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REVIEW

The developmental hourglass model: a predictor of the basic body plan?

Naoki Irie[1,*] and Shigeru Kuratani[2,*]

ABSTRACT

The hourglass model of embryonic evolution predicts an hourglass-like divergence during animal embryogenesis – with embryos being more divergent at the earliest and latest stages but conserved during a mid-embryonic (phylotypic) period that serves as a source of the basic body plan for animals within a phylum. Morphological observations have suggested hourglass-like divergence in various vertebrate and invertebrate groups, and recent molecular data support this model. However, further investigation is required to determine whether the phylotypic period represents a basic body plan for each animal phylum, and whether this principle might apply at higher taxonomic levels. Here, we discuss the relationship between the basic body plan and the phylotypic stage, and address the possible mechanisms that underlie hourglass-like divergence.

KEY WORDS: Body plan, Evo-devo, Evolution, Hourglass model, Phylotype, Transcriptome

Introduction

Animals that belong to the same phylum or to the same (usually large) phylogenetic group are considered to share a basic body plan (Jane et al., 2013; Wallace, 2000), which is key to grouping different animal species together (see Box 1). For example, despite the various niches inhabited (aquatic, terrestrial and aerial environments) and the over 1000-fold difference in body size [the smallest vertebrate is a frog less than 1 cm long (Rittmeyer et al., 2012) and the largest is the 30 m blue whale], all vertebrates share a set of anatomical features, such as a dorsally located central nervous system, segmented trunk muscles, vertebrae along the anteroposterior axis, a complex head with sensory organs (eyes, inner ear and nose), and organs such as the brain, heart and liver (Benton, 2004). Similarly, various other animal groups are also said to have a conserved basic body plan within their phyla. But what underlies this conservation across evolution? Why should basic anatomical features but not, for example, body mass or colour, be conserved across evolution? Is this anatomical conservation inevitable?

Why is the body plan conserved?

Considering hundreds of millions of years of animal evolution, with highly variable interactions between organisms and their environment, it is not easy to explain why the basic body plan is conserved within each phylum. The concept that the basic body plan arises from a set of conserved morphological elements found at a specific stage of embryogenesis (Fig. 1A,B) was suggested by classical morphological studies, and has recently been lent support by molecular analyses. Various concepts have been advanced to explain why specific embryonic patterns should be conserved (Fig. 1C). For example, the idea of 'developmental constraint' argues that the nature of the developmental process imposes certain limitations on phenotypic variability, leading to evolutionarily conserved phenotypes (Maynard Smith et al., 1985). Similarly, the concept of 'developmental burden' (Riedl, 1978) suggests that, because developmental processes are hierarchically organized and interdependent (e.g. somite segmentation requires the prior establishment of the anteroposterior axis), strong conservation is inevitable (discussed further below). As Garstang proposed in his 'stepping-stone model' (Garstang, 1922; Holland, 2011), we often see that specific embryonic structures at one developmental stage require structures or developmental processes that arise or occur at an earlier stage. This type of dependency could have been a force that ensured conservation of embryonic structure.

Although these concepts have been around for several decades, no consensus has been reached as to whether these mechanisms actually take place to conserve basic body plans. Meanwhile, recent analyses at the genomic and transcriptomic level are providing new insights into the problem, identifying the conserved mid-embryonic period from a molecular perspective and allowing scientists to extract shared morphological patterns that are conserved in this molecularly identified period, which may perhaps serve as a source of the basic body plan. In this Review, we first introduce the current models in an attempt to lay out a generalized relationship between animal development and evolution – focussing particularly the 'developmental hourglass model' (Fig. 2; discussed further below) that potentially explains the conservation of animal body plans. We then evaluate the predictions of and questions raised by the hourglass model, especially in terms of how a basic animal body plan can be explained on the basis of this model.

Conservation of embryonic development

Despite their widely divergent final appearance, all vertebrates go through a broadly similar set of developmental stages, starting from a single-celled fertilized egg, proceeding through broadly

Box 1. Basic body plan and the phylotypic period

Basic body plan has been defined as 'an assemblage of morphological features shared among many members of a phylum-level group' (Valentine, 2004). Chordate basic body plan, for example, includes anatomical features such as notochord, pharyngeal gill slits, brain, dorsal hollow nerve cord and post-anal tail. For unknown reasons, body size, coat colour or body weight are not well conserved in phylum-level animal groups, and are much less commonly characterized as morphological features of basic body plans. The developmental hourglass model hypothesizes that basic body plans are established at the most conserved embryonic period, or phylotypic period – which occurs during mid-embryogenesis (see also Fig. 2).

[1]Department of Biological Sciences, School of Science, University of Tokyo, Tokyo 113-0033, Japan. [2]Group for Morphological Evolution, RIKEN Center for Developmental Biology, Kobe 650-0047, Japan.

*Authors for correspondence (irie@bs.s.u-tokyo.ac.jp; saizo@cdb.riken.jp)

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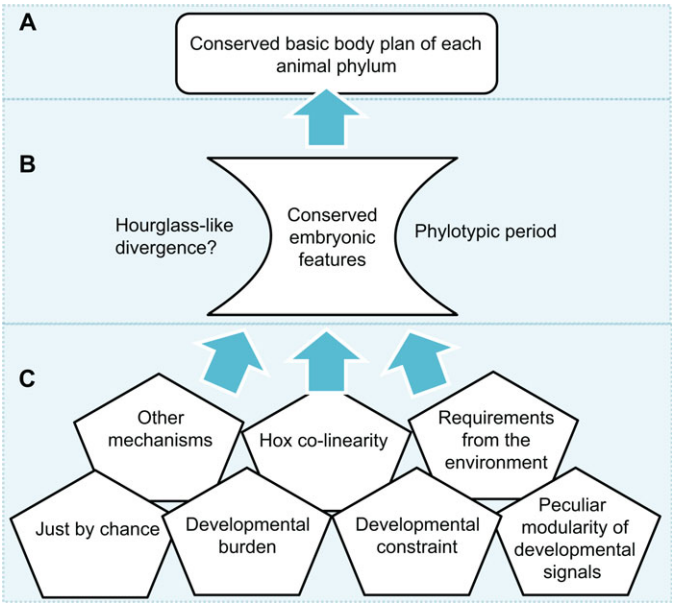

Fig 1. The logic underlying the hourglass model-predicted body plan hypothesis. (A) The basic body plan is conserved within each animal phylum but no consensus has been reached as to how or why this is the case. (B) Of the various possible reasons that may explain the shared basic body plan, the contribution of the conserved embryonic pattern is now gaining interest due to studies that support the developmental hourglass model. In brief, a set of conserved, anatomical features found at the mid-embryonic period contribute to the basic body plan of a given animal phylum (or other taxonomic group). However, it is largely unknown why the mid-embryonic period should be conserved. (C) Many hypotheses have been proposed, which may not be mutually exclusive, but no consensual empirical evidence has been obtained.

equivalent blastula, gastrula, neurula and pharyngular stages, before later stages define the complex anatomical structures that lead to the adult form.

Early conservation models

During the 19th century, von Baer proposed a parallel relationship between the morphological features observed during embryogenesis and a hierarchical structure of taxonomy – noting particularly that earlier embryos show features shared by a larger animal group (von Baer, 1828). Haeckel, however, proposed that embryogenesis is a quick replay of evolutionary history (Haeckel, 1866), a view popularized as 'ontogeny recapitulates phylogeny' and one with which von Baer, who did not accept the concept of evolution, strongly disagreed. Although these classical concepts are no longer accepted in modern biology (Gould, 1977), some aspects of these ideas have persisted and are recognized as part of an 'early conservation model' or 'funnel model' (Rasmussen, 1987; Riedl, 1978; von Baer, 1828; Wallace, 1984; Wimsatt, 1986). In brief, this model predicts that the earlier stages of embryogenesis reflect a more ancestral state – with more conserved features – than later stages. A possible underlying mechanism for this early stage conservation is developmental burden (Riedl, 1978), as mentioned above.

Developmental burden suggests that all developmental patterns are dependent on preceding developmental processes, and therefore that more-conserved patterns and mechanisms should be found in the earlier stages of development. Similarly, the idea of 'generative entrenchment' predicts early stage conservation in development (Wimsatt, 1986). In brief, generative entrenchment proposes that an upstream element responsible for generating downstream information tends to be evolutionarily conserved, because changes to this upstream information result in major developmental abnormalities; thus, it has less probability of being changed (entrenched). Both ideas are based on the fact that the later stages of embryogenesis are dependent on basic information established during earlier stages, through a causal relationship, and further imply that earlier stages of embryogenesis are difficult to change. However, these proposals have been difficult to investigate empirically.

The hourglass model

In the 1990s, based on the observation that early embryonic processes (e.g. cleavage and gastrulation) of different vertebrates are rather divergent, as well as the discovery of conserved Hox cluster gene expression along the anteroposterior axis of animal embryos, Duboule (1994) proposed the developmental hourglass model (Fig. 2). This model argued for evolutionarily diverged early and late stages, with an intermediate period of embryogenesis being most conserved. A similar concept was also proposed by Raff (1996). The hourglass model predicts that the most conserved embryonic stage of animal phyla (Duboule, 1994; Raff, 1996) is not the earliest, fertilized egg stage, but a mid-embryonic period called the 'phylotypic period', during which the common anatomical features of the basic body plan are defined (see Box 1). In addition, these seminal papers also proposed possible evolutionary mechanisms that could explain the hourglass-like pattern of conservation during embryogenesis. Duboule (1994) proposed that the spatial and temporal co-linearity of Hox cluster gene expression would make fundamental changes to the organization along the embryonic anteroposterior axis unlikely, further leading to body plan conservation. Meanwhile, Raff (1996) proposed that the highly inter-dependent molecular signalling among developmental modules in the mid-embryonic stages makes this period developmentally constrained, thus leading to evolutionary conservation (Fig. 2B). In brief, these two hypothetical mechanisms (which currently lack empirical verification) attribute mid-embryonic conservation to the fundamentally important nature of the developmental system itself; changes in the molecular network during this mid-embryonic period could have fatal consequences, thus leading to evolutionary conservation.

In response to the proposal of the hourglass model, the quest to uncover the relationship between development and evolution was renewed. Some researchers proposed different models for different animal groups (Salazar-Ciudad, 2010), whereas others tested possible models by evaluating evolutionary divergence during embryogenesis based on quantitative measurements of morphologically homologous traits of various vertebrate embryos (Bininda-Emonds et al., 2003; Poe, 2006; Poe and Wake, 2004; Richardson et al., 1997, 1998; Richardson and Keuck, 2002; Galis and Metz, 2001; Hall, 1997). However, these analyses have proved inconclusive, and it has remained controversial whether the hourglass model holds.

A number of alternatives have been proposed. For example, based on comparative morphological analyses, Richardson et al. suggested an alternative model, the 'adaptive penetrance model', that questions the existence of a conserved mid-embryonic stage (Richardson et al., 1997). The model attributes the highly divergent mid-embryonic, organogenesis stages to a higher tendency for beneficial mutations, because this is the period when the basic body plan is established and when there is the potential to generate adult innovation. Others have proposed an 'ontogenetic adjacency model' that does not assume a temporal difference of evolutionary conservation during development (Poe and Wake, 2004). Based on the observed lack of trends in heterochronic changes during embryogenesis, the authors suggest that 'evolutionary change is easier between ontogenetically adjacent events'.

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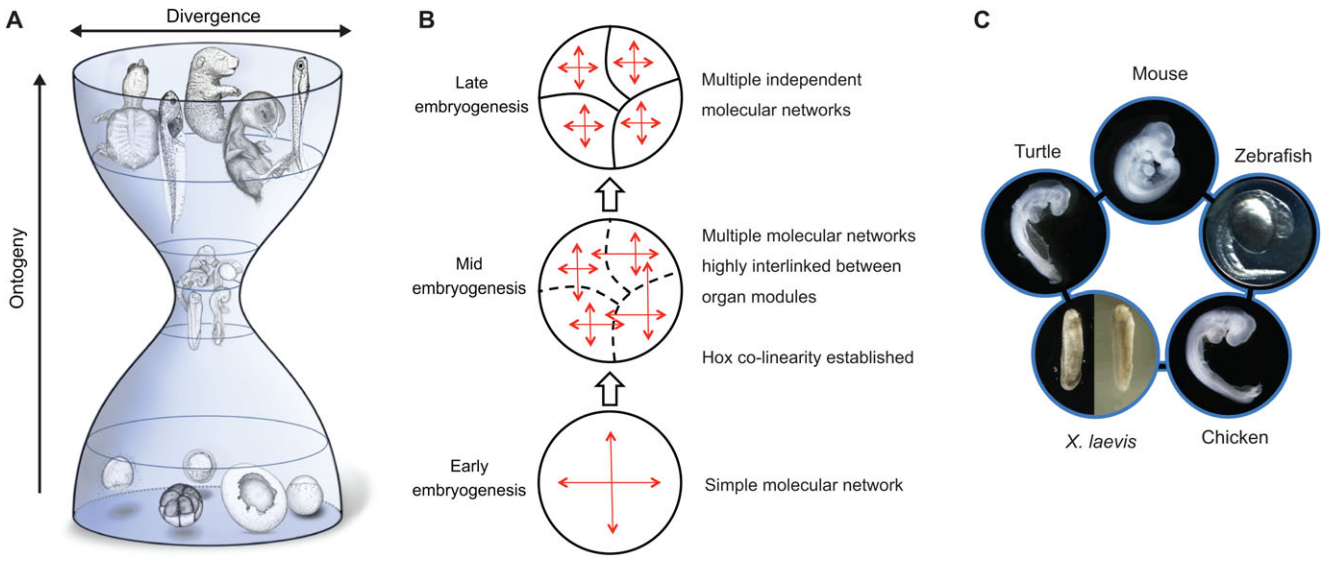

Fig 2. The developmental hourglass model. (A) The developmental hourglass model predicts that mid-embryonic organogenesis stages (phylotypic period) represent the period of highest conservation, and that the phylotypic period is the source of the basic body plan at a phylum level. Adapted, with permission, from Wang et al. (2013). (B) Hourglass-like divergence has been proposed to result from the spatial temporal co-linearity of Hox cluster genes (Duboule, 1994), from the existence of highly interdependent molecular networks at the phylotypic stage (Raff, 1996). (C) Potential phylotypic period for vertebrates. Two stages of *X. laevis* are shown, as there was no statistically significant difference between these two stages. Adapted, with permission, from Wang et al. (2013).

The confusion relating to the timing of evolutionary divergence is primarily associated with the difficulty in using morphological characteristics to evaluate quantitatively the degree of conservation at each developmental stage given qualitatively different morphological features. For example, although one can quantitate differences in, for example, blastomere number at an early stage there is no method for quantitatively comparing this variation against the variations of somite number at a later stage. Meanwhile, some researchers have tested the models by investigating developmental features that may potentially result in certain evolutionary conservation (e.g. Raff proposed that the phylotypic period of the hourglass model arises because the organogenesis period is constrained, with changes during this period tending to result in a lethal or less adaptive phenotype). In concordance with this viewpoint, some researchers have tested the models by reviewing the studies that applied teratogens to rodent embryos (reviewed by Galis and Metz, 2001), to identify the stages that are particularly sensitive to these treatments. They identified enhanced sensitivity (in terms of a higher frequency of abnormality of lethality) to teratogens during the organogenesis stages. However, it is not surprising that teratogens cause more malformations during the period of organogenesis because these agents are, by definition, chemicals that cause abnormal organogenesis. Therefore, it remains to be clarified whether the organogenesis stages are actually most susceptible to failure or lethality due to genetic mutations, and whether this can cause an hourglass-like divergence in evolutionary timescale. Similarly, Roux and Robinson-Rechavi pointed out that genes expressed in early stages are often indispensable (Roux and Robinson-Rechavi, 2008); however, the data were not comprehensive and, as argued by Kalinka and Tomancak (2012), loss-of-function analysis does not address the issue of evolvability of different periods of development.

Testing models with molecular approaches

Since the models discussed above were introduced to explain the divergence of embryos over hundreds of millions of years (Myr) of evolutionary time (~550 Myr ago for vertebrates), clarifying the possible evolutionary mechanism underlying embryonic body plan conservation is far more challenging than evaluating the degree of conservation. Traditionally, studies assessing divergence/conservation have used morphological approaches, but the rise of sequence-based analysis for evolutionary studies, based on genomic or transcriptomic data, has provided a new tool for researchers in this field. Thus far, and not surprisingly given the challenges mentioned above, most molecular studies have focused on evaluating the divergent or conserved nature of embryos by quantitatively measuring expression profiles of genes during animal embryogenesis.

Pioneering studies took advantage of expression profiles from single species (Hazkani-Covo et al., 2005; Irie and Sehara-Fujisawa, 2007), and more recently from multiple species to make cross-species comparisons of expression profiles (Kalinka et al., 2010; Irie and Kuratani, 2011; Levin et al., 2012; Yanai et al., 2011; Wang et al., 2013; Schep and Adryan, 2013). Measuring orthologous gene expression profiles from whole embryonic RNA samples discards all the morphological information, but the measured expression similarity between samples can be regarded as an index that reflects the degree of similarity in cellular composition between embryos. Therefore, it is potentially a useful alternative approach to identify conserved embryonic stages. Moreover, such molecular studies seem to have some advantages over morphological approaches because they more directly assess the inherited entities (the DNA sequences and transcribed information). This is similar to what has happened in the field of phylogenetics, where molecular phylogeny now dominates morphology-based data.

The pioneering molecular studies (Hazkani-Covo et al., 2005; Irie and Sehara-Fujisawa, 2007; Artieri et al., 2009; Cruickshank and Wade, 2008) had two major limitations. First, they were based on a limited number of genes [e.g. they used EST (expressed sequence tag) data]. Second, their evaluation was largely based on the sequence conservation of expressed genes in single species, such as mice (Hazkani-Covo et al., 2005; Irie and Sehara-Fujisawa, 2007) or *Drosophila* (Artieri et al., 2009; Cruickshank and Wade, 2008), rather than incorporating data from multiple species. It is important to bear in mind that these experimental animals were

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selected as models because they share useful features, such as a short generation period and quick developmental time, and that this may represent a highly derived mechanism of development compared with other animals – thus potentially having limited use for evolutionary studies (Hall, 1997). Rapid advancements in technology, such as microarray and massively parallel sequencing, have addressed the first limitation mentioned above. However, the second limitation still applies to many studies (Domazet-Loso and Tautz, 2010; Piasecka et al., 2013; Quint et al., 2012; Roux and Robinson-Rechavi, 2008). Although analysing the expression profiles of single species [e.g. using the ancestor index (Irie and Sehara-Fujisawa, 2007) and the transcriptome age index or TAI (Domazet-Loso and Tautz, 2010)] does not directly identify whether the evolutionarily equivalent genetic program is working in other species, these studies have stimulated debate on this long-standing problem. Many of these single-species-based expression analyses show that the expression profiles at mid-embryogenesis stages have the highest ratio of sequence-conserved genes, thus supporting the hourglass model (Cruickshank and Wade, 2008; Domazet-Loso and Tautz, 2010; Hazkani-Covo et al., 2005; Irie and Sehara-Fujisawa, 2007; Quint et al., 2012). However, some studies favour the funnel model (Artieri et al., 2009; Roux and Robinson-Rechavi, 2008), and the authors of these studies have proposed a co-existence model using zebrafish that incorporates both the hourglass model for morphological variations and the funnel model for molecular processes (Comte et al., 2010; Piasecka et al., 2013). It should be noted, however, that one of the most detailed morphological studies on vertebrate embryos is not consistent with the hourglass model (Bininda-Emonds et al., 2003).

Considering that the estimation of evolutionary conservation using extant animal taxa largely relies on the observation of shared features between different species, it is essential to include cross-species evaluation of shared gene expression in these types of molecular studies. Ancestor Index (Irie and Sehara-Fujisawa, 2007) and TAI (Domazet-Loso and Tautz, 2010) analyses, on the other hand, can evaluate only the abundance of sequence-conserved genes in embryos of each species, and these do not necessarily tell us whether the evolutionarily equivalent genetic program is commonly taking place among different species. More recently, a number of studies have included cross-species comparisons of orthologous gene expression (Table 1). In general, these have supported hourglass-like divergence, with the most conserved expression profile at mid-embryonic stages. Importantly, these cross-species studies clearly indicated that hourglass-like divergence can be found irrespective of platform (microarray or massively parallel sequencers), normalizations and signal calculations, and similarity calculation methods. However, the diversity of measurements used also means that consensus has yet to be reached. Given that these robust studies included cross-species comparisons of shared expression of orthologous genes, it seems reasonable to accept that hourglass-like divergence, which is a prerequisite for the developmental hourglass model, can be observed in a variety of animal species.

Table 1. Summary of cross-species transcriptome comparisons that test the hourglass model

Compared species (groups)	Divergence time (Myr ago)	Reference
Five nematode species	30*	Levin et al., 2012
Six *Drosophila* species	40‡	Kalinka et al., 2010
X. tropicalis and *X. laevis*	50-81§	Yanai et al., 2011
P. sinensis (turtle) and chicken	248-268¶	Wang et al., 2013
A. gambiae and *Drosophila*	240**	Schep and Adryan, 2013
X. tropicalis and *D. rerio*	476‡‡	Schep and Adryan, 2013
Mouse, chicken, *X. laevis* and *D. rerio*	476‡‡	Irie and Kuratani, 2011
Mouse, chicken, *Xenopus*, *D. rerio* and *A. gambiae*	993§§	Irie and Kuratani, 2011
X. tropicalis and *C. elegans*	1177§§	Levin et al., 2012

*Cutter, 2008; ‡Kalinka et al., 2010; §Evans et al., 2004; ¶Wang et al., 2013; **You et al., 2013; ‡‡Blair and Hedges, 2005; §§Benton and Ayala, 2003.

The hourglass model and the basic body plan

A discussed above, most available data support a model in which animal embryogenesis shows hourglass-like divergence. Given this premise, an important question to ask is which embryonic stages can be regarded as the 'bottleneck' of the hourglass, or the most conserved or representing the phylotypic period? This is because the essential prediction of the hourglass model resides at the bottleneck stage – the phylotypic period, during which the body plan for vertebrates (Duboule, 1994) and each phylum (Raff, 1996) is expected to be established. Three major issues must be resolved to verify this prediction. First, assuming that the phylotypic period represents the basic body plan for each phylum, what morphological elements can be found during this period, and how can we define the basic body plan from the embryonic point of view? To solve this issue, studies based on sufficient coverage of phylum-wide species must be carried out. Second, we still do not know the range of species or phylogenetic groups, that can be explained by this model: does it apply at the level of the phylum, as was initially proposed, or can it explain conservation within smaller or larger taxonomic groups? Third, what mechanisms might the conservation during embryogenesis predicted by the hourglass model. Below, we discuss each of these challenges in turn.

The morphological features of the phylotypic period

Comparing embryonic expression profiles among six *Drosophila* species that split 40 Myr ago, Kalinka et al. (2010) found that the extended germband stage has the most conserved expression profile; this stage is generally considered to be the arthropod phylotypic period (Sander, 1976). Meanwhile, by comparing *D. melanogaster* and *A. gambiae* and using expression analysis limited to transcription factor-coding genes, Schep and Adryan (2013) reported that there are two periods of high conservation peaks between these species, one at the extended germband stages as Kalinka et al. reported, and the other at a later stage (stage 17 in *Drosophila*). *Drosophila* embryos at this later stage have a more-complex set of features than at the extended germband stage: they have undergone head involution and various structures – including the atrium, the ventral nerve cord, the proventriculus, the hindgut and the posterior spiracles – have formed (Campos-Ortega and Hartenstein, 2013). Which stage could be regarded as the potential phylotypic period for arthropods? In other words, which of these stages contain the morphological elements that explain the three primary features of the arthropod body plan (Jane et al., 2011): anteroposteriorly segmented structures, the external skeleton and articulated limbs? Given that imaginal discs that later become adult limbs already exist at the extended germband stage, and segmented trunk of this stage contributes to adult segments, it can be said that two out of three features of the arthropod body plan can be traced back to an extended germband stage. However, the other key feature of the arthropod body plan, the external skeleton,

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cannot be explained by morphological elements found at either of these stages. Cells that later produce external skeleton at these embryonic stages contribute to the exoskeleton of only the pupa, rather than that of the adult. However, both these studies have limitations: the first (Kalinka et al., 2010) was based on a shallower degree of evolutionary depth (only 40 Myr since the *Drosophila* species diverged, compared with 250 Myr since *Drosophila* and *Anopheles* diverged), while the latter (Schep and Adryan, 2013) was based on only a subset of genes – transcription factors. Therefore, a more comprehensive study is warranted to cover the arthropod phylum.

The situation is similar for studies on chordate species. By using the gene expression profiles of four vertebrate species (mouse, chicken, *X. laevis* and zebrafish) that separated 400 Myr ago, we previously identified the pharyngular embryo as a potential vertebrate phylotypic period (Irie and Kuratani, 2011). This was achieved by making all-to-all comparisons of the developmental stages from each species and identifying the most conserved set of embryonic stages from the four vertebrates in terms of expression profile similarity. More recently, we have added another vertebrate species, the Chinese soft-shell turtle (*Pelodiscus sinensis*), to the analysis (Wang et al., 2013). Although the turtle has a very different body plan to that of other vertebrates (Nagashima et al., 2009), we found that the most conserved embryonic period between turtle and chicken in terms of expression profiles matched the same vertebrate phylotypic period that had been identified previously (Irie and Kuratani, 2011) (Fig. 2C).

Do these stages share the anatomical features that can explain the basic body plan of the vertebrates, or chordates, as predicted by the hourglass model? Based on the hypothetical form of the vertebrates' common ancestor deduced from paleontological studies (Benton, 2004), the shared morphological features of vertebrates that constitute the basic body plan can be defined as: the notochord; dorsal nerve cord; head with nostril, eye, ear, muscle blocks, horny teeth and pharynx with slits; trunk with the heart, liver, stomach, gonad and kidney; and tail with anus. Almost all of these features, or at least organ primordia that later give rise to most of these features, can be found in the identified phylotypic period, except for horny teeth. However, as in studies with arthropods, we have to admit that this result may not be conclusive, as these studies did not include early diverged vertebrates such as cyclostomes (lamprey and hagfish). The features found at the identified phylotypic period of the vertebrates are much more complex than the defined key elements of the chordate body plan: the notochord, pharyngeal gill slits, brain, dorsal hollow nerve cord and post-anal tail (Jane et al., 2011). One might therefore imagine that including invertebrate chordate species in the analysis would shift the most conserved stage to an earlier phase of development, thus defining a different phylotypic period for chordates than for vertebrates. Obviously, further studies with more species are needed to investigate this apparent discrepancy.

As described above, it seems too early to conclude whether the anatomical elements found in the most molecularly conserved embryonic stages of arthropods and chordates correspond to the basic body plans of the group concerned, and two issues need to be addressed to test this phylotypic period hypothesis. The first is that the number of species covered by these studies is still not broad enough to cover phylum-wide animals; the second is that the developmental stages investigated in these studies are not comprehensive. For example, none of the studies listed above that focused on identifying the arthropod phylotypic period covered stages later than the embryonic period, such as metamorphosis stages. Thus, there still remains the possibility that even later stages show the most conserved expression profiles. Addressing both these issues is feasible with current technologies, and should shed significant light on the degree to which the developmental hourglass model can explain evolutionary conservation.

Phylogenetic units and the hourglass model

Can the hourglass model be applied to groups of animals that are wider, or narrower, than the phylum? Although more than 30 animal phyla are known to exist (Carroll et al., 2001), only three phyla (chordates, arthropods and nematodes) have been the focus of cross-phylum molecular studies during embryogenesis (see also Table 1). Levin et al. performed a cross-phylum comparison by using embryonic expression profiles of *C. elegans* and *X. tropicalis* (Levin et al., 2012), and reported that the ventral enclosure stage of *C. elegans* and the *X. tropicalis* tailbud stage showed the highest expression similarity, further indicating that the phylotypic period overlaps with the stage when the body plan for the whole animal is established, as has been hypothesized Slack et al. (1993). Our previous study also indicated a similar implication based on the finding that the segmentation stage of *A. gambiae* showed the highest similarity with the expression profiles of mid-embryonic (around gastrula to organogenesis) stages of four vertebrate species (mouse, chicken, *X. laevis* and zebrafish; Irie and Kuratani, 2011). These studies, together with attempts to identify conserved molecular modules (Gerstein, 2014), may provide a way to investigate what molecular and morphological features the urbilaterian ancestor possessed (Hejnol and Martindale, 2008), though the number of species studied so far is limited and further investigation with a broader range of animals is needed.

With respect to the experimentally identified conserved period of the hourglass, it is of particular interest that these organogenesis periods (i.e. the pharyngular stage for vertebrates) were found to be maximally conserved irrespective of the phylogenetical distance of the species being compared. For example, the most molecularly conserved period found in *X. laevis* when compared with three other vertebrates (mouse, chicken and zebrafish) was the pharyngular stage (Irie and Kuratani, 2011); this stage (the tailbud stage of *Xenopus* – stages 28-31) was also found to be most conserved when compared against the phylogenetically closer species *X. tropicalis* (Yanai et al. (2011). Why should this be? The observation suggests that the anatomical features of the most conserved embryonic stages do not necessarily reflect shared, adult anatomical features of the species being compared. In other words, anatomical features of the conserved embryos always show features of the body plan of that phylum. In fact, the most highly conserved stage identified in various comparisons between tetrapods is, as discussed above, the pharyngular stage (Irie and Kuratani, 2011; Wang et al., 2013) – before limbs develop. This is despite the fact that the adults forms of all embryos compared have limbs. Importantly, this tendency – so-called 'persistent conservation' – was also observed in other phyla, nematodes and arthropoda. The highest conservation among nematode species was at the ventral enclosure stages, and these stages also showed the highest expression similarity to the *X. laevis* tailbud stages (Levin et al., 2012). Similarly, the highest conservation between fly species was at the extended germband stage, showing much simpler morphological features than those commonly found in *Drosophila* adults. In the next section, we will further discuss this 'persistent conservation' in the context of underlying mechanism for the hourglass model.

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Evolutionary mechanisms underlying the hourglass model

Despite the recent advances in the field, the issue of why hourglass-like divergence is observed remains unresolved. Some researchers have proposed that divergence found in early and late embryonic stages is the result of adaptation to particular types of reproductive strategy (Slack et al., 1993), or to diverse ecological niches (Kalinka and Tomancak, 2012); these imply that the phylotypic period is just in between diversifying stages, and is a 'period of calm' with much less selective pressure. However, considering rapid neutral evolution, it would be interesting to determine why the phylotypic period has remained both molecularly and morphologically conserved after hundreds of million of years of evolution. Moreover, it would be interesting to know how animals could have tolerated changes in early developmental stages while conserving the phylotypic period (discussed by Irie and Kuratani, 2011). Similarly, we still do not know why the most conserved period should be the organogenesis phase. In contrast to ideas proposed by Slack et al., and as mentioned above, Duboule and Raff ascribed this conservation to characteristics of embryogenesis:, Duboule (1994) attributed the conservation of the phylotypic period to the co-linearity of Hox cluster gene expression, whereas Raff (1996) attributed it to the particularly complex signalling modularity within organ primordia found in the phylotypic period (Raff, 1996).

In accordance with this, the 'persistent conservation' of the potential phylotypic period irrespective of the species being compared suggest that evolutionary diversification of this embryonic period has been strictly limited throughout the hundreds of millions of years of evolution, and that this constraint might still apply extant animal embryos. In this context, it would be interesting to see whether the variations among inbred strains, natural populations and genetically identical individuals are also the smallest during the phylotypic period.

If the intrinsic characteristics of embryogenesis are indeed the main reason for this persistent conservation of the phylotypic period, two opposite possibilities can still be considered: fragility or robustness of phylotypic period embryos. For example, hypotheses proposed by Duboule and Raff suppose the fragility, or a limited flexibility, of developmental systems during the mid-embryonic period – such that no species could withstand genetic mutations that lead to drastic changes in the phylotypic period. As mentioned above, studies reviewed by Galis and Metz (2001) also seem to support this idea: organogenesis stages of rodents were prone to die when treated with teratogen, although there are caveats to these studies. The other possibility is that the phylotypic period is 'robust' against induced changes, thereby conserving molecular and morphological features, as proposed, for example, for the segment polarity network (von Dassow et al., 2000). Mechanistically, such robustness could potentially be implemented by a capacitor such as heat-shock protein HSP90 (Rohner et al., 2013), which buffers the effects of mutation-induced misfolding of proteins and eventually masks the abnormal phenotype. No empirical evidence has been obtained so far, however, and quantitative measurements of the fragility/robustness of phylotypic stage embryos in various animals would provide insights to this problem.

Conclusions and future perspectives

In conclusion, although recent molecular studies have demonstrated hourglass-like divergence in various animal species, and the identified bottleneck periods – the potential phylotypic periods – show morphological similarities across species, it is still perhaps premature to conclude that the phylotypic period really represents the body plan for a given animal group. The range of species studied, and the developmental stages covered, are limited; further analysis is required to fill these gaps. The observed 'persistent conservation' of the phylotypic period, as discussed above, suggests this period could conceivably reflect the basic body plan at a higher taxonomic level than the species being compared in any particular analysis, but whether or not the phylotypic periods represent a phylum-specific body plan is not yet clear. In addition, it is still not clear why embryonic evolution exhibits hourglass-like divergence, and this is an important issue that needs to be addressed. Not only do we not know whether hourglass-like divergence arises from developmental constraints, but we still have no effective tools to measure these developmental forces that may conserve embryonic patterns. Comprehensive measurements of embryonic gene expression profiles have fostered this field in recent years, but intervening experiments such as adding mutations and fluctuations to embryonic stages are required to test the concept, and to investigate the relationship between the robustness or fragility of embryos and evolutionary conservation. Answers to these problems should shed light on the issue of why no new animal phylum has appeared since the Cambrian explosion, and help us to better understand our own body plan and how it relates to our vertebrate relatives.

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Competing interests

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References

Artieri, C. G., Haerty, W. and Singh, R. S. (2009). Ontogeny and phylogeny: molecular signatures of selection, constraint, and temporal pleiotropy in the development of Drosophila. *BMC Biol.* **7**, 42.

Benton, J. M. (2004). In *Vertebrate Palaeontology*, 3rd edn. pp. 14. West Sussex, UK: Wiley.

Benton, J. M. and Ayala, F. J. (2003). Dating the tree of life. *Science* **300**, 1698-1700.

Bininda-Emonds, O. R. P., Jeffery, J. E. and Richardson, M. K. (2003). Inverting the hourglass: quantitative evidence against the phylotypic stage in vertebrate development. *Proc. R. Soc. Lond. B* **270**, 341-346.

Blair, J. E. and Hedges, S. B. (2005). Molecular phylogeny and divergence times of deuterostome animals. *Mol. Biol. Evol.* **22**, 2275-2284.

Campos-Ortega, J. A. and Hartenstein, V. (2013). *The Embryonic Development of Drosophila melanogaster*, 2nd edn. Heidelberg, Germany: Springer.

Caroll, B. S., Grenier, K. J. and Weatherbee, D. S. (2001). *From DNA to Diversity*. Oxford, UK: Blackwell Publishing.

Comte, A., Roux, J. and Robinson-Rechavi, M. (2010). Molecular signaling in zebrafish development and the vertebrate phylotypic period. *Evol. Dev.* **12**, 144-156.

Cruickshank, T. and Wade, M. J. (2008). Microevolutionary support for a developmental hourglass: gene expression patterns shape sequence variation and divergence in Drosophila. *Evol. Dev.* **10**, 583-590.

Cutter, A. D. (2008). Divergence times in Caenorhabditis and Drosophila inferred from direct estimates of the neutral mutation rate. *Mol. Biol. Evol.* **25**, 778-786.

Domazet-Lošo, T. and Tautz, D. (2010). A phylogenetically based transcriptome age index mirrors ontogenetic divergence patterns. *Nature* **468**, 815-818.

Duboule, D. (1994). Temporal colinearity and the phylotypic progression: a basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony. *Development* Suppl. 135-142.

Evans, B. J., Kelley, D. B., Tinsley, R. C., Melnick, D. J. and Cannatella, D. C. (2004). A mitochondrial DNA phylogeny of African clawed frogs: phylogeography and implications for polyploid evolution. *Mol. Phylogenet. Evol.* **33**, 197-213.

Galis, F. and Metz, J. A. J. (2001). Testing the vulnerability of the phylotypic stage: on modularity and evolutionary conservation. *J. Exp. Zool.* **291**, 195-204.

Garstang, W. (1922). The theory of recapitulation: a critical re-statement of the biogenetic law. *Linn. J. Zool.* **35**, 81-101.
Gerstein, M. B., Rozowsky, J., Yan, K. K., Wang, D., Cheng, C., Brown, J. B., Davis, C. A., Hillier, L., Sisu, C., Li, J. J., et al. (2014). Comparative analysis of the transcriptome across distant species. *Nature* **512**, 445-448.
Gould, J. S. (1977). *Ontogeny and Phylogeny*. Harvard University Press.
Haeckel, E. (1866). *Generelle Morphologie der Organismen*. *Allgemeine GrundzuXge der organischen Formen-Wissenschaft, mechanisch begruXndet durch die von Charles Darwin reformirte Descendenz-Theorie*. Berlin: Georg Reimer.
Hall, B. K. (1997). Phylotypic stage or phantom: is there a highly conserved embryonic stage in vertebrates? *Trends Ecol. Evol.* **12**, 461-463.
Hazkani-Covo, E., Wool, D. and Graur, D. (2005). In search of the vertebrate phylotypic stage: a molecular examination of the developmental hourglass model and von Baer's third law. *J. Exp. Zool. B Mol. Dev. Evol.* **304B**, 150-158.
Hejnol, A. and Martindale, M. Q. (2008). Acoel development supports a simple planula-like urbilaterian. *Phil. Trans. R. Soc. B* **363**, 1493-1501.
Holland, N. D. (2011). Walter Garstang: a retrospective. *Theory Biosci.* **130**, 247-258.
Irie, N. and Kuratani, S. (2011). Comparative transcriptome analysis reveals vertebrate phylotypic period during organogenesis. *Nat. Commun.* **2**, 248.
Irie, N. and Sehara-Fujisawa, A. (2007). The vertebrate phylotypic stage and an early bilaterian-related stage in mouse embryogenesis defined by genomic information. *BMC Biol.* **5**, 1.
Jane, B. R., Lisa, A. U., Michael, L. C., Steven, A. W., Robert, B. J., Peter, V. M. and Neil, A. C. (2011). *Campbell Biology*, 9th edn. Benjamin Cummings.
Jane, B. R., Lisa, A. U., Michael, L. C., Steven, A. W., Robert, B. J. and Peter, V. M. (2013). *Campbell Biology*, 10th edn. Benjamin Cummings.
Kalinka, A. T. and Tomancak, P. (2012). The evolution of early animal embryos: conservation or divergence? *Trends Ecol. Evol.* **27**, 385-393.
Kalinka, A. T., Varga, K. M., Gerrard, D. T., Preibisch, S., Corcoran, D. L., Jarrells, J., Ohler, U., Bergman, C. M. and Tomancak, P. (2010). Gene expression divergence recapitulates the developmental hourglass model. *Nature* **468**, 811-814.
Levin, M., Hashimshony, T., Wagner, F. and Yanai, I. (2012). Developmental milestones punctuate gene expression in the Caenorhabditis embryo. *Dev. Cell* **22**, 1101-1108.
Maynard Smith, J., Burian, R., Kauffman, S., Alberch, P., Campbell, J., Goodwin, B., Lande, R., Raup, D. and Wolpert, L. (1985). Developmental constraints and evolution: a perspective from the mountain lake conference on development and evolution. *Q. Rev. Biol.* **60**, 265-287.
Nagashima, H., Sugahara, F., Takechi, M., Ericsson, R., Kawashima-Ohya, Y., Narita, Y. and Kuratani, S. (2009). Evolution of the turtle body plan by the folding and creation of new muscle connections. *Science* **325**, 193-196.
Piasecka, B., Lichocki, P., Moretti, S., Bergmann, S. and Robinson-Rechavi, M. (2013). The hourglass and the early conservation models–co-existing patterns of developmental constraints in vertebrates. *PLoS Genet.* **9**, e1003476.
Poe, S. (2006). Test of Von Baer's law of the conservation of early development. *Evolution* **60**, 2239-2245.
Poe, S. and Wake, M. H. (2004). Quantitative tests of general models for the evolution of development. *Am. Nat.* **164**, 415-422.
Quint, M., Drost, H.-G., Gabel, A., Ullrich, K. K., Bönn, M. and Grosse, I. (2012). A transcriptomic hourglass in plant embryogenesis. *Nature* **490**, 98-101.
Raff, A. (1996). *The Shape of Life*: *Genes*, *Development*, *and the Evolution of Animal Form*. Chicago, IL: University of Chicago Press.
Rasmussen, N. (1987). A new model of developmental constraints as applied to the Drosophila system. *J. Theor. Biol.* **127**, 271-299.
Richardson, M. K. and Keuck, G. (2002). Haeckel's ABC of evolution and development. *Biol. Rev. Camb. Philos. Soc.* **77**, 495-528.
Richardson, M. K., Hanken, J., Gooneratne, M. L., Pieau, C., Raynaud, A., Selwood, L. and Wright, G. M. (1997). There is no highly conserved embryonic stage in the vertebrates: implications for current theories of evolution and development. *Anat. Embryol. (Berl.)* **196**, 91-106.
Richardson, M. K., Minelli, A., Coates, M. and Hanken, J. (1998). Phylotypic stage theory. *Trends Ecol. Evol.* **13**, 158.
Riedl, R. (1978). *Order in Living Organisms*. West Sussex, UK: Wiley-Interscience.
Rittmeyer, E. N., Allison, A., Gründler, M. C., Thompson, D. K. and Austin, C. C. (2012). Ecological guild evolution and the discovery of the world's smallest vertebrate. *PLoS ONE* **7**, e29797.
Rohner, N., Jarosz, D. F., Kowalko, J. E., Yoshizawa, M., Jeffery, W. R., Borowsky, R. L., Lindquist, S. and Tabin, C. J. (2013). Cryptic variation in morphological evolution: HSP90 as a capacitor for loss of eyes in cavefish. *Science* **342**, 1372-1375.
Roux, J. and Robinson-Rechavi, M. (2008). Developmental constraints on vertebrate genome evolution. *PLoS Genet.* **4**, e1000311.
Salazar-Ciudad, I. (2010). Morphological evolution and embryonic developmental diversity in metazoa. *Development* **137**, 531-539.
Sander, K. (1976). Specification of the basic body pattern in insect embryogenesis. *Adv. Insect Physiol.* **12**, 125-238.
Schep, A. N. and Adryan, B. (2013). A comparative analysis of transcription factor expression during metazoan embryonic development. *PLoS ONE* **8**, e66826.
Slack, J. M. W., Holland, P. W. H. and Graham, C. F. (1993). The zootype and the phylotypic stage. *Nature* **361**, 490-492.
Valentine, J. W. (2004). *On the Origin of Phyla*. Chicago, IL: University of Chicago Press.
von Baer, K. E. (1828). *Uber Entwickelungsgeschichte der Thiere*. Koenigsberg: Beobachtung und Reflektion.
von Dassow, G., Meir, E., Munro, E. M. and Odell, G. M. (2000). The segment polarity network is a robust developmental module. *Nature* **406**, 188-192.
Wallace, A. (1984). *Mechanisms of Morphological Evolution*. *A combined Genetic Development and Ecological Approach*. Chichester: John Wiley & Sons.
Wallace, A. (2000). *The Origin of Animal Body Plans: A Study in Evolutionary Developmental Biology*. Cambridge: Cambridge University Press.
Wang, Z., Pascual-Anaya, J., Zadissa, A., Li, W., Niimura, Y., Huang, Z., Li, C., White, S., Xiong, Z., Fang, D. et al. (2013). The draft genomes of soft-shell turtle and green sea turtle yield insights into the development and evolution of the turtle-specific body plan. *Nat. Genet.* **45**, 701-706.
Wimsatt, W. C. (1986). *Integrating Scientific Disciplines* (ed. P. W. Bechtel). Heidelberg, Germany: Springer.
Yanai, I., Peshkin, L., Jorgensen, P. and Kirschner, M. W. (2011). Mapping gene expression in two Xenopus species: evolutionary constraints and developmental flexibility. *Dev. Cell* **20**, 483-496.
You, M., Yue, Z., He, W., Yang, X., Yang, G., Xie, M., Zhan, D., Baxter, S. W., Vasseur, L., Gurr, G. M. et al. (2013). A heterozygous moth genome provides insights into herbivory and detoxification. *Nat. Genet.* **45**, 220-225.