan organisms in which the hatchling is indeed a miniaturised adult, if we just look close enough.

Yet, if we compare two species we can, of course, recognise the relative similarities. A silverfish hatchling (to go back to the "ametabolous" insect example) is much more similar to its adult form than what the hatchling of a butterfly, i.e. a caterpillar, is to its adult. Haug and Haug (2013) have discussed that we will most likely be unable to find a distinct threshold for the term metamorphosis, but only be able to tell via comparison which developmental pattern is more metamorphic. The same seems to apply here: the absolute case might not exist at all, but in a comparative way the expression might still be useful. This might well also apply to all the other terms and phrases that follow.

Direct/indirect development

It makes most sense to discuss these two terms as a pair, as they are used to address two opposites. Concerning synonymy, direct development has been considered to be identical to 'hatching as small adult' (e.g., Rabalais and Gore, 1985, and references therein; Hanken, 2007; Arenas-Mena, 2010). To my understanding of the phrase this is incorrect. 'Hatching as small adult' addresses clearly cases in which we have no (or more likely only few) changes of morphology, hence a stable position within the same location in the morphospace during the entire post-embryonic ontogeny. Direct development in my view indicates

changes in morphology, i.e., changes in the position in the morphospace over time, and is therefore distinctly different from 'hatching as small adult'. Direct development describes in my view that there is a change and makes a statement about the process of this change.

Concerning morphospace, development can be best understood as a line in morphospace between hatching and adulthood. However, at this point one has to be aware that hatching does not mean the same developmental condition in different animals, as, for instance, Minelli *et al.* (2006) demonstrated. For simplicity, I assume that the hatching event is further towards the bottom in morphospace and the reaching of adulthood further to the top. If the connecting line between these two points does not drop below the morphospace of the larva and rise above that of the adult, i.e. remains within the morphospace delineated by the two, it should be considered as direct development.

This could, yet, also be true for developmental patterns that involve stages that are generally considered as larvae, and larval development has usually been used as synonymous to indirect development. I have argued elsewhere (Haug *et al.*, in press-b) that indirect development could be understood in an evolutionary view, when an immature stage evolves new characters (one way to evolve a larva). Yet, concerning morphospace such cases may still be considered direct.

There are in fact cases in which the developmental curve leaves the area of the morphospace delineated by hatchling and adult. The hatching larvae of mantis lacewings are mobile organisms roughly comparable to the larvae of ladybugs (campodeiform larva). Concerning body organisation and legs they are already reminiscent to their adults (e.g., Redborg and MacLeod, 1985; Hoffmann and Brushwein, 1992; Ohl, 2011). Yet, the second stage larva that is often immobile and appears grub-like, is much less similar to the adult and clearly occupying another zone of the morphospace (e.g., Redborg and MacLeod, 1985; Hoffmann and Brushwein, 1992; Ohl, 2011). Such a developmental pattern could indeed be described as indirect. Comparable cases occur in other insects with so called hypermetabolous development, e.g., in strepsipterans or certain beetles (e.g., Pinto, 2009; Bologna and Di Giulio, 2011).

Yet, this way of presentation is, of course, an oversimplification. As pointed out above, my morphospace example is a multi-dimensional space projected into a single dimension. When considering the entire multidimensional space, every deviation from a straight direct connection of the two points (hatching and reaching of adulthood) could be considered as indirect. Yet, in such a case we would most likely face the problem that there is simply no example of a totally straight direct connection and all developmental patterns would need to be considered to be indirect.

In summary, it seems wise to specify a bit more precisely what direct versus indirect development should mean, either in an evolutionary way or as morphogenetic pattern. Similarly to the suggestion concerning metamorphosis (Haug and Haug, 2013), it seems most precise to not understand the phrases as absolute cases but rather in a comparative way, i.e. 'the developmental pattern for species x is more indirect than that of species y'.

Furthermore important: as outlined, direct development does neither have to be equivalent to 'hatching as small adult' nor to 'development without larvae'. Indirect development is not necessarily equivalent to 'developmental pattern involving larval stages'. Probably, all patterns of indirect development include stages that are generally considered as larvae, but not all developmental patterns comprising larvae have to be indirect. Yet, it seems that all cases of hypermetabolous development known so far may be considered indirect development.

Gradual/saltatory development

These two terms are often involved in discussions about metamorphosis, with saltatory development used as synonym to metamorphic development, and gradual development as opposing the two. As shown above, indirect development and metamorphosis are usually considered as being synonymous, which would indicate that indirect development is also saltatory, and direct development is gradual. Yet, this also does not seem to be necessarily the case (see below).

As for metamorphosis, the question where the threshold between gradual and saltatory lies is most likely impossible to identify and should be better approached in a comparative way (see above). Metamorphic development could indeed represent an equivalent to saltatory development, with being gradual as the opposing condition. Yet, indirect and direct development indicate different aspects of the developmental pattern and are not necessarily correlated to gradual or saltatory development. Direct/indirect development addresses the area of morphospace occupied throughout post-embryonic development, while gradual/saltatory development addresses how fast changes in the morphospace occur.

Summarising

I strongly argue for a system quite different from the two-box system above. It is more of a partly encaptic system (umbrella categories including further differentiated sub-categories, all based on inclusive criteria not on exclusive criteria; comparable to the concept of monophyly ["monophylly s.str.", "holophy-

ly"] in phylogenetic systeamtics), and it is always embedded in a comparative way of thinking. On a first level, there is a distinction between developmental patterns in which no post-embryonic change occurs (hatching as small adult) on the one hand, and patterns in which developmental change occurs on the other hand (Fig. 1). In a comparative framework this means nothing less than that there are patterns in which we have more changes and those in which we have less changes.

On a second level, i.e. within the second first level category, the ways in which changes occur can be described. This is outlined by a two-dimensional frame with four corners (Fig. 1):

1) In the upper left corner, we have developmental patterns that are more gradual and direct than others. Yet, they may still include stages that are generally considered larvae. The development of the brine shrimp *Artemia salina* is an example for such a case: its development is much more gradual and direct than that of many other crustaceans, still its early post-embryonic stages are generally accepted as being larvae (e.g., Olesen, 2014).
2) In the upper right corner, we have developmental patterns that are also direct, but more saltatory. An example is the development of extant polyneopteran insects. They develop their structures in a very direct way compared to holometabolous insects. Yet, due to evolutionary changes the development of the wings is very much postponed and then occurs in a more saltatory process compared to early representatives of the lineage (Haug *et al.*, 2016).
3) In the lower left corner, we have indirect, but gradual development. An example is the development of holothurian echinoderms. As in many other echinoderms, their development is more indirect than that of their closer relatives, such as hemichordates. After their stage as auricularia-type larva (roughly equivalent to the tornaria in hemichordates) they transform into a doliolaria, a stage absent in hemichordates (indirect in evolutionary frame), which also lacks a functional mouth (indirect in morphospace aspect) (e.g., Lacalli and West, 2000). Yet, the overall transformation, also further into the final adult form, occurs much more gradually than in other echinoderms.
4) In the lower right corner, we have saltatory indirect patterns. As already lain out hypermetabolous insects are an ideal example. As holometabolous insects they are more saltatory in development compared to many other arthropods; with their new specialised larvae they are more indirect (evolutionary and morphospace frame) than other holometabolous insects. Applying this frame would reduce ambiguities in communicating differences in developmental patterns tremendously. Yet, the most important mes-

sage still remains that we should stop searching for seemingly synonymous terms to improve the language style. If we would use a single well-outlined expression for the same aspect to be described, discussion as outlined here may become unnecessary.

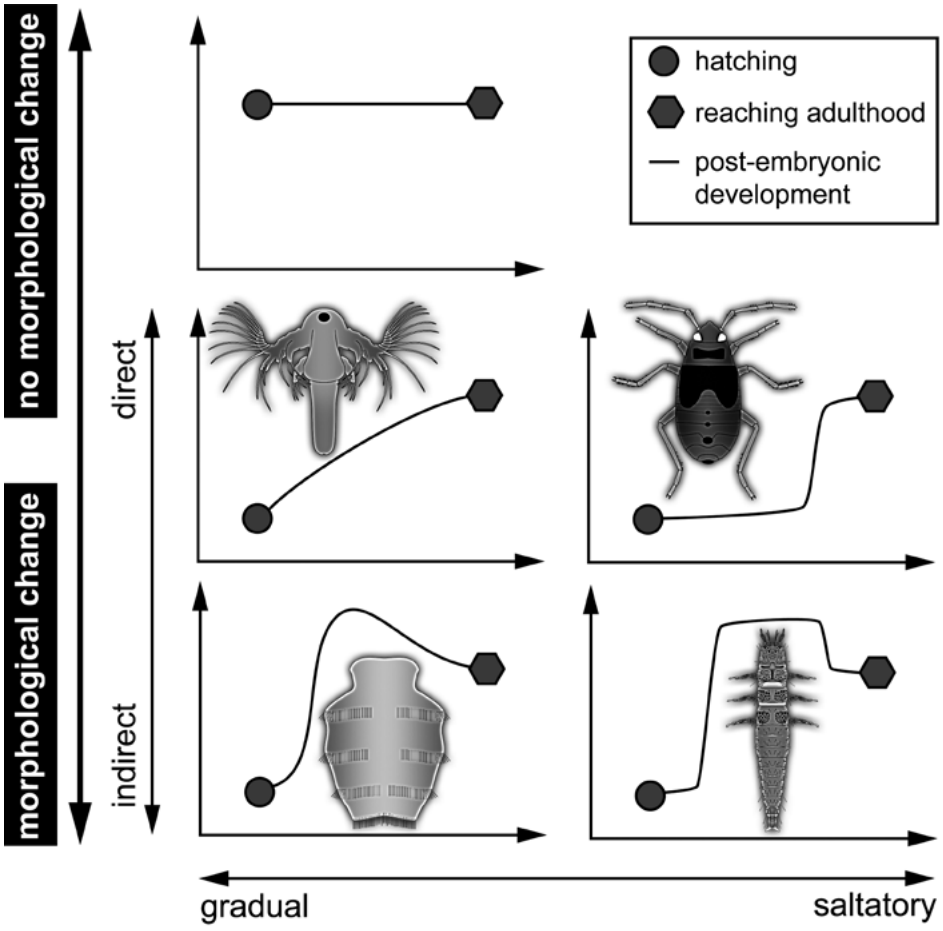

Figure 1. Different developmental patterns illustrated as changes in morphospace. In all graphs, the x-axis represents developmental time, the y-axis represents the morphospace (corrected for body size). Top: no morphological change during development ('hatching as small adult'). Bottom: four different types of developmental patterns in which morphological change occurs; upper left: direct and gradual development, example: nauplius larva of *Artemia salina*; upper right: direct and saltatory development, example: fifth nymphal stage of a firebug; lower left: indirect and gradual development, example: doliolaria larva of holothurian echinoderm, simplified from Lacalli and West (2000); lower right: indirect and saltatory development, example: first stage larva of the mantis lacewing *Mantispa pulchella*, simplified from Redborg and MacLeod (1985).

Acknowledgments

I am kindly supported by the Volkswagen Foundation. I am thankful to many colleagues for inspiring discussions.

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Evolving understanding of trilobite development: Recapitulation to adaptationism

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Abstract

The rich record of trilobite ontogeny offers the potential to examine the relationship between ontogeny and phylogeny in an ancient arthropod clade. Here I argue that the advent of population genetics and its effects on evolutionary biology changed the way in which palaeontologists viewed the record of trilobite ontogeny, reorienting it from a central role in the search for evolutionary processes toward a more rigorous but more circumscribed charge within an evolutionary biology then focused on adaptation.

Introduction

Trilobite exoskeletons likely became biomineralized shortly after hatching accounting for the unusually good record of development of this exclusively Palaeozoic arthropod group. "Unusually good" should be viewed in context: juvenile growth stages are known only for several hundred of the 20,000+ trilobite species described to date, and many are known from few specimens representing fewer stages. The great majority of species are known only in their mature form. This bias toward maturity is mostly because smaller specimens were fragile and therefore rarely preserved. Where immature growth stages are preserved, the original exoskeleton has generally dissolved and, on occasion, been replaced by another mineral. The fidelity of such replacement can be extremely high, but such specimens are almost always disarticulated, meaning they comprise isolated sclerites (component pieces of the exoskeleton) separated from other parts of the same carcass or exuvium.

Despite these drawbacks, the ontogenetic record of trilobites can, in certain cases, be of exceptional fidelity. Outstanding examples include the famous upper Cambrian *orsten* deposits in which articulated, 3D, immature exoskele-

G. Fusco (ed.) *Perspectives on Evolutionary and Developmental Biology.*

tons of putative trilobites are preserved along with casts of their limbs (Müller and Walossek, 1987). Faithful replacement of trilobite sclerites in silica has contributed enormously both to the resolution of higher level phylogenetic relationships among trilobites (e.g., Fortey and Chatterton, 1988; Chatterton *et al.,* 1990, 1999; Adrain, 2011), and in assessing aspects of developmental control in some species of the group (e.g., Webster and Zelditch, 2011a,b). Thousands of co-occurring articulated exoskeletons spanning a wide range of developmental stages have allowed for detailed exploration of the control of trilobite trunk segment generation and growth (Fusco *et al.,* 2004, 2014). Together, these varied cases of unusually favourable preservation permit a variety of insights into the development of a clade extinct for over 250 million years. This prospectus offers a brief summary of how earlier knowledge of trilobite development itself evolved, and the relationship between this and the development of evolutionary biology. The subject matter is rich and will merit more comprehensive review, especially in the light of the recent interest in "paleo-evo-devo" in which biologists are starting to explore ancient controls of developmental patterns (e.g., Hughes *et al.,* 2017).

Before ontogeny

Significant ontogenetic change within trilobite species was first recognised some 30 years after the systematic study of trilobites began. During the "pre-ontogenetic" interval representatives of most of the major trilobite clades known today had been described, and various classificatory schemes were proposed (Brongniart, 1822); some emphasising cephalic characters (e.g., Dalman, 1827; Goldfuss, 1843) and others based primarily on those of the trunk (e.g., Milne Edwards, 1840; Emmrich, 1845; Burmeister, 1846). At this time it was appreciated that specimens belonging to the same species could vary in size, but little attention was given to size-related shape changes that might link forms of different size with different morphologies.

Trilobite ontogeny discovered

The diligent and skilled worker Joachim Barrande arranged sequences of specimens by size and discovered their progressive shape change. In 1852 he published the first of his series of taxonomic monographs describing the lower Palaeozoic fauna of the Bohemian massif, with trilobites as its subject (Barrande, 1852). The recognition of size-related shape change was likely especially satisfying to Barrande, because five years earlier Hawle and Corda (1847) published a volume on Bohemian trilobites that would have robbed Barrande of taxonomic

priority for many species but for the latter's peremptory note (Barrande, 1846). In their volume, Hawle and Corda named and illustrated numerous species based on small specimens that Barrande (1849, 1852) showed to be the earlier stages of common mature forms.

Barrande discovered that as juvenile trilobites moult and grow, their increase in size was accompanied by the addition of segments in the trunk region (Fig. 1). He understood that a subsequent phase of grow began when the animals ceased adding new trunk segments, although growth and moulting continued. This biphasic growth pattern is generally known as hemianamorphic (Minelli *et al.,* 2003). Barrande recognised four main styles of trilobite ontogeny based on the development of exoskeletal segmentation, but there is no record of his considering trilobite ontogeny in the light of von Baer's laws and, to the end of his career, Barrande believed in the immutability of species (Kříž and Pojeta, 1974, p. 490).

Meanwhile in evolutionary biology…

Darwin referred to Barrande's demonstration of progressive stratigraphic succession of fossil form in later versions of the *Origin*, but not to his discovery of ancient ontogeny. The approximately 50 year interval between the publication of the *Origin* and the establishment of population genetics saw vigorous debate about whether variation in natural populations was continuous or discontinuous, and different fields of biology jostled, each assessing its own relevance to the new evolutionary framework (Provine, 2001). Anatomists greatly expanded documentation of living and past biological diversity, and also sought to detect patterns within it. It was at this time that Ernst Haeckel (1866) proposed his "biogenetic law" which stated that new evolutionary innovations appear in the final ontogenetic stages of development, and thus that during their development descendent species passed through or "recapitulated" the developmental stages of their ancestors. According to this view, evolutionary lineage could be read from ontogenetic growth series.

Trilobite recapitulation

Haeckel's advocacy of deterministic parallels between ontogeny and phylogeny encouraged palaeontologists working on extinct groups because it gave the group's demise unique value: just as a complete ontogeny could be studied, so could a complete phylogeny, and in this context a group's extinction could be considered an advantage. It also engendered a focus on what Gould (1977a) called the "internal" causes of evolution such as the ideas that evolution was

guided by an unspecified mechanistic process as Haeckel himself suggested, or by an innate property of life itself (e.g., Osborn, 1934).

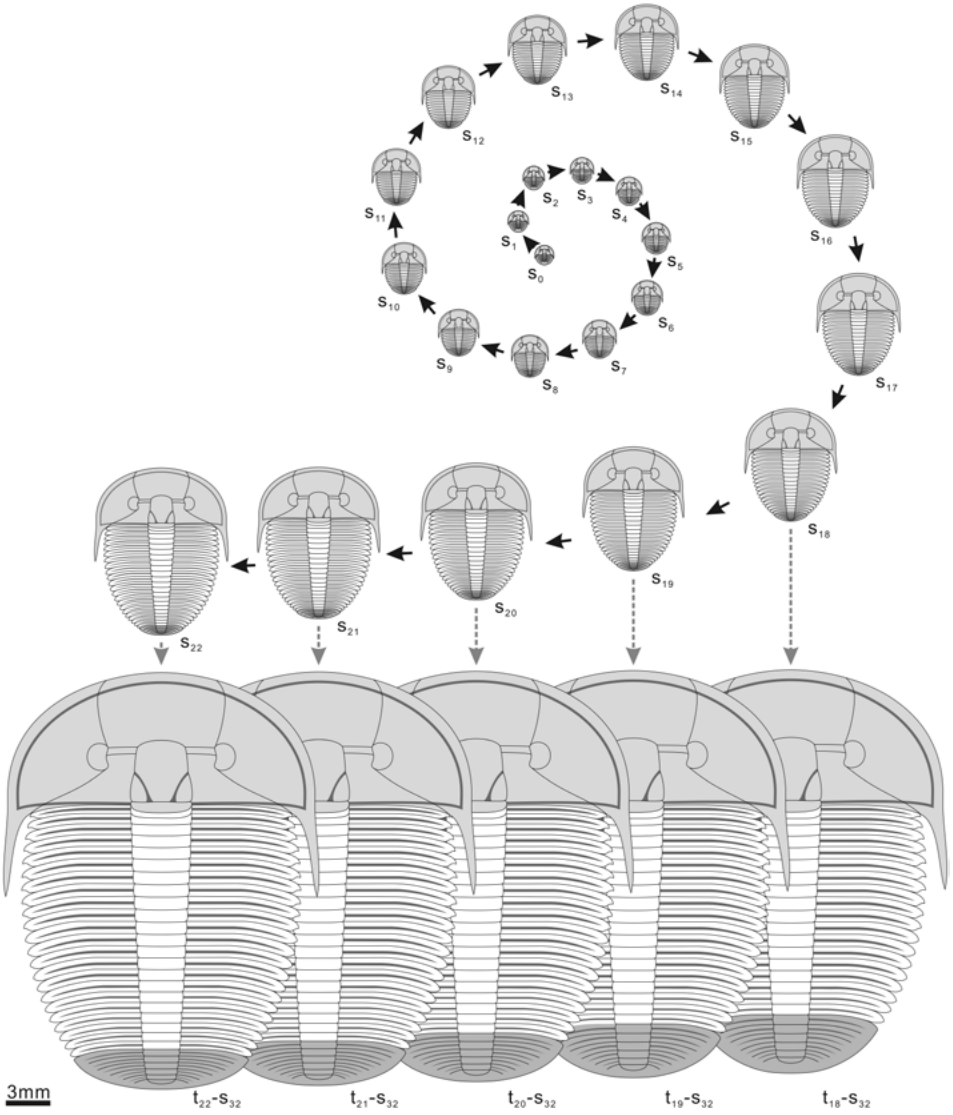

Figure1. Ontogeny of the 429 million year old Czech trilobite *Aulacopleura koninckii* (after Hughes *et al.*, 2017), first described by Barrande in 1852. In this species there are five mature morphotypes (t_{18}-t_{22}), each with a different number of developmental stages with thoracic segment addition (up to s_{18}-s_{22}). At the bottom of the figure are the five morphotypes at stage s_{32}. The example illustrates how variation in segment developmental schedules resulted in intraspecific morphological variation. All stages are shown at the same scale with respect to body length. For a detailed explanation of this ontogeny see Hughes *et al.* (2017) and references therein.

With respect to trilobites, Charles Beecher's examination of limb-bearing specimens from New York State and their ontogeny (see Beecher, 1895) led ultimately to a broader consideration of how ontogeny might inform trilobite phylogeny (Beecher, 1897a,b). Beecher rejected Haeckel's (1896, p. 653) own attempts at trilobite classification, but argued that the prolonged hemianamorphic development of trilobites (including a postembryonic phase of addition of trunk segments, see Hughes *et al.,* 2006) indicated both their status as primitive arthropods and their potential for demonstrating the validity of the biogenetic law. In particular, Beecher claimed that Barrande's four styles of trilobite ontogeny represented a progressive increase in both the number of trunk segments and the overall morphological complexity of the earliest preserved developmental stage – the *protaspis* – in derived taxa. He attributed this to the evolutionary acquisition of derived characters late in ontogeny, which putatively had the effect of compressing characters with early origins in phylogeny into progressively earlier ontogenetic stages. Beecher's claims received attention for their apparent promise of an evolutionary basis for constructing trilobite high-level systematics. However, the evidence provided for character pre-displacement in trilobites was modest. His phylogenetic arguments were based on the fact that some early groups lacked dorsal facial sutures throughout life, just as some derived groups do in their earliest growth phases. This view was partly supported by others such as Warburg (1925) and Poulsen (1927). Beecher (1897a) also introduced the term "gerontic" for the latest phase of trilobite ontogeny, in alignment with the recapitulatory idea that lineages, like individuals, reach senescence prior to extinction. He did not specify any characteristics that would identify such forms, but it is noteworthy that ideas of gerontism or "racial old age" persisted long after the demise of recapitulation as the primary motif of trilobite evolution (see, for example, Stubblefield, 1959, p. 58). In keeping with recapitulatory thought Beecher and his student Raymond (1920, p. 141) envisaged the ancestral trilobite to be protaspid-like, with a small number of trunk segments. Accordingly, just as new trunk segments appeared sequentially during ontogeny through "terminal addition" (see Jacobs *et al.,* 2005), so too were segments added phylogenetically. Both ontogenetic and phylogenetic styles of "terminal addition" were thus seen as linked processes in trilobites. Nevertheless, despite this putatively straightforward connection, Beecher and his sympathizers offered scant evidence of lineages in which phylogenetic terminal addition trend could be traced.

A bolder argument for recapitulation in trilobites derived from a detailed ontogenetic study of the late Cambrian trilobite *Leptoplastides salteri* (Raw, 1925). In seeking to place this study in wider context, Raw advocated common

names for stages of both the ontogeny of this species and of the phylogeny leading to its evolution (Raw, 1925, 1927a,b). The result was that the developing *L. salteri* was suggested to pass through stages represented by adults in species of *Olenelloides* and *Ctenoype,* although no species of either genus was considered to be ancestral to *L. salteri.* Although Raw's 1925 paper saw the introduction of the terms *meraspid* and *holaspid*, which have been widely used in trilobite ontogeny ever since, his evolutionary ideas have seen little subsequent support (see Whittington [1957, pp. 450-453] for an overall review of Raw's thoughts).

Revealingly, although Raw was a strong proponent of recapitulation, his views on the segmental condition of the basal trilobite were the opposite of those of Beecher and Raymond, for he long argued (1925, p. 255; 1953) that trilobites were derived from a multisegmented, annelid-like ancestor. To account for this, Raw (1925, p. 255) suggested that trunk segment number in trilobites effectively operated independently of the "biogenetic law" despite being subject to obvious ontogenetic increase in all trilobite groups then known. This disenfranchise of the character providing the easiest recognised marker of ontogenetic change within species, and thus also of potential heterochronic change between species, is striking although it is true that greatest range in the number of mature trunk segments in trilobites occurs among earliest Cambrian representatives (Hughes, 2007). Accordingly, proponents of both the segment-poor and segment-rich basal trilobite condition could point to examples of stratigraphically basal species fitting their preferred condition for the ur-trilobite.

Trilobite capitulation[1]

Beecher and Raw both recognised that, despite trilobites developing in an incremental and progressive fashion well suited for exploring recapitulation or any other heterochronic pattern, the phylogenetic distributions of many characters did not fit recapitulatory scenarios. Novel features appearing in early ontogeny, which contradicted recapitulatory expectations, were labelled "adaptive" and thus not of phylogenetic significance, but without further explanation. A more basic problem was that few specific cases of recapitulation were advanced and that phylogenies underlying the few supposed examples were shown to be unsound. Thus, recapitulation foundered on the fact that its predicted pattern was at odds with the morphological diversity evident in trilobite ontogeny and phylogeny. These are reasons why, despite their apparent advantages, trilobites never provided strong putative examples of recapitulation.

[1] I use the term "capitulation" here in the sense of a set of agreements, not in the sense of defeat.

A seminal paper on trilobite ontogeny published by Stubblefield in 1926 revealed the locus of where new segments (the segments added during postembryonic development) were first expressed in the developing trilobite trunk. While Stubblefield did touch on the implication of this discovery for assessing relationships with other arthropods, the paper is notably restrained in its conclusion. Its succinct, empirical approach extended to other important works, such as Stubblefield's (1936) synoptic study of trilobite facial sutures, in which he presented arguments for the role of paedomorphosis in the evolution of certain distinctive trilobites (perhaps prompted by the publication of de Beer's book *Embryos and Ancestors* [de Beer, 1930, see also Gould, 1977b]). Stubblefield's main focus, however, was on a comprehensive review of the form of the facial suture and its relationship to other characters considered important in assessing higher level phylogenetic relationships among trilobites.

The contrast between Raw's (1925, 1927a,b) and Stubblefield's (1926) approaches to trilobite ontogeny may reflect a wider reappraisal of the role of palaeontology within evolutionary biology that was taking place at that time. It coincided with the transition from palaeontologists thinking of themselves as custodians of the most secure authority on both evolutionary pattern and process – the fossil record – to a more rigorous but much circumscribed vision of the role of the discipline. Stubblefield was apparently aware that rapid advances in population genetics (see Provine, 2001) discouraged the idea that "internal" factors play a substantive role in guiding evolution. His response was to advocate rigour in morphological studies, sharpening and widening consideration of the phylogenetic implications of such studies amongst Trilobita (although interestingly, as noted above, ideas of phyletic gerontism persisted in his thought). The rigour of Stubblefield's approach was welcome, but by focusing strictly on the specifics of trilobite skeletal anatomy, integration of trilobite ontogeny with that of other arthropods became more challenging. For example, the articulation-based ontogenetic stages recognised in trilobites and so aptly applicable within the group do not apply outside it and thus have hindered comparison of trilobite development with that of other arthropods (Hughes *et al.*, 2006, p. 621).

Expanding database and adaptionism

The discovery of extensive silicified trilobite ontogenies, particularly in North America and more recently also in South America, Australia and Korea, along with more exacting standards of specimen description and illustration, has greatly widened the database on trilobite development. Such studies have figured prominently in considerations of trilobite higher level taxonomy, with several major clades having synapomorphies expressed in larval stages in ac-

cordance with von Baer's laws, but not with Haeckel's. (Although, as Beecher pointed out in 1897, the protaspids of some derived groups are both character-rich, and bear a more developed trunk region, than those of basal clades, suggesting some pattern of peramorphic evolution). By 1957 such studies were already showing that simple heterochronic explanations, whether peramorphic or paedomorphic, were unable to comprehensively account for evolutionary relationships at a variety of taxonomic scales. This led Whittington (1957, p. 460) to write that "Adaptation to particular environments – at different depths and on different types of sea bottoms – would seem to have been important in trilobite evolution, and selection may have been in favour of particular organs and their associated muscles, or of a larger pygidium and fewer thoracic segments, as providing better protection when enrolled." This quote encapsulates trilobite palaeontology's new alignment with the prevailing adaptation-focused evolutionary biology of that era (see Williams, 1966). Thus the adaptations that Beecher (1897a, p. 98) saw as inconvenient disrupters of phylogenetic history, had now became key to its resolution.

Postscript

My purpose here has been to place the earlier history of studies of trilobite ontogeny within a wider context, in an attempt to show how changing thought in evolutionary biology influenced the discipline. Since 1957 the publication of studies of trilobite ontogeny and specifically of heterochrony have accelerated markedly, particularly at low taxonomic levels (e.g., McNamara, 1986). But even at such levels it has been challenging to demonstrate that differences between putative ancestor and descendent species are the results of changes in developmental timing alone, as opposed to more subtle patterns of allometric repatterning (e.g., Webster *et al.*, 2001; Hunda and Hughes, 2007). The greatest phylogenetic challenges remaining involve understanding the origins of the novelties that define the major derived clades. The role of ontogeny in doing so has yet to be resolved.

Analogous perhaps to the shift from the recapitulatory to adaptationist approaches has been the trend toward increased analytical rigour in establishing phylogenetic relationships, and in quantifying differences between ontogenies, when evaluating the role of heterochrony. But a broader trend, and particularly since the establishment of evolutionary developmental biology as a discipline in its own right, has been the opportunity to better integrate studies of trilobite ontogeny with those of the development of living arthropods (e.g. Minelli *et al.,* 2003). Here the hope of providing critical data on the ancestral states of developmental characters identifies a unique role for palaeontology, focused on its

connections to the living, rather than that of the recapitulatory prognosticator of doom.

Acknowledgments

With respect to my own efforts in trilobite developmental palaeobiology, Sandro Minelli has been the leading light. Thank you, Sandro, for the opportunities and friendships you have given me.

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Genetics makes more sense in the light of development

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Abstract

In this chapter, I consider the *genes as blueprints* metaphor, that is, that genes are blueprints of the adult forms of organisms. I first explain the conceptual problems of this metaphor, and how they support false conceptions such as genetic determinism. Then I show how a careful consideration of development can help both address this false conception and provide the grounds for new and better metaphors, such as that genes contain a generative plan for the adult form. Genes do not determine traits but they are implicated in their development. I conclude by suggesting that it is easier to understand the role of genes if it is taught and presented in the context of developmental processes.

Introduction

Metaphors have a central place in scientific discourse. They are useful for conveying meaning and understanding, and in some cases they are irreplaceable. Metaphors have been extremely useful in genetics research. As John Avise has put it: "Evocative metaphors can distill an ocean of information, whet the imagination, and suggest promising channels for navigating uncharted genetic waters" (Avise, 2001, p. 86). Some examples of metaphors that have been extensively used in genetics are the metaphors of the genome as a "blueprint", a "book" or a "software". For instance, the announcement of the first sequence of the human genome in 2000 was presented in BBC under the title "Reading the book of life," actually using all of these metaphors in the first lines: "The blueprint of humanity, the book of life, the software for existence – whatever you call it, decoding the entire three billion letters of human DNA is a monumental achievement."[1]

Several other metaphors have been used to describe the properties and roles of DNA. Thus, DNA sequencing has been described as "reading"; DNA rep-

[1] http://news.bbc.co.uk/2/hi/indepth/scitech/2000/humangenome/760893.stm

G. Fusco (ed.) *Perspectives on Evolutionary and Developmental Biology.*

lication has been described as "copying"; RNA synthesis has been described as "transcription"; protein synthesis has been described as "translation"; RNA modification has been described as "editing"; and there is more. Such metaphors are not inherently wrong and can actually help us make sense of the respective phenomena. But we should always keep in mind that metaphors are a means of representation, and nothing more. "Books," "software," "reading," "editing," and so on are all human inventions and thus have an inherent dimension of anthropomorphism. This needs to be made explicit, or we should otherwise avoid any unnecessary use of expressions of this kind. However, this is not always easy to do, and misunderstandings can thus occur. In this chapter, I focus on the problems with the metaphor of *genes as blueprints* (for a similar argument, from a different perspective, see Pigliucci, 2010).

Genes as blueprints

In order to understand the origins of the *genes as blueprints* metaphor, we need to consider the meaning of the term "development". The term literally means unfolding something that already exists preformed somewhere. It is no coincidence that the same term is used for the process of printing on paper the image in a photographic film (Lewontin, 2000, p. 5). During the eighteenth century there were two major competing theories of development: preformation and epigenesis. Preformation theories suggested that a germ capable of development already possessed a certain structure that somehow preconditioned the adult form. In contrast, the theories of epigenesis suggested that a germ capable of development was unformed (Maienschein, 2012; Müller-Wille and Rheinberger, 2012).

Richard Lewontin has argued that it is actually the preformation view that has dominated genetics. This does not mean, of course, that anyone thinks that the adult form is preformed in the first cells from which an organism develops. Nevertheless, the idea of a "blueprint" that contains the necessary information for the production of the adult form is quite similar, because it accepts that there exists some fixed genetic essence inside the organism that causally determines its form. Thus, the role of the external environment is limited to certain conditions that may be necessary to trigger the developmental process and to allow it to proceed along a more or less predetermined path (Lewontin, 2000, pp. 6, 10–13). In short, the blueprint metaphor assumes that DNA determines traits by containing a detailed plan, like those used in engineering and architecture. This entails that the outcome of development is already specified in detail there, in the same way that the structure of a building is specified in an architectural plan.

This results in a very problematic discourse that attributes full powers to single genes. Consider the following statements from a best-selling book about the *SRY* (sex determining region Y) gene:

> In genetic terms, this suggests a peculiar paradox. Sex, one of the most complex of human traits, is unlikely to be encoded by multiple genes. Rather, a single gene, buried rather precariously in the Y chromosome, must be the master regulator of maleness. (Mukherjee, 2016, pp. 359-360)
>
> Is all of sex just one gene, then? Almost. (Mukherjee, 2016, p. 362)

This kind of discourse, and the idea of "genes as blueprints", implicitly supports three independent but interrelated misconceptions about genes (Kampourakis, 2017, p. 6):

- *Genetic essentialism*: genes are fixed entities, which are transferred unchanged across generations and which are the essence of what we are by specifying characters from which their existence can be inferred.
- *Genetic determinism*: genes invariably determine characters, so that the outcomes are just a little, or not at all, affected by changes in the environment or by the different environments in which individuals live.
- *Genetic reductionism*: genes provide the ultimate explanation for characters, and so the best approach to explain these is by studying phenomena at the level of genes.

But is it so bad to use the blueprint metaphor? A study aimed at assessing public interpretations of popular discourse about genetics, by providing participants with sample genetics news articles and asking for their interpretations of the "blueprint" metaphor. From the 137 college students who participated in this study, 58 provided responses that were explicitly nondeterministic and 39 provided explicitly deterministic ones (the other participants provided mixed or irrelevant responses). Interestingly, nondeterministic views were based on interpretations of the "blueprint" metaphor that referred to genes as operating in a partial and probabilistic fashion, as well as being malleable and not determining one's destiny (Condit, 1999). This study therefore suggests that the blueprint metaphor does not necessarily result in genetic determinist thinking.

However, a more recent study that explicitly contrasted the "blueprint" metaphor with the "instruction" metaphor gave different results. In this case, 324 adults were given a definition of genes that used either an "instruction" metaphor or a "blueprint" metaphor. The "instruction" metaphor suggested that "A unit of DNA is called a "gene." Every person has the same 30,000 genes, but a person can have different versions of these 30,000 genes in comparison to others. Each gene provides instructions for a specific chemical substance that the body uses. Not all instructions are followed all the time. As with any set of

instructions, how they are followed or whether they are followed depends on many factors." In contrast, the "blueprint" metaphor suggested something less flexible: "Genes are working subunits of DNA. DNA is a vast chemical information data base that carries the complete set of instructions for making all the proteins a cell will ever need. Each gene contains a particular set of instructions, usually coding for a particular protein." The researchers found that the "blueprint" metaphor compared to the "instruction" metaphor promoted stronger essentialist beliefs, that genes are "a causal, powerful, deterministic driver of health", which aligned with more intense positive attitude towards the efficacy of genetic research and human health (Parrott and Smith, 2014).

Therefore, it is possible that the use of the "blueprint metaphor" in public discourse about genetics enhances genetic essentialist and genetic determinist views. However, if one considers what happens during development, it becomes clear why the "blueprint" metaphor is actually a very bad one.

Genes are not blueprints but are implicated in development

The development of tissues and organs, and eventually the production of the adult form, is not controlled by genes or DNA but by the exchange of signals among cells. These signals consist of gradients of signaling proteins. Whatever a cell does depends on the kinds of signals it receives from its immediate environment. Therefore, neighboring cells are interdependent, and it is local interactions among cells that drive the developmental processes. These localized processes also make the development of different organs relatively independent, which allows for changes in each organ independently from other organs. During development, cells multiply, differentiate, and migrate to various parts of the developing organism. This happens in a coordinated manner, but without any centralized coordination of development; cells simply respond to signals from their local environment. What genes do is that they are involved in the production of proteins that are in turn involved in signal production, signal reception, and signal response. Genes are therefore implicated in this unconscious coordination of development, but they in no way determine its course and its outcomes (Davies, 2014, pp. 132, 251-252).

An appropriate way to conceptualize the role of genes in development is to think of an organism as an origami (Wolpert, 2011, p. 11). According to this, the DNA of the fertilized ovum is not a blueprint that contains the plan of the final form of the adult organism. Rather, it contains a set of instructions for making the organism, which will affect cell proliferation and differentiation. These instructions are about "how to make" the adult organism, not about "how the adult organism will look." Therefore, the DNA of the fertilized ovum contains a

generative plan, not a descriptive plan. According to this analogy, what is available are the instructions about when, where, and how to fold the paper in order to make a structure. A description of how the origami structure will look would be entirely useless, because it would provide no clear clues about how to generate it. In the same sense, a description of how the adult form will look is useless; what is needed is a set of instructions about how to generate it. Therefore, what happens during development is not that genes containing the blueprint for the adult form express themselves and thus the organism is constructed to resemble this blueprint. What actually happens is that cells follow the generative plan encoded in genes and the signals they receive from their environment. It is from the combination of numerous local signals coming from the intracellular and the intercellular space that cell division, proliferation, and differentiation take place during development. Appropriate signals will drive the production of the anticipated "normal" outcome, whereas "bad" signals can make things go wrong and bring about developmental defects. Thus, from a single fertilized ovum an adult organism develops.

Therefore, in order to provide a better understanding of genetics and to address the problems that the use of the blueprint metaphor might bring about, it is necessary to talk about genetics alongside development. Contrary to what Mukherjee (2016) attributed to the *SRY* gene, as shown above where he practically described it as the gene for sex, it makes more sense and it is far more accurate to present the role of *SRY* in its developmental context. It is certainly the case that a mutation in the *SRY* gene is enough to make an XY individual develop as a female with underdeveloped reproductive organs (Jäger *et al.*, 1990). It has also been found that a translocation of part of the Y chromosome including the *SRY* gene onto the X chromosome in humans makes an XX individual develop as a "true hermaphrodite" (a medical term for a form of *intersexuality*, i.e., carrying both male and female gonadal tissues) (Margarit *et al.*, 2000). But these instances do not justify the statement that *SRY* is the gene for sex.

The *SRY* gene is a gene on the Y chromosome indeed related to the development of male features. The default developmental outcome for the human embryo is to become a female. The expression of the *SRY* gene is what makes the difference in the outcome, because it affects a pathway that guides the development of the male or the female sexual organs. In this sense, *SRY* makes a difference for the development of sex. Embryos carrying the Y chromosome and the *SRY* gene develop testes and a male reproductive system, whereas those not having either the Y chromosome or the *SRY* gene develop ovaries and a female reproductive system (Davies, 2014, pp. 147–151). However, if one looks carefully at the details of the process, several proteins (and therefore genes) are involved

in the process of sex differentiation. The bi-potential precursor of gonads (testes and ovaries) is established by various proteins including SF1 and WT1, the early expression of which might also initiate that of SOX9 in both sexes. b-catenin can begin to accumulate at this stage, and in XX cells its levels can repress SOX9 production. However, in XY cells, increasing levels of SF1 activate the production of SRY that, along with SF1, enhances SOX9 expression. If SRY activity is weak, low or late, there is no SOX9 expression as b-catenin levels accumulate and shut it down. In the testis, SOX9 promotes the testis pathway, and it can do so even in the absence of SRY (Sekido and Lovell-Badge, 2009). Therefore, the *SRY* gene does nothing on its own. Sex is the outcome of a complex developmental process that involves several factors, and to understand their effect one has to consider the whole process of sex development.

Conclusion

My suggestion therefore is that we should always be explicit about the limits of the metaphors we use. We can say that genes "encode" some "functional" products, insofar as we clearly explain that this is just a way of representing the informational properties of DNA. These properties are not inherent, and they make sense only in the cellular context in which they can in turn be used as a resource for the production of molecules that contribute to the maintenance and the roles of self-regulated, living systems. We should also explain that often metaphors are used because we ignore the details and so they have a heuristic value both in explaining the respective phenomena and in guiding further research. It seems that we have to rely on metaphors, therefore we need to use ones that are more inclusive and represent more accurately the respective phenomena. For example, we need to stop thinking in terms of genes only and start thinking in terms of genomes (or genetic material) that include genes and various other sequences. We also need to replace the concept of gene action with that of gene interaction. This means that we should refrain from talking about genes that do this or that, and refer to genes that interact with other genes and with their environment. With simple changes like these we can at least give a better sense of the complexity of these phenomena. Metaphors will always be there, and we can make an appropriate use of good metaphors that will help nonexperts make sense of genes, and more broadly of genetics research.

To achieve all this, I suggest that we need to carefully consider development in teaching and in public discourse about genetics. Elsewhere, Alessandro Minelli and I have argued that evolution makes more sense in the light of development (Kampourakis and Minelli, 2014). In this chapter, I have argued that the same is the case about genetics. Genetics makes more sense in the light of devel-

opment because it is one way to show that genes are not blueprints of the adult forms, and that they are implicated in the development of traits. This makes the writings of Alessandro Minelli valuable because he has provided a deep understanding of developmental processes, and therefore of genetics. I find no better way to conclude this chapter by quoting Alessandro Minelli himself, citing two of his landmark books:

> Problems arise when one attempts to attribute a greater importance to genes that they probably actually have. […] Even though it is impossible to deny that genes have very important control functions in the development of the organism and the explication of its everyday activities, it would be prudent to distance ourselves from a view of living beings that is too exclusively (I was about to write: obtusely) focused on the gene. (Minelli, 2009, pp. 61-61)

> […] the question is whether it is genes that have generated new forms or if, instead, already existing forms have been attractors for the expression of genes that have eventually acquired, with time, a major function in the perpetuation of those phenotypes. The latter option, although perhaps counterintuitive and hardly reconcilable with the common view of DNA as the blueprint for the phenotype, is arguably closer to evolutionary history, as a rule at least. (Minelli, 2018, p. 82)

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Overcoming the constraint-adaptation dichotomy: Long live the constraint-adaptation dichotomy

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Abstract

There are good reasons to spurn the constraint-adaptation dichotomy in biological theory. Organismal trait distributions always involve both adaptation and "constraint" (however defined), so attributing any given pattern to one or the other is not a faithful reflection of biological process. Moreover, once enough information is in hand regarding the causes underlying limited variation, then the vague term "constraint" becomes unnecessary. The situation is different for an empricial biologist wishing to explain a specific pattern of trait variation. A necessary first step is to understand whether "empty space" morphologies can be produced in development, i.e. whether empty spaces are produced by "constraint" or adaptation, making the constraint-adaptation dichotomy a necessary empirical first step, regardless of the dichotomy's lack of ability to play a meaningful role in scientific theories.

Introduction

If a single characteristic epitomizes the living world, it is its magnificent variety, and yet biologists spend much of their time concerned about limited variation (Fusco, 2001; Minelli, 2009; Bateson, 2016). For all the vastness that the span of organismal form describes, it is still much smaller than the array of easily, and even plausibly, imaginable variation (McGhee, 2007). One of the central questions of biology is therefore why, of the array of imaginable morphologies, are only some observed, and of those an even smaller subset are commonly observed?

In modern biology, questions regarding why a given organism has the morphology that it does are often addressed by appealing to a dichotomy: adaptation versus constraint (Palmer *et al.*, 1999; Pigliucci *et al.*, 2000; Schwenk *et al.*, 2004; Shanahan, 2008). By definition, adaptation involves selection of a sub-

G. Fusco (ed.) *Perspectives on Evolutionary and Developmental Biology.*

set from among a wider field of developmental contenders. Therefore, selection explains why some variants are observed and why some others aren't. But when studying a pattern of narrow biological variation, it isn't clear whether alternatives to the observed morphologies are commonly produced, or are even possible. Finding that the field of developmental alternatives to the common morphologies is sufficiently wide that different configurations could be produced under selection is consistent with selection narrowing the field of possibilities and leading to the common configurations. However, if alternatives to the common configurations are developmentally impossible, or are very rare, then this opens the door to further investigation to identify the causes biasing the variation that can be produced. Because, among other reasons, faithful explanations of the processes leading to bounded variation always will include aspects shaped by selection and others limited by non-adaptive or not obviously adaptive causes, many authors declare the constraint-adaptation dichotomy to be at best better addressed by focus on other phenomena such as modularity or plasticity (Müller, 2007), regarding the dichotomy as largely passé. Others find the dichotomy to be simply meaningless because constraints and selection can't be separated into two distinct evolutionary phenomena (Arnold, 1992; Schwenk *et al.*, 2004; Shanahan, 2008; Badyaev, 2011; Losos, 2011). Still other authors cautiously find a useful distinction, but only to the extent that the dichotomy invites careful reflection on the processes involved in generating constraints and on the interaction between constraints and selection (Pigliucci *et al.*, 2000; Fusco, 2001). I summarize some of the reasons often given for the inadequacy of the dichotomy for thinking about evolution in general terms. I also show why, from the point of view of the empirical researcher, the dichotomy is a necessary and permanent part of any efforts to construct explanations of organismal trait distributions.

Why the constraint-adaptation dichotomy is passé

There are three main reasons to reject the constraint-adaptation dichotomy (Arnold, 1992; Schwenk *et al.*, 2004). The first is that "constraint" is a vague term that does not designate a salient phenomenon in nature. The second reason is that, whatever the definition of constraint that one adopts, both constraint and adaptation are involved in the production of any given structure. The third is that when sufficient mechanistic details are in hand regarding the causes of trait distribution, no additional useful information is added by declaring the pattern to be preponderantly caused by one or the other. I turn to these three considerations now.

The blanket term "constraint" does not refer to any distinct phenomenon in nature

The first reason to reject the constraint-adaptation dichotomy is that there is no definition for the blanket term "constraint." Most definitions imply some bias or limitation to the variation that is observable in nature (Maynard Smith *et al.*, 1985; Fusco, 2001). A major division between conceptions of constraint involve those that define it so broadly as to include natural selection (because selection limits or "constrains" the domain of the commonly observed) (Congdon *et al.*, 1987) versus those that exclude selection. What benefit regarding selection as constraint might bring is not clear, with the only thing gained from replacing the already-difficult but reasonably precise vocabulary of natural selection with the vague one of constraint being a loss of clarity and invitation to cross-talk (Antonovics *et al.*, 1991; Schwenk, 1995). Though shrouded in vague language, at least on examination *selective constraint* is clear about causality, with other definitions being supremely unclear regarding their postulated causes, as in the case of *allometric constraints*. In some cases, authors clearly assume that allometry is caused by selection favoring certain trait proportionalities (West *et al.*, 2005; Conner *et al.*, 2011), whereas others assume that unspecified causes obligate organisms to fall within a restricted portion of morphospace (Fodor *et al.*, 2011). *Phylogenetic constraints* are probably the ones whose definitions are most mysterious, with authors often invoking phylogeny as "explaining" trait distributions (Senior *et al.*, 2016; Volf *et al.*, 2018). The forces causing similarity across diverged species are rarely if ever specified in such studies. Instead, the tendency for closely related species to resemble one another (including the phenomenon of taxic homology/synapomorphy, (*sensu* Patterson, 1982) seems best regarded as a pattern to be explained, not an explanatory process (Crisp *et al.*, 2012). Along these lines, evo-devo studies do try to discover the shared developmental properties that lead to similarities across species (Wagner, 1989; Minelli, 2003, 2010; Cracraft, 2005; Müller, 2007; Scotland, 2011). Another common conception of constraint are the patterns of covariation known as *quantitative genetic constraints*, as represented in indices such as the G matrix (Arnold, 1992; Mitteroecker, 2009; McGlothlin *et al.*, 2018). The G matrix is a representation of the degree to which multiple traits can vary independently of one another or not, owing to "genetic" covariation (though see Pigliucci, 2006; Wang *et al.*, 2012). Many other notions of constraint can be found in the literature, but the ones mentioned here are sufficient to illustrate that the fan of meanings of the term is very wide. In practical terms the fan of meanings is so wide that simply using the term "constraint" usually leads to authors either disagreeing explicitly on the meaning of the term or, more commonly, unwittingly talking

past each other because each understands different but unarticulated meanings (Antonovics *et al.*, 1991). So, from the point of view of its semantic content, the "constraint-adaptation" dichotomy is an unsatisfactory one.

All organismal features are shaped by adaptation as well as "constraint," however construed

Second, the dichotomy is unsatisfactory even when picking a single meaning of constraint and applying it consistently to a given system. This is because, under any conception of constraint, it is possible to find ways that both factors, adaptation and constraint, participate in generating any given organismal trait distribution (Fisher, 1985; Minelli, 2010). It is also possible to find ways in which both adaptation and constraint, however construed, mutually shape one another in evolution. Cell size provides an example of how constraint and selection both impose limitations on observed variation. On the one hand, selection clearly favors different cell sizes in different situations. The tiny cells of some microorganisms and the massive ones of bird eggs certainly illustrate that the developmental possibilities for cell volumes are very wide (National Research Council (U.S.), 1999). In the wood of trees, meristematic cells called fusiform cambial initials give rise to alternating cell types (Montes-Cartas *et al.*, 2017). To take as an example conifers, the group that includes the oldest trees on earth, the same fusiform initial year after year produces conductive cells called tracheids, which during maturation elongate well beyond the length and width of the fusiform cambial initial that gave rise to them. Tracheids can be 10,000 μm long and 90 μm in diameter (Wilson *et al.*, 2010). Occasionally, a fusiform cambial initial produces cells called axial parenchyma. These not only do not elongate beyond the length of the fusiform cambial initial that gave rise to them, as a tracheid would do, but they also divide into strands of several cells. Axial parenchyma cells are much smaller than tracheids, often less than 100 μm long. This illustrates that from the same embryonic cell, cells of different mature volumes can be produced. While the developmental possibilities with regard to cell volume are very wide in the derivatives of the fusiform cambial initials, selection favors different cell sizes in different functional contexts. Selection favors long conductive cells because passage of water through the cellulose matrix that makes up the cell membrane in tracheids imposes resistance. Longer cells mean more flow through lumina and fewer transits through membrane, and therefore higher energetic efficiency of conduction (Sperry *et al.*, 2006; Wilson *et al.*, 2010). Parenchyma cells are the site of storage of sugars and starch, which are mobilized in the osmotic regulation of conduction (Zwieniecki *et al.*, 2009), and conifer parenchyma cells are often involved in resin production. Less theory

is available to explain why small cell sizes are favored in these cells, but the production of gradients involved active loading of sugars or resins between adjacent cells is more readily achieved by small cells. Presumably well below the size ranges of tracheids and axial parenchyma cells are the minimum cellular volumes that can be produced given the geometry of lipid bilayers and cellulose cell walls. Even if such tiny cell volumes were favored in selection, they would not be achievable. Thus, while selection clearly seems to impose maximum and minimum diameters over the range of cell sizes routinely and not so routinely produced in development, the space of developmental possibility is bounded by biophysical possibility. In this way, constraint and selection are both responsible for the boundedness of organismal form.

Along these same lines, even more inextricable are examples in which constraints seem clearly shaped by selection. Conner *et al.* (2011) examined the relationship between floral tube length and filament length in a type of wild radish. Filaments are the slender stalks that anthers, the pollen bearing structures of flowers, are borne on. Short filaments hide the anthers inside the floral tube, whereas long ones dangle them well outside the tube opening. Unless the position is just right, deposition of pollen on insect visitors, and transfer to the female surface of other flowers, will not be successful. Selection would therefore be expected to favor precise relative positioning of filament length, the length of the floral tube, and the length of the pollen-receiving female organ, and this is exactly what is observed. Yet a great deal of developmentally possible variation can be observed within and across individuals. Conner *et al.* found that floral tube length is highly variable, as was filament length. But, the relative variability between tube and filament length is highly predictable: when tubes are short, so are the filaments, and when tubes are long, the filaments are as well, in exactly such a way that the relative position of anther and tube opening is maintained despite absolute size variation. Many different relative positions are observed across the flowering plants, but in the case of wild radish the anthers just peek out of the floral tube. From a quantitative genetics perspective, the tube length and filaments are highly genetically correlated (Conner *et al.*, 1993, 2011). Also, from the point of view of quantitative genetics, genetic correlations are regarded as constraints on evolution because selection acting on one trait will drag the trait values of the other, thus limiting their mutual evolutionary independence. Indeed, Conner *et al.* documented marked genetic variance along the axis of covariation between the two traits. That is, developmental variants are common along the line of proportionality. Variation perpendicular to the line of proportionality is very limited, however. This would seem to offer a classic example of a developmental bias, in this case a clearly

adaptive one. Conner *et al.* showed that heritable developmental variants with regard to tube lengths are commonly produced. But these variants much more often than not have filaments that exactly maintain the relative position of the anthers. Therefore, if selection were to favor relatively longer or shorter filaments, the low genetic variance perpendicular to the filament-tube scaling line would give little variation for selection to act on, a classic quantitative genetic constraint on the potential for adaptation. However, Conner *et al.* found that in just nine generations of artificial selection, they were able to generate wild radish lines in which the stamens were very long, clearly bearing the anthers well outside the floral tube, as well as lines with the anthers hidden well within it. They showed that the constraint of adaptively biased variation was easily malleable under selection, and clearly shaped by it. Because it will always be advantageous for organisms in their development to favor some outcomes over others, a major part of the evolution of development is the favoring of situations in which certain outcomes are produced dependably and others are produced infrequently or not at all (Willmer, 2003; Arthur, 2004; Blumberg, 2010). A standard view of natural selection is that unfavorable developmental variants can be completely extinguished from populations, closing the door to the production of those variants and biasing production in favor of others (Haldane, 1927). From this point of view, especially from the point of view of quantitative genetics, constraints themselves are favored by selection, further making the dichotomy meaningless.

The goal is explanation of organismal form, not the upholding of opposing banners of externalism or internalism

Third, a final set of examples will suffice to make the point that the notion of opposing sets of distinct constraint-adaptation processes is difficult defend biologically. The general lessons of these examples is that once the biological mechanisms underlying the production of different sets of developmental variants is elucidated, the need for vague terms such as "constraint" evaporates, because we have in hand a precise description of mechanism. Application of a constraint label adds no additional insight. One such example is provided by the evolution of sex combs in *Drosophila* by Malagón *et al.* (2014). The legs of both male and female *Drosophila* species are covered with bristles, which are arranged in rows that are more or less transverse with respect to the proximo-distal axis of the leg. In male *Drosophila,* some of these bristle rows are arranged longitudinally and, rather than fly-like and unkempt, are of sufficiently regular shape, length, and even of dark pigmentation, so as to look like small black combs arrayed with their long axes parallel to the limb main axis. These play some ineffable

role in titillating or at least maneuvering female flies (Ng *et al.*, 2008; True, 2008; Malagón *et al.*, 2014). In *D. melanogaster*, the sex combs form transversely and rotate in development to their mature longitudinal position. The number of teeth in a sex comb, and therefore how long it is, responds readily to selection and varies markedly under mutational provocation. But the longer a comb, the more likely it is in its rotation to blunder up against one of the transverse rows of normal bristles. Selection or mutation leading to longer combs does not automatically mean that the transverse bristles will make way for the sex comb as it rotates in the pupa to its mature, longitudinal position. As a result, the proximal ends of longer combs become distorted as they crowd against the transverse rows. This distortion leads to combs that have lowered functionality. In this example, adaptationists will see the all-powerful hand of selection at work: of the developmentally possible variants, some work better than others and are favored. Fans of constraint will instead see the crucial role of development in driving evolution: the course of development, indifferent to function, involves a competition for developmental resources, in this case space, and a bent sex comb grudgingly abutting and distorted by a transverse bristle row is the result. Adjudicating between these scenarios is unnecessary because they are both necessary for describing why sex combs evolve as they do. Declaring the pattern of *Drosophila* sex comb diversity and distortion as the result of selection or constraint adds nothing to the detailed description that Malagón *et al.* (2014) offer. Galis *et al.* (2006) (Minelli, 2009; de Bakker *et al.*, 2013; Kavanagh *et al.*, 2013) describe a similar situation with regard to the seven cervical vertebrae of mammals, again illustrating that when the mechanisms leading to a given trait distribution are elucidated, no additional insight is gained by adjudicating in favor of selection or constraint as the main cause of a given trait distribution.

Many other cases could be offered, but these are sufficient to make the point. A dichotomy of opposing factors designated by the general terms "constraint" versus "selection" is unhelpful in explaining trait distributions across living things. The vagueness of the term "constraint," the inseparability of constraint and adaptation, and their clear mutual shaping means that there is nothing to be gained by declaring either one as solely operative in a given situation. The dichotomy is clearly passé.

The constraint-adaptation dichotomy lives

And yet there is a situation in which the dichotomy is supremely useful, indeed essential, for taking meaningful empirical steps (Olson, 2012; Olson *et al.*, 2015). This is the situation in which an empirical worker is confronted with nothing but a restricted pattern of trait variation. "Restricted" in this context means that

some sector of plausibly occupiable morphospace is apparently empty. Allometric scaling patterns are common examples (Fig. 1). Organismal trait values are commonly found along the scaling line but rarely or never in the space immediately above and below the line.

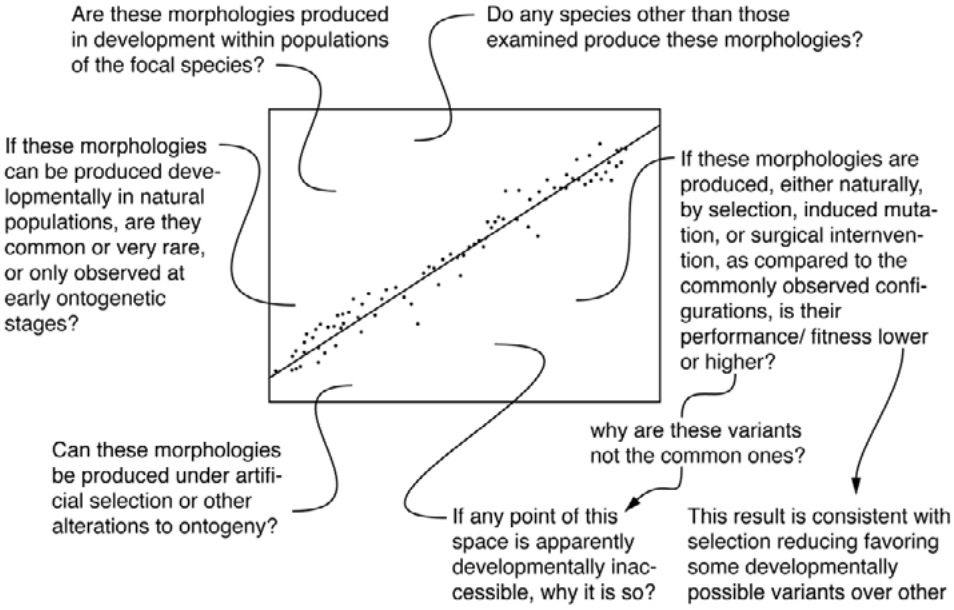

Figure 1. Constraint or adaptation? Exploring empty morphospace. There are reasons to question the usefulness of the constraint-adaptation dichotomy in thinking about evolution in general terms. However, empirical efforts to explain specific patterns of trait distribution are a different matter. The example above represents an allometric relationship between two features across the members of a species. Understanding why some areas of the space are filled and others are empty requires asking whether the empty space morphologies can be produced developmentally or not, such that the range of developmental possibility would permit relationships different from the commonly observed one. This process involves exploring natural variation in development, teratology, and artificial selection and other perturbations to development. Finding that the "empty space" morphologies can be readily produced and are of lower fitness than the common ones is consistent with standard accounts of selection favoring some variants over others. Finding that morphologies plausibly adjacent to the observed ones apparently cannot be produced, or that surgically produced "empty space" morphologies are of higher fitness than the common ones, points to the need to explore the developmental causes of this apparent inaccessibility. In practice, therefore, asking whether empty spaces can be occupied ("constraint"), and how their performance compares to the common morphologies ("adaptation"), is an essential step in exploring the causes of trait distributions.

Confronted with such a situation, there is nothing for the empirical worker to do but to ask whether the empty spaces can be readily produced in development or not (Sinervo *et al.*, 1991; Conner *et al.*, 1993; Galis *et al.*, 2006; McGhee, 2007; Minelli, 2009; Niklas, 2009; Frankino *et al.*, 2010; Flores-Renteria *et al.*, 2011). This starting point makes itself welcome by opening a host of doors for further research. Finding that the morphologies corresponding to apparently empty spaces can be produced means that they can be readily compared to the common combinations. The expectation that the common combinations should have higher performance or fitness than the empty space ones can be tested directly. Finding that they apparently cannot be produced spurs surgical and other manipulations to force morphologies into unoccupied space (Sinervo *et al.*, 1991; Beldade *et al.*, 2002; Frankino *et al.*, 2007; Polak *et al.*, 2010; Conner *et al.*, 2011; Malagón *et al.*, 2014). Finding that surgically created "empty space" morphologies have higher performance or fitness than the common morphologies strongly suggests that these parts of morphospace are of difficult or impossible developmental access, spurring the search for the developmental mechanisms underlying this inaccessibility. Finding instead that the common morphologies have the highest fitness is congruent with the notion that selection has extinguished variation or established biases making the maladaptive combinations rare or impossible. As a result, the constraint-adaptation dichotomy lives on, in the laboratory, where it is a useful and welcome empirical first step.

Evidence for developmental inaccessibility opens the door to detailed investigation of developmental mechanisms

Upon finding that a plausibly occupied portion of morphospace is apparently developmentally inaccessible (Fig. 1), a biologist's logical next step is to understand *why* the space is inaccessible. Distinguishing between situations in which a lack of variation is adaptive because the unoccupied spaces are of low fitness versus situations in which the lack is indifferent with respect to adaptation is a central locus of investigation. In general, finding that morphologies corresponding to unoccupied spaces (produced by surgical intervention) adjacent to the occupied ones are of lower performance or fitness than the commonly observed morphologies is consistent with the notion that selection has extinguished that variation. Producing it with nudges from mutagens is also consistent with the notion that such variation is indeed developmentally possible, just not currently observed. Likewise, a comparative approach often shows that, whereas in the study system variants are not produced in a given sector of morphospace, they are routinely produced in differing selective contexts (Olson, 2012; Swartz and Middleton 2008). So, one possibility is that the variants that are plausibly

imagined but not observed are likely not produced because selection has biased the production of variants with better performance or fitness, a very useful outcome from developmental study, but in the end not one that is particularly surprising from the point of view of traditional adaptation studies.

However, there are other cases that so strongly question the traditional assumptions regarding the variation available for selection to act on that they demand the integration of studies of developmental variation into all studies of adaptation; indeed, they have led precisely to the dissolution of the adaptation-constraint dichotomy described here. Although the biologists of the Modern Synthesis recognized that in development some variants were produced more commonly than others, and that there could be some "holes" or biases in morphological space (Fisher, 1930; Simpson, 1955; Dobzhansky, 1970), they argued that the evolutionarily relevant variation was the random variation (i.e., not predictable with respect to timing, location in the genome, or with respect to the direction of phenotypic change) of small phenotypic effect (Fisher, 1930; Wright, 2000; Conticello *et al.*, 2001; Shanahan, 2004; Jablonka and Lamb, 2005). It is understandable that in the early Synthesis biologists sought to justify simplistic assumptions, and indeed assuming that the variation that can be produced in development falls neatly around the observed mean in a (multivariate) normal distribution greatly simplifies the mathematization of population genetics. As so often happens, much analytical power is gained by sacrificing, tentatively, some biological reality. This is an exercise in which biologists ask what-if, in this case, to what degree is it possible to describe observed phenomena and predict them given these assumptions. These assumptions, so convenient from an analytical point of view, tacitly specify that essentially all parts of morphospace are accessible. If selection were to favor movement in a given direction, then the mean will shift, and the variance with it, putting some variants in previously unoccupied quadrants of morphospace. If in a succeeding generation selection were again to favor movement in the same direction, the mean will again shift, and again some variants will fall in previously unoccupied morphospace. This process can in principle continue indefinitely to whatever parts of morphological space are favored by selection. The assumption of multivariate normal random variation is license to ignore and black-box all of the dynamics of development: large developmental jumps in morphospace are possible, but they are irrelevant evolutionarily; there are no holes, gaps, or biases in the variants produced developmentally in each generation. Therefore, there is no reason to study the variants than can or can't be produced in development.

But, if it could be shown that some parts of plausibly occupied morphospace are inaccessible, then the assumption of multivariate normal and random vari-

ation would have to be relinquished. But because this assumption is so superlatively expedient, it would take very compelling evidence indeed to motivate its abandonment. Such evidence in fact abounds, a prominent example being the regularity in human teratologies. Some variants are produced with some frequency whereas others, plausibly imagined and adjacent to the observed ones, are apparently never observed (Alberch, 1989; Blumberg, 2009). These examples are presented as evidence that the variation available for selection is bounded, biased, and discontinuous. As such, it cannot be black-boxed and ignored but must become a central focus of biological research. However, many cases can be dismissed by appeals to selection. For example, it can be argued that, yes, two-headed individuals *can* be produced but their production is rare because selection acts against them. The same goes for the unobserved morphologies: it is likely that trichotomies of the anterior axis in vertebrates were possible at one time, but it is so clear that they would be of such low fitness that the ability to produce them has been extinguished. So, what was required to underscore the importance of developmental possibility in all studies of organismal form was a case in which developmental biases were so clearly inexplicable by appeal to selection that there was no way of explaining them away. Such a case was forthcoming.

This case was one of biased developmental variation so opaque to explanation by the traditional Synthesis assumptions that to this day it helps motivate the study of developmental possibility in studies of organismal form at large throughout biology, and it is the case of segment number in geophilomorph centipedes. Geophilomorpha is a large order of mostly ground-dwelling centipedes that have elongate bodies and, crucially for our discussion, many segments. In 1988, Alessandro Minelli and his student Stefano Bortoletto assembled a prodigious dataset on segment number variation in these animals. Together with previously published data, they emphasized a point recognized by myriapodologists but essentially unknown to biologists at large. This was that leg-bearing segment number in the geophilomporphs varies across species from 29 to 191 (subsequent studies have expanded this range to 27-191, Minelli *et al.,* 2000), and that within this range, the number of segments is stubbornly odd. To be sure, centipede taxonomists had counted segment numbers previously, but Minelli and Bortoletto brought together these data scattered throughout the literature to argue that in apparently no case had any centipede specialist come upon even a single individual with an even number of segments. Moreover, Minelli and Bortoletto adduced additional evidence that even within species that vary in segment length between individuals, no even-segmented individuals were ever found, even in embryos and even in teratological individuals. Inspired by

these studies, subsequent workers examined the causes of the segmentation and the contingent developmental processes that make even numbers so difficult to produce (Arthur and Farrow, 1999; Chipman *et al.* 2004; Vedel *et al.*, 2010). In one case, biologists found an apparently otherwise normal even-segmented adult (Lesńiewska et al. 2009), suggesting that the developmental bias could be overcome, with its rarity suggesting that only in most unusual situations. Whereas other cases of developmental bias seem at least potentially dismissed as reflecting the action of selection, the example of odd segment numbers in the geophilomorphs stubbornly resists it. To date, no biologist has been able to propose a plausible scenario in which selection would favor individuals with, say, 189 or 191 segments but not 190. Instead, the possible morphospace of geophilomorph segment number is starkly unlike the multivariate normal traditionally envisioned for developmental possibility. Instead of essentially all plausible spaces being occupiable via minute continuous steps, geophilomorph segment space has a rigidly regular series of occupiable and non-occupiable states. Crucially, this regularity manifestly has nothing to do with developmental processes forged by selection biasing the production of viable morphologies over less viable ones. As a result, this example helped provide inspiration to biologists who are interested in organismal form generally. This inspiration has led to the battery of empirical questions summarized in Fig. 1 (Olson, 2012).

Conclusion

Motivated by examples such as the one provided by Minelli and Bortoletto (1988; see also Minelli, 2009), explanations accounting for trait distributions in nature have been irrevocably expanded. With counterexamples as compelling as segment number in the geophilomorphs, it is no longer possible in any given case to assume, untested, that the variation that can be produced in development and be subject to selection is continuous throughout plausibly occupied morphospace. Instead, it is clear that understanding how developmental space is filled and why is a crucial locus of research in constructing explanations of the variation in organismal form. As discussed above, when provided with rich biological detail, such explanations gain no additional clarity by declaring variation to be accounted for by constraint or selection in any given case. However, it is precisely this dichotomy that provides the essential and fertile empirical path for investigating the causes of empty morphospace, a path that ultimately makes the distinction itself obsolete.

Acknowledgements

Thanks to Wallace Arthur and Giuseppe Fusco for their careful readings and helpful suggestions. Special thanks to Sandro Minelli for his inspiration, insight, and warmth.

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The continued mystery of the phylotypic stage

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Abstract

Evo-devo research has created a renewed interest in understanding what is nowadays called the *phylotypic* stage. While there are new datasets from genomic and transcriptomic comparisons that support the reality of this stage, it is still very much an open question why it exists. Here I ask whether the general notion, namely that the stage represents a particularly constrained phase of development, is actually supported by sufficient data. It appears that the alternative view, namely that the stage is shielded from environmental influences and is therefore less likely to be subject to new adaptations, has not been ruled out.

The development of the concept

The initial concepts around the *phylotypic* stage have been around since more than 200 years (Meckel, 1811; von Baer, 1828), while the term itself was only adopted step by step (Sander, 1976; Duboule, 1994; Raff, 1996). But, as with so many biological concepts, the exact definition of what a phylotypic stage is, and by which criteria it should be delimited, remains elusive. In a very general sense, it refers to the fact that embryonic and larval stages between species can look more similar to each other than adult stages. In a more narrow sense, it refers to a stage of the transition from the germ layer specification stage to the organ development, called *pharyngula* in vertebrates or *extended germband* in arthropods. This stage appears to be characteristic for phyla, which is the reason for including "phylo" in the term. But apart of the fact that also the concept of a phylum is elusive (Hejnol and Dunn, 2016), the generality of a phylotypic stage keeps being discussed (Hall, 1997; Richardson *et al.*, 1998; Bininda-Emonds *et al.*, 2003; Kalinka and Tomancak, 2012; Abzhanov 2013; Cridge *et al.*, 2016), although there is a consensus that the general pattern as such exists.

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In evolutionary biology we are used to infer processes from patterns and the phylotypic stage is certainly one of those patterns that are highly recurrent. However, while there is an ongoing controversy around the definition of the phylotypic stage, there is a surprising consensus about the likely underlying evolutionary mechanisms, i.e. the WHY question. Almost without exception, authors state that this must be a particularly constrained stage that does not allow evolutionary variation, a point that has been particularly elaborated by Raff (1996). He proposed that the complex interactions between the developing organs would imply that even small mutational changes would lead to pleiotropic consequences at later stages, thus effectively precluding evolutionary changes at this stage. But this is only a verbal argument and proof for this common assumption is actually rather scarce.

Darwin's view

One of the first speculations on the evolutionary reasons for relatively more conserved embryonic or larval stages came from Darwin. He took the relative similarity of embryonic forms as a proof for the common descent of species from each other. He also believed in the recapitulation concept that was most forcefully put forward by Haeckel, but there is no need to discuss these two aspects here. Instead, it is interesting to see that Darwin used an adaptation argument to explain the relative conservation of earlier forms. He writes in the *Origin of Species*:

> It deserves notice that it is of no importance to a very young animal, as long as it remains in its mother's womb or in the egg, or as long as it is nourished and protected by its parent, whether most of its characters are acquired a little earlier or later in life. It would not signify, for instance, to a bird which obtained its food by having a much-curved beak whether or not whilst young it possessed a beak of this shape, as long as it was fed by its parents. (p. 391, ed. 6th, 1872)

and:

> Let us take a group of birds, descended from some ancient form and modified through natural selection for different habits. Then, from the many slight successive variations having supervened in the several species at a not early age, and having been inherited at a corresponding age, the young will have been but little modified, and they will still resemble each other much more closely than do the adults. (p. 393, ed. 6th, 1872)

This argument is very different from claiming a strong constraint for the conservation of embryonic forms. Just the opposite: the claim is that there is no

need for specific adaptations at these early stages and hence there is no positive selection for them. One could thus view the embryonic stages as a kind of "living fossils". They would have been optimized in evolution to adjust to the conditions under which they develop, but would not show many further adaptations to environmental conditions under which they would live as adults, simply since they are protected from the environment. In turn, this means that embryonic stages are only seemingly constrained and they could change quickly in case the conditions require this. In fact, Richardson *et al.* (1997) have made the point that even the vertebrate pharyngula is much more variable than usually depicted.

The genic view

So we have two competing hypotheses. Either the phylotypic stage is considered as a highly constrained phase during development, i.e. under strong purifying selection that does not allow changes. Or it is a stage with little environmental interactions that could trigger new adaptations, i.e. positive selection does not act as much on it as it does on other stages. How can one test these alternative views?

Our own route towards this question was a bit indirect. In 2007 we proposed that one can harness information from the then upcoming full genome sequences by paying attention towards the time at which specific genes have arisen (Domazet-Loso *et al.*, 2007). We called this procedure *phylostratigraphy* and applied it to general biological questions (Domazet-Loso and Tautz, 2008, 2010b). One of them was to correlate the average expression of genes throughout ontogenetic stages with their average age of the corresponding genes in the whole transcriptome. We called this a *transcriptome age index* (TAI) and applied it to data from zebrafish development that we generated for this purpose. The results were rather striking: the relatively oldest genes were found to be expressed during the phase of development that would correspond to a phylotypic stage (Domazet-Loso and Tautz, 2010a). Both the transcriptomes before and after this stage were younger, implying a correspondence to the *developmental hourglass* pattern that was suggested before (Duboule, 1994). This particular pattern was later challenged by claiming that one should have done a log-transformation of the expression data to reduce the impact of outliers (Piasecka *et al.*, 2013). But while we do not endorse this view, even in the analysis of the transformed dataset, one can see that adults express the younger set of genes. Further, comparable patterns were later found also in a number of other cases, including plants and fungi (Irie and Kuratani, 2011; Yanai *et al.*, 2011; Quint *et al.*, 2012; Cheng *et al.*, 2015; Cridge *et al.*, 2016).

But what has this to do with the above competing hypothesis? The argument is as follows: newly evolved genes, called *orphan genes* or *taxonomically restricted genes*, are thought to reflect lineage-specific adaptations (Khalturin *et al.*, 2009; Tautz and Domazet-Loso, 2011). Hence, finding fewer such genes being expressed at embryonic stages argues for a lowered need for specific adaptations. In fact, at least in the zebrafish data, we saw also fewer young genes being expressed in very old animals, possibly because the efficacy for new adaptations is also reduced in them, due to dwindling reproductive success (Domazet-Loso and Tautz, 2010a).

In a paper published in parallel to the one discussed above, Kalinka *et al.* (2010) took a somewhat different approach. They studied divergence patterns of the transcriptomes between *Drosophila* species and found lower divergence and higher constraints at the phylotypic stage. Further, it was reported that genes expressed in the *Drosophila* embryo and larvae show lower substitution rates than those expressed in adults (Domazet-Loso and Tautz, 2003; Artieri *et al.*, 2009). Along these lines, Drost *et al.* (2015) generated an analogue of the transcriptome age index, namely a *transcriptome divergence index* (TDI), which measures evolutionary rates between closely related species and they found also lower rates at the phylotypic stage. However, while these observations are compelling, they still do not provide a clear answer to whether they reflect higher constraints, or less environment-specific adaptations.

Testing the alternatives

A direct test for special constraints at the phylotypic stage would be to ask whether experimental perturbations or mutations would have a particularly strong effect at this stage. Galis and Metz (2001) were the first to screen the literature for reports on the effect of perturbations at different times of pregnancies in mammals. They concluded from these reports that the phylotypic stage is indeed particularly vulnerable, which would seem to support the constraint hypothesis. However, since the original studies had not specifically been designed to answer this question, they had insufficient data on the stages preceding the phylotypic stage. Uchida *et al.* (2018) revisited this question by systematically studying zebrafish, frogs and chicken at all stages. They found that the earliest stages up to gastrulation are the most sensitive ones, not the later phylotypic stage. This argues against a special constraints status at the phylotypic stage.

Zalts and Yanai (2017) took a different approach. They compared mutation accumulation lines in *C. elegans* and found that less variation is generated in mid-development than in early or late development. This would suggest that mutations at mid-development are more likely to be deleterious. However, the

results do also not rule out the possibility of more positive selection in the other stages.

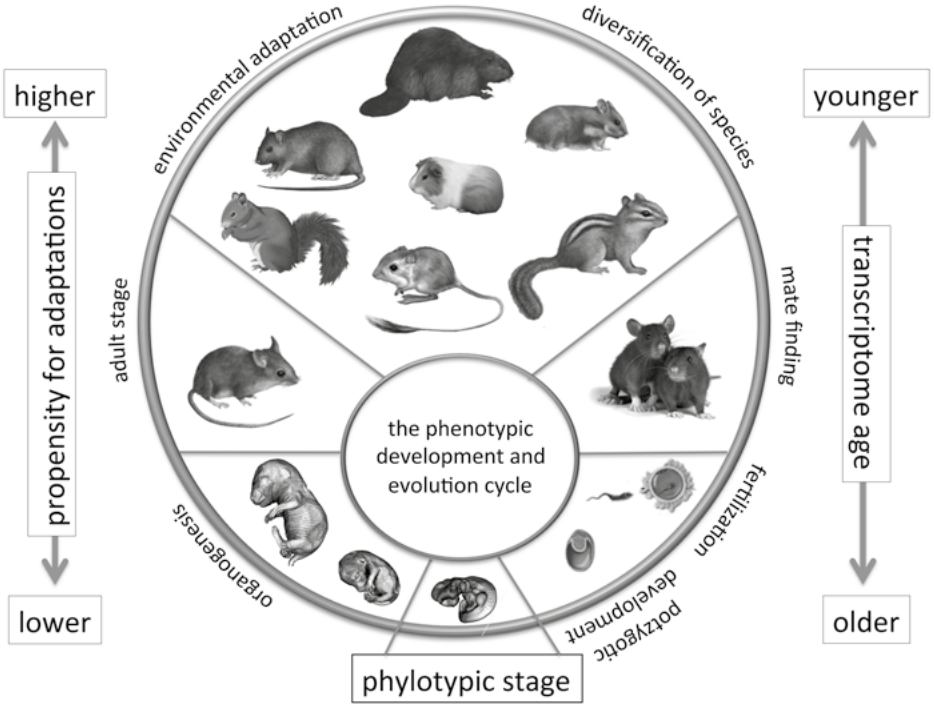

Figure 1. Depiction of a development and evolution cycle of mammals. It follows the asymmetric wheel model proposed by Tautz and Schmid (1998). The two circles are most narrow around the phylotypic stage and widen towards adult stages. The latter are represented as multiple species of rodents to symbolize the range of adult adaptations. Different phases of the life cycle are depicted around the circle.

The argument of constraints in regulatory networks for the conservation of the phylotypic stage is in part derived from the fact that the genes regulating early developmental patterning tend to be highly conserved. In fact, one can even make an argument that the phylotypic stage should be equated with the expression of the anterior-posterior Hox gene cluster (Slack *et al.*, 1993). However, only a small fraction of the whole transcriptome is involved in these early regulatory interactions. Hence, since the TAI or TDI is based on whole transcriptome data, the reality of a phylotypic stage cannot be explained by this small set of genes alone.

Maybe we still need to dig deeper and design new experiments and/or analysis schemes to come closer to answering the WHY question. But we might

also broaden the scope of the problem a bit. The hourglass or funnel analogies are almost invariably drawn in a linear fashion. But it should really be a circle, since there is a new embryogenesis after the adult stage. Abzhanov has juxtaposed these depictions in Figure 6 of his review on the topic (Abzhanov, 2013). But even this depiction omits an important aspect. The adult phase should better be depicted as the evolutionary divergence phase in the form of different species. We have previously provided such a depiction for insects (Tautz and Schmid, 1998). Figure 1 here shows an analogous circle for rodents. Note that this is more than just a question of representation. By including the species diversification aspect, it implies that the transcriptomic age differences between embryo and adult actually represent recent evolutionary divergences of adults in different taxa.

Outlook

Note that the fact that one finds generally a "younger transcriptome" in adults implies that there are also more orphan genes expressed at these stages. Cell biology and developmental biology have so far focused very much on conserved gene sets, while lineage-specific orphan genes are much less represented in genetic studies (Tautz and Domazet-Loso, 2011). These conserved genes have often a regulatory role, involved in specifying axes and cell types. In contrast, genes and genetic processes that generate the actual three dimensional phenotype are still only poorly known. In fact, we do not expect anymore that phenotypes are generated by single genes only. Rather, they are governed by the principles of complex trait genetics. Deep new insights into the genetic architecture of complex traits have been obtained in the past few years. The most radical view is that almost all genes may be involved in specifying a given phenotype (Boyle *et al.*, 2017), or in other words, all genes affect all phenotypes to some degree. We are currently only at the brink of understanding the consequences of this newly discovered genetic architecture of complex traits. Hence, the speculations about pleiotropic consequences of possible mutations at any stage of development are just that: speculations. We are still far away from understanding how three dimensional forms are generated by the genetic system and hence the postulation of constraints seems still premature. Only through studying complex trait genetics, we will eventually have a chance to provide a deeper understanding of the evolutionary mechanisms behind the patterns of the phylotypic stage. Until then, it will remain a continued mystery.

Acknowledgements

Our work in the Evo-Devo field had originally started with asking questions on the homology of segmentation, based on what we had learned in the *Drosophila* system. Sandro Minelli was the first zoologist who visited us (at that time in Munich) and discussed this with us. I will always be indebted to him for giving us the introduction into classic and new ideas on patterns of segmentation and evolution.

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Part III Disclosing the tree of life

The molecularization of centipede systematics

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Abstract

The injection of molecular data over the past 20 years has impacted on all facets of centipede systematics. Multi-locus and transcriptomic datasets are the source of a novel hypothesis for how the five living orders of centipedes interrelate but force homoplasy in some widely-accepted phenotypic and behavioural characters. Molecular dating is increasingly used to test biogeographic hypotheses, including examples of ancient vicariance. The longstanding challenge of morphological delimitation of centipede species is complemented by integrative taxonomy using molecular tools, including DNA barcoding and coalescent approaches to quantitative species delimitation. Molecular phylogenetics has revealed numerous instances of cryptic species. "Reduced genomic approaches" have the potential to incorporate historic collections, including type specimens, into centipede molecular systematics.

Introduction

Centipedes – the myriapod Class Chilopoda – are an ancient group of soil predators, with a >420 million year fossil history and about 3150 described extant species (Minelli, 2011). They are of interest to students of arthropods more broadly for conserved elements of their relatively compact genome (Chipman *et al.*, 2014), for their insights into the position of myriapods in Arthropoda (Rehm *et al.*, 2014), and for the data available on their mechanisms of segment proliferation (e.g., Brena, 2014), in light of the systematic variability in their numbers of trunk segments and modes of postembryonic development (Minelli *et al.*, 2000). These questions have all been profoundly impacted by conceptual and technological advances in molecular biology, and the same is true of other fields of biology, including systematics. This essay explores the impact of molecular methods on reconstructing the evolutionary relationships of centipedes, dating centipede diversification, and recognizing species.

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Just over a decade ago, we reviewed the evolutionary biology of centipedes (Edgecombe and Giribet, 2007) and developments in centipede systematics over the previous 25 years (Edgecombe, 2007). At the time, the higher – level phylogeny of centipedes was considered a given and a textbook example of the congruence between morphology and molecules. The past 10 years have been marked by an uptake in molecular approaches to centipede systematics that have made the field more integrative and informed by novel sources of data, increasingly on a scale vastly larger than was formerly the case. But the new datasets have also brought disagreement for a few critical nodes in the centipede tree – nodes that have forced us to rethink the evolutionary trajectory of major characters, including postembryonic addition of segments and maternal care.

Inferring phylogenies: the impact of Next-Gen methods

Centipede phylogenetics exemplifies a pattern across evolutionary inference as a whole since the mid-2000s, a shift from traditional Sanger sequencing to Next-Generation approaches. To date, this shift has been witnessed mostly at relatively deep phylogenetic levels, such as the question of the interrelationships of the five extant orders of Chilopoda (see Fig. 1).

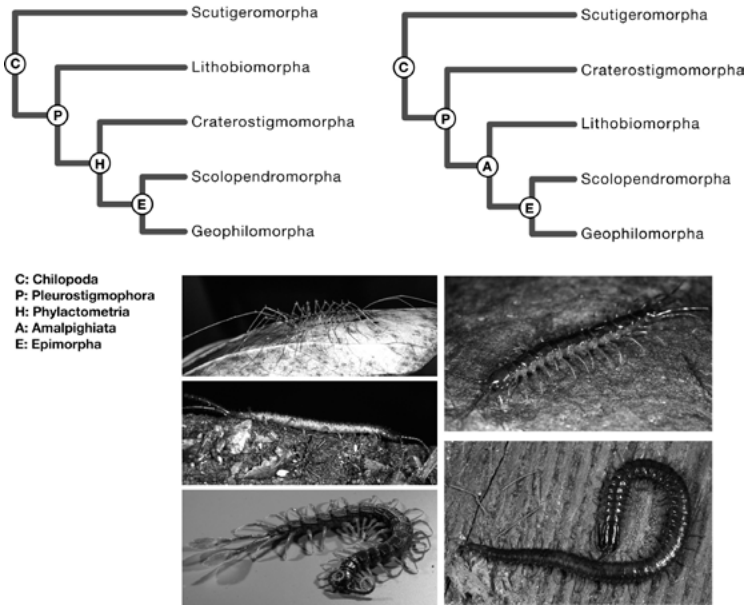

Figure 1. Relationships between the five extant centipede orders based on morphology (Phylactometria hypothesis, at left) versus transcriptomic datasets (Amalpighiata hypothesis, at right), and exemplars of Scutigeromorpha (*Sphendononema guildingii*), Craterostigmomorpha (*Craterostigmus crabilli*), Scolopendromorpha (*Alipes* sp.) (left, from top), Lithobiomorpha (*Paralamyctes levigatus*) and Geophilomorpha (*Mecistocephalus* sp.) (right, from top).

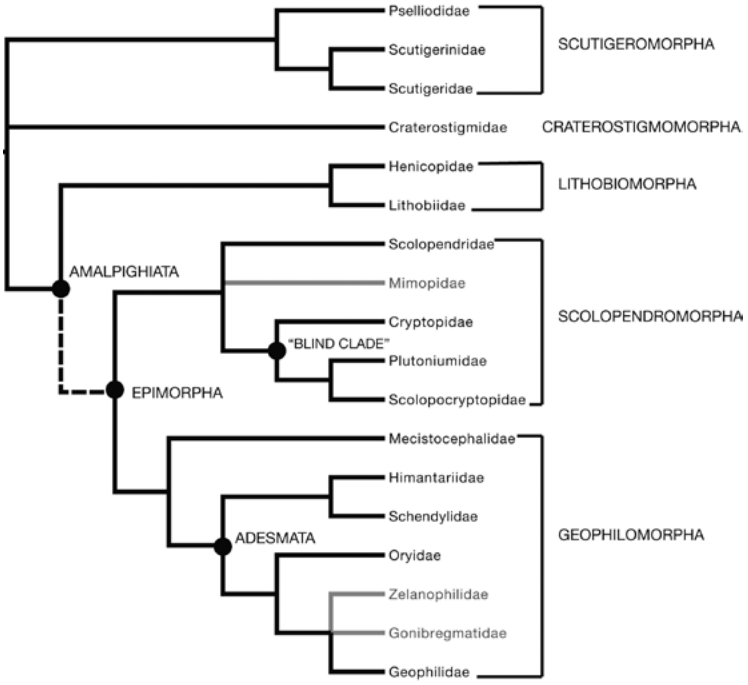

Figure 2. Summary of relationships between family-level groupings of centipedes (after Fernández *et al.*, 2016). Families lacking transcriptomic data shown in light font.

Molecular systematic studies of centipedes were launched in the late 1990s, originally targeted largely at relationships between the five orders, aiming to test such questions as whether centipedes with epimorphic development (hatching with the complete adult segment number) form a clade and the position of the Tasmanian–New Zealand Craterostigmomorpha, an order composed of just two species. These analyses used two or three nuclear protein-coding genes (Regier *et al.*, 2005), the small and large nuclear ribosomal RNAs (Edgecombe *et al.*, 1999) the latter combined with a few mitochondrial loci (Edgecombe and Giribet, 2004), or all of these markers together, with or without morphological data (Giribet and Edgecombe, 2006a). By the mid 2000s or early part of the present decade, taxonomic sampling for these Sanger-sequenced markers was dense enough within each of the four large centipede orders to allow many lower-level taxonomic hypotheses to be tested (see Fig. 2). The limits of the small set of genes used in these early analyses for resolving deep nodes have been apparent, these sometimes being weakly supported and unstable, at least in the larger clades. Nonetheless, some parts of the centipede tree were stable and well supported based on "first generation" molecular data. Scutigeromorpha divides into the Neotropical/tropical African Pselliodidae as sister group to the south-

ern African/Malagasy Scutigerinidae and the globally-distributed Scutigeridae (Edgecombe and Giribet, 2006; Butler *et al.*, 2010; Giribet and Edgecombe, 2013). Lithobiomorph monophyly is well established molecularly, the clade composed of two monophyletic families, the mostly Laurasian Lithobiidae and the mostly temperate Gondwanan Henicopidae (Edgecombe and Giribet, 2003). The latter has been especially well sampled, and taxonomic studies on Australian diversity have been accompanied by molecular data applied to phylogenetic placement of new species and Gondwanan biogeography (e.g., Giribet and Edgecombe, 2006b). Phylogenies for Geophilomorpha depict the basal split into Placodesmata (consisting of only one family, Mecistocephalidae) and Adesmata as in morphological classifications, and allow the larger clade Adesmata to be carved into subgroups (Bonato *et al.*, 2014) (Fig. 2). Scolopendromorpha divide into a blind clade and a clade whose members have the eye organized as a cluster of four ocelli, but deep nodes within species-rich groups, such as the subfamilies Scolopendrinae and Otostigminae, are often unstable and weakly supported (Vahtera *et al.*, 2012, 2013).

This last problem has partly been rectified by the greatly expanded gene samples available from transcriptomes (Fernández *et al.*, 2014; Fernández *et al.*, 2016, 2018). Phylogenetic resolution within orders is strongly supported and stable across a broad range of tree-building approaches and models. In Scolopendromorpha, the division into an ocellate clade (Scolopendridae) and a blind clade composed of the three families Cryptopidae, Scolopocryptopidae and Plutoniumidae is extremely robust (Fig. 2). Deep scutigeromorph and lithobiomorph relationships are the same as had been resolved in Sanger analyses, though to date only one or two species per family have transcriptomic data publicly available.

A reopened question, however, is the position of Craterostigmorpha. Morphological studies had settled on a sister group relationship between *Craterostigmus* and Epimorpha (=Scolopendromorpha + Geophilomorpha), and this hypothesis was formalized as the taxon Phylactometria (Edgecombe and Giribet, 2004) (Fig. 1, right). The name refers to a putative shared derived character – the mother brooding the eggs and hatchlings – in these three orders. This behavioural character is congruent with numerous other putative shared derived characters from varied organ systems. Some early molecular studies recovered support for Phylactometria (Edgecombe *et al.*, 1999) but others found novel placements for *Craterostigmus*. A recurring pattern is *Craterostigmus* as sister group to a clade composed of Lithobiomorpha and the two orders of Epimorpha (Fig. 1, left). That result was recovered in analyses of 62 nuclear protein-coding genes (Regier *et al.*, 2010) as well as with transcriptomic datasets (Fernández *et*

al., 2014; Fernández *et al.*, 2016). A putative clade composed of the three orders to which *Craterostigmus* is sister group was named Amalpighiata (Fernández *et al.*, 2014), its name signalling one of the few potentially diagnostic anatomical characters, a lack of supernumerary Malpighian tubules. *Craterostigmus* is alternatively recovered as sister group of Scutigeromorpha in a subset of analyses, a morphologically implausible grouping. Given that the morphological support for Phylactometria substantially outstrips that for Amalpighiata, it remains an open question whether *Craterostigmus* is spuriously attracted to Scutigeromorpha as a result of long branch attraction or other phylogenetic biases. However, applying substitution models intended to counter such systematic error fails to repel the "pull" of *Craterostigmus* towards the base of the centipede tree (Fernández *et al.*, 2016, 2018).

Molecular dating

Best practice for dating the tree of life using fossil-calibrated time trees is a topic of lively discussion in recent literature. Centipedes have been the subject of several molecular dating studies, some of them aimed at estimating divergence dates between deep (ordinal and familial-level) branchings, but also others that deal with shallower nodes to test biogeographic hypotheses.

The fossil record of Chilopoda is highly incomplete, but minimum divergence dates for several nodes in the crown group are constrained by Palaeozoic fossils. Incorporating these fossils under standard node calibration approaches – where prior densities for ages of nodes in a molecular tree are constrained by known fossils – recovers dated trees in which numerous family-level divergences within Chilopoda are inferred to have occurred in the Palaeozoic (Murienne *et al.*, 2010; Giribet and Edgecombe, 2013; Fernández *et al.*, 2016).

In the case of Scutigeromorpha, fossils constrain the crown-group (i.e., the clade derived from the most recent common ancestor of the group's living members) to the Late Silurian. The three extant families have ancient (Devonian to Permian) stem-groups but younger (Triassic-Jurassic) crown-group diversifications (Giribet and Edgecombe, 2013). Diversification rates show only minor shifts throughout the clade's 400 million-year history, and the dated tree is consistent with deep divergences, such as between African and Neotropical Pselliodidae and between Australian and New Caledonian Scutigeridae, probably resulting from vicariance rather than geologically recent dispersal.

Dated species-level phylogenies are increasingly being used to model vicariance and dispersal. A time tree for the scolopendrid genus *Digitipes* in the Western Ghats of peninsular India, including molecular data for numerous specimens of each species together with niche modelling, provided a basis for

evaluating how geological history may have shaped the group's diversification and distribution (Joshi and Karanth, 2012; Joshi and Karanth, 2013). Deep divergence dates and ancestral area reconstructions are consistent with the hypothesis that species inhabited refugia during Late Cretaceous vulcanism of the Deccan traps (Joshi and Karanth, 2013). Dated trees for Indian diversity of Scolopendridae more broadly show Cretaceous divergences within other genera as well (Joshi and Karanth, 2011). A dated tree using sequence data for four genes for 16 species of the Northern Hemisphere geophilomorph genus *Strigamia* recovered largely exclusive European and East Asian clades, each of which diversified over the past 30 million years (Bonato *et al.*, 2017).

Dating studies have also touched on comparative phylogeography of centipedes on continental and oceanic islands. Using likelihood mapping and neighbour nets for three loci, *Cryptops pictus*, endemic to New Caledonia (a continental island) was found to have a higher level of genetic structure and diversity than populations of *C. niuensis* from across Fiji and Vanuatu, geologically younger oceanic islands (Murienne *et al.*, 2011).

Species delimitation and integrative taxonomy

A number of studies on centipedes have used either sequences for the standard "DNA barcode" locus, cytochrome *c* oxidase subunit I (COI), or phylogenetic analysis based on COI or together with a few other widely-sampled Sanger-sequenced markers to identify species. This has the advantage in that species can be delimited based on the criterion of monophyly, which was often not the case based on morphological approaches, in which species could be distinguished with no phylogenetic context or could be non-monophyletic.

The German Barcode of Life program (www.bolgermany.de) has included analyses of a few centipede groups. Divergences within putative specimens of *Stenotaenia linearis* indicate the likely existence of cryptic species that are not clearly geographically separated from each other (Wesener *et al.*, 2015), and a similar pattern of molecularly distinct cryptic lineages is found in Italian populations of this genus (Del Latte *et al.*, 2015). COI phylogeny and pairwise distance comparisons for German populations of *Cryptops* likewise revealed a greater than expected number of species, including within long-known morphospecies (Wesener *et al.*, 2016). Notably, three geographically separate subgroups within *Cryptops parisi* signal likely cryptic species. COI barcodes have also been applied to identifying introduced species in groups that are challenging to determine to the species level morphologically, such as the lithobiomorph *Lamyctes* (Decker *et al.*, 2017) and scolopendromorph *Cryptops* (Reeves, 2017).

COI sequences and phylogenetic analyses that include them have been generated in some studies describing new species, such as for a new *Eupolybothrus* from Croatian caves (Akkari *et al.*, 2017). Molecular phylogenetics based on COI data unite two troglomorphic species of *Eupolybothrus* from the Balkans as each other's closest relative, and two different quantitative methods of molecular species delimitation allow them to be distinguished in a manner compatible with secondary sexual modifications of the males. Species descriptions have been accompanied by multi-locus sequence data (Siriwut *et al.*, 2015b; Kang *et al.*, 2017) or even transcriptomic data (Stoev *et al.*, 2013) for various centipede groups.

Cryptic species detected with molecular data have proven in some cases to be identified morphologically. In *Digitipes* from the Western Ghats, India, several clades with genetic distances indicative of species (Joshi and Karanth, 2012) were subsequently the target of morphological study, with some but not all putative new species being diagnosable based on morphological characters (Joshi and Edgecombe, 2013). Coalescent and Bayesian approaches to species delimitation allow five species of *Ethmostigmus* to be recognised in the Western Ghats, three of which form a clade in which morphological differentiation is subtle (Joshi and Edgecombe, 2018).

Similarly, trees based on COI sequences for putative material of *Lithobius* (*Monotarsobius*) *crassipes* from Germany, France and northern Spain revealed a distinct clade in Spain that was deemed a "pseudo-cryptic" species (Voigtländer *et al.*, 2017). It can be diagnosed morphologically, though based on characters (including some described using geometric morphometrics) that would likely have been dismissed as intraspecific variation under traditional species concepts, which were biased towards an assumption that species are geographic widespread and polymorphic.

The two species of *Craterostigmus*, despite each being monophyletic and distributed allopatrically in Tasmania and New Zealand, are also subtly distinguishable morphologically, but are easily diagnosed by species-specific substitutions or indels in the nuclear RNA (Edgecombe and Giribet, 2008; Giribet *et al.*, 2009; Vélez *et al.*, 2012). Geographic structure within *Craterostigmus tasmanianus* involves little variation in two mitochondrial loci within populations but considerable distance between geographically separated clusters of populations (Vélez *et al.*, 2012).

Although cryptic species are thus a common discovery in centipede analyses, in some cases molecular phylogeny is readily reconciled with traditional taxonomy based on external morphological characters. Comparisons of mitochondrial COI and 16S rRNA for populations of *Scolopendra cingulata* across

its geographic range in central/southern Europe depict clear gaps in intra- and interspecific distances (Oeyen *et al.*, 2014). In Southeast Asian species of *Scolopendra*, clades identified from molecular phylogeny inferred from three markers are compatible with groupings in geometric morphometric analyses, and species are diagnosed using traditional external taxonomic characters, with little change in nomenclature (Siriwut *et al.*, 2015a, 2016). This approach has been followed for Chinese species of *Scolopendra* (Kang *et al.*, 2017), and Southeast Asian species of *Rhysida* (Siriwut *et al.*, 2018). In the latter example, the molecular trees established that diagnostic characters of a monotypic genus, *Alluropus*, are actually secondary sexual characters of males, the females having been classified as a different genus and species.

Incongruence between morphological diagnoses and molecular phylogeny is pervasive in the scolopendromorph *Newportia*, a Neotropical radiation. Some geographically widespread morphospecies appear to be polyphyletic groups, with specimens of different species from the same geographic areas uniting with each other rather than with their putative conspecifics from other areas (Edgecombe *et al.*, 2015). This does not appear be common in centipede taxonomy. Morphospecies may underestimate species diversity (because cryptic species are undetected) but they are not usually in significant conflict with molecularly-delimited species.

Where to next?

It is not possible to accurately predict the future, but we can guess new directions for centipede systematics judging from what we are currently seeing in other fields of arthropod systematics and genomics. Centipedes have been at the forefront of phylotranscriptomics, but these have an important limitation – the requirement of fresh tissue preserved for RNA work. Even other PCR-based methods have limitations of sample availability, as they were not well suited for working with old museum samples, which often have degraded (i.e., highly fragmented DNA). However, new shotgun "reduced genomic approaches", namely target enrichment (e.g., Lemmon *et al.*, 2012) and ultraconserved elements, UCEs (e.g., Faircloth *et al.*, 2012), are amenable not only to specimens collected recently and preserved especially for DNA work, but also for old museum specimens (Sproul and Maddison, 2017). This opens the door to novel approaches to centipede taxonomy, perhaps the most exciting being the ability to incorporate genomic-level data from historic type material, circumventing the need to use morphological and geographic proximity of a fresh voucher to the types as the basis for fixing taxonomic names.

At the population-level, PCR-amplification of a few selected markers is now transitioning towards genomic subsampling. A popular technique is ddRAD-seq (double digest RAD sequencing) (Peterson *et al.*, 2012), a method for SNP discovery and genotyping, yet no study has applied it to the study of centipede populations. Additional SNP discovery methods include extending UCEs towards the least conserved areas (Starrett *et al.*, 2016), which could generate similar data to ddRADseq, but in a more repeatable manner.

Finally, from a genomics perspective, centipede genomes can now be sequenced with relative ease, although only that of *Strigamia maritima* is available (Chipman *et al.*, 2014). We expect a growth in centipede genomics, especially with the growing interest in their venom (Undheim *et al.*, 2016). The strength and stability of support for deep nodes in such challenging groups as Scolopendromorpha in transcriptome-based phylogenies (Fernández *et al.*, 2016) suggests a similar approach may also stabilize recalcitrant nodes at shallower taxonomic levels as more species are sequenced for large numbers of genes.

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"Perspectives in Animal Phylogeny and Evolution": A decade later

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Abstract

Refinements in phylogenomic methods and novel data have clarified several controversies in animal phylogeny that were intractable with traditional PCR-based approaches or early Next Gen analyses. An alliance between Placozoa and Cnidaria has recently found support. Data from newly discovered species of *Xenoturbella* contribute to Xenacoelomorpha being placed as sister group of Nephrozoa rather than within the deuterostomes. Molecular data reinforce the monophyly of Gnathifera and ally the long-enigmatic chaetognaths with them. Platyzoa was an artefactual grouping, and deep relationships within Spiralia now depict Rouphozoa (= Gastrotricha + Platyhelminthes) as sister group to Lophotrochozoa, and Gnathifera (plus Chaetognatha) their immediate sister group. A "divide and conquer" strategy of subsampling clades to optimize gene selection may be needed to simultaneously resolve the many disparate clades of the animal tree of life.

Introduction

In the preface to his textbook *Perspectives in Animal Phylogeny and Evolution*, Minelli (2009) formulated a simple, clear question based on a summary of some "unexpected and arguably controversial hypotheses" in a paper then just co-authored by us (Dunn *et al.*, 2008). He asked, "Will these three phylogenetic hypotheses eventually replace those presented in this book, which have been distilled from the evidence available until last week?", and concluded that "at the moment there is, arguably, nothing like a single best tree for the metazoans." This chapter addresses the major changes over the decade, in relation to our understanding of animal phylogeny and evolution. These changes did not happen in a vacuum, but rather at the interface between amplicon-based (us-

G. Fusco (ed.) *Perspectives on Evolutionary and Developmental Biology.*

ing PCR) and non-targeted gene sequencing paradigms. In the former, a few markers were selected, and primers designed to amplify them. In the second approach, genes were sequenced from cDNA libraries randomly. The Dunn *et al.* (2008) analysis combined – as other papers did at the time – whole genomes of a selected number of model organisms with a few ESTs (expressed sequence tags), on the order of hundreds to a few thousand, for a growing number of metazoans. This approach was later succeeded by denser gene sampling using next generation sequencing platforms (first Roche's 454 and then Illumina). Today, Illumina and other techniques are routinely producing large numbers of genomes and rather complete transcriptomes. Some of the discussions below focus on recent developments in the field of phylogenomics.

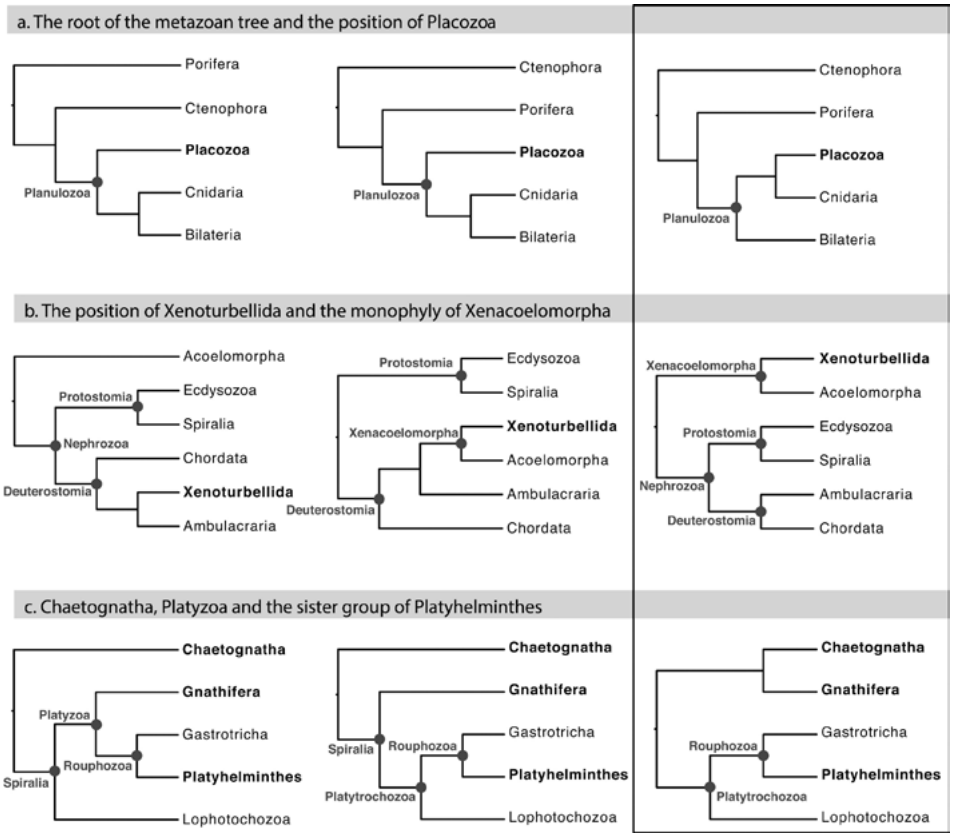

Figure 1. Alternative molecular hypotheses of selected metazoan clades in relation to the base of the animal tree (a), the position of Xenoturbellida (b) and selected spiralian relationships (c). Hypotheses on the left were generally supported by PCR-based phylogenies or early phylogenomic analyses. Rightmost hypotheses are preferred, as they are based on phylogenomic analyses with increased taxon and gene sampling and more sophisticated analytical methods for orthology selection and phylogenetic analyses.

Novel results

Many novel results have been proposed in the past decade, but not all have withstood scrutiny in the same way. Because the question about the position of ctenophores and sponges (which of the two is sister group to all other metazoans) has been debated to exhaustion without firm conclusion – each new paper claiming that the debate has been finally settled – we refrain here from revising such controversy, but refer the reader to recent reviews and the latest analyses (e.g., Dunn *et al.*, 2014; Dunn *et al.*, 2015; Feuda *et al.*, 2017; Shen *et al.*, 2017). Some other controversies have also been discussed, but unlike that of sponges–ctenophores, the addition of new data has provided new insights. Such is the case of the position of Xenacoelomorpha, the clade that includes Xenoturbellida and Acoelomorpha (= Acoela + Nemertodermatida). In addition, new hypotheses are emerging with the addition of genomes, as discussed below for Placozoa. Finally, results related to some clades of Spiralia are also discussed below in reference to Chaetognatha and Gnathifera, the paraphyly of Platyzoa, and the sister group of Platyhelminthes. Some of these hypotheses and alternative views are summarized in Figure 1.

Placozoa and the new animal phylogeny

Placozoans (currently represented by two genera, *Trichoplax* and *Hoilungia*) (Eitel *et al.*, 2018), have traditionally been placed as one of the earliest animal lineages due to their body plan simplicity, yet molecular phylogenetics has, since its early days, placed placozoans as more derived than their morphology suggested (Fig. 1a). In fact, most molecular analyses, and nearly all phylogenomic analyses suggested Placozoa to be the sister group to Cnidaria + Bilateria (= sometimes called Planulozoa[1]) (e.g., Srivastava *et al.*, 2008; Hejnol *et al.*, 2009; Pisani *et al.*, 2015; Simion *et al.*, 2017; Whelan *et al.*, 2017). However, a recent analysis including genomes of four new placozoans found strong support for a sister group relationship between Placozoa and Cnidaria (Laumer *et al.*, 2018), contradicting most previous phylogenomic analyses that supported the monophyly of Cnidaria + Bilateria when only the genome of *T. adhaerens* was sampled (e.g., Hejnol *et al.*, 2009; Feuda *et al.*, 2017). The new analyses suggest that such a grouping may be an analytical artifact, as a majority of genes supporting Cnidaria + Bilateria show evidence of compositional heterogeneity. Further research will be necessary to continue to test this hypothesis, and this should be

[1] There is confusion in the literature about the names Planulozoa and Parahoxozoa, which sometimes are used interchangeably and sometimes Planulozoa is a subclade of Parahoxozoa. Here we follow Wallberg et al. (2004) in identifying Planulozoa as composed of Placozoa, Cnidaria and Bilateria (see their Fig. 2).

facilitated by the recent availability of multiple placozoan genomes (Eitel *et al.*, 2018; Kamm *et al.*, 2018; Laumer *et al.*, 2018).

The position of Xenacoelomorpha in the animal tree

While the monophyly of Xenacoelomorpha is now well accepted, this has not always been the case. Two of its subclades, Acoela and Nemertodermatida, had traditionally been classified within Platyhelminthes (e.g., Karling, 1974), while the position of Xenoturbellida had long been debated, and included an affinity to Platyhelminthes, among many other groups (e.g., Reisinger, 1960; Haszprunar *et al.*, 1991). Early amplicon-based approaches showed that Acoela, first, and Nemertodermatida, later, were not part of Platyhelminthes, but closer to the bilaterian root, as sister group to Nephrozoa (e.g., Ruiz-Trillo *et al.*, 1999; Jondelius *et al.*, 2002; Ruiz-Trillo *et al.*, 2002; Telford *et al.*, 2003). This position seemed settled until *Xenoturbella* came back into play. Molecular accounts of the only accepted species of Xenoturbellida at the time (after a troubled earlier history of contaminations) seemed to suggest that Xenoturbellida was a deuterostome, most probably related to Ambulacraria (Bourlat *et al.*, 2003; Bourlat *et al.*, 2006; Bourlat *et al.*, 2009). This idea resonated with the epidermal ultrastructure of *Xenoturbella*, which bears resemblance to that of hemichordates (Pedersen and Pedersen, 1986, 1988). Subsequent work adding phylogenomic-scale data, mitogenomes and microRNAs of *Xenoturbella* and Acoelomorpha were used to switch the position of acoels from being the sister group of Nephrozoa, to becoming another deuterostome, as the sister group of Xenoturbellida (Philippe *et al.*, 2011). This position was in fact not supported by the microRNA data, which favour Xenacoelomorpha as the sister group to Nephrozoa, and the mitogenomic data only provided marginal support to the deuterostome affinity of Xenacoelomorpha. Much larger mitogenomic sampling has more recently suggested that Xenacoelomorpha are not nested within Deuterostomia, but rather that they are their sister group (Robertson *et al.*, 2017), with marginal nodal support. Deuterostome affinities for Xenacoelomorpha were not supported by other phylogenomic work (Hejnol *et al.*, 2009; Cannon *et al.*, 2016; Laumer *et al.*, submitted), notably when additional species of *Xenoturbella* are added to the analyses (Rouse *et al.*, 2016), and now Xenacoelomorpha is widely regarded as the sister group to Nephrozoa (Brauchle *et al.*, 2018). Since then, Xenoturbellida has now become a clade of six species (Nakano *et al.*, 2017), and many more probably await to be discovered in the deep ocean. They may well turn into a novel model to understand early bilaterian evolution, complementing acoels. Additional genomic data will contribute to definitively place this important animal lineage, and while the weight of phylogenetic evidence has shifted to a

sister group relationship with Nephrozoa (Fig. 1b), mitochondrial gene order and the presence of some genes in xenoturbellids and ambulacrarians have been suggested to support the position of xenoturbellids among deuterostomes (M.J. Telford, pers. comm.).

Chaetognatha and Gnathifera

The monophyly of Gnathifera – a clade uniting Rotifera, Gnathostomulida and Micrognathozoa – has been supported with morphology (Ahlrichs, 1993; Sørensen, 2003) and suspected using molecular approaches that lacked data from micrognathozoans (Witek *et al.*, 2009; Struck *et al.*, 2014). It has only recently been well established that gnathiferans form a clade that is well supported molecularly as well as morphologically (Laumer *et al.*, 2015a), and they constitute the sister group to all other spiralians (Struck *et al.*, 2014; Laumer *et al.*, 2015a) (Fig. 1c). Chaetognaths, on the other hand have been much more difficult to place reliably on the animal tree using molecular approaches, especially due to the long branch separating them from other protostomes (Marlétaz *et al.*, 2006; Matus *et al.*, 2006). A recent analysis of *Hox* genes across metazoans proposed novel synapomorphies between chaetognaths and rotifers, including loss of the *lox5*-parapeptide and the presence of the *MedPost* gene, found in no other animal groups examined to date, suggesting a possible relationship between Chaetognatha and Gnathifera (Fröbius and Funch, 2017). Such *Hox* signatures, however, remain unstudied in Gnathostomulida and Micrognathozoa. Novel phylogenomic analyses using the CAT+GTR model, including substantive data on all gnathiferan phyla (including the first gnathostomulid genome) and new chaetognath sequences, provide support for a sister group relationship between Gnathifera and Chaetognatha (Laumer *et al.*, submitted). This relationship should encourage future research on putative morphological synapomorphies, perhaps those related to the feeding apparatus of gnathiferans and chaetognaths, and additional genome signatures that may help further test this relationship (Fröbius and Funch, 2017; Laumer *et al.*, submitted).

Rouphozoa: Discovering the sister group of flatworms

Not totally unrelated to the resolution and position of Gnathifera and even Acoelomorpha, is the phylogenetic placement of Platyhelminthes – and the dismissal of a clade named Platyzoa (see Struck *et al.*, 2014; Laumer *et al.*, 2015a) proposed by Cavalier-Smith (1998) and endorsed in early amplicon-based (Giribet *et al.*, 2000) and EST-based (Hejnol *et al.*, 2009) analyses. Platyhelminthes have changed membership a few times (see for example the case of Acoelo-

morpha above), but are now understood to be composed of two main clades, Catenulida and Rhabditophora (e.g., Egger *et al.*, 2015; Laumer *et al.*, 2015b). However, their closest relative has been elusive for some time, and they have often been allied to a diversity of acoelomate animal groups, or to nemerteans (which have a coelom but have been considered as functionally acoelomate). Gastrotrichs, on the other hand, have been often grouped with other "aschelminths" due to their cuticle and nervous system of the cycloneuralian type. Using the newest phylogenomic data (well sampled and mostly Illumina-based datasets), results have settled on Gastrotricha being the closest living relative of Platyhelminthes (Struck *et al.*, 2014; Laumer *et al.*, 2015a; Kocot *et al.*, 2017; Laumer *et al.*, submitted) (Fig. 1c), a clade named Rouphozoa by Struck *et al.* (2014) as a derivation of the Greek word *rouphao*, for "ingesting by sucking", referring to the preferred feeding mode of platyhelminths and gastrotrichs. As in many other higher clades, synapomorphies are difficult to identify for these sister taxa, as many shared characters seem to be symplesiomorphic traits for Spiralia, such as lack of coeloms, complete or nearly complete body ciliation, and protonephridia. The presence of a duo-gland organ system (Tyler and Rieger, 1980) may constitute a true synapomorphy of Rouphozoa, even though this was once considered a striking case of convergence between platyhelminths and gastrotrichs (Tyler, 1988).

Discussion of "new old" results

The debate about whether Ctenophora or Porifera constitutes the sister group to all other animals was probably what made Minelli choose the Dunn *et al.* (2008) paper to open his book and to question how long novel results such as the ones presented in that paper might last. A decade later, the number of phylogenetic papers addressing this particular issue, and no doubt more importantly, the amount of research on both Ctenophora and Porifera has grown considerably, at least in non-taxonomic journals. While debate about the phylogenetic position of particular taxa may seem frustrating to many non-systematists who just desire a stable tree, at least in this case it has served to raise interest in nearly all aspects of the biology of sponges and ctenophores.

Many other key aspects have been consistently resolved since, sponge monophyly being one of them. While no PCR-based approach was able to recover monophyly of sponges, nearly all phylogenomic data sets now support the monophyly of Porifera (e.g., Pick *et al.*, 2010; Whelan *et al.*, 2015; Simion *et al.*, 2017; Laumer *et al.*, submitted). The implications of sponge paraphyly were especially relevant for understanding the last common ancestor of Metazoa, especially in light of the "choanoblastaea" theory (Nielsen, 2008), and therefore,

the dismissal of sponge paraphyly has been an important contribution of phylogenomics. The segregation of Homoscleromorpha from Demospongiae is also broadly accepted (Gazave *et al.*, 2012).

The revival of the old taxon Lophophorata (Bryozoa, Brachiopoda and Phoronida) is another contribution of the newest generation of phylogenomic data (Nesnidal *et al.*, 2013; Laumer *et al.*, 2015a; Laumer *et al.*, submitted), although the position of Entoprocta (sometimes allied to Bryozoa, as supported by Nielsen), has introduced some instability to this clade, especially when Cycliophora are introduced in the analyses (Laumer *et al.*, 2015a; Kocot *et al.*, 2017; Laumer *et al.*, submitted). Resolving whether Entoprocta and Cycliophora belong with Lophophorata (possibly as their sister group?), constituting the clade Polyzoa (Hejnol *et al.*, 2009; Laumer *et al.*, submitted), or whether Polyzoa may be artefactual (Nesnidal *et al.*, 2013), remains to be resolved.

Future directions

As eloquently stated recently by Laumer (2018), "Contemporary phylogeneticists enjoy an embarrassment of riches, not only in the volumes of data now available, but also in the diversity of bioinformatic tools for handling these data." These riches thus require more than just brute force, as we now see in most contemporary phylogenomic analyses, where sets of genes are carefully selected according to their properties, taxa need be judiciously selected according to the particular hypothesis to be tested, and methods are thoroughly tested and thoughtfully selected. Yet some questions remain recalcitrant to such treatments, especially when trying to infer relationships of such disparate sets of taxa as Metazoa. Some of our work has thus re-focused towards subsampling clades in order to optimize gene selection and to maximize gene and taxon representation for particular subsets of taxa, whether these are metazoan phyla (Laumer *et al.*, submitted), or subclades of crustaceans (Schwentner *et al.*, 2018). This strategy of divide-and-conquer may seem at odds with much phylogenetic thinking that aimed to build phylogenies as large as possible and may be more allied with some of the supertree aims. The future may decide which strategy is better suited for simultaneously resolving the phylogenetic position of groups such as ctenophores and chaetognaths.

A final reflection has to do with the integration of genome-level data and morphology, a topic that has been debated in several contexts, especially for the integration of fossil and phylogenomic data (e.g., Giribet, 2015; Pyron, 2015), which is essential for "total evidence" dating methods (Ronquist *et al.*, 2016). However, this integration has to date almost entirely been conducted in a Bayesian framework employing standard Markov models of evolution (the Mk

model; Lewis, 2001) that behave well for molecular characters, but not for morphological ones, a debate that has yet to be resolved (e.g., Goloboff *et al.*, 2018; O'Reilly *et al.*, 2018b, a). Some advances have recently been made in modeling transitions between plesiomorphies and apomorphies for morphological characters in a more appropriate manner than assuming equal frequencies through time. The MkA model (for "asymmetrical"), for example, limits reversal in morphological characters (Pyron, 2017). Continuing these efforts to devise better models for morphological characters could be a promising step forward in "total evidence" phylogenetics.

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Humans of the Middle Pleistocene: an evolutionary scenario for the origin of *Homo sapiens*

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Abstract

Looking at the human fossil record of the Middle Pleistocene (between 781 and 126 thousand years BP) a somewhat puzzling scenario emerges, with a considerable phenetic diversity, which may be referred to the existence of a single and multiregional species. It is probable that after one million years BP a new kind of humanity appeared and dispersed across Africa and Eurasia. The most appropriate taxonomic denomination for this widespread taxon is *Homo heidelbergensis*. During the Middle Pleistocene different lineages of this species are recognized, suggesting the identification of geographic varieties, or subspecies (i.e., incipient species), which respectively gave rise to distinct allopatric speciation events, including those of *Homo neanderthalensis* (in Europe), *Homo sapiens* (Africa) and the so-called Denisovans (Asia).

Introduction

African representatives of the genus *Homo* dated to about one million years BP – i.e., fossil specimens from sites such as Bouri (Daka), Buia and Olorgesailie – maintain morphological affinities with the "archaic" human species *H. ergaster (*where *H.* stands for *Homo)*, as pointed out by Manzi *et al.* (2003) among others. In this perspective, these hominins of the late Early Pleistocene are distinct from the more abundant fossil record of a few thousand years later. In the Middle Pleistocene (781-126 thousand years BP, or ka), in fact, African specimens like Bodo and Kabwe – as well as many other in Europe and mainland Asia – exhibit a new phenotype referred to the species *H. heidelbergensis*.

Generally speaking, the "new" humans of the Middle Pleistocene – widely distributed and spanning Africa and a large part of Eurasia, apparently with the exception of Indonesia (i.e., Java) – appear different from the diversity of phenotypes that derived from the earliest dispersal of the genus *Homo* out of Africa

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(Fig. 1), which included a variety of species: *H. ergaster*, *H. antecessor*, *H. erectus* (*sensu stricto*), *H. naledi* and the diminutive hominins from the island of Flores: *H. floresiensis*. This observation suggests that a taxonomic and phylogenetic discontinuity ranges across the Matuyama- Brunhes magnetostratigraphic boundary of about 780 ka (Manzi, 2004; Profico *et al.*, 2016).

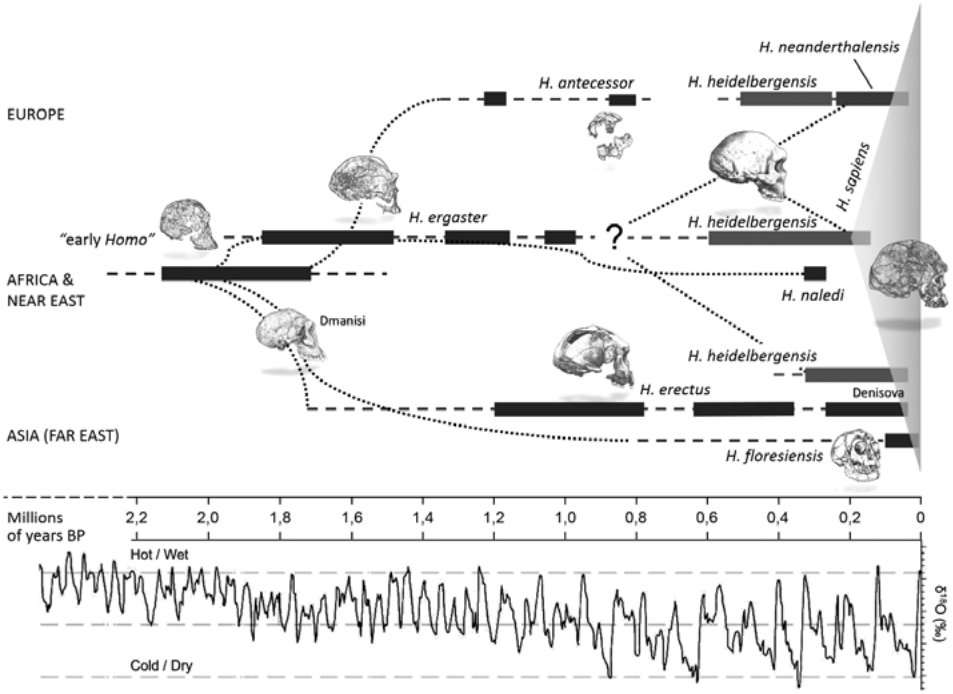

Figure 1. Schematic representation of the evolution of the genus *Homo*, displaying chronology and topology of the fossil record (bold lines = hard fossil evidence; dashed lines = inferred hominin occurrence); tentative trajectories of diffusion and/or phylogenetic relationships between species are also pointed out (dotted lines). It is reported below a curve of global climatic variations, based on marine isotopic stages. Redrawn and modified from Manzi *et al.* (2011) and Manzi (2012).

The phenetic distance observed across this discontinuity, preceding in turn the appearance of more derived humans such as *H. neanderthalensis* and *H. sapiens*, requires a distinction at the species level. Morphological features and advanced morphometric analyses (e.g., Mounier *et al.*, 2009, 2011) demonstrate the uniqueness of the human fossil record that is more recent than 780 ka. Its distinctiveness reflects the retention of some plesiomorphic features (mostly related to the architecture of an elongated cranial vault), combined with apomorphic traits (including larger cranial capacities, less flattened midsagittal profiles,

and a peculiar morphology of the supraorbital torus, etc.). Such a mosaic morphology fills the empty morpho-space between more "archaic" species of the Early Pleistocene, some still persisting throughout the Middle Pleistocene (Fig. 1), and more recent and derived varieties, including Neanderthals and modern humans. This suggests the existence of a distinctive species during the Middle Pleistocene, which was polymorphic and widely dispersed. This species should be referred to as *H. heidelbergensis*.

Despite controversy (Rightmire 1998, 2008; Hublin, 2009; Stringer, 2012; Arsuaga *et al.*, 2014; Balter, 2014), *H. heidelbergensis* – named after the discovery in 1907 of the Mauer mandible, near Heidelberg in Germany (Schoetensack, 1908) – has been resurrected at the end of the last century as a crucial reality (Rightmire, 1996), stating the existence of a discrete taxon that appeared in the late Early Pleistocene, largely distributed during the Middle Pleistocene and antedating the speciation of both *H. neanderthalensis* and *H. sapiens* (for a review, see Manzi, 2012, 2016).

There is at present a relatively rich fossil record that may represent the hypodigm (*i.e.*, the fossil record referred to a given species) of *H. heidelbergensis*. It embraces the following list of specimens and samples (Fig. 2):

a) in Africa, there are middle Middle Pleistocene crania (e.g., Bodo, Kabwe, Elandsfontein) that are typical of this taxon, but there are also more derived samples of the late Middle Pleistocene (e.g., Florisbad, Ngaloba, Omo Kibish II, Eliye Springs, Djebel Irhoud), which are close to the emergence of the modern human species;
b) in Europe, there are relevant specimens that antedate the Neanderthals and phylogenetically are (at least in part) their forerunners; these samples range from Northern latitudes (e.g., Swanscombe in England; Mauer, Bilzingsleben and Steinheim in Germany) to the Mediterranean regions (e.g., the impressive material from Atapuerca Sima de los Huesos in Spain; Arago in Southern France; Petralona in Greece; Ceprano, Venosa and Visogliano in Italy);
c) in mainland Asia, all the "non-*erectus*" specimens, formerly considered by some workers as "archaic *H. sapiens*", both from India (e.g., Narmada) and China (e.g., Dali, Jinniushan) belong to *H. heidelbergensis*; it is probable that these humans are the ancestors of the so-called "Denisovans" (see below).

Despite the relative abundance of the fossil record in the Middle Pleistocene, the origin of *H. heidelbergensis* is still unclear. At present, we do not know the provenance of the penecontemporaneous appearance of humans that are referred to this taxon. We know (or, better, we may infer) that they soon spread

geographically in Africa and Eurasia, evolving in regional lineages during the Middle Pleistocene; ultimately, they were ancestral to both Neanderthals and modern humans (Rightmire, 2008; Hublin, 2009; Stringer, 2012).

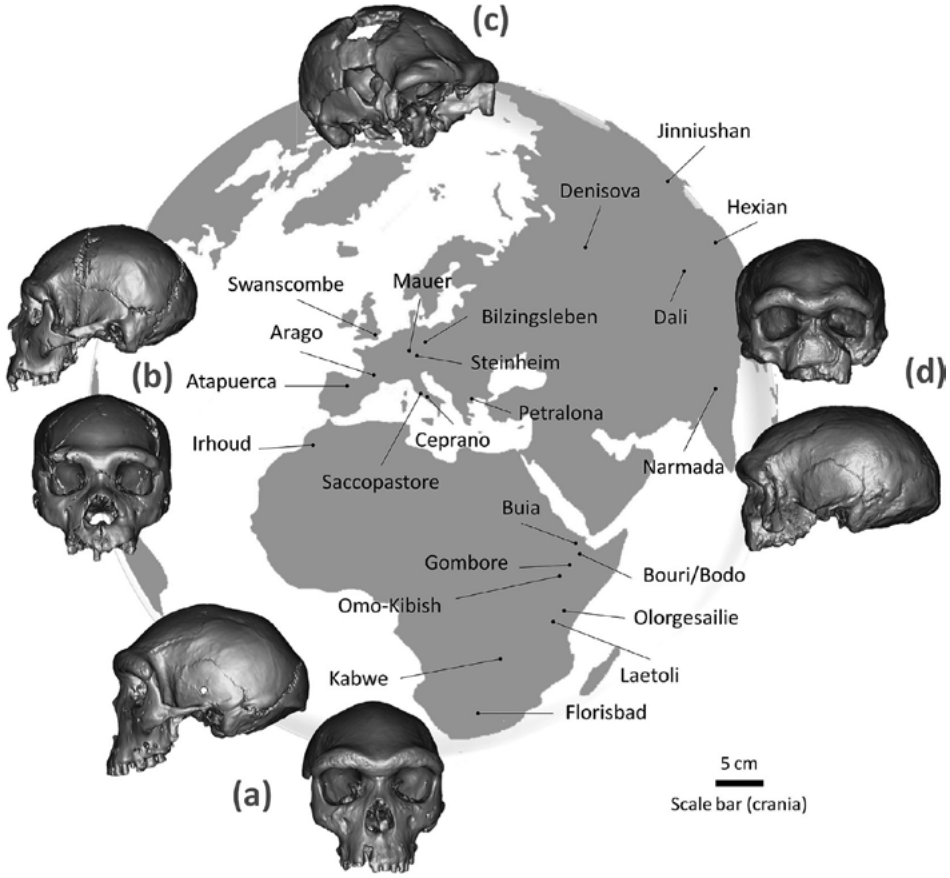

Figure 2. Distribution of sites mentioned in the text where human fossil specimens have been found, with examples of their variability in cranial morphology (CT-based reconstructions) between approximately 600 and 200 ka in Africa and Eurasia: a) Kabwe 1 (Zambia); b) Sima de los Huesos cranium 5 (Atapuerca, Spain); c) Ceprano (Italy); d) Dali (China). Redrawn from Manzi (2016).

Reasonably, it may be assumed that *H. heidelbergensis* emerged from Africa (e.g., Profico *et al.*, 2016), although an origin in the Near East cannot be excluded (Martinon-Torres *et al.*, 2007; Dennel *et al.*, 2011; Stringer, 2012; Bermúdez de Castro and Martinon-Torres, 2013). The African origin is also consistent with the observation that the occurrence in the fossil record of *H. heidelbergensis*

mostly relates to the dispersal from Africa into Eurasia of the Mode 2 (Acheulean) techno-complexes of the Lower Paleolithic (e.g., Asfaw *et al.*, 1992; Lycett, 2009). Unfortunately, however, in sub-Saharan Africa – as well as in Eurasia – the pertinent period is very poor in fossil evidence (Fig. 1), despite the occurrence of scattered and fragmentary specimens that are nonetheless of great interest, like the partial cranium from Gombore II MK(1-2) (Profico *et al.*, 2016).

Something happened between about 900 ka and 600 ka that led to a new and more encephalised kind of humanity. It is referred here to a single, widespread and progressively polymorphic species, whose more appropriate taxonomic denomination is *H. heidelbergensis*.

Origin and trajectories

A possible answer about the origin of *H. heidelbergensis* comes from the mitochondrial DNA (mtDNA) extracted from a single fragment of human phalanx that has been found within the Denisova cave in the Altai mountains, Southern Siberia; it has been dated to 48-30 ka (Krause *et al.*, 2010).

In the context of episodic occupations of this site during the Late Pleistocene, the stratigraphic sequence where the small specimen was found contains both Middle and Upper Paleolithic assemblages, which are commonly referred to Neanderthals (whose geographical distribution reached this area of Northern Asia) and modern humans (which at that time were already spreading in large part of Eurasia) respectively.

Surprisingly, the sequenced mtDNA that had been extracted from the Denisova phalanx pointed to humans that were different from both *H. neanderthalensis* and *H. sapiens*, but that shared with them a common ancestor around one million of years BP (Krause *et al.*, 2010). As a working hypothesis, this suggested that the Denisova phalanx might represent a still unknown variety of humans – provisionally named Denisovans – that originated before the beginning of the Middle Pleistocene, interestingly (let me add) this time span just antedates the appearance of *H. heidelbergensis* in the fossil record (Fig. 1).

As a matter of fact, given the chronological framework that is obtained in combining fossil and molecular data, we may speculate that the Denisovans were in relationships with a "non-*erectus*" occupation of mainland Asia (Krause *et al.*, 2010). Thus, excluding *H. erectus* from the scope of possibilities, we need to look to other humans that were in that continent during the late Middle Pleistocene and in the early Late Pleistocene, focusing on specimens such as Dali and Jinniushan, which in the past had been ascribed to *H. sapiens daliensis* (Wu, 1981) and are currently considered by various authors as representatives of the Easternmost populations of *H. heidelbergensis* (e.g., Stringer, 2012), corresponding to specimens mentioned in the Introduction.

Further analyses led the researchers to publish additional data, including those on the exceptionally preserved nuclear DNA from the same phalanx (Reich *et al.*, 2010), which suggested affinities with the Neanderthals, even closer than those expected from the mtDNA. The scenario that has been inferred from these new data suggested that the Denisovans were a sister group of the Neanderthals, "with a population divergence time of one-half to two-thirds of the time to the common ancestor of Neanderthals and modern humans" (Reich *et al.*, 2010, p. 1057). However, Reich *et al.* (2010, p. 1057) also admit that, "other, more complex models could explain the data". As a matter of fact, in my view, the occurrence of gene flow across Eurasia, between the ancestors of both Neanderthals and the Denisovans is a better explanation of their affinities in nuclear DNA (Fig. 3). This is also indirectly suggested by the subtle signals coming from the mitochondrial and nuclear DNA extracted from specimens belonging to the European sample from Sima de los Huesos in the Sierra de Atapuerca, Spain (Meyer *et al.*, 2014, 2015).

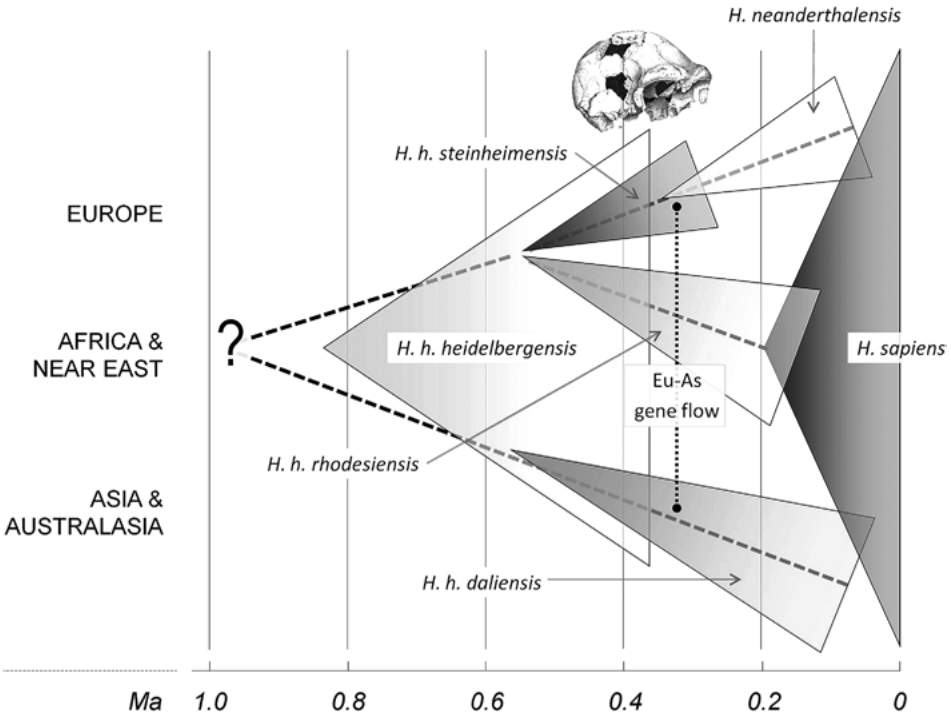

Figure 3. *H. heidelbergensis* and its subspecies in time and space, as suggested in this paper. Each triangle represents a subspecies of this taxon or a derived species (i.e., *H. neanderthalensis* and *H. sapiens*); evolutionary trajectories (dashed lines) and possible Eurasian gene flow (dotted line) between lineages are shown. A drawing of the cranium from Ceprano (Italy, 400 ka ca.) is also reported; it represents a proxy for the original morphology of *H. heidelbergensis*. Redrawn from Manzi (2016).

We should add that the observed genetic diversity between Neanderthals and modern humans points to a coalescence around 500 ka (Green *et al.*, 2008; Briggs *et al.*, 2009; Endicott *et al.*, 2010), substantiating previous conclusions based on morphology and paleogeography, which suggest isolation and divergence between the European and African lineages during the Middle and the early Late Pleistocene (after Santa Luca, 1978; see Fig. 3). Looking at the hypodigm of *H. heidelbergensis* as a whole, in fact, a considerable amount of variability occurred during the Middle Pleistocene, suggesting that the populations of this species bore regional features – in Africa, Asia and Europe respectively – due to a well-known phenomenon referred to as "isolation by distance" (Wright, 1943).

Evolving subspecies

To resume, looking at the fossil record of the Middle Pleistocene in Africa and Eurasia, a disruption is observed between the late representatives of early-established variants of the genus *Homo* (including *H. ergaster, H. erectus* and other, more local and rather derived, species), on one hand, and *H. heidelbergensis*, on the other hand, which were the humans of the Middle Pleistocene in Africa and Eurasia.

The latter species may be viewed as a widely-diffused and derived taxon, which was ancestral in turn to Neanderthals, Denisovans and modern humans. Only a few specimens among the potential hypodigm of this species – particularly, the calvarium from Ceprano, Italy (Ascenzi *et al.*, 1996; Manzi *et al.*, 2001; Manzi, 2016) – display a combination of features that are suitable to represent the ancestral morphotype of this species. Moreover, the hypodigm of *H. heidelbergensis* shows a considerable variability (Mounier *et al.*, 2009, 2011), given that significant phenotypic variations are observed on a wide geographical horizon and even locally.

Combining all the available elements, *H. heidelbergensis* may be considered as a species that probably originated in Africa (Figs. 1, 3) and then geographically diffused, showing a progression of phenetic (and genetic) diversification. Given this intraspecific variability, it may be useful to make further distinctions at the sub-specific level, assuming also the possibility of interbreeding among different demes.

In this light, the use of sub-specific ranks within *H. heidelbergensis* appears to me mandated and useful. According to Mayr (1942: p. 155): "every species that developed through geographic speciation had to pass through the subspecies stage." As a matter of fact, in my view, *H. heidelbergensis* clearly includes

regional incipient species, which apparently anticipates the allopatric speciation of *H. neanderthalensis* (in Europe), *H. sapiens* (in Africa) and, possibly, that of the Denisovans.

I have suggested elsewhere (Manzi, 2012, 2016; Manzi and Di Vincenzo, 2012) the introduction of a trinomial nomenclature for this taxon. My suggestion is to distinguish a stem variety and three geographical lineages, as in the schematic representation shown in Figure 3. Using names already available in the literature – i.e., according to rules of the International Code of Zoological Nomenclature (see at http://www.nhm.ac.uk/hosted-sites/iczn/code) – the proper denominations for these subspecies, consistently with their respective distribution in time, space and morphology, should be:

- *H. heidelbergensis heidelbergensis* (Schoetensack, 1908) – this subspecies represents the ancestral and still largely unknown variety of *H. heidelbergensis* that might be represented by the name-bearing type, the mandible from Mauer, and other specimens that are either demonstrably archaic and/or not clearly involved in any regional lineage; this group would include fossil crania such as Arago and Ceprano in Europe, Gombore II MK(1-2) in Africa and, possibly, Hexian in Asia;
- *H. heidelbergensis daliensis* (Wu, 1981) – this is the Asian non-*erectus* sample that is chronologically interposed between Dali (China, type specimen of this subspecies) and the meagre, but paleogenetically much informative material from Denisova; it includes therefore crania such as those from Narmada (India), as well as Dali and Jinniushan (China);
- *H. heidelbergensis rhodesiensis* (Woodward, 1921) – this sub-species include the African fossil record of the Middle Pleistocene preceding the appearance of modern humans (*H. sapiens*); it is represented by the type specimen from Kabwe (or Broken Hill 1) and other penecontemporaneous specimens (Bodo, Elandsfontein), but also later samples from various part of the continent sometimes referred to as "archaic *H. sapiens*" (Brauer, 1984; Hublin *et al.*, 2017), such as Djebel Irhoud, Florisbad, Eliye Springs, Ngaloba and Omo Kibish II;
- *H. heidelbergensis steinheimensis* (Berckhemer, 1936) – eventually there is the European lineage of the Middle Pleistocene leading to the Neanderthals (*H. neanderthalensis*), including the type specimen from Steinheim, other crania such as Petralona, Reilingen, Swanscombe and, most notably, the extremely reach assemblage from Atapuerca Sima de los Huesos.

Acknowledgements

This is my personal homage to Alessandro Minelli; it is devoted to his seventy

years of life, largely dedicated to a remarkable and internationally renowned contribution to studies in evolutionary and developmental biology. Congratulations Sandro, for many more years of active life on this path! At the same time, it represents a brief overview of the approach I followed in understanding human evolution before the origin of *H. sapiens*. For these combined reasons, I am grateful to both the editor of this volume, for his appreciated invitation, and to all the authorities, institutions and colleagues that have been fundamental in my previous research on this issue.

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Phylo-evo-devo, tardigrades and insights into the evolution of segmentation

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Abstract

The concept of phylo-evo-devo highlights the benefits of reciprocal illumination between studies of phylogeny and of developmental biology when studying character evolution. Here we consider the case of the evolution of a segmented body plan within the major animal clade of Ecdysozoa. Specifically, we consider developmental studies supporting the homology of segmentation between Arthropoda, Onychophora and Tardigrada. In parallel, we examine inconclusive results regarding the possible phylogenetic affiliations of the tardigrades. If tardigrade arthropod and onychophoran segmentation is indeed homologous, molecular phylogenies supporting a sister group relationship between tardigrades and nematodes imply a loss of segmentation in the latter. To progress, we need both fully resolved phylogenies, and more developmental studies of 'lesser' groups including tardigrades and even more obscure, segmented ecdysozoan clades such as the Kinorhyncha (mud dragons).

Introduction

The term *phylo-evo-devo* was coined to highlight the potential for reciprocal illumination between the fields of developmental biology and phylogenetics (Minelli, 2009). Minelli's original example of the working of phylo-evo-devo concerned the evolutionary developmental origin of the reduced forewings of the Strepsiptera (twisted wing flies). Early phylogenetic analyses suggested that these unusual parasitic insects were closely related to the Diptera (whose hind wings are themselves reduced to halters) (Whiting *et al.*, 1997). This phylogenetic relationship suggested the intriguing possibility that the common ancestor of Diptera and Strepsiptera, both of which have a single pair of wings, was itself

G. Fusco (ed.) *Perspectives on Evolutionary and Developmental Biology.*

two winged and that the reduced wings were an homologous character between the two groups. The really intriguing inference from this scenario stems from the observation that the pair of wings that are reduced in the two orders (fore wing in Strepsiptera versus hind wing in Diptera) are not on homologous segments, implying that the reduced wings, if homologous, must have swapped segmental position between the two groups by some amazing homeotic change (Whiting and Wheeler, 1994). Subsequent phylogenetic studies solved the problem by showing that the apparent link between Strepsiptera and Diptera was a tree reconstruction artefact stemming from the unequal rates of evolution amongst the insect lineages (Wiegman *et al.*, 2009). New data and improved analyses showed that the fast evolving Strepsiptera were in fact related to the Coleoptera (as morphologists had originally suggested) and that the reduced forewings of Strepsiptera might therefore be related to the tough elytra (modified forewings) seen in the beetles.

Here we focus on a similar phylo-evo-devo problem involving questions of both phylogeny and of homology. We consider the likely homology of segmentation between Tardigrada (the water bears), Onychophora (velvet worms) and Arthropoda (Chelicerata (arachnids, horseshoe crabs and pycnogonids), Myriapoda (centipedes, millipedes and their allies), and Pancrustacea (crustaceans including insects)). Alongside this, we discuss the, still contentious, question of the phylogenetic position of the tardigrades (Fig. 1). While the solution to the first part of this two-sided problem seems fairly straightforward – phylogeny, morphology and developmental genetics all suggest the homology of tardigrade and arthropod segmentation (and therefore its presence in their common ancestor), a solution to the second part of the problem, a precise phylogenetic placement for the tardigrades, is still lacking. This results in a lack of understanding of the deep evolutionary history of segmentation.

Homology of segmentation in Arthropoda and Tardigrada

As is typical for a phylo-evo-devo question, when considering whether the segmentation in Tardigrada, Onychophora and Arthropoda is homologous, we first need to know whether these lineages are closely related. Phylogenetic proximity implies homology through the simple argument that the distribution of a putatively homologous character can be parsimoniously reconciled with the known phylogeny (Telford and Budd, 2003). As we will see in the following section, whatever the unknowns regarding the exact position of the tardigrades, they are nevertheless generally agreed to be relatively closely related to the Arthropoda and Onychophora, even if not necessarily their sister group (e.g. Telford *et al.*, 2008).

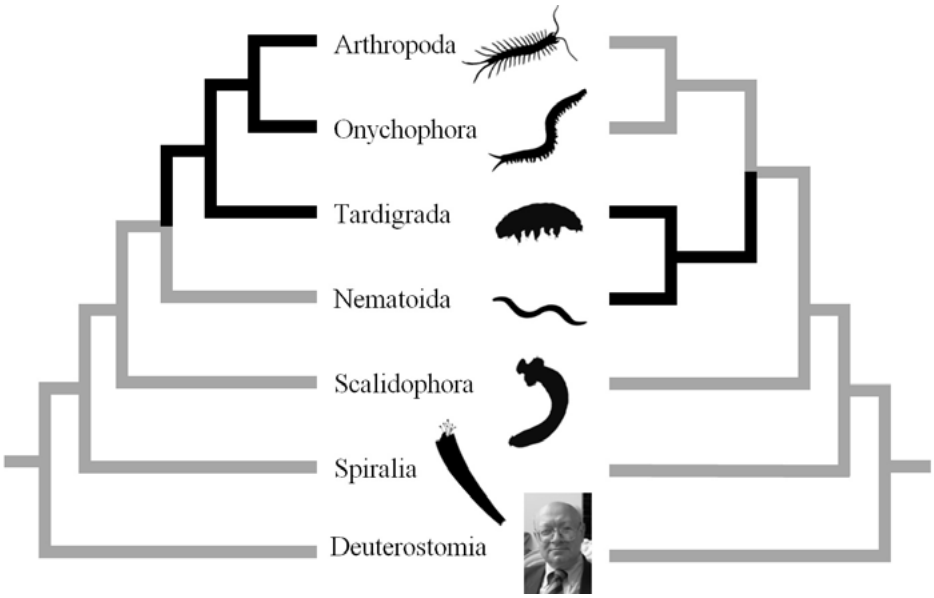

Figure 1. Two hypotheses of Tardigrada affinity. On the left they are shown as sister to the other Panarthropoda, on the right as sister to Nematoida. Animal silhouettes are from PhyloPic.org (Creative Commons, CC0 1.0 Universal).

The second part of the determination of homology depends on a consideration of the degree and detail of similarity of segmentation between the groups at a morphological, embryological and developmental genetic level. Again, detailed similarities would be most parsimoniously explained by homology rather than convergence (Telford and Budd, 2003). On this score, although there are still limited data from the Tardigrada, there is every reason to believe that arthropod and tardigrade segmentation is homologous. While the details of plausibly common features of segmentation vary (and there is reason to believe that tardigrades have changed a lot from the ground state due to miniaturisation), Arthropoda and Tardigrada have similar arrangements of similar components within their segments suggesting homology. Tardigrada, Onychophora and Arthropoda all possess paired ventro-lateral appendages with terminal claws associated with a subset of their segments, all have segments in which the segment polarity gene *engrailed* is expressed in the dorsal portion of each segment (Gabriel and Goldstein, 2007) and there are clear detailed similarities between the segmental ganglia of Tardigrada and Arthropoda at least (Mayer *et al.*, 2013). Finally, albeit a less direct correspondence, the expression domains of orthologous Hox genes coincide with segmental boundaries in a similar manner across all putative panarthropod groups (Smith *et al.*, 2016). Ultimately, the linking of

Arthropoda, Onychophora and Tardigrada within Ecdysozoa and the detailed similarities of their segmental make up mean there is little doubt that their common ancestor was segmented.

The phylogenetic relationships of the segmented animal phyla

Tardigrada, Onychophora and Arthropoda were traditionally grouped in the Articulata (Cuvier, 1817), alongside the other coelomate, segmented protostome phylum, the Annelida (earthworms, leeches, etc.). Articulata was a surprisingly long-lived concept and perhaps the most significant early victim of the application of numerical cladistic methods and molecular phylogenetics (see Minelli 1993 for an overview). Eernisse *et al.* (1992) were the first to find strong morphological evidence for the separation of Annelida from the other segmented phyla. Soon after the study of Eernisse *et al.* (1992), Aguinaldo *et al.* (1997) provided the first molecular evidence (using 18S rRNA) for a clade of ecdysing (i.e., moulting) animals, that they named the Ecdysozoa. The study of Aguinaldo *et al.* (1997) was seminal because of its careful taxon selection and the sophisticated phylogenetic methodologies (including maximum likelihood) it implemented. The same clade was soon after also recovered using broad taxon sampling, i.e., adding many nematodes including "long branched" ones, more ecdysozoan phyla, and using other tree reconstruction methods (Giribet and Ribera, 1998). Ecdysozoa includes a diversity of segmented phyla (Arthropoda, Tardigrada, Onychophora and also the Kinorhyncha or mud dragons, in which segments are referred to as zonites) and four non-segmented phyla (Priapulida – penis worms, Nematoda – roundworms, Nematomorpha – Gordian worms, and Loricifera – the loricated animals).

While the general rejection of Articulata was almost immediate, the monophyletic status of the Ecdysozoa was initially debated, as many early studies failed to find support for the inclusion of the nematodes in Ecdysozoa (e.g., Blair *et al.*, 2002; Wolf *et al.*, 2004; Philip *et al.*, 2005; Zheng *et al.*, 2007). Improved taxon sampling, the development and application of more sophisticated evolutionary models and methods, analyses of rare genomic changes and the presence of specific genes have now broadly confirmed the monophyly of Ecdysozoa (e.g., Telford, 2004; Telford *et al.* 2015; Philippe *et al.*, 2005; Irimia *et al.*, 2007; Holton *et al.*, 2010). While Ecdysozoa is now universally considered a valid lineage, the relationships between the phyla constituting the Ecdysozoa have proven harder to resolve, with the relationships of the Tardigrada being particularly contentious.

Within Ecdysozoa, Priapulida, Kinorhyncha and Loricifera may constitute a monophyletic lineage – the Scalidophora (Schmidt-Rhaesa *et al.*, 1998), al-

though there remains uncertainty over the inclusion of the long branched and poorly sampled Loriciferans within this group (e.g., Yamasaki *et al.*, 2015; Giribet *et al.* 2017). Similarly, Nematoda and Nematomorpha are generally grouped together as the Nematoida (e.g., Dunn *et al.*, 2008; Campbell *et al.*, 2011; Borner *et al.*, 2014; Yoshida *et al.*, 2017). Finally, it is generally agreed that Onychophora and Arthropoda share a common ancestor to the exclusion of Nematoida and Scalidophora (e.g., Dunn *et al.*, 2008; Campbell *et al.*, 2011; Borner et al., 2014; Yoshida *et al.*, 2017). The relationships between Tardigrada, Onychophora + Arthropoda and Nematoida, however, are still debated (Fig. 1). While morphology clearly links the segmented, jointed-legged tardigrades to the Onychophora + Arthropoda in a monophyletic Panarthropoda, molecular phylogenetic analyses have been ambiguous, with some studies recovering Panarthropoda (Campbell *et al.*, 2011; Rota-Stabelli *et al.*, 2011) but most others resolving Tardigrada as the sister group of Nematoida (Dunn *et al.*, 2008; Borner *et al.*, 2014; Yoshida *et al.*, 2017). Considering the long branches leading to both the Nematoida and Tardigrada there is a suspicion that the Nematoida plus Tardigrada grouping is a long branch attraction artefact. Borner *et al.* (2014), for example, found that the signal for Nematoida plus Tardigrada was preferentially found in fast evolving sites (see Philippe *et al.*, 2001; Campbell *et al.*, 2011; Rota-Stabelli *et al.*, 2011). The situation is further complicated by the fact that, while Campbell *et al.* (2011), Borner *et al.* (2014) and one of the analyses of Yoshida *et al.* (2017) found Nematoida plus Tardigrada to be the sister group of Arthropoda, Dunn *et al.* (2008) found Tardigrada to be the sister group of Nematoida within the context of a monophyletic Cycloneuralia (i.e., Scalidophora plus Nematoida).

Tardigrade genomes and implication for phylogeny

A recent important new contribution to the question of Tardigrade affinities came from the analysis of their genomes. Compared with most other animals, tardigrades possess rather compact genomes (55-104 Mb). Interpreting tardigrade genomics in an evolutionary context, however, proved to be challenging, and the results of these interpretations seem to be nothing short of enigmatic. The first genome revealed an unprecedented high level (17%) of genes acquired from other organisms through Horizontal Gene Transfer (Boothby *et al.*, 2015), but subsequent reanalyses and new genomes showed that this estimate was heavily biased by a poor filtering of contaminants (Koutsovolous *et al.*, 2016; Bemm *et al.*, 2017).

Tardigrade genomes seem to contain contradictory phylogenetic signals. While phylogenies based on concatenated genes tend to support tardigrade as sister to Nematoida (even though this result is model dependent), analyses using rare changes, such as presence of specific orthologs, support tardigrades as

sister group to arthropods (Hashimoto *et al.*, 2016; Bemm *et al.*, 2017; see also Borner *et al.*, 2014). Considering that tardigrades and nematodes are obvious candidates for being affected by long branch attraction (Campbell *et al.*, 2011), it is clear that the use of well-fitting models and testing for specific artifacts (as in Campbell *et al.*, 2011 and Feuda *et al.*, 2017) is key to using tardigrade genomic data for phylogenetic analyses.

Discussion

We have seen that, despite limited data from tardigrades, segmentation seems highly likely to have been present in the common ancestor of Tardigrada and Arthropoda. However, and fittingly in this examination of phylo-evo-devo, we need new knowledge of both phylogeny and development if we are to understand the evolution of segmentation in the arthropods better (and its potential loss in Nematoida and perhaps elsewhere).

First is the pressing need to establish the true relationships between major ecdysozoan groups, most obviously to establish whether the Tardigrada are the sister group of Arthropoda + Onychophora or Nematoida. Assuming homology of tardigrade and arthropod segmentation, this latter possibility would force us to conclude that segmentation has been lost in Nematoida.

Second is the wish to know more about the relationship between segmentation in arthropods and other protostomes. Are zonites in kinorhynchs homologs of arthropod segments, making the ecdysozoan ancestor segmented? Could the old concept of homology of annelid and arthropod segmentation be correct, making the protostome ancestor segmented (Balavoine, 2014)?

Minelli observed (Minelli, 2009, p. 2) that "We now have more and more robust phylogenies and deeper insights into evolutionary variations of developmental mechanisms, but the challenge is to understand the data in an integrated phylo-evo-devo framework." What we have seen in this examination of segmentation and phylogeny in Ecdysozoa is that we have yet more work to do to achieve truly robust phylogenies (Telford *et al.*, 2015) and we must gain even deeper insights into developmental mechanisms from more fascinating, if obscure, animal groups such as water bears and mud dragons.

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Part IV Evolving organism features

Hyper-epigyny is the ultimate constraint on orchid floral morphology and an ideal model for testing the Extended Synthesis

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Abstract

The developmental morphology and genetics of the orchid flower is described in order to explore the evolutionary 'no man's land' that separates the Extended Synthesis from the Modern Synthesis. The gynostemium, ubiquitous among orchids and developed through congenital fusion (and dorsal suppression) of fertile reproductive organs, is an unbreakable evolutionary constraint of high burden. We agree with Mondragón-Palomino and Theissen that the fundamentally tripartite bauplan of six tepals of three morphologies constitutes a remarkably robust 'super-organ' but we would include the gynostemium in the super-organ concept. Within the fundamental constraint of this super-organ, intersecting clines of expression overlain by extensive pleiotropy are hypothesised to cause sufficient mosaicism and heterochrony to permit the evolution of vast numbers of trivially distinct species. We cannot yet estimate the contribution to evolvability of natural selection – directly through adaptation and indirectly through exaptation – relative to the non-aptations that must by definition reflect the many other causes of evolution.

Preamble: constraint versus adaptation

Few terms in evolutionary biology are employed as frequently as 'constraint' or explicitly defined as rarely. Even a cursory examination of the literature reveals that there is little consistency among authors in the meaning attributed to this term (e.g., Antonovics and van Tienderen, 1991). The near-random spectrum of definitions presented as Appendix A even differ radically in the identity of the adjective that precedes and qualifies 'constraint.' Using 'developmental constraint' or 'phylogenetic constraint' at least narrows the intended meaning to specific disciplines within biology, though surprisingly, these two disciplines

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were viewed as synonymous by West-Eberhard (2003, p. 25). Recognising an 'evolutionary constraint' arguably encompasses an unmanageably wide suite of possible meanings, and 'biological constraint' is too ridiculously broad in scope to offer any real utility. In addition, the definitions of 'constraint' summarised in Appendix A differ in their primary target; most apply to either a demographic unit (typically the population level: i.e., a collection of related organisms) or a character state/trait (i.e., one or more features of those organisms).

Interestingly, the one apparent common denominator of these contrasting definitions lies in identifying adaptation – the downstream hand-maiden of neoDarwinian evolution – as the converse of constraint. It might therefore prove easier to agree a definition of 'constraint' if we can first agree upon a definition of its supposed antithesis, adaptation. Unfortunately, doing so proves equally problematic. Consider, for example, the definition of 'adaptation' presented in Wikipedia (as accessed in August 2018): "the dynamic evolutionary process that fits organisms to their environment [...] [and] a phenotypic trait with a functional role in each individual organism that is maintained by, and has been evolved by, natural selection." This statement is effectively two definitions rather than one; the first makes 'adaptation' a verb representing an evolutionary process, whereas the second makes 'adaptation' a noun representing a character state/trait of an organism. We assume that the trait in question is expected to be the product of the underlying evolutionary process. In most definitions, this causative process is specified to be natural selection (presumably directional or disruptive selection, given that stabilising selection is by definition a force for stability rather than change).

We now focus briefly on the definition of adaptation that features a trait rather than a process, seeking practical means of identifying adaptations. Conventional wisdom states that recognition of an adaptive feature of an organism requires that the feature in question should satisfy all four of the following criteria: it must be (1) functional, (2) heritable, (3) increase organismal fitness, and (4) have originated through natural selection. While provisionally accepting these criteria, we note from the outset that ticking all four of these boxes is no easy task for the evolutionary biologist. When presented with a trait that apparently fulfils the readily demonstrated criteria (1) and (2) and the far less readily demonstrated criterion (3), it is tempting to simply assume congruence with the extremely challenging criterion (4).

In his stimulating and provocative essay *Grand challenges in evolutionary developmental biology*, Minelli (2015, p. 3) argued that "It is true that factors other than natural selection, such as developmental constraints, can plausibly account for the unequal filling of morphospace. However, whenever developmental biology is able to demonstrate that morphologies that would occupy currently

empty parts of the morphospace can nevertheless be readily produced, this will turn into a rejection of the constraint hypothesis and lend instead support to hypotheses of adaptation." Comments made later in his essay add nuance to this statement, but many other evolutionary biologists lack such nuanced views and simply assume adaptation. Our primary concern is that the widespread perception of evolution as merely a balance between the yin of natural selection (as manifested through adaptation) and the yang of constraint leaves no room for the additional evolutionary mechanisms that provide the ongoing impetus to the first (fairly) explicit manifesto of the evolutionary-developmental genetics community, the Extended Synthesis (*sensu* Pigliucci and Müller, 2010).

Here, we have chosen to explore evolution in general – and the relationship between constraint and adaptation in particular – through the lens of current evolutionary-developmental knowledge of the orchid flower. To assist this goal, we have attempted our own definitions of key evolutionary terms in Appendix B.

The nature of the orchid flower

Compared with the ongoing "mystery" posed by the evolutionary origin of the angiosperm flower, students of the origin of the orchid flower benefit from a wealth of relevant information obtained from the extant flora (admittedly, the situation contrasts strongly in the fossil record, where evidence of orchids is remarkably sparse: e.g., Ramirez *et al.*, 2007; Gustafsson *et al.*, 2010; Poinar and Rasmussen, 2017). Research has been encouraged by the distinctiveness and complexity of the flowers, multiple symbiotic relationships, and ever-increasing horticultural importance of the orchid family. Its (crudely) estimated 18,000–25,000 extant species encompass a predictably large spectrum of both floral and vegetative diversity that is distributed among five increasingly well-delimited subfamilies (Rudall and Bateman, 2002; Deng *et al.*, 2015). Moreover, comparison with the closest outgroups suggests that the few members of the earliest diverging of the five subfamilies possess morphological features that indicate a primitive and potentially near-ancestral condition for the family, despite the estimated 25–45 Myr of evolution that separate the stem node, dated to the Early Cretaceous, and crown node, dated to the Late Cretaceous at *ca* 80 Ma (Gustafsson *et al.*, 2010; Eguchi and Tamura, 2016; Zhang *et al.*, 2017).

After gymnosperms had spent the first *ca* 250 Myr of their existence maximising the spatial distance separating the 'male' and 'female' reproductive functions, the evolutionary origin of (morphologically recognisable) angiosperms condensed the two genders into the single organ complex that constitutes the typical angiosperm flower (e.g., Bateman *et al.*, 2006, 2011; Endress, 2006; Rudall *et al.*, 2011). This profound rapprochement between 'male' and 'female' func-

tions was later taken to its extreme expression with the origin of the orchid family, when fusion of organs of the two genders formed their characteristic, arguably unique gynostemium – such comprehensive fusion has been termed by us hyper-epigyny (Rudall and Bateman, 2002; Rudall *et al.*, 2013). It involves the congenital integration of reproductive structures from their inception onward with concomitant loss of organ boundaries, such profound synorganisation (or "congenital union" *sensu* Sattler, 1978; Verbeke, 1992), leaving organ homologies highly cryptic. The resulting distinctive floral phenotype fits well Minelli's (2015, 2017) category of "misfits by synorganisation." The gynostemium and its contiguous inferior ovary have been the occasional subject of ontogenetic investigations via microscopy, but as targeted gene studies currently give way to data-rich genomic studies, the processes underpinning this important evolutionary transition are being brought into sharper focus.

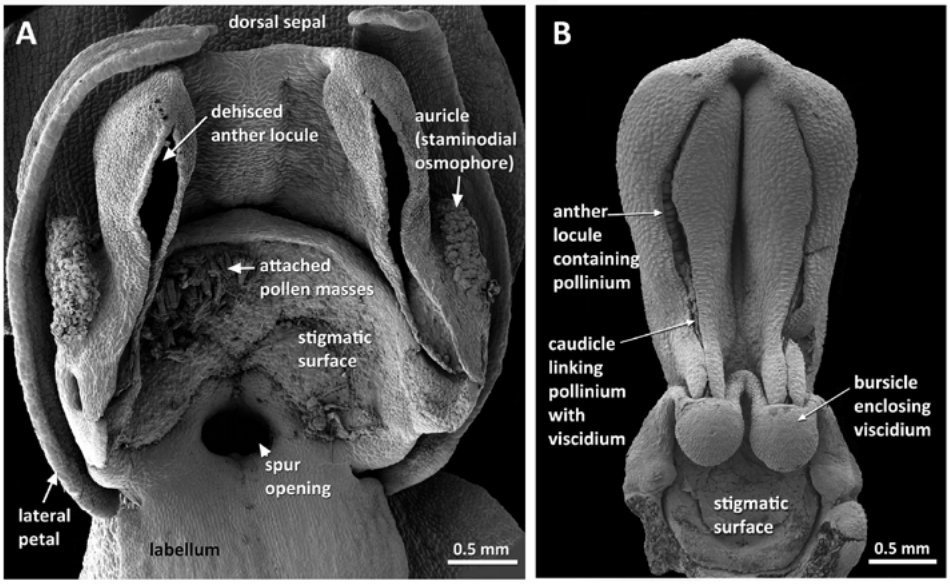

Figure 1. Labelled scanning electron micrographs of (A) the gynostemium and basal portions of the perianth of *Platanthera chlorantha* and (B) the gynostemium of *Ophrys speculum lusitanica* (*vernixia*) (both Orchidaceae: Orchidoideae).

Although most structural ontogenetic studies are reasonably holistic, most genetic explorations have focused heavily on the perianth of the orchid flower, which famously consists of two whorls, closely spaced along the primary axis of the flower and located distal to the ovary, each consisting of three segments that are often collectively termed tepals (Figs. 1A, 2A). In a typical orchid flower there is evident at least modest differentiation between the two whorls and also

between the single median and two lateral members of each whorl, the morphological distinction typically being less within the proximal sepal whorl than within the (slightly) more distal petal whorl. The median petal, termed the labellum and often misrepresented as being unique to the orchid family, is in almost all orchids the most morphologically complex of the six perianth segments. The 180° resupination achieved by the pedicel of most orchid flowers places the labellum lowermost, where it can best function as a landing stage for potential pollinators. The tricarpellate inferior ovary contains numerous minute seeds that lack endosperm. Most orchid flowers are profoundly zygomorphic distal to the ovary, the bilateral symmetry being most clearly evident in the comparative complexity of the labellum (Fig. 2A). It is less often noted that bilateral symmetry is also fundamental to the morphology of the gynostemium in terms of the positioning relative to the vertical plane of the stigmatic surface, the fertile (i.e., pollinaria-generating) anthers and the infertile staminodes (homologous with suppressed stamens, which are differentially suppressed in contrasting orchid subfamilies; Rudall and Bateman, 2002) (Fig. 1).

Here, we explore the implications for both adaptation and constraint of the origin of the orchid flower, keeping at the back of our minds throughout the discussion our particular interest in the evolutionary-developmental origin of the all-important gynostemium.

The MADS-box era

At the beginning of the 21st Century, building on extensive morphological and developmental knowledge, and on a more limited range of developmental genetic observations largely based on candidate-gene studies that began with Lu *et al.* (1993) and were achieved via Sanger sequencing, we (Rudall and Bateman, 2002) and others (e.g., Johansen and Frederiksen, 2002) deliberately laid out a blueprint for evolutionary-developmental genetic (evo-devo) studies of the orchid flower. Resource constraints meant that the majority of such studies merely described a single phenotype rather than comparing two or more phenotypes, and thereby qualified only as developmental genetic studies rather than evolutionary-developmental studies. Parallel technological constraints meant that, during the late 1990s and 2000s, most investigations used Sanger sequencing to target candidate genes. Inevitably, these studies focused on the ancient, transcription-coding MADS-box gene family. In particular, within the comparatively evolutionarily conservative Type II clade, MIKC^c-type MADS-box genes had already been demonstrated, through studies of model organisms such as *Arabidopsis* and *Antirrhinum*, to have major impacts on floral phenotypes (Coen and Meyerowitz, 1991, *et seq.*).

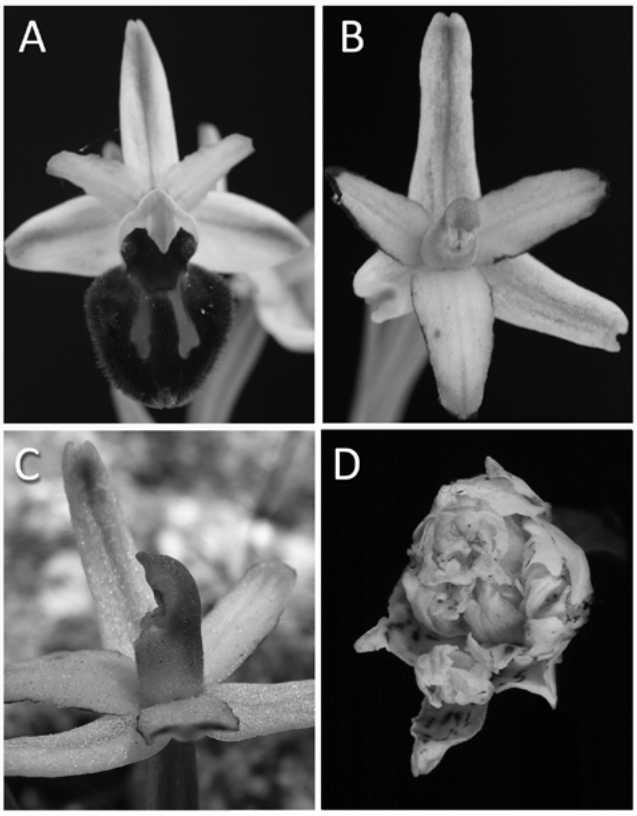

Figure 2. Radical phenotypic shifts due to presumed mutations in two orchidoid orchids. (A–C) compare a wildtype (A) and a peloric (B, C) flower of *Ophrys sphegodes panormitana*, illustrating the developmental robustness of the gynostemium in the face of radical simplification of the perianth. (D) shows a flower of *Dactylorhiza fuchsii* that has entirely lost determinacy, consequently attempting to produce an infinite spiral of increasingly poorly-developed flowers. Much wider ranges of orchid floral mutants were illustrated and discussed in an evo-devo context by Bateman *et al.* (2006), Duttke *et al.* (2012), Mondragón-Palomino (2013), Huang *et al.* (2017), Su *et al.* (2018).

Given that evidence was rapidly accumulating of extensive, potentially evolutionarily crucial, whole-genome duplications across the land-plant clade (e.g., De Bodt *et al.*, 2005; Tank *et al.*, 2015), it was inevitable that discussions of MADS-box genes would focus on the number and phylogenetic relationships of genes in particular gene subfamilies and be couched in terms of the now (in)famous 'ABC(D)E' model of floral developmental control – a model derived from equally model organisms. Framed simplistically, A-function genes alone specify sepals, A- plus B-function specify petals, B- plus C-function specify stamens and C-function alone specify carpels (D function, impacting primarily on

ovules, can usefully be regarded as subfunctionalisation of the closely similar C-function). The ensuing search for co-factors to the readily dimerised ABC-encoded proteins led to the quartet model of multimeric protein complexes (Theissen and Saedler, 2001), which required further (E-factor) proteins to dimerise the ABC proteins throughout the flower. Later research effectively downgraded the perceived significance of A-factor genes, showing that they are actually phylogenetically localised within the angiosperm clade and that E-function proteins alone are sufficient to generate sepals. Each of these four classic gene functions (A–C, E) is fulfilled by a different gene family, two distinct subfamilies being recognised within each of the B and E function families that together lie at the core of flower development (Melzer *et al.*, 2010; Pan *et al.*, 2014; Theissen *et al.*, 2016; Dodsworth, 2017).

Botanical MADS-box studies soon graduated from the initial model organisms to consider less tractable groups, not least orchids. Inevitably, given their horticultural importance, early evo-devo studies of orchids focused on horticulturally dominant tropical epiphytes of subfamily Epidendroideae such as *Phalaenopsis*, *Oncidium*, *Cymbidium* and *Dendrobium* (e.g., Tsai *et al.*, 2004; Chang *et al.*, 2009, 2010; Mondragón-Palomino and Theissen, 2011; Pan *et al.*, 2011, 2014; Wang *et al.*, 2011; Hsu *et al.*, 2014). Progressive understanding of floral bauplan development in orchid flowers is well documented in the works of Mondragón-Palomino and Theissen (2008, 2009, 2011; Mondragón-Palomino *et al.*, 2009; reviewed by Mondragón-Palomino, 2013), who christened their conceptual model the 'orchid code'. Expression in orchid flowers of most classes of MADS-box genes is fairly conventional. Class A (*FRUITFULL*-like), C (*AGAMOUS*-like) and ovule-determining D (*SEEDSTICK*-like) genes are expressed primarily in the gynostemium, often extending proximally into the ovary, whereas class E (*SEPALLATA*-like, here considered to include the *AGAMOUS*-like 6 clade) and class B subclass *GLOBOSA*-like (= *PISTILLATA*-like) genes are expressed throughout the flower. The orchid code is actually constructed primarily upon increasing evidence that orchids reliably maintain several functional class B subclass *DEFICIENS*-like (= *APETALA3*-like) genes, which are presumed to be the products of gene duplication. The model requires four clades of such genes, clades 1 and 2 being expressed substantially in all perianth segments except the labellum, whereas clades 3 and 4 are reputedly expressed at comparatively low levels in the lateral petals and higher levels in the labellum. Expression of these *DEF*-like genes was explored in a more dynamic, developmental context by Pan *et al.* (2011), Hsu *et al.* (2014) and Acri-Nunes-Miranda and Mondragón-Palomino (2014), who noted that expression of clade 3 and 4 genes became localised on the flower far earlier in ontogeny than that of clades 1 and 2. However, more recently, Hsu *et al.* (2015) argued that clade 4 genes do not significantly affect

perianth differentiation in *Oncidium*, thereby placing greater emphasis on clade 3 B-function genes.

The great potential explanatory power of comparing wild-type and teratological orchid flowers was laid out by Rudall and Bateman (2002), elaborated by Bateman and Rudall (2006), tested by Mondragón-Palomino and Theissen (2009), and more recently reviewed by Mondragón-Palomino (2013), who cleverly titled the (epi)mutants 'helpful monsters.' The first developmental genetic comparison of such morphs, published by Tsai *et al.* (2004), provided an early indication of the respective roles fulfilled by the multiple *DEF*-like genes, as well as offering a useful reminder of how minute genetic changes can underpin radical phenotypic shifts. A subsequent study by Mondragón-Palomino and Theissen (2009) developed credible hypotheses to explain all of the categories of terata established by Bateman and Rudall (2006), and a further investigation of *Phalaenopsis* flowers demonstrated how homeotic replacement of the paired lateral petals with additional labella required expanded expression of both clade 3 and clade 4 *DEF*-like genes (Mondragón-Palomino and Theissen, 2011). Similarly, Wang *et al.* (2011) demonstrated that functional copies of both of the C-class genes detected in the epidendroid genus *Cymbidium* were necessary if a gynostemium were to develop rather than the early-stage bud grading ontogenetically into a many-tepalled, essentially indeterminate structure.

Note that, other than stating which MADS-box genes have been shown to be expressed in the gynostemium, we have as yet given little indication regarding whether or not those genes appear to be responsible for the stamen-pistil congenital fusion or stamen suppression patterns that confer on this structure its profound integration and thereby its strongly expressed bilateral symmetry. When viewed within the context of the ABCE model, such integration appears counter-intuitive. We will therefore conclude this section by deviating briefly from MADS-box genes in order to consider the TCP gene family, which has been shown to induce strong dorsiventral clines of expression in some strongly zygomorphic flowers such as *Anthirrhinum* but for many years escaped detailed examination in orchids (e.g., Mondragón-Palomino and Theissen, 2009; Mondragón-Palomino, 2013; Rudall *et al.*, 2013). A gene duplication event early in land-plant evolution is believed to have led to the distinct roles played by the TCP Class I subfamily of putative transcriptional activators and the TCP Class II subfamily of putative transcriptional repressors. In *Antirrhinum* and other core eudicots, the much-discussed Class II genes *CYCLOIDEA* and *DICHOTOMA* famously establish the dorsiventral cline in the perianth of the strongly zygomorphic flowers, operating antagonistically to the MYB family genes *RADIALIS* and *DIVARICATA*. It seemed to us that TCP family genes offered the best prospect of solving the mystery of gynostemium formation through synorganisation.

The long wait was ended by a detailed exploration of TCP genes in the derived epidendroid orchid *Cattleya* (Madrigal *et al.*, 2017). No less than 18 TCP homologues were detected, Class I *CINCINNATA*-like and Class II *PROLIFERATION CELL FACTOR*-like genes being especially well represented. In contrast, *CYC*-like genes are represented by only one copy in *Cattleya* (Madrigal *et al.*, 2017) and in the orchidoid genus *Orchis* (De Paolo *et al.*, 2015) but by two copies in *Phalaenopsis* (Lin *et al.*, 2016). Moreover, *CYC* expression levels proved to be low compared with non-orchid families and its expression to be more generalised within the flower. In particular, similar levels of expression in the labellum and lateral petals suggest that the *CYC*-like gene does not play a significant role in the development of zygomorphy in the flower, contrary to its behaviour in taxonomic families that are as closely related to the orchids as gingers and grasses (e.g., Bartlett and Specht, 2011). The relative unimportance of *CYC*-like genes in determining orchid flower morphology is further suggested by contrasting reports of where within the flower expression (though low) is highest: lateral petals and labellum in *Cattleya*, labellum and dorsal sepal in *Phalaenopsis*, and most remarkably, leaves in *Orchis* (reviewed by Madrigal *et al.*, 2017). Similarly generalised and low-key expression patterns have since been obtained from *Cymbidium* (Su *et al.*, 2018). Such inconsistency and pleiotropy of expression across the three genera also suggest a somewhat reduced role for *CYC*-like genes in determining floral morphology within the orchid family. In summary, present evidence suggests that TCP genes will not move us closer to understanding the evolutionary origin of the gynostemium.

Whole-genome sequencing: panacea or barely relevant distraction?

Over the last few years several analytical techniques collectively termed next-generation sequencing (NGS: e.g., Olson *et al.*, 2016; Dodsworth, 2017) have increasingly replaced Sanger techniques as the 'go to' methods for nucleic acid sequencing. The net result has been a vast increase in the percentage of the total nuclear genome that can readily be sequenced, but also unavoidably entails complex, fully automated data filtration and analysis. Analytical decision-making now arguably depends more heavily on statistical and information technology choices than on purely biological criteria.

To our knowledge, at the time of writing, near-complete genome sequences have been published for only three orchid species. Inevitably, the first two genome-wide sequences were obtained from the evolutionarily derived, species-rich 'model' epidendroids *Phalaenopsis* (Cai *et al.*, 2015) and *Dendrobium* (Zhang *et al.*, 2016), but happily, the third and most recent genome was obtained from *Apostasia*, one of two species-poor genera that constitute the earli-

est-diverging orchid subfamily, Apostasioideae (Zhang *et al.*, 2017). In addition, transcriptomes of large numbers of orthologous low-copy nuclear genes were recently obtained from 13 orchid species that together span all five subfamilies and include all five genera accepted within subfamily Cypripedioideae (Unruh *et al.*, 2018).

Reading some of the more passionate advocacies of NGS approaches would suggest that all our questions regarding orchid evolution will soon be answered. However, the few conclusions so far reached serve mainly to reinforce inferences made by earlier pre-NGS authors. Estimated sequence completeness in these studies has reliably exceeded 90%, the *ca* 1 Gb of sequence data recovered being dominantly repetitive DNA, mainly retrotransposons. Introns are also unusually long, but nonetheless the data predict numbers of protein-coding genes typical of angiosperms: *ca* 29,000 in both *Phalaenopsis* and *Dendrobium* but only *ca* 22,000 in *Apostasia*. The results supported previous assertions that a whole-genome duplication occurred on the branch immediately subtending the orchid crown-group node (dated to 71–81 Ma by Cai *et al.*, 2015), potentially opening the door to extensive neofunctionalisation and subfunctionalisation (e.g. Zhang *et al.*, 2017). We view previously suggested correlation with the Cretaceous–Tertiary boundary event as rather fanciful, given the breadth of the error bars on the estimated date; the possible correlation with the most recent common ancestor of the crown group represents a more credible, and more relevant, speculation.

It was inevitable that MADS-box genes (helpfully cross-correlated in outline with orthologues of other model angiosperms by Callens *et al.*, 2018, their table 1) would once again figure prominently in discussion and that their numbers would be interpreted within the context of whole-genome duplications (Zhang *et al.*, 2017; Unruh *et al.*, 2018). Functional MADS-box genes proved to be less numerous in orchids than in other model angiosperms such as *Arabidopsis* and *Oryza*, estimated total numbers being 63 plus 12 pseudogenes in *Dendrobium*, 51 plus nine pseudogenes in *Phalaenopsis*, and just 36 in *Apostasia* (cf., Cai *et al.*, 2014; Zhang *et al.*, 2016, 2017). Numbers of Type II MADS-box genes detected in the three respective genera were 35 (7 × E-class, 4 × C/D, 4 × A, 1 × B-GLO, 4 × B-DEF), 29 (9 × E, 5 × C/D, 1 × A, 1 × B-GLO, 4 × B-DEF) and 27 (5 × E, 4 × C/D, 2 × A, 1 × B-GLO, 2 × B-DEF). An additional B_{sister} gene present in each genus is thought to promote ovule development.

The MIKC* clade is sister to the MIKC^c clade and is divided into two monophyletic groups, S and P. All three orchids possess a single S-group gene, but *Apostasia* lacks the single P-group gene found in the more derived genera *Dendrobium* and *Phalaenopsis* (Zhang *et al.*, 2017). As these genes are expressed

during pollen development, it appears possible that loss of the P-group gene early in the initial diversification of the orchid family contributed to the formation of the almost unique cohesive pollen masses (pollinia – the apical portions of more complex pollen dispersal structures termed pollinaria) that characterise the vast majority of orchid species. Although the Type I MADS-box clade has generated less excitement than the Type II clade among orchid geneticists, one of its three subclades, Mβ, is not represented in the genome of any of the three orchid genera. Given that in most angiosperms Mβ genes play important roles in development of the endosperm, both its initiation and subsequent cellularisation, their absence may explain the early-stage failure of endosperm development in the 'dust-seeds' of orchids (Zhang *et al.*, 2016, 2017).

The smaller number of B-DEF genes found in the early-divergent genus *Apostasia* relative to the highly derived epidendroids *Dendrobium* and *Phalaenopsis* correlates with its exceptionally simple floral morphology. However, this correlation does not demonstrate causation, especially when similar teratological morphs – presumably reflecting small mutations (perhaps as small as the two-base pair mutation in the *CsAP3-2* gene of *Cymbidium sinense*) or epimutations – can be found within species of the most derived orchid subfamilies. And in the case of *Apostasia*, there is the additional complication that its sister-genus within this first-divergent subfamily, *Neuwiedia*, possesses features more typical of derived orchid subfamilies compared with *Apostasia*, which lacks resupination, a differentiated labellum and the A1 stamen (located opposite the labellum), and – most importantly, in the context of this essay – shows only partial fusion of the stamens to the pistil (Kocyan and Endress, 2001; Rudall and Bateman, 2002). Consequently, Rudall and Bateman (2002) felt obliged to present two optimisations of major morphological features of the orchid flower, one of which treated the comparative morphological simplicity of *Apostasia* as primitive and thus potentially reflective of the most recent common ancestor of the crown group, the other of which viewed its simplicity as representing secondary losses. Secondary simplification is our preferred optimisation, and is certainly the scenario that allows a stronger interpretation of the mass of genomic data recently derived from *Apostasia* by Zhang *et al.* (2017). Hopefully, *Neuwiedia* is now the highest priority for similar NGS treatment.

Returning to the topic of 'helpful monsters' briefly discussed in the previous section of this paper, the wider availability during the past few years of 'whole-transcriptome' sequencing has allowed us to delve deeper into the underlying causes of floral terata. We recognised six kinds of perianthic mutants that could be rationalised into two broader categories, peloria (when the perianth is transformed from strongly zygomorphic to actinomorphic) and

pseudopeloria (when the transformation occurs only from strong zygomorphy to weak zygomorphy) (Rudall and Bateman, 2002, 2004; Bateman and Rudall, 2006). A seventh kind was added by Mondragón and Theissen (2009). A study of pseudopeloric *Phalaenopsis* mutant lineages by Huang *et al.* (2016) showed four alternatively spliced C-terminal variants of AGL6b proteins to be competing, the three novel variants increasing in expression levels at the expense of a *ca* 50% reduction in expression of the wild-type transcript. The morphological outcome of these protein-protein interactions was an expanded labellum more closely resembling the lateral petals.

This study led in turn to an impressively synthetic comparison of one wild-type, two peloric and three pseudopeloric morphs of *Cymbidium sinense* by Su *et al.* (2018), synthesising data from whole-transcriptome sequencing, quantitative RT-PCR, RNA *in situ* hybridisation, *Arabidopsis* transformation, and SEM studies of floral ontogeny. Even more impressively, for each morph they compared expression in vegetative organs with four contrasting stages of floral ontogeny. When interpreting their results, the authors inevitably homed in on MADS-box genes, arguing that when exploring other gene families "it is difficult to tell how these differences [in enrichment reactions] are correlated with the flower morphology" (p. 3). They identified 27 MIKC^c factors (20 highly expressed) forming seven clades. SEM study revealed the expected early-stage retardation of ventral organogenesis (affecting the median sepal and lateral petals), the gynostemium being the last major feature to be initiated via a primordium. Late-stage buds showed enhancement of C-class and B-GLO-class genes but diminished A-class expression. Su *et al.*'s most exciting discovery was that among the seven SEP and AGL6 genes detected, one labellum-specific E-class (strictly, AGL6 clade) gene, *CsAGL6-2*, was upregulated in both peloric morphs but downregulated in all three pseudopeloric morphs. Moreover, of five B-function genes encountered, one (*CsAP3*-2) that is expressed in all three petals showed a similar expression pattern. A mere two base-pair deletion in the first exon caused a frameshift of sufficient consequence to radically alter the morphology of the flower, for example allowing features of the labellum to be expressed in lateral petals or sepals. An equally small genetic change, on this occasion involving insertion of a retrotransposon into the first exon of an E-class gene, caused loss of function and so yielded a broadly similar phenotype in a mutant *Habenaria* orchid (Mitoma and Kanno, 2018).

But here's the rub. The belief of Su *et al.* (2018, p. 2) that their aggregate "results unravel zygomorphic floral development of *C. sinense*" appears to us decidedly premature; this particular Gordian Knot has so far been teased rather than cut. Even their excellent integrative study had little to say about the gynostemi-

um or subjacent ovary; we learn only that one AGL6-clade gene is preferentially expressed in the gynostemium, and that in the two peloric morphs the gynostemium is developmentally modified to the point of being sterile. More generally, our above account of genetic research into orchid floral development is littered with adjectives of uncertainty. The truth is that, with the arguable exception of the B-class genes (*DEF*-like and *GLO*-like) and perhaps certain *AGL6*-like genes, we really do not know what the protein products of these genes actually do within the plant, and we certainly do not know how they do it – at least, not in orchids. Even when played at the level of whole-genome sequencing, genetics is currently largely a numbers game, causation remaining a distant goal.

Perianth versus gynostemium: the allure of the tractable?

Why has the gynostemium – supposedly the focus of our essay – featured so little in the above discussion? In truth, one of our key points is to emphasise how little we have learned about synorganisation and the resulting hyper-epigyny through application of the battery of developmental genetic techniques that have been applied to the orchid family. In our opinion, it is the ambiguous homologies caused by the profound 'male'-'female' synorganisation that render the gynostemium particularly resistant to evo-devo approaches. In the words of Minelli (2015, p. 1), albeit written in a subtly different context: "we can hardly hope to get meaningful and interesting results from a study of a system whose boundaries have not been meaningfully fixed." It is no coincidence that most of the progress made in understanding the orchid flower has been made on the six perianth members. With the exception of the species-poor Subfamily Cypripedioideae and a few members of Subfamily Epidendroideae, which have lateral sepals more-or-less completely congenitally fused via intercalary growth to form a synsepal (Kurzweil, 1993), the six perianth members of orchids are reliably unfused for most or more commonly all of their length. Combined with the fact that they usually encompass at least three distinguishable morphologies, this makes deviation from the wild-type phenotype unusually easy to identify in the case of the perianth.

In contrast, phenotypic deviations in gynostemium morphology require far closer scrutiny to recognise and even closer scrutiny if they are to be correctly interpreted. Rudall *et al.* (2013) suggested that the exceptional loss of organ boundaries displayed by orchid flowers are likely to be the result of increased overlap in expression of several developmental genes. In compound organs such as the orchid gynostemium that are integrally united from inception, and enlarge by means of intercalary or zonal growth, the boundaries of the contributing organs are never fully specified throughout floral ontogeny. More broadly,

the evolutionary history of the angiosperm flower *per se*, and the concomitant evolution of novel structures distal to and including the carpel, are increasingly perceived as a product of the changing patterns of gene expression resulting from loss of organ boundaries caused by congenital and/or postgenital fusion – that is, from synorganisation (Endress, 2006, 2016; Rudall, 2013; Specht and Howarth, 2015; Minelli, 2018; Sokoloff *et al.*, 2018).

Organ boundaries and leaf margins are known to specifically express distinct boundary genes that promote organ separation, such as the *CUP-SHAPED COTYLEDON* (*CUC*) genes, which encode NAC domain transcription factors (Aida *et al.*, 1997). Also expressed at organ boundaries are *LATERAL ORGAN FUSION* (*LOF*) genes, which encode MYB transcription factors that help to regulate lateral organ separation, partly through interaction with *CUC* and the *KNOX*-like gene *STM* (Lee *et al.*, 2009). Mutations of these genes in *Arabidopsis* resulted in fused organs that displayed substantial changes in cellular organisation. Additional gene families have been implicated in organ boundaries in model eudicots such as *Arabidopsis* and *Petunia* (reviewed by Specht and Howarth, 2015).

We believe that the downstream consequences of the high level of synorganisation needed to produce the gynostemium have been widely under-appreciated. For example, septal nectaries are highly characteristic of the majority of insect-pollinated monocot lineages and are always correlated with postgenital carpel fusion. However, they are entirely absent from both Orchidaceae and the taxonomic order Liliales, apparently precluded by the congenital carpel fusion. Thus, carpel fusion appears to impose an unbreakable evolutionary constraint on the development of septal nectaries. We presume that this constraint stimulated the evolution of novel tepal-based nectaries in orchids (Rudall, 2002; Rudall and Bateman, 2002; Remizowa *et al.*, 2010; Endress, 2011). These tepal-based nectaries take many forms in orchids, the most common developing as adaxial invaginations in the proximal region of the labellum that are termed spurs (Fig. 1A). Widely regarded as crucial to pollinator interest (despite being a moving target for selection due to ongoing ontogenetic expansion during anthesis overlain by ecophenotypy: Bateman and Sexton, 2008), the development of labellar spurs is fine-tuned downstream of B-function genes by *KNOX*-like genes (Box *et al.*, 2012).

The earliest evo-devo studies of orchids sought, fairly fruitlessly, to address the complexities of orchid pollinarium formation (Lu *et al.*, 1993; Johansen and Frederiksen, 2002). However, the pollinaria, stigma and associated morphological elaborations of the gynostemium have barely merited comment in subsequent evo-devo studies, at least partly reflecting their recalcitrance to de-

velopmental-genetic study due to their intimate fusion as elements of the gynostemium. The pollinarium is rendered especially developmentally complex by essentially being a hybrid structure; in most orchids the adhesive viscidial disc is reputedly generated by the rostellum (essentially a modified median stigma lobe), often enclosed within a bursicle, and linked to the pollen-generating pollinarium via a caudicle that is partly composed of tapetal and pollen wall remnants (e.g., Kurzweil, 1987; Dressler, 1993) (Fig. 1B). We speculate a possible downstream influence of P-group MIKC* genes on *QUARTET* genes, which have been shown to promote pectin production (Lora *et al.*, 2014). Given that pectin-like substances are essential components of the viscidium, caudicle and rostellum of a typical orchid pollinarium, and probably also contribute to the stigmatic 'glue' found in many orchids, these presumably pleiotropic genes may play important roles in orchid reproduction. Nonetheless, we find it ironic that the pollinaria that heralded the onset of evo-devo studies of orchids are proving far from tractable, being arguably the most challenging of all features of an orchid flower to interpret within an evo-devo context.

Does a 'super-organ' mark the boundary between evolutionary constraint and lability?

Mondragón-Palomino (2013, p. 1) expressed an opinion held by many orchid researchers when she began her review by arguing that "the unique diversification of flower morphology in Orchidaceae has taken place in the framework of a relatively conserved structure." Yet four years earlier, Mondragón-Palomino and Theissen (2009, p. 592) had argued convincingly that "evolution of the four classes of paralogous *DEF*-like genes 'modularized' the orchid perianth in such a way that the inner tepals could evolve semi-independently of the outer ones and the lip semi-independently of the lateral inner tepals. In this way, evolution of the paralogous *DEF*-like genes may have 'deconstrained' a lily-like floral perianth that was limited in its evolutionary potential by the pleiotropic interdependence of tepals. Once these constraints were reduced by modularization, the different classes of tepals thus generated were capable of evolving in a semi-independent way". Taken together, these statements imply that the orchid family originated by breaking a previous constraint – specifically, radial symmetry of the perianth – but that the ongoing possession of the ancestral six-part perianth remains a constraint on evolvability within the orchid family.

Both radial symmetry and a six-part perianth characterise all of the potential sister groups of Orchidaceae listed by Rudall and Bateman (2002). Orchids and their relatives do not play meristic games *sensu* Ronse De Craene (2016). Moreover, orchid terata that deviate from the six-tepal bauplan are rare, most

gaining tepals as a result of having lost determinacy (Fig. 2D), and such terata have never achieved the stability needed to establish a widely recognised species. Such observations led Melzer and Theissen (2016) to describe the orchid floral bauplan as showing developmental robustness (persistence of an organismal trait under perturbations; Appendix B) and to argue that although such "robustness may reduce the morphological disparity at one level, it may be the basis for increased morphological disparity and for evolutionary innovations at another level, thus fostering species diversity" (p. 725). We would argue that hyper-epigyny is an equally robust, constrained element in the floral bauplan of orchids, and is an even more fundamental influence on their famously diversified reproductive biology. Even when bilateral symmetry is lost from the perianth it often persists in the gynostemium of mutant phenotypes that deviate radically from wild-type (Bateman and Rudall, 2006; Mondragón-Palomino, 2013) (Fig. 2A–C). This observation suggests that any significant developmental disruption of the gynostemium would almost certainly affect both stamens and stigma, thereby at least reducing and more likely eliminating reproductive competence. The gynostemium is evidently a compound feature of exceptional burden.

We would further argue that the predilection of the orchid flower for producing typically a few thousand ovules and many thousands of pollen grains (often remaining in permanent tetrahedral tetrads), massed in pollinia and in in most cases firmly consolidated by tangles of viscin threads, constitutes a subtly different kind of evolutionary constraint – one that requires an approximate functional balance between the number of ovules present in the ovary and the number of pollen grains brought into proximity with that ovary via a pollinating animal. Orchids with few ovules but many pollen grains, or many ovules but few pollen grains, are unknown. Here, the underlying constraint is presumably the lack of an endosperm, which in practice condemns orchids to operate with many minute seeds dependent on fungal infection for early growth rather than fewer larger seeds that carry their own initial food reserves. If further evidence is gained that supports the hypothesis that the lack of an endosperm simply reflects the lack of any Mβ subclade genes, that gap in their genetic armoury may well be the underlying cause. If so, we are witnessing a genetic constraint underlying a functional constraint that in turn dictates both the life history and the reproductive biology of an entire species-rich family. Futuyma (2010, p. 1869) argued that "adaptation may be slow if it requires coupled change in multiple, genetically independent but perhaps functionally interdependent characters." The orchid family actually suggests that for high-burden, highly robust traits, adaptation can be precluded rather than merely "slowed."

These observations take on even greater significance when viewed in the light of recent studies across the flowering plants. Sauquet *et al.* (2017) compiled a massive database of many phenotypic traits in 792 extant species of flowering plants, before using a series of phylogenetically constrained mathematical analyses to reproduce the most statistically probable combination of traits possessed by the conceptual ancestral species occupying the crown node. However, Sokoloff *et al.* (2018a) noted that, in the case of the comparative merism of the perianth, androecium and gynoecium, the authors had reconstructed a combination of traits that does not exist in any of the *c*a 300,000 extant angiosperm species. Sokoloff *et al.* therefore suggested that the developmental shift from spiral to whorled phyllotaxy required by the hypothesised transition from sepal to petal whorl actually represents a seemingly unbreakable constraint.

Viewed in this context, constraints begin to at least superficially resemble Russian matryoshka dolls; evolution may eventually break one constraint but it will immediately face another constraint within a nested sequence that may ultimately prove infinite. In the case of the orchid flower, the inferior tripartite ovary rich in minute ovules, the six-part perianth and the highly integrated gynostemium are all components of an extreme form of synorganisation that constitutes an apparently unbreakable constraint (Rudall and Bateman, 2002; Bateman and Rudall, 2006; Rudall *et al.*, 2013; Endress, 2016). In the case of orchid flowers, the next challenge for evolution was the ventral suppression of between three and five of the six ancestral stamens, the resulting patterns delimiting clades at approximately the subfamily level. Interestingly, the vast majority of species in the family occur in subfamilies that possess only one fertile stamen. Melzer and Theissen (2016) argued that the six-part zygomorphic perianth of the orchids constitutes a 'super-organ'. We readily adopt their terminology but believe that it is the floral bauplan as a whole, rather than merely the internally differentiated six-part perianth, that operates as a 'super-organ.' We argued long ago (Rudall and Bateman, 2002; see also Rudall *et al.*, 2013) that the bauplan reflects the intersection of proximal–distal and dorsal–ventral clines together generating zones of overlapping gene expression, and we believe that subsequent data are consistent with this hypothesis. In combination, they generate a robust super-organ that provides a consistent framework within which develop the phenotypic details that dictate the overall functionality of the individual flower.

We agree with Melzer and Theissen (2016) that subfunctionalisation among these genes is probably largely responsible for the fine balance achieved by the orchid family between developmental robustness and evolvability at the lower hierarchical levels of genus and species. The list of more labile morphologi-

cal features that have permitted the evolution of several hundred genera (most recircumscribed using molecular data) and many thousands of species (sadly, most *not* recircumscribed using molecular data) is predictably long. Flower number and size span vast spectra of variation. As noted by many observers, the constraint of a ubiquitous six-petal, two-whorl, three-morph bauplan of the orchid perianth nonetheless permits the evolution of an almost infinite variety of overall shapes. Meanwhile, expression of cell-determining gene classes such as MYB experiments wildly with smaller-scale epidermal textures, assisted by myriad varieties of background colours and superimposed markings to create a symphony of mosaicism. TCP-class genes such as *CYC* also appear to operate with less predictable effects, both within the floral bauplan and among comparatively closely related genera (Mondragón-Palomino and Theissen, 2009; Madrigal *et al.*, 2017). We have already noted the remarkable diversity of potentially nectariferous spurs (e.g., Box *et al.*, 2012), and the myriad of pollinaria differ greatly in number, size, architecture, robustness and adhesion capability (well-illustrated by Dressler, 1993; Claessens and Kleynen, 2011). The three or more sterilised stamens present in at least vestigial form in all orchid flowers can also be put to good use, variously functioning as barriers between pollinia and stigma, as secretors of viscidial glue, or as sources of volatile fragrances. Even floral resupination via 180° torsion is frequently evolutionarily reversed (thereby challenging Dollo's Law of irreversibility) or, more rarely, nullified via a further 180° twist of the pedicel plus ovary (thereby either challenging Dollo's Law or supporting it, depending on whether one chooses to prioritise the cause – no twist versus two twists – or effect – in both cases the labellum is presented uppermost on the flower). Clearly, even within the super-organ constraint, there remains plenty of heritable variation in phenotypic features that readily act as playthings for ongoing evolution.

Admittedly, one of the difficulties in interpreting which aspects of the orchid family drive its remarkable species-level diversity is the paucity of genuinely comparable clades. We previously compared orchid flowers with those of *Corsia* (Corsiaceae) and *Pauridia* (Hypoxidaceae) (Rudall and Bateman, 2002), but as noted by Rutishauser (pers. comm. 2018), the Australasian subfamily Stylidioideae also offers at least a superficial comparison. Its members have several features in common with orchids: flowers are resupinate, have inferior ovaries and the single adaxial pair of anthers is adnate to the style to form a structure referred to by some authors as a gynostemium (Carlquist and Lowrie, 1991). Members of the subfamily have six sepals and most have four petals held in two pairs, though some species produce a fifth, morphologically differentiated petal that is sometimes termed a labellum. Thus, tepal number is less effectively constrained than in orchids; also, in contrast with orchids, the sepals and petals

are fused for much of their length. Despite having existed for approximately the same period of time as the species-rich orchid subfamily Epidendroideae, and similarly possessing specialised pollination mechanisms, Stylidioideae have generated a modest five genera and *ca* 240 species. Whatever advantage in speciation rate Epidendroideae possess over Stylidioideae remains open to debate.

A miscellany of constraints on the study of constraints

Writing a decade ago, mainstream evolutionist Douglas Futuyma (2010, p. 1878) argued that "So far, the expectation that evolutionary developmental biology (evo–devo) would describe and explain constraints has been largely unmet." While viewing this general statement as somewhat exaggerated, we agree that the focused attention of many gifted scientists has so far yielded only an outline understanding – perhaps best described as a well-founded predictive framework – of how the distinctive orchid flower originated and diversified, or how it maintains strong developmental canalisation in bauplan while nonetheless permitting sufficient morphological diversification to permit extensive speciation. But what can be done to further enhance understanding?

Even among the best-known MADS-box gene families, predictions of the function of the gene products based on phylogenetically related species are proving unreliable. Intensively investigated eudicot models such as *Arabidopsis* and *Antirrhinum* are certainly limited guides to orchid evolution. The apparently critical interactions between B-function and E-function proteins differ substantially between orchids and their relatives, even with closely related petaloid monocot families such as Zingiberaceae (gingers). And in terms of TCP family gene expression, there appear to be not only radical differences between orchids and their relatives and other families but also significant contrasts within the orchid family. We therefore have little doubt that the immediate future will bring in-depth studies of additional orchid species, presumably built in part on whole genomes/transcriptomes.

A further lesson provided by numerous recent studies is that, unsurprisingly, the expression levels and patterns of key developmental genes often vary enormously during floral ontogeny. The greatest difficulty is discerning patterns in early ontogeny, where the extreme synorganisation of orchid flowers, and consequent blurring of physical barriers (and conceptual homologies) between floral organs, makes it extremely difficult to interpret developmental patterns, irrespective of the technology being applied. New cell-lineage tracking and three-dimensional visualisation methods might have promise in this regard if combined with micro-expression techniques (e.g., Bartlett *et al.*, 2008; Bassel and Smith, 2016).

Turning from the empirical to the conceptual, we wrote in an earlier section of the "allure" of the perianth as a research focus. Here, we note a much broader distortion of scientific endeavour, the allure of the dynamic transition. Speciation is a far more charismatic topic than evolutionary stasis, just as most current earth science research in stratigraphy explores the boundaries separating major stratigraphic units at the expense of examining the vast, apparently unexciting tracks of intervening strata. These seemingly tedious strata actually need to be better understood, if only as a null hypothesis for comparison with the radical changes supposedly captured at the stratigraphic boundaries. Evolutionary constraints are by definition a study in stasis and stasis lacks obvious charisma.

In reviewing the current literature, we noted that developmental studies that combine simultaneous in-depth exploration of several developmental phases by examining both genotype and phenotype are disappointingly rare, and that even fewer compare at least two contrasting phenotypes to investigate transitions involving a diversity of traits (cf., Pan *et al.*, 2011, 2014; Hsu *et al.*, 2015; Su *et al.*, 2018). We were especially disappointed to realise that most studies relevant to orchid evo-devo remain inadequately integrated. In particular, the vocabulary of macroevolution that we regard as essential to link presumed genotypic cause to presumed phenotypic effect – adaptation vs. exaptation (e.g., Gould and Vrba, 1982; Gould, 2002), robustness vs. evolvability (e.g., Melzer and Theissen, 2016; Minelli, 2017, 2018), heterochrony vs. heterotopy (e.g., Alberch *et al.*, 1991; Bateman, 1994) – is often absent from, or merely receives lip-service in, hard-core genetics studies. This observation leads us onward to our final topic.

How far does the Extended Synthesis stretch beyond the Modern Synthesis?

As enshrined in the near-ubiquitous Modern Synthesis (e.g., Huxley, 1942; Mayr, 1963; Stebbins, 1966; Dobzhansky, 1974), neoDarwinism has long exhibited the amoeboid property of rejecting surrounding particles (in this analogy, novel evolutionary ideas) for protracted periods. Eventually confronted by incontrovertible evidence, those conceptual particles are eventually ingested by the neoDarwinian amoeba amid claims that it remains essentially unchanged by the assimilation process. Even (fairly) radical evolutionary guru Stephen Jay Gould eventually succumbed to this phenomenon (Gould, 2002). This trend has seemingly continued with the evo-devo-inspired Extended Synthesis, processes that were brought into sharper focus by Pigliucci and Müller (2010) soon being rationalised as business as usual by the majority of the few evolutionary lu-

minaries who even realised that a meaningful challenge had been issued (e.g., Wray *et al.*, 2014; Futuyma, 2017).

Selecting just a few inferences from the above review is sufficient to challenge such complacency. There seems little doubt that the orchid family originated as the direct or indirect result of a whole-genome duplication event (Zhang *et al.*, 2016, 2017; Minelli, 2018), which by definition was not the direct result of natural selection (i.e., an adaptation). Nor can the duplication event *per se* readily be viewed as an exaptation, because by definition an exaptation also must, at some point earlier in its history, have been the result of natural selection (Appendix B). Switching from a wholesale genetic change to a minute one, within the constraint of the perianth-gynostemium super-organ, many of those floral features that remain evolutionarily malleable within the orchid lineage can be massively disrupted by as little as the two base-pair deletion detected in the MADS-box gene *CsAP3-2* by Su *et al.* (2018). In the light of such observations, it becomes more difficult to deny the potential for saltational evolution or to reject its likely role in generating key innovations (Bateman and DiMichele, 2002; Theissen, 2006, 2009; Minelli, 2015, 2017, 2018; Rutishauser, 2019 this volume). In between these extremes lies the six-tepalled zygomorphic floral bauplan of the orchid. Endress (2006, 2011) argued that zygomorphy is an exaptation when viewed across the angiosperms; it originated frequently from within actinomorphy, but some of the derived zygomorphic lineages subsequently diversified greatly whereas others did not.

The majority of phenotypic transitions that mark speciation events in the orchid family occur *within* individual organs such as the labellum and gynostemium, most commonly taking the form of broadly heterochronic shifts in the timing of gene expression, though the phenotypic outcomes can be interpreted as showing heterotopy as one structure converges on the appearance of another (Rudall *et al.*, 2013). Such events lie less in phylogenetic trees derived from the realm of classical taxic homology and more in the much greyer area variously termed homoiology, latent homology or underlying synapomorphy – in other words, in the expression of developmental genes heavily mediated (and often masked) by a wide range of epigenetic, ontogenetic and ecophenotypic influences to generate a diversification trend rather than an irreversible evolutionary threshold. Although occasionally appropriated for botanical use (e.g., Rutishauser and Moline, 2005), the concept of homoiology has thus far had greatest impact on discussions regarding hominin evolution (e.g., Collard and Wood, 2007).

In our opinion, simultaneous fractal division of phenotype and niche to form numerous trivially distinct species, all but the derived autogams seeking

spectra of pollinating animals compatible with their floral phenotypes, is the less exciting level in the evolutionary-systematic hierarchy within Orchidaceae, despite the attention it has garnered from Darwin (1862) onwards (see also Minelli, 2016). We recognise that many of the evolutionary events that occur downstream of major phenotypic shifts are likely to involve natural selection, but we question its often tacitly assumed ubiquity. The frequently isolated populations and sporadic, biased reproduction of orchid populations make them unusually good targets for drift (Tremblay *et al.*, 2005), while several heritable epigenetic phenomena have in recent years either been discovered or recognised as being unexpectedly widespread; these also routinely impact on orchid populations (e.g., Paun *et al.*, 2010). Perhaps most intriguingly, as predicted by Bateman (2012), the spotlight of selection pressure has indeed proven to play on particular aspects of orchid reproductive function for far less than the time required to generate directionality. In one noteworthy example, Scopece *et al.* (2017) recently reported year-on-year fluctuations in selection gradients and differentials within *Orchis* populations based on measurement of several floral features. Such observations mesh well with Bateman's (1999) insistence that strong, persistent selection pressures are needed to overcome the effects of the inevitable environmental perturbations that, in reality, transform a theoretical adaptive landscape into an actual fitness seascape of comparative instability and unpredictability.

It particularly interests us that, for most of the key terms underpinning evolutionary biology that are defined in Appendix B, we were able to find in the literature many contrasting definitions. Previous authors have made similar observations while expressing varying degrees of concern (e.g., Gould and Vrba, 1982; Antonovics and van Tienderen, 1991; Bateman and DiMichele, 2002; Gould, 2002; Minelli, 2015, 2017). In most cases, we could find contrasting definitions of key terms that variously rendered natural selection obligatory (the majority of definitions), optional, or failed to mention it at all. Even among the latter, the proportion of definitions requiring selection as an obligatory element would increase still further if one accepts the mainstream definition of an adaptation as a trait that can only have arisen directly through the action of natural selection. For example, in his characteristically thoughtful critique of the Extended Synthesis, Futuyma (2017, p. 1) nonetheless stated categorically that "adaptations are attributable to the sorting of genetic variation by natural selection, which remains the only known cause of increase in fitness."

This statement begs several important questions. Firstly, to state the obvious, competition among conspecific plants is at best indirect; it can only target either shared resources such as light, water and nutrients, or symbiotic part-

ners such as mycorrhizae or pollinators. Given that orchid species (1) never dominate the ecosystems that host them and (2) can generate vast numbers of potential progeny from a single pollination event, they appear especially uncompetitive even for land-plants. Their fight for survival is with their immediate environment through time. Secondly, most studies of orchids that reputedly demonstrate adaptation in particular traits do not assess fitness, and those studies that do assess fitness do so only as far as production of viable seed by a single generation of plants. Such short-term assessments operate at the mercy of the fitness seascape and so should be viewed as incurring massive margins of error. But thirdly, what proportion of the functional traits of an orchid are actually *bona fide* adaptations?

In their much-cited classic paper, Gould and Vrba (1982) coined the term exaptation to enable explicit distinction from adaptation of what had previously been largely termed pre-adaptation. Understandably, the term exaptation has subsequently achieved much traction in the literature, and Gould's expansion of his ideas occupied *ca* 80 pages of his later *magnum opus* (Gould, 2002). Less understandably, the evolutionary biology community has virtually ignored the companion term for adaptation and exaptation that was coined by Gould and Vrba (1982), specifically non-aptation. Non-aptation encompasses phenotypic traits that have not originated either directly (adaptation) or indirectly (exaptation) via natural selection. Gould and Vrba (1982, p. 12) argued that non-aptations can be exapted, noting that "Exaptations that begin as non-aptations represent the missing concept [in neodarwinian evolution]; they are not covered by 'preaptation' [= pre-adaptation], for they were not adaptations in ancestors." They further argued that "adaptations and non-aptations provide an enormous pool of variability, at a higher level than mutations, for co-optation as exaptations." We agree, perceiving much of the phenotypic variation observed among the (literally) countless number of orchid species as most likely being non-aptive. The greatest promise of the Extended Synthesis lies in its ability to finally fully acknowledge the collective importance of the diverse evolutionary processes that contribute to that most neglected of concepts, non-aptation.

Acknowledgements

We thank Giuseppe Fusco for inviting us to contribute to this festschrift and Rolf Rutishauser for his thorough, well-informed review. It is our great pleasure to explicitly acknowledge Sandro Minelli's substantial and original contributions to evolutionary-developmental biology.

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Appendix A

Contrasting definitions of the term 'constraint' when placed within a broadly evolutionary context

- "evolutionary constraint = any limit on the rate and direction of adaptive evolution" (Futuyma, 2010, p. 1865)
- "evolutionary constraint = when a character fails to change in an adaptive manner due to preventative factors or mechanisms" (Burt, 2001, p. 515)
- "biological constraint = when a trait is precluded from reaching, shifted away from, or slowed down in its approach to a defined selective optimum" (Hansen, 2014)
- "evolutionary constraint = factors that retard or prevent a population from reaching its immediate adaptive peak on an ecological timescale" (Bjorklund, 1996, p. 423)
- "developmental constraint = the conservative and directive influence of development on evolution; any developmental bias in the production of variants, [including] canalised pathways [and] cascades of effects [...] constraints are [often] contrasted with selection as factors responsible for the form of observed traits [...] developmental constraints and 'phylogenetic constraints' amount to the same thing" (West-Eberhard, 2003, pp. 25–26)

Appendix B

Glossary of evolutionary concepts as defined for the present discussion

- natural selection = differential survival and reproduction of individuals due to contrasts in one or more phenotypic traits
- adaptation (noun) = a heritable phenotypic trait with a functional role that directly reflects its origin through natural selection
- exaptation (pre-adaptation, co-option) = a heritable phenotypic trait with a functional role that differs substantially from that for which it originated through natural selection

- non-aptation = a heritable functional phenotypic trait that did not originate either directly or indirectly through natural selection
- evolvability = rate of acquisition by a lineage of heritable phenotypic traits that do not decrease the overall fitness of the lineage
- constraint (evolutionary) = a heritable phenotypic trait (or set of functionally linked phenotypic traits) that limit the ability of a lineage to respond to perturbation
- robustness (developmental = canalisation) = ability of a phenotypic trait to persist in a perturbed lineage
- pleiotropy (evolutionary) = ability of a single gene to influence two or more unintuitively related phenotypic traits
- mosaicism (evolutionary) = partially or wholly independent evolution of multiple heritable phenotypic traits occurring within individual organisms
- burden (evolutionary) = functional responsibility of a heritable phenotypic trait as measured by the number and magnitude of dependent traits
- heterochrony = temporal change in the expression of a heritable phenotypic trait between putative ancestor and putative descendant
- heterotopy = spatial (positional) change in the expression of a heritable phenotypic trait between putative ancestor and putative descendant
- homoiology (latent homology, underlying synapomorphy) = apparently homologous heritable phenotypic traits in two (typically closely related) lineages differentially expressed due to ontogenetic, epigenetic and/or ecophenotypic influences

Becoming segmented

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Abstract

Fully segmented body plans are found in three phyla. These phyla are among the most successful on earth. The segmented body plan is not a single character, but a complex phenomenon, which evolved through a series of evolutionary steps. I suggest a hypothetical reconstruction of the steps involved in the appearance of segmented body plans, starting from the evolution of bilateral symmetry and ending with spatially coordinated segments that are generated from segmental precursors during development. Each step is built on the previous one, and a selective advantage is suggested for each step. The evolution of segmented body plans paved the way for significant diversity in the phyla wherein it evolved.

Introduction

The segmented body plan is the body plan found in the largest number of species among bilaterians. It consists of repeating morphological units along the anterior-posterior axis of the animal, with each of these units (or segments) including components from a number of different organ systems in register with each other. A hallmark of the segmented body plan is that the segments are formed ontogenetically, mostly during embryonic development, as distinct but undifferentiated units (sometimes referred to as "somites"), and the elements of the different organ systems develop within the distinct segmental units (Scholtz, 2002).

Fully segmented body plans, as described above, are found among members of three animal phyla: Chordata, Annelida and Arthropoda. However, examples of partial segmentation –repetition of only some organ systems, or incomplete integration among the different systems and the segmental register – are found both in sister taxa to segmented animals and in the fossil record of their stem

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groups (Minelli and Fusco, 2004; Chipman, 2010). This indicates that the segmented body plan is not a single all-or-none character (Fusco, 2008), and it suggests that the segmented body plan evolved gradually, with different aspects appearing sequentially. The three fully segmented phyla are among the most successful groups of animals, and it is highly likely that their success is linked to the segmented body plan.

The aim of this essay is to suggest a hypothetical series of steps that led to the evolution of the segmented body plan and to the success of the segmented phyla. The guiding principle of this evolutionary reconstruction is that each step must provide a selective advantage in its own right, and must build upon the previous step. Segmentation is believed to have evolved convergently in the three segmented phyla (Seaver, 2003), thus, the description of the steps will not go into phylum-specific details, to allow it to be generalizable for all three cases.

Step 1: Cephalization and directionality

Segmented animals are of course all bilaterians. Thus, the prerequisite for the evolution of a segmented body plan is the evolution of bilaterality. The appearance of bilateral symmetry is a result of the appearance of an anterior pole in the organism, as part of the evolution of a novel lifestyle, which involved directional movement. The first step in the long road towards a segmented body plan is the transition from a radially symmetrical sessile or planktonic ancestor to an animal with a preferred movement direction and a concentration of feeding, sensory and neural structures at the leading edge of the organism.

This transition is in itself not a single step, but is made up of several sub-steps, and organisms representing intermediate stages in this transition can be found today. The first sub-step involves a breaking of the symmetry of the radial organism, while still maintaining a mostly radial structure. This can be seen in the existence of a "directive axis" in anthozoan cnidarians (Genikhovich and Technau, 2017), which has been suggested to be indicative of a first step towards bilateral symmetry (although this may be convergent with Bilateria). Others suggest that the crucial symmetry breaking event might be the shift of the gastrulation site in an ancestral cnidarian-like ancestor, leading to a shift of the oral-aboral axis (Martindale, 2005; Martindale and Hejnol, 2009).

Based on the structure of the Xenacoelomorpha, which are widely regarded as being the sister group to all other bilaterians (Cannon *et al.*, 2016), the next step towards bilaterality involved creating a true directional axis and the evolution of mesoderm (contributing to directional locomotion), but not the appearance of a through gut or a centralized nervous system (Hejnol and Pang, 2016).

These only appeared with the evolution of Nephrozoa; a group that includes the fully bilaterally symmetrical animals with functional heads.

This gradual attainment of true bilaterality highlights the difficulty of discussing the characteristics of the hypothetical bilaterian ancestor or Urbilateria (Kimmel, 1996; Butts *et al.*, 2008; Hejnol and Martindale, 2008). The discussion is muddied by the fact that different authors actually mean different things by Urbilateria. If it is the first bilaterally symmetrical organism, then it should be a cnidarian-like organism with a shifted axis. If it is the common ancestor of all extant bilaterians, it should have the relative simplicity of a xenacoelomorphan. If it is the common ancestor of complex bilaterians (e.g., flies and frogs), or the protostome-deuterostome ancestor (PDA), then it is clearly more complex. I will return to this question at the end of the essay.

Step 2: Axial elongation

Once an anterior-posterior axis has been established, there is a general trend in many taxa for this axis to elongate, giving rise to numerous cases of generalized "worms" (Moore, 2006). The selective advantages of an elongated main body axis are obvious. Elongating the axis improves locomotion, both on the surface and in the substrate, as well as when swimming actively (usually with the addition of swimming appendages). A longer axis also allows a longer digestive system, potentially making feeding more energetically efficient and usually associated with differentiation of the gut into distinct regions. Indeed, the prevalence of the worm-like form in so many distantly related phyla implies that it has evolved numerous times independently.

Axial elongation through terminal growth is considered to be a defining feature of Bilateria (Jacobs *et al.*, 2005; Martindale and Hejnol, 2009) or even a more ancient character of stem group Bilateria (Gold *et al.*, 2015). In almost all cases of animals with an extended body axis, the developmental basis of this extension was found to be through a process of terminal growth (Jacobs *et al.*, 2005), or more precisely sub-terminal growth, with the added tissue being anterior to the very end of the embryo. The selective advantage for this mode of axial elongation could be the requirement of an ancestral larva to begin feeding as early as possible in its life history. Pushing all of the axial extension to the posterior allows the mouth to form early, without feeding interfering with continued growth (Martindale and Hejnol, 2009). It also allows anterior sensory structures to continue developing and differentiating as the larva grows.

There are several well-studied examples of non-segmental organisms that exhibit axial elongation through terminal growth, most notably hemichordates

and molluscs (Martin and Kimelman, 2009; Nakamoto *et al.*, 2010; Fritzenwanker and Lowe, 2014; Darras *et al.*, 2018). Therefore, while terminal growth is often linked to segmentation, it is clear that it represents a separate process that apparently evolved independently from segmented body plans.

Step 3: Distribution of structures along the axis

An elongated body axis presents several challenges for organismal integration. Locomotion in a worm-like organism can no longer rely on ciliary action, and striated muscles are needed to flex the body in a spatially distributed pattern. Repeated muscle units probably appeared along the body axis concomitantly with its elongation, since an elongated body without spatially distributed muscle units could not function. However, as the body grows longer, the coordination of these muscles becomes more complex, and lag times of neural messages from a centralized anterior brain become significant. There would be a strong selective advantage to evolving localized regulation centers along the anterior-posterior axis. These regulation centers – clusters of neurons – are the precursors of axially distributed ganglia found in many bilaterian animals. Initially, these ganglia do not have to have a one-to-one correspondence with repeated muscular units, and there are examples of non-correspondence in extant organisms, such as onychophorans (Mayer and Harzsch, 2007; Mayer and Whitington, 2009).

The excretory system also functions more efficiently when it is distributed along the axis in a long organism. Thus, the evolution of individual nephridia in distinct positions from anterior to posterior would provide a clear selective advantage. Here too, there are many examples of non-segmental organisms with serially repeated metanephridia, e.g. polyplacophoran and monoplacophoran molluscs (Giribet *et al.*, 2006; Nielsen, 2012).

Several other structures are found as serially repeated units in diverse taxa, including serially repeated gonads, external cuticular rings or spicules, gut diverticula and more (Minelli and Fusco, 2004). These are not necessarily linked to repeated units of other organ systems, and are not therefore considered to be true segmental units in the strict definition of the term. In all cases, the selective advantage presumably has to do either with more efficient function of the respective organ system in a long organism, or with mechanical considerations related to locomotion.

Step 4: Spatial coordination of different structures

The most important step towards the evolution of true segmentation is the spatial coordination of serially repeated structures, which initially evolved inde-

pendently to answer different selective pressures resulting from an elongated body axis. Spatial coordination brings the different repeated structures into register, so that repeated units of one organ system maintain a consistent spatial relationship to repeated units of a different system.

The selective advantage to such coordination is obvious in some cases, but not immediately so in others. In organ systems related to locomotion, there is a clear mechanical advantage to having the different organs in register. Each muscle unit will thus activate a single skeletal unit (or connect two adjacent units), and will be controlled by motor neurons relaying from a single ganglion. A single or paired ganglion can control the muscles on both sides of the organism, thus allowing bilaterally simultaneous contraction of muscles for peristaltic movement or alternate contractions for serpentine movement.

Similarly, when a liquid filled celomic cavity is essential for locomotion (as in many annelids), septa separating cavities would be in register with muscles and body wall units to allow efficient coordination of movement. A secondary outcome of the physically separate coelomic cavities is a selective advantage to having cavity-specific nephridia, thus bringing the excretory system into register with muscles and ganglia.

The explanation for spatial coordination of unrelated organ systems is not necessarily strictly selective, but may be related to inherent properties of development. A possible source of such a coordination is the recruitment of an existing positional information system to patterning additional organs. Thus, sensory organs could be in register with motor neurons, not because there is a direct advantage to such an arrangement, but because they are both responding to the same developmental signals.

Step 5: Temporal coordination and inter-segmental dissociation

The process of spatial coordination among different organ systems leads to an organism that is functionally fully segmented. However, most extant segmented organisms are also characterized by a developmental process, in which segmental units are formed first, before the appearance of the repeated organ systems that will develop from them, and then differentiate to give repeated units of different organ systems. For instance, a vertebrate somite is formed as an undifferentiated block of mesoderm, which only later splits into distinct domains that will give rise to muscles, skeleton etc. The hypothetical sequence of events described in the previous section does not necessarily lead to this type of developmental segmentation. Indeed, there is no a-priori constraint on the different segmental organ units developing individually at different stages of development.

It is this temporal coordination of the development of the different organ systems, and the dissociation of the development of individual units from each other within a given system, that is the true hallmark of morphological segmentation. This is the most interesting stage in the evolutionary process leading to modern segmented organisms, and it is the most difficult to explain. It's important to stress that all known examples of animals with spatially coordinated repeated structures that are formed during embryogenesis develop in a temporally coordinated fashion from undifferentiated somitic units. Therefore, although I have listed these as two separate steps in the evolutionary process, there is no direct evidence that they indeed occurred independently. Note that in animals with post-embryonic segmentation (e.g., some arthropods), this is not always true (Minelli and Fusco, 2013).

Nonetheless, the selective pressures hypothesized to lead to spatial coordination of repeated structures do not lead to their temporal coordination during development, so one must try to find an independent selective or developmental advantage to its evolution. The question is especially germane, since undifferentiated segmental precursors are found in all three segmented phyla (Balavoine, 2014; Graham *et al.*, 2014; Williams and Nagy, 2017), despite significant differences in their ultimate segmental pattern.

The simplistic explanation for the advantage to generating undifferentiated somites is that it is the most efficient mode of generating coordinated repeated units. However, evolution rarely favors the most efficient option. It is more likely that the true reason for the parallel evolution of somites lies in pre-existing mechanisms for generating a repeated pattern that gradually recruited additional target structures into the early phases of pattern generation. Unfortunately, in the absence of examples for intermediate stages in this process, there is no direct way to explore it experimentally.

Step 6: Evolvability and modularity

Once the segmental body plan has been established in evolution, and the fundamental developmental process generating it has been assembled, both the process and the resulting morphology are inherently highly evolvable. The characteristics of the segmental body plan that make it evolvable and have led to the success of the segmented phyla are unrelated to the selective forces that initially led to its evolution. The nascent segment or somite is a repeated modular unit. While the process generating individual segments can be conserved, the downstream morphological differentiation of each segment can diverge independently, under the control of regional signals such as Hox genes. The level of divergence between segments is primitively low, but in some cases, most

notably in arthropods (e.g., malacostracan crustaceans), every segment can be morphologically distinct and bear specific unique appendages. This ability for each segment to evolve semi-independently from others is what underlies the fantastic diversity of arthropods. The diversity of vertebrates is less dependent on variation in individual segment identity, but more on the number of segments allocated to each of a limited number of types of vertebrae, and on the morphology of a small number of appendages and of the head. Annelid diversity is mostly unrelated to segmental differentiation, and may have to do with the varied modifications of the segmental body plan itself.

Concomitantly, the developmental process of segmentation can evolve, leading to homologous structures that are generated through very different mechanisms. This is most notable and has been studied to the greatest extent within arthropods (Peel *et al.*, 2005), and specifically within insects (Stahi and Chipman, 2016). Arthropod segments are formed in different cellular contexts, at different temporal scales and using highly variable gene regulatory networks (Fusco and Minelli, 2013; Auman and Chipman, 2017). Nonetheless, the outcome is always a series of undifferentiated segmental units. This phenomenon is referred to as developmental systems drift, in which the end product of a developmental process remains more or less constant despite gradual changes in the individual components and steps of the process.

There is significantly less diversity in vertebrate somitogenesis. There are differences in the relative rate of different processes (Gomez *et al.*, 2008), and minor differences in the specific genes involved (Oates *et al.*, 2012), but the general process is conserved among all studied vertebrates. Much less is known about the diversity of annelid segmentation processes, since there have been fewer model species studied. From what we do know, there seem to be significant differences between the development of the segments in different taxa (Balavoine, 2014), including differences in the identity of the cells involved and the genetic networks active during the process.

The origin(s) of segmented body plans

The question of the evolution of the segmented body plan is related to a larger question regarding the nature of the first bilaterian organisms. In the past 20 years or so, there has been a tendency to assign fairly complex characters to the common ancestor of Bilateria, based on perceived similarities among extant organisms (Kimmel, 1996; Cornec and Gilles, 2006). Others have argued for a simple ancestor and convergence of many complex characters (Hejnol and Martindale, 2008; Chipman, 2010). As mentioned earlier, part of the complexity of this debate rests upon the question of which bilaterian ancestor is actually

being discussed. However, even if one discusses the ancestor of complex bilaterians (the protostome-deuterostome ancestor), a fully segmented ancestor is highly unlikely. In this essay, I have argued that segmented body plans must have evolved in a step-wise fashion, with each step dependent on the previous one. The existence of intermediate steps in extant organisms within Nephrozoa indicates that this step-wise process has taken place since the divergence of protostomes and deuterostomes. Thus, their common ancestor could not have been a truly segmental organism. It is not entirely clear where in the steps described herein the ancestor was. Based on the data reviewed here, I suggest that ancestor of Bilateria had started step 2 (axial elongation), whereas the ancestor of Nephrozoa was somewhere between the completion of step 2 and step 3 (distribution of structures along the axis). Breaking down the evolution of segmented body plan into constituent steps and trying to identify when and where each step took place (often in more than one branch) will ultimately help clarify the evolution of this central morphological feature.

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Space and time in Hox/ParaHox gene cluster evolution

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Abstract

Hox/ParaHox genes have been an enduring paradigm in evolutionary developmental biology (evo-devo). Amongst the many insights into mechanisms of evo-devo these genes have provided, the links between gene regulation and morphological evolution have been prominent. The phenomenon/phenomena of colinearity in Hox/ParaHox gene expression has/have been central to much of this Hox/ParaHox research. Temporal colinearity has gained prominence in explanations of colinearity. Recent data and hypotheses have focused on Sub-cluster rather than Whole-cluster Temporal Colinearity. A brief overview of these ideas is provided here.

Introduction

The rapid expansion of evolutionary developmental biology (evo-devo) from the mid 1980s onwards was in large part stimulated by the molecular discoveries that revealed a surprising level of conservation ("deep homology") of the genetic control mechanisms of animal development. The Hox genes figured prominently in this renaissance, since they are conserved across nearly all animals and seem to be operating in a comparable fashion to control position along the anterior-posterior axis of developing embryos, whether the animal is a mouse, a fly, a worm or perhaps even a sea anemone (DuBuc *et al.*, 2018). The flip-side of this surprisingly widespread conservation of developmental control genes is the question of how the huge diversity of animal form can evolve whilst using largely similar genes. The Hox genes have had a prominent role in understanding the evolution of this diversity of form as well (Akam, 1998; Pick and Heffer, 2012), and as they have revealed both deep homology as well as providing case studies of the molecular contributions to morphological evolution they have remained a central paradigm within evo-devo.

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Despite the enduring interest in Hox gene research, numerous major questions remain about how these genes function and what precisely is conserved and divergent across the animal kingdom. A key aid to resolving these issues is the improving degree of taxon-sampling, with research extending beyond the traditional study species for developmental genetics, such as the fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans* and a small selection of vertebrates such as the mouse *Mus musculus*. The improving spread into various additional study species has an important role in helping to deduce what are the fundamental features of these important developmental control genes and, conversely, what are lineage-specific oddities.

In addition to the Hox genes it is also useful to consider the ParaHox genes. This is because the ParaHox genes (*Gsx, Xlox/Pdx, Cdx*) are the evolutionary sisters to the Hox genes and, importantly in the current context, they are also often clustered and exhibit colinearity – the order of the genes along the chromosome corresponds to their order of expression (Brooke *et al.*, 1998; Ferrier and Minguillón, 2003). The ParaHox genes are also notable for their roles in patterning the development of the central nervous system and the gut (e.g., Hui et al., 2009 and references therein). Thus, when we consider the interplay between the Hox genes, their organisation and evo-devo then there are likely to be equivalent insights to be gleaned from also considering the ParaHox genes.

The challenge has always been to determine what aspects of Hox/ParaHox organisation and expression are retained from an ancestral state (such as the bilaterian ancestor or even the ancestor of all animals) and at what point in the evolution of any particular lineage the genes have started to depart from this ancestral arrangement and modify some process in a lineage-specific way. This problem of deducing ancestral characteristics versus lineage-specific derivations and novelties from comparisons of living organisms is a common issue for evolutionary biology and certainly figures prominently in evo-devo. Thinking about Hox/ParaHox gene activity can thus provide us with useful case studies with very wide-ranging implications for how we understand evo-devo in general.

In this context, the expression of the Hox/ParaHox genes in staggered domains along the embryonic anterior-posterior axis in an order that tends to match the order of the genes along the chromosome (the phenomenon of *spatial colinearity*) has been an enduring puzzle (Lewis, 1978; Duboule and Morata, 1994; Monteiro and Ferrier, 2006; Duboule, 2007; Deschamps and Duboule, 2017). Ideas about colinearity have been many and varied over the years and improved taxon-sampling has made significant contributions to these ideas, particularly in recent years with the progress in genome sequencing. The original

formulation of colinearity was that centred on spatial expression of the genes (spatial colinearity), but subsequent ideas have included "quantitative colinearity", "macro- and micro-colinearity", and even "virtual colinearity" (Montavon *et al.*, 2008; Durston, 2018). However, besides spatial colinearity it is *temporal colinearity* (whereby the order of gene activation progresses along the chromosome through the gene cluster) that has remained the major area of focus.

Wider taxon-sampling with regards to determination of the genomic organisation of Hox/ParaHox genes as well as their expression has clearly shown that the Hox/ParaHox genes do not need to be clustered in order to retain their staggered expression along the anterior-posterior axis. This means that whatever the mechanism(s) is/are that produce spatial colinearity they do not by necessity require an intact, ordered gene cluster for their operation. The situation is less clear with regards to temporal colinearity. It has been hypothesized that the mechanism of temporal colinearity leads to, or constrains, the organisation of the Hox/ParaHox genes in an intact ordered cluster (Duboule, 1994; Ferrier and Minguillón, 2003; Monteiro and Ferrier, 2006; Duboule, 2007; Deschamps and Duboule, 2017). Conversely, in those species in which temporal colinearity is not utilised, for example in cases in which embryogenesis is too rapid for a temporal progression of Hox/ParaHox gene initiation to be accommodated, then an alternative mechanism of Hox initiation had to evolve, which coincided with the release of the constraint on clustering that the temporal colinearity imposed. However, with increased taxon-sampling, several possible contradictions to the temporal colinearity hypothesis have now appeared, with intact ordered clusters that contain genes that are not activated in a temporal order that matches the order of the genes along the chromosome. However, a closer examination of these exceptions highlights that we still need to proceed with caution before we dispense with the temporal colinearity hypothesis. A particularly important recent development questions whether we should still be considering colinearity across the cluster as a whole (Whole-cluster Temporal Colinearity, WTC), or instead Sub-cluster Temporal Colinearity (STC) is a more important principle.

Temporal is more important than spatial (at least for organisation)

Although spatially restricted expression of the Hox/ParaHox genes is integral to their function in axial patterning, spatial colinearity does not seem to be the key to understanding the link between cluster organisation and gene function. This is because there are now numerous examples of animals with dispersed Hox genes that still retain aspects or remnants of the spatially restricted expression of the genes (Monteiro and Ferrier, 2006; Lemons and McGinnis, 2006;

Ferrier, 2012). The most extreme example is probably that of the appendicularian *Oikopleura dioica* in which no two Hox genes are clustered in the genome, but the Hox genes are still expressed in anterior-posterior staggered domains (Seo *et al.*, 2004). So 'spatial colinearity' does not necessarily require an intact, ordered cluster to operate, although of course it is a moot point as to whether one can talk about colinearity at all if the cluster is not intact (Monteiro and Ferrier, 2006) (but see Duboule (2007) for a proposal of a classification scheme for cis- and trans-colinearity that tries to accommodate clustered and non-clustered genes).

Where there does seem to be a stronger link is between temporal colinearity and cluster organisation (Ferrier and Minguillón, 2003; Monteiro and Ferrier, 2006; Garstang and Ferrier, 2013). One of the outstanding issues though is whether the mechanism or mechanisms of temporal colinearity are in any way universal and homologous (i.e., were present in the bilaterian ancestor or even earlier), or instead have arisen via parallel evolution, arising independently in distinct lineages in which temporal colinearity is found (e.g., vertebrates, the annelid *Capitella teleta* and the crustacean *Parhyale hawaiensis*; Fröbius *et al.*, 2008; Serano *et al.*, 2016).

Temporal colinearity tends to be found in animals that have a progressive anterior-posterior development, perhaps involving a growth zone (Deschamps and Duboule, 2017). This seems to be the ancestral condition for many groups for which we know the most about Hox/ParaHox gene expression and organisation, e.g. chordates, arthropods, molluscs, annelids. It seems reasonable to suppose that this progressive anterior-posterior development was also present in the ancestral bilaterian, although this point could be moot. Turning to the fossil record, for example, as a way to resolve the characteristics of the bilaterian ancestor is venturing into controversial territory (e.g., *Dickinsonia* as a segmenting bilaterian; Gold *et al.*, 2015; Hoekzema *et al.*, 2017).

Furthermore, temporal colinearity leading to spatially restricted expression has been highlighted as an intuitively elegant way in which to produce spatial colinearity (Duboule, 1994; Deschamps and Duboule, 2017), via a temporal sequence of initiation being translated into a spatial coordinate system of expression during progressive anterior-posterior growth. In fact, Deschamps and Duboule (2017) go so far as to say there is such a benefit and necessity of implementing the mechanisms of temporal colinearity to produce spatial colinearity in progressively elongating embryos that there is a difficulty in evolving an alternative strategy. This raises the prospect that this restriction might make such a system prone to evolution via convergence, thus 'muddying the waters' of our attempts to deduce ancestral states (see below and Kmita and Duboule, 2003; Duboule, 2007).

However, a progressive anterior-posterior ontogenesis does not necessarily impose a temporal colinearity. For example, leeches undergo a very temporally regimented mode of development via the array of teloblasts that progressively 'bud-off' successive band cells (Shankland and Savage, 1997). Nevertheless, the Hox genes of leeches are dispersed (Simakov *et al.*, 2013), but retain the anterior-posterior staggered expression (Kourakis *et al.*, 1997). So progressive anterior-posterior embryogenesis does not inherently require a temporal colinearity mechanism that in turn imposes an intact, ordered cluster. Leeches thus provide a relatively clear exception – of a progressively elongating embryo specifying its tissues in an anterior-to-posterior sequence that is not using a temporal colinearity mechanism to produce spatially-restricted Hox expression. There is, however, a possible complication in interpreting the leech Hox data, which is that there have been extensive duplications of leech Hox genes and loci, with consequent unknown possibilities for cross-regulation of the genes.

A further striking observation is that temporal colinearity of ParaHox genes is inverted relative to Hox genes. This goes against hypotheses of an anterior-to-posterior progression of expression being enforced onto such clusters due to gradual posterior elongation or the timing of tissue specification, since in the case of the ParaHox cluster it is the 'posterior' gene (*Cdx*) that is activated first whilst the 'anterior' *Gsx* gene patterns aspects of anterior central nervous system development (Hui et al. 2009, and references therein), with odd exceptions (e.g. Fröbius and Seaver, 2006). Much more work is needed on the regulation of the ParaHox genes in order to distinguish the similarity, or otherwise, of the colinearity generating mechanisms relative to those of the Hox cluster. The urge to posit an elegant, all-encompassing process versus diverse, messy biology impacts Hox/ParaHox thinking just as in other areas of biology.

To try to distinguish all-encompassing elegance from messy diversity we need to know much more about how temporal colinearity is actually produced. The clearest mechanistic data comes from vertebrates. There is progressive 'opening' of Hox clusters via chromatin modulation (Chambeyron *et al.*, 2005), but whether this is permissive or instructive for Hox activation remains an open question (Deschamps and Duboule, 2017). Additional mechanisms involve long-range multigenic enhancers (Kmita and Duboule, 2003), operating within the context of a growing embryo and a dynamically changing intercellular signalling environment. Both progressive chromatin modulation and long-range multigenic enhancers place an obvious constraint on cluster retention. How widespread such mechanisms are across the rest of the animal kingdom, particularly in those species with intact Hox/ParaHox clusters, remains to be determined.

One important issue that is sometimes overlooked when considering colinearity, whether temporal or spatial, is the need to focus on the initial activation

of the genes in deducing whether colinearity is operating. Breaks in spatial colinearity later in development have been known for quite some time, an extreme example being HoxC13 in mouse whisker development, with a Posterior Hox gene now specifying the development of one of the most anterior cell types (Godwin and Capecchi, 1998). This perhaps occurs once the genes are not necessarily imparting positional information, but instead simply acting as 'master control' genes for certain cell types. Determining when a gene is activated, to determine whether temporal colinearity is present or not, can also be technically demanding, requiring both a fine-scale resolution of ontogenesis and available material as well as a sensitive method for detecting gene expression.

The technique used to analyse expression and assess whether temporal colinearity is occurring is important. Analyses using transcriptome data tend to focus on peak expression levels, which are calculated according to normalisation with general expression levels, whereas in situ hybridisation experiments look at individual genes in a non-quantitative way. Arguably it is the in situ hybridisation approach that is most suited to the temporal colinearity question, because it is far from clear that the levels of Hox expression (or gene expression levels more generally) are the key to their function. That is, genes can be functional at relatively low expression levels. More importantly in the current context, temporal colinearity is more focused on when the gene expression is initiated as a marker for indicating the activity of the regulatory elements and promoters of each gene, with function of the gene almost being a secondary issue. Again, this does not need to be quantitative, but instead can simply be assessed according to whether any transcripts from a particular gene can be detected by whatever technique (notwithstanding that there can be leaky expression from some promoters, which could be misleading if our detection is 'too sensitive'!).

Should the focus be on sub-cluster rather than whole-cluster?

The recently sequenced genome of the scallop, *Patinopecten yessoensis*, revealed a Hox/ParaHox organisation with very little, if any, derivation from the hypothetical ancestral state for protostomes and deuterostomes (Wang *et al.*, 2017). Furthermore, detailed analyses of expression, including both transcriptome and in situ hybridisation data, revealed a form of temporal colinearity not previously considered – Sub-cluster Temporal Colinearity (STC). Wang and colleagues (2017) described how the Hox cluster of this mollusc could be subdivided into four sections, each of which initiates expression at the same ontogenetic time with the succeeding genes following on in their activation in order along the chromosome. A close inspection of the Wang *et al* (2017) data shows that *Lox4* is

expressed at gastrulation along with the other primary sub-cluster genes. This *Lox4* expression is detected at low levels in the transcriptome data, but is very clear in the in situ hybridisation data (see the supplementary information in Wang *et al.*, 2017). Thus, are there five sub-clusters rather than four, at least in these bivalves? Regardless of this, Wang and colleagues show that the sub-cluster divisions can vary slightly over large inter-phyletic distances (see Figure 4 of Wang *et al.*, 2017), but this is perhaps not so surprising given the evolutionary timespans involved. The authors attribute this to possible lineage-specific modifications linked to the evolution of lineage-specific body plans.

One of the clearest examples of invertebrate temporal colinearity is found in another protostome, *Capitella teleta* (whilst interestingly exhibiting a few minor breaks from spatial colinearity for the genes *pb*, *Hox3* and *Antp*) (Fröbius *et al.*, 2008). What is perhaps of more significance here is that the Hox gene expression of this annelid can be divided into four distinct groups in terms of their activation (*labial/proboscipedia/Hox3*; *Deformed/Sex combs reduced*; *Lox5/Antennapedia/Lox4*; *Lox2/Post2*), thus exhibiting a form of sub-cluster structure to the activation of expression, which Wang *et al.* (2017) call Subcluster-based Whole-cluster Temporal Colinearity (S-WTC). This division into four sub-clusters matches the sub-cluster boundaries in the scallop *P. yessoensis*, except at the Posterior end of the cluster where the sub-cluster 'boundary' is between *Lox2* and *Post2* in the scallop but between *Lox4* and *Lox2* in *C. teleta*. This shift also correlates with a difference in the organisation of the Posterior end of the Hox clusters in these two species, with the Post1 gene being lost from the Hox cluster in *C. teleta* and perhaps annelids more generally (Fröbius *et al.*, 2008; Hui *et al.*, 2012), but still being within the cluster in the scallop (Wang *et al.*, 2017).

Turning back to the vertebrates, perhaps they are not as representative of the prototypical state for Hox/ParaHox expression as often proposed. Gaps in all vertebrate Hox clusters due to gene loss is one obvious departure. There are early departures from spatial colinearity as well. Mouse Hox2 genes are expressed more anteriorly than Hox1 genes in the central nervous system. Also, zebrafish and dogfish Hox2 expression is more anterior than Hox1 expression (Prince, 1998; Pascual-Anaya *et al.*, 2018). In fact, this may be a general feature that is found widely across bilaterian animals (Fröbius *et al.*, 2008).

In the context of potential sub-cluster structuring of Hox clusters, there may even be evidence of a degree of sub-cluster regulation in the vertebrate Hox clusters, with *Wnt3/3a* activating *Hox 1-3*, *Cdx* (under control of *Wnt*) acting on *Hox4-10*, then *Gdf11/TGF-Beta* on *Hox 11+* (reviewed in Deschamps and Duboule, 2017). Potentially informative future experiments could thus address whether a vertebrate cluster can be broken apart into these hypothetical

sub-clusters and still act normally, at least in trunk axial development? However, there is the possible problem with cross-regulation across the paralogous vertebrate clusters, which always hinders interpretation of the vertebrate work. Thus, a more long-term experiment would be to determine whether the same signalling systems regulate the single amphioxus Hox cluster and, once techniques are available, to make genomic rearrangement mutations in amphioxus that split this cluster, without the confounding issue of cross-cluster regulation and redundancy. This prospect is getting closer now that it is possible to maintain successive generations of amphioxus in the laboratory as well as engineer mutations (Li *et al.*, 2017).

Another informative recent genome sequence is that of the ascidian *Halocynthia roretzi* (Sekigami *et al.*, 2017). Strikingly, the *H. roretzi* Hox cluster is separated into similar sub-components as *Ciona intestinalis*, even though these two ascidian species are in quite distant tunicate groups (Delsuc *et al.*, 2018). Therefore, there is the possibility that there were functional sub-clusters in the ascidian ancestor that have constrained the positions at which the cluster can be broken apart and dispersed (Sekigami *et al.*, 2017). As intra-phylum sampling increases hand-in-hand with the general increase in taxon sampling, then we will obtain a clearer picture of whether Hox/ParaHox cluster breaks tend to occur in similar patterns across species within the same larger clade. In the long term it will be necessary to then determine the regulatory landscape over these cluster remains, in parallel to deducing the mechanisms operating in intact clusters, to discover whether sub-cluster mechanisms act as a significant constraint on cluster dispersal. This could be the case because even if sub-cluster rather than whole-cluster mechanisms are prevalent, this will greatly constrain break-up of the cluster as there are so few locations at which viable breaks can form.

Homology versus convergence

Distinguishing between homology and convergence is a problem that strikes to the core of evo-devo and evolution in general. The view of Deschamps and Duboule (2017) is that the vertebrate colinearity mechanisms may well have evolved specifically in these animals, consolidating the Hox clusters (see Duboule (2007) for an explanation of this consolidation). There is certainly an element of truth in this view, as Hox regulation does appear to be mechanistically more complex in these animals (for example, extra Topologically Associated Domains (TADs) and new long-range enhancers; Deschamps and Duboule, 2017). However, we should perhaps be cautious about then jumping to the conclusion that in invertebrates it has been a free-for-all during evolution, such that any sort of regulatory mechanism could evolve so long as the spatial staggering of the Hox genes was the output.

First, there had to have been an ancestral starting point for the bilaterian divergence of mechanisms, and depending on the nature of this starting point in terms of Hox/ParaHox control mechanisms (e.g., sub-cluster or whole-cluster), then there could have been very different levels of constraint. Second, the deduction that there was some sort of constraint comes from a consideration of the Hox/ParaHox clusters being very ancient entities, possibly arising at the origin of the animals (Mendivil-Ramos *et al.*, 2012; Fortunato *et al.* 2014; Ferrier, 2016a). Also, although the cnidarians have certainly produced several lineage-specific Hox duplications they do nevertheless have distinct groups of Hox/ParaHox genes that have homology with the distinct bilaterian groups that are now often clustered (Ryan *et al.*, 2007; Hui *et al.*, 2008). Thus, the Hox/ParaHox clusters presumably were preserved either from the origin of the animals, or at least from the divergence of the cnidarian-bilaterian lineages through to the origin(s) of all of the main bilaterian lineages. The alternative is that the Hox/ParaHox genes arose by duplication (presumably tandem duplications) and were scattered around the ancestral animal genome, then secondarily came together again either in the bilaterian ancestor (which still gives us the question as to why they came together and also what was the regulatory state) or in any subsequent ancestor leading to lineages in which we currently see intact Hox/ParaHox clusters (e.g., chordates, arthropods, molluscs). Intuitively we have tended to put aside this seemingly unparsimonious hypothesis of secondary clustering; however, life and evolution do not necessarily need to be parsimonious and there may be data accumulating that indicates that secondary clustering is possible (Ferrier, 2016b). The resolution of this issue will hinge on a much better understanding of the dynamics of genome evolution, in concert with mechanisms of gene regulation, both across a diversity of lineages. Only then can we hope to deduce any generalities.

A significant issue in this regard is that the genomes of different species/lineages almost certainly evolve at different rates and in different ways. This is clearly evident in characteristics like genome size, fast clock/slow clock rates of nucleotide change, prevalence of repetitive transposable elements and, perhaps most significantly here, in the extent of conserved synteny (the linkage of genetic loci on the same chromosome). The discovery of conserved ancient synteny has perhaps been as shocking as the initial discovery of deep homology of developmental control genes in the 1980s and 1990s, as it transforms our views on evolutionary diversity and the variable rates at which evolution can act (from very rapid inter-population changes to exceptionally ancient almost kingdom-wide conservation) (Putnam *et al.*, 2007; Putnam *et al.*, 2008; Chipman *et al.*, 2014; Wang *et al.*, 2017). Hox/ParaHox cluster evolution must be considered in concert with the genome in which these clusters reside and hence the

background nature of rearrangement to which the clusters can be subjected, this being dramatically different depending on the species being considered.

The ParaHox cluster also presents an interesting opportunity in the context of determining the extent of homology versus convergence in colinearity mechanisms and ancient ancestral states. In terms of the Hox and ParaHox genes, temporal colinearity is observed in both types of cluster, although it is reversed in orientation, progressing from the 'posterior' *Cdx* gene to the 'anterior' *Gsx* gene in the ParaHox cluster. Is this then indicative of any homology of regulatory mechanisms between the two types of cluster, or instead is this evolutionary convergence? The resolution of this question requires a much better understanding of ParaHox gene regulation along with further work on the variety of Hox regulatory mechanisms.

Returning to amphioxus, one of the animals that has retained amongst the most archetypal Hox/ParaHox clusters, an important issue raised is whether the ancestral mechanism(s) are still operating, even in these archetypally organised clusters. Pascual-Anaya *et al.* (2018) provided *Branchiostoma belcheri* Hox transcriptome data that is consistent with the previous *B. lanceolatum* data showing a small number of genes breaking from Whole-Cluster Temporal Colinearity (WTC) (Pascual-Anaya *et al.*, 2012). The point made by Pascual-Anaya *et al* (2018) is that amphioxus could fit with the model of Sub-cluster Temporal Colinearity (STC), but importantly the boundaries between the sub-clusters are in different places than those for other invertebrates like the scallop. One interpretation is that this indicates an ancestor of chordates that may well have had WTC and that amphioxus has evolved STC independently from the STC of protostomes like the scallop. What is not clear is whether the bilaterian ancestor had WTC or STC. Consequently, even in those taxa like amphioxus that have Hox/ParaHox clusters conforming to the ancestral organisation, the regulation of the genes may well have evolved away from the ancestral mechanism at some point along the lineage. Wide taxon-sampling paired with mechanistic data is obviously key to resolving these issues.

Conclusions

The importance of deducing the evolution of the Hox/ParaHox regulatory mechanisms is that any resulting regulatory constraints may in turn act as constraints on body plan evolution within clades of animals. Deschamps and Duboule (2017) hypothesise that the temporal colinearity mechanisms in vertebrates have constrained their bauplan/body plan. This could well also be the case for other phyla or large clades, with the restricted possibilities for viable rearrangements of the Hox/ParaHox genes producing limits within which their

regulation can evolve and change, with consequences for limitations on how these genes can then change the development and body plan of the respective animals.

It is certainly true that the role of the Hox/ParaHox systems is to produce an array of proteins that control the transcription of downstream 'effector' genes to impart the different regional fates and cell types (although this might not be universally true for bilaterians; e.g., Ikuta *et al.*, 2010). How the genes are regulated in order to produce this output is a significant question for evo-devo. Determining what the ancestral state was provides us with the starting point for the subsequent diversification and, more importantly, how this could have happened across the animal kingdom. What constraints has the Hox/ParaHox regulatory system(s) placed on the evolution of these genes and hence the evolution of animal development? Have any lineages evolved ways to release themselves from this/these constraint(s) (the appendicularian *Oikopleura* being a prime example) and how has this happened? What are the widely conserved, fundamental mechanisms, if any? And how far can we go in jumping between different animal species to use as models for Hox/ParaHox regulation and function in general? Although we have certainly come a long way since the initial discovery of the homeobox in the Hox genes of a handful of animals (including humans), many questions remain and there is much still to be done. Hox/ParaHox research thus has a long future with inevitable impacts on developmental biology, biomedicine, evolution and molecular genetics.

It is clear that regulatory mechanisms that produce spatial colinearity do not necessarily act as a constraint on cluster organisation, whereas mechanism(s) controlling the temporal time-course of Hox expression do have a greater role in constraining the Hox/ParaHox clusters. The extent of this constraint remains unclear however. In a 'strong' version of the temporal-colinearity-constraining hypothesis, which involves a whole cluster mechanism, we can only hope to determine this mechanism in intact, well-ordered Hox clusters, which are very rare. The situation is further complicated by the possibility that even though an extant cluster might be intact and well-ordered it does not necessarily follow that the ancestral temporal colinearity mechanism is still in operation – it could have been lost on the evolutionary lineage to the particular extant animal possessing the cluster in question, but the cluster may have remained intact and ordered due to evolutionary inertia, with no viable cluster rearranging mutations having been produced. This may be the situation for amphioxus, for example, explaining its breaks from temporal colinearity. However, perhaps a more appealing scenario is a 'weaker' version of the temporal-colinearity-constraining hypothesis, in which STC rather than WTC is in operation. This certainly fits

with the types of data in Wang *et al.* (2017) and may well be more generally applicable when we consider the data in other lophotrochozoans, amphioxus, ascidians and even vertebrates. Whether STC or WTC was present in the ancestral bilaterian is still, however, an open question (Pascual-Anaya *et al.*, 2018).

Acknowledgements

The author thanks Giuseppe Fusco for the opportunity to contribute to this book, and Sandro Minelli for many valuable and interesting discussions over the years that have supplemented the numerous instances of thought-provoking writing that have originated from Sandro's 'pen'. The author would also like to thank Juan Pascual-Anaya for discussion of his amphioxus Hox expression data and for highlighting the importance of the methodology of interpreting the transcriptome data, and Joanne Ferrier for comments on the manuscript. Work in the author's laboratory is funded by the Leverhulme Trust, BBSRC and the School of Biology, University of St Andrews.

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Homology of endites and palps in insect mouthparts: Recent advances based on gene expression studies

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Abstract

Insect mouthparts comprise several shorter and longer branches, generally termed endites and palps. The serial homology of these branches within the different mouthparts is discussed controversially, especially in the mandibles. The *telognathic* concept and the *gnathobasic* concept of the mandible are briefly summarized. Recent results supporting the gnathobasic concept and the idea of a "single-endite" mandible are reviewed. A hypothetical scenario for the evolution of insect head appendages from a common ground-state head appendage is presented.

Endites – a primitive component of arthropod limbs

The eponymous character of the arthropods is their segmented limbs (from ancient greek *arthron*, "a joint" and *podos*, "foot, limb"). The jointed limbs are also among those features of the arthropod bodyplan that have contributed most significantly to the evolutionary success of the group. Their morphology has been adapted to a multitude of functions and arthropod limbs therefore rank among the most morphologically diverse organs in the animal kingdom.

This diversity, however, makes it difficult to devise a viable scenario for how the many extant limb types have evolved from the limbs of the last common ancestor of the arthropods, and how these ancestral limbs might have looked like (reviewed in Minelli, 2003). It is generally accepted that limb components present in most arthopod groups are phylogenetically old and might even trace from the ancestral limb (the "ground-state appendage"). Endites are outgrowths on the ventral side of the appendages and a very good candidate for an ancient limb component. They are usually rigid and stout structures used for crushing, cutting and grinding food (hence they are often also more specifically called

G. Fusco (ed.) *Perspectives on Evolutionary and Developmental Biology.*

"gnathendites"). Most importantly, endites are present in all extant arthropod groups and are known from most fossil representatives as well (reviewed in Boxshall, 2004). Therefore, endites represent a phylogenetically ancient element of arthopod limbs and might even be regarded as a component of the ground-state appendage of the ancestral arthropod.

Insect mouthparts and their endites

In some arthropod groups, endites occur on most appendages along the entire body axis, e.g. in many crustaceans. In insects, however, endites are not a prominent feature of the body plan, and clear-cut endites are only present in two appendage pairs of the head. The head is the anterior-most tagma of the insect body plan (Fig. 1a) and comprises five segments (plus an anterior portion, the composition of which is highly controversial) (Fig. 1b). Undisputed endites are only present on the maxillae and second maxillae which are the appendages of the posterior two head segments and mainly serve as mouthparts (gnathalia). Other outgrowths on the insect body have at times been regarded as remnants or modifications of endites as well (for example several types of abdominal outgrowths), but a discussion of these appendages is beyond the scope of this short contribution.

The mouthparts in insects are frequently modified as an adaptation to new, highly specific food sources and feeding behaviour (e.g., the proboscis of lepidopterans, or the rostrum in heteropterans). These adaptations often change mouthpart morphology dramatically and obscure the primitive composition (i.e. primitive in arthropods) of these appendages. This primitive composition, however, is best seen in insect species with a non-specialised chewing-biting feeding strategy (Fig. 1c). In these species the maxillae and second maxillae comprise a proximal portion and a distal, multisegmented palp. The endites in these appendages grow from the ventral side of the proximal portion. There are usually two endites per appendage. These endites are termed *lacinia* and *galea* in the maxilla, and in the second maxilla the two endites are called *glossa* and *paraglossa* (not to be confused with components of the hypopharynx that are frequently, but incorrectly, also called glossa and/or paraglossa). Note that the pair of maxillae (left and right maxilla) is always separate, but the left and right second maxilla are always fused along the basis of their proximal portion and thus form a composite "lower lip", the so-called *labium* (Fig. 1c).

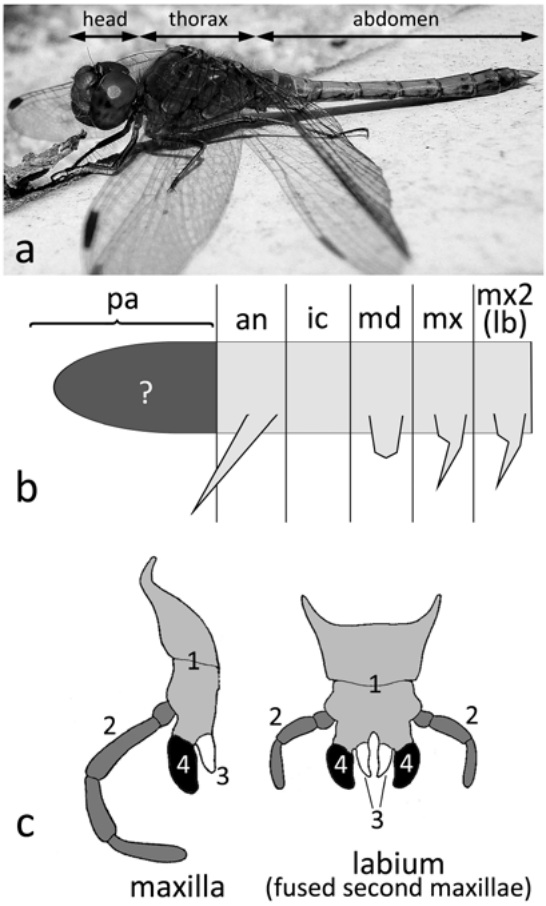

Figure 1. The insect bodyplan and explanation of important morphological terms. (a) The insect body (the image shows a dragonfly) comprises three tagmata: head, thorax and abdomen. The head usually forms a heavily sclerotised head capsule that obscures the segmental composition of this tagma. (b) Embryological studies have revealed that the head comprises five segments: antennal (an), intercalary (ic), mandibular (md), maxillary (mx) and labial (=second maxillary) segment (lb, mx2). All head segments, except for the intercalary segment, bear paired appendages. The head portion anterior to the antennal segment, the pre-antennal region (pa), might represent non-segmental tissue, or comprise additional cryptic segments, or consist of a combination of both, but this is still unclear (indicated by the questionmark (?)). (c) The mouthpart appendages maxilla and second maxilla are morphologically very similar and are of the same substructure, except that the maxillae are separate whereas the left and right second maxilla are fused together to form the labium. The shades of gray plus the numbers indicate serially homologous parts. 1 (light gray) denotes the bipartite basis of these appendages. On this basis there are three outhgrowths: the palp (2, dark gray), and two endites (3 and 4, black and white). In the maxilla these endites are called lacinia (3) and galea (4), and in the second maxillae/labium these endites are termed glossa (3) and paraglossa (4).

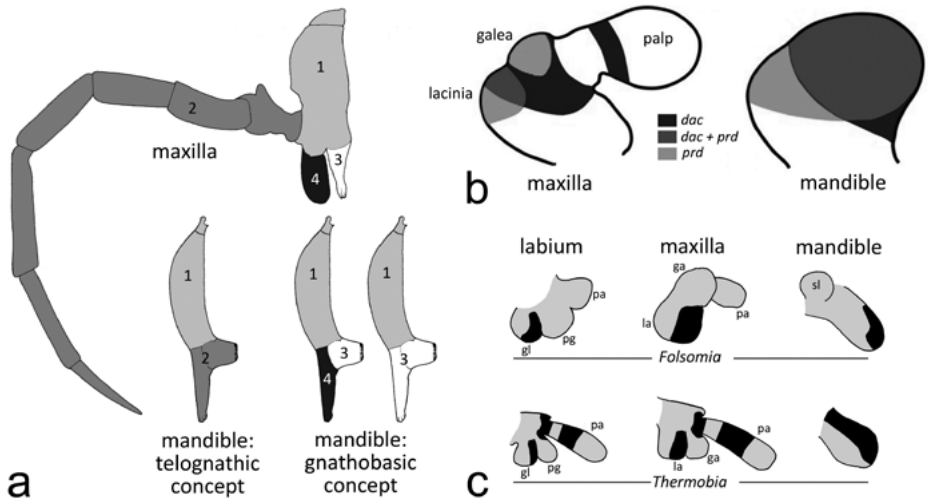

Figure 2. Serial homologies of endites in insect mouthparts. (a) Two major hypotheses for serial homologies between maxilla and mandible. The shades of gray and the numbers denote homologised components. The telognathic concept posits that the mandible corresponds to the entire maxilla and therefore contains a serial homologue of the maxillary palp (dark gray, 2). The gnathobasic concept proposes that the mandible lacks the palp and homologises the endites of the maxilla with the toothed lobes of the mandible (black and white, 3 and 4). The left image shows the interpretation after Machida (2000), the right image shows the modified interpretation after Coulcher and Telford (2013) (see panel b) (images modified after Machida, 2000). (b) Co-expression patterns of *dachshund* (*dac*) and *paired* (*prd*) strongly suggest that the mandible is serially homologous only to the lacinia endite in the maxilla, thus supporting the gnathobasic concept, but adding a significant modification to the interpretation of endite homology (images modified after Coulcher and Telford, 2013). (c) The expression patterns of *dachshund* (indicated in black in the drawings) in the mouthparts of *Folsomia candida* (top row) and *Thermobia domestica* (bottom row) support the modified gnathobasic concept. In both species, the *dac* gene is expressed in half of the glossa (gl), lacinia (la) and mandible, but not in the paraglossa (pg) and galea (ga). Further abbreviations: pa, palp; sl, superligua (a tiny appendage present in the vicinity of the mandible in *Folsomia*, but not present in *Thermobia* (images modified after Schaeper *et al.*, 2013).

Serial homology of insect mouthparts

All segmental appendages along the insect body are serially homologous (i.e., they derive from an ancestral appendage by the duplication or addition of segments, quasi an evolutionary "copy-and-paste" mechanism). Thus, the basic components should also be homologous between the appendages on different segments. Apart from the fact that the second maxillae are fused to form the labium, second maxillae and maxillae are morphologically very similar, and it is straightforward to homologise their main components (Fig. 1c). Also the serial homology of the two endites of each appendage type is not controversial: the

maxillary lacinia is homologous to the labial glossa, and the maxillary galea is homologous to the labial paraglossa (e.g., Snodgrass, 1935; Matsuda, 1965). However, the extension of homology hypotheses to include further head appendages, for example the mandible, is more difficult and has led to considerable controversy.

The *mandibles* are the gnathal appendage pair in the segment anteriorly adjacent to the maxillae. They are usually strongly sclerotised, tooth-like appendages, with little morphological subdivision apart from two toothed lobes, termed *molar* and *incisor* (alluding to the teeth of mammals). Two homology hypotheses have been proposed (Fig. 2a): (1) the so-called telognathic concept regards the mandible as a complete appendage with both proximal and distal components (Manton, 1973). Therefore, all chewing/biting components (incisor, molar) are regarded as the distal portion of the mandible and are homologized with the distal element of maxilla and labium, the palp. (2) The gnathobasic concept does not consider the mandible to be a full appendage. Instead, the gnathobasic concept assumes that all distal elements have been lost in the mandible, i.e. the mandible once had a palp like maxilla and labium, but this palp has been lost entirely during evolution (Snodgrass, 1935; Kukalova-Peck, 1992). The two lobes of the mandible are then homologized with the two endites in the maxilla and the labium (but see below for a modification of this idea).

Gene expression and serial homology

Interestingly, to solve this dispute about serial homology between maxilla/labium and mandible, for the first time in the history of zoological research, gene expression patterns have been exploited to aid the identification of serial homologs. The gene *brista*/*Distal-less* (*Dll*) has been discovered in *Drosophila melanogaster* and has been found to be essential for the development of the distal portion of the legs (Sunkel and Whittle, 1987; Cohen *et al.*, 1989). Expression of Dll protein has been detected in the distal portion of the limbs of diverse arthropods, but not in the mandible of insects (Panganiban *et al.*, 1994; Panganiban *et al.*, 1995). This was interpreted as very strong support for the gnathobasic concept of the insect mandible (Popadic *et al.*, 1996). If the mandible contained distal elements as proposed by the telognathic concept, then it should have expressed *Dll* as well. The lack of *Dll* expression thus agreed well with the lack of distal portions proposed by the gnathobasic concept. However, it was later discovered that *Dll* is not only expressed in the distal portion of all limbs, but also in endites and other outgrowths (e.g., epipodites, gills) (e.g., Giorgianni *et al.*, 2004; Jockusch *et al.*, 2004). Therefore, the lack of *Dll* expression in the insect mandible is less informative than previously thought. However, the gnathobasic

concept of the mandible has also received support from careful re-studies of *Dll* expression in insects, crustaceans and myriapods (Popadic *et al.*, 1998; Scholtz *et al.*, 1998; Mittmann and Scholtz, 2001), and also from morphological and embryological studies (Kukalova-Peck, 1992; Machida, 2000).

Strong support for the gnathobasic concept was provided subsequently by a second marker gene, *dachshund* (*dac*), that was used for the assessment of serial homology between maxilla and mandible parts. A study in the flour beetle *Tribolium castaneum* showed that the *dac* gene has a bi-partite expression in the embryonic legs: there is a ring-shaped domain in the distal portion of the legs, but there is also a separate patch of *dac* expression in the proximal portion of the legs (Prpic *et al.*, 2001). This bi-partite expression pattern is also found in the maxilla, but not in the mandible, where only a single domain is present that fills the entire tip. Thus, this single domain in the mandible could either correspond to the distal ring or to the proximal patch in the maxilla and the legs. In *Tribolium* a mutant is available that does not express a functional Dll protein and, therefore, in these mutant animals all appendages are forced to develop without Dll similar to the mandible in the wildtype (Beermann *et al.*, 2001). In these *Dll* mutants, the legs are shortened and thus similar to the mandible of the wildtype. The distal *dac* ring is deleted together with all distal leg tissue, but intriguingly the proximal *dac* domain is still present and now fills the tip of the leg stump highly reminiscent of *dac* expression in the wildtype mandible (Prpic *et al.*, 2001). This suggests that the *dac* domain in the wildtype mandible is homologous to the proximal *dac* domain in the maxilla and legs, and thus *dac* expression supports the gnathobasic concept of the insect mandible.

Especially since the careful embryological study in a basal flightless insect species, the bristletail *Pedetontus unimaculatus* almost two decades ago (Machida, 2000), the two lobes of the mandible are considered homologous to the two endites of the maxillae/second maxillae. The molar is homologised with the lacinia/glossa and the incisor is homologised with the galea/paraglossa. It came, therefore, as a sizeable surprise that a recent study (Coulcher and Telford, 2013) provided compelling evidence for the homology of the entire mandible with only a single endite, namely the lacinia/glossa. The study added a third molecular marker to the assessment of serial homology in the insect mouthparts, the gene *paired* (*prd*). The exact co-expression pattern of *dac* and *prd* in the lacinia of *Tribolium* is virtually identical to these patterns in the mandible (Fig. 2b). Although these results are surprising and do not agree with embryological results (Machida, 2000), the hypothesis of a "single-endite" nature of the insect mandible has been proposed earlier by Demoulin (1960) based on a careful analysis of the musculature in the mouthparts. In addition, the single endite nature of

the mandible is supported by recent results with *dac* expression in two basal insects, the springtail *Folsomia candida* and the firebrat *Thermobia domestica* (Schaeper *et al.*, 2013). In these two species, *dac* is expressed only in one of the two endites, namely in the glossa and the lacinia (Fig. 2c). It is also expressed in the mandible of both species. This alone would not yet be strong support for the single-endite hypothesis, but if one also takes into account the exact spatial pattern of *dac* expression, the similarities between glossa, lacinia, and mandible are striking: in all cases *dac* expression fills only one half of the structure; in *Thermobia* the glossa and the lacinia even look like smaller versions of the mandible.

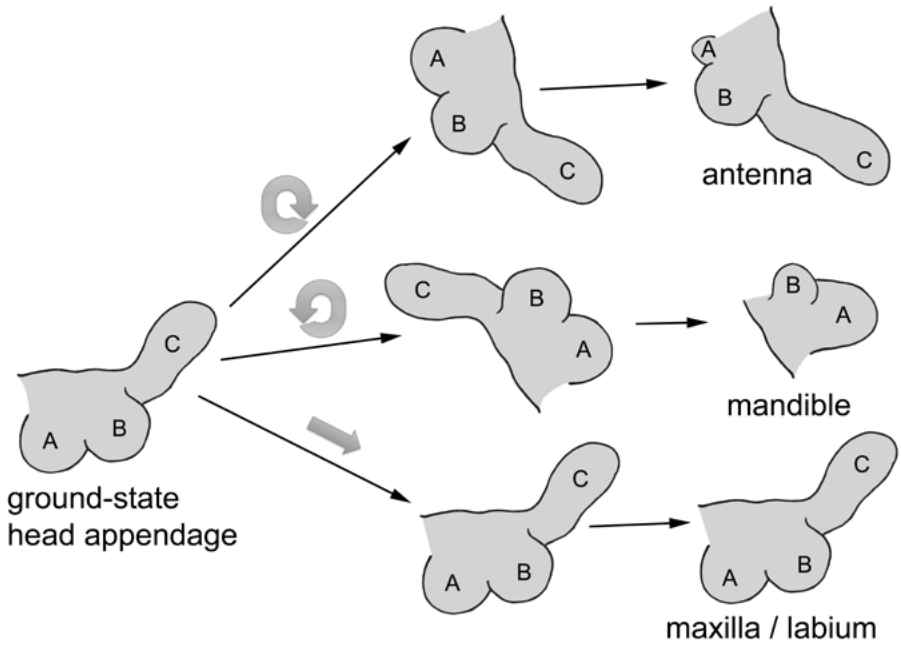

Figure 3. Hypothetical scenario of insect head appendage evolution from a common ground-state head appendage. The ground-state head appendage comprises a basal portion from which three outgrowths arise: two endites (A, B) and a palp (C). The anterior head is subject to morphogenetic movements leading to moderate shifts of the appendages; the antennal precursor is rotated clockwise, the mandible precursor is rotated counter-clockwise. Top row: the final antenna is hypothesised to be the palp. Endite A of the antennal precursor becomes reduced and forms the so-called "pre-antenna", a tiny bud that has been described in a few insect embryos, and endite B is hypothesised to form the labral buds that fuse to form the labrum proper (see Kimm and Prpic, 2006). Centre row: the palp of the mandibular precursor is lost entirely, the final mandible is hypothesised to derive from endite A, whereas endite B forms the superlingua that is present in several insect species. Bottom row: the final maxilla and second maxilla (labium) are derived from the ground-state appendage with minimal modification.

A ground-state head appendage?

In summary, recent advances in expression studies of developmental genes in insect mouthparts strongly support the gnathobasic concept of the mandible, but also introduce a significant modification of the concept, namely that the mandible is homologous only to a single endite in the other gnathal appendages. All available data support an ancestral gnathal appendage that looked similar to the maxilla/second maxilla in extant insects with chewing-biting feeding strategies (see also Matsuda, 1965). The modern gnathal appendages are derived from this ancestral gnathal appendage with either minimal modification (maxilla, labium) or moderate counter-clockwise rotation and the loss of the palp and the galea/paraglossa endite (mandible) (Fig. 3). Finally, one could take the idea of the ancestral gnathal appendage one step further and assume that it also represents the ground-state for all head appendages (Fig. 3). Starting with an appendage with a palp and two endites, the antenna can be derived from it by a moderate clockwise rotation and the loss of the two endites; the antenna itself would then essentially correspond to the palp of the ground-state head appendage. The loss of the endites need not be entire: if we assume that the original endites can also be retained in a reduced form, then these endite relics provide the basis for additional hypotheses of serial homology that could be tested in the future by the discovery of additional molecular markers. In the case of the antenna (Fig. 3, top row), a tiny relic antennal lacinia/glossa could actually account for previous reports of enigmatic "pre-antennal" limb buds in a few insect embryos (e.g., Leunziger *et al.*, 1926). And a relic antennal galea/paraglossa could be homologised with another disputed outgrowth in the vicinity of the antenna: the labrum. In the adult insect, the labrum is a single (i.e., not paired) structure and its appendicular nature is therefore controversial (reviewed in Janssen, 2017), but in some species, e.g. in *Tribolium castaneum*, the labrum develops from two separate limb bud-like primordia that fuse during embryogenesis (similar to the labium) (Kimm and Prpic, 2006), and there is actually an earlier hypothesis that derives the labrum from fused endites (albeit from the intercalary segment, rather than from the antennal segment) (Haas *et al.*, 2001). In the case of the mandible (Fig. 3, centre row), the palp is lost entirely, the lacinia/glossa homolog is enlarged and becomes the mandible proper, and the relic galea/paraglossa homolog might well correspond to the superlinguae, enigmatic appendages that are present in several insect groups and that have been attributed either to their own (purely hypothetical) "superlingual segment" (Hansen, 1893) or to the mandibular segment (Hoffmann, 1911; Snodgrass, 1935). The lack of *Dll* and *dac* expression in the superlinguae of *Folsomia candida* (Schaeper *et al.*, 2013) is actually compatible with a galea/paraglossa

homology of the superlinguae, because like the superlinguae neither the galea nor the paraglossa express *Dll* and *dac*, whereas both genes are expressed in the lacinia and the glossa of *Folsomia candida*.

Acknowledgements

I would like to thank Giuseppe Fusco for giving me the opportunity to contribute to this Festschrift on the occasion of Alessandro Minelli's 70th birthday, and for his tremendous effort to bring it all together. I also thank Giuseppe Fusco and Gerhard Schlosser for helpful comments on the manuscript. Finally, I thank Alessandro Minelli for his continued influence on the fields of evolutionary developmental biology and animal phylogeny; he has provided me with vital thought-provoking impulses throughout my entire scientific career.

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What have we learned about the evolutionary relationships of neural structures in arthropods?

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Abstract

Arthropods are the most diverse phylum of the animal kingdom, which makes them an excellent biological system for the evo-devo research field. The evolution of arthropod body shapes and their nervous systems have been extensively studied over the past decades and large data sets of structural, developmental and gene expression data are available nowadays. Here I discuss if these new data have brought us closer to understanding the evolutionary relationships of neural structures, using arthropod mushroom bodies as an example.

Introduction

The arthropod nervous system shows several conserved features that have been used for classification: a tripartite brain consisting of protocerebrum, deutocerebrum and tritocerebrum, and a ladder-like ventral nerve chord (Bullock and Horridge, 1965; Strausfeld, 2012). Furthermore, the brain contains specialised centres, which are associated with specific neurological functions, such as learning and memory (Strausfeld, 2012). However, the homology of the brain centres between the various arthropod groups has been debated, as has been the homology of the different subdivisions of the brain and the ventral nerve cord – questions which are directly linked with the "arthropod head problem" and the homologisation of gnathal and trunk segments between taxa (reviewed by Scholtz, 2016). Over the past decades, the analysis of potentially homologous neural structures has achieved increasingly higher resolution due to significant advances in imaging and sequencing technologies. However, rather than joining all pieces of the puzzle, the new data sets have made the term homology even more complex. We are now left with the problem at which and how many

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levels we have to consider homology in order to identify evolutionary relationships of morphological structures. In the following I will discuss this problem using arthropod mushroom bodies (MBs, a paired structure of the brain) as an example. This short essay is by no means a comprehensive review of the field but discusses a few recent advances and their importance in understanding the evolution of homologous and convergent neural structures.

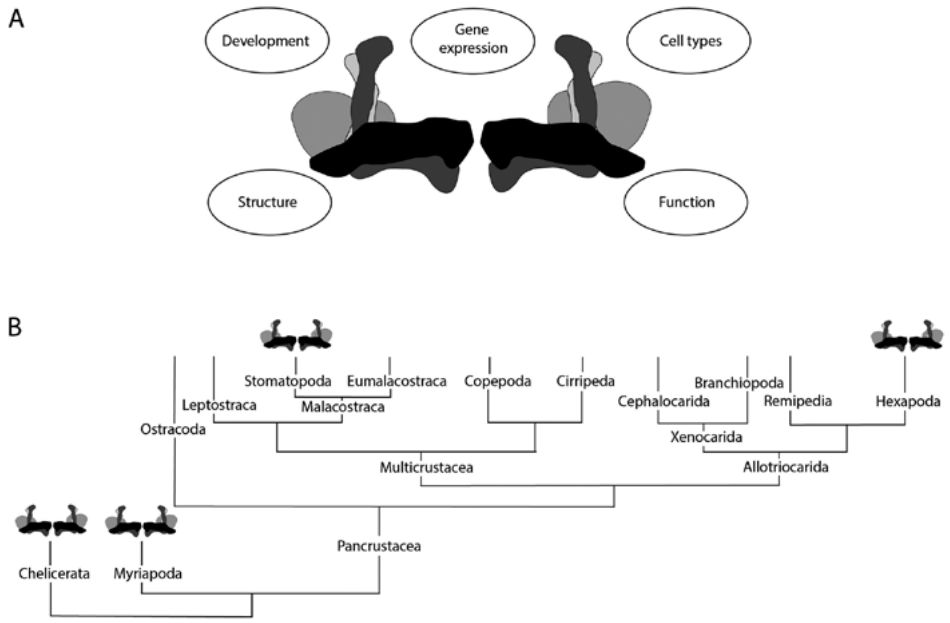

Figure 1. Mushroom body structure and distribution in arthropods. (A) Schematic drawing showing the structure of the *Drosophila melanogaster* mushroom body (black: β' lobe; dark grey: βlobe (bottom), α lobe (top); middle grey: calyx; light grey: α' lobe) and the different characteristics discussed here. (B) Arthropod phylogeny (modified after Wolff *et al.*, 2017) indicating groups with mushroom bodies. Please note that the mushroom body symbol does not depict the divergent structures.

Evolutionary relationships of arthropod mushroom bodies

MBs have first been described in insects and are centres for learning and memory (Strausfeld *et al.*, 1998). These second-order sensory neuropils are located in the most anterior part of the brain, the protocerebrum. The name-giving structures are a pair of calyces, cap-like neuropile regions, connected to a stalk, the pedunculus (Figure 1A). The pedunculus consists of a large amount of parallel axons projecting towards the front of the brain, where they divide into a ver-

tical and medial lobe (Strausfeld *et al.*, 1998; Strausfeld, 2012). The pedunculus and lobes contain many thousands of parallel fibers that originate from the Kenyon cells, which are grouped together around the calyces (Kenyon, 1896). Although brain structures with properties similar to insect MBs are present in most arthropod groups, differences in shape and innervation patterns have led to debates about their homology. Can we now solve the homology problem by considering recent data on all available features of these arthropod brain structures (e.g., development, gene expression, cell types, greater species coverage) (Figure 1A,B)?

Does comparison of structure and function solve the homology problem?

Within arthropods, MBs have been identified in hexapods, chelicerates and myriapods (Strausfeld 2012). Based on currently accepted molecular phylogenies, which suggest a sister group relationship of myriapods and pancrustaceans (insects and crustaceans), together called Mandibulata, and place chelicerates as sister group to the Mandibulata (Regier *et al.*, 2010) (Figure 1B), MBs must have been present in the last common ancestor of arthropods and thus represent homologous structures. However, there are problems with this interpretation. MBs were thought to be absent in all crustacean groups (Maza *et al.*, 2016). Since crustaceans are paraphyletic and insects (and the remaining hexapods) are nested within crustaceans in the arthropod tree (Regier *et al.*, 2010), the question arises of how insect MBs are related to the MBs of the remaining arthropod groups and how learning and memory centres have evolved in arthropods.

One hypothesis suggests that the hemiellipsoid bodies of malacostracan crustacens (e.g., crayfish, shrimps, crabs) might correspond functionally to the insect MBs (Maza *et al.*, 2016). Like insect MBs, the crustacean hemiellipsoid bodies are second order olfactory centres, which receive input from thousands of projection neurons originating in the olfactory lobe (Sullivan and Beltz, 2004; Wolff and Strausfeld, 2015). Furthermore, recent behavioural studies combined with in vivo calcium imaging in the crab *Neohelice granulate* showed training-dependent changes in neuronal responses of the hemiellipsoid bodies (Maza *et al.*, 2016). These findings are further supported by the expression of proteins required for learning and memory formation and enriched in the *Drosophila melanogaster* MBs, e.g. protein kinase A catalytic subunit alpha and phosphorylated calcium/calmodulin-dependent protein kinase II (Wolff *et al.*, 2012; Stemme *et al.* 2016; Wolff, *et al.*, 2017).

Historically, only a few criteria were used for identifying MBs, such as hundreds of clustered minute globuli cells (Kenyon cells in insects) whose processes generate the peduncle, lobes and calyces. However, in many insects as well

as in myriapods and chelicerates the calyces are absent (Wolff and Strausfeld, 2015). Wolff *et al.* (2017) therefore dissected the neuroanatomical structure into smaller units and compared over a dozen characters (e.g., density of globuli cells, axonal/dendritic arrangement, presence/absence of specific neurotransmitters) in several insect and malacostracan species. The authors document the surprising finding that Stomatopoda (mantis shrimps, the sister group of eumalacostracans) possess insect-like MBs since their hemiellipsoid bodies share all analysed morphological characters with insects, while less than half of the insect MB features can be found in the equivalent brain centre of the remaining malacostracans (Figure 1B).

This poses a problem as current molecular phylogenies suggest that crustacean taxa lacking MBs are most closely related to insects (Regier *et al.*, 2010; von Reumont *et al.*, 2012; Oakley *et al.*, 2013) (Figure 1B). Thus, rather than resolving the homology issue, the detailed recent studies leave us again with two scenarios – either MBs are homologous between all arthropod groups (where they are present) or they have evolved convergently. In the first scenario MBs would have been present in the last common ancestor of pancrustaceans and almost all crustacean taxa would have lost many (or all) of the typical features. Only Hexapoda and Stomatopoda would have retained the ancestral structure. In the second scenario, MBs would have been absent in the pancrustacean ancestor, and hexapod and stomatopod MBs would have evolved convergently (Wolff *et al.*, 2017). Consequently, hexapod and stomatopod MBs would not be homologous to chelicerate/myriapod MBs.

Which scenario is more likely? In order to address the question from a different angle, we can assess the similarities between chelicerate, myriapod and insect (stomatopod) MBs. The homology of MBs in these three arthropod groups has been debated because of differences in structure, function and position (e.g., Loesel *et al.*, 2013; Battelle *et al.*, 2016). For example, in chelicerates and myriapods calyces are absent and in a large group of chelicerates, the araneans (spiders), the MBs do not receive olfactory input, rather, they are visual neuropils (Strausfeld and Barth, 1993). However, these differences do not exclude homology since there are high variations in MB morphology even within taxa. Calyces, for example are extremely variable across insects and are lacking in some genera altogether (e.g., anosmic insects; Strausfeld *et al.*, 1998). Furthermore, differences in function might be due to variations in the position of primary sensory neuropils and the way sensory information is processed. For example, in spiders the olfactory glomeruli are not located in the brain but distributed metamerically in the ganglia of body segments carrying olfactory sense organs (Strausfeld and Barth, 1993).

In support of homology, Wolff and Strausfeld (2015) compared anterior brain centers across invertebrate phyla and identified a few characters, which they found to be present in all analysed species: a cluster of globuli cells, which form parallel intrinsic processes; an orthogonal network formed with extrinsic neurons and centrifugal cells that loop back at more distal levels forming feedback pathways, and enriched expression of proteins required for learning and memory (e.g., PKA catalytic subunit α, CaMKII). While these features could represent the MB ground pattern and thus support a common protostomian origin, one could argue that there are developmental constraints restricting the arrangement of higher order association centres and that the learning and memory proteins have been independently recruited. What is known about the development of MBs in arthropods and do the data help determine the evolutionary relationships of MBs in arthropods?

Adding complexity – development, gene expression and cell types

Unfortunately, our knowledge of arthropod MB development and cellular subpopulations is fragmentary, except for insects. There are no data available on brain centre development in myriapods and crustaceans; however, two comparative studies have been published on representatives of spiders and onychophorans, the sister group of arthropods (Doeffinger *et al.*, 2010; Eriksson and Stollewerk, 2010). Spider and insect MB development share several similarities. (1) In both cases MBs arise from bilateral clusters of neural precursors (Farris and Sinakevitch, 2003; Urbach and Technau, 2003, Döffinger *et al.*, 2010). (2) Both in insects and spiders, the *achaete-scute* homologues (*ASH*) and the transcription factor *dachshund* (*dac*) are strongly expressed in the MB precursors and (3) in both cases the neural progenitors continue to proliferate and show a distinct proliferation pattern. They are arranged concentrically and spread posteriorly after delamination. This tangential extension of neural precursors is unusual as neural precursors normally segregate into increasingly deeper layers (Urbach and Technau, 2003; Döffinger *et al.*, 2010).

On the other hand, there are fundamental differences. (1) In insects the MBs originate from a variable number of neural stem cells (neuroblasts), ranging from one neuroblast in moths to 500 in bees (Farris and Sinakevitch, 2003; Urbach and Technau, 2003). In spiders, neuroblasts are absent and the MBs are generated from neural precursors that invaginate and form large bilateral vesicles (Döffinger *et al.*, 2010). (2) Despite the partially similar arrangement of neurons and their processes, the composition of neuronal subtypes must be different in insects and spiders since spider MB neurons do not form calyces and are exclusively part of the visual circuit, while the neurons of insect MBs are

mainly integrated in the olfactory pathway and in addition process gustatory and visual information. Furthermore, onychophorans do not show any similarities to the development of arthropods MBs, i.e. the procephalic neuroectoderm does not show subdivisions into vesicles or clusters at the formation site of MBs. The areas of MB formation can also not be distinguished by enhanced *ASH* expression.

Although only derived from a few species, the developmental data show that approximately the same number of differences counterbalances similarities in developmental processes. One could argue, however, that the differences in the developmental origin of MBs are related to the overall differences in neurogenesis in the various arthropod groups. Under this assumption, the bilateral clusters of insect MB neuroblasts and their progeny could be homologous to the bilateral vesicles of neural precursors in spiders, for example. Thus, the developmental differences could simply be the result of over 500 Mio years of divergent evolution. This still leaves us with the problem that we must assume that spider MB precursors do not generate the same neuronal subpopulations as in insects because of the different functions of these brain centres.

This argumentation also links to the question if there is a common arthropod or even protostomian origin of a learning and memory centre. In insects, there is substantial and continuously accumulating evidence that MBs fulfil this role (e.g., Crocker *et al.*, 2016; Saumweber *et al.*, 2018). One recent study suggests a similar function of hemielliposoid bodies, at least in malacostracans (Maza *et al.*, 2016). The fact that myriapod, chelicerate (except araneans) and onychophoran MBs receive afferent fibers from primary olfactory centres indicates that MBs are sensory association centres (Strausfeld, 2012); however, that does not make them learning and memory centres per se. Learning and memory processes might occur in various other brain areas. This is supported, for example, by the ability of isopods to learn olfactory cues despite the lack of MB-like structures (Linsenmair, 1987) and the lack of consistent correspondence between the size/elaboration of MBs and the complexity of behaviour (Strausfeld, 2012). Although proteins used as indicators for learning and memory processes are present in MB(-like) structures across phyla, they might have different roles as they show pleiotropic functions (Wolff and Strausfeld, 2015). Thus one would need additional markers for learning and memory processes and a better understanding of the neuronal subpopulations and how they are generated in order to unambiguously determine if there is a common origin of MBs in arthropods (or even protostomians). Such studies could uncover a deep homology of neurons, which developed the functional features necessary for a role in learning and memory processes early in protostomian evolution. What is known about the neuronal subpopulations of MBs and how are they generated in arthropods?

In *Drosophila*, the MBs consist of over 2000 neurons. On the neuroanatomical level, MB neurons can be subdivided into intrinsic neurons (mainly composed of Kenyon cells), which arborize exclusively within the MBs, and extrinsic neurons connecting the MB with other brain areas. In *Drosophila melanogaster*, Kenyon cells arise from four MB neuroblasts and are generated in a specific temporal sequence from embryonic to pupal stages. They can be assigned to three classes (γ, α/β, α'/β'), which can be further subdivided into seven cell types by gene expression and morphology/innervation patterns (Tanaka *et al.*, 2008; Kunz *et al.*, 2012; Aso *et al.*, 2014). The extrinsic neurons have been classified into 21 MB output neuron types and 20 dopaminergic neuron types (Aso *et al.*, 2014). Similar Kenyon cell subpopulations have been detected in other insects (e.g., Schatton and Scharff, 2017) and the presence of distinct lobular structures in the MBs of myriapods and chelicerates suggests also neuronal diversity among the globuli cells, although detailed studies are not available (Wolff and Strausfeld, 2015).

In *Drosophila* the embryonic MB neuroblast lineages can be distinguished by the expression of specific combinations of transcription factors (*dac*, *eyeless* (*ey*), *retinal homeobox* (*rx*)) (Kunz *et al.*, 2012). The lineages contribute distinct numbers and subpopulations of intrinsic and extrinsic neurons to the MB. Such detailed analysis is not available in other arthropods. Based on a few publications covering MB gene expression outside of insects, it seems that MB precursor gene expression is highly variable. In *Drosophila*, the neuroectodermal areas giving rise to the MB neuroblasts express *dac* and *ey* (a *Pax6* homologue) but neither *sine oculis* (*six1/2* homologue) nor *eyes absent* (*eya*) (Urbach and Technau, 2003; Kunz *et al.*, 2012). The absence of the latter two markers and the expression of a combination of other genes that were assumed to be uniquely expressed in two of the four *Drosophila* MB neuroblasts (*achaete, lethal of scute, tailless, seven-up, sloppy-paired* and *orthodenticle* (*otd*)) was used to infer homology between *Platynereis dumerilii* and *Drosophila melanogaster* MBs (Tomer *et al.*, 2010). However, a later publication on MB neuroblast lineages showed that only one of the cells expressing the above combination is a MB neuroblast (Tomer *et al.*, 2010). The same is true for the bilateral expression domains in the anterior brain anlage of *Platynereis dumerilii*: not all cells expressing the above combination of genes contribute to MBs. Furthermore, in contrast to *Drosophila melanogaster sine oculis* homologues are expressed in the developing MBs of the beetle *Tribolium castaneum* (*six3*) and the spider *Cupiennius salei* (*six1b, six3b*). Although some markers are present in MB precursors across taxa/phyla (eg., *dac, otd*) (Urbach and Technau, 2003; Döffinger *et al.*, 2010, Tomer *et al.*, 2010), they are not exclusively expressed in the MB anlagen and MB precursors express these markers in various combinations with other neural markers. Taken together the data suggest that, so far, there is no unambiguous molecular finger-

print of MB progenitors that might help resolve the evolutionary relationship of higher order association centres in arthropods (and beyond).

This raises the question if we just need more data to discover unique combinations of gene expression in MB neurons. A promising approach is single cell RNA sequencing (scRNAseq), which has greatly advanced the field and led to the identification of unique gene expression profiles of various neuronal cell types. These include neurons previously identified by morphology, function and/or marker gene expression (e.g. Kenyon cells in *Drosophila melanogaster*; Croset *et al.*, 2018), as well as entirely new neuronal subtypes (e.g., Usoskin *et al.*, 2014; Tasic *et al.*, 2016; Yang *et al.*, 2016; Cao *et al.*, 2017; Croset *et al.*, 2018; Hochgerner *et al.*, 2018). Furthermore, the RNAseq method has led to identification of different states of differentiation in neuronal populations that were previously thought to be homogenous (e.g., hippocampal progenitor cells; Hochgerner *et al.*, 2018). An excellent example of evolutionary cell tracing is the recent comparative study by Tosches and co-workers (Tosches *et al.*, 2018) on brain areas (pallium) required for learning and memory in amniotes (birds, reptiles, mammals). The authors discovered clusters of adjacent neurons in the reptilian hippocampus by scRNAseq, which show similar molecular identities to the mammalian CA1, CA3 and dentate gyrus neuronal populations. These findings together with previous morphological and physiological data support the hypothesis that mammalian-like subdivisions were already present in the last common ancestor of amniotes (Shen and Kriegstein, 1986; Medina *et al.*, 2017, Reiter *et al.*, 2017). (In this context it should be mentioned that a common origin of protostomian MBs and the vertebrate pallium has been proposed but this debate is beyond the scope of the review; for references and discussion see e.g. Tomer *et al.*, 2010; Wolff and Strausfeld, 2016).

ScRNAseq could also be a powerful tool for identifying functionally equivalent brain structures if combined with behavioural studies. In an elegant study, Crocker and co-workers (Crocker *et al.*, 2016) combined cell type-specific transcriptome analysis with single-fly learning and memory assays in *Drosophila melanogaster*. They identified 390 genes, which are differentially expressed in 3 distinct MB cell types after memory induction. Performing similar experiments in other arthropods would resolve the question, if evolutionary conserved neuronal cell types with comparable activity dependent gene expression changes are present in MB(-like) structures across all taxa and thus would bring us closer to solving the homology problem.

However, on the other hand RNAseq data show that neurons are transcriptionally very diverse and that this diversity does not necessarily correlate with morphological and functional variations (e.g., their role in neural circuits) (Cao

et al., 2017). For example, in *C. elegans*, where each of the 302 neurons have been identified individually, classification of neurons by scRNAseq does largely not align with the 118 morphologically distinct neuronal cell types (Hobert *et al.*, 2016; Cao *et al.*, 2017). An example for the negative correlation between gene expression and morphological outcome in arthropods is the early development of the ventral nerve cord (Biffar and Stollewerk, 2014; Biffar and Stollewerk, 2015). In insects the neural progenitors (neuroblasts) that generate the conserved axonal scaffold of the ventral nerve cord are arranged in a fixed pattern in each segment. The arrangement is similar in all insects analysed. The 60 neuroblasts per segment are individually identifiable and produce fixed lineages of neural precursors, among others so-called pioneer neurons required for the formation of the conserved pattern of axonal tracts. However, a comparison between *Drosophila melanogaster* and *Tribolium castaneum* revealed that the expression profiles differ significantly in most neuroblasts (44 of 60 per segment) and that despite the molecular differences, neuroblasts in comparable positions produce the same set of Even-skipped positive pioneer neurons. Recent studies in the optic lobe of *Drosophila melanogaster* confirm that different sets of transcripton factors can activate the same neural effector genes. Desplan and co-workers (Konstantinides *et al.*, 2018) showed by scRNAseq and functional studies, that the expression of specific neurotransmitters can be regulated by different transcription factors in different neuronal cell types. This has previously also been shown in *Caenorhabditis elegans* for glutaminergic, cholinergic and GABAergic neurons (Zhang *et al.*, 2014; Pereira *et al.*, 2015; Grendel *et al.*, 2016).

Conclusion

Taken together the presented examples show that research has produced fascinating results from single cell expression profiles to structural and functional data. On the other side, the large and complex data sets have challenged the ways of inferring evolutionary relationships. At which level do we consider homology of neural structures? It seems that neither structural, cellular, developmental nor gene expression data can unambiguously solve the problem. We also have to ask the questions of how useful developmental gene expression data are in assessing homology, if neural developmental programmes tolerate considerable molecular variations in order to ensure the formation of functional phenotypes, and if different (convergent) morphological phenotypes are produced using conserved genetic tools. ScRNAseq data and other neural lineage studies show that there is a much greater diversity of gene expression in neurons than previously thought. Establishing comparative brain atlases to trace the evolutionary origin of neuronal cell types across taxa and phyla will be a

challenge considering the high variation in neural gene expression not only based on divergence but also influenced by different neuronal 'states' over time, such as life and reproductive cycle stages, cellular aging, environmental influences and activity status.

It seems that there are many different ways of making similar biological structures and cell types, which might, among others, be due to the lack of selective pressure on restricting developmental variations. This in turn has undoubtedly facilitated the evolution of diversity but also hampers our attempts to identify morphological and molecular fingerprints to understand the evolution and origin of neural structures.

Acknowledgements

Many thanks to Sandro Minelli whose work and enthusiasm for evo-devo and arthropods has greatly inspired my research. Many thanks also to Giuseppe Fusco for inviting me to contribute to Sandro's book.

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Ecological Developmental Biology and global ocean change: brachyuran crustacean larvae as models

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Abstract

We discuss several forms of developmental plasticity exhibited by marine crabs, in the context of ecological developmental biology (EcoDevo), and seek to motivate research in EcoDevo by addressing some key questions of the field. We summarise the diversity of plastic developmental responses exhibited during crab development, identify gaps in knowledge and highlight the importance of EcoDevo research in the light of current climate change. Marine crabs show a suite of plastic responses including transgenerational plasticity (e.g., maternal effects), as well as developmental plasticity both within the larval phase and across the larval-juvenile life history transition (e.g., latent effects). Given the potential ecological and evolutionary consequences we think that there is much potential for research in the field of EcoDevo using brachyuran crabs as model organisms.

Introduction

Marine crustaceans comprise one of the most diverse group of animals on our planet. As many other marine organisms, they represent a challenge for research in ecology and developmental biology; yet, the known diversity of phenotypic responses as well as the potential for adapting to the vast, deep and sometimes unknown confines of the sea, also ensure for very exiting avenues of research. Many habitats found in the ocean are characterised by important variability in environmental factors or extreme conditions: as the consequence of the tides, sessile crustaceans in intertidal zones, and those inhabiting estuaries, experience tremendous variations in temperature and salinity at a daily

G. Fusco (ed.) *Perspectives on Evolutionary and Developmental Biology.*

scale. At the abyssal plains, crustaceans have adapted to develop under high pressures; in the vicinity of hydrothermal vents organisms have evolved to take nourishment from sources of energy not linked to solar radiation. Copepods and larval stages of most crustaceans have evolved to live in suspension in the water column. Because working in marine habitats or replicating aquatic environments in the laboratory impose tremendous logistical challenges, we are still uncovering the suit of phenotypic plastic responses exhibited by marine crustaceans (as well as other groups).

We write this chapter in order to answer some questions posed in Eco-EvoDevo research and motivate interdisciplinary research integrating marine ecologists, physiologists and developmental biologists. We feel that there is a "deep trench" in knowledge and understanding, lying somewhere between our ecological approach to phenotypic variation and that of developmental biologists, at least for marine crustaceans. For a handful of species, we know much about the endocrinology, neurogenesis and other physiological processes occurring at time scales encompassing single moult cycles to the whole development under optimal conditions. Likewise, we have a comparatively large amount of information on plastic responses on some life history traits, such as growth rate, body size, and developmental time. Yet, we lack information and understanding of epigenetic mechanisms linking the environment with the subsequent phenotypic responses and their consequence in terms of fitness. This chapter is an invitation to the reader, to dive into the diversity of potential mechanisms driving developmental plasticity of marine organisms with crustaceans as examples.

We start the chapter by briefly outlining questions and basic ideas around the study of phenotypic plasticity and the field of EcoEvoDevo. We then present a series of studies about phenotypic plasticity viewed from the perspective of marine physiologists and ecologists and finally we attempt to highlight known mechanisms of developmental regulation as the candidates for explaining the observed phenotypic responses, focussing in brachyuran crabs as model species.

Concepts of EcoDevo and EcoEvoDevo

> Ecological developmental biology is the science that studies the interactions between developing organisms and their environmental contexts. It studies development in the 'real world' of predators, competitors, symbionts, toxic compounds, temperature changes and nutritional differences. (Gilbert, 2017)

> Ecological development or 'eco-devo' examines the mechanisms of developmental regulation in real-world environments, providing an integrated approach for investigating both plastic and canalized aspects of phenotypic expression. (Sultan, 2007)

Ecological developmental biology, "EcoDevo", seeks to study the dependency of developmental mechanisms and their resulting phenotype in an environmental context as seen in "real world environments" and to explore how developmental pathways incorporate environmental cues to generate context-dependent phenotypes including resulting fitness differences (Bosch *et al.*, 2014; Gilbert, 2017; Sultan, 2017). The term "EcoEvoDevo" integrates developmental biology and ecology into the evolutionary theory (Abouheif *et al.*, 2014) to acknowledge that phenotypic plastic responses are products of the evolutionary process and past selection so that they reflect phylogenetic, genetic and biochemical constraints (Sultan, 2017). "Environment" includes known abiotic and biotic factors (e.g., temperature, food, conspecifics, predators), but also endocrine disruptors (i.e., environmental compounds that can disrupt normal development by changing gene expression) and teratogens (i.e., compounds causing birth defects) modifying normal development (Gilbert, 2017).

Some key points addressed by EcoEvoDevo research comprise how environmentally responsive developmental systems evolve and how developmental plasticity does affect the process of adaptive evolution (Sultan, 2017). In addressing these points, Nijhout (2003) suggested that responses to environmental variation have evolved either to minimize disrupting effects of particular environmental variables on phenotypes ("canalization") or to modulate such responses in order to generate differently adaptive phenotypes. Along these lines, possible costs of evolving relatively plastic *versus* constant reaction norms is an intensively discussed aspect. Recent line of evidence suggests that the evolution of adaptively plastic developmental systems is not constrained by unique costs so that plasticity should not be seen as a special case of development (Sultan, 2017). Concerning underpinning mechanisms, EvoDevo research includes studies at different level of organization. On molecular and cellular levels, EcoDevo analyses the environmental input on regulatory systems such as hormonal transduction pathways or molecular changes such as DNA methylation that modify gene expression ("epigenetics"). Such epigenetic regulation allows environmental signals to be integrated at the genome level (Bosch *et al.*, 2014).

In addition, there are two other important questions addressed by EcoDevo. The first focusses on how development mediates the effects of anthropogenic climate change on performance of organisms and ultimately determines which species will adapt to climate change. The second question concentrates on whether adaptive norms of reaction are limited to certain well-studied types of organisms or are widespread among life histories and phylogenetic groups. These two questions are tightly related because the former requires a quantification of the magnitude and the nature of interspecific variation in transgener-

ational or developmental plasticity. We address the question of climate change in the following section and the question of non-model organisms in the last three sections.

EcoDevo meets global ocean change biology: emerging questions

Sultan (2007) suggested EcoDevo approaches as crucial to understand responses of organisms that are increasingly confronted with anthropogenic altered environments. A contemporary line of research in this field aims at gaining insights into the immediate tolerance of organisms and their evolutionary potential to adapt to the changing physical and biotic environmental conditions created by anthropogenic climate change (Sultan, 2007, 2015, 2017). For example, evidence obtained from the analysis of long term data series (Wiltshire *et al.*, 2010; Boersma *et al.*, 2016) indicates that global ocean change already has a major impact on marine ecosystems including plankton communities (Beaugrand *et al.*, 2009; Burrows *et al.*, 2011; Poloczanska *et al.*, 2013; García Molinos *et al.*; 2016; Boyd et a., 2018). In particular, semi-enclosed seas will be increasingly affected by rising surface temperatures (Meier, 2006; Grawe *et al.*, 2013; Hiddink *et al.*, 2015; Robins *et al.*, 2015). For a given species, one way of reacting to environmental change is shifting its range. In the North European seas, climate models predict that various animal and plant species will extend their range northwards, in agreement with current observations (Burrows *et al.*, 2011; Poloczanska *et al.*, 2013). Species, which are not able to track their preferred environment in space, may adapt *in situ* to avoid extinction when the rate of environmental change is high.

Phenotypic plasticity and evolutionary adaptation are currently discussed as essential mechanisms for organisms to adapt to environmental change both in marine (Reusch, 2013; Boyd *et al.*, 2018) and terrestrial ecosystems (Chevin *et al.*, 2010; Hoffmann and Sgro, 2011; De Meester *et al.*, 2018; Somero, 2010; Franks and Hoffmann, 2012). Evolutionary or genetic adaptation, a change in one or more heritable traits, is seen as an important way for natural populations to counter rapid climate change or to realize ecological opportunities arising from climate change (Hoffmann and Sgro, 2011). Local adaptation may however be reduced through gene flow, even in fragmented populations. In the latter case, population connectivity, *via* genetic exchange of adults, larvae, seeds or spores, leads to inflow of new genotypes (Cowen and Sponaugle, 2009). Spatially divergent selection leads to local adaptation whereas gene flow has homogenizing effects away from adaptive changes. The balance between these two effects may differ between different types of habitats and ecosystems and strongly depends on the realized dispersal of a species (Reusch, 2013). Realized dispersal, among

contrasting habitats, and a multitude of other factors, may be influenced by phenotype-environment mismatch of dispersing propagules called "selection against immigrants" (Reusch, 2013). Phenotypic plasticity (i.e., the capacity of a genotype to adjust phenotypic values depending on the environment without genetic changes; West-Eberhard, 2003) provides organisms with the ability to adapt within their lifetimes (through developmental plasticity) or within a very low number of generations (through transgenerational plasticity). Adaptive plasticity comprises beneficial adjustments expressed in response to specific environmental cues (Sultan, 2017). Disentangling whether changes in traits found e.g., in longitudinal studies of single populations have evolved (are genetic) or instead have occurred through phenotypic plasticity is an essential problem in predicting future species distribution (Hoffmann and Sgro, 2011; Reusch, 2013).

Multi-population comparisons have emerged as one experimental approach to indirectly address the extent of local genetic adaptation *versus* phenotypic plasticity and disentangle the confounding effects of a complex genetic architecture, genetic drift, or a complex demographic history (De Villemereuil *et al.*, 2016). So called "synchronous studies" compare populations coming from divergent habitats in laboratory settings ("space-for-time substitution") or reciprocal transplant approaches (Reusch, 2013). Experiments using the space for time substitutions compare populations of organisms located across spatial environmental gradients; here a spatial (e.g, temperature) gradient represents the temporal changes to be experienced by local populations at some extreme of the gradient (e.g., at the coolest extreme). Such experiments may be devised as a common garden experiment where the genetic basis of a trait is quantified by comparing the phenotypes generated by different genotypes under the same environment (e.g., increased temperature). Reciprocal transplant experiments compare the survival rate of local and non-local genotypes to examine local adaptation. Hence, both synchronic approaches test the end result of past evolution.

Brachyuran larvae: life cycle, metamorphosis, morphology

The Decapoda are a highly diverse taxon of malacostracan crustaceans and include well known representatives such as crayfish, clawed and spiny lobsters, hermit crabs and true crabs. The most species-rich infraorder of the Decapoda are the true crabs, the Brachyura. Adults are characterized by a very short pleon that is entirely hidden underneath the flattened cephalothorax. Similar to the majority of marine benthic invertebrates, brachyuran crabs develop through a biphasic life-cycle (Fig. 1). The larval phase occurs in the pelagic environment, followed by the juvenile/adult phase, mostly in the benthos. Larval dis-

persal takes place in the pelagic environment followed by larval settlement in the benthos. Furthermore, juvenile growth into adulthood, mating, extruding of embryos, embryonic development, and larval hatching (Fig. 1) occur in the benthic environment (reviews Williamson, 1982; Anger, 2001, 2006; Martin *et al.*, 2014; Haug and Haug, 2015; Jirikowski *et al.*, 2015; Møller *et al.*, 2019). Brachyuran crustaceans hatch as zoeae (Fig. 2) that differ in their appearance from the adults. These larvae grow by successive moults, and the number of zoeal instars is species-dependent (Zeng *et al.*, 2019). Most zoeae of marine species actively feed (Jeffs and O'Rorke, 2019) and possess a wide range of organ systems necessary for autonomous development in the plankton (Spitzner *et al.*, 2018). After a first metamorphosis to a semi-benthic megalopa, they develop further through a second metamorphosis and settle as a benthic juvenile (Gebauer *et al.*, 2019). These continue to grow through several moults into adulthood. This "double metamorphosis" of brachyurans involves major transitions in habitat, behaviour, locomotion, feeding, morphology and ecology (Haug, 2019).

In general, the pelagic larval phase of marine invertebrates is vital for meroplanktonic species as a means of dispersal and therefore affects gene flow, and population structure and connectivity (Pechenik, 1999; Strathmann *et al.*, 2002; Cowen *et al.*, 2007; Cowen and Sponaugle, 2009; Morgan, 2019). However, larval development encompasses risks due to higher vulnerability through predation, higher sensitivity to abiotic factors or overdrift into unsuitable habitats for settlement (Fig. 1; McEdwards, 1995; Pechenik, 1999). In addition, larval development, without parental care, requires that brachyuran larvae are adapted to survive and grow in the plankton. Such morphological and behavioural adaptations are related to movement, nutrition, sensing, etc. (Anger, 2001, 2006). The zoeal cephalothorax possesses rostral, dorsal and lateral spines that serve as defensive structures against predators (Fig. 2). The first and second maxillipeds of planktonic zoeae are used for handling food items and also fulfil a natatory function to control the vertical position of the larvae within the water column. During the first metamorphosis, the locomotory function shifts to the pleopods which emerge gradually as embryonic anlagen in the zoeal stages and become functional after metamorphosis to the megalopa (Spitzner *et al.*, 2018). Megalopae use the pleopods for swimming and the pereiopods for walking as an adaptation to the semi-benthic life style. The pleopods lose their natatory function to become part of the reproductive system after the second metamorphosis to benthic juveniles.

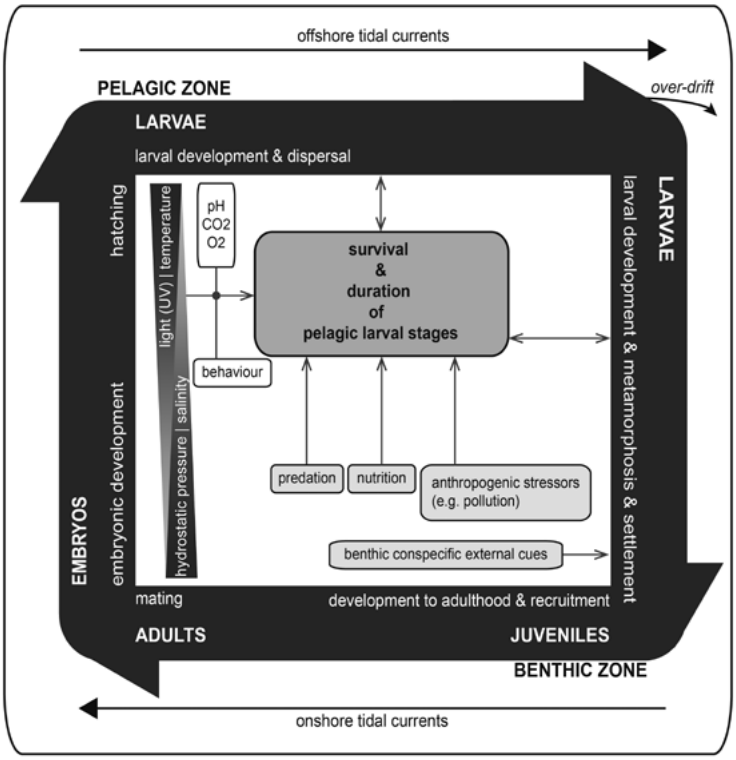

Figure 1. Schematic representation of the biphasic life cycle of brachyuran crabs including abiotic and biotic factors affecting larval development (modified from Eckman 1996 and Anger 2001). Development comprises an embryonic phase, a larval phase (pelagic zoeae and a semi-benthic megalopa), and a benthic juvenile/adult phase (see text for explanation). Females of brachyuran crabs carry the embryos: larvae hatch and are released into the pelagic environment. Dispersal occurs mainly during the larval phase and results from the combination of larval behaviour (driven by environmental cues such as light) and hydrodynamics (tidal currents). The megalopa settles in the benthic habitat and metamorphoses into a juvenile crab; settlement and metamorphosis are, in some species, promoted by conspecific cues. Individuals grow and reach adulthood in the benthic environment where they mate and reproduce. Some key factors driving larval performance are shown (e.g., abiotic factors: temperature, salinity, anthropogenic stressors; biotic factors: predation, food availability/quality).

Zoeae display a rich behavioural repertoire that allows for responses to variations in environmental factors such as light, hydrostatic pressure, tidal currents, and temperature (Forward, 2009; Epifanio and Cohen, 2016; Cohen and Epifanio, 2019). Highly developed larval sensory systems include compound eyes as well as abundant chemo- and mechanosensory sensilla (Spitzner *et al.*, 2018), for instance to sense chemical cues from conspecifics to choose suitable habitats for settlement (Gebauer *et al.*, 2019). Complex larval behavioural pat-

terns, such as active vertical migration in response to tidal currents, allow the larvae to avoid predators and to control their horizontal dispersal (Epifanio and Cohen, 2016; Cohen and Epifanio, 2019).

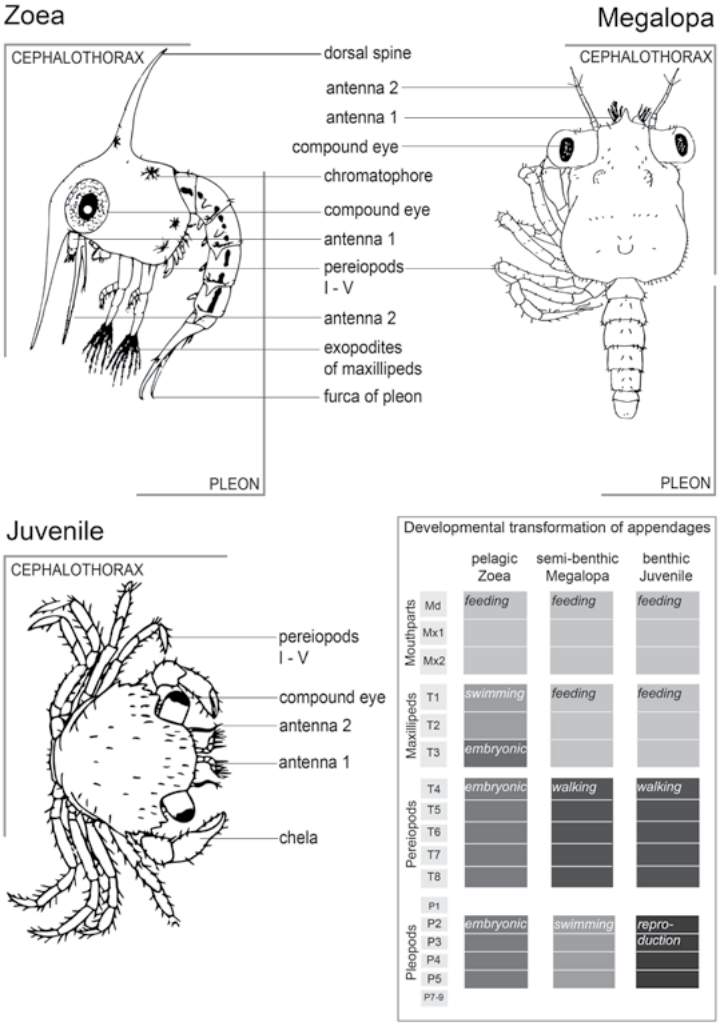

Figure 2. Generalized morphology of early life history stages in brachyuran crabs: lateral view of a zoea (top left), frontal view of a megalopa (top right) and a juvenile (bottom left). A summary of the ontogenetic changes in the function of appendages after each metamorphosis is given in the box located in the bottom right corner (after Harzsch *et al.* 1998 and Spitzner *et al.* 2018). Illustrations are modified from Williams (1968), Rice and Ingle (1975), and Martin *et al.* 2014. Abbreviations: Md – mandibular segment, T1-3 – thoracomere 1-3 with appended 1st to 3rd maxilla, T4–8 - thoracomere 4-8 with appended pereiopod one to five, P1-9 – pleomeres 1-9 with appended pleopods on segments 2-5.

Contributions of brachyuran larvae to EcoDevo research

Coastal crabs constitute good model systems for EcoDevo research because, in their natural habitat, they are exposed to important variations in several environmental factors, such as temperature, salinity, food levels, and pollutants. This is particularly relevant for those species inhabiting estuaries where e.g., salinity varies at several time scales, due to weather variability and tidal cycles. Over the past 50 years, experimental research has uncovered a suit of phenotypic plastic responses in coastal crabs, occurring over a single moulting cycle or spanning over several moulting stages. Some include responses to the maternal environment (maternal effects), others are short-term responses to conditions experienced at early stages or early in the moult cycle. These responses include changes in the acclimation state, timing of moulting, growth rate, chemical composition, body size and switches from short to long developmental pathways usually characterised by additional zoeal stages (summarised in Giménez, 2019). In the following sub-sections, we will cover maternal effects, developmental plasticity and latent effects crossing the metamorphic transition.

Maternal effects

Maternal effects consist of effects of the maternal environment or phenotype on offspring performance (Marshall and Uller, 2007). Experiments manipulating the maternal environment (Giménez and Anger, 2001, 2003; Torres *et al.*, under review) in coastal estuarine crabs have uncovered a number of pre- and post-zygotic maternal effects on larval traits. Here, we focus on the best-known pre-zygotic effects (i.e., occurring before fertilization), consisting of changes in provision of reserves (e.g., protein and lipids) into embryos. However, some effects are surely occurring through maternal provision of hormone precursors and microRNAs (Lachaise *et al.*, 1992; Chung and Webster, 2004). There is a large number of examples showing intraspecific variation in allocation of nutritional reserves into eggs in marine crabs (Giménez, 2019); however, only few studies have investigated the mechanisms driving such variation in the laboratory. Using an estuarine crab (*Neohelice granulata*) as model species, Giménez and Anger (2001, 2003) showed that females that experienced reduced salinities, similar to their natural habitat (saltmarshes located in estuaries and coastal lagoons of the Atlantic South America), allocated more reserves into eggs than those growing in seawater (= 32-33 salinity). This response was interpreted to be adaptive, because subsequently embryos experiencing reduced salinities, lost more carbon and nitrogen than those developing in seawater. Such losses reduced the amount of reserves and the survival rates of the freshly hatched lar-

vae. Hence, in this particular case, one may hypothesise that the positive correlation between larval survival and nutritional reserves has led to the evolution of adaptive mechanisms starting well before egg fertilization, whereby increased egg reserves compensate for subsequent energy losses during embryogenesis. Changes in larval reserves driven by salinity and other environmental factors may be widespread and have been found in other crustaceans (Giménez, 2019). A key and yet unanswered question is which are these mechanisms.

Post-zygotic maternal effects (i.e., occurring after fertilization but before larval hatching) have been also studied regarding the effects of salinity, temperature and PCO_2 (e.g., Laughlin and French, 1989; Giménez and Anger, 2003; Ituarte *et al.*, 2005; González-Ortegón *et al.*, 2014; Schiffer *et al.*, 2014; Torres *et al.*, under review). In estuarine crabs (*N. granulata*: Giménez and Anger, 2003) the salinity conditions experienced during embryogenesis modify the larval capacity to tolerate low salinities. For example, embryos exposed to moderately low salinities (> 50% that of full seawater) develop into larvae capable of withstand salinities as low as 5, while larvae hatching from embryos developing in seawater do not survive the first 24 h at such low salinity. At the population level, changes in the tolerance to low salinity can have important ecological consequences. For instance, in/at the coast of Uruguay, local populations of *N. granulata* are found in estuaries where larvae are likely to develop in the open coast and hence to be transported towards other estuaries by coastal currents, thereby contributing to the metapopulation connectivity. Using maps of salinity fields, Giménez (2003) showed that embryonic acclimation could have important effects on initial larval survival, a key contributor to self-recruitment and connectivity. Post-zygotic effects are also important from the standpoint of how larvae respond to climate driven environmental change (Torres *et al.* under review). For example, zoea I of the shore crab *Carcinus maenas* show thermal amelioration of osmotic stress, an antagonistic response whereby the detrimental effects of low salinity on survival are mitigated if the larvae are reared at moderately high temperatures. However, the magnitude of the mitigation is modulated by the temperature and salinity experienced during embryogenesis (Torres *et al.*, under review).

We know little about the mechanistic basis for post-zygotic effects occurring in crustaceans. In *N. granulata*, the increase in the capacity to tolerate low salinities is based on a considerable increase in the larval capacity to osmoregulate (Charmantier *et al.*, 2002). Osmoregulation is a compensatory mechanism to buffer blood osmolality (i.e., the concentration of osmotically active substances, e.g., Na^+), from variations in the osmolality of the environment (see e.g., Henry *et al.*, 2013). Osmoregulation is based on the development of a special type of cell

called ionocyte, which in zoeae are located in the branchial chamber (Cieluch *et al.*, 2007). Ion uptake is driven by the activity of the enzyme Na^+-K^+-ATPase, located at the basal membrane of the ionocytes, which pumps Na^+ from the cell cytoplasm into the haemolymph generating a gradient that favours the passive entrance of Na^+ from the environment. We know that exposure to low salinity in juveniles and adults can result in increased activity of Na^+-K^+-ATPase, and higher number of mitochondria within the ionocytes as well as increased number of ionocytes located in the ion-transport epithelia (e.g., Lovett *et al.*, 2006; Torres *et al.*, 2007; Rivera-Ingraham *et al.*, 2016). However, we do not know whether these mechanisms operate during embryogenesis and result in larvae characterised by enhanced osmoregulatory capacity. From the perspective of plasticity and EcoDevo, the mechanisms leading to proliferation of ionocytes may be one of the key processes to study, because such proliferation can take place several days after low salinities are experienced (Lovett *et al.*, 2006) and this rate may somehow constraint the capacity to respond to abrupt changes of salinity. In embryos, cell proliferation may be restricted to advanced stages during the period of formation of the zoea.

Developmental plasticity

Within the larval phase, plasticity can involve compensatory responses within each moult cycle, comprising several moulting stages and/or including the development through alternative pathways (Giménez, 2019). One of the best studied cases concerns the effect of food limitation on moulting and morphogenesis. Within a moult cycle, decapods are able to delay moulting time, when they are exposed to initial starvation periods (Anger *et al.*, 1981): for instance, zoea I stages exposed to initial starvation for a day, take an extra day to moult to the second stage. This response enables larvae to feed for an extra day in order to recover reserves needed for the moulting process. By contrast, when exposed to late starvation periods, zoea I do not delay moulting but moult to the second stage with a reduced body mass (Anger, 1987). In this latter case, the moulting period of the zoea II may also be extended; this type of plastic response, consisting on compensatory responses over consecutive stages is likely to reduce effects of stressors on the larval reserves for the subsequent moulting (Anger and Spindler, 1987).

Temperature and salinity do not necessarily reduce growth rates as in the case of food limitation. Increased temperature leads to the shortening of the moult cycle and produces a unimodal response in larval body mass, with biomass peaking at intermediate temperatures (Anger, 2001). The acquired body mass must result from the interplay of developmental and growth rate, which

depend on the energy available to the organisms in terms of nutritional reserves and oxygen. Increased temperatures also lead to increases in metabolic demands which are compensated by increased ventilation and heart rates; however, at some critical temperature (known as the pejus temperature) the function of the circulatory and respiratory systems is compromised and provision of oxygen to the tissues decreases considerably (Storch *et al.*, 2011). Oxygen limitation is currently considered a critical driver of reduced body mass in aquatic organisms living under increased temperatures (Horne *et al.*, 2015; Walczyńska *et al.*, 2015).

A major issue in estuarine coastal crabs is the effect of reduced salinity on growth and development. A central point here is the osmoregulatory capacity (i.e., quantifying how well the concentration of osmotically active substances present in the haemolymph is kept constant irrespective of that found in the environment); this capacity varies among species and developmental stages depending on the life history and migration patterns exhibited in the larval phase (Anger *et al.*, 2008). In crab larvae, we observe a suite of adaptive responses, in terms of accumulation of nutritional reserves such as lipids and proteins (Torres *et al.*, 2011). Crab larvae accumulate lipids especially early in the moult cycle and use such reserves partly to sustain the process of moulting and morphogenesis. In strong osmoregulators, which tolerate a wide range of salinities, neither growth nor development is impaired by moderately reduced salinities. By contrast, in weak osmoregulators with a narrower salinity tolerance, reductions in the accumulation of lipids (but not proteins) are observed. In osmoconformers, with a very limited tolerance to low salinity, net losses of both protein and lipids as well as delays in the timing of moulting may be observed. Overall, the strong link between osmoregulation and growth suggests that plastic responses associated to osmoregulation safeguards growth from variation in salinity, but if needed, lipid reserves may be used as a buffer to keep protein levels constant. At yet lower salinities, larvae may need to delay development or moult with reduced body mass.

Some species of brachyurans (in addition to caridean shrimps) are able to develop through alternative developmental pathways characterised by a variable number of stages (Anger, 2001). In response to nutritional or osmotic stress, or depending on the temperature experienced early in development, larvae of many brachyurans may follow developmental pathways characterised by an additional zoeal stage. For instance, in the estuarine crab *N. granulata*, such response is triggered at the first to third zoeal stage, as the latter may moult into alternative forms of zoea IV (Ostrensky *et al.*, 1997). This pattern of developmental plasticity is understood as an adaptive strategy to prioritise survival

and growth over morphogenesis, as in non-brachyurans, moulting can occur without any evident morphological progress (Knowlton, 1974).

Knowledge regarding the mechanisms driving developmental responses in larvae is variable. We would expect regulation of development driven by mechanisms ranging from molecular epigenetic changes to signals carried through hormonal transduction pathways. We know that increases in the length of the moult cycle in response to food limitation are associated to a delay in the appearance of peaks in ecdysteroids responsible for the process of moulting (Anger and Spindler, 1987). We do not know the mechanisms driving changes in body size of brachyurans in response to temperature, but research on insects suggest limited oxygen levels constitute the signal or cue of a hormonal cascade driving body size (Ghosh *et al.*, 2013, Kivelä *et al.*, 2016). Responses to low salinity are also under hormonal control (Charmantier and Charmantier-Daures, 2001; Chung and Webster, 2004) and moulting hormones are also involved in the larval development through alternative pathways (Gross and Knowlton, 2002).

Latent effects

A third form of plasticity observed in brachyuran crabs (and in other decapods) are latent effects (Giménez, 2006, 2019); i.e. effects of environmental conditions experienced by larvae on the size and development after metamorphosis (Pechenik, 2006). Latent effects may consist of reduced body size at metamorphosis in response to food limitation (Giménez, 2010) or lack of cues indicating for optimal substratum type at the time of metamorphosis (Gebauer *et al.*, 2003, 2019). In addition, differences in body mass existing at the megalopa and originated in developmental variability, can be carried over to the juvenile stages (Giménez *et al.*, 2004). In the latter case, we see the consequences of larval developmental plasticity extending beyond the metamorphic boundary.

There is still no information available about the mechanisms underlying latent effects and uncovering them will require targeted research in order to better understand how environmental conditions modify processes occurring during metamorphosis. From the eco-evolutionary perspective, latent effects are interpreted as developmental trade-offs; as such, effects such as reduction in body size are expected to impact juvenile performance and recruitment (Torres *et al.*, 2016). In brachyurans, size dependent cannibalism is a critical process driving recruitment (Moksnes, 2004) and latent effects may impact juvenile survival by exposing individuals to increased risk of being cannibalized. Understanding latent effects is therefore of great ecological importance.

EcoDevo of brachyuran larvae and understanding effects of global ocean change

Crustacean larvae play a central role in connecting established populations and in founding new populations to expand a species' range (Cowen *et al.*, 2007; Cowen and Sponaugle, 2009; Burgess *et al.*, 2015; Morgan, 2019). They represent the most sensitive stage of the crustacean life cycle and therefore are strongly affected by fluctuations in the animal's environment. Therefore, quantifying larval responses to variations in abiotic factors such as temperature and salinity (reaction norms; see the previous section on EcoEvoDevo concept) is important because temperature controls the dispersal potential through changes in the length of the dispersal phase (longer at lower temperatures) and through effects on larval growth and survival (Fig. 2; Anger, 2006; Epifanio, 2013; Anger *et al.*, 2015). Predictions of the distribution of crab species in the future ocean, so far, have been primarily based on thermogeography, the analysis of heat tolerance and potential for thermal acclimation of adult animals (e.g., Compton *et al.*, 2010; Paganini *et al.*, 2014; Tepolt and Somero, 2014). Nevertheless, some studies have used information on reaction norms of brachyuran larvae to determine the likelihood of range expansion or range shifts of crab species in response to ocean change (Sanford *et al.*, 2006; deRivera *et al.*, 2007).

There is a long tradition of examining the reaction norms of decapod crustacean larvae to changes in single environmental drivers such as temperature, salinity and food availability. In the past, these data have been mainly discussed in the broad context of larval biology including aspects of life history cycles, supply-side ecology, biogeographic distribution, population connectivity, and invasion biology (reviews in e.g., Anger, 1987, 1998, 2001, 2006; Jeffs and O'Rorke, 2019; Zeng *et al.*, 2019) but not primarily in the context of global ocean change. An EcoDevo perspective may be critical to understand and predict the future of organisms in a changing world (Sultan, 2007). Today, understanding synergism and antagonism among multiple environmental drivers is seen, more and more, as essential to predict future species distribution (Boyd *et al.*, 2018; Galic *et al.*, 2018, Schäfer and Piggott, 2018; Piggott *et al.*, 2015). Sultan (2017) calls for reaction norm experiments "designed around real-world systems" because combinatorial treatments are more ecologically meaningful than those varying only single environmental drivers as in traditional developmental biology. In general, brachyuran larvae are well suited to analyse such effects of combined multiple drivers. In crustacean larvae, both temperature and salinity are known to operate in combination leading to multiple stressor effects on larval performance (e.g., González-Ortegón *et al.*, 2013). Furthermore, available studies have analysed the pH sensitivity of brachyuran larvae to address ocean acidification

(OA: a decline in ocean surface pH by absorption of elevated atmospheric CO_2; Carter *et al*., 2013, Ceballos-Osuna *et al*, 2013, Schiffer *et al*., 2014). For instance, although embryos of *Petrolistes cinctipes* are regularly exposed to naturally fluctuating hypercapnic water in the intertidal, sustained exposure to low pH may be detrimental on embryos and larvae (Ceballos-Osuna *et al*., 2013). Metabolic responses of larvae reared according to year 2300 predictions of OA showed lower metabolism and dry weight. This study showed differences among broods indicating maternal effects (Carter *et al*., 2013). Exposure of larvae of the spider crab *Hyas araneus* to the combined effect of elevated levels of CO_2 in seawater and heat-shock affected the gene expression of heat shock proteins, resulting in a decrease in the thermal limits on the whole animal.

Common garden experiments compare populations from divergent habitats in laboratory settings to analyse the extent of local genetic adaptation *versus* phenotypic plasticity. This approach that analyses the genetic basis of a trait by comparing the phenotypes generated by different genotypes under the same environment has not yet been widely explored in crustacean larvae but was applied in studies on non-malacostracan crustaceans. Data on rearing larvae of the mud fiddler crab *Uca pugnax* from different populations under different controlled temperatures were analysed to explain the geographic range limits of this species (Sanford *et al*., 2006). Comparing the thermal and salinity tolerance of larvae of the barnacle *Balanus improvisus* from Baltic *versus* Atlantic populations revealed that the Baltic population may be favoured by near-future seawater warming (Nasrolahi *et al*., 2016). Furthermore, populations of this barnacle species taken from the Baltic salinity gradient (Baltic, Kattegat, and Skagerrak) were cultured in a common garden approach to test phenotypic traits. This study suggested that in this species plastic responses are more likely than evolutionary tracking, in order to cope with future changes in coastal salinity (Wrange *et al*., 2014). Thermal tolerance quantified in laboratory rearing and selection experiments of the tidepool copepod *Tigriopsis californicus* from eight populations, covering a wide longitudinal gradient suggested that plasticity and adaptation have limited capacity to buffer isolated populations against future increases in temperature indicating a high extinction risk in species with strong local adaptation (Kelly *et al*., 2012).

Future directions

This review has attempted to answer/discuss a key question of EcoDevo: do non-model species show adaptive reaction norms? Our answer is yes, non-model species show a large diversity of adaptive responses, many of which are perhaps not even present in model species. Hence, exploring responses in non-model

species, considering variation within and among populations, in order to better understand the potential interplay between plasticity and local adaptation through genetic evolution is a warranting enterprise for future researchers. In addition, such information would be critical to elucidate the mechanisms by which biodiversity/diverse organisms responds to global change. From the perspective of development, changes in biodiversity and community structure, as well as the dynamics of populations, emerge from adaptive responses of organisms to environmental variables, conspecifics and other species, as organisms develop in their natural habitat. Hence, quantifying interspecific variation in the nature of plastic responses as well as its dynamics, can give us important insights into how communities may emerge from the process of adaptation and extinction.

Concerning marine crustaceans, the interplay between plasticity and local adaptations involves the study of pelagic dispersive larvae. For instance, plasticity in developmental rates are central to understand patterns of larval dispersal. Changes in body size and physiological state will be important to understand patterns of larval survival in the sea. Larval dispersal and survival are necessary conditions for population connectivity (i.e., dispersal of individuals among subpopulations that survive to reproduce), which in turn determines the capacity of local populations to recover from extinction and contributes to population persistence. Survival and dispersal depend strongly on behavioural and physiological performance. Key behaviours are vertical migration, escape responses, and movements to capture food. Such activities are driven by the availability of reserves and the aerobic metabolic capacity which depend on processes occurring at the systemic, organ and tissue level. Physiological performance depends on the existence of compensatory mechanisms that buffer the organism from environmental variation (e.g., osmoregulation).

Another area of research should focus on quantifying how developmental mechanisms mediate the effect of the environment on phenotypes, both during larval and juveniles stages in marine crustaceans. For instance, we need more information on how the responses to the environment at the molecular, hormonal, tissue and organ level are coupled to lead to the formation of the phenotype. In addition, we still have limited information about environmental effects at the level of organs and tissues. We have information available on how hormonal cascades mediate environmental effects, but (to the best of our knowledge) a large gap lies beyond that level down to the potential epigenetic effects.

Acknowledgements

The writing of this essay was supported by DFG RTG 2010 and the Alfred Wegener Institute.

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Part V Modelling evolution and development

Evo-devo beyond development: The evolution of life cycles

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Abstract

In principle, development is only a part of a life cycle, as a life cycle can include more than one ontogeny and reproductive phase. Here I argue that evolution should be viewed as the "change of life cycles with time", rather than ontogenies, as the basic evo-devo rationale is generally summarized. Each different segment of a life cycle can provide scope for evolutionary change, and the articulation of life cycles into multiple segments can itself vary and evolve, as the diversity of life cycles in the tree of life shows. This more inclusive perspective may have valuable consequences for the evo-devo research agenda.

Introduction

As a very general statement, the evolutionary developmental biology (*evo-devo*) approach to the study of evolutionary patterns and processes is motivated by the idea that, in order to explain evolution, it is crucial to take development into consideration (Hall, 1992; Amundson, 2005; Minelli and Fusco, 2008). Considering the processes of sorting of extant variation operated by natural selection and random drift, in association with the generative processes of variation that derive from the developmental systems, provides more complete explanations of observed evolutionary patterns (Müller, 2007). This idea is often synthesized by a formula that states that *evolution is the change of ontogenies with time*, rather than simply the modification of genotypes and phenotypes (Gilbert *et al.*, 1996).

Here I will argue that this formulation should be replaced by a more inclusive one, that substitutes *life cycles* for *ontogenies*, thus acknowledging that development – which comprises all the transformations of an individual, from its onset until disappearance – is often only a segment of an organisms' life

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cycle. In many taxa, the life cycle involves a sequence of more than one individual, each with its own developmental processes and reproductive phases. Every different segment of a life cycle can provide scope for evolutionary change, and the articulation of life cycles into multiple segments can itself vary and evolve, as the diversity of life cycles in the tree of life shows.

Life cycles[1]

Everybody has an intuitive idea of what a life cycle is. It is the series of transformations and events which, from a given life stage of a given organism, leads to the same stage in a next generation of the same organism: from a zygote to a zygote, but also from an adult to an adult, or from an embryo to an embryo. In a cyclic process, the choice of the 'initial stage' cannot be other than an arbitrary or conventional choice, as the egg-chicken dilemma beautifully illustrates.

That said, the description of a cycle has necessarily to start somewhere, and as an example let us concisely describe the life cycle of the fruit fly *Drosophila melanogaster*, starting from the zygote stage. Within the egg case, the zygote proliferates by mitosis during embryonic development and builds up, through complex and highly coordinated morphogenetic processes, the body of a worm-like individual that at some point will be ready to interact with the external world. Hatching occurs after about 12 h of embryonic development at 25 ℃. During the subsequent free-living larval period (about 4 days at 25 ℃), the insect grows by feeding on rotten fruit while moulting twice (after about 24 and 48 h from hatching), so that the larval period is partitioned by moult into three stages (or instars). The third larval stage develops into the pupa stage, which, while sheltered by the exoskeleton of the final larval stage (the puparium), undergoes a four-day-long metamorphosis. This is a process of profound transformation of the individual, where large parts of its body lose the larval organization and a completely new body organization is built. Once the metamorphosis is complete, an adult fly emerges from the puparium. Fruit flies reproduce sexually: males and females mate, and from the fusion of their gametes the zygotes of the next generation are produced. The development of a fly, from zygote to adult, is considered to be complex, because metamorphosis separates two very different segments of the insect's post-embryonic life, the larva and the adult. For this reason it qualifies as a kind of *indirect development*.

However, despite the complex developmental path from zygote to adult, in the panorama of the diversity of life cycles, that of the fruit fly actually appears to be relatively simple, because the whole cycle is traversed by a single devel-

[1] This section draws extensively from Fusco and Minelli (in press)

oping and reproducing individual. This is not the case for a multitude of plants, animals, fungi and microorganisms.

As an example of a more complex life cycle, let us concisely describe that of a fern, like *Polypodium*, starting from the better known phase of a macroscopic plant, with roots, stem and fronds. A mature leafy fern plant (a diploid phase called *sporophyte*), reproduces sexually (by means of recombination) and uniparentally (i.e., without the need of a partner) by producing haploid spores by meiosis. Spores disperse and germinate on the ground, each developing into a tiny multicellular haploid plant called *prothallus* (*gametophyte* phase). Prothallia, which bear both male and female reproductive organs, reproduce sexually (through fertilization) and biparentally (i.e., through cross-breeding) by producing gametes that will fuse to form diploid zygotes, the founding cells of the sporophytes of the next cycle. During early development, the sporophyte is retained on the parent gametophyte that nourishes it, until it produces the first leaves and roots and becomes independent. In the cycle of a fern there are at least two generations (a sporophyte and a gametophyte), which constitute two distinct *organizational forms*, i.e. two kinds of individual of the same species, each with its own ontogeny. In the case of the fern, one form starting from a zygote develops into a macroscopic diploid leafy plant, the other form starting from a spore develops into a haploid tiny thallus. The two generations are separated by two reproductive phases: the production of spores by the sporophyte and the production and the fusion of gametes of the gametophyte.

The cycle of the fruit fly is an example of a *monogenerational life cycle*, that is, a cycle in which the same developmental phase (e.g., the first larval stage) of the single organizational form of the organism is repeated after one generation. In contrast, the cycle of the fern is an example of a *multigenerational life cycle*, because the cycle passes through a given developmental stage (e.g., the fully-formed thallus) of a given organizational form (in this case, the gametophyte) after more than one generation, in this case two (Minelli and Fusco, 2010). In multigenerational life cycles there are reproductive phases where offspring are generated that are not of the same kind (of the same organizational form) as the parent(s), so that more than one generation is required to return to a starting form.

Multigenerational life cycles, also called *cycles with alternations of generations*, are widespread in the tree of life. In addition to the aforementioned cycles with an alternation of haploid and diploid generations, which are found in many groups of algae and in all land plants, there are cycles with alternation of sexual and asexual generations (*metagenetic cycle*s; e.g., many cnidarians, cestodes, polychaetes, tunicates), alternation of amphigonic and parthenogenetic

generations (*heterogonic cycles*; e.g., monogonont rotifers, cladocerans, aphids), alternation of unicellular and multicellular generations (e.g., mycetozoans) and many others (review in Fusco and Minelli, in press). Really complex, multigenerational life cycles include multiple organizational forms which can exhibit a different genetic make-up (e.g., haploid vs. diploid in mosses), a different morphology (e.g., winged vs. wingless in aphids), a different living environment (e.g., a different host in parasitic flatworms), a different mode of reproduction (e.g., sexual vs. asexual in pelagic tunicates), and/or a different kind of development (e.g., direct vs. indirect in cnidarians). In many organisms, the route through which the life cycle closes on itself can be very tortuous.

Evolutionary change

The central claim of evo-devo, that to better explain evolutionary change, development has to be taken into account (Robert, 2004), has been synthesises in various ways. For instance by stating that *evolution is the change of ontogenies with time* (McKinney and Gittleman, 1995), or that *evolution proceeds by developmental repatterning* (Arthur, 2011).

Recognizing development as a part of the life cycle, and acknowledging the "life cycle as a unit of evolution" (Minelli, 2009, p. 155), both of the above claims can be rewritten by substituting "life cycle" for "development", thus reading either *evolution is the change of life cycles with time,* or *evolution proceeds by life cycle repatterning*.

The rationale behind these two new claims has not changed with respect the those centred on development. The main idea remains that the production of variation can significantly affect the direction of evolution, no less than selection and drift (Stern, 2000). This is possible because such variation is structured, rather than isotropic, and thus instructive – i.e., potentially able to influence the direction of evolution – rather than merely permissive – i.e., only necessary for evolution under natural section (Fusco, 2015; Jaeger, 2019 this volume). From the "more elevated" view point of the life cycle, however, it is possible to contemplate the possible source of variation more inclusively. There are many kinds of evolutionary change that cannot be qualified as changes in developmental pathways or their control, and that are instead modifications of specific features of the structure of the life cycle, such as its articulation into one or more organizational forms, or the specific mode of reproduction of one of these to the next.

Life cycle evolution

In the view of a variational approach to the study of evolutionary change (Wagner and Altenberg, 1996; Salazar-Ciudad, 2006), one of the directions of development of the so called *extended evolutionary synthesis* (Müller, 2017), the evolution-of-life-cycle perspective exposes a multidimensional space of variation that goes beyond the already vast space of developmental variation with its genetic and environmental modulations (Moczek, 2019 this volume; Gilbert, 2019 this volume), and that obviously includes it as a subspace.

Selectable variation can emerge at any developmental stage of any organizational form of the organism. Sorting at the level of variation within an organizational form is standard (although developmentally informed) phenotypic evolution. However, other changes at the level of the whole cycle can occur. Here are a few examples.

- *A new organizational form can be added to the cycle*. The life cycle of many red algae (e.g., *Polysiphonia*) includes three generations: gametophyte, carposporophyte and tetrasporophite. The diploid carposporophyte, which develops from a fertilized egg cell and asexually generates tetrasporohytes by means of diploid (unreduced) carpospores, intercalates between a gamete-producing gametophyte generation and a (meio)spore-producing sporophyte generation. This three-generation life cycle is thought to have evolved from a primitive cycle with biphasic alternation between gametophyte and sporophyte generations (Yang *et al*., 2016). While the tetrasporophyte seems to correspond to the primitive sporophyte, actually the carposporophyte qualifies as an evolutionary novelty (Minelli and Fusco, 2005). However, homologies in life cycle traits, especially if a strictly historical concept of homology is applied (see Minelli and Fusco, 2013), are not easy to establish (see DiFrisco, 2019 this volume)
- *A primitive organizational form can be suppressed in the cycle*. In some brown algae (e.g., *Fucus*), the primitive haplodiplontic cycle with alternation of generations has evolved into a monogenerational diplontic cycle. The gametophytic organizational form has been suppressed and the diploid sporophyte, by meiosis, produces haploid gametes, rather than spores, in a cycle that structurally does not significantly differ from that of mammals. In the cnidarian class Cubozoa (box jellyfish), the primitive multigenerational metagenetic cycle, with an alternation of a sexually reproducing medusa and an asexually reproducing polyp, has evolved into a monogenerational cycle. Cubozoan polyps go through a metamorphosis and become medusae, rather than asexually generating them. In these cnidarians the polyp generation has been assimilated into the medusa generation as an early develop-

mental phase of the latter. The polyp organizational form as such has been suppressed.

- *The relative predominance (however defined) of different organizational forms can be altered*. In modern angiosperms, the sporophyte generation (the generally macroscopic, autotrophic plant) is dominant with respect to the generation of the gametophyte, that at maturation consists only of the few cells of the embryo sac (female gametophyte) and pollen grain (male gametophyte). Both male and female gametophytes conduct a non-autonomous existence, protected and nourished by the parental sporophyte. However, according to the so called *antithetic theory* of land plant evolution (see Haig, 2008), this condition has evolved from the opposite condition, where the dominant generation was that of the gametophyte, with the sporophyte actually parasitic on it (a condition similar to that found in extant mosses) (Kenrick, 2017).
- *The reproductive mode of one or more organizational forms can be modified*. Parthenogenesis has evolved independently multiple times from amphigonic reproduction in monogenerational (e.g., fish, amphibians and squamate reptiles) and multigenerational (e.g., cladocerans among crustaceans and aphid among the insects) life cycles of animals (Simon *et al.* 2003), as well as in plants, where processes related to parthenogenesis are commonly referred to as apomixis (e.g., *Hieracium* and *Taraxacum*; Van Dijk, 2009).

There are important taxonomic groups in which interspecific diversity is largely a matter of variation in life cycle. Among these are the green algae (Gastineau *et al.*, 2014), red algae (Lee, 2008), cnidarians (Fautin, 1992) and trematodes (Galaktionov and Dobrovolskij, 2003). In all these cases, speaking about a "typical life cycle" is more a mystification that a simplification.

Difficulties around the corner

The characterization of a life cycle, a necessary step for any comparative analysis in an evolutionary context, strongly depends on the possibility of distinguishing the reproductive events, with the value of a transition to a new generation, from the developmental processes, which are instead transformations of the individual. This is not always as easy as it might seem (see DiFrisco, 2019, this volume; Fusco and Minelli, in press), and cases that are difficult to classify are not rare. Evolutionary processes of change are evidently not compelled to respect the limits imposed by our categories. For instance, there are situations where metamorphosis (a developmental process) can fade into asexual reproduction, when, as in many marine invertebrates, most of the larval body is discarded and the young derives from a small number of *set-aside cells*, or

even, as in the bivalve *Mutela bourguignati*, from a larval bud (Fryer, 1961). Are there one or two generations in the cycle of this bivalve? Yet another example is when reproduction blends into development, a very common situation found in many colonial marine invertebrates, like bryozoans or corals. Here, the asexual reproduction of the zooids actually takes on the meaning of growth at the level of the whole colony. A final example will further highlight how much our pre-established classifications can condition the interpretation of an organism's life cycle. All mammals are considered to have a monogenerational diplontic life cycle. However, the armadillos of the genus *Dasypus* exhibit obligate polyembryony, i.e. more than one embryo constitutively develops from a single zygote. If one considers polyembryony as a form of asexual reproduction at a very early (embryonic) stage of development, these mammals actually exhibit an alternation of sexual and asexual generations, a metagenetic cycle not different from that of most cnidarians.

Conclusions

Beyond introducing a new slogan for evo-devo, "life cycles evolve", this more inclusive view on the "unit of evolution" has some more profound implications. It exposes the fact that both development and reproduction are incomplete causal factors in the continuity of life through generations. At the same time, it shows that the way development and reproduction are associated in the life cycle, and the capacity of the life cycle to change in time (life cycle evolvability) are actually the means through which living systems can persist across vast spans of time. The life cycle and its evolution is the core of the persistence of life.

A sparse but lively literature demonstrates that life cycle evolution is a challenging subject of study (Valero *et al.*, 1992), where different kinds of evidence and theories meet, from palaeobiology (e.g., Taylor *et al.*, 2009), life-history trait evolution (e.g., Louhi *et al.*, 2013), gene expression (e.g., Bowman *et al.* 2016; Kenrick, 2017), natural selection (e.g., Mable and Otto, 1998; Szövényi *et al.*, 2013; Rescan *et al.*, 2016; Scott and Rescan, 2017), to evolvability (e.g., Minelli and Fusco, 2010). A merger between these diverse lines of investigation, together with a new awareness of the place of development within the broader context of the life cycle in ongoing research in developmental evolution, may have valuable consequences for the evo-devo research agenda.

Acknowledgments

My profound gratitude goes to my mentor and friend Sandro Minelli, with whom I have been discussing, and continue to discuss, many topics about evo-

lution, development and much more. In a different context, I would certainly have asked him to co-author this contribution (and the text would have benefitted from that), as many of the contents are mine only in part, nurtured by frequent and iterated discussion with him through many years of intense interaction and fruitful collaboration. I apologize for that. James DiFrisco, Nigel Hughes and Johannes Jaeger provided valuable comments on an earlier version of the manuscript.

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Towards a theory of Extended Development

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Abstract

General evolutionary theories have not yet successfully included a general theory of units of development, nor reconciled functional characterizations of development with its formal and material conditions, mechanisms and operations. I characterize what it means to "extend development," appealing to a key bridge concept of "developmental scaffolding," as a prelude to developing an extended developmental theory that might be incorporated into an extended evolutionary synthesis (EES). I distinguish *theory extension*, *domain expansion*, and *practice integration* as three different modes of "synthesis." I discuss how various "centrisms" biasing the perspectives and principles of development needed to fully articulate an EES – adultocentrism, genocentrism, and finalism – can be avoided.

> Debates on the need (or not) to extend the Synthesis somewhat resemble the question of whether the glass is half full or half empty. Both the Darwinian Revolution and the Modern Synthesis were built on huge black boxes. […] As far as I can see, such debates can go on forever. If, as seems obvious to me, the Synthesis has no essence, its extensions are negotiable. I consider this a Good Thing.
>
> (Callebaut, 2010, pp. 457–458)

Perhaps, however, reality is different.
(Minelli, 2009, p. 62)

Introduction

Whether the modern evolutionary synthesis (MES) needs extending in an extended evolutionary synthesis (EES) or can remain loyal to MES's core "standard evolutionary theory" (SET), is probably not a decidable question, given

G. Fusco (ed.) *Perspectives on Evolutionary and Developmental Biology.*

Callebaut's remark, quoted above (compare Laubichler, 2010 vs. Minelli, 2010; Laland *et al.*, 2014 vs. Wray *et al.*, 2014). This is partly due to the facts that:

1. social change (including among scientists) is rarely understandable from the inside while it is happening,
2. what counts as overcoming a substantial empirical or theoretical challenge worthy of labels such as "revised," "extended," or "revolutionary" depends on one's perspective, agenda, training, tools, and resources, not merely on an objective "fact of the matter" (and there are many different perspectives and so forth among the sciences where the MES vs. EES debate takes place), and
3. semantically, terms used by the many debate parties, such as "supplemented," "modified," "extended," "integrated," not to mention "theory" and "synthesis," are meant in a wide variety of sometimes incompatible ways.

In particular, whether extension aims at an expanded *domain of phenomena* or a modified, enhanced, or somehow different *theory* of evolution than SET is masked by describing ESS as involving extension, integration, expansion, or the like without regard to the substantial differences in claims regarding concepts, theories, domains, practices, specialties, or disciplines that can all fall under the heading of "kinds of things one might synthesize."

Talk of environmental engineering, niche construction, multiple levels of selection, beyond-gene inclusive inheritance, phenotypic plasticity, developmental constraint, evolvability, novelty, innovation, or modularity is often ambiguous between calls for change of theory, domain or practice because the terms themselves are so flexible in application and meaning. Not that theories, domains and practices aren't intertwined. But claims (and counter-claims) should still be clear and clearly distinguished for the sake of not talking past one another (unless talking past others for the sake of rhetorical gain is the goal).

In this essay, I seek to do three things: (1) express a view of "extension" as a technical term to be applied to theory-revision and theory-building, (2) articulate a not very standard view of what theories are, and (3) push beyond the rhetorical contest over whether SET is too hot, too cold, or just right to sustain MES. What's too hot, too cold, or just right is a subjective matter open to many different correct answers (Callebaut, 2010; Minelli, 2010).

To try to make good on (3), I pursue a very abstract theory of development, one designed to connect to evolutionary theory (and evolutionary theory to it). This theory is so abstract that it will likely be as unsatisfying to nearly everyone as Darwin's (1859) bare characterization of evolution as "descent with modification" or its more principled modern expression by Lewontin (1970) as a process occurring whenever there is heritable variation in fitness among the members

of a population. I offer it, not as an answer, but as an abstract principle from a specific theoretical perspective to prompt a fresh look at old models and to entertain new and different styles of modeling that might be found in either the MES or EES "traditions" (if the latter can yet be called that).

A key to making an abstract principle less unsatisfying is to show where it affords the development of bridge concepts that might do the work of linking abstract theories of evolution, development, and ecology in a way that, eventually, might lead to better theoretical models, suggest new ways of looking at old phenomena, make sense of new phenomena, and guide exploration of new domains. I propose that "developmental scaffolding" is one such bridge concept linking evolutionary considerations of *functional* arrangements in the service of evolutionary adaptation beyond the body of a developing "unit" (for want of a better abstraction from "organism") with *form* in the service of developmental construction, again beyond the body of some developing "unit."

Decentering centrisms

My general assessment of the state of EES (reviewed in Müller, 2017) is that it has done a very good and interesting job of pointing to proposed or in-the-works extensions – albeit ambiguous between theory, domain and practice – of, or beyond, or side-stepping SET to consider multiple levels of *selection* (group selection, gene selection, kin selection, species selection, community selection), *ecology* (ecological engineering, niche construction, phenotypic plasticity), and *inheritance* (epigenetic, behavioral/neuronal, cultural). It has oddly not done that much to extend concepts of *development*. It is true that developmental thinking within evo-devo has expanded beyond initially narrower starting points, mechanisms, and research methods (classical embryology, morphology, developmental genetics), but one doesn't have a sense of a general theory or principle of what development *is* beyond the slogans of decades or centuries past. This seems to me a lacuna in EES.

My plan is to offer one way of characterizing what "extended development" might mean and what the project of extending development might contribute to a version of EES. I advance ideas about extending development prompted by a perspective on development and evo-devo due in significant degree to Minelli (2003, 2009, 2010, 2011, 2014, and elsewhere). Minelli (2009, p. 65) urged that development is a science which explores "[…] the border between possible and impossible forms; on those laws or rules whose existence we begin to suspect when our expectations are so blatantly proved wrong." It is a key insight of those who seek a dialogue or rapprochement of evolution and development that developmental biology is a science of what variations can be produced and thus

what variation can be available to evolution. It is a science of both construction and constraint. But on what? What are the "units" of development that figure in a theory of development which might connect with a theory of evolution?

A second feature of Minelli's perspective is that developmental biology should follow evolutionary biology's lead and rid itself of "finalism." This preparatory step is needed for a coherent synthesis of evolutionary and developmental theories, domains, practices, disciplines (or whatever it is synthesis is to be about). Finalism in evolution took the form of a teleological tendency, carried over in the transformation of Paleyan design-based explanations of adaptation into Darwinian selection-based accounts. Although modern neo-Darwinism proclaimed itself free of teleology, it took a long time for biologists to stop appealing to "the good of the species" or to evolutionary outcomes as explanations of the causes of the evolution of adaptive traits. Popular expressions of evolutionary ideas still have not all done so, and Gould and Lewontin (1979) were still complaining about. In developmental biology and even more so in embryology proper, finalism often takes the form of what Minelli calls "adultocentrism," treating stages of development prior to the achievement of adulthood as merely preparatory rather than confronting "the developmental phenomena for what they are, *hic et nunc*, without unwarranted projections toward a future (the adult condition) […]" (Minelli, 2009, p. 90).

A third feature of Minelli's perspective is shared with many who endorse EES, a rejection of "genocentrism." Although the history of evo-devo is as much anchored in comparative evolutionary morphology as it is in molecular developmental genetics (Love, 2003), the latter – conceptually – only shifted genocentrism circa the modern synthesis away from its emphasis on "structural genes" and point mutation as sole sources of variation with its placement of almost all causal weight on selection (modulo drift) for explaining the directionality of evolution, toward an emphasis on gene expression and regulation, but with no clear theoretical insight into how to construct a "source law" (Sober, 1984) for the "guided," "facilitated," "constrained" variation that emerges in development.

Expanding domains, extending theories, integrating practices

Entertaining a role for developmental biology in an EES faces the immediate challenge that words commonly used to describe extension and synthesis and why an extended synthesis is needed (Müller, 2017) mean such a wide variety of things to authors with such a wide variety of goals and perspectives, that it is hard to pin down the project(s). As with the architects and proponents of MES, from Dobzhansky, to Mayr, to Simpson, to Stebbins, (not to mention Huxley, Haldane, Wright, Fisher, Hogben, Chetverikov, Krementsov, Rensch, Schmal-

hausen – see Callebaut, 2010, p. 453; Wray *et al.*, 2014), it is not at all clear that proponents of EES, any more than those who support continued allegiance to MES, march under the same programmatic orders even if they fly the same flag. I will use the term "extending" for theories, "expanding" for domains of phenomena or subjects of study, and "integrating" for practices. Theory extension works by building out from (and maybe modifying) a core theory to incorporate new theoretical principles (perhaps from another theory). Domain expansion adds phenomena to the scope of a field of study, altering the subject matter of a line of work, specialty, or discipline. Practice integration incorporates a practice into a workflow from other lines of work, specialties, disciplines, possibly changing what work is produced or changing interpretations.

I think one challenge to EES is to *not* think of theories strictly or solely in terms of the mathematical equations of population/quantitative genetics. Although mathematical equations are often labeled "theory," it may be more appropriate to treat the math as models of/for a theory (see Griesemer, 2013). A somewhat more eclectic notion of "theory" as: (1) a set of principles expressed in terms of core concepts, together with (2) a family of models and (3) a perspective (that shapes model-building, phenomenon identification or construction, and delimits a domain of study) can also describe the mathematized theories familiar from textbooks. A virtue of this looser, distributed view of "theory" as comprising these three kinds of components that together do the work of theories traditionally conceived as "laws of nature," is that theory extension can be seen to occur via a variety of modes (addition, subtraction, modification, recombination) of operation on concepts, principles, models, or the perspectives that coordinate activities relating models to phenomena. To make a claim of theory extension clear, the mode (addition, subtraction, modification, recombination) and target (concept, principle, model, perspective) must be specified.

It is not part of theory change, on this picture of theories, to expand (or contract) the domain of study. Noting that celestial mechanics also applies to terrestrial motions is not theory change in that the equations of motions are not altered, but if it is part of the perspective of the mechanics that guides application only to extremely massive objects, the discovery that the very same equations of motion explain smaller terrestrial object motions involves not only a change of domain, but a change of perspective, which is a form of theory change on the present account of theories. Some EES proponents seem to call for theory change on the grounds that they think the domain of evolutionary theory should include the so-called extended phenomena but only assume this would entail or demand a change of perspective (on how to build models, on what aspects of phenomena are relevant to extended evolutionary explanation,

and so forth). Some MES defenders regard these phenomena as "covered" already by SET, so that no change of perspective or perhaps even any significant change of models is required to "accommodate" such phenomena as part of a domain expansion, but not as a theory extension. This seems to mark one difference, for example, between Laland *et al.* (2014) and Wray *et al.* (2014).

Extended synthesis can thus mean three kinds of things: (1) *theory extension* from some starting theoretical core to incorporate or accommodate other *theories* (or components of theories), (2) *domain expansion* to include other *phenomena* to study under the umbrella of a theory and guidance of its perspective, or (3) *practice integration* to include methods, protocols, procedures (with their tools, instruments, and funding streams) within a workflow. Each of these modes of synthesis can proceed independently of the others, though more often they involve mutual adjustment and inter- or multi-disciplinary conversation and negotiation to succeed.

A fuller treatment of these distinctions would fit well the "population genetics synthesis" (Provine, 1971) preceding the MES, extending Mendelian transmission genetics to phenotype change by "reconciliation" of Mendelism and Darwinian selection theory. It would also fit well the various ways in which "inclusive inheritance" theories from group selection to Price covariance equations to epigenetic inheritance to generalized transmissible inheritance extend beyond SET by adapting the well-established framework of quantitative evolutionary genetics (Wade, 2016, Price, 1970, Frank, 1995, Tal *et al.*, 2010, Danchin *et al.*, 2011). But that is a story for another essay.

Extended evolutionary synthesis

Extended evolutionary synthesis extends core neo-Darwinian principles – Darwin's Principles of variation, heritability, and fitness differences – interpreted through the lens of population genetics (Lewontin 1970) – to multiple levels of compositional organization (multi-level selection theory, Wade, 2016), to environments (niche construction theory, Odling-Smee *et al.*, 2003), to multiple levels of inheritance (group and community as well as individual and family heritability, Goodnight, 1990; reviewed in Wade, 2016), to multiple inheritance systems or modes (epigenetic, behavioral, and cultural, as well as genetic, Jablonka and Lamb, 2005) (see Pigliucci and Müller, 2010; Laland *et al.*, 2015; Müller, 2017).

As I interpret "extended synthesis," the open theoretical question is to what extent and in what ways the core of neo-Darwinian theory must be modified to accommodate various *theory* extension(s). We have seen various theory extensions that involved changes to the core population genetics theory: from

simple allele frequency change to include trait change, from additive to include non-additive phenotypic effects of alleles, from single level (organism) to multi-level selection and heredity, from fixed fitness to frequency, density, and environment-dependency. What about more radical changes, such as to the concept of the genotype-phenotype map itself (Wagner and Altenberg, 1996; Wagner, 2014)? What about changing the assumption of simple randomness: in the generation of variation beyond simple random point mutation or simple probabilities of chromatin rearrangements (recombination, inversions, deletions, translocations)? What about changing assumptions of uncontrolled, unregulated generation of variation from "above" (by the cell, by the organism, by the environment)? A different question, typically debated in the literature, concerns whether core neo-Darwinism can be *applied* without modification to phenomena outside its classically understood domain (Minelli, 2010; Wray *et al.*, 2014) or not (Laubichler, 2010, Laland *et al.*, 2014). Often these questions are conflated. Surely the latter is true, as traditionalists point out, because in a sense the equations of population genetics are analytic truths: all manner of un-tracked, non-additive causal factors are "included" in the "error" or "environment" terms of population genetic equations. The question really is what we learn from such claims of "extension." If all the action is in the non-additive terms, or indeed, if the values of the additive terms change as a function of the non-additive ones, then while "applicable," the traditional equations don't tell us much.

While evo-devo and eco-evo seem to extend evolutionary theory to development and ecology, at least in the former case, the *theory* of development still seems tethered to a traditional notion of ontogeny from egg or seed to adult organism. This is not only incongruous, it is theoretically limiting. The units of investigation in an extended evolutionary synthesis should include many sorts of biological "systems" that are not "organisms" in the traditional sense. Holobionts, for example, are living systems comprised of "macrobes" (the host macro-organism) together with their symbiotic microbes (e.g. Gilbert, 2019 this volume) and practitioners take these to be units of investigation (Lloyd, 2017). Whether holobionts have a "development" of their own should be an askable question within an extended evolutionary framework. While this emerging line of work or specialty seems intent on including ecological and genetic interactions of microbes and "macrobes" in the development of each, the question of collective *development* of holobionts is barely asked (but see e.g. Griesemer, 2017).

Extending development

Here's a principle of development that extends development in space, time and functional role beyond their traditional scopes of the organism (life trajectory from egg/seed to adult) and production of "adult" form. "Development is the recursive acquisition (over a compositional hierarchy of parts and wholes) of a capacity to reproduce. Recursion bottoms out in 'null development' in which progenerated [component] entities are born 'ready-made' with a capacity to develop, rather than having to acquire a capacity to develop" (Griesemer, 2014, p. 187). An entity is progenerated (propagule generated) if it is made from material parts of a pre-existing entity ("parent") and (at least some of) the material parts convey developmental capacities to the new entity ("offspring") via the transfer of parts.

The materiality of the parts anchors the account as a spatial, compositional concept and differentiates it from views that permit development to be driven by transmission/propagation of purely formal information rather than transfer of material parts. But unlike traditional views of development as an unfolding, or growth/maturation, or differentiation of parts, or even an informing upon parts to produce adult form, the spatial extent of development is not limited to cellular units nor their multi-cellular compositions: the spatial extent of development can be smaller in spatial scale – non-cellular entities such as molecules can develop – and so can supra-organismal entities such as (some) groups of organisms and possibly even more inclusive (social) entities (see Griesemer, 2000, 2013, 2014). Because development on this view is recursive, it envisions development at multiple levels of compositional organization, not necessarily at a single level, such that, say, only cell-based organisms develop. The view does not suppose a cell-centrism about its "units."

The view envisions development as a process of acquiring a capacity (to reproduce). The realization of the capacity to reproduce is perhaps necessary for the *fulfillment* of the developmental process, but not necessarily for its instantiation, so developmental processes can be completed on a shorter time scale than some traditional views of what it takes to achieve "adult form" (e.g., including sexual maturation). While this may appear to narrow the concept of development, by tying it to reproduction, the way reproduction is handled by the account suggests this is not so: any process that provides a *path* to propagule generation, whether or not progeneration occurs, can count as a "development." And the recursion can be rather shallow: cell division is a developmental process leading to the progeneration of two daughter cells from an ancestral one. The "null development" in cell division is simply the autocatalytic production of the molecules necessary for cell growth and division.

Moreover, some developmental processes (in complex life cycles) may take more than a single "organism" generation to be fulfilled, so the time scale of development may be longer than traditionally conceived within-generation life trajectories and involve more temporally extensive developmental lineages (see Griesemer, 2016). While it is more standard to consider that some (multigenerational) life cycles include more than one individual (and individual development) (see Minelli and Fusco, 2010; DiFrisco, 2019 this volume; Fusco 2019 this volume; Fusco and Minelli, in press), here I allow development to be "stretched" beyond organism life trajectories to acknowledge that some developments of groups or complexes of organisms (as well as "multi-generational" parasite life cycles within single host life trajectories). (Thanks to Giuseppe Fusco for emphasizing this contrast.)

The view envisions the function of development in terms of reproduction rather than in terms of a specific morphology or form to be achieved, while the materiality of the process insures that some form or other may be a typical or evolved form that achieves a developmental outcome. The functional characterization permits development to range over functional forms much broader than current or traditional concepts of development anchored in exemplary study or model organisms. The concept of development is abstracted from any particular cellular or mechanistic mode or form of outcome, so one can imagine modeling development in non-cellular processes such as proto-cell evolution, non-biological processes of cultural development resulting from social interactions among biological (and other) constituents, and can even envision inorganic systems of artifacts as developmental entities (see Griesemer, 2014 for discussion; also Wimsatt and Griesemer, 2007).

As a principle of development, the account on offer can be considered a component of a theory of development, which would need a family of models and a perspective to round out the theory. That is a task for a different essay. But it is important to note that this principle of development is embedded in a more inclusive account of reproduction (Griesemer, 2000, 2013, 2014, 2016, and elsewhere). Reproduction is a central concept and process in all of biology and in evolutionary biology in particular. Darwin's background Malthusianism driving his view of evolution as a fundamentally competitive process has at its heart a concept of multiplication or "increase" which can be grounded in reproduction. So the principle of development offered here can be linked to evolutionary theory through an account of reproduction.

A key idea that makes these extensions in space, time and function more concrete is the concept of a *developmental scaffold* – an entity involved in facilitating a developmental process but which is typically conceived as part of the

environment of a developing entity, yet must be in intimate contact and interaction with a scaffolded entity to play a scaffolding role. An example of development that can be interpreted as scaffolded is reported in Rampho *et al.* (2011). It concerns the green sea slug, *Elysia chlorotica*, which feeds on the filamentous alga *Vaucheria litorea*. Sultan (2015, pp. 32-33) describes it as a case of "co-construction" of the organism and its environment, which it certainly is. The larval sea slugs feed on the alga, sucking out the contents, and in the process acquire intact chloroplasts from their prey which become incorporated intracellularly in the digestive diverticula cells of the sea slug. The sea slug becomes photosynthetic for the rest of its 10 month life span. Although this incorporation marks a change in developmental trajectory for the sea slug (from heterotroph to facultative autotroph), it can also be understood as a case of scaffolded development in which a developmental transition is facilitated by the alga. According to Rampho *et al.* (2011, p. 306), in their artificial seawater culture studies:

> Successful planktotrophic development was recorded for all developing larvae that were fed a unicellular algal diet of *Isochrysis galbana*. Metamorphosis of larvae to the juvenile stage requires the presence of *V. litorea* filaments. Immediately following metamorphosis, the juveniles begin feeding on the filamentous alga, engulfing plastids and turning green. A transient nature to the plastid symbiotic association is observed in recently metamorphosed juvenile sea slugs if removed from the presence of *V. litorea* too soon (less than ~6 days); this also results in cessation of their morphological development. Plastid uptake until the establishment of irreversible kleptoplasty appears to be required for full adult development and survival, although one report of 'albino ghost' *E. chlorotica* was documented in 1986 (Gibson *et al.*, 1986). Establishment of the kleptoplastic association involves specific recognition processes that comprise at least two steps: (1) planktonic larvae require *V. litorea* filaments to be present for settlement and metamorphosis to the juvenile stage, and (2) adult development requires uptake and retention of *V. litorea* plastids by cells lining the digestive diverticula.

Thus, although it appears early developmental stages of the sea slug can subsist on other algal species (*I. galbana*), feeding on *V. litorea* not only provides food, but also facilitates metamorphosis. Although Rampho *et al.*'s studies show this scaffolding interaction necessary for metamorpohsis, one can imagine that early in the evolution of this symbiosis, the alga aided/facilitated metamorphosis and subsequently evolved into an obligate symbiosis.

Expanding form and function

Maps (Winther, in press), models (van Fraassen, 2006; Giere, 2007) and scaffolds (Bickhard, 1992) are all characterized functionally – in terms of what they do

and what they are for, e.g. for representation/navigation, representation/exemplification, or construction/facilitation – rather than in terms of what they are made of or how they are put together. The maddening thing about functionally characterized concepts is that *anything* can be one – provided they are used in a way appropriate to fulfilling the role. Salt and pepper shakers may not be *very good* models of Sun and Earth to demonstrate planetary motion, but they *can* be used as such. A puddle in the driveway may not be a very good habitat for a tadpole to develop in, but it can serve the purpose. The realizers have structures (forms, organizations), of course, and some structures are more suited to play the functional role than others, given the particularities of target, context, purpose of use, and audience for any material realizer of a map, model or scaffold.

We pick out the salient structure/form/organization of a map, model, or scaffold once the function(ing) has been identified. Form follows function, one might say, in terms of the order of discovery – we see how the arrangement of parts serves the function. When it appears otherwise, it is likely that the observer is already familiar with the characteristic forms used to fulfill a function or perform a functional activity. That said, a designer of a map, model or scaffold likely has a function already in mind and goes straight to organizing materials into a suitable structure so as to serve the function. Function follows form, one might say, in terms of the order of construction.

Eco-devo (Gilbert, 2001; Gilbert and Epel, 2009; Sultan, 2015), ecological developmental biology, recognizes that environments are critical and substantial players in development. In addition to providing mates, competitors, predators, prey, habitat and all the usual "services" described by ecologists, environments provide triggers, scaffolds and prostheses as aides, facilitators and enhancers of developmental processes (Griesemer, 2014, p. 186). Light may trigger, a substrate may orient, or an abandoned shell may enhance development.

Developmental scaffolds are facilitators of development. Developing entities often depend on order provided by their environments to organize aspects of their development. Developmental scaffolds can be anything from a mineral substrate to an abandoned nest hole to a parent organism to a peer teacher to a member of another species in symbiotic or mutualistic interaction, so long as the functional role affects development. "Scaffolding refers to facilitation of a process that would other-wise be more difficult or costly without it, and which tends to be temporary – an element of a maintenance-, growth-, development-, or construction process that fades away, is removed, or becomes "invisible" even if it remains structurally integral to the product" (Griesemer, 2014b, p. 26; following Bickhard, 1992).

These ideas suggest an *expansion* of the domain of developmental phenomena, as has already been suggested by eco-devo and evo-devo: to consider phe-

nomena in the environment as potential aspects and causes of development, to consider entities above and below organism level as possible units of development, and to consider processes shorter and longer in time scale than life trajectories from egg/seed to adult. The view of development offered here also suggests *extending* the concept of development, and thus the principle of development, and thus (one kind of) theory of development beyond traditional notions as well.

The unit of development suggested by my account that is relevant for evolution may be a strange hybrid entity: the developer plus scaffold. Although often a rather temporary "group," a developer and scaffold form intimate bonds (as intimate as those between human parent(s) and children, or infecting parasite and host, see Griesemer, 2014b and 2014a, respectively). Because, as argued elsewhere, following Bickhard (1992 and elsewhere), developmental scaffolds can have fitness consequences, they can be directly connected to Darwinian, neo-Darwinian, modern synthetic, and extended synthetic theories of evolution, though the argument is far beyond the scope and limits of this brief essay (see Griesemer, 2014a, 2014b, 2016).

'Scaffold' is thus a bridging concept for a possible extension of evolutionary theory and expansion of evolutionary biology's domain. It extends SET by characterizing a functional, fitness-altering role for parts of environments that become (temporarily) parts of developing units but which are not recognized as parents in the usual genetic sense of cellular entities like gametes or buds carrying genomes to offspring. Scaffolds play a role in the constructional processes generating and changing form and ultimately, if successful, in generating in the developing unit a capacity to reproduce.

It remains to be seen if new forms of mathematical model can be built along lines of the principle of development and concept of developmental scaffold described here. Previous work has articulated a "reproducer perspective" that might help guide such work. It seems plausible that models mimicking some aspects of the form of niche construction models might be appropriate for developmental scaffolding. Both involve feedback from environments that alter selection coefficients and fitness functions, both involve active roles of developing entities in shaping the environments that provide the feedback. In the case of scaffolding, this active construction involves whatever developers do to enroll, engage, or recruit scaffolds. Scaffolds appear "ephemeral" compared to genomes (though I have argued that genomes themselves may, in effect, be viewed as "internalized" scaffolds incorporated into cell dynamics over the time scale of evolution from proto-cells to fully cellular life, see Szathmáry and Griesemer, 2009). In that respect, scaffolding dynamics may be more like epigenetic inheri-

tance systems than genomic systems. In any case, a full theory of development relying on the principle of development and concept of developmental scaffolding require a family of novel, distinctive models.

Extension of SET along these lines should lead to an "etho-eco-devo-evo," since behaviors of developer and scaffold, as well as their ecological interactions and impacts on the trajectory and fitness consequences of development, are central to their functional roles in scaffolding interactions.

Conclusion

I offered a principle of development and concept of developmental scaffolding to indicate one path to a full theory of development. I argued that a theory of development is needed if SET is to be extended to include development, given my use of 'extension' as a technical concept applying to theory development rather than to domain expansion or practice integration.

Perhaps the proposed path is radical, in that it accommodates a kind of hybrid entity or unit – developer plus scaffold – that biological scientists would perhaps not consider even worthy of exploration and certainly not worth committing valuable and limited time, money or effort to develop. I would point out, however, that domain expansion to include such hybrids is compatible with treating the narrower domain of traditional biology covered by SET as a special case. Given the present state of theory in biology, the main tension is with the perspectives that have guided the development and applications of SET. It remains to be seen whether models of transmission and expression for such hybrid systems, analogous to genetic models of Mendelian transmission and molecular gene expression, would be compatible with SET models as special cases. It is hard to envision a "genetics" with such radical violations of Mendelism as are implied by the notion that a "parent" could be an artifact or a stone on a river bank. On the other hand, Johannsen wasn't sure that genes were material objects at all when he coined the term "genotype" (see Johannsen, 1911), nor was Darwin (1859) sure of the "laws of life" when he based his selection account of "modification" on a completely black-boxed assumption of laws of inheritance. So a theory of development that is open to non-cellular, non-adultocentric, non-finalistic accounts of developing entities as developer-scaffold hybrids and their role in an extended evolutionary theory may not be so radical after all.

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Dynamic structures in evo-devo: From morphogenetic fields to evolving organisms

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Abstract

Evolution does not act on particular stages in the life of an organism. Instead, it alters developmental processes and life cycles in response to environmental conditions to bring about phenotypic change. The structure of these processes determines evolvability, the capacity of organisms to adapt. These structures are intrinsically dynamic. The organisational principles underlying organisms and the morphogenetic fields that constitute their ontogeny actively remodel themselves over time. How this occurs, and how it influences the rate and direction of evolutionary change, are central questions for biology. They lead us to fundamentally reconsider the active role of organisms in evolutionary change, which raises the possibility of a new agent-based theory of evolution in which organisms and their perceived environments co-construct each other in a radically innovative dialectic dynamic.

Evolving life cycles

In order to achieve a modification in adult form or behaviour, evolution must alter the ontogenetic processes responsible for producing that form or behaviour (Bonner, 1974; Horder, 1989; Fusco, 2001; Amundson, 2005). If we do not understand ontogeny, we cannot arrive at a satisfactory explanation of phenotypic evolution. Our explanation will remain causally incomplete. This is called the causal completeness principle (Amundson, 2005), variously attributed to Goldschmidt and Waddington (Gilbert *et al.*, 1996), Garstang (Raff, 1996), or de Beer (Johnston and Gottlieb, 1990). Arguably, it is the most fundamental assertion of evolutionary developmental biology (evo-devo), and its many structuralist historical predecessors (Amundson, 2005).

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Changes in ontogeny cause evolutionary change. What do I mean by "ontogeny" in this context? Often, the term is used interchangeably with "development." There are several problems with this. First, evolution can act at any point within the life cycle – the trajectory of an organism from coming into being to reproducing (and senescence beyond that if we consider life history strategies as evolving traits) (Fusco, 2001; Minelli, 2003, 2014). However, development (in the conventional sense of the term) is restricted to a specific time interval covering specific stages within the cycle. To make things worse, it is not evident where development ends or where it begins (this issue is extensively discussed in Minelli and Pradeu, 2014; see also Oyama 2000, Minelli, 2003, 2011, Pradeu *et al.*, 2011). Multigenerational life cycles can be composed of several phases of development (DiFrisco 2019 this volume; Fusco 2019 this volume; Fusco and Minelli, in press). Finally and foremost, it is not only developmental processes that contribute to the successful closure of the life cycle. Therefore, I will use the term "ontogeny" in a general sense here to include the sum of all metabolic, physiological, developmental, and behavioural processes that contribute to such closure.

The life cycle is composed of numerous intricately interwoven ontogenetic processes (see, for example, Jaeger and Monk, 2015; Nicholson and Dupré, 2018). They come in diverse forms, involve various chemical and physical substrates, occur at a wide range of different time scales, and typically depend on the intra- and extra-organismic environment. What all of these processes have in common is that they are transient and dynamic. They constantly arise from activities and interactions of other processes, transmuting, or fading as their lifetime expires and their contribution is achieved. Nothing remains constant in life. Static explanations, such as network graphs, are clearly inadequate to capture the ever-changing nature of ontogeny. Even phenotypes must ultimately be considered to be dynamic (Fusco, 2001). For this reason, we require genuinely processual explanations for ontogeny and phenotypic transitions. The evolution of ontogeny is a process consisting of processes within processes, a monumental dynamic hierarchy (Riedl, 1975; Riedl, 1977). In this chapter, I examine the structure of this hierarchy of processes and find that it itself is constantly changing over time. This has important conceptual and methodological repercussions for evo-devo.

Morphogenetic fields as dynamical modules

My structuralist approach aims to understand the organisational principles that define the space-time order of the generative processes underlying ontogeny (Waddington, 1957; Waddington, 1970; Thom, 1976; Oster and Alberch, 1982; Goodwin, 1982a,b; Webster and Goodwin, 1996; Goodwin, 1999). These organisational principles are captured by the structure of morphogenetic fields. The

morphogenetic field was the main explanatory concept of classical embryology before its decline and eclipse during the second half of the 20th century (Goodwin, 1982a,b; Webster and Goodwin, 1996; Gilbert *et al.*, 1996). Morphogenetic fields are not necessarily identifiable with specific cells or tissues. Instead, they describe the relations of various physico-chemical processes responsible for generating specific ontogenetic patterns. They are spatially and temporally bounded, with given initial and boundary conditions. Within each field, global order arises from local interactions. Like the magnetic fields that inspired them, they are able to maintain a global pattern under various perturbations, such as stochastic fluctuations, truncation or fusion of fields. Morphogenetic fields are organized in a hierarchical manner during ontogeny and interact with each other in various ways. Specific examples of morphogenetic fields include the primary embryonic field of insect segment determination (Akam, 1987; Rosenberg, 2009; Jaeger, 2009, 2011, 2012, 2018), or more specific organ-forming fields, such as the vulval field in nematodes (Hoyos *et al.*, 2011; Félix and Barkoulas, 2012; Corson and Siggia, 2012), the field responsible for dorso-ventral patterning in the vertebrate neural tube (Dessaud *et al.*, 2008; Balaskas *et al.*, 2012, Zagorski *et al.*, 2017), or Turing patterning generators, such as the one responsible for digit formation and growth of the vertebrate limb (Marcon and Sharpe, 2012; Raspopovic *et al.*, 2014; Onimaru *et al.*, 2016).

One central characteristic of morphogenetic fields is their robustness against many kinds of perturbations (Goodwin *et al.*, 1993; Webster and Goodwin, 1996). This implies that they can be implemented by a wide range of molecular mechanisms, which differ between almost every individual organism. In other words, a field represents a class or population of individual ontogenetic mechanisms that all share an equivalent structure and parameters defining their patterning activity (Oster and Alberch, 1982; Goodwin, 1982a; Alberch, 1991). In this sense, the morphogenetic field closely resembles the concept of a developmental type, which has nothing to do with essentialist typology (Amundson, 2005; Wagner, 2014). Fields as developmental types are reliably and robustly accessible features of evolving species (robust in the sense of Wimsatt, 2007) that can be observed, measured, and/or inferred from empirical data (Jaeger and Crombach, 2012). They mediate the mapping of genotypic to phenotypic variation under the influence of the environment (Waddington, 1970; Burns, 1970; Oster and Alberch, 1982; Goodwin, 1982a,b; Alberch, 1991; Pigliucci, 2010), constituting what Waddington called the epigenotype (Waddington, 1942, 1953) (Fig. 1). While individual mechanisms map to a given field, parameters of the field will not correspond to any specific molecular configuration in turn. The relationship between mechanism and field is therefore asymmetric and degenerate – many mechanisms for

one field – which implies that it is not sufficient to study ontogeny at the level of molecular mechanisms to understand developmental evolution. In addition, we must focus our attention on the level of the epigenotype: the morphogenetic field, with its defining structure and parameters (Waddington, 1957, 1970; Oster and Alberch, 1982; Goodwin, 1982a,b; Webster and Goodwin, 1996; Jaeger *et al.*, 2015; Jaeger and Monk, 2015).

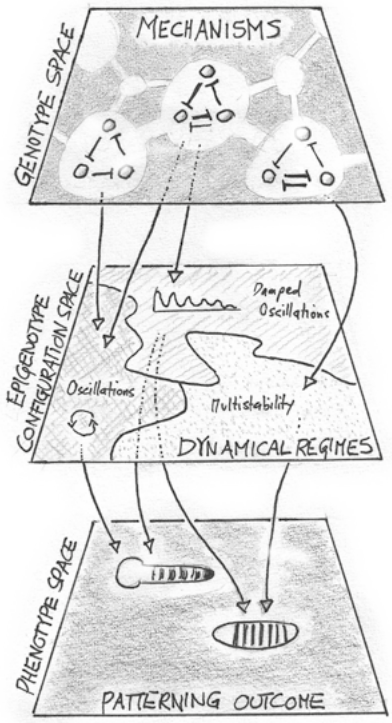

Figure 1. The epigenotype mediates the mapping from genotypic to phenotypic variation, determining the non-random probabilities of phenotypic transitions. Top: genotype space. Genotypes of individuals in evolving populations determine ontogenetic mechanisms through specific molecular implementations of gene regulatory networks. Each network differs in its molecular details. Mechanisms are connected by mutations, constituting what is called a "genotype network" (a meta-network of networks; Wagner, 2011). Middle: configuration space. Individual mechanisms map to regions of configuration space that correspond to specific dynamical regimes (e.g. oscillations, damped oscillations, or multistability, i.e. switch-like behaviour). The same mechanism can produce different dynamical regimes in different contexts, and different mechanisms can produce the same dynamical regime due to the degenerate asymmetrical relationship between molecular mechanisms and the control parameters defining the dynamical regime. Bottom: phenotype space. Different dynamical regimes, mediated by dynamical modules, combine in complex regulatory networks to produce a patterning outcome. Depending on the stability of the underlying dynamical regimes, random changes in molecular mechanisms lead to non-random probabilities of phenotypic transitions.

Morphogenetic fields affect the rate and direction of evolution through their role in mapping genetic to phenotypic variation (Fig. 1). In this way, they determine the evolvability of an ontogenetic process, its capacity to produce adaptive change (Dawkins, 1989; Goodwin, 1982b; Wagner and Altenberg, 1996; Hendrikse *et al.*, 2007; Pigliucci, 2008). The structure of the genotype-phenotype map determines the variational properties of a trait and, therefore, the probability of phenotypic transitions, providing a map of the possible underlying evolution by natural selection (Alberch, 1991; Wagner and Altenberg, 1996; Wagner, 2011; Francois and Siggia, 2012; Jaeger *et al.*, 2012; Jaeger and Monk, 2014; Wagner, 2014; Jaeger *et al.*, 2015).

I find one aspect of this structure particularly striking. The fact that ontogeny can be subdivided into individual morphogenetic fields – each responsible for a distinct observable dynamic pattern – implies a deep underlying dynamical modularity (Kauffman, 1993; Irons and Monk, 2007; Verd *et al.*, 2018b; Jaeger, 2018). Modularity limits pleiotropic effects (Wagner and Alternberg, 1996; Wagner and Zhang, 2011), and is often seen as a prerequisite for adaptive evolution (Wagner and Altenberg, 1996; Raff, 1996; von Dassow and Munro, 1999; Schlosser and Wagner, 2004; Wagner *et al.*, 2007; Callebaut and Rasskin-Gutman, 2007; Wagner, 2014). It allows us to identify components and interactions within a developmental process that contribute to a particular patterning activity, or dynamical regime (Irons and Monk, 2007; Verd *et al.*, 2018b). Dynamical regimes represent qualitatively different types of patterning behaviour: Turing modes producing spots or stripes, for example (see, for example, Murray, 1981; Meinhardt, 1982; Meinhardt and Gierer, 2000; Murray, 2003; Kondo and Miura 2010; Marcon and Sharpe, 2012), or multistable switch-like behaviour (Monod and Jacob, 1961; Gardner *et al.*, 2000; Wang *et al.*, 2011; Furusawa and Kaneko, 2012, Ferrell, 2012, Verd and Jaeger, 2014), or the generation of damped or sustained oscillations and waves (Goodwin, 1963, Goodwin and Cohen, 1969, Goldbeter, 1997, Novak and Tyson, 2008; Maroto and Monk, 2009; Verd *et al.*, 2018a).

The behaviour of distinct dynamical modules shows different degrees of robustness, or structural stability (Thom, 1976; Verd *et al.*, 2018b). In other words, different modules show different sensitivities to structural changes: some are very robust, while others are in a state of criticality, which means that even small alterations in certain aspects of their structure can lead to large and abrupt changes in their dynamical behaviour. The system is poised on what Stuart Kauffman (1993) has called "the edge of chaos," likely to change its behaviour in certain ways, while maintaining it overall structural integrity. This is how dynamical modularity enables some evolutionary transitions while preventing others, therefore affecting the evolvability of ontogenetic processes and the phenotypes they generate (Verd *et al.*, 2018b; Jaeger, 2018).

The dynamic structure of modular fields

To understand the behaviour of a morphogenetic field, we must understand how its structure relates to the dynamics it can generate. We achieve this by using the powerful tools of dynamical systems theory (Hirsch *et al.*, 2012; Strogatz, 2014). In this mathematical framework, the structure of the system is represented by a set of rules governing how it proceeds from its initial to its final state. Typical state variables for ontogenetic processes are concentrations of metabolites or gene products, signalling activities, membrane potentials, or other biophysical indicators such as cytoplasmic viscosity or tissue-level strain (see, for example, Noble, 2002; Forgacs and Newman, 2005; Jaeger, 2009; Jaeger *et al.*, 2012; Kicheva *et al.*, 2012; Morelli *et al.*, 2012; Heisenberg and Bellaïche, 2013; Briscoe and Small, 2015; Gilmour *et al.*, 2017), but they can also be abstract growth parameters modelling morphogenesis (e.g. McGhee, 2006; Mitteroecker and Huttegger, 2009). The rules governing change in these variables can be encoded by differential equations or logical rules. The precise mathematical formalism used is of no importance to our argument. What is important is that the rules determine the type and strength of regulatory interactions, the production rates and lifetimes of system components. In addition, the system must be bounded: we must specify which components and interactions are included (and which ones are left out), and we must define and limit the spatio-temporal domain over which the systems applies. Influences from outside the system must be given by boundary conditions. Internal rules and boundary conditions together represent the structure of the system.

The structure of the system determines the range of dynamical regimes it can generate, that is, its dynamical repertoire (Jaeger and Crombach, 2012; Jaeger and Monk, 2014; Strogatz, 2014; Jaeger *et al.*, 2015). Nearly all biological regulatory systems are non-linear. This implies that small changes to the structure can lead to large changes in the repertoire. Dynamical regimes can be created or annihilated through bifurcations (Hirsch *et al.*, 2012; Strogatz, 2014). Monostable systems have a very restricted repertoire with only one dynamical regime, while multistable systems can show a wide range of different behaviours depending on the initial conditions of the system. In any case, initial conditions and structure of the system together fully determine the dynamical behaviour of the system, given by its trajectory through state or configuration space (Thom, 1976; Jaeger *et al.*, 2012; Jaeger and Monk, 2014; Strogatz, 2014; Verd and Jaeger, 2014; Jaeger *et al.*, 2015).

On the one hand, this type of formalism is very powerful and well-suited for the study of modular morphogenetic fields. Equations and parameters not only define what the system does, but also what it can do under various circumstanc-

es, and how it can change over time. On the other hand, the dynamical systems approach depends on two conditions that severely restrict its applicability in biology. First, we need to be able to precisely delimit the boundaries of the system. This is never an easy task. In reality, no module is an isolated system. Modularity is always a matter of degree. Herbert Simon called this widespread feature of complex systems near-decomposability (Simon, 1962, 1973). Which components and interactions to include in a module, and which to ignore, is often a matter of subjective judgment and depends on the nature of the problem being addressed (see, for example, Chu *et al.*, 2003; Chu, 2011). Second, the structure of the system is assumed to remain constant over time. State variables change, but the parameters and rules governing their dynamics are supposed to remain constant. This assumption is rarely warranted in biology. Growth and tissue rearrangements alter the spatio-temporal domain. External factors – from inductive signals to changes in environmental conditions – modify the regulatory structure of morphogenetic fields (see, for example, Kicheva *et al.*, 2012; Corson and Siggia, 2012; Verd and Jaeger, 2014; Verd *et al.*, 2017). During ontogeny, fields arise and disappear, they split and merge (Goodwin, 1982a; Webster and Goodwin, 1996; Jaeger and Monk, 2015). This fundamental transience of ontogeny limits the applicability of the dynamical systems framework to short time frames, and only those modular fields that can be identified and bounded in space and time with reasonable precision.

Many difficulties arise, as soon as we look beyond this limited context. It becomes increasingly challenging to delimit the system precisely. The fact that its structure constantly changes raises the question at what point the system ceases to be the same process. And even though some mathematical tools exist to deal with time-dependent system structure (Mesarovic and Takahara, 1975; Rasmussen, 2007; Kloeden, 2011; Verd and Jaeger, 2014), it becomes increasingly difficult to accurately represent the changing structure of the system and analyse its behaviour in terms of dynamical systems theory. Although there is a growing sample of morphogenetic fields for which good models exist, we seem to be a long way away from understanding the complete ontogeny of any organism. This is not simply an empirical limitation, a matter of more research being done, but requires novel concepts and mathematical tools to deal with the transient and dynamic structure of ontogenetic processes.

Organisms and organisational closure

The problem gets much worse – or more interesting, depending on your point of view – once we consider whole organisms as dynamical systems (Varela *et al.*, 1974; Varela, 1979; Varela *et al.*, 1991; Rosen, 1991; Saunders, 1993; Gilbert

and Sarkar, 2000; Gantí, 2003; Thompson, 2007; Moreno and Mossio, 2015). It is impossible to capture the causal structure, often called organisation, of a living organism with a formalism that requires a strict distinction between internal rules and boundary conditions or, in other words, a strict distinction between the system itself and its external environment. The reason for this is that organisms are not only bounded in space and time, but also show organisational closure (Piaget, 1967; Varela *et al.*, 1974; Rosen, 1991; Letelier *et al.*, 2011; Moreno and Mossio, 2015; Montévil and Mossio, 2015). Organisational closure reflects the fact that each part of an organism is both means and end, as both Aristotle and Kant already recognised. Not only does the whole owe its existence to its parts, but each part of an organism only exerts its function in the context and for the sake of the whole. This makes organisms fundamentally different from machines, or any other non-living system (Letelier *et al.*, 2011; Nicholson, 2013, 2014). In fact, some authors have argued that closure is *the* defining characteristic of life (Varela *et al.*, 1974; Rosen, 1991; Moreno and Mossio, 2015).

Organisational closure implies "mutual dependence between a set of constituents which could not exist in isolation, and which maintain each other through their interactions" (from Piaget, 1967, translated in Montévil and Mossio, 2015). Organisational closure is complementary to thermodynamic openness in living systems, and is directly related to the self-determination and agency of living beings (Jonas, 1966; Piaget, 1967; Varela *et al.*, 1974; Varela, 1979; Ruiz-Mirazo and Moreno, 2004; Moreno and Mossio, 2015). Robert Rosen (1991), was the first to distinguish material and efficient causation in this context: material causes provide the chemical building blocks for the structure of the living system, efficient causes define its organisation. Organisms are open with regard to the former, but closed with regard to the latter. Every functional component of the organism's organisation is produced and maintained from within the system. This leads to autopoiesis – self-making, self-maintenance, and self-renewal of the system (Varela *et al.*, 1974; Varela, 1979; Letelier *et al.*, 2003) . Rosen (1991) used category theory to formally prove that systems closed to efficient causation cannot be captured by traditional mathematical formalisms such as dynamical systems theory.

Rosen's abstract top-down scheme for closure to efficient causation is not easy to map to actual biophysical cellular components (Letelier *et al.*, 2003; Wolkenhauer and Hofmeyr, 2007; Piedrafita *et al.*, 2010; DiFrisco, 2014; Hofmeyr, 2017). More recent work uses a bottom-up approach to link organisational closure to the underlying principles of far-from-equilibrium thermodynamics. It distinguishes between physico-chemical processes and their constraints, which determine how energy is released and propagated through a living sys-

tem (Moreno and Ruiz-Mirazo, 1999; Kauffman, 2000; Ruiz-Mirazo and Moreno, 2004; Moreno and Mossio, 2015; Montévil and Mossio, 2015; Mossio *et al.*, 2016). Such constraints are an essential component of the structure of a living system. They reduce the degrees of freedom of the processes on which they act, canalising energy flow into work-constraint cycles (Kauffman, 2000; Gantí, 2003). Catalytic enzymes are one important example of biological constraints: they alter the flow of metabolic processes without being affected by it, at least at the timescale of metabolism itself. On longer timescales, constraints need to be replaced, repaired, and maintained. This is achieved through mutual dependence: organisational closure can be interpreted as a closure of constraints, where each constitutive constraint in the system is both dependent on and generative of at least one other constraint (Fig. 2) (Moreno and Mossio, 2015; Montévil and Mossio, 2015). This leads to a system with an organisation that creates the conditions for its own continued existence (Moreno and Mossio, 2015; Mossio *et al.*, 2016). The function of a component process is defined by its contribution to this self-maintaining dynamic (Mossio *et al.*, 2009; Saborido *et al.*, 2011; Moreno and Mossio, 2015). The autopoietic nature of the system, in turn, explains the autonomy, agency and self-determination of a living organism as the causes for its continued self-maintenance and -propagation are immanent within the system, not externally imposed on it (Varela *et al.*, 1974; Varela, 1979; Bickhard, 2000; Christensen and Hooker, 2000; Moreno and Mossio, 2015; Mossio and Bich, 2017; Walsh, 2015, 2018).

In dynamical systems terms, constraints typically correspond to boundary conditions of the physico-chemical processes or subsystems that comprise a living system. Closure of constraints therefore implies that each constitutive boundary condition of an organism must be generated and maintained by other processes and their constraints within the system (Moreno and Mossio, 2015; Montévil and Mossio, 2015; Mossio *et al.*, 2016). This creates two interesting problems when considering the entire organism as a dynamical system. First, closure of constraints leads to a system with is bounded, yet without constitutive boundary conditions, as these are now caused within the limits of the organism. Such a system cannot be encoded in terms of differential equations (Rosen, 1991).

More importantly in this context, the structure of the system – the rules governing processes and constraints – are constantly and radically changing. Self-maintenance is achieved through a fundamentally dynamic organisation, as constraints modify each other and keep on rerouting the underlying physico-chemical processes. Without this dynamic, closure cannot be achieved. Even worse, changes can be unpredictable. They can happen with many degrees of

freedom as long as they do not disrupt the closure of constraints (see, Soto *et al.*, 2016a; Mossio *et al.*, 2016; Montévil *et al.*, 2016a; Soto *et al.*, 2016b, and other articles in the same special issue). Classical dynamical systems theory is ill-suited to represent such open-ended structural dynamics. If structure is allowed to change, it does so in predictable ways without closure (Rasmussen, 2007; Kloeden, 2011). Radically new approaches and methods will be required to capture this most fundamental aspect of evolving organisms in mathematical terms.

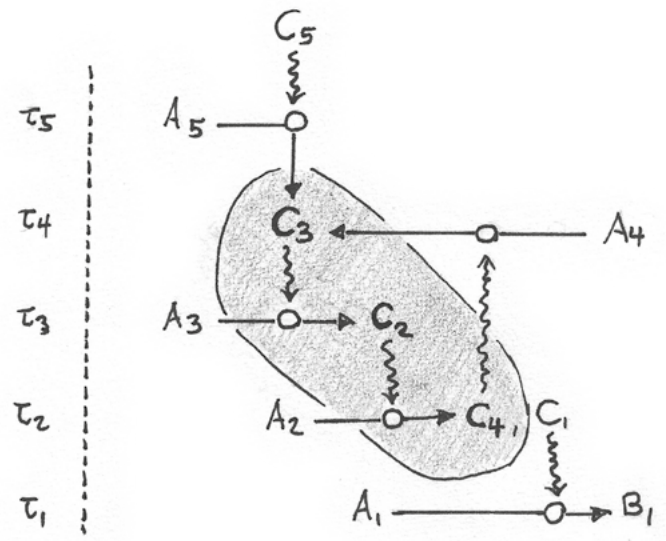

Figure 2. Organisational closure as closure of constraints. Physico-chemical processes are shown as solid arrows representing transformation of an input A to an output B. Constraints C act on these processes (wiggly arrows), redirecting energy flows. Constraints remain unaffected at the time scale of the constrained process (τ, column on the left). However, constraints can be generated by processes under constraints at different time scales. For instance, constraint C_2 (the product of a process at time scale τ_3) is dependent on C_3 (at τ_4) and is generative of C_4 (at τ_2). C_2–C_4 form a network with organisational closure (indicated by grey background) as all constraints are accounted for by other constraints produced within the system. Organisationally closed systems are always materially and energetically open, indicated by their interactions with external processes and constraints that are not part of the system itself (C_1 and C_5, for example). Diagram adopted from Montévil and Mossio, 2015; reproduced with permission.

Dependent co-constitution

The fact that organisms are dynamical systems generating their own constitutive boundary conditions has interesting consequences concerning the

traditional notion of configuration space. Configuration space is an abstract multi-dimensional space defined by axes that consist of the system's state variables and parameters (Thom, 1976; Jaeger *et al.*, 2015). In traditional dynamical systems theory, this space is pre-given: its geometry remains fixed, determined by externally imposed rules that govern the trajectory of the system through its configuration space. The idea that the world is structured in this way has been called the *Newtonian paradigm* by physicist Lee Smolin (2013), with the solar system as a classical example, where planets follow trajectories imposed on them by the laws of gravity. The dynamics of organisms are radically different, as the rules of change are immanent to the system: they originate with the self-determined activities of living beings and are constantly altered while the system unfolds (Varela *et al.*, 1974, Varela, 1979; Varela *et al.*, 1991; Rosen, 1991; Kauffman, 2000; Thompson, 2007; Letelier *et al.*, 2011; Moreno and Mossio, 2015; Walsh, 2015). Subsystems are created or eliminated, state variables and parameters are reconfigured, as a consequence of the interplay between processes and their constraints within organisational closure. Put more directly, the very existence of the system and its configuration space become mutually dependent. This type of co-dependence goes far beyond mere mutual causation between two separately existing processes (Walsh, 2015, 2018). Neither the organism nor its configuration space exist without each other. They are dependently co-originated or co-constituted in a dialectical relationship through the autonomous activities of the living system (Lewontin and Levins, 1985; Walsh, 2015, 2018; Gilbert, 2018).

Autopoietic systems cannot exist without thermodynamic openness (Jonas, 1966; Piaget, 1967; Varela *et al.*, 1974; Varela, 1979; Ruiz-Mirazo and Moreno, 2004; Moreno and Mossio, 2015; Montévil and Mossio, 2015), and a tight structural coupling with their environment (Varela *et al.*, 1974; Varela, 1979; Varela *et al.*, 1991; Thompson, 2007). The dialectical relationship presented above extends to this ecological realm. The experienced environment of an organism – also called umwelt (von Uexküll, 1909) or lebenswelt (Husserl, 1936) – is dependently co-constituted through the interactions of the living system with its surroundings (Thompson, 2007; Walsh, 2015). It is distinct from the physical environment and does not have any independent existence in the absence of the organism. Walsh (2015) illustrates this by comparing how paramecia and porpoises experience their aqueous environment. At the scale of the paramecium, locomotion through water takes the form of burrowing through a very viscous fluid. For the porpoise, moving is a problem of hydrodynamics and laminar flow. This leads to the evolution of very distinct locomotory strategies despite the fact that both organisms live in the same physical medium.

The outcome of evolution, ultimately, depends on a myriad of such individual interactions between organisms and their experienced environment (Walsh,

2015, 2018). These interactions are fundamentally dialectic. Living agents perceive affordances (Gibson, 1986) – opportunities to pursue, or obstacles to avoid – that determine the success or failure of their autonomous actions. In this way, the organism and its experienced environment are intimately commingled (Walsh, 2015). The organism not only co-constitutes the boundary conditions that lead to organisational closure, but also those features of the environment that present opportunities for successful survival and reproduction. Hence, the distinction between the living system and its configuration space also breaks down at the level of its ecological interactions. Both ontogeny and evolution are governed by a fundamentally dialectical dynamic that depends on constant changes to its underlying structure. This leads to truly open and unpredictable evolutionary dynamics: novel dialectical interactions emerge at different moments in time – within the organism and between the organism and its experienced environment – resulting in a ceaseless exploration of what Stuart Kauffman calls the adjacent possible, a region of configuration space not only unexplored, but often also non-existent just a short moment before (Kauffman, 1996; Longo *et al.*, 2012).

Whither dynamical systems biology?

My argument reveals a basic dilemma for current biology. On the one hand, dynamical systems theory is a powerful approach to study the behaviour of complex regulatory systems. On the other hand, traditional dynamical systems theory is fundamentally limited when dealing with the co-constituting dynamic structures of living systems. Using our current methods, we can only study subsystems with a very narrow scope. A radically new approach is needed to overcome this limitation.

Simulation-based studies, rather than mathematical analysis, offer a partial solution. Existing computational frameworks – such as agent-based modeling (see, for example, Klügl and Bazzan, 2012; Wilensky and Rand, 2015) and related approaches – allow us to implement evolving rules for the co-dependent emergence of system and configuration space. Unfortunately, there are two drawbacks. First, rules and context must still be provided externally, falling short of the radically immanent dialectic dynamics outlined above. The massive evolutionary simulation frameworks of artificial life, from Tierra (Ray, 1991) to Avida (Adami, 1998; Lenski *et al.*, 2003), have met with limited success in capturing the truly open-ended innovative dynamic leading to ever-increasing complexity in evolution. Even these extremely ambitious and complex simulations are still missing essential aspects of evolving living systems. Second, purely simulation-based approaches become increasingly difficult to understand as their

complexity increases. They expose us to the classic pitfall of systems biology: replacing a natural complex system we do not understand with a computational complex system we do not understand. Simulation generates prediction, but not necessarily insight. Our aim should not be to reproduce life in a computer, but to recognize and explain what life is and how it evolves in the natural world.

Ultimately, a deep understanding of living systems requires approaches that strike a compromise between analytical tractability and accuracy/completeness. It is not yet clear what such a theory would look like, but certain efforts point in the right direction. One example is René Thom's (1976) study of morphogenesis in terms of structural stability and bifurcations. Others are Robert Rosen's (1991) treatment of biological organisation using category theory, and Mesarovic and Takahara's (1976) generalisation of systems theory. These top-down theoretical efforts need to be combined with empirically grounded studies of particular ontogenetic systems, using novel modelling approaches and conceptual frameworks (see, for example, Verd and Jaeger, 2014; Montévil *et al.*, 2016b; Verd *et al.*, 2018a,b). This requires a serious and sustained trans-disciplinary effort of the research community. It poses a grand challenge for the life sciences in the 21st Century. Its aim is no less than to put the organism back at the heart of biology again.

Acknowledgments

I wish Sandro Minelli all the best for his 70th birthday. That you may remain as productive as ever! I have greatly profited from your encyclopaedic knowledge throughout the years we have been running the Venice Summer School together. Grazie mille, Sandro, for your intellectual influence on my career and your continued support. I thank Berta Verd, whose modelling work and mathematical analyses catalysed my thinking about the fundamental time-variance of biological organisation and the limitations of dynamical systems theory. Many of the arguments in this chapter grew out of discussions we had about her work. I thank James DiFrisco, Nick Monk, Scott Gilbert, and Denis Walsh for discussions and comments on the manuscript. I thank Maël Montévil and Matteo Mossio for letting me use a caricature of their figure. I thank Jannie Hofmeyr who greatly helped me understand Robert Rosen's work, and Olaf Wolkenhauer who achieved the same for Mesarovic and Takahara. I am grateful to the many fellows and visitors who I had the pleasure to meet during my time at the Konrad Lorenz Institute (KLI). They introduced me to many new aspects of the topics discussed here. It was a wonderfully inspiring time. It is a shame it could not last.

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The origin of evolutionary storytelling

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Abstract

Phylogenetics emerged in the second half of the nineteenth century as a discipline dedicated to constructing descriptive and explanatory narratives that traced the evolutionary origins of taxa and traits. Because ancestors and evolutionary transformations are empirically inaccessible, phylogeneticists had no choice but to use their more or less informed imagination to gain access to this epistemic hinterland. The explanatory power of phylogenetic hypotheses resides in their ability to trace back traits to their evolutionary origins. Hypothetical ancestors therefore became important epistemic tools as they were deliberately equipped with characters that could function as suitable evolutionary precursors for traits of interest. I argue that the precursor potential of hypothetical ancestors therefore became the first, more or less objective, phylogenetic optimality criterion.

Something missing?

When you read the historical sketches in systematics textbooks, you sometimes get the impression that something is missing. After starting with Aristotle and arriving in the nineteenth century, Wheeler (2012) dutifully praises Ernst Haeckel for coining the word 'phylogeny' and drawing such beautiful trees, before vaulting straight to the evolutionary taxonomists of the 1930s and 40s. Baum and Smith (2013) similarly laud Haeckel for his artful phylogenies, but without mentioning that he founded the discipline and coined much of its terminology. They get from Haeckel to Hennig in just three sentences. And although Haeckel figures in the index to Felsenstein's book *Inferring phylogenies* (2004), this is scarcely deserved as his presence is limited to an uninformative remark where we learn that phylogenies "were discussed by Darwin and Haeckel" (Felsenstein, 2004, p.123). What the first generations of phylogeneticists did around the turn of the nineteenth century is apparently of little interest or relevance to

modern readers, and perhaps this is so. You don't need to know anything about the history of phylogenetics to become a competent phylogenomicist. But there is another explanation for this historical blind spot.

Reconstructing systematic relationships has always been the core business of systematists, both before and after the spread of evolutionism. But the first phylogeneticists developed a different agenda. They were evolutionary biologists, chiefly zoologists studying comparative morphology and embryology, who were interested in the evolution of body plans (Nyhart, 1995; Bowler, 1996; Amundson, 2005). Like systematists they wanted to discover the relationships between taxa, but not as an end in itself, or to prop up classifications. Systematic relationships could guide phylogeneticists in their attempts to trace evolution, although systematic ignorance was hardly a barrier to phylogenetic speculation. Instead of pursuing the systematists' goal of constructing classifications to crystalize relationships located on the synchronic surface of systematics, phylogeneticists wanted to delve into the diachronic depths of geological time to tell evolutionary stories. Their primary goal was not to sharpen systematic tools, but to discover the phylogenetic events that had produced the natural system. Textbooks can therefore jump straight from Haeckel to Hennig.

Yet, the founding of phylogenetics as a discipline of evolutionary storytelling is a distinctive and noteworthy event in the history of biology, and one that has received comparatively little attention in the historical literature. In this essay I will have a brief look at the kind of storytelling that arose with the origin of phylogenetics, and the epistemic challenges that it posed. What follows is excerpted from a longer narrative that I develop in my forthcoming book on *Ancestors and the science of evolutionary storytelling*, to be published by Cambridge University Press in the Systematics Association Special Volume Series.

The consequences of descent theory

The scientific discipline of evolutionary storytelling originated in the second half of the nineteenth century. Comparative biologists and palaeontologists had long before amassed copious data on the morphology and development of organisms, but they didn't integrate these into historical narratives. Their research produced essentially static syntheses. The first grand narratives of biology only emerged after the advent of evolutionism, especially in the wake of the metaphysical revolution of Darwinism. Before that time systematic relationships didn't have a reading direction that could support a narrative arc. The unity of type that embryologists and morphologists had detected underneath nature's surface diversity was essentially static, and dynamic type concepts, such as Goethe's plant archetype, were based in mental metamorphoses

of form, rather than concrete, historical transformations. Even palaeontologists weren't motivated to connect their fossil dots into unbroken genealogies of ancestors and descendants on the canvas of deep time. The theory of common descent changed all that forever.

The science of storytelling was born when the arrow of time penetrated biology. It gave systematic relationships a reading direction from past to present. It brought ethereal archetypes down to earth as concrete ancestors who were linked to modified descendants through the material bonds of ancestry. It turned biological and palaeontological evidence into witnesses of a historical process, and made evolutionary biology a narrative discipline dedicated to the telling of origin stories. Ernst Haeckel called (t)his new science *Phylogenie* (Haeckel, 1866a). Its foundation was Darwin's key insight that the observable patterns of biodiversity are the products of an unseen process of descent. Edmund Beecher Wilson nicely captured the exciting prospects of the new evolutionary biology:

"The central question in every morphological investigation became twofold: it was no longer simply *what is?* it was also *how came it to be?* And this second question, be it observed, is not properly a speculative matter at all, but an historical one; it related not to an ideal or hypothetical mode of origin, but to a real process that has actually taken place in the past and is to be determined like any other historical event. "Speculative zoology" thus, by slow degrees, became the guide and leader of research, and every morphological inquiry became, in the last analysis, a genealogical one" (Wilson, 1891, p. 54; italics in original).

An important consequence of the realization that biological patterns were produced by evolutionary processes was that it required phylogeneticists to shift their focus to the gaps of the natural system. It became clear that systematic relationships did not map neatly onto the phylogenetic relationships that could explain them. Before the late-nineteenth century systematic diagrams generally depicted relationships between taxa as lines drawn directly between them. However, such lines cannot trace genealogical relationships unless taxa were interpreted as ancestors and descendants. To properly depict phylogenetic relationships systematic diagrams had to be redrawn. Consequently, the range of shapes of systematic diagrams published during the decades following the publication of the *Origin of species* decreased sharply, coinciding with the rise of tree-like branching diagrams (Pietsch, 2012; Morrison, 2014). Importantly, this did not reflect a sudden improvement of systematic tools to better reveal trees, but rather it recorded the imposition of the Darwinian expectation that relationships should generally be branching if descent with modification was indeed the process that produced biological diversity. Hence, in a relatively short period systematic diagrams that directly connected taxa were replaced by branching tree-like diagrams that connected taxa indirectly via hypothetical

common ancestors, so that the lines connecting taxa could trace the flow of evolutionary events through time. But gaining access to these events posed an epistemological challenge.

The imagination as an epistemic tool

Haeckel first outlined the goals and procedures of phylogenetics in his *Generelle Morphologie*. He distinguished two branches in the science of morphology: *Anatomie* was the science of the *vollendenten* (completed) form of organisms, while *Morphogenie* was the science of the *werdenden* (becoming, developing) form of organisms. Within *Morphogenie* he defined two subdisciplines, *Ontogenie* (or *Embryologie*), the science of the embryonic development of organismal form, and *Phylogenie*, the science of the evolutionary history of organismal form. For Haeckel they were intimately related as descriptive and explanatory sciences that aimed, respectively, to uncover the "developmental history of concrete morphological individuals", and the "evolutionary history of abstract genealogical individuals" (Haeckel, 1866a, p. 60). Their conceptual unity was underlined by Haeckel's use of the term *Entwicklungsgeschichte* for both. The goal of phylogeny was to "investigate the connected chain of forms of all those organic individuals that have branched off from one and the same shared stem-form" (Haeckel, 1866a, p. 30). Phylogeneticists, therefore, were to seek the explanation of evolutionary origins in the lineages of ancestors that underpinned every observable tip in the tree of life.

Like embryology, phylogenetics located its explanatory power in the tracing of origins. As Haeckel put it in the *Generelle Morphologie*, "Jedes Sein wird nur durch sein Werden erkannt" [Every being can only be understood by its becoming"] (Haeckel, 1866a, p. 23). This explanatory ambition further demarcated the emerging field of phylogenetics from systematics, which remained a primarily descriptive discipline strongly rooted in the empirical trinity – observation, description, and comparison – that had been its foundation ever since natural history emerged as a distinct scholarly subject in the sixteenth century (Ogilvie, 2006). However, this three-pronged tool can't penetrate the phylogenetic barrier. Ancestors and evolutionary processes exist in an epistemic hinterland that is empirically inaccessible. Only by floating free from pure observation and following their more or less informed imagination could phylogeneticists hope to enter this realm with their mind's eye. Haeckel approvingly quoted his friend and scientific idol Johannes Müller's defense of the imagination as an epistemic tool: to the nature researcher "fantasy is an indispensable good" (Haeckel, 1866a, p. 74). In the following decades explicit endorsements of the imagination as a phylogenetic tool became commonplace. Haeckel's student Anton Dohrn

put it like this in a letter to E. B. Wilson in 1900: "Phylogeny is a subtle thing, it wants not only the analytical powers of the "Forscher" [researcher], but also the constructive imagination of the "Künstler" [artist], – and both must balance each other, which they rarely do, – otherwise the thing does not succeed" (Dohrn in Groeben, 1985, p. 16).

Hypothetical ancestors as central subjects in scenarios

The phylogeneticist's job was to imaginatively interpret different sources of evidence to construct evolutionary narratives (scenarios) to account for the origin of focal traits and taxa. The fossil record was of course considered to be the ideal source of evidence since it really was located in the past. But its promise was compromised by the realization that it was a "completely gappy and torn up patchwork" ("*vollständig lückenhaftes und zerrissenes Flickwerk*") (Haeckel, 1866b, p. 307). And even when a fossil was unearthed that seemed to have ancestral traits, one typically couldn't be sure that it was a lineal ancestor rather than a closely related collateral relative, as Haeckel realized all too well (Haeckel, 1891, p. 466). The primary documents that phylogeneticists could use to guide their mind's eye into the evolutionary past were therefore the development and morphology of extant organisms. Haeckel's most distinctive and infamous strategy was to try to replay the tape of evolution from the reel of ontogeny. His biogenetic law declared that ontogeny recapitulates phylogeny, and its application allowed him to turn developmental stages into hypothetical ancestors. The tiny cup-shaped animal ancestor that Haeckel called *Gastraea* was a pure product of recapitulationist reasoning. He identified it from the evolutionary afterimage that he thought was retained as the invagination gastrula found in the development of several animal phyla.

Gastraea anchored one of the most influential, controversial, and enduring phylogenetic hypotheses ever conceived (Haeckel, 1874, 1877). Despite its simple structure *Gastraea*'s morphology was key to its explanatory power. Its invaginated archenteron and its separate ecto- and endodermal cell layers represented the phylogenetic origin of the gut and germ layers of all animals. Haeckel used these homologies to tie together the entire animal kingdom into a single monophyletic clade that he christened Metazoa. This achieved the unification of the four major animal types – Vertebrata, Radiata, Articulata and Mollusca – that had stood in isolation ever since von Baer and Cuvier had declared their incompatibility on developmental and functional morphological grounds. *Gastraea* was the fifth of two dozen ancestors that Haeckel identified as being part of the ancestral lineage of humans (Haeckel, 1895, p. 631). This lineage of hypothetical ancestors represented the central subject in Haeckel's historical

scenario for human origins. Central subjects are the foci around which stories, including historical narratives (Hull, 1975), are organized, and that provide them with unity and continuity. Importantly, lineages of hypothetical ancestors do all the explanatory work in phylogenetic scenarios.

The foundation of phylogenetic explanatory power

Much of the pre-cladistic literature on animal body plan evolution was dedicated to the construction of hypothetical ancestors to explain the origin of novel traits. The explanatory power of hypothetical ancestors resides entirely in their ability to root the evolutionary origins of traits. To accomplish this they need to possess precursors of homologous characters identified in descendant taxa. Traits that cannot be traced back to ancestors necessarily fall outside the explanatory umbrella of phylogenetics. Evolutionary morphologists understood this well. E. Ray Lankester called this insight "one of the fundamental principles of phylogeny, viz. that new organs do not arise *de novo* as new parts, but by the modification of pre-existing parts" (Lankester, 1881, p. 646). This core principle – as much an ontological commitment as an epistemological necessity – underpins the explanatory power of all phylogenetic morphology (Huxley, 1858, p. 382; Dohrn, 1875, p. 21; Lankester, 1875, p. 480; 1876, p. 54; Hubrecht, 1887, p. 644; MacBride, 1895, p. 342; Meyrick, 1895, p. 10; Patten, 1912, p. 253; Crampton, 1916, p. 2; Bock, 1959, p. 210; Raw, 1960, p. 500; Rensch, 1960, p. 275; Ghiselin, 1969, p. 114; Willmer, 1974, p. 327; Ghiselin, 1991, p. 292; 1994, p. 11; Nyhart, 2003, p. 165; Cracraft, 2005, p. 354; Gudo, 2005, p. 194; Kluge, 2007, p. 217, 224; Arthur, 2014, p. 232; Brunet *et al.*, 2015, p. 836; Havstad *et al.*, 2015; Minelli, 2016, p. 42).

Because phylogenetic explanations require the identification of ancestral precursors, the principle of 'no *de novo* origins' points the way to what I think is the first, more or less objective, phylogenetic optimality criterion: the precursor potential of hypothetical ancestors. It specifies what ancestral traits a hypothetical ancestor possesses from which descendant traits of interest could have evolved, and how plausible the necessary evolutionary transformations were thought to be. Because a consensus on higher-level metazoan relationships that could constrain speculations has only emerged during the last few decades, evolutionary morphologists often deliberately chose or designed hypothetical ancestors so as to maximize their precursor potential for explaining the phylogenetic origins of traits. It is in this sense that hypothetical ancestors were used as an epistemic tool in evolutionary narratives.

An example of how this was done is provided by the independent, yet near simultaneous, phylogenetic speculations of turbellarian expert Otto Steinböck,

cnidarian expert Jovan Hadži, and protist expert Earl Hanson in the mid-twentieth century. They sharply rejected the widespread consensus that animals had evolved from colonial flagellates, and instead proposed that bilaterian animals had descended from ciliate ancestors (Hadži, 1953, 1958, 1963; Hanson, 1958, 1963, 1977; Steinböck, 1958, 1963). Although their phylogenetic views are complex and do not agree in all details, the crux of their joint preference for ciliate ancestors was their superior precursor potential. The simple morphologies of the *Blastaea*- and *Gastraea* ancestors conjured by Haeckel's recapitulationist interpretation of development provided preciously few cues to explain the origin of distinctive animal traits such as protonephria, mesoderm, muscles, and more. Hadži, Steinböck and Hanson thought that they could locate homologues of these and other traits in ciliates. Hypothetical ciliate ancestors therefore had the precursor potential needed to explain the origin of simple turbellarian body plans. Alas, despite loud applause in some quarters of the zoological community (de Beer, 1954, 1958), this heterodox hypothesis never found widespread approval.

Because phylogenetic explanatory power resides in the precursor potential of hypothetical ancestors it can be used as a guide for understanding phylogenetic debates. For example, Holland *et al.* (2015) tabulate no fewer than 124 phylogenetic scenarios for the origin of vertebrates, with hypothetical ancestors drawn from many branches of the animal tree. However, they don't discuss why so many scenarios were proposed. Several factors are involved, but a key one is that different workers chose different explanatory foregrounds to which they tried to fit specific hypothetical ancestors. Three early scenarios illustrate these points. Anton Dohrn proposed annelid ancestors because he placed chordate segmentation in the explanatory foreground of his scenario. Annelid segments and segmental appendages provided all the precursors he needed to fashion vertebrate traits, from ribs to penis and post-anal tail (Dohrn, 1875). However, he relegated the notochord to the explanatory background as he could not find any annelid precursor. His scenario also required an inversion of the dorso-ventral axis to maintain homology of the annelid and chordate nerve cords, and the evolution of a new mouth as the old one degenerated. Ambrosius Hubrecht considered such events a "fata morgana" (Hubrecht, 1887, p. 641). He sought the origin of chordates in nemerteans (Hubrecht, 1883, 1887) precisely because they seemed to possess a precursor structure for the trait that had earned them their name: he derived the notochord from the coelom surrounding the nemertean proboscis. However, since nemerteans lack segmentation, Hubrecht had to resort to unconvincing arguments to explain the origins of the segmented aspects of the vertebrate body plan. Importantly, it was Hubrecht himself who didn't

put too much trust in these aspects of his scenario. Any arguments that went beyond the few characters in his explanatory foreground were "merely the sequel in a train of thoughts" (Hubrecht, 1883). Adam Sedgwick (1884) subsequently placed chordate segmentation back in the explanatory foreground, but considered it "exceedingly improbable that an animal should lose it mouth and develope a new one" (Sedgwick, 1884, p. 75). He therefore rejected Dohrn's annelid ancestors, and instead traced chordate somites back to the gut pouches of an anthozoan-like ancestor. These examples show that phylogenetic scenarios were devised as evolutionary origin narratives, each of which is characterized by a specific explanatory focus rooted in a unique combination of hypothetical ancestors and evolutionary intuitions.

Narrative phylogenetics today

The cladistic revolution of the mid-twentieth century delegitimized the use of deliberately fashioned hypothetical ancestors as epistemic tools in phylogenetic narratives. Modern approaches award logical priority to the reconstruction of patterns of systematic relationships, which tightly constrain speculations about ancestors and evolutionary processes. Nevertheless, systematic relationships do not properly become phylogenetic relationships without explicit hypotheses about ancestors and evolutionary character transformations. Indeed, in research areas where a consensus about systematic relationships is emerging, evolutionary storytelling often makes a comeback.

A recent issue of the *Journal of Experimental Zoology B (Molecular and Developmental Evolution)* (issue 6 in volume 6 in 2015) offers a perfect illustration. It collects together responses to a recent paper (Pyron and Burbrink, 2014) that claimed to have discovered that viviparity evolved early in squamate reptiles, and reversed to oviparity multiple times. Although the study was based on a comprehensive phylogenetic analysis and the use of sophisticated models for ancestral state reconstruction, its results were questioned because they conflicted with the respondents' intuitions about what is and isn't likely to happen during evolution. They used data and ideas from genetics, development, anatomy, physiology and ecology to argue that it is exceedingly unlikely that viviparous ancestors could have re-evolved oviparity. The merit of these arguments isn't important here. What is important is that all authors used their more or less informed evolutionary intuitions to diagnose that something must be wrong with the study of Pyron and Burbrink (2014). In bringing their biological intuitions to bear in this way, biologists today stay true to the original spirit of phylogenetics as a storytelling discipline, whether they realize it or not.

Coda

One of the most important events in my intellectual ontogeny was seeing a copy of Sandro and Fred Schram's 1994 paper titled 'Owen revisited: a reappraisal of morphology in evolutionary biology' on the desk of my undergraduate supervisor André van Loon at the University of Utrecht in 1995. It finalized my resolve to pursue a career in evolutionary biology, which I started by doing a PhD under Fred Schram's excellent guidance at the University of Amsterdam. In the years since I've absorbed Sandro's ideas through the never abating avalanche of papers and books that he continues to produce. Sandro, I hope your fount of inspiration will never run dry. May it long continue to fertilize our thinking about all things evolving.

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The relationship between genetics, epigenetics and epigenesis in evolution and development

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Abstract

In addition to DNA, reproduction and development require of epigenetic factors. Here we discuss on the nature of these epigenetic factors and their non-reducibility to genetics. Development can be understood as a sequence of reiterative interactions between genetics and epigenetic factors. These epigenetic factors can be traced back to the origins or very early evolution of life. Variation in epigenetic factors is usually non heritable and this may seem to suggest that they are less important to understand the relationship between genetic and phenotypic variation and evolution. However, since development involves a reiterative interaction between genetic and epigenetic factors, the latter need to be taken into account to understand development, phenotypic evolution and the evolution of development.

Introduction

The 20th century can be seen as the century of genetics. We have learned that phenotypes and most of its variation have, ultimately, a specific genetic basis. We know what is at the bottom, the genome, and what is at the top, the phenome, but we do not understand well enough the processes in between to explain which changes at the genetic level lead to which specific changes at the phenotypic level (and why to those changes and not to others). It is our perception, and that of many others (Houle and Omholt, 2010), that the early 21th century biology would largely be about understanding this genotype-phenotype map.

G. Fusco (ed.) *Perspectives on Evolutionary and Developmental Biology.*

When the phenotype considered is morphology (i.e., the spatial distribution of cells, cell types and extracellular matrix in space), the genotype-phenotype map is, to a large extent, determined by development (Alberch, 1991). At the most general conceptual level, development can be described as the process by which specific arrangements of cell types, what we call *developmental patterns* (Salazar-Ciudad *et al.*, 2003*)*, transform into other developmental patterns. Over developmental time, these pattern transformations occur constantly. In fact, they lead from a simple developmental pattern, the zygote, to a quite complex one, the adult organism.

This complex phenotype arises because genes interact in networks that regulate each other's expression, as well as, cell behaviors (e.g., cell division, cell adhesion, cell contraction, etc.) and cell mechanical properties. We call *developmental mechanism* each network that regulates cell behaviors and mechanical properties and leads, as a consequence, to developmental pattern transformations. Some of the gene products in those developmental mechanisms may be extracellular diffusible signals which can alter the behavior or gene network dynamics of neighboring cells. As a result of these interactions, cells change their location in space and, through extracellular diffusible signals, affect back which genes are expressed in which cells over space. Then, to understand the association between a specific mutation in a gene and a morphological change, one needs to understands how this mutation affects the dynamics of the network in which the gene is embedded and how that, in turn, affects, the cell signaling and tissue bio-mechanics that build the body.

By eliminating some specific phenotypes but not others, natural selection determines how the phenotypes present in populations change over time. This is, for example, how limbs get longer or shorter, how they, at the same time, become wider or, more in general, how they change their shape over evolution. In other words, natural selection is a crucial factor determining the direction of evolutionary change. Natural selection, however, can only eliminate among existing phenotypic variation. If, in a given generation, phenotypes cannot vary in every conceivable direction, then, those directions of change that are possible have also a strong influence on how phenotypes evolve over generations (Alberch, 1982; Salazar-Ciudad, 2006). In fact, it has been repeatedly proposed that phenotypic variation has a structure: Some phenotypic variants are common, some are rare and some are just not observed (Alberch, 1982; Salazar-Ciudad, 2006). Thus, it is never the case that all conceivable phenotypic variation, even if small, is possible in a given generation or even in the short term.

In the case of morphology it is development, through its networks of gene and cell interactions, that determines which phenotypic variation arises as a result of genetic variation in each generation. The direction of evolutionary

change is then determined by both natural selection and development in each successive generation. This applies to the variation possible in each generation and to the variation possible in the short-term, that is, while development itself does not evolve much. What is possible at the phenotypic level may change over longer time periods as development itself evolves. Even at this level there are some rules about how development can change, not everything is possible (Newman and Comper, 1990; Newman and Müller 2000; Salazar-Ciudad *et al.*, 2003, 2004; Salazar-Ciudad, 2010).

Heritable phenotypic variation arises, ultimately, from genetic variation but the paragraph above implies that the phenotypic consequences of genetic variation are only understandable from development. In the rest of this chapter we will explain how development itself is not reducible to the interactions between gene products. Development involves, in addition, a set of epigenetic factors that extensively interact with gene products, but that are not fully explainable by them.

Epigenesis, epigenetic factors and preformation

The concept of epigenetic factors either refers to anything that is not in the DNA sequence but that is heritable and has a causal role in development (such as the patterns of methylation on the DNA) or to the adjective form of the substantive "epigenesis" (Haig, 2004). In this latter case epigenetic would simply mean "of epigenesis or related to epigenesis". Epigenesis is one of two alternative views Aristotle proposed about embryonic development. Epigenesis is the view that during development new organization arises from previously existing organization that was not equal or trivially similar to it (Müller, 2007). The other view is preformationism: the view that nothing really new arises in development and that most body parts and organization are already present, at a smaller scale, within the parent's gametes.

Nowadays it is clear that there are not many organizational similarities between an oocyte and an adult (e.g., in humans) and, that in fact, most of the organization in the adult has to be built de novo from that present in the gametes. In fact, development can be described as a sequence of transformations between what we have called developmental patterns. Most of these transformations are not non-trivial, otherwise development would be easy to understand and it is certainly not. Embryonic development can then be said to be in agreement with the Aristotelian view of epigenesis. There is something, however, that does indeed remain constant during development and that is faithfully copied between generations: the genotype. In that sense, embryonic development is epigenesis but is also, for the part of the "phenotype" we call genotype, preformationist. As

we will explain development, an in fact the whole life cycle, can then be understood as an interaction between this preformed part and the epigenetic part of the organism at any given time (Fig. 1).

Epigenetic factors and development

From the above one can define as genetic all that is copied, such as the DNA, and as epigenetic all that is not copied between generations but has a causal role in the understanding of development.

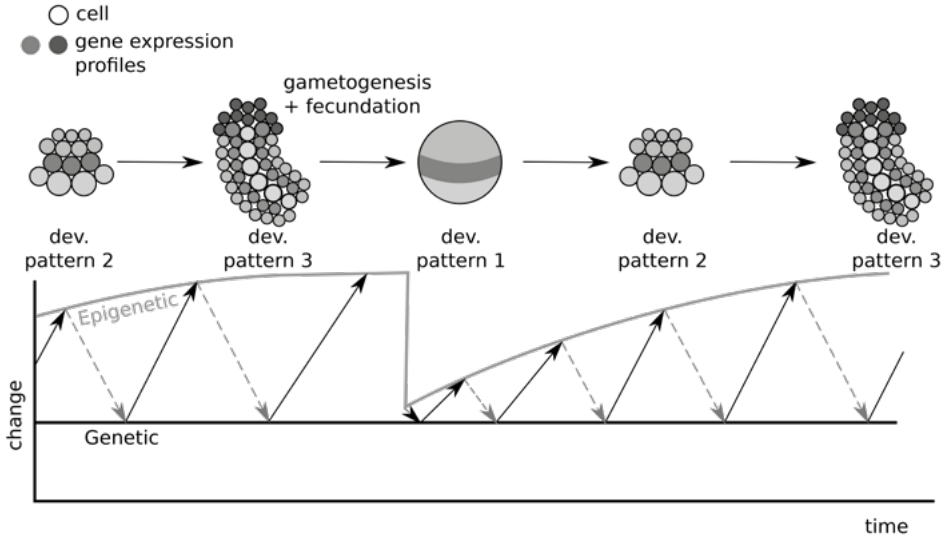

Figure 1. Schema of the interplay between genetic and epigenetic factors along successive stages of organismal development. The top row shows different developmental patterns, different distributions of cell types in space, from the zygote to the adult of one organism and its parent. In the case of the zygote, the developmental pattern comes from the asymmetric distribution of gene products mediated by the mother during gametogenesis. The plot below shows the amount of genetic and epigenetic accumulated change within an individual and between generations in respect to the zygote. The genotype does not change *per se* during development, it is the interplay between it and the epigenetic factors (black solid and green dashed arrows) that builds the developing organism. This interaction (the arrows) should be understood, as discussed in the text, as different epigenetic factors affecting where and when different genes get expressed, while at the same time, which epigenetic factors are encountered in each time and place in the embryo depends on previous genetic and epigenetic interactions, as the arrows abstractly depict).

A developmentally important example of epigenetic factor is the asymmetric spatial distribution of many proteins and RNAs in the oocytes of many species (Newman, 2011). These distributions are relatively simple, most animals

have an asymmetry along the animal-vegetal pole and many of them have also an asymmetry along what would become the dorso-ventral axis of the embryo (Gilbert and Raunio, 1997; Gilbert and Barresi, 2016). These asymmetries are absolutely required for development, if they are experimentally disturbed, embryos become symmetric and their development gets arrested very early (Kandler-Singer and Kalthoff, 1976). These spatial asymmetries arise from spatial asymmetries present in the gonads of the parents (or, in some species, in the environment), typically their cell-level apical-basal asymmetries. These are either inherited from the epithelium as the oocyte gets extruded from it (Bastock and St. Johnston, 2008), or imprinted to the oocyte from an apical-basally polarized epithelium through short-range signaling (Neuman-Silberberg and Schupbach, 1993; Roth and Lynch 2009).

One may argue that the asymmetries in the mother's gonads are due to gene product interactions in the earlier development of the mother. This is indeed the case, but these asymmetries in the mother's gonad required also that the oocyte that gave rise to the mother had the same spatial asymmetries, otherwise, the mother's development would have arrested early on. The spatial asymmetries in the oocyte are, thus, not reducible to, or completely explainable from gene product interactions.

This causal interdependence between oocyte's asymmetries and gene product interactions can be traced backwards through generations in evolution. In fact it can be found even in bacteria. Bacterial reproduction requires the pre-existence of a cell with a cell membrane, RNA and DNA polymerases, ribosomes, tRNA-acetyltransferases, etc. In other words, the machinery for DNA replication and protein synthesis has to be present in the cell for genes to have any causal effect. In addition, there has to be some spatial asymmetries within the cell too. The membrane, for example, is polarized by some sort of bacterial cytoskeleton (Celler *et al.*, 2013) so that in binary fission sister cells grow apart and split in specific directions. Although the experiments have not been done, it is unlikely that this cytoskeleton, or cell structure in general, could re-organize itself into a viable cell if the spatial structure of the cell would be scrambled, even if just internally. Most likely, as in the case of the oocyte, this spatial organization is strictly required and not reducible to genes. As in the case of oocyte asymmetries also, these cell asymmetries are inherited but not copied. They are simply re-built in each generation from gene networks and these same asymmetries in the mother.

One alleged exception to this epigenetic-genetic causality chain across generations would be some hard version of the RNA world hypothesis on the origins of life (Orgel, 1968). According to this hypothesis life would have arisen

from naked RNA molecules of some sort. This is a specially popular hypothesis in genetics textbooks (Griffith, 2002) and among researchers non-specialized in the origins of life. This hypothesis is probably popular because it fits the gene-center line of though according to which genetic factors are the most fundamental and that anything else stems from them. Among origins of life researchers, however, there are many other, equally or even more popular, hypotheses ranging from metabolism-first (Huber and Wächterhäuser, 1998; Smith and Morowitz, 2004) to cell-first (Oparin, 1938; Segré *et al.*, 2001; Hunding *et al.*, 2006) hypotheses or even hypotheses in which the genetic-epigenetic causality chain will be quintessential of life (Salazar-Ciudad, 2013).

Another example of epigenetic factor are the *developmental patterns* themselves. Gene product interactions are crucial in determining which developmental patterns arise from which previously existing developmental patterns in each stage during development, but so are these previously developmental patterns themselves. The same developmental mechanism (i.e., gene network plus cell behaviours and mechanical properties) can lead to different final developmental patterns depending on which previous developmental pattern it acts on (Salazar-Ciudad *et al.* 2000, 2003) and, thus, these patterns are also explanatory about which pattern transformations are observed. In each developmental stage, existing developmental patterns depend, however, on previous gene product interactions acting on previous developmental patterns (Fig. 1). Thus, these patterns, starting from the asymmetries in the oocyte, are both a consequence and a cause of developmental dynamics. In that sense embryonic development can be described as a chain of interactions between genes and epigenetic factors. This chain extend across generations since the asymmetries in the oocye require the developmental pattern of the adult mother and some gene networks in it, as explained above. The complex process of embryonic development can then be seen as a process of rebuilding adult complexity from the spatial asymmetries in the zygote and its genotype. In this process genes and epigenetic factors are intricately interdependent, but not reducible to each other (Fig. 1).

The same chain applies to the development arising from asexual reproduction. In some examples of asexual reproduction, such as in gemmation among cnidaria, the contribution of epigenetic factors is even more evident since offspring does not develop from a simple cell but from part of an adult that is, inevitably, more complex than an oocyte. In terms of Figure 1, this sort of asexual reproduction would be like if the lowest point in the plot (that corresponds to the gametes in sexual reproduction) would be at higher position and then less phenotypic changes are required from there to reach the adult.

Other epigenetic factors relevant for embryonic development include the mechanical properties of cell collectives (Newman and Comper, 1990; Newman and Müller, 2000) and basic cell behaviors such as cell division, cell adhesion, apoptosis, extracellular signal secretion, etc. (Salazar-Ciudad *et al.*, 2003). All these factors are often regulated by gene products, but their existence is not due to, or merely reducible to, genes or genetic interactions. In fact, many cells and tissue mechanical properties that are relevant to understand morphogenesis are also found, in some rudimentary form, in liposomes devoid of proteins and other non-biological natural and artificial systems (Newman and Comper, 1990) and in cell aggregates and simple metazoans containing only subsets of the developmental genes of multicellular organisms (Newman, 2012). Some of these may have been present in early life evolution too (Segré *et al.*, 2001; Hunding *et al.*, 2006). From this perspective, gene product interactions, simply act to more finely regulate properties and behaviors that are intrinsic to cell clusters and whose existence is affected but not totally explained by them. Understanding those cell properties and behaviors is fundamental to understand development, as it has been widely discussed (Newman and Comper, 1990; Beloussov, 1998; Oyama, 2000; Newman, 2011; Guillot and Lecuit, 2013) under different names and slightly different concepts: epigenetic mechanisms (Newman and Müller, 2000), soft-matter properties (Newman and Comper, 1990), developmental resources (Oyama, 2000), phenogenetic (Weiss and Fullerton, 2000), and epigenotype (Waddington, 1942). These views are simply concrete specifications of the view that there is a gene-centered bias in current developmental biology and evolutionary biology (Lewontin, 2000; Minelli and Pradeu, 2014).

The inheritance of variation and evolution

Although both genetic and epigenetic factors are heritable, only variation in the former is also heritable (Salazar-Ciudad, 2008). Thus, for example, environmental perturbations leading to changes in oocyte's asymmetries are very unlikely to give rise to viable offspring. Even if they would, for those changes to be heritable, they would need to give rise to mothers that produce oocytes with the same perturbed oocyte asymmetries. This seems very unlikely, especially for complex multicellular organisms. In some protists, however, something very similar has been observed. In some ciliates, the grafting of the mouth (an invagination of the cell membrane specialized in feeding) from one individual to another gives rise to a two-mouthed individual. By fission, this cell gives rise to cells that, over generations, keep having to mouths (Sonneborn, 1964).

Other examples of heritable variation in the epigenetic factors has been reported (Jablonka and Lamb, 2005; Frankel, 2008) but, in general, genetic vari-

ation is much more likely to be heritable than epigenetic variation. In fact, that may be the ancestral *raison d'être* of genetic factors (i.e., DNA), its capacity to accumulate heritable variation (Salazar-Ciudad, 2013). In contrast with the above example of oocyte asymmetries, any change in DNA, as long as it leads to one of the four bases after repair, is heritable. If the DNA can affect the phenotype, that is what is visible to natural selection, then heritable adaptive changes should accumulate and lead to long-term adaptive evolution (Salazar-Ciudad, 2013). This is important because then evolution would be based on those changes that are heritable and can be accumulated over time. This means phenotypic changes that are associated with specific genetic changes, at least statistically, just as classical evolutionary theory states. From the perspective of which variation is heritable, then, genetic and epigenetic factors are not totally equivalent, as claimed by the proponents of Developmental Systems Theory (Oyama, 2000).

Since variation in epigenetic factors is not usually heritable (but see Jablonka and Lamb, 2005), one could argue that epigenetic factors are not necessary to understand the genotype-phenotype map. After all, this map is defined as the association between genetic variation and phenotypic variation. However, as we come to explain, genetic and epigenetic factors need to interact for complex phenotypes and their variation to be possible at all. Even if only genetic variation is heritable, their effect on the phenotype is only explainable from the interaction between genes and existing epigenetic factors (e.g., developmental patterns, starting from the spatial asymmetries in the oocyte). These epigenetic factors may change in evolution as a result of genetic variation but how they change is not determined by the latter but by the interaction between the latter and the former. In other words, since any change in phenotypic evolution is first a change in development and development can only be understood from the interaction between genetic and epigenetic factors, evolution can only be understood too, from the interaction between epigenetic factors and the variation in genetic factors. In a way, genetic and epigenetic factors will always be inextricably interdependent in life. The phenotypic effect of any genetic change will depend on how the epigenetic factors are in a given moment, and these will depend on how epigenetic factors were in the past and how they interacted with genetic factors then. In summary, thus, genetic factors, epigenetic factors and their interaction need to be taken into account to understand development, phenotypic evolution and the evolution of development.

Acknowledgements

We thank J. Jernvall, I. Salvador-Martinez, A. Matamoro, P. Hagolani, F. Oliveira, T. Välikangas, K. Ruiz-Mirazo, A. Moreno, S. A. Newman and R. Zimm for comments. This research was funded by the Finnish Academy (WBS 1250271) and the Spanish Ministry of Science and Innovation (BFU2010-17044) to I.S.C.,

the Spanish Ministry of Science and Innovation (BES2011-046641) to M.B.U., and the Generalitat de Catalunya (2013FI_B00439) to M.M.R.

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Treacherous trees: Trials and tribulations in tracing the trajectories of traits

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Abstract

Evolutionary trajectories of individualized characters can be represented as phylogenetic trees, similar to species or gene trees. However, character trees may not always adequately represent the evolutionary trajectories of characters for two reasons. First, novel characters may originate by recombination of old parts rather than by duplication and divergence resulting in trajectories that are more network- than tree-like. Second, characters represented on different trees (or different branches of the same tree) may be genetically or functionally coupled reflecting incomplete individualization. This leads to co-evolution of the characters and correlations between different trees. Thus, character trees can provide us with an invaluable tool to understand origins and diversification of characters, but only if these caveats are born in mind.

Introduction

Well, I may have listened to too much Wagner these days (Richard, not G*ü*nter) but the contrived title of this short essay still tries to make a serious point. "Tree-thinking" as it has been called, is a central element of evolutionary theory (Baum *et al.*, 2005). Darwin first proposed that the genealogical relationships of all species should be presentable as a single "Tree of Life", the branching pattern of which can explain the hierarchical nesting of taxonomic categories (species, genus, family etc.) long recognized in systematics (Doolittle and Bapteste, 2007). The metaphor of the "Tree of Life" is still serving us well. The phylogenetic relationships of most groups of animals and plants, in which speciation occurs predominantly by the splitting of lineages is best represented as a tree. However, it also has become clear that for other groups of organisms this metaphor is quite inappropriate. Due to the prevalence of horizontal gene transfer in pro-

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karyotes, their relationships are better represented as a network of diverging and converging lineages rather than as a tree (Doolittle and Bapteste, 2007). Occasionally, horizontal gene transfer or the origin of new species by hybridization (e.g., by allopolyploidy) can lead to fusion of branches also in animals and plants, tainting the ideal picture of a phylogenetic tree with purely diverging branches also for these groups.

Species trees and character trees

The tree-like pattern of species evolution is a direct consequence of "descent with modification": the organisms of one generation give rise to progeny of the same kind but subject to occasional subtle heritable variations. The separation of descendants exhibiting different heritable traits (e.g., due to reproductive isolation between two populations acquired after geographic separation) results in lineage divergence, i.e. tree-like branching. While phylogenetic trees were first used to depict species relationships, it was soon realized that the evolutionary trajectories of other units which faithfully reproduce from generation to generation can also be shown as trees. In particular, genes which faithfully replicate with only occasional mutations form a tree-like pattern of relationships. Like species trees, gene trees branch during speciation events because the genes from different species don't mix and recombine. However, genes may also duplicate without speciation, producing two or more copies in a single genome, and subsequently diverge by the accumulation of different mutations in each copy (Fitch, 1970). Consequently, gene trees are embedded within the species tree but can have additional branching points due to duplication and divergence (Fig. 1).

It has recently been emphasized that phylogenetic trees can not only represent the evolution of species or genes but also of characters such as particular cell types or organs (Geeta, 2003; Oakley, 2003; Serb and Oakley, 2005; Wagner, 2014). Like gene trees, character trees are embedded within the species tree but may be more highly branched due to character duplication and divergence independent of speciation events (Fig. 1). However, in contrast to organisms and genes, the "progeny" of a character in the next generation is not easily identifiable as outcome of a simple reproduction or replication event. Instead, characters are constructed anew during development of each generation. Genealogical continuity can thus only be established for characters which clearly retain their identity across generation boundaries. Such characters need to remain recognizable and distinct from other characters even in the face of heritable variation. It has been proposed that a core network of regulatory genes (so-called character identity network or ChIN), ensures stable character identity through-

out evolution, whereas downstream of this core network a battery of "realizer genes" determines character state, which is subject to variation (Wagner, 2007, 2014). This hierarchical view of characters has been successfully applied to cases like cell type evolution (Arendt *et al.*, 2016).

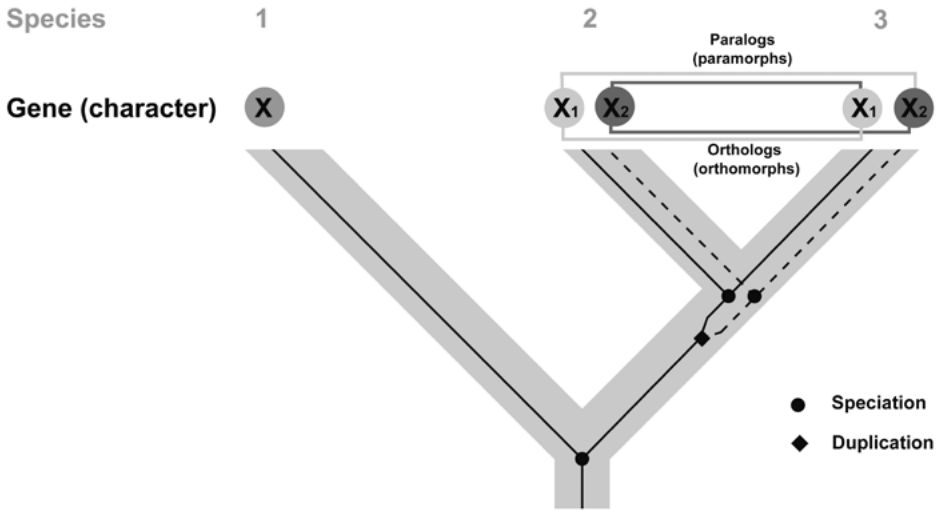

Figure 1. Character and species trees. Genes or characters can duplicate and diverge independent of speciation events. The resulting gene or character trees (black) are embedded within the species tree (grey). Genes may be derived from a common ancestor through speciation (orthologs) or through duplication (paralogs) and a similar distinction can be made for characters (orthomorphs vs. paramorphs; see (Wagner, 2014)). Thus X_1 in species 2 and 3 are orthologs/orthomorphs as are X_2 in species 2 and 3, while X_1 and X_2 are paralogs/paramorphs.

Although it is still unresolved whether this hierarchical model is applicable to all types of characters, it is clear that only individualized parts of the organism, i.e. those parts which evolve as an integrated unit but relatively independently from other parts have sufficient autonomy to qualify as proper characters (Schlosser, 2002; Wagner, 2001, 2014). Such individualized characters (also referred to as units or modules of evolution) follow an evolutionary trajectory relatively independently from other characters and this trajectory can be depicted as a phylogenetic tree. Two conditions have to be met to allow the quasi-independent evolution of individualized characters. First, they have to have at least a partially different genetic basis from other characters allowing them to be subject to heritable variations that affect only those, and not other characters

(genetic individuation) (Wagner, 2014). And second, they must have at least a partially separable function from other characters and make an independent fitness contribution (Schlosser, 2002, 2004). While the first condition is widely recognized, the second one is often overlooked. It is nevertheless essential because fitness epistasis tends to tie together the evolutionary fate of parts which cooperate in the fulfilment of a common function resulting in internal selection and coevolution (Schlosser, 2002; Wagner and Schwenk, 2000).

The new perspective of considering characters as units with an independent evolutionary trajectory has been fruitfully applied to the evolution of organs such as plant leaf primordia and animal eyes (Geeta, 2003; Oakley, 2003) as well as to the evolution of cell types (Arendt, 2003, 2008; Arendt *et al.*, 2016). For example, it has provided us with valuable new insights into the evolution of two types of photoreceptors – ciliary and rhabdomeric. These differ with respect to the subcellular location of membrane expansions which carry photopigments, but rely on evolutionarily related opsins and phototransduction mechanisms indicating that they have evolved as so-called "sister cell types" by duplication and divergence from an ancestral photoreceptor (Arendt, 2003; Plachetzki *et al.*, 2010).

However, similar to the "Tree of Life", which does not always accurately capture the complex evolutionary trajectories of species, character trees have their limitations and do not always adequately represent the evolutionary trajectories of traits. In fact, because characters are always only partially independent from each other, such limitations are much more prevalent and potentially misleading for character trees than for species trees. Only when these limitations are properly acknowledged, can we appreciate both the potential and the pitfalls of character phylogenies. I will consider two important caveats here. First, evolutionary trajectories may be convergent as well as divergent and, consequently, can be more like a network than like a tree. Second, different character trees or even different branches of a single character tree may not be independent from each other because of genetic or functional coupling between characters (reflecting incomplete character individualization) leading to their co-evolution.

Trees versus networks

The first problem (network vs. tree-like trajectories) arises, because novel characters can arise either by duplication and divergence of existing characters or "de novo" (Wagner and Lynch, 2010). However, evolution mostly "tinkers" with what is already there rather than creating new structures out of thin air (Jacob, 1977). "De novo" origin of characters, thus typically involves the redeployment

and recombination of pre-existing components into new structures (Minelli and Fusco, 2005; Oakley, 2017). Only when characters evolve by duplication and divergence will their evolutionary trajectory be tree-like. In contrast, origin of new characters by recombination of old components is better represented by a network in which lineages can converge (fuse) as well as diverge (Fig. 2). Moreover, the connections between different nodes in such a network will often be non-equivalent, because various old characters may make qualitatively and quantitatively different contributions to a novel character.

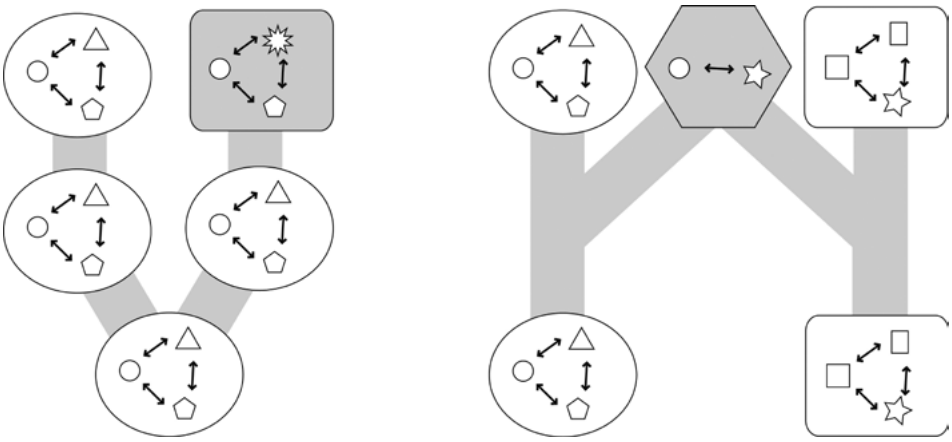

Figure 2. Origin of novel characters. Novel characters may arise either by duplication and divergence (left) or by recombination (right). Characters are depicted as large symbols, their components which interact to form a character identity network as small symbols. Modified from (Oakley, 2017).

The evolution of novel cell types provides some nice examples here. Although duplication and divergence was clearly at work during the evolution of some cell types such as the photoreceptors discussed above, other cell types (e.g., neurons in eumetazoans, osteocytes in jawed vertebrates) most likely originated by the recombination and redeployment of old genes and proteins into new regulatory networks. The origin of stinging cells (cnidocytes) in cnidarians is a case in point (Tardent, 1995). These cells which are only found in cnidarians release the contents of a venom filled capsule - the cnidocyst – in response to mechanical stimuli. Although cnidocytes are developmentally and possibly evolutionarily related to cnidarian neurons, they are specified by transcription factors which are not expressed in neurons (e.g., PaxA, Mef2), some of which were possibly redeployed from other cell types. In addition, they use cnidaria-specific proteins (e.g., minicollagens) and the enzyme poly-γ-glutamate synthase

acquired by horizontal gene transfer from bacteria for building the cnidocyst (Babonis and Martindale, 2014, 2017).

Correlation between trees due to co-evolution of characters

The second problem (co-evolution) arises because characters are never completely independent of other characters resulting in correlated changes in different character trees or in duplicated and diverged characters comprising different branches of the same tree. Because the individualization of characters depends on two different conditions (genetic individuation and functional separation), there can be two different reasons, why two characters may not evolve fully independently from each other: incomplete genetic individuation or incomplete functional separation.

On the one hand, development of different characters may only partly rely on different genes, while other genes have a shared role for both characters (incomplete genetic individuation). The two characters are, thus, to some extent genetically (or generatively) coupled. Some mutations in their shared genes will have pleiotropic effects on both characters. The variability of both characters is therefore to some extent linked imposing developmental constraints (Maynard Smith et al., 1985; Schlosser, 2007; Wagner and Schwenk, 2000). Heritable changes in the common genetic basis of two characters will, thus, result in correlated changes in their character trees, also known as concerted evolution (Musser and Wagner, 2015). The possibility of concerted evolution poses a challenge to our "tree-thinking" abilities since it makes character trees quite unlike genealogical trees, where different branches are independent. It would be very strange indeed if, for example, the appearance of longer noses in the descendants of one family, would mirror the appearance of longer ears in the descendants of another.

The evolution of fore- and hindlimbs in jawed vertebrates can serve as an example here. Fore- and hindlimbs are partially genetically individuated due to the expression of different transcription factors (e.g., Tbx5 in forelimbs vs. Tbx4/Pitx1 in hindlimbs) which help to channel development of fore- and hindlimbs into different pathways (Ouimette *et al.*, 2010). This allows their accumulation of independent changes during evolution; in some groups, such as birds and bats, this can result in widely diverging morphologies of forelimbs (wings) and hindlimbs (legs). However, most of the genetic machinery is shared between fore- and hindlimbs (Tickle, 2015) and this results in pervasive concerted evolution. The latter is reflected in correlated patterns of character state changes in fore- and limbs as has recently been well documented for the phylogenetic pattern of toe loss in fore- and hindlimbs of squamate reptiles (Brandley *et al.*,

2008). The correlated changes in trunk and antennal segmentation of centipedes (Minelli *et al.*, 2000) most likely present another case of concerted evolution although the shared genetic basis of these traits is currently unknown.

On the other hand, characters may also co-evolve not because they are genetically linked but because they functionally cooperate at least occasionally (incomplete functional separation). The fitness of one character then depends on the state of the other character with which it functionally cooperates (fitness epistasis). This implies that heritable changes in one of the characters can change the fitness landscape for the second character, for example by changing the relative fitness ranks of various character states. This will induce selection on the second character and affect the direction of its evolutionary trajectory. The fitness effects of character state changes are therefore to some extent linked between the two characters imposing functional constraints and resulting in reciprocal internal selection (Schlosser, 2007; Wagner and Schwenk, 2000). As a consequence, there will be co-adaptation of characters, which will be the more pronounced, the more functionally intertwined the two characters are.

Co-evolution of functionally cooperating characters can be observed at all levels of the biological hierarchy. Organs (e.g., limb buds) will to some degree co-evolve with those other organs (e.g., the axial skeleton) and cells (e.g., limb muscle cells) will co-evolve with those other cells (e.g., limb cartilage and bone cells) with which they work together during development and in the adult. Similarly, on a molecular level, functionally interacting proteins will co-evolve. The co-evolution of ligands and their receptors in various signaling pathways illustrate this point particularly nicely. Because a certain signaling function can only be fulfilled when ligands are bound by their receptors and induce the appropriate conformational change, evolutionary changes of ligands result in selection of receptor variants which bind the modified ligand more effectively and vice versa. The resulting co-adaptation of receptor and ligand is reflected in correlated evolutionary rates and branching patterns between receptor and ligand ((Pazos and Valencia, 2008); note, however, that correlations between evolutionary rates can have other causes as well).

A recent scenario on the evolution of G protein-coupled receptors mediating signaling by hormones related to Gonadotropin releasing hormone (GnRH) in protostomes may serve to illustrate this point (Hauser and Grimmelikhuijzen, 2014). Based on a large body of gene sequence data, it proposes that duplication of an ancestral GnRH-like receptor gene led to corazonin-receptor-like receptors (CRZR) and adipokinetic hormone-receptor-like receptors (AKHR) in ancestral protostomes. In parallel, the ligand for this ancestral receptor duplicated and diverged to give rise to CRZ- and AKH-like ligands, each of which

specifically binds to its respective receptor. A subsequent duplication of the gene encoding AKHR in arthropod ancestors was mirrored by duplication of its ligand AKH leading to the evolution of a third receptor-ligand signaling system (the AKH/corazonin-related peptide or ACP system) co-existing with CRZ- and AKH- signaling in arthropods.

Not all characters are functionally as closely tied to each other as a receptor and its ligand. In fact, I have argued above that only units which behave as quasi-independent modules (i.e., which evolve in an integrated fashion but relatively independently from other units), qualify as proper characters and these follow an evolutionary trajectory relatively independent from other characters. However, the quasi-independence of modules is a matter of degree and modules occasionally need to interact in the service of organismal integrity, possibly forming higher order modules. As I have elaborated elsewhere, this is reflected in a certain co-evolution probability of modules or characters (Schlosser, 2002, 2007), which decreases with the degree to which characters are individualized and develop and function independently from each other.

Conclusion

Applying "tree-thinking" not only to the relationships between species – the "Tree of Life" – but also to genes and characters is without doubt a very illuminating strategy to trace the evolutionary trajectory of traits. It provides us with an invaluable tool to conceptualize and graphically represent the evolutionary history of genes and characters. However, I argue here that we have to use this tool with caution and avoid the pitfalls of stretching the analogy too far. Whenever novel characters originate by recombination of old parts rather than by duplication and divergence, this will result in a very un-tree-like fusion of branches. Whenever different characters are genetically or functionally coupled, changes will tend to accumulate in different trees or different branches of the same tree in a correlated fashion, very different from a family tree representing a genealogical lineage of descent. But when we carefully consider these caveats, trees of traits can be true treasures.

Acknowledgments

I thank Giuseppe Fusco and Nikola-Michael Prpic-Schäper for the critical reading of the manuscript. This essay is dedicated to Alessandro Minelli on occasion of his 70th birthday. May his splendid spirit sparkle for many years to come!

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Alessandro Minelli short biography

A biographical sketch of Alessandro Minelli, with a list of selected publications

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Alessandro (Sandro) Minelli was born in Treviso, about 30 km north of Venice, on December 20th 1948. A passionate naturalist from early youth (particularly fond of beetles and dragonflies at that time), his interests and knowledge grew up under the guidance of a local amateur naturalist, Milo Burlini, towards whom, on several occasions, he expressed his debt of gratitude (for a personal account of Sandro's career, see Minelli, 2009a). In 1963, at age 15, Minelli published his first scientific article, a short note on the first Italian record of a species of chrysomelid beetles (Minelli, 1963).

He graduated in Natural Sciences at the University of Padua in 1970, with a thesis on "A cybernetic approach to oriented movements in organism behaviour and morphogenesis", under the supervision of the distinguished Italian biologist Pietro Omodeo. He subsequently developed all his academic career at the same University, where he became Full Professor of Zoology in 1987. He retired in 2011. Retirement, however, was not the end of his scientific activity, as testified by nearly 40 articles in international academic journals, six authored or edited scientific books and several other publications of different kinds (editorials, book reviews, letters, book chapters, encyclopaedia articles, etc.), for a total of nearly 200 publications since then.

His research activity can be roughly divided into two periods. A first one, until the mid-1990s, was dominated by his interest in the principles and methods of biological systematics (Minelli, 1993) and by his work in modern evolutionary taxonomy, including phylogenetics, with a specific focus on arthropods, in particular myriapods. From mid-1990s onwards, his main interest turned towards evolutionary developmental biology (evo-devo), with special regard to the origin and evolution of animal body architectures (Minelli, 2003, 2009b) and a focus on development and evolution of serial structures, such as arthropod segments and appendages. Very recently, he has also ventured into the evo-devo of plants (Minelli, 2018).

As a taxonomist, he described species and revised the classification of taxa at different levels in planarians, leeches, beetles, and, much more extensively, myriapods, in particular geophilomorph centipedes, for which he became a reference specialist for the international community (Minelli, 2011, 2015). He also directed the creation of the world reference Global Taxonomic Database for the Chilopoda (*ChiloBase*, first release 2006). Beyond myriapodology, in the wider context of animal taxonomy and faunistics, he was chief editor in the publication of a complete checklist of the species of the Italian fauna (Minelli *et al.*, 1993-1995), which was the first complete animal checklist of a country in the world. He was member (1988-2013) and the President (1996-2001) of the *International Commission on Zoological Nomenclature* (*ICZN*) and in this capacity he coordinated the drafting of the fourth edition of the *International Code of Zoological Nomenclature* (1999), which is still in force. He is currently a member of the *Comitato Scientifico per la Fauna d'Italia* of which he served as President from 1991 to 1998.

As an evolutionary biologist, his main contributions relate to the origin and evolution of animal body architecture (including body axes, tagmosis and segmentation), the periodization of development (in particular postembryonic) and its evolution. These questions have been mostly investigated through the comparative method applied both to morphology and morphological development. Pivotal principles in his research are an avoidance of typological thinking, a disbelief in the application of excessively rigid categories to biological phenomena, and the rejection of adultocentrism and finalism in development and evolution. Among his original conceptual contributions we can mention those of *combinatorial homology*, the idea that homology is not an all-or-nothing relation, but rather a complex and multidimensional one (Minelli, 1998; Minelli and Fusco, 2013), *axis paramorphism*, the hypothesis that body appendages, such as arthropod and vertebrate limbs and chordate tails, are evolutionarily divergent duplicates (paramorphs) of the main body axis (Minelli 2000, 2003), and *temporal phenotype*, to formally incorporate the often overlooked variation in phenology in modelling the evolution of organismal life cycles (Minelli and Fusco, 2012; Minelli, 2018). He was founding member (1987) and vice-president (1997-99) of the *European Society for Evolutionary Biology* and in 2006 he organized the first European Workshop on Evolutionary Developmental Biology. From 2014 to 2017 he was Editor-in-chief of the section Evolutionary Developmental Biology in the journal *Frontiers in Ecology and Evolution* and still figures in the editorial board of several academic journals. He organized five editions (2009-2017) of the biennial Venice Summer School on Evolutionary Developmental Biology, devoted to the conceptual foundations of evo-devo.

Alessandro Minelli is a very prolific biologist. Up today (20.12.2018) the list of his publications includes 1125 titles. Beyond obvious professional products of his scientific activity (articles in scientific journals, contributed chapters in scientific books, scientific books; see Appendix) the list includes numerous edited monographs, book prefaces, articles in newspapers and popular journals, popular scientific books, book translations, book reviews and even children's books on wildlife.

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Appendix

Alessandro Minelli's selected international publications

Books as an author

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Dedicated to Alessandro Minelli, distinguished zoologist and evolutionary biologist, this essay collection spans a wide range of approaches to the study of development and evolution, especially, but not exclusively, at their fertile interface, evolutionary developmental biology (or, evo-devo). Discussed topics include the analysis of the development and evolution of specific features in plants and animals, phylogenetic inference, historical and philosophical revision of key aspects of the disciplines, theoretical elaborations on fundamental notions, and conceptual modelling of developmental and evolutionary dynamics. While being mainly aimed at professional researchers in the field, the book could also provide material for discussion groups in undergraduate and graduate university courses.

ISBN 978-88-6938-140-9

€ 15,00