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The evolution of Hox genes in Spiralia

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Abstract

The decoding of genomes of a larger number of animal species have provided further insights into the genomic Hox gene organization and with this indicated the evolutionary changes during the radiation of several clades. The expansion of gene expression studies during development and life history stages of more species, complete the picture of the relationship between cluster organisation and temporal and spatial correlation of the Hox activity. Now these results open the opportunity to look deeper into the regulatory pathways that form these patterns and identify what exact changes caused the evolution of the application of this iconical gene set for the evolution of new larval forms and new structures. Here we review recent progress of Hox gene related research in the large clade Spiralia, that comprises Annelida, Mollusca, Lophophorata, Platyhelminthes, Nemertea and others. Albeit their relationship to each other is not resolved yet, there are emerging patterns that indicate that Hox genes are mainly used for patterning late, adult body parts and that Hox genes are often not expressed on the larval stages. Hox genes seem also often recruited for the formation of morphological novelties. Together with the emerging genomic information Hox genes show a much more dynamic evolutionary history than previously assumed.

Keywords: hox genes; gene cluster; larva; evolutionary novelties; Spiralia; Lophotrochozoa; Annelida; Rotifera

Introduction

After thorough studies in arthropods (Ecdysozoa) and chordates (Deuterostomia), a better understanding of the evolution and function of Hox genes in Spiralia—one of the three major lineages of bilaterian animals—is beginning to emerge, qualifying, as we will demonstrate in this review, many of the previous assumptions about the roles of these iconic genes in animal development and evolution. Spiralia (or Lophotrochozoa) is a diverse and fascinating clade of animals that comprise an incredible diversity of animal forms, from tiny interstitial protostomes (e.g., gastrotrichs and rotifers) to many worm-shaped creatures (e.g., nemerteans, annelids) and shell bearing groups (mollusks and brachiopods). Their last common ancestor also gave rise to the impressive cephalopods, with a larger body plan and complex nervous systems. Spiralia is, however, keeping the secret of how this morphological, functional, and ecological diversity evolved, as well as the set of characters that were present in their last common ancestor. Given the conserved role of Hox genes in patterning animal body plans, it is thus tempting to study the role of these genes in Spiralia and the interplay between changes in the genomic organization, regulation and expression of these genes and the diversification of spiralian body plans. An extensive body of work has been conducted over the last two decades and the results are as diverse as the spiralian body plans themselves. While the first comparative studies between fruit flies and vertebrates offered a seemingly consistent pattern of Hox gene organization and role during development, the addition of an increasing number of spiralian species is delivering a complex, but captivating picture of the role of Hox genes in animal evolution. Yet the limited understanding of the internal phylogenetic relationships of Spiralia is also complicating this endeavor, sometimes hampering the identification of the direction of evolutionary change. Here, we summarize the current knowledge of and more recent findings on the evolution and roles of spiralian Hox genes, putting forward some evolutionary conclusions and working hypotheses that we hope might help clarify and advance the study of Hox genes in this and other animal groups.

Hox cluster evolution

The genomic arrangement of Hox genes in some animal lineages is intimately connected to the temporal and spatial expression of these genes during embryogenesis—and is known as temporal and spatial collinearity [1-4]. The genomic organization and chromatin 3D architecture of the Hox genes influences their expression during development. Changes in the complement and genomic linkage of these genes, as well as deviation from a temporal/spatial collinearity correlate with morphological and developmental evolution in animals [5-9]. The advent of more affordable, high throughput sequencing technologies has facilitated genomic investigations in a broader taxonomic span of spiralian lineages, ultimately providing a better resolved picture of Hox gene evolution in this bilaterian clade. Currently, there is transcriptomic and/or genomic data on Hox genes for 11 of the 15 major animal groups that comprise Spiralia (Figure 1), with data missing for Gnathostomulida, Micrognathozoa, Gastrotricha and Cycliophora. Together, these new datasets clarify the ancestral Hox gene complement of Spiralia—likely comprising *Hox1*/*lab*, *Hox2*/*pb*, *Hox3*, *Hox4*/*Dfd*, *Hox5*/*Scr*, *Lox5*, *Antp*, *Lox4*/*Lox2*, and at least one posterior Hox—, demonstrating that distinct patterns of central and posterior Hox evolution co-occurred with the phylogenetic split of Spiralia into Gnathifera and Lophotrochozoa (Figure 1).

Gnathifera comprises Chaetognatha, Gnathostomulida, Micrognathozoa, and Rotifera [10, 11], but information on Hox complements and their expression only exist for Chaetognatha and Rotifera [12-15]. Our understanding of Hox complements in chaetognaths—arrow worms—is currently based on transcriptomic data and targeted searches [14], and thus whether Hox genes are organized in a cluster in this group is unknown. Genomic data is available for rotifers however, indicating that a Hox cluster is absent in this group [15], which correlates with a lack of temporal and spatial collinear expression of rotiferan Hox genes [12]. Both chaetognaths and rotifers share the presence of a unique type of Hox gene phylogenetically related to both Medial/Central and Posterior Hox genes in other non-gnathiferan taxa, referred to as MedPost genes, as well as a unique motif in the *Hox6*/*Lox5* group [12, 16]. Other than that, Hox gene complements differ significantly between these two lineages, with chaetognaths apparently lacking *Hox2* and rotifers missing *Hox7*/*Antp*, *Hox8*/*Lox4*/*Lox2*, and posterior genes (Figure 1). Altogether, the shared idiosyncratic signatures of Hox gene complements observed in chaetognaths and rotifers backs the phylogenetic relationships among gnathiferan clades, suggesting that lineage-specific diversification of Hox complements might underpin phenotypic evolution in Gnathifera [12].

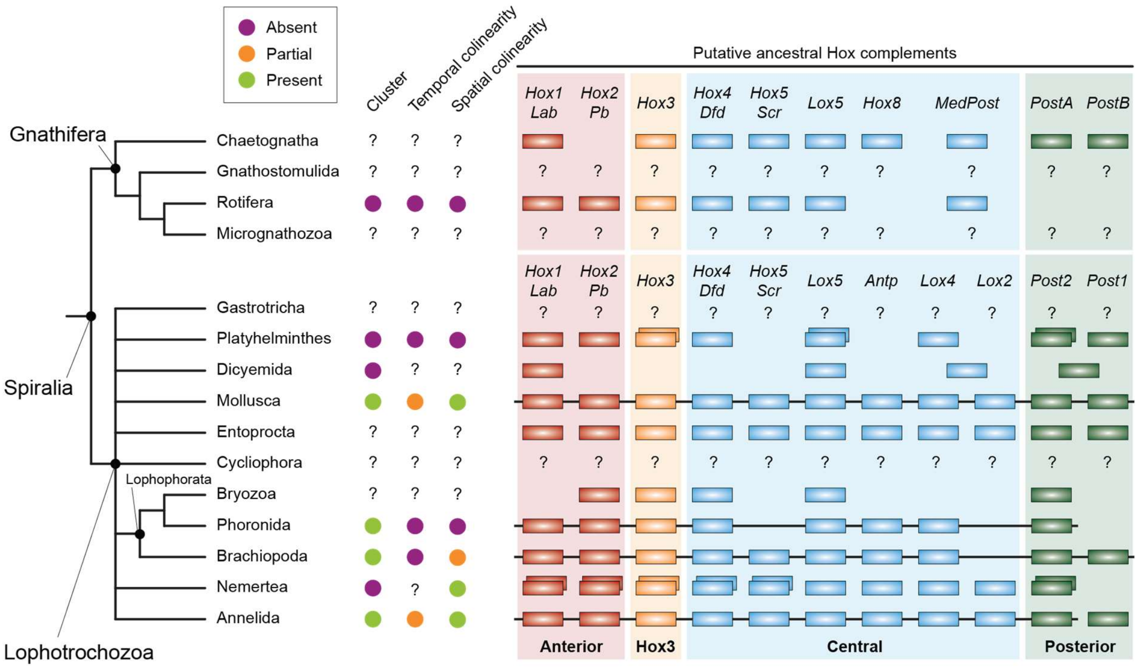

Figure 1 – The Hox gene complement in Spiralia. On the left, spiralian phylogeny indicating the ancient split into Gnathifera and Lophotrochozoa. The internal phylogeny of the latter is still poorly resolved. On the right, schematic representation of the putative ancestral Hox complements for the 15 major spiralian clades. Note that Gnathifera and Lophotrochozoa experienced distinct dynamics of central and posterior Hox evolution. In the middle, presence of a cluster of Hox genes and temporal/spatial collinear expression of these genes along the anteroposterior axis. Expansions are shown as two stacked boxes. See main text for references.

Lophotrochozoa (*sensu* [10]) is the second major lineage of spiralian taxa, and its internal phylogenetic relationships are still debated [10, 11, 17-19]. Gastrotricha, Platyhelminthes and Dicyemida have often been related in phylogenomic analyses [19, 20]. These are molecularly fast-evolving lineages, which is also reflected in their highly divergent Hox gene complements (Figure 1). While data for Gastrotricha is absent, genome sequencing of the dicyemid *Dicyema japonicum* uncovered only 4 Hox genes belonging to three orthogroups: *Hox1*, *Lox5* and Posterior, with no genomic linkage [21, 22] (Figure 1). Platyhelminthes have lost *Hox5*, *Antp*, and *Lox2*, with recent analysis identifying both *Post1*—previously thought to be absent in flatworms—and *Post2* genes in the planarian *Schmidtea mediterranea* [23-25] (Figure 1). Expression data is only available for free-living planarian flatworms, which do not exhibit temporal and spatial collinear deployment of Hox genes along the anteroposterior axis [23]. However, planarians exhibit a medial—instead of anterior—oral opening and lack an anal opening, which are morphological divergences along the main body axis that might account for the lack of spatial collinearity in Hox genes. Altogether, this data indicates that the evolution of these fast-evolving lophotrochozoan lineages appears to correlate with the disintegration of the ancestral Hox cluster, the loss of a variable number of Hox genes (followed by the duplication of others [23]) and possibly the loss of spatial collinearity (Figure 1).

The lophotrochozoan groups Bryozoa, Brachiopoda and Phoronida are often grouped within Lophophorata [10, 11, 26, 27], with the latter two being the most studied clades. Studies on Bryozoan genomes are scarce [28] and have not focused on Hox gene organization. Transcriptomic and targeted searches have, however, revealed the likely loss of several Hox gene classes in this group, including *Hox1*, *Hox5*, *Antp*, *Lox4*, *Lox2* and potentially *Post1* [29] (Figure 1). The absence of *Lox2* is shared with Phoronida and Brachiopoda [7, 30-32] (Figure 1), however it remains unclear whether the gene was lost in the common ancestor of Lophophorates, or originated after split of them from the remaining Lophotrochozoa [32]. Phoronids seem to have lost *Post1* and *Scr* [30, 32]. Regardless of these losses, an ordered Hox cluster appears ancestral for both Brachiopoda and Phoronida, with the lineage of the brachiopod *Lingula* having experienced a genomic inversion of the cluster [7, 30, 31]. In some phylogenomic analyses, Nemertea has been related to Brachiopoda and Phoronida, in the group Kryptochozoa [33], yet a more recent phylogeny places this group together with Annelida and Platyhelminthes [10]. The nemertean *Notospermus geniculatus* exhibits expansions of nearly all Hox gene classes (*Hox1*, *Hox2*, *Hox3*, *Hox4*, *Hox5*, and *Post2*) and lacks *Post1* and a Hox cluster [30]. Transcriptomic searches in other nemerteans—*Pantinonemertes californiensis* and *Micrura alaskensis*—uncovered a more reduced complement of Hox genes, which exhibit spatial collinearity during juvenile development [34, 35] (Figure 1). Similarly, traces of spatial collinearity are observed in Brachiopoda [7, 36] (Figure 1), demonstrating that coordinated expression of Hox genes has been retained in this spiralian clade—at least during certain stages of their life cycle—and despite changes in the complement and genomic organization of Hox genes.

Mollusca and Annelida are two of the most species-rich and morphologically diverse spiralian lineages. As the spiralian taxon with more sequenced genomes, the evolutionary history of Hox genes in Mollusca is well understood. The ancestral molluscan condition was an intact Hox gene cluster with at least one copy of each lophotrochozoan Hox orthogroup and signs of spatial and sub-cluster temporal collinearity [37, 38] (Figure 1). Interestingly however, closely related molluscan species show distinct genomic organizations of Hox genes (e.g., intact vs broken Hox clusters), indicating that weak selective pressures act on maintaining Hox genes clustered in this group. Recently, Entoprocta has been phylogenetically associated with Mollusca [10]. Although both genomic information and expression data are not available for entoprocts, transcriptomic analyses indicate that all lophotrochozoan Hox orthogroups were present in their last common ancestor [39] (Figure 1). Therefore, if the Mollusca + Entoprocta association holds stable, a full Hox gene repertoire was likely ancestral for this clade.

In Annelida, the species *Capitella teleta*, *Alitta virens*, *Platynereis dumerilii* [40-42] and *Owenia fusiformis* (unpublished data) have complete Hox gene complements and exhibit an ordered Hox cluster, except for *Post1*, which is separate (Figure 1). This is likely the ancestral annelid condition, and signs of spatial and temporal collinearity has been described for *Capitella teleta*, *Alitta virens*, *Platynereis dumerilii* [40, 41, 43] (Figure 2) and *Owenia fusiformis* (unpublished data). Similar Hox complements are also observed in two lineages of morphologically divergent annelids: Vestimentifera, which have an intact Hox cluster but lack *Antp* [44]; and *Dimorphilus gyrociliatus*, which exhibits an intact Hox cluster lacking *Lox2* and *Post1* despite its miniaturized genome [45]. However, Clitellata—leeches, earthworms, and allies—has divergent Hox complements with both multiple independent duplications (e.g., *Hox1*, *Hox3*, *Hox4*, *Hox5*, *Lox5*, *Lox4*, *Lox2*) and losses (*Hox2* and an unclear orthology of posterior Hox genes) and no Hox cluster [46, 47]. Recently, the parasitic group Orthonectida has been assigned to Annelida [48]. As with the parasitic Dicyemida, orthonectids have a reduced Hox complement only comprising *Hox2*, *Hox4*, and an ortholog to *Lox5*/*Antp*/*Hox8*/*Lox4*/*Lox2*, without a clustered organization [49, 50]. Therefore, the ancestral annelid condition suggests a genomic organization and expression similar to that observed in Mollusca, with subsequent morphological and ecological diversification in annelids occurring with either conservation of this Hox arrangement or the complete disintegration of the Hox gene cluster.

In summary, the recent genomic characterization of a broader array of spiralian lineages has uncovered two different dynamics of Hox evolution in this group, concomitant to the Gnathifera/Lophotrochozoa split (Figure 1). The presence of an ordered Hox cluster with 11 orthogroups exhibiting spatial collinearity and temporal collinearity in blocks—as observed in other major bilaterian lineages—is probably ancestral to Lophotrochozoa, yet the poor understanding of the internal phylogenetic relationships of this group makes reconstructing the exact evolutionary history of Hox genes difficult. Interestingly, *Lox2* and *Post1* have been repeatedly lost during spiralian evolution, which as discussed below might be associated with the cooption of these Hox genes to morphological novelties.

Ancestral and divergent roles of Hox genes in Spiralia

Hox genes represent one of the most comprehensively studied families of animal transcription factors and their expression has been investigated in numerous Spiralia [25], representing diverse evolutionary lineages, such as flatworms [23, 51, 52], rotifers [12], chaetognaths [13], dicyemids [53], nemerteans [34, 35], lophophorates [7, 32, 36, 54], annelids [40, 41, 43, 45, 55-58] and mollusks [37, 38, 59-66]. However, the function of particular Hox genes in those studied species is inferred only from expression patterns, since among spiralians the functional studies of Hox genes has been conducted thus far only on adult planarians [52]. The level, to which Hox expression has been studied varies a lot from clade to clade and for some spiralians expression of only single orthogroups has been investigated thus far (e.g., for chaetognaths, dicyemids and bryozoans), while for others the expression of full, or almost full Hox complements has been described throughout several developmental stages (e.g., for rotifers, nemerteans, brachiopods, phoronids, annelids and mollusks; Fig. 2). Fortunately, the comprehensively studied species are widely spread across phylogeny, which allows insight into evolution of the Hox function in the morphologically diverse clade of Spiralia.

In several nemertean and annelid species Hox genes generally show typical staggered expression along the anterior-posterior (A-P) axis (Fig. 2) and are expressed predominantly in the ectoderm and developing nervous system [34, 35, 40, 41, 43, 45, 55, 56], which possibly reflects the ancestral Hox expression in Bilateria [12, 67, 68]. Moreover, in the nereid annelids the colinear Hox gene expression is retained in the adult worms and it has been suggested that during postmetamorphic growth Hox genes provide positional information for the morphologically homonomous segments [43] and play a role in establishing segmental identity during posterior regeneration [57, 69, 70]. The latter function has been also reported in other annelids based on in situ RNA hybridization [71] and regeneration stage-specific transcriptomics [72]. Traces of the A-P staggered expression are also evident in rotifers, where Hox genes are expressed almost exclusively in the developing nervous system [12]. In mollusks, the Hox genes seem to have dual function (Fig. 2; [38]): several of them are expressed in the non-colinear way in the dorsal domain, where they are involved in the patterning of the shell field [38, 60, 61, 64-66], while ventrally they are expressed in the ectoderm, neuroectoderm and mesoderm and, at least in some of the developmental stages of some species, they show canonical staggered expression along the A-P axis [37, 38, 62-64]. The lophophorates show more derived Hox expression patterns. The Hox genes seem to not be

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expressed in any neural structures neither in phoronids nor brachiopods (which probably correlates with the extensive reduction of the central nervous system in those sessile animals) and instead they are expressed in various ectodermal and mesodermal domains in the non-colinear manner (Fig. 2; [7, 32, 36]). Interestingly, three Hox genes – *pb*, *Hox3* and *Dfd*, show staggered mesodermal expression along the A-P axis in larval brachiopods [7], while after metamorphosis they are expressed in specific muscles [36], indicating cooption of a subset of the Hox gene system into mesodermal A-P specification in this clade. Additionally, several of the Hox genes are expressed in specific morphological structures of both brachiopods (*lab* and *Post1* in chaetal sacs, *Scr* in shell field [7, 36]) and phoronids (*pb* in protonephridia and *Lox4* at the junction between midgut and proctodeum [32]) suggesting that the Hox genes, released from their putative ancestral function in the patterning of neuroectoderm, became widely and independently coopted into morphogenesis of various organs in Lophophorata. Another interesting example of the evolution of new function for Hox genes can be found in planarians. Although at least *HoxD* plays a role in the A-P patterning during planarian embryogenesis [51], the Hox genes are also widely expressed in the adult worms, some of them in the A-P axis and others in radial gradients [23]. Recently, it has been demonstrated that Hox genes play a role in the asexual reproduction of *Schmidtea mediterranea*, by regulating behavior and tissue segmentation in this well studied planarian species [52].

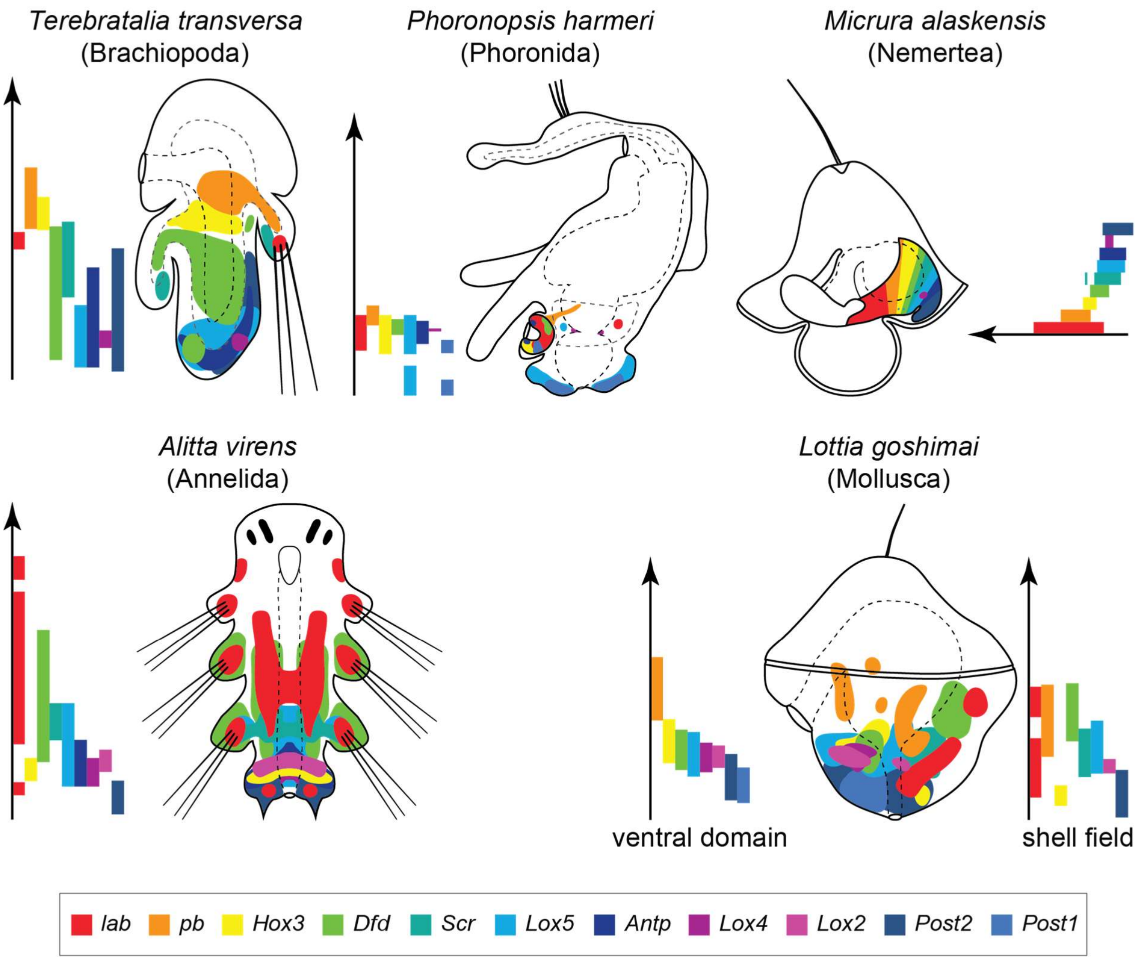

Figure 2. Comparison of Hox gene expression in selected spiralian larvae. For each larva the antero-posterior Hox gene expression gradients along the body axis is indicated. All larvae are shown from the left side (with exception of *Alitta virens*, which is shown in the dorsal view) with the anterior (apical) side oriented to the top. Note, that in brachiopod larva *pb*, *Hox3* and *Dfd* exhibit staggered A-P expression, while *lab* is expressed in chaetal sacs. In Phoronida and Nemertea the Hox genes are not expressed in the larval tissues but only in the rudiments of adult worms. Additionally, the nemertean Hox genes show staggered expression along the A-P axis of the worm rudiment (note, that larval and adult body axes are not aligned). In the annelid *A. virens* most of the Hox genes are expressed in colinear manner while *lab* is additionally expressed in the developing parapodia of each segment. In *Lottia goshimai* Hox genes show spatial collinearity within the ventral neuroectoderm but not in the dorsal shell field. See main text for references.

Altogether, comparison of Hox gene expression across Spiralia (Fig. 2) shows that unprecedented morphological diversity of this clade correlates well with evolutionary lability of Hox gene expression and function. In several spiralian clades the Hox genes are expressed not only in ectoderm, but also in the mesoderm and its derivatives. However, detailed comparison of mesodermal Hox expression shows that the set of Hox genes expressed in the mesodermal derivatives differs markedly from clade to clade and that their temporal transcription in the mesodermal tissues can occur at very different developmental stages. Therefore, it remains likely that Hox genes become coopted many times independently into patterning of the mesoderm and its derivatives in Spiralia [32, 36]. Clades that exhibit canonical expression of Hox genes along the A-P axis also seem to retain the ancestral function of Hox genes in the patterning of ectoderm and central nervous system. On the other hand, the co-option of Hox genes into morphogenesis of novel structures or regulation of postembryonic processes exemplifies evolution of new, less constrained functionalities for the Hox system in Spiralia. In the case of lophophorates, the evolutionary reduction of the central nervous system correlates with the diversification of Hox genes within this clade. However, the processes that favored analogous evolutionary trends in other spiralians remain largely unknown.

Hox genes and spiralian novelties

The cooption of Hox genes into the morphogenesis of particular structures and organs is an important evolutionary mechanism well studied in numerous animals. The Hox genes might be re-wired into patterning of the preexisting organs but they can also contribute to the origin of morphologically and molecularly novel structures, the so-called evolutionary novelties [73, 74]. The latter, as evident from the preceding summary of the Hox expression in Spiralia, is also a widespread phenomenon in this animal clade. The most famous examples of Hox gene cooption into spiralian morphological novelties can be found in mollusks, where Hox genes have been coopted into patterning of the shell in Conchifera [38, 60, 61, 64-66] and subsequently into patterning of brachial crown, funnel tube and stellate ganglia in cephalopods [59].

The hard, external shell is a hallmark of the mollusk body plan, however, a similar structure evolved independently also in lophophorates and is present in all extant brachiopod species. The investigation of Hox gene expression in larval, juvenile and adult brachiopods showed that the Hox gene *Scr* is expressed in the epithelial cells that produce both larval [7] and adult shell [31, 36], indicating that *Scr* has been coopted for the patterning of the shell-forming epithelium in brachiopods. Even though extant phoronids, the other lophophorates, for which Hox gene complement and expression have been studied, lack any shell-like structures, their closest known fossil relative, a tommotid *Eccenthrotheca*, possessed a hard mineralized exoskeleton, which reinforced a tube in which the animal dwelled [75, 76]. This indicates that the biomineralization capacities have been lost in the lineage of phoronids, which coincides with the absence of *Scr* in the otherwise well conserved phoronid Hox cluster [30, 32].

Hox expression studies in annelids revealed that expression of *Post1* deviates from the neuroectodermal A-P expression witnessed for other Hox genes and instead the gene is expressed in the developing chaetal sacs [40, 41, 55], the morphological structures that secrete chaetae, stiff bristles used by annelids for locomotion and protection. Expression of *Post1* in the chaetae-related territories has also been reported in brachiopods [7, 36] in concert with the morphology-based hypothesis on the homology of chaetae in both clades [77-79]. In brachiopods another Hox gene, *lab*, is also expressed in the chaetal sacs [7, 36], however, this cooption seems to be restricted only to the brachiopod lineage. Since chaetae-like structures are also present in some fossil mollusks [80], it seems plausible that chaetae were already present in the last common ancestor of annelids, mollusks and brachiopods and that *Post1* was already coopted for the patterning of the chaetal sacs in the lineage leading to this hypothetical ancestral animal. Interestingly, *Post1* is missing from the genomes of phoronids [30, 32], which – according to this evolutionary scenario – have secondarily lost the chaetae forming apparatus. This suggests that the gene, and the morphological structure patterned by it, had been lost in unison in the phoronid lineage.

Another possible example of cooption of Hox genes into patterning of evolutionary novelties can be found in the serpulid annelid, *Spirobranchus lamarcki*. As for other Serpulidae, *S. lamarcki* possess an operculum – an unpaired head appendage with a biomineralized shield used to plug the tube where the worm dwells [81]. The operculum is considered an evolutionary novelty of serpulids and many species are capable of its regeneration. Analysis of gene expression during operculum regeneration in *S. lamarcki* indicates that among many homeotic genes expressed in the regenerating organ, there is also a Hox gene *Antp* [82]. While the role of *Antp* in operculum formation is still unclear, these observations highlight the plasticity of Hox gene expression in spiralians, even in those clades exhibiting a marked and conserved spatial collinearity.

As Hox expression is studied in more spiralian species in the future, additional examples of Hox cooption into patterning of morphological novelties are expected to be found. However, even those which are currently known, help to understand evolution of such important spiralian novelties as shells or chaetae. Combination of Hox expression studies, comparative morphology and paleontology provides a comprehensive picture of evolution of these complex characters and points toward possible evolutionary mechanisms which lead to the reduction of morphological characters in certain clades. Most importantly, the functional analyses, using e.g., RNAi or CRISPR gene editing, are still needed to confirm that expression of particular Hox genes in the developing morphological structures is really indispensable for their morphogenesis.

Hox genes and life history stages

Studies of Hox gene expression during development of animals with complex life cycles can also inform about the evolution of life histories [83]. Spiralia are a particularly interesting clade in this respect, since many of them develop through distinct larval types [84, 85], some of which represent clade specific innovations (e.g. pilidium of pilidiophoran nemerteans, actinotrocha of phoronids or mitraria of oweniid annelids), while others are highly conserved over long phylogenetic distances (e.g. trochophore present in numerous annelids, kamptozoans and mollusks).

In the pilidiophoran nemertean *Maculaura (Micrura) alaskensis* the Hox genes are not expressed until late developmental stages when their expression is initiated in the ectodermal rudiments, from which the juvenile worm develops (Fig. 2; [35]). Comparison of Hox gene expression between *M. alaskensis* and the direct developing hoplonemertean *Pantinonemertes californiensis* showed that juveniles of both species are patterned in a similar way, while the pilidial development represents the derived condition [34]. This observation, combined with other gene expression studies and phylogenetic distribution of pilidium larvae, indicates that the pilidium represents a new body plan likely intercalated into an ancestral, more direct nemertean life cycle [34, 35]. Since Hox genes were already used for the patterning of the adult body plan, another, yet unknown, molecular system had to be deployed in the pilidiophoran ancestor for the patterning of this new life stage [35].

Hox genes are also not expressed during development of the actinotrocha, the highly specialized phoronid larva [32]. Their expression is delayed until later larval stages with most of the Hox genes being expressed in the rudiment of the adult trunk and in other posterior structures, which contribute to the adult body after metamorphosis (Fig. 2). A similar expression dynamic of Hox genes is observed in the mitraria larva of the palaeoannelid *Owenia fusiformis* (Martín-Durán, unpublished data), which also undergoes catastrophic metamorphosis [86]. The broad expression of head-specific genes in the larval phoronid indicates that the actinotrocha represents a so-called head-larva [32]. This would explain why the actinotrocha forms without input from the Hox patterning system – in many animals Hox gene expression is restricted to the post-head body regions, while heads develop from the anterior, Hox-free territory [6, 30, 34, 87-89]. The evolution of new larval types by precocious development of head structures or delayed development of the trunk has been proposed for several other animal clades, e.g. crustaceans and hemichordates [reviewed recently in 90]. In the latter clade the tornaria larva also develops without expression of Hox genes, which become activated only after onset of the trunk development [6], showing convergent expression dynamics of Hox genes between indirectly developing hemichordates and phoronids.

Lack of Hox gene expression during development of pilidium, actinotrocha and mitraria contrasts with the regular Hox expression in larval brachiopods [7] and trochophores of mollusks and annelids [37, 38, 40, 61-63, 65], in which both larval and adult bodies exhibit similar Hox gene patterns. The fact that those larvae are patterned by the conserved Hox system, as well as their phylogenetic distribution, suggest that all of those larval stages are eventually derived from the ancestral larval type present in the last common lophotrochozoan ancestor. Therefore, the future investigation of Hox gene expression during embryonic and larval development of other indirectly developing spiralians could help to resolve whether their larvae represent modification of the ancestral spiralian larva or more recent evolutionary innovations resulting from the intercalation of new head-larvae. This would be especially interesting in the cases of some strange spiralian larvae, whose evolution and homology to the other larval types remain obscure, such as the Müller’s larva of polyclads, creeping larva of kamptozoans or cyphonautes of gymnolaemate bryozoans.

Conclusions

A high conservation of Hox clusters in some lineages, together with the dissociation of the cluster and loss of certain genes in some sublineages characterizes the evolution of Hox genes in Spiralia. It remains unclear however, which forces prevent the Hox cluster from atomizing in this animal group, yet genome regulatory aspects have likely played a major role. Despite the conservation of the Hox cluster in major spiralian lineages, neofunctionalization of Hox genes—even of those existing as single copies—is not uncommon. Therefore, Hox genes are often used for axial patterning, but also deployed in novel structures in later stages of development. Contrary to what is generally observed in arthropods and chordates, spiralians exhibit many cases in which Hox clusters repeatedly disintegrate without reproducible patterns, nor are Hox genes connected to specific germ layers, body regions or cell types. When looking at the life cycle, many spiralian larva do not involve Hox genes in their patterning, or if they do, then only in tissues that are transferred to the adult body plan during metamorphosis. Together, the evolution and diversification of Spiralia for more than 500 million years is a showcase of Hox gene evolution, where defining a general pattern is difficult. Moreover, Spiralia teaches us a lesson about the importance of using more taxon sampling to test—and sometimes reject—hypotheses based on observations on a few animal lineages. In this context, the diverse patterns of Hox expression and genomic organization that we find in Spiralia provide a novel resource to discover new mechanisms of genome regulation and organization, and the interplay between the two, along with the correlation of these phenomena with morphological evolution.

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References

1. Duboule D: **The rise and fall of Hox gene clusters**. *Development* 2007, **134**(14):2549-2560.
2. Duboule D: **Temporal colinearity and the phylotypic progression: a basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony**. *Dev Suppl* 1994:135-142.
3. Ferrier DEK, Minguillon C: **Evolution of the Hox/ParaHox gene clusters**. *Int J Dev Biol* 2003, **47**(7-8):605-611.
4. Monteiro AS, Ferrier DEK: **Hox genes are not always Colinear**. *Int J Biol Sci* 2006, **2**(3):95-103.
5. Smith FW, Boothby TC, Giovannini I, Rebecchi L, Jockusch EL, Goldstein B: **The Compact Body Plan of Tardigrades Evolved by the Loss of a Large Body Region**. *Curr Biol* 2016, **26**(2):224-229.
6. Gonzalez P, Uhlinger KR, Lowe CJ: **The Adult Body Plan of Indirect Developing Hemichordates Develops by Adding a Hox-Patterned Trunk to an Anterior Larval Territory**. *Curr Biol* 2017, **27**(1):87-95.

7. Schiemann SM, Martin-Duran JM, Børve A, Vellutini BC, Passamaneck YJ, Hejnol A: **Clustered brachiopod Hox genes are not expressed collinearly and are associated with lophotrochozoan novelties**. *Proc Natl Acad Sci U S A* 2017, **114**(10):E1913-E1922.
8. Mallo M: **Reassessing the Role of Hox Genes during Vertebrate Development and Evolution**. *Trends Genet* 2018, **34**(3):209-217.
9. Mallo M, Wellik DM, Deschamps J: **Hox genes and regional patterning of the vertebrate body plan**. *Dev Biol* 2010, **344**(1):7-15.
10. Marlétaz F, Peijnenburg K, Goto T, Satoh N, Rokhsar DS: **A New Spiralian Phylogeny Places the Enigmatic Arrow Worms among Gnathiferans**. *Curr Biol* 2019, **29**(2):312-318 e313.
11. Laumer CE, Bekkouche N, Kerbl A, Goetz F, Neves RC, Sorensen MV, Kristensen RM, Hejnol A, Dunn CW, Giribet G *et al*: **Spiralian phylogeny informs the evolution of microscopic lineages**. *Curr Biol* 2015, **25**(15):2000-2006.
12. Fröbius AC, Funch P: **Rotiferan Hox genes give new insights into the evolution of metazoan bodyplans**. *Nat Commun* 2017, **8**.
13. Papillon D, Perez Y, Fasano L, Le Parco Y, Caubit X: **Restricted expression of a median Hox gene in the central nervous system of Chaetognaths**. *Dev Genes Evol* 2005, **215**(7):369-373.
14. Papillon D, Perez Y, Fasano L, Le Parco Y, Caubit X: **Hox gene survey in the chaetognath Spadella cephaloptera: evolutionary implications**. *Dev Genes Evol* 2003, **213**(3):142-148.
15. Flot JF, Hespeels B, Li X, Noel B, Arkhipova I, Danchin EGJ, Hejnol A, Henrissat B, Koszul R, Aury JM *et al*: **Genomic evidence for ameiotic evolution in the bdelloid rotifer *Adineta vaga***. *Nature* 2013, **500**(7463):453-457.
16. Mauer KM, Schmidt H, Dittrich M, Fröbius AC, Hellmann SL, Zischler H, Hankeln T, Herlyn H: **Genomics and transcriptomics of epizoic Seisonidea (Rotifera, syn. Syndermata) reveal strain formation and gradual gene loss with growing ties to the host**. *BMC Genomics* 2021, **22**(1):604.
17. Kocot KM: **On 20 years of Lophotrochozoa**. *Organisms Diversity & Evolution* 2016, **16**(2):329-343.
18. Kocot KM, Struck TH, Merkel J, Waits DS, Todt C, Brannock PM, Weese DA, Cannon JT, Moroz LL, Lieb B *et al*: **Phylogenomics of Lophotrochozoa with Consideration of Systematic Error**. *Syst Biol* 2017, **66**(2):256-282.

19. Struck TH, Wey-Fabrizius AR, Golombek A, Hering L, Weigert A, Bleidorn C, Klebow S, Iakovenko N, Hausdorf B, Petersen M *et al*: **Platyzoan paraphyly based on phylogenomic data supports a noncoelomate ancestry of spiralia**. *Mol Biol Evol* 2014, **31**(7):1833-1849.

20. Lu TM, Kanda M, Satoh N, Furuya H: **The phylogenetic position of dicyemid mesozoans offers insights into spiralian evolution**. *Zoological Lett* 2017, **3**:6.

21. Lu T-M, Kanda M, Furuya H, Satoh N: **Dicyemid Mesozoans: A Unique Parasitic Lifestyle and a Reduced Genome**. *Genome Biology and Evolution* 2019, **11**(8):2232-2243.

22. Zverkov OA, Mikhailov KV, Isaev SV, Rusin LY, Popova OV, Logacheva MD, Penin AA, Moroz LL, Panchin YV, Lyubetsky VA *et al*: **Dicyemida and Orthonectida: Two Stories of Body Plan Simplification**. *Front Genet* 2019, **10**:443.

23. Currie KW, Brown DD, Zhu S, Xu C, Voisin V, Bader GD, Pearson BJ: **HOX gene complement and expression in the planarian *Schmidtea mediterranea***. *EvoDevo* 2016, **7**:7.

24. Koziol U, Lalanne AI, Castillo E: **Hox genes in the parasitic platyhelminthes Mesocestoides corti, Echinococcus multilocularis, and Schistosoma mansoni: evidence for a reduced Hox complement**. *Biochem Genet* 2009, **47**(1-2):100-116.

25. Barucca M, Canapa A, Biscotti MA: **An overview of Hox genes in Lophotrochozoa: Evolution and functionality**. *Journal of developmental biology* 2016, **4**(1):12.

26. Nesnidal MP, Helmkampf M, Meyer A, Witek A, Bruchhaus I, Ebersberger I, Hankeln T, Lieb B, Struck TH, Hausdorf B: **New phylogenomic data support the monophyly of Lophophorata and an Ectoproct-Phoronid clade and indicate that Polyzoa and Kryptrochozoa are caused by systematic bias**. *BMC Evol Biol* 2013, **13**:253.

27. Emig C: **On the origin of the Lophophorata**. *Journal of Zoological Systematics and Evolutionary Research* 1984, **22**(2):91-94.

28. Rayko M, Komissarov A, Kwan JC, Lim-Fong G, Rhodes AC, Kliver S, Kuchur P, O'Brien SJ, Lopez JV: **Draft genome of Bugula neritina, a colonial animal packing powerful symbionts and potential medicines**. *Sci Data* 2020, **7**(1):356.

29. Passamaneck YJ, Halanych KM: **Evidence from Hox genes that bryozoans are lophotrochozoans**. *Evol Dev* 2004, **6**(4):275-281.

30. Luo YJ, Kanda M, Koyanagi R, Hisata K, Akiyama T, Sakamoto H, Sakamoto T, Satoh N: **Nemertean and phoronid genomes reveal lophotrochozoan evolution and the origin of bilaterian heads**. *Nat Ecol Evol* 2018, **2**(1):141-151.

31. Luo YJ, Takeuchi T, Koyanagi R, Yamada L, Kanda M, Khalturina M, Fujie M, Yamasaki S, Endo K, Satoh N: **The *Lingula* genome provides insights into brachiopod evolution and the origin of phosphate biomineralization**. *Nature Communications* 2015, **6**.

32. Gąsiorowski L, Hejnol A: **Hox gene expression during development of the phoronid *Phoronopsis harmeri***. *Evodevo* 2020, **11**:2.

33. Hejnol A, Obst M, Stamatakis A, Ott M, Rouse GW, Edgecombe GD, Martinez P, Baguna J, Bailly X, Jondelius U *et al*: **Assessing the root of bilaterian animals with scalable phylogenomic methods**. *Proc Biol Sci* 2009, **276**(1677):4261-4270.

34. Hiebert LS, Maslakova SA: **Expression of Hox, Cdx, and Six3/6 genes in the hoplonemertean *Pantinonemertes californiensis* offers insight into the evolution of maximally indirect development in the phylum Nemertea**. *EvoDevo* 2015, **6**.

35. Hiebert LS, Maslakova SA: **Hox genes pattern the anterior- posterior axis of the juvenile but not the larva in a maximally indirect developing invertebrate, *Micrura alaskensis* (Nemertea)**. *BMC Biol* 2015, **13**.

36. Gąsiorowski L, Hejnol A: **Hox gene expression in postmetamorphic juveniles of the brachiopod *Terebratalia transversa***. *EvoDevo* 2019, **10**:1.

37. Wang S, Zhang J, Jiao W, Li J, Xun X, Sun Y, Guo X, Huan P, Dong B, Zhang L: **Scallop genome provides insights into evolution of bilaterian karyotype and development**. *Nature ecology & evolution* 2017, **1**(5):1-12.

38. Huan P, Wang Q, Tan S, Liu B: **Dorsoventral decoupling of Hox gene expression underpins the diversification of molluscs**. *Proc Natl Acad Sci U S A* 2019, **117**(1):503-512.

39. Merkel JW, A.; Lieb, B.: **Novel and Conserved Features of the Hox Cluster of Entoprocta (Kamptozoa)**. *J Phylogenetics Evol Biol* 2018, **6**(1):194.

40. Kulakova M, Bakalenko N, Novikova E, Cook CE, Eliseeva E, Steinmetz PRH, Kostyuchenko RP, Dondua A, Arendt D, Akam M *et al*: **Hox gene expression in larval development of the polychaetes *Nereis virens* and *Platynereis dumerilii* (Annelida, Lophotrochozoa)**. *Dev Genes Evol* 2007, **217**(1):39-54.

41. Fröbius AC, Matus DQ, Seaver EC: **Genomic Organization and Expression Demonstrate Spatial and Temporal Hox Gene Colinearity in the Lophotrochozoan *Capitella* sp I**. *Plos One* 2008, **3**(12):e4004.

42. Hui JH, McDougall C, Monteiro AS, Holland PW, Arendt D, Balavoine G, Ferrier DE: **Extensive chordate and annelid macrosynteny reveals ancestral homeobox gene organization**. *Mol Biol Evol* 2012, **29**(1):157-165.

43. Bakalenko NI, Novikova EL, Nesterenko AY, Kulakova MA: **Hox gene expression during postlarval development of the polychaete *Alitta virens***. *Evodevo* 2013, **4**.

44. Sun Y, Sun J, Yang Y, Lan Y, Ip JC, Wong WC, Kwan YH, Zhang Y, Han Z, Qiu JW *et al*: **Genomic signatures supporting the symbiosis and formation of chitinous tube in the deep-sea tubeworm Paraescarpia echinospica**. *Mol Biol Evol* 2021, **38**(10):4116- 4134.

45. Martin-Duran JM, Vellutini BC, Marletaz F, Cetrangolo V, Cvetesic N, Thiel D, Henriet S, Grau-Bove X, Carrillo-Baltodano AM, Gu W *et al*: **Conservative route to genome compaction in a miniature annelid**. *Nat Ecol Evol* 2021, **5**(2):231-242.

46. Simakov O, Marlétaz F, Cho SJ, Edsinger-Gonzales E, Havlak P, Hellsten U, Kuo DH, Larsson T, Lv J, Arendt D *et al*: **Insights into bilaterian evolution from three spiralian genomes**. *Nature* 2013, **493**(7433):526-531.

47. Zwarycz AS, Nossa CW, Putnam NH, Ryan JF: **Timing and Scope of Genomic Expansion within Annelida: Evidence from Homeoboxes in the Genome of the Earthworm *Eisenia fetida***. *Genome Biol Evol* 2016, **8**(1):271-281.

48. Schiffer PH, Robertson HE, Telford MJ: **Orthonectids Are Highly Degenerate Annelid Worms**. *Curr Biol* 2018, **28**(12):1970-1974 e1973.

49. Mikhailov Kirill V, Slyusarev Georgy S, Nikitin Mikhail A, Logacheva Maria D, Penin Aleksey A, Aleoshin Vladimir V, Panchin Yuri V: **The Genome of Intoshia linei Affirms Orthonectids as Highly Simplified Spiralians**. *Current Biology* 2016, **26**(13):1768-1774.

50. Slyusarev GS, Starunov VV, Bondarenko AS, Zorina NA, Bondarenko NI: **Extreme Genome and Nervous System Streamlining in the Invertebrate Parasite Intoshia variabili**. *Curr Biol* 2020, **30**(7):1292-1298 e1293.

51. Martín-Durán JM, Amaya E, Romero R: **Germ layer specification and axial patterning in the embryonic development of the freshwater planarian Schmidtea polychroa**. *Developmental Biology* 2010, **340**(1):145-158.

52. Arnold CP, Lozano AM, Mann FG, Nowotarski SH, Haug JO, Lange JJ, Seidel CW, Alvarado AS: **Hox genes regulate asexual reproductive behavior and tissue segmentation in adult animals**. *Nature Communications* 2021, **12**(1):6706.

53. Kobayashi M, Furuya H, Wada H: **Molecular markers comparing the extremely simple body plan of dicyemids to that of lophotrochozoans: insight from the expression patterns of Hox, Otx, and brachyury**. *Evolution & Development* 2009, **11**(5):582-589.

54. Fuchs J, Martindale MQ, Hejnol A: **Gene expression in bryozoan larvae suggest a fundamental importance of pre-patterned blastemic cells in the bryozoan life-cycle**. *EvoDevo* 2011, **2**(1):13.

55. Kulakova MA, Kostyuchenko RP, Andreeva TF, Dondua AK: **The Abdominal-B-like gene expression during larval development of *Nereis virens* (polychaeta)**. *Mechanisms of Development* 2002, **115**(1-2):177-179.

56. Irvine SQ, Martindale MQ: **Expression patterns of anterior Hox genes in the polychaete *Chaetopterus*: Correlation with morphological boundaries**. *Dev Biol* 2000, **217**(2):333-351.

57. Novikova EL, Bakalenko NI, Nesterenko AY, Kulakova MA: **Expression of Hox genes during regeneration of nereid polychaete Alitta (Nereis) virens (Annelida, Lophotrochozoa)**. *EvoDevo* 2013, **4**(1):14.

58. Kourakis MJ, Master VA, Lokhorst DK, Nardelli-Haefliger D, Wedeen CJ, Martindale MQ, Shankland M: **Conserved Anterior Boundaries of Hox Gene Expression in the Central Nervous System of the LeechHelobdella**. *Developmental Biology* 1997, **190**(2):284-300.

59. Lee PN, Callaerts P, de Couet HG, Martindale MQ: **Cephalopod Hox genes and the origin of morphological novelties**. *Nature* 2003, **424**(6952):1061-1065.

60. Samadi L, Steiner G: **Involvement of Hox genes in shell morphogenesis in the encapsulated development of a top shell gastropod (*Gibbula varia* L.)**. *Dev Genes Evol* 2009, **219**(9-10):523-530.

61. Samadi L, Steiner G: **Expression of Hox genes during the larval development of the snail, *Gibbula varia* (L.)-further evidence of non-colinearity in molluscs**. *Dev Genes Evol* 2010, **220**(5-6):161-172.

62. Fritsch M, Wollesen T, de Oliveira AL, Wanninger A: **Unexpected co-linearity of Hox gene expression in an aculiferan mollusk**. *BMC Evol Biol* 2015, **15**.

63. Fritsch M, Wollesen T, Wanninger A: **Hox and ParaHox Gene Expression in Early Body Plan Patterning of Polyplacophoran Mollusks**. *J Exp Zool Part B* 2016, **326**(2):89-104.

64. Wollesen T, Monje SVR, de Oliveira AL, Wanninger A: **Staggered Hox expression is more widespread among molluscs than previously appreciated**. *P Roy Soc B-Biol Sci* 2018, **285**(1888).

65. Salamanca-Díaz DA, Calcino AD, de Oliveira AL, Wanninger A: **Non-collinear Hox gene expression in bivalves and the evolution of morphological novelties in mollusks**. *Scientific Reports* 2021, **11**(1):3575.

66. Hinman VF, O'Brien EK, Richards GS, Degnan BM: **Expression of anterior Hox genes during larval development of the gastropod *Haliotis asinina***. *Evol Dev* 2003, **5**(5):508-521.

67. Hejnol A, Martindale MQ: **Coordinated spatial and temporal expression of Hox genes during embryogenesis in the acoel *Convolutriloba longifissura***. *BMC Biol* 2009, **7**.

68. Deutsch J, Le Guyader H: **The neuronal zootype. An hypothesis**. *Cr Acad Ssci III-Vie* 1998, **321**(9):713-719.

69. Kostyuchenko RP, Kozin VV: **Comparative Aspects of Annelid Regeneration: Towards Understanding the Mechanisms of Regeneration**. *Genes* 2021, **12**(8):1148.

70. Pfeifer K, Dorresteijn AW, Fröbius AC: **Activation of Hox genes during caudal regeneration of the polychaete annelid *Platynereis dumerilii***. *Dev Genes Evol* 2012, **222**(3):165-179.

71. de Jong DM, Seaver EC: **A Stable Thoracic Hox Code and Epimorphosis Characterize Posterior Regeneration in *Capitella teleta***. *PLoS One* 2016, **11**(2):e0149724.

72. Ribeiro RP, Ponz-Segrelles G, Bleidorn C, Aguado MT: **Comparative transcriptomics in Syllidae (Annelida) indicates that posterior regeneration and regular growth are comparable, while anterior regeneration is a distinct process**. *BMC Genomics* 2019, **20**(1):855.

73. Müller GB, Wagner GP: **Homology, Hox Genes, and Developmental Integration**. *American Zoologist* 1996, **36**(1):4-13.

74. Wagner GP, Lynch VJ: **Evolutionary novelties**. *Current Biology* 2010, **20**(2):R48-R52.

75. Skovsted CB, Brock GA, Paterson JR, Holmer LE, Budd GE: **The scleritome of *Eccentrotheca* from the Lower Cambrian of South Australia: Lophophorate affinities and implications for tommotiid phylogeny**. *Geology* 2008, **36**(2):171-174.

76. Skovsted CB, Brock GA, Topper TP, Paterson JR, Holmer LE: **Scleritome construction, biofacies, biostratigraphy and systematics of the tommotiid *Eccentrotheca helenia* sp. nov. from the early Cambrian of South Australia**. *Palaeontology* 2011, **54**(2):253-286.

77. Gustus RM, Cloney RA: **Ultrastructural Similarities Between Setae of Brachiopods and Polychaetes 1**. *Acta Zoologica* 1972, **53**(2):229-233.

78. Lüter C: **Brachiopod larval setae–a key to the phylum's ancestral life cycle?** In: *Brachiopods.* CRC Press; 2001: 60-69.

79. Hausam B, Bartolomaeus T: **Ultrastructure and development of forked and capillary setae in the polychaetes Orbinia bioreti and Orbinia latreillii (Annelida: Orbiniidae)**. *Invertebrate Biology* 2001, **120**(1):13-28.

80. Thomas RD, Runnegar B, Matt K: **Pelagiella exigua, an early Cambrian stem gastropod with chaetae: lophotrochozoan heritage and conchiferan novelty**. *Palaeontology* 2020, **63**(4):601-627.

81. Bok MJ, Porter ML, Ten Hove HA, Smith R, Nilsson D-E: **Radiolar eyes of serpulid worms (Annelida, Serpulidae): structures, function, and phototransduction**. *The Biological Bulletin* 2017, **233**(1):39-57.

82. Barton-Owen TB, Szabó R, Somorjai IML, Ferrier DEK: **A Revised Spiralian Homeobox Gene Classification Incorporating New Polychaete Transcriptomes Reveals a Diverse TALE Class and a Divergent Hox Gene**. *Genome Biology and Evolution* 2018, **10**(9):2151-2167.

83. Hejnol A, Vellutini BC: **Larval Evolution: I'll Tail You Later ...** *Curr Biol* 2017, **27**(1):R21-R24.

84. Haug JT: **Why the term "larva" is ambiguous, or what makes a larva?** *Acta Zoologica* 2020, **101**(2):167-188.

85. Wanninger (ed.) A: **Evolutionary developmental biology of invertebrates 2: Lophotrochozoa (Spiralia)**: Springer; 2015.

86. Wilson DP: **IV. On the Mitraria Larva of *Owenia fusiformis* Delle Chiaje**. *Philosophical Transactions of the Royal Society of London Series B, Containing Papers of a Biological Character* 1932, **221**(474-482):231-334.

87. Lowe CJ, Wu M, Salic A, Evans L, Lander E, Stange-Thomann N, Gruber CE, Gerhart J, Kirschner M: **Anteroposterior patterning in hemichordates and the origins of the chordate nervous system**. *Cell* 2003, **113**(7):853-865.
88. Steinmetz PR, Urbach R, Posnien N, Eriksson J, Kostyuchenko RP, Brena C, Guy K, Akam M, Bucher G, Arendt D: **Six3 demarcates the anterior-most developing brain region in bilaterian animals**. *EvoDevo* 2010, **1**(1):1-9.
89. Aronowicz J, Lowe C: **Hox gene expression in the hemichordate *Saccoglossus kowalevskii* and the evolution of deuterostome nervous systems**. *Integrative and Comparative Biology* 2006, **46**(6):890-901.
90. Strathmann RR: **Multiple origins of feeding head larvae by the Early Cambrian**. *Canadian Journal of Zoology* 2020, **98**(12):761-776.