

# Hox gene expression during development of the phoronid *Phoronopsis harmeri*

Ludwik Gąsiorowski

Universitetet i Bergen

Andreas Hejnol (✉ [andreas.hejnol@uib.no](mailto:andreas.hejnol@uib.no))

<https://orcid.org/0000-0003-2196-8507>

---

## Research

**Keywords:** Lophophorata, Spiralia, biphasic life cycle, intercalation, life history evolution, body plan, indirect development, Lox2, head, brain

**Posted Date:** December 3rd, 2019

**DOI:** <https://doi.org/10.21203/rs.2.17983/v1>

**License:**  This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

---

**Version of Record:** A version of this preprint was published at EvoDevo on February 10th, 2020. See the published version at <https://doi.org/10.1186/s13227-020-0148-z>.

## Abstract

**Background:** Phoronida is a small group of marine worm-like suspension feeders, which together with brachiopods and bryozoans form the clade Lophophorata. Although their development is well studied on the morphological level, data regarding gene expression during this process are scarce and restricted to the analysis of relatively few transcription factors. Here we present a description of the expression patterns of Hox genes during the embryonic and larval development of the phoronid *Phoronopsis harmeri*.

**Results:** We identified sequences of 8 Hox genes in the transcriptome of *Ph. harmeri* and determined their expression pattern during embryonic and larval development using whole mount *in situ* hybridization. We found that none of the Hox genes is expressed during embryonic development. Instead their expression is initiated in the later developmental stages, when the larval body is already formed. The Hox genes are expressed in the non-collinear manner in the posterior body of the larvae: in the telotroch and the structures that represent rudiments of the adult worm, which emerges through the process of drastic metamorphosis. Additionally, we found that certain head-specific transcription factors are expressed in the oral hood, apical organ, anterior digestive system and developing larval tentacles, anterior to the Hox-expressing territories.

**Conclusions:** The lack of Hox gene expression during early development of *Ph. harmeri* indicates that the larval body develops without positional information of the Hox patterning system. Such phenomenon might be a consequence of the evolutionary intercalation of the larval form into an ancestral life cycle of phoronids. The observed Hox gene expression can also be a consequence of the actinotrocha representing a “head larva”, which is composed of the most anterior body region that is devoid of Hox gene expression, which is supported by the expression of head-specific transcription factors. This implies that the Hox patterning system is used for the positional information of the trunk rudiments and is, therefore, delayed to the later larval stages. We propose that a new body form was intercalated to the phoronid life cycle by precocious development of the anterior structures or by delayed development of the trunk rudiment in the ancestral phoronid larva.

## Background

Hox genes encode a family of transcription factors present in Bilateria and Cnidaria(1–4), which bind with their conserved homeodomain directly to regulatory regions of downstream genes and activate or suppress their expression (e.g. (5–7)). In many clades, Hox genes are differentially expressed in the early developmental stages along the anterior-posterior axis of the developing embryo, being one of the important components of molecular patterning of axial identities(4–6, 8–10). The diversity of Hox genes present in extant Bilateria originated likely by multiple duplication events, which resulted in the physical linkage of Hox genes in the genomes of many Bilateria, the so called Hox clusters (e.g. (9, 11, 12)). It is possible to discriminate organized, split and disorganized Hox clusters, depending on the level of their organization(7, 12) and in certain Bilateria the Hox genes are expressed in roughly the same order as they are located in the cluster: a phenomenon referred to as collinearity(6, 9, 11). The correspondence between position of the gene in cluster and onset of its expression might have a temporal (during development) or spatial (along body axis) character and accordingly it is possible to discriminate between the temporal and spatial collinearity. It has been proposed that collinearity, especially the temporal one, is a major factor responsible for conservation (or maybe even formation) of the ordered Hox cluster in the genome(9, 11–16).

Although expression of Hox genes has been described during embryonic and larval development of many animals representing diverse evolutionary lineages(4, 16–49), there are still some clades for which information about Hox expression during development is lacking. Among them are phoronids, marine, sessile worms, which feed using a specialized filter apparatus, the so-called lophophore (*lp* in Fig. 1B). Due to the presence of lophophore, Phoronida have been traditionally united with two other clades—Ectoprocta (Bryozoa) and Brachiopoda—into the group called Lophophorata(50, 51), which recently gained support as a valid clade from several transcriptomic and phylogenomic studies(52–55). Although originally the Lophophorata were considered as deuterostomes(50, 51), molecular data showed their protostome affinity(56) and currently the lophophorates occupy a well-supported position within the clade of Spiralia(52–55, 57). Most phoronids develop through a distinctive planktotrophic larval stage, called actinotrocha(58–61). After a prolonged planktonic life, the actinotrocha larva settles and undergoes drastic metamorphosis (Fig. 1C), during which the rudiment of the body wall of the adult worm, the so called metasomal sac (ms, Fig. 1C), is everted and the rudiments of the adult internal organs descent from the larval body to the newly formed juvenile worm (Fig. 1C)(60, 61). The only exception from this pattern is *Phoronis ovalis*, which is a sister group to the remaining phoronids(62–64) and which develops through the creeping slug-like larva(60). After few days of development the active larva of *P. ovalis* settles and acquires a smooth hemispherical shape(60). Unfortunately, the degree of the metamorphosis-related remodeling of internal structures in *P. ovalis* remains poorly examined.

The phoronid development has been well studied on the morphological level (e.g. (58–61, 65–85)), including preliminary cell lineage, blastomere ablation and fate mapping studies(86–88). However, information about the molecular patterning is limited to the single study of 9 transcription factors (which include anterior, posterior and endomesodermal markers) during the development of *Phoronopsis harmeri*(85). Importantly, information about expression of Hox genes during development of any phoronid species is still lacking(40, 59).

Recently, Luo et al. have demonstrated that in phoronid *Phoronis australis* a Hox cluster is highly organized with all of the 8 phoronid Hox genes forming a single cluster that retains the ancestral spiralian order of genes ((89), also Fig. 1A). This is in contrast to brachiopods, the putative close relatives of Phoronida, where various level of Hox cluster disorganization is shown (Fig. 1A) and temporal and spatial collinearity is missing (16, 40, 89, 90). Therefore, it remains important to examine whether phoronid Hox genes are also expressed in the spatio-temporally collinear manner during development, which would correspond with the retention of the organized Hox cluster shown in this clade.

Phoronids exhibit a biphasic life cycle with planktotrophic larvae that transform into the juvenile in a catastrophic metamorphosis event (Fig. 1C; e.g. (59, 60, 73, 75, 81, 82)), which is much more drastic than relatively gradual metamorphosis of most Spiralia. Importantly, the A-P axis of the larva is profoundly altered during metamorphosis(60, 77, 81, 82) and results in the U-shaped organization of the internal structures of the juvenile worm (Fig. 1C). In animals with pronounced metamorphosis Hox genes might exhibit noticeable differences in the expression patterns during development of larval and adult bodies. In pilidiophoran nemerteans and indirectly developing hemichordates it has been demonstrated that Hox genes are involved in patterning of only adult bodies(37, 38), while in tunicates and sea urchins different sets of Hox genes are expressed during larval and adult body development(21, 22, 44, 47). On the other hand, in animals with more gradual metamorphosis (e.g. cephalochordates, mollusks, annelids or brachiopods), the Hox genes seem to pattern both the larval and adult body plans in a relatively similar way(31, 39, 40, 46, 48). However, studies focusing on metamorphosis-related differences of Hox gene expression in Bilateria are still limited to a relatively few evolutionary lineages(40, 92). Therefore, the comparison of Hox gene expression between the embryonic and larval development and the development of the metasomal sac in phoronids might shed new light into the understanding of the evolution of differential genetic control of the axis patterning in animals with extreme metamorphosis.

In this study we investigated the Hox genes complement and their expression patterns during the development of the phoronid *Phoronopsis harmeri*, for which the extensive data on the morphological aspects of the development and some molecular data on the A-P axis are available(66, 72, 75–78, 80–82, 84, 85)). Our aim was to answer whether phoronid Hox genes show staggered expression along the A-P axis at any of the developmental stages as well as to examine if there are traces of temporal collinearity that could hint to the presence of a Hox cluster as described for another phoronid *P. australis*(89). We also wanted to investigate whether there are differences in the Hox gene expression (and possibly in the patterning of the A-P axes) between the larva and the rudiment of the forming juvenile worm and compare our findings with other species that exhibit extreme metamorphosis.

## Results

### *Hox complement and gene orthology*

We identified 8 Hox genes in the transcriptome of *Ph. harmeri* and our phylogenetic analysis allowed their assignment to particular orthology groups (Fig. 2). Those genes represent orthologues of the genes *labial (lab)*, *proboscipedia (pb)*, *hox3*, *deformed (dfd)*, *lox5*, *antennapedia (antp)*, *lox4* and *post2* (Figs. 1A and 2). Moreover, in addition to the gene *cdx* reported by Andrikou et al.(85), we identified two other paraHox genes in the transcriptome of *Ph. harmeri*: *gsx* and *xlox*. Most of the Hox orthologues form distinct clades in our phylogenetic tree (Fig. 2). Sequences from the two orthologues (*sex combs reduced (scr)* and *antp*) do not form clades but rather grades of similar sequences (Fig. 2), which nevertheless allow exact orthology assessment. We found that the gene identified by Luo et al. as *lox2* in the genome of *P. australis*(89) and its orthologue in *Ph. harmeri* do not fall into the clade containing *lox2* sequences from other Spiralia, but instead they group in the grade containing *antp* sequences. Accordingly, sequence of those two phoronid genes lack most of the residues proposed as signature of *lox2* by de Rosa et al. (Additional File 1: Fig. S1; (93)).

### *Embryonic and larval development of Ph. harmeri*

Embryos and larvae of *Ph. harmeri* are relatively transparent and many aspects of their morphology can be easily observed with the light microscope using Nomarski interference contrast (Fig. 3). At 9°C the blastula stage is reached at about 6–8 hours post fertilization (hpf). Around 12 hpf a swimming blastula with a large blastocoel (*bc*) is formed (Fig. 3 A, A'). At 20 hpf the gastrulation process is initiated, which leads to the formation of the gastrula (Fig. 3 B, B') that displays a distinctive blastopore (*bp*), the archenteron (*ar*) and the anterior mesoderm (*am*). Subsequently, the embryo (including the archenteron) elongates along the A-P axis and the oral hood (*oh*) develops anteriorly leading to the formation of the early larval stage, at approximately 40 hpf (Fig. 3C, C'). In the posterior part of the early larval gut

the intestine (*in*) develops, which merges with the posterior ectoderm and forms an anus. Laterally to the intestine the first undifferentiated rudiments of the protonephridia are present (*pr* in Fig. 3C, C'). At 60 hpf the pre-tentacle larval stage is reached (Fig. 3D, D'), which possesses a tripartitioned digestive tract (with esophagus, *es*; stomach, *st* and intestine, *in*), an apical organ (*ao*), protonephridial rudiments (*pr*) and rudiments of the first three pairs of tentacles (*rt*). 3 days post fertilization (dpf) larvae can be already identified as early 6-tentacle actinotrocha (Fig. 3E, E') due to the presence of three pairs of well-defined tentacles (*te*). At this stage the larval protonephridia reach their definite branching form (*pn*, Fig. 3E), the rudiments of posterior mesoderm are morphologically distinguishable (*pm*, Fig. 3E) and the posterior telotroch starts to form around anal opening (*tt*, Fig. 3E'). At 5 dpf (Fig. 3 F, F') the telotroch is fully formed, while the posterior mesoderm forms rudiments of the posterior coelom compartment (metacoel), displacing the terminal organs of larval protonephridia to the more anterior position. The actinotrocha reach the 8-tentacle stage at 7 dpf (Fig. 3G, G'). At this stage the post-tentacular region of the body elongates and the metasomal sac, a rudiment of the body wall of the prospective adult worm, is formed (*ms*, Fig. 3G, G'). The metasomal sac at this stage appears as an ectodermal thickening located on the ventral side between tentacle bases and the telotroch. The thickening is concave and it is possible to discriminate between its opening (which will represent the anterior part of the adult trunk), and bottom (which after eversion will contribute to the posterior ampulla). The morphological details of the embryonic and larval development of *Ph. harmeri* are well described elsewhere (66, 72, 75–78, 80–82, 84, 85), therefore we did not examine further the embryonic and larval morphology.

## Hox gene expression

We did not detect expression of any of the Hox genes in blastula and gastrula stages (Additional File 1: Fig. S2). Expression of the anterior Hox gene *lab* is detected for the first time during development at the late 6-tentacle actinotrocha stage (Fig. 4A g and h). The gene is expressed in the ventro-posterior ectodermal domain, between the tentacle bases and the telotroch (black arrowhead, Fig. 4A g and h) and in the paired domains of the dorso-lateral posterior mesoderm (red arrowheads, Fig. 4A g and h). Both of the expression domains persist to the 8-tentacle actinotrocha stage (Fig. 4A i and j). At this developmental stage the ectodermal domain is part of the metasomal sac, where *lab* is expressed in the cells of the anterior and bottom portion of the sac (Fig. 5A and A').

The second anterior Hox gene, *pb*, is the earliest expressed among all Hox genes in *Ph. harmeri* as its expression can be already detected in the early larva stage (42 hpf) in the cells at the border of intestine and anus (red arrowheads, Fig. 4B a and b). This expression domain remains in the pre-tentacle stage (56 hpf, 4B c and d) and later it is restricted to the small lateral domains (Fig. 4B e and f), which in late 6-tentacle actinotrochae expands and the gene labels lateral, mesodermal sheet-like structures, extending along posterior ectoderm (red arrowheads Fig. 4B g and h). In 8-tentacle actinotrochae *pb* is expressed in two mesodermal stripes, which extends from the metasomal sac to the base of tentacles (Fig. 4B i and j, 5G) and corresponds to the telotroch flexor muscles, as described in the late actinotrocha of *Ph. harmeri* based on phalloidin staining (81).

*Hox3* expression is detected in the late 6-tentacle actinotrochae in an ectodermal domain between the tentacle bases and telotroch (black arrowhead, Fig. 4C g and h). At the 8-tentacle actinotrocha stage *hox3* is uniformly and exclusively expressed in the ectodermal cells of the metasomal sac (black arrowheads, Fig. 4C i and j; Fig 5B and B').

*Dfd* expression initiates only at the 8-tentacle actinotrocha stage (Fig. 4D i and j), where the gene is expressed in a small, anterior subset of the metasomal sac (Fig. 5C and C').

Transcripts of the gene *lox5* are detected first in the early 6-tentacle actinotrocha in posterior cells of the developing telotroch (green arrowhead, Fig. 4E e and f). Later on, *lox5* remains expressed in the telotroch, expanding its expression domain to the entire structure (green arrowheads, Fig. 4E g–j). Two additional expression domains of *lox5* also appear: the metasomal sac rudiment (black arrowhead, Fig. 4E h), which later encompasses the entire metasomal sac (black arrowheads Fig. 4E j and inset between i and j; Fig 5D and D'), and an asymmetric domain in the left ventro-lateral posterior mesoderm, located between metasomal sac, midgut and left body wall (red arrowheads Fig. 4 i and j and inset between g and h; Fig. 5H, I).

Expression of *antp* is not detected until the 8-tentacle actinotrocha stage. Transcripts of the gene are found in ectodermal cells around the opening of the metasomal sac (black arrowheads, Fig. 4F i and j; Fig. 5E and E'), which in a dorso-ventral view look like a ring on the ventral body surface between the tentacles base and the telotroch (Fig. 4F i).

Similarly, *lox4* expression is not detected until the 8-tentacle actinotrocha stage, where the gene is exclusively labeling the ring of the endodermal cells at the junction between stomach and intestine (blue arrowheads, Fig. 4G i and j; Fig. 5J).

The only posterior Hox gene, *post2*, is expressed from the early 6-tentacle actinotrocha (3dpf) in the telotroch (green arrowheads, Fig. 4H e and f), initially in the posterior portion of the organ but later on the expression domain is uniformly surrounding the anus (green arrowheads, Fig. 4H g–j). However, compared to *lox5* expression (which is also demarcating the telotroch), *post2* labels only the inner ring of epidermal cells of the organ (compare Fig. 4E g–j and 4H g–j) and not the entire structure. At the 8-tentacle actinotrocha stage the gene *post2* is additionally expressed in the small posterior portion of the metasomal sac (black arrowhead, Fig. 4H j; Fig 5F and F').

## Head-specific genes

In addition to the investigation of Hox genes we tested expression of several head-specific genes in the early larva (42 hpf) and advanced 8-tentacle actinotrocha. The genes, which expression we investigated, were *foxG* (also known as brain factor–1 or BF–1), *foxQ2*, *six3/6*, *otx* and *pax4/6*, all commonly considered as head markers (38, 45, 89, 94–98). One of the two *foxG* paralogues (see methods section for details), which expression we managed to detect, *foxGa*, is expressed in the early larva in the epidermal cells, from which the tentacular crown will develop (Fig. 6A–C). In 8-tentacle stage the gene expression was not detected (data not shown). We managed to clone one of two *foxQ2* paralogues, *FoxQ2b*, and detected its exclusive expression in the apical organ of both early and 8-tentacle stage larvae (Fig. 6D–G). *six3/6* is expressed in the early larvae in the apical organ, hood mesoderm, postoral ectoderm and in the anterior portion of the digestive tract (Fig. 6H and I) as previously reported(85). In 8-tentacle actinotrocha *six3/6* is expressed in the apical organ, hood muscles, some cells associated with dorso-anterior part of digestive system and in the stripe of mesodermal cells along ventral side of anterior digestive system (Fig. 6J–M). In early larvae *otx* is expressed in the apical organ, ventral preoral ectoderm, anterior portion of the digestive tract and in two spots in the ventro-posterior ectoderm, which lay in the prospective tentacular crown territory (Fig. 6N–P), following the expression pattern described before(85). In the 8-tentacle stage *otx* is expressed in the apical organ, rim of the oral hood, anterior portion of the digestive tract and in the small spots close to the tips of each tentacle (Fig. 6Q–U). *pax4/6* is expressed in the early larvae in the two stripes of cells which extend along ventral side of the larva, from mouth to about half of the body length (Fig. 6V, W) and which correspond with the position to the tentacular neurite bundles (78). In 8-tentacle stage *pax4/6* expression is detected along frontal side of each larval tentacle (Fig. 6X and Y), which also corresponds to the tentacular innervation(78). In general the head specific genes are broadly expressed in both developmental stages in the body structures anterior to the Hox-expressing territory (Fig. 6Z).

## Discussion

### Hox gene complement in Phoronida

Similar to the results of the investigation of *P. australis* genome, we identified 8 Hox genes in *Ph. harmeri*, which represent single copies of the conserved orthologues of the spiralian Hox genes (Figs. 1A, 2). Luo et al.(89) reported that *P. australis* lacks *scr* and *post1* orthologues and we also did not identify orthologues of those two genes in the transcriptome of *Ph. harmeri*, strengthening the idea they were already absent in the common ancestor of all phoronids.

In their paper Luo et al. (89) suggested that *scr*, which is expressed in the shell forming tissues of brachiopods(16, 40), might be lost in Phoronida due to the evolutionary reduction of the shell in this clade. Such interpretation is in accordance with paleontological data, as a fossil cambrian tommotiid, *Eccentrotheca* sp., which has been proposed as a stem group phoronid(99, 100), possessed a mineralized external tube-shaped skeleton. Recent studies are favoring a sister group relationship between phoronids and ectoprocts(52–55, 101), the latter of which possess a mineralized external skeleton, similarly as brachiopods. However, the Hox gene survey using degenerate polymerase chain reaction primers in ectoproct *Crisularia (Bugula) turrita* did not retrieve a *scr* sequence(102), which questions the possible correlation between loss of this gene and the reduction of shell secreting tissues in phoronid lineage. Yet, since it is difficult to recover the full hox complement with degenerate polymerase chain reaction, further studies on bryozoan hox genes, utilizing genomic or transcriptomic data, are needed to ascertain.

The gene that was identified as *lox2* by Luo et al.(89) in the genome of *P. australis* (and its orthologue in *Ph. harmeri*) was recovered in our gene orthology analysis as orthologue of *antp* (Fig. 2). Inspection of the phylogenetic tree available in Luo et al. shows that the assessment of the orthology of this gene was tentative, since the gene was actually placed outside of the well-defined clade of *lox2* in their analysis(89). Identification of this gene as *antp* instead of *lox2* is further supported by its position in the genome of *P. australis*, which corresponds to the *antp* position in the spiralian species with conserved, organised Hox cluster (Fig. 1A). Additionally, alignment of those phoronid genes with *antp* and *lox2* shows that they lack typical signatures of *lox2*(93) and instead are more similar to the *antp* sequence (Additional File 1: Fig. S1). Consequently, both phoronid species lack an orthologue of *lox2*, the absence, which is apparently shared by Phoronida with other Lophophorata(16, 89, 90, 102) as well as with some other Spiralia—i.e. Rotifera(34, 103) and Platyhelminthes(42, 104). *Lox2* was originally described from leeches(105, 106) to be later proposed as an evolutionary innovation of Lophotrochozoa ((93), sensu = Spiralia(107)).

However, its orthologues are so far identified only in annelids (e.g. (27, 46, 91, 93, 105, 106, 108)), nemerteans(89), molluscs (e.g. (30, 36, 41, 91, 93, 109–112)) and possibly kamptozoans(113) (however, in the latter the *lox2*-like sequence lacks most of the residues considered as *lox2* signature; Additional File 1: Fig. S1). This indicates that *lox2* evolved only after split of the common ancestor of those clades from remaining Spiralia and does not belong to the ancestral hox complement of all Spiralia(16). Whether absence of *lox2* in lophophorates is plesiomorphic or represents evolutionary reversal depends on the position of Lophophorata within Spiralia, which is still debatable and not fully resolved(52–55, 101).

## Hox genes in Phoronida do not show traces of collinear expression

When assuming the presence of a similar gene order in the Hox cluster of *Ph. harmeri* as in *P. australis* then the former does not show any traces of temporally (Fig. 4) or spatially (Fig. 7) collinear expression of Hox genes. This is in stark contrast to other Spiralia, in which at least some of the Hox genes shows staggered expression along A-P axis (Fig. 7). The lack of collinear Hox expression in phoronids is especially intriguing taking into account that *P. australis* has highly organized Hox cluster and collinear expression (especially in its temporal aspect) has been proposed as a main evolutionary factor responsible for conservation of Hox cluster organization(9, 11–16, 49). Therefore, either another mechanism is responsible for Hox cluster conservation in Phoronida or the two discussed phoronid species varies greatly in the cluster organization and/or Hox gene expression patterns.

6 out of 8 identified Hox genes are expressed in the metasomal sac (*pb* and *lox4* being the only two, which expression was not detected in the structure) and already at the stage of 8-tentacle actinotrocha some of those genes (*lab*, *dfd*, *antp*, *post2*) show differentiated expression in a particular region of the sac (Fig. 5), although without any clear pattern along the future A-P axis. However, it is possible that in the competent larvae (when the metasomal sac is a fully formed, elongated structure; (81, 82)), the expression of particular Hox genes is restricted to the different regions of the trunk rudiment and shows some traces of staggered expression along the future A-P axis of the worm body. Hence, the future investigation of the Hox expression in competent larvae and freshly metamorphosed juveniles can reveal spatial collinearity obliterated in the early stages of metasomal sac development or eventually confirm a lack of collinear Hox expression throughout entire development of phoronids.

## Germ layer-specific expression of Hox genes in Spiralia

Although Hox genes in Bilateria are predominantly expressed in the ectoderm (including nervous system) and their ectodermal expression is often considered as an ancestral feature(14, 28, 34), in various spiralian species certain Hox genes are also expressed in mesoderm, endoderm and clade-specific structures like chaetal sacs or shell fields (e.g. (16, 23, 24, 27, 29, 31, 35, 36, 39–41, 46); Tab. 1). Inclusion of the data on Hox expression in Phoronida gives some new insight into the understanding of the evolution of germ-layer specific Hox expression in Spiralia. *Ph. harmeri*, similarly as two investigated brachiopod species(16, 40), seems to lack expression of any of the Hox genes in the nervous system, the peculiarity that might actually represent an apomorphy of Lophophorata. Three of the Hox genes—*pb*, *hox3* and *dfd*were shown to be differentially expressed along the A-P axis in the mesoderm of brachiopod larvae (Fig. 7; (16)). Out of those three genes, only *pb* (which mesodermal expression is actually lacking in craniiformean *Novocrania anomala*(16)) is expressed mesodermally in *Ph. harmeri*, indicating that cooption of *hox3* and *dfd* into mesoderm patterning occurred after the split of brachiopods and phoronids. Interestingly, the expression of *pb* shows a remarkable similarity between *Ph. harmeri* and the rynchonelliformean brachiopod *Terebratalia transversa*. In both species the gene is initially expressed in a broader mesodermal domain, while later its expression is restricted to the single pair of muscles (telotroch flexors in *Ph. harmeri* and anterior shell adductor muscles in *T. transversa*(40)). Taking into account that in advanced phoronid larvae *pb*-positive cells are exclusively mesodermal, the cells at the border between hindgut and anus which express *pb* in early larval stages of *Ph. harmeri* might represent early precursors of the posterior mesoderm. This corresponds to the morphological observation that posterior mesoderm originates in early larvae from the posterior part of digestive tract after formation of protonephridial rudiments(76). Comparison of Hox gene expression across Spiralia (Tab. 1) allows the observation that *pb* is mesodermally expressed in many species and it is likely that mesodermal expression of *pb* represents an ancestral condition in Lophotrochozoa (*sensu stricto*(107)). Last but not least, the endodermal expression of *lox4* in *Ph. harmeri* is a peculiar and derived feature as this gene is expressed in other Spiralia in ectoderm, nervous system or mesoderm. In general, among investigated Spiralia, the Hox genes are rarely expressed in the endodermal structures (Tab 1.).

## Hox gene expression and the nature of actinotrocha larvae

We showed that in *Ph. harmeri* Hox genes are not expressed during embryogenesis, when the larval body is formed, but instead they are expressed mainly in prospective adult structures, namely in the metasomal sac (which will contribute to the adult trunk epidermis), posterior

mesoderm (which contributes to the mesodermal structures in the adult trunk), the small posterior portion of the endoderm (which during metamorphosis descent into the trunk rudiment forming loop of the U-shaped intestine) and the larval telotroch. In most of the investigated Bilateria, Hox genes are expressed already during early developmental stages and, if a biphasic life cycle is present, they are involved in the formation of both larval and adult body plans (e.g. (16, 27, 29–31, 40, 41, 45, 46, 48)). However, there are some animals that, similarly as phoronids, deviate from this general pattern. Specifically, in piliophoran nemerteans(37) and indirectly developing hemichordates(38), the larvae develop without expressing any of the Hox genes, which instead patterns only the adult body rudiment.

Two evolutionary processes have been proposed to explain these observations. According to the first hypothesis, based on the results from piliophoran nemerteans, the new larval form, a piliidium, was intercalated into to the ancestral life cycle of gradually developing nemertean(37, 45). This intercalation of a larval form caused that Hox genes patterning is only retained in the adult worm. In contrast the new larval form, which body axis is not aligned with the adult one (Fig. 7), uses another molecular mechanism to provide primary positional information to the cells of developing body(37, 45).

Another concept was proposed to explain phenomenon observed during larval development of a hemichordate *Schizocardium californicum*(38, 92). Although metamorphosis in this species is not so drastic(114) and the body axes of both stages are congruent, the larva develops without expression of any Hox genes. Instead, they are expressed only late during larval development and only in the most posterior region of the competent larvae, from which the trunk of the juvenile worm will develop during metamorphosis(38, 114). Because the larva expresses genes that are usually expressed in the bilaterian head throughout its body, the so-called “head larva”-hypothesis was

**Table 1.** Expression of Hox genes in spiralian species.

species	clade	reference	Hox gene												
			Lab	Pb	Hox3	Dfd	Scr	Lox5	Antp	Lox4	Lox2	Post2	Post1		
<i>Phoronopsis harmeri</i>	Phoronida	this study	ectoderm, mesoderm	mesoderm	ectoderm	ectoderm	gene absent	ectoderm, mesoderm	ectoderm	ectoderm	endoderm	gene absent	ectoderm	gene absent	
<i>Terebratalia transversa</i>	Brachiopoda	(16, 40)	chaetal sac	ectoderm, mesoderm	ectoderm, mesoderm	ectoderm, mesoderm	shell field	ectoderm	ectoderm	ectoderm	ectoderm	gene absent	ectoderm	chaetal sac	
<i>Novocrania anomala</i>	Brachiopoda	(16)	chaetal sac	ectoderm	ectoderm, mesoderm	ectoderm, mesoderm	shell field	ectoderm	ectoderm	unknown	gene absent	unknown	gene absent		
<i>Capitella teleta</i>	Annelida	(46)	ectoderm, nervous system	ectoderm, nervous system	ectoderm, nervous system	ectoderm, nervous system	ectoderm, nervous system	ectoderm, nervous system	ectoderm, nervous system	ectoderm, nervous system	ectoderm, nervous system	ectoderm, nervous system	ectoderm, nervous system	chaetal sac	
<i>Alitta virens</i>	Annelida	(27, 31)	ectoderm, chaetal sac, nervous system	ectoderm, mesoderm	ectoderm	ectoderm, nervous system	ectoderm, nervous system	ectoderm, nervous system	ectoderm, nervous system	ectoderm, nervous system, mesoderm	ectoderm, nervous system	ectoderm, nervous system, mesoderm, endoderm	ectoderm, nervous system, mesoderm, endoderm	chaetal sac	
<i>Chaetopterus variopedatus</i>	Annelida	(23)	ectoderm, nervous system	ectoderm, nervous system, mesoderm	ectoderm, nervous system	ectoderm, nervous system	ectoderm, nervous system	unknown	unknown	unknown	unknown	unknown	unknown	unknown	
<i>Acanthochitona crinita</i>	Mollusca	(35, 36)	ectoderm, mesoderm	mesoderm	mesoderm	ectoderm, mesoderm	mesoderm	ectoderm, mesoderm	mesoderm	ectoderm, mesoderm	mesoderm	ectoderm, mesoderm	mesoderm	ectoderm, mesoderm	gene absent
<i>Antalis entalis</i>	Mollusca	(39)	shell field, nervous system	ectoderm, nervous system	ectoderm, shell field, nervous system, mesoderm	nervous system, mesoderm	nervous system, mesoderm	ectoderm, nervous system	gene absent	ectoderm, nervous system, mesoderm	unknown	ectoderm, nervous system	ectoderm, nervous system	ectoderm, shell field, nervous system	
<i>Lottia goschimai</i>	Mollusca	(41)	shell field, nervous system	shell field, nervous system	shell field, nervous system	shell field, nervous system	shell field, nervous system	shell field, nervous system	nervous system	shell field, nervous system	ectoderm, shell field, nervous system	shell field, nervous system	nervous system	nervous system	
<i>Brachionus manjavacas</i>	Rotifera	(34)	gene absent (?)	nervous system	nervous system	nervous system	unknown	nervous system	gene absent (?)	gene absent (?)	gene absent (?)	gene absent (?)	gene absent (?)	gene absent (?)	

proposed which states that the larval body represents the homologue of only the head region of the future animal, while the trunk is added later during post-larval development(38). It has been proposed that ancestrally in Bilateria Hox genes were involved only in the patterning of the trunk, while head developed from the anterior, Hox-free region, the condition, which is still retained in numerous bilaterian lineages (38, 45, 89, 94, 95). That would explain why tornaria, as a larva composed solely of the head, develops without expression of the Hox genes, which become activated only after the onset of trunk development and pattern only the adult body(38).

Both of those hypotheses (intercalation and “head-larva”) might be applied to explain the Hox expression patterns we observed in *Ph. harmeri*. According to the first hypothesis, the specific actinotrocha larva would represent an evolutionary novelty in the life cycle of phoronids, which was intercalated in the phoronid lineage and that is why it is not patterned by an ancestral Hox gene system. Such idea is supported by the fact, that the actinotrocha body plan does not bear obvious homology to those of any other spiralian larvae(80, 115, 116). Additionally, similarly as in case of pilidium, most of the larval tissues are lost during drastic metamorphosis event and larval A-P axis is not aligned with the juvenile one(60, 72, 77, 81, 82). Moreover, the actinotrocha is lacking in *P. ovalis*(60), which is the sister species to all remaining phoronids(62–64), suggesting that the actinotrocha was not even present in the most recent ancestor of all Phoronida, but instead appeared during the evolution after split between *P. ovalis* and the remaining phoronids.

On the other hand, from the morphological point of view, the tentacles of actinotrocha larvae are corresponding to the tentacles of the lophophore in the adult worm ((73, 82, 116); Fig. 1C), and the adult lophophore exhibits the molecular signature of a bilaterian head(89). As tentacles are positioned posteriorly in the early actinotrocha, one can conclude that on a morphological basis the actinotrocha is mostly composed of the head region. Following such interpretation, all of the Hox genes are expressed in the structures, which will contribute to the adult trunk tissues but are not expressed in the developing future head (and hence in the largest portion of the larval body). Accordingly, based on a body region specific transcriptome, it has been demonstrated that in adults of *P. australis* Hox genes are not expressed in the lophophore, while their expression is detectable in the trunk and posterior ampulla(89). Similarly, in rhynchonelliformean and craniiformean brachiopods none of the Hox genes are expressed in the larval anterior lobe(16, 40), which contributes to the lophophore after metamorphosis(40, 116). A lack of Hox expression in the adult lophophore tissue (as opposed to the remaining body regions) was also shown for the linguliformean *Lingula anatina*, based on the tissue specific transcriptomics(89). Additionally, our study shows that two of the Hox genes (*lox5* and *post2*) are expressed in the telotroch, which represent a truly larval structure, that is lost during metamorphosis(73, 82), therefore Hox genes are indeed, albeit to only a limited degree, involved in the larval development. Hox gene expression in the larval telotroch is a result of telotroch representing a truly “posterior” structure, which belongs to the post-head body region even in the earliest, “head dominated” actinotrocha. The “head larva” interpretation is additionally strengthened by our results of the expression of several head-specific genes in *Ph. harmeri*. Those genes are broadly expressed in the early larvae and 8-tentacle stage, but only in the structures located anteriorly to the Hox-expressing territory (Fig. 7Z), resembling conditions in developing tornaria(38).

It is, however, important to stress that the hypotheses of the “larval intercalation” and “head larva” are not mutually exclusive. It is possible that the actinotrocha was intercalated into life cycle of Phoronida after split of *P. ovalis* lineage and that it originated from the precocious development of the head structures or delayed development of the adult trunk.

## Conclusions

We identified 8 Hox genes (*lab*, *pb*, *hox3*, *dfd*, *lox5*, *antp*, *lox4* and *post2*) in the transcriptome of *Phoronopsis harmeri* showing that Hox complement in this species is the same as in *Phoronis australis*, which genome was recently described. Both phoronid species lost genes *scr* and *post1*, which belong to the ancestral Hox cluster of Spiralia. Phoronids, similarly as closely related brachiopods and bryozoans, lack gene *lox2*. This absence indicates that *lox2* evolved in the common ancestor of annelids, nemerteans molluscs and possibly entoprocts, while its lack is plesiomorphic among Spiralia.

Hox gene expression is activated late during the development of *Ph. harmeri*. The larval body develops without expressing any of the Hox genes, which instead are expressed in the tissues of the prospective rudiment of the adult worm and in the telotroch. Such expression might result either from the intercalation of actinotrocha larva into the ancestral life cycle of phoronids or from the fact that the early larva of phoronids represents a “head larva”, which develops without expressing any Hox genes. Our investigation of expression of head-specific genes confirms, that most of the larval body exhibits head-specific gene expression profile. Those two explanations are not mutually exclusive and we propose that actinotrocha was intercalated to the phoronid life cycle by precocious development of the anterior structures or by delayed development of the trunk rudiment in the ancestral phoronid larva. Such hypothesis can be tested in future by investigation of the Hox gene expression during the development of *Phoronis ovalis*, a sister group to all remaining Phoronida, which lacks the actinotrocha larva stage and develops through a creeping, worm-like larva.

## Methods

### Animal collection and fixation

Gravid females of *Ph. harmeri* Pixell, 1912 were collected in Bodega Bay (38° 18' 51.9012" N 123° 3' 12.3012" W) in California during April and May. Although the California population of *Phoronopsis* is sometimes referred to as separate species *Ph. viridis*(84, 117), we followed the widely accepted interpretation of Joan Rattenbury Marsden, that *Ph. viridis* is in fact a younger synonym of *Ph. harmeri*(118). The

animals were opened in the laboratory and eggs (fertilized during dissection by sperm stored in the coelom of females) were transferred to the clean cultures with filtered sea water (as described in e.g. (78, 84, 85)). Embryos are initially lecithotrophic, but, after formation of the gut, larvae require feeding, hence concentrated *Rhodomonas* or *Rhinomonas* algae were added to the cultures. Water in the larval cultures was exchanged every 2–3 days, followed by the addition of fresh algae. Embryos and larvae on desired developmental stages were relaxed with 8% MgCl<sub>2</sub>, fixed in 3.7% formaldehyde and subsequently washed in phosphate buffer with 0.1% Tween–20. Fixed animals were stored in 100% methanol in –20° C.

## Hox genes identification and orthology assessment

We searched transcriptome of *Ph. harmeri* (<https://doi.org/10.18710/89HNMI>) with reciprocal TBLASTN using 8 Hox protein sequences from *Phoronis australis*. The top 10 homeodomain-containing BLAST hits from each search were blasted back against the protein database at NCBI (<http://blast.ncbi.nlm.nih.gov/>) and if any Hox gene was a top reciprocal hit, the sequence was considered to be a putative Hox gene. We identified 8 sequences, which passed this reciprocal test and translated them to the proteins using CLC Main Workbench 7. Orthology of particular phoronid Hox genes was assessed based on the results of phylogenetic analysis. In order to construct alignment, amino acid sequences of Hox transcription factors and nucleotide sequences of Hox genes from several spiralian species were obtained from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>), ENSEMBL genome data base (<https://www.ensembl.org/index.html>) and website of Marine Genomics Unit of Okinawa Institute of Science and Technology (<http://marinegenomics.oist.jp>). For the nucleotide sequences ORF were determined based on BLAST results at NCBI and sequences were translated into proteins using CLC Main Workbench 7. All spiralian sequences used in this study with their source and accession number are provided in the Additional File 1: Table S1.

The spiralian Hox protein sequences, including putative Hox genes of *Ph. harmeri*, were aligned in CLC Main Workbench 7 and then the alignment was manually trimmed to contain the conserved homeodomain (60 amino acids), five aa 5' of the homeodomain, and eight aa 3' of the homeodomain (the trimmed alignment in FASTA format is available in the Additional File 1). ProtTest3 (119) was used to determine the best-fitting substitution model (JTT+I+G). Bayesian analysis was run in MrBayes v3.2.6 (120, 121) with the JTT+I+G substitution model in two independent runs, each with 4 Markov chains (three heated and one cold) with 3,000,000 generations sampled every 500 generations. The *Gsx* sequence of *Capitella teleta* was designated as outgroup. The first 25% of samples were discarded as burn-in and the remaining trees were used to calculate posterior probability values and construct consensus tree, which was visualized and adjusted in FigTree v1.4.3.

All new sequences obtained and identified in this study were uploaded to the GenBank (accession numbers MN443105–MN443114).

## Gene cloning and probe synthesis

Fragments of each Hox gene were amplified from cDNA libraries from mixed larval and adult tissues using gene specific primers (provided in Additional file1: Table S2) designed in MacVector 11.0.4 based on the sequences found in the transcriptome. PCR products were cloned into pGEM-T Easy vectors (Promega, USA) and then transformed into competent *Escherichia coli* cells. Plasmid DNA was isolated and sequenced in both forward and reverse directions using T7 and SP6 primers. Labeled antisense RNA probes were transcribed from linearized DNA using digoxigenin–11-UTP (Roche, USA) according to the manufacturer's instructions.

## Head-specific genes

Additionally, we searched transcriptome of *Ph. harmeri* in order to identify head-specific genes—*foxG*, *foxQ2* and *pax6*. We identified two potential paralogues of both *foxG* and *foxQ2* and called them correspondingly *foxGa*, *foxGb*, *foxQ2a* and *foxQ2b*. The vertebrate genes *pax4* and *pax6* originated through the vertebrate-specific duplication(122–124) and accordingly their protostome orthologue should be called *pax4/6*, same as in case of the other invertebrate Pax genes (*pax3/7*, *pax1/9* and *pax2/5/8*). Therefore, despite the fact that *pax6* is often used to refer to this gene in other protostomes, we decided to name the identified gene *pax4/6* in order to stress its co-orthology to both vertebrate genes. We run phylogenetic analyses to make sure that identified phoronid genes truly represent orthologues of the genes of interest. For Fox genes we aligned phoronid Fox sequences with published alignment of Fox domains (98), while for Pax4/6 we assembled alignment from sequences available in genBank (see Additional file1 for alignments and list of used sequences). The alignments were trimmed in trimAl software(125) (using the gappyout option) and the phylogenetic trees were calculated with FastTree v2.1(126) (using the LG amino acid substitution model). The obtained trees were visualized and adjusted in FigTree v1.4.3 and confirmed identity of all identified phoronid genes (Additional file1: Figs. S3 and S4). Sequences of phoronid Fox genes and *pax4/6* were deposited in the GenBank (accession numbers MN734372–MN734376). Probes against head-specific genes were synthesized in the same way as described for Hox genes

(although we did not manage to clone *foxQ2a*).. Additionally we used the same dig-labeled probes against *otx* and *six3/6* as in Andrikou et al. (85) (see method section in there for more details).

## In situ hybridization and light microscopy

Single whole-mount in situ hybridization was performed following an established protocol(127) with proteinase K digestion time of 2 minutes. Probes were hybridized at a concentration of 1ng/μl at 67°C for approximately 72 h, detected with anti-digoxigenin-AP antibody in 1:5000 concentration in blocking buffer and visualized with nitroblue tetrazolium chloride and 5-bromo–4-chloro–3-indolyl phosphate. Embryos and larvae were mounted in 70% glycerol and examined with Zeiss AxioCam HRc connected to a Zeiss AxioScope Ax10 using bright-field Nomarski optics.

## Image processing and figure preparation

Light micrographs were adjusted in Adobe Photoshop CS6 for contrast and assembled in Adobe Illustrator CS6. All figures and drawings were prepared in Adobe Illustrator CS6.

## Declarations

### *Ethics approval and consent to participate*

Studies of phoronids do not require ethics approval or consent to participate.

### *Consent for publication*

Not applicable.

### *Availability of data and material*

Sequences generated and analyzed in this study have been deposited in NCBI's GenBank database under accession numbers MN443105–MN443114 and MN734372–MN734376. All remaining data generated or analyzed during this study are included in this published article or its supplementary materials.

### *Competing interests*

The authors declare that they have no competing interests.

### *Funding*

Research was supported by the European Research Council Community's Framework Program Horizon 2020 (2014–2020) ERC grant agreement 648861 and the Norwegian Research Council FRIPRO grant 815194 to AH.

### *Authors' contributions*

Both authors conceived the study, analyzed data and contributed to writing. LG took part in sample collection, conducted gene search and orthology assessments, performed in situ hybridization, arranged figures and drafted the manuscript. Both authors read and approved the final manuscript.

### *Acknowledgements*

We thank Yale Passamaneck, who contributed with phoronid collection and spawning. Chris Lowe from Hopkins Marine Station of Stanford University is greatly acknowledged for hosting LG in his lab in April – May 2018 and for sharing a transcriptome of *Ph. harmeri* before it was made public. We also would like to thank Carmen Andrikou and Andrea Orús Alcalde as well as Chris Lowe and Paul Bump for contributing with animal collection in April – May 2018. Carmen Andrikou and Chris Lowe are additionally greatly acknowledged for reading and commenting on the early version of the manuscript and providing probes against *otx* and *six3/6*. We also thank Nadya Rimskaya-Korsakova, who cloned and prepared the probe against *pax4/6*.

## References

1. Yanze N, Spring J, Schmidli C, Schmid V. Conservation of Hox/ParaHox-related genes in the early development of a cnidarian. *Dev Biol.* 2001;236(1):89-98.
2. Finnerty JR. Cnidarians reveal intermediate stages in the evolution of Hox clusters and axial complexity. *Am Zool.* 2001;41(3):608-20.
3. Ryan JF, Pang K, Program NCS, Mullikin JC, Martindale MQ, Baxeavanis AD. The homeodomain complement of the ctenophore *Mnemiopsis leidyi* suggests that Ctenophora and Porifera diverged prior to the ParaHoxozoa. *Evodevo.* 2010;1(1):9.
4. He SN, del Viso F, Chen CY, Ikmi A, Kroesen AE, Gibson MC. An axial Hox code controls tissue segmentation and body patterning in *Nematostella vectensis*. *Science.* 2018;361(6409):1377.
5. McGinnis W, Krumlauf R. Homeobox genes and axial patterning. *Cell.* 1992;68(2):283-302.
6. Pearson JC, Lemons D, McGinnis W. Modulating Hox gene functions during animal body patterning. *Nat Rev Genet.* 2005;6(12):893-904.
7. Lemons D, McGinnis W. Genomic evolution of Hox gene clusters. *Science.* 2006;313(5795):1918-22.
8. Krumlauf R. Hox Genes in Vertebrate Development. *Cell.* 1994;78(2):191-201.
9. Garcia-Fernandez J. The genesis and evolution of homeobox gene clusters. *Nat Rev Genet.* 2005;6(12):881-92.
10. Mallo M, Wellik DM, Deschamps J. Hox genes and regional patterning of the vertebrate body plan. *Dev Biol.* 2010;344(1):7-15.
11. Ferrier DEK, Minguillon C. Evolution of the Hox/ParaHox gene clusters. *Int J Dev Biol.* 2003;47(7-8):605-11.
12. Duboule D. The rise and fall of Hox gene clusters. *Development.* 2007;134(14):2549-60.
13. 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.* 1994:135-42.
14. Deutsch J, Le Guyader H. The neuronal zootype. An hypothesis. *Cr Acad Sci III-Vie.* 1998;321(9):713-9.
15. Monteiro AS, Ferrier DEK. Hox genes are not always Colinear. *Int J Biol Sci.* 2006;2(3):95-103.
16. Schiemann SM, Martin-Duran JM, Børve A, Vellutini BC, Passamaneck YJ, Hejnal 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-E22.
17. Lewis EB. A gene complex controlling segmentation in *Drosophila*. *Nature.* 1978;276(5688):565-70.
18. Beeman RW. A Homeotic Gene-Cluster in the Red Flour Beetle. *Nature.* 1987;327(6119):247-9.
19. Graham A, Papalopulu N, Krumlauf R. The Murine and Drosophila Homeobox Gene Complexes Have Common Features of Organization and Expression. *Cell.* 1989;57(3):367-78.
20. Kenyon C, Wang B. A Cluster of Antennapedia-Class Homeobox Genes in a Nonsegmented Animal. *Science.* 1991;253(5019):516-7.
21. Arenas-Mena C, Martinez P, Cameron RA, Davidson EH. Expression of the Hox gene complex in the indirect development of a sea urchin. *Proc Natl Acad Sci USA.* 1998;95(22):13062-7.
22. Arenas-Mena C, Cameron AR, Davidson EH. Spatial expression of Hox cluster genes in the ontogeny of a sea urchin. *Development.* 2000;127(21):4631-43.
23. Irvine SQ, Martindale MQ. Expression patterns of anterior Hox genes in the polychaete *Chaetopterus*: Correlation with morphological boundaries. *Dev Biol.* 2000;217(2):333-51.
24. 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):508-21.
25. Lee PN, Callaerts P, de Couet HG, Martindale MQ. Cephalopod Hox genes and the origin of morphological novelties. *Nature.* 2003;424(6952):1061-5.
26. Aronowicz J, Lowe CJ. Hox gene expression in the hemichordate *Saccoglossus kowalevskii* and the evolution of deuterostome nervous systems. *Integr Comp Biol.* 2006;46(6):890-901.
27. Kulakova M, Bakalenko N, Novikova E, Cook CE, Eliseeva E, Steinmetz PRH, 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.
28. Hejnal A, Martindale MQ. Coordinated spatial and temporal expression of Hox genes during embryogenesis in the acoel *Convolutiloba longifissura*. *BMC Biol.* 2009;7.
29. 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):523-30.
30. 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):161-72.

31. Bakalenko NI, Novikova EL, Nesterenko AY, Kulakova MA. Hox gene expression during postlarval development of the polychaete *Alitta virens*. *EvoDevo*. 2013;4.
32. Janssen R, Eriksson BJ, Tait NN, Budd GE. Onychophoran Hox genes and the evolution of arthropod Hox gene expression. *Front Zool*. 2014;11.
33. 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):224-9.
34. Fröblius AC, Funch P. Rotiferan Hox genes give new insights into the evolution of metazoan bodyplans. *Nat Commun*. 2017;8.
35. 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.
36. 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.
37. 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.
38. 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.
39. 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).
40. Gąsiorowski L, Hejnoj A. Hox gene expression in postmetamorphic juveniles of the brachiopod *Terebratalia transversa*. *EvoDevo*. 2019;10:1.
41. Huan P, Wang Q, Tan S, Liu B. Dorsoventral dissociation of Hox gene expression underpins the diversification of molluscs. *BioRxiv*. 2019;603092.
42. Currie KW, Brown DD, Zhu S, Xu C, Voisin V, Bader GD, et al. HOX gene complement and expression in the planarian *Schmidtea mediterranea*. *EvoDevo*. 2016;7:7.
43. Seo HC, Edvardsen RB, Maeland AD, Bjordal M, Jensen MF, Hansen A, et al. Hox cluster disintegration with persistent anteroposterior order of expression in *Oikopleura dioica*. *Nature*. 2004;431(7004):67-71.
44. Ikuta T, Yoshida N, Satoh N, Saiga H. *Ciona intestinalis* Hox gene cluster: Its dispersed structure and residual colinear expression in development. *Proc Natl Acad Sci U S A*. 2004;101(42):15118-23.
45. Hiebert LS, Maslakova SA. Expression of Hox, Cdx, and Six3/6 genes in the hoplonemertean *Pantionemertes californiensis* offers insight into the evolution of maximally indirect development in the phylum Nemertea. *EvoDevo*. 2015;6.
46. Fröblius 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).
47. Mooi R, David B. Radial Symmetry, the Anterior/Posterior Axis, and Echinoderm Hox Genes. *Annu Rev Ecol Evol S*. 2008;39:43-62.
48. Pascual-Anaya J, Adachi N, Alvarez S, Kuratani S, D'Aniello S, Garcia-Fernandez J. Broken colinearity of the amphioxus Hox cluster. *EvoDevo*. 2012;3(1):28.
49. Pascual-Anaya J, Sato I, Sugahara F, Higuchi S, Paps J, Ren YD, et al. Hagfish and lamprey Hox genes reveal conservation of temporal colinearity in vertebrates. *Nat Ecol Evol*. 2018;2(5):859-66.
50. Emig C. Un nouvel embranchement: les Lophophorates. *Bull Soc Zool France*. 1977;102:341-4.
51. Emig C. On the origin of the Lophophorata. *J Zool Syst Evol Res*. 1984;22(2):91-4.
52. Laumer CE, Bekkouche N, Kerbl A, Goetz F, Neves RC, Sørensen MV, et al. Spiralian phylogeny informs the evolution of microscopic lineages. *Curr Biol*. 2015;25(15):2000-6.
53. Laumer CE, Fernandez R, Lemer S, Combosch D, Kocot KM, Riesgo A, et al. Revisiting metazoan phylogeny with genomic sampling of all phyla. *Proc Biol Sci*. 2019;286(1906):20190831.
54. 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):312-8 e3.
55. Zverkov OA, Mikhailov KV, Isaev SV, Rusin LY, Popova OV, Logacheva MD, et al. Dicyemida and Orthonectida: Two Stories of Body Plan Simplification. *Front Genet*. 2019;10:443.
56. Halanych KM, Bacheller JD, Aguinaldo AMA, Liva SM, Hillis DM, Lake JA. Evidence from 18s Ribosomal DNA That the Lophophorates Are Protostome Animals. *Science*. 1995;267(5204):1641-3.

57. Dunn CW, Hejnal A, Matus DQ, Pang K, Browne WE, Smith SA, et al. Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature*. 2008;452(7188):745-U5.
58. Emig CC. Embryology of Phoronida. *American Zoologist*. 1977;17(1):21-37.
59. Santagata S. Phoronida. *Evolutionary Developmental Biology of Invertebrates 2*: Springer; 2015. p. 231-45.
60. Silén L. Developmental biology of Phoronidea of the Gullmar Fiord area (west coast of Sweden). *Acta Zool*. 1954;35(3):215-57.
61. Hermann K. Phoronida. In: Harrison FW, Woollacott RM, editors. *Microscopic anatomy of invertebrates volume 13: lophophorates, Entoprocta, and Cycliophora*. New York: Wiley-Liss; 1997. p. 207-35.
62. Temereva EN, Chichvarkhin A. A new phoronid species, *Phoronis embryolabi*, with a novel type of development, and consideration of phoronid taxonomy and DNA barcoding. *Invertebrate Systematics*. 2017;31(1):65-84.
63. Santagata S, Cohen BL. Phoronid phylogenetics (Brachiopoda; Phoronata): evidence from morphological cladistics, small and large subunit rDNA sequences, and mitochondrial cox1. *Zool J Linn Soc*. 2009;157(1):34-50.
64. Hirose M, Fukiage R, Katoh T, Kajihara H. Description and molecular phylogeny of a new species of *Phoronis* (Phoronida) from Japan, with a redescription of topotypes of *P. ijimai* Oka, 1897. *Zookeys*. 2014(398):1-31.
65. Nezlin LP. The development of monoaminergic elements of the nervous system in the actinotroch - planktonic larvae of *Phoronopsis harmeri*. *Zh Evol Biokhim Fiziol*. 1988;24(1):76-80.
66. Temereva EN, Tsitrin EB. Development and organization of the larval nervous system in *Phoronopsis harmeri*: new insights into phoronid phylogeny. *Front Zool*. 2014;11:3, 1-25.
67. Bartolomaeus T. Ultrastructure and Relationship between Protonephridia and Metanephridia in *Phoronis muelleri* (Phoronida). *Zoomorphology*. 1989;109(2):113-22.
68. Hayschmidt A. Catecholamine-Containing, Serotonin-Like, and Fmrfamide-Like Immunoreactive Neurons and Processes in the Nervous-System of the Early Actinotroch Larva of *Phoronis vancouverensis* (Phoronida) - Distribution and Development. *Can J Zool*. 1990;68(7):1525-36.
69. Hayschmidt A. Distribution of Catecholamine-Containing, Serotonin-Like and Neuropeptide Fmrfamide-Like Immunoreactive Neurons and Processes in the Nervous-System of the Actinotroch Larva of *Phoronis muelleri* (Phoronida). *Cell Tissue Res*. 1990;259(1):105-18.
70. Lacalli TC. Structure and Organization of the Nervous System in the Actinotroch Larva of *Phoronis vancouverensis*. *Philos T Roy Soc B*. 1990;327(1244):655-&.
71. Bartolomaeus T. Ultrastructure and formation of the body cavity lining in *Phoronis muelleri* (Phoronida, Lophophorata). *Zoomorphology (Berlin)*. 2001;120(3):135-48.
72. Santagata S, Zimmer RL. Comparison of the neuromuscular systems among actinotroch larvae: systematic and evolutionary implications. *Evol Dev*. 2002;4(1):43-54.
73. Santagata S. Structure and metamorphic remodeling of the larval nervous system and musculature of *Phoronis pallida* (Phoronida). *Evol Dev*. 2002;4(1):28-42.
74. Santagata S. Larval development of *Phoronis pallida* (Phoronida): implications for morphological convergence and divergence among larval body plans. *J Morphol*. 2004;259(3):347-58.
75. Temereva EN, Malakhov VV. Development of excretory organs in *Phoronopsis harmeri* (Phoronida): From protonephridium to nephromixium. *Zool Zh*. 2006;85(8):915-24.
76. Temereva EN, Malakhov VV. Embryogenesis and larval development of *Phoronopsis harmeri* Pixell, 1912 (Phoronida): dual origin of the coelomic mesoderm. *Invertebr Reprod Dev*. 2007;50(2):57-66.
77. Temereva EN. The digestive tract of actinotroch larvae (Lophotrochozoa, Phoronida): anatomy, ultrastructure, innervations, and some observations of metamorphosis. *Can J Zool*. 2010;88(12):1149-68.
78. Temereva E, Wanninger A. Development of the nervous system in *Phoronopsis harmeri* (Lophotrochozoa, Phoronida) reveals both deuterostome- and trochozoan-like features. *BMC Evol Biol*. 2012;12:27pp.
79. Temereva EN, Malakhov VV. Embryogenesis in phoronids. *Invertebr Biol*. 2012;9(1):1-39.
80. Temereva EN. Ventral Nerve Cord in *Phoronopsis harmeri* Larvae. *J Exp Zool Part B*. 2012;318B(1):26-34.
81. Temereva EN, Tsitrin EB. Development, organization, and remodeling of phoronid muscles from embryo to metamorphosis (Lophotrochozoa: Phoronida). *BMC Dev Biol*. 2013;13:14.
82. Temereva EN, Malakhov VV. Metamorphic remodeling of morphology and the body cavity in *Phoronopsis harmeri* (Lophotrochozoa, Phoronida): the evolution of the phoronid body plan and life cycle. *BMC Evol Biol*. 2015;15:229.

83. Temereva EN. Ground plan of the larval nervous system in phoronids: Evidence from larvae of viviparous phoronid. *Evol Dev.* 2017;19(4):171-89.
84. Rattenbury JC. The embryology of *Phoronopsis viridis*. *J Morphol.* 1954;95(2):289-349.
85. Andrikou C, Passamaneck YJ, Lowe CJ, Martindale MQ, Hejnal A. Molecular patterning during the development of *Phoronopsis hameri* reveals similarities to rhynchonelliform brachiopods. *EvoDevo.* 2019;in press.
86. Pennerstorfer M, Scholtz G. Early cleavage in *Phoronis muelleri* (Phoronida) displays spiral features. *Evol Dev.* 2012;14(6):484-500.
87. Freeman G. The Bases for and Timing of Regional Specification during Larval Development in *Phoronis*. *Dev Biol.* 1991;147(1):157-73.
88. Freeman G, Martindale MQ. The origin of mesoderm in phoronids. *Dev Biol.* 2002;252(2):301-11.
89. Luo YJ, Kanda M, Koyanagi R, Hisata K, Akiyama T, Sakamoto H, et al. Nemertean and phoronid genomes reveal lophotrochozoan evolution and the origin of bilaterian heads. *Nat Ecol Evol.* 2018;2(1):141-51.
90. Luo YJ, Takeuchi T, Koyanagi R, Yamada L, Kanda M, Khalturina M, et al. The *Lingula* genome provides insights into brachiopod evolution and the origin of phosphate biomineralization. *Nature Communications.* 2015;6.
91. Simakov O, Marlétaz F, Cho SJ, Edsinger-Gonzales E, Havlak P, Hellsten U, et al. Insights into bilaterian evolution from three spiralian genomes. *Nature.* 2013;493(7433):526-31.
92. Hejnal A, Vellutini BC. Larval Evolution: I'll Tail You Later ... *Curr Biol.* 2017;27(1):R21-R4.
93. de Rosa R, Grenier JK, Andreeva T, Cook CE, Adoutte A, Akam M, et al. Hox genes in brachiopods and priapulids and protostome evolution. *Nature.* 1999;399(6738):772-6.
94. Lowe CJ, Wu M, Salic A, Evans L, Lander E, Stange-Thomann N, et al. Anteroposterior patterning in hemichordates and the origins of the chordate nervous system. *Cell.* 2003;113(7):853-65.
95. Steinmetz PRH, Urbach R, Posnien N, Eriksson J, Kostyuchenko RP, Brena C, et al. Six3 demarcates the anterior-most developing brain region in bilaterian animals. *EvoDevo.* 2010;1.
96. Santagata S, Resh C, Hejnal A, Martindale MQ, Passamaneck YJ. Development of the larval anterior neurogenic domains of *Terebratalia transversa* (Brachiopoda) provides insights into the diversification of larval apical organs and the spiralian nervous system. *EvoDevo.* 2012;3.
97. Kumamoto T, Hanashima C. Evolutionary conservation and conversion of Foxg1 function in brain development. *Development Growth & Differentiation.* 2017;59(4):258-69.
98. Fritzenwanker JH, Gerhart J, Freeman RM, Lowe CJ. The Fox/Forkhead transcription factor family of the hemichordate *Saccoglossus kowalevskii*. *EvoDevo.* 2014;5.
99. 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):171-4.
100. 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;54:253-86.
101. Bleidorn C. Recent progress in reconstructing lophotrochozoan (spiralian) phylogeny. *Org Divers Evol.* 2019:1-10.
102. Passamaneck YJ, Halanych KM. Evidence from Hox genes that bryozoans are lophotrochozoans. *Evol Dev.* 2004;6(4):275-81.
103. Flot JF, Hespeels B, Li X, Noel B, Arkhipova I, Danchin EGJ, et al. Genomic evidence for ameiotic evolution in the bdelloid rotifer *Adineta vaga*. *Nature.* 2013;500(7463):453-7.
104. Wasik K, Gurtowski J, Zhou X, Ramos OM, Delás MJ, Battistoni G, et al. Genome and transcriptome of the regeneration-competent flatworm, *Macrostomum lignano*. *Proc Natl Acad Sci USA.* 2015;112(40):12462-7.
105. Wysocka-Diller JW, Aisemberg GO, Baumgarten M, Levine M, Macagno ER. Characterization of a Homolog of Bithorax-Complex Genes in the Leech *Hirudo medicinalis*. *Nature.* 1989;341(6244):760-3.
106. Nardellaefliger D, Shankland M. Lox2, a Putative Leech Segment Identity Gene, Is Expressed in the Same Segmental Domain in Different Stem-Cell Lineages. *Development.* 1992;116(3):697-+.
107. Hejnal A. A twist in time—the evolution of spiral cleavage in the light of animal phylogeny. *Integr Comp Biol.* 2010;50(5):695-706.
108. 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):271-81.
109. Albertin CB, Simakov O, Mitros T, Wang ZY, Pungor JR, Edsinger-Gonzales E, et al. The octopus genome and the evolution of cephalopod neural and morphological novelties. *Nature.* 2015;524(7564):220-+.
110. Belcaid M, Casaburi G, McAnulty SJ, Schmidbaur H, Suria AM, Moriano-Gutierrez S, et al. Symbiotic organs shaped by distinct modes of genome evolution in cephalopods. *Proc Natl Acad Sci USA.* 2019;116(8):3030-5.

111. Zhang GF, Fang XD, Guo XM, Li L, Luo RB, Xu F, et al. The oyster genome reveals stress adaptation and complexity of shell formation. *Nature*. 2012;490(7418):49-54.
112. Barucca M, Olmo E, Canapa A. Hox and paraHox genes in bivalve molluscs. *Gene*. 2003;317(1-2):97-102.
113. Merkel JW, A.; Lieb, B. Novel and Conserved Features of the Hox Cluster of Entoprocta (Kamptozoa). *J Phylogenetics Evol Biol*. 2018;6(1):194.
114. Gonzalez P, Jiang JZ, Lowe CJ. The development and metamorphosis of the indirect developing acorn worm *Schizocardium californicum* (Enteropneusta: Spengelidae). *Front Zool*. 2018;15:26.
115. Haug JT. Why the term "larva" is ambiguous, or what makes a larva? *Acta Zool*. 2018.
116. Kuzmina TV, Malakhov VV. Structure of the brachiopod lophophore. *Paleontol J*. 2007;41(5):520-36.
117. Hilton W. Phoronidea from the coast of southern California. *Journal of Entomology and Zoology*. 1930;22:33-5.
118. Rattenbury Marsden J. Phoronidea from the Pacific coast of North America. *Canadian Journal of Zoology*. 1959;37(2):87-111.
119. Darriba D, Taboada GL, Doallo R, Posada D. ProtTest 3: fast selection of best-fit models of protein evolution. *Bioinformatics*. 2011;27(8):1164-5.
120. Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*. 2001;17(8):754-5.
121. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*. 2003;19(12):1572-4.
122. Balczarek KA, Lai ZC, Kumar S. Evolution and functional diversification of the paired box (Pax) DNA-binding domains. *Molecular Biology and Evolution*. 1997;14(8):829-42.
123. Manousaki T, Feiner N, Begemann G, Meyer A, Kuraku S. Co-orthology of Pax4 and Pax6 to the fly eyeless gene: molecular phylogenetic, comparative genomic, and embryological analyses. *Evolution & Development*. 2011;13(5):448-59.
124. Wang W, Zhong J, Wang YQ. Comparative genomic analysis reveals the evolutionary conservation of Pax gene family. *Genes & Genetic Systems*. 2010;85(3):193-206.
125. Capella-Gutierrez S, Silla-Martinez JM, Gabaldon T. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics*. 2009;25(15):1972-3.
126. Price MN, Dehal PS, Arkin AP. FastTree 2-Approximately Maximum-Likelihood Trees for Large Alignments. *Plos One*. 2010;5(3).
127. Hejnal A. In situ protocol for embryos and juveniles of *Convolutiloba longifissura*. *Protoc Exch*. 2008;201.

## Figures

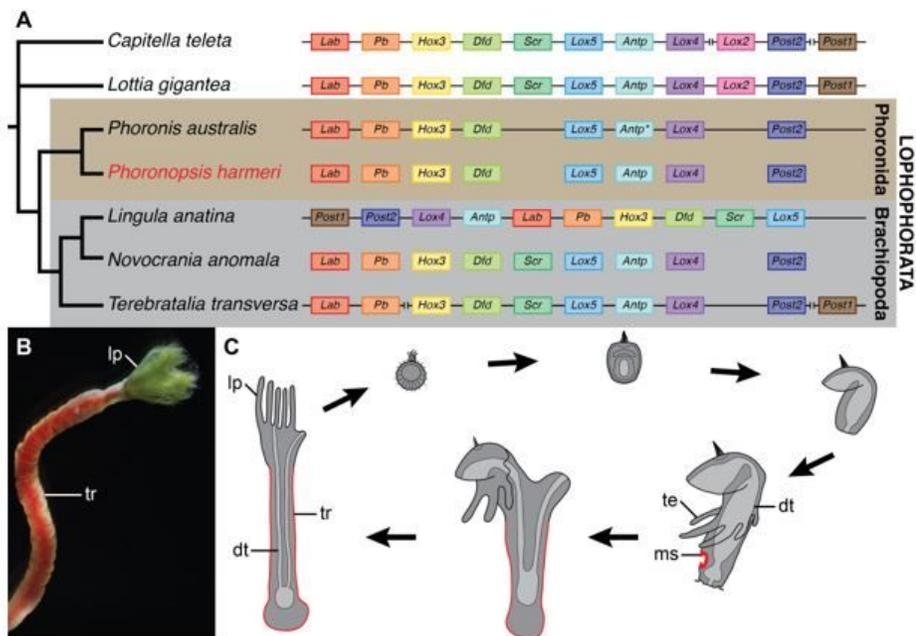
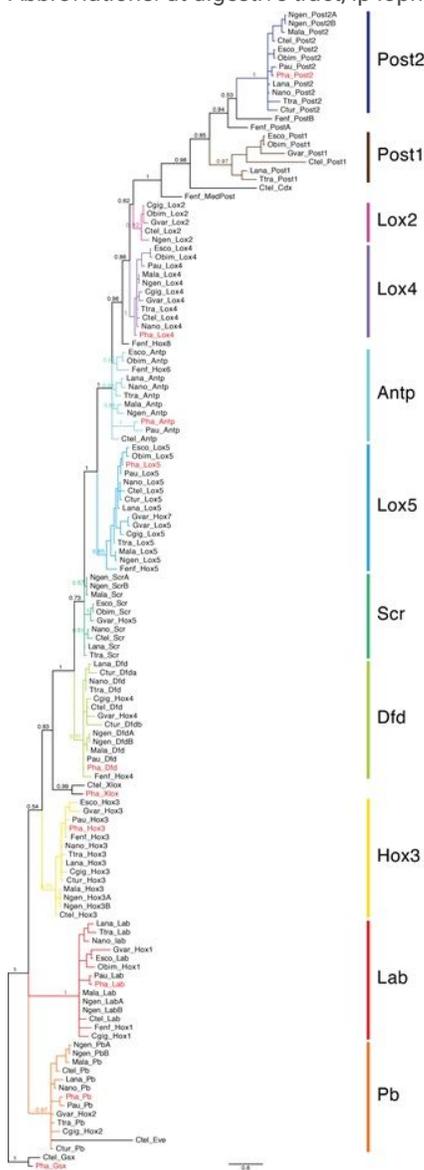


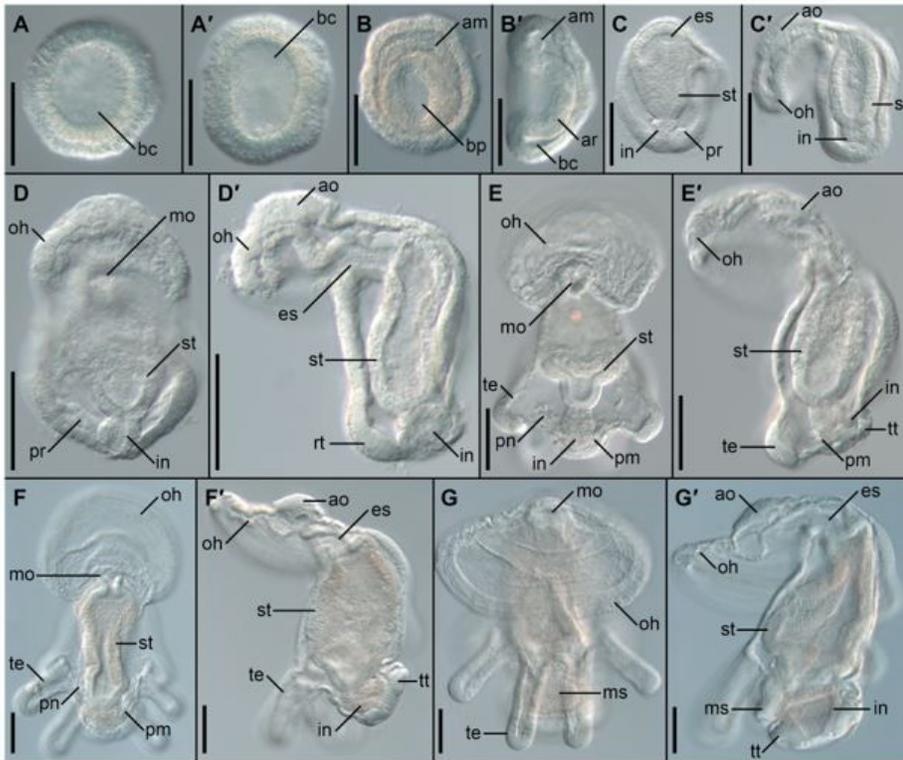
Figure 1

Hox gene cluster organization in various Spiralia (A), *Phoronopsis harmeri* gross morphology (B) and details of its life cycle (C). Hox cluster organization and Hox gene complement in A are taken from (16, 89, 91). Gene *antp* from *Phoronis australis* (marked with asterisk) was originally described as *lox2* (see text for discussion). Metasomal sac and adult trunk originating from it are marked in red in C. Abbreviations: dt digestive tract, lp lophophore, ms metasomal sac, te larval tentacles, tr trunk.



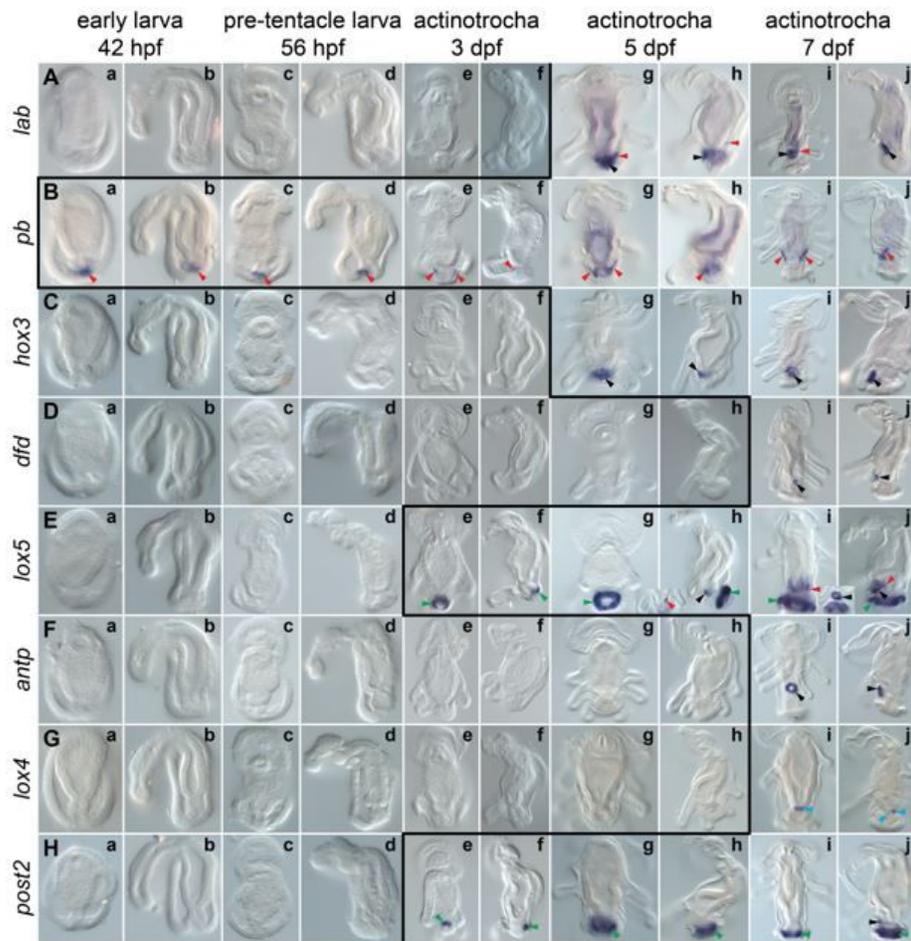
**Figure 2**

Bayesian phylogeny under JTT+I+G substitution model of the amino acid sequences of spiralian Hox genes homeodomains, including phoronid sequences. Genes from *Ph. harmeri* are marked in red. Posterior probability values are shown for important clades. Full species names and sequences accession number are provided in Additional file 2: Tab. S1.



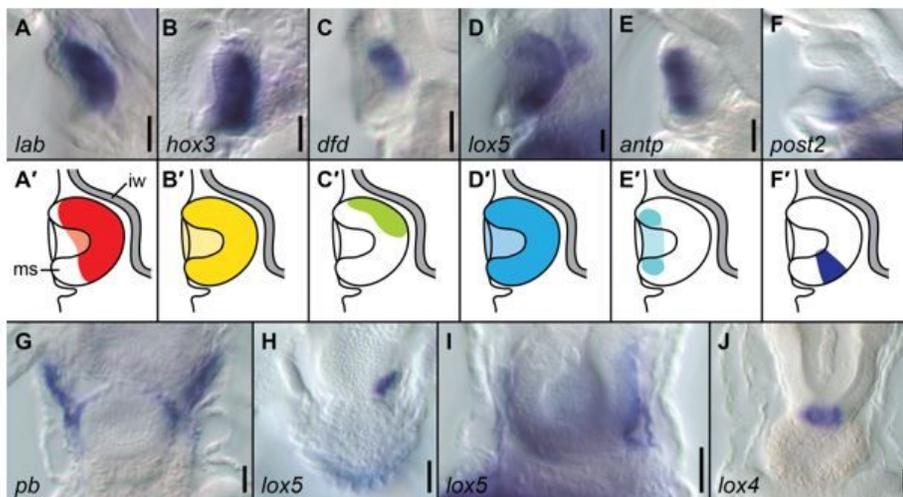
**Figure 3**

Development of *Phoronopsis harmeri*. Blastula, 12 hpf (A, A'); gastrula, 24 hpf (B, B'); early larva, 42 hpf (C, C'); pre-tentacle larva, 56 hpf (D, D'); actinotrochae: 3 dpf (E, E'), 5 dpf (F, F') and 7 dpf (G, G'). For each developmental stage the left panel shows embryo or larvae in dorso-ventral view and right panel (marked as ') in lateral view with ventral to the left; anterior is to the top on all panels. Scalebars 50 $\mu$ m. Abbreviations: am anterior mesoderm, ao apical organ, ar archenteron, bc blastocoel, bp blastopore, es esophagus, in intestine, mo mouth opening, ms metasomal sac, oh oral hood, pm posterior mesoderm, pn protonephridium, pr protonephridial rudiment, rt tentacle rudiment, st stomach, te tentacle, tt telotroch.



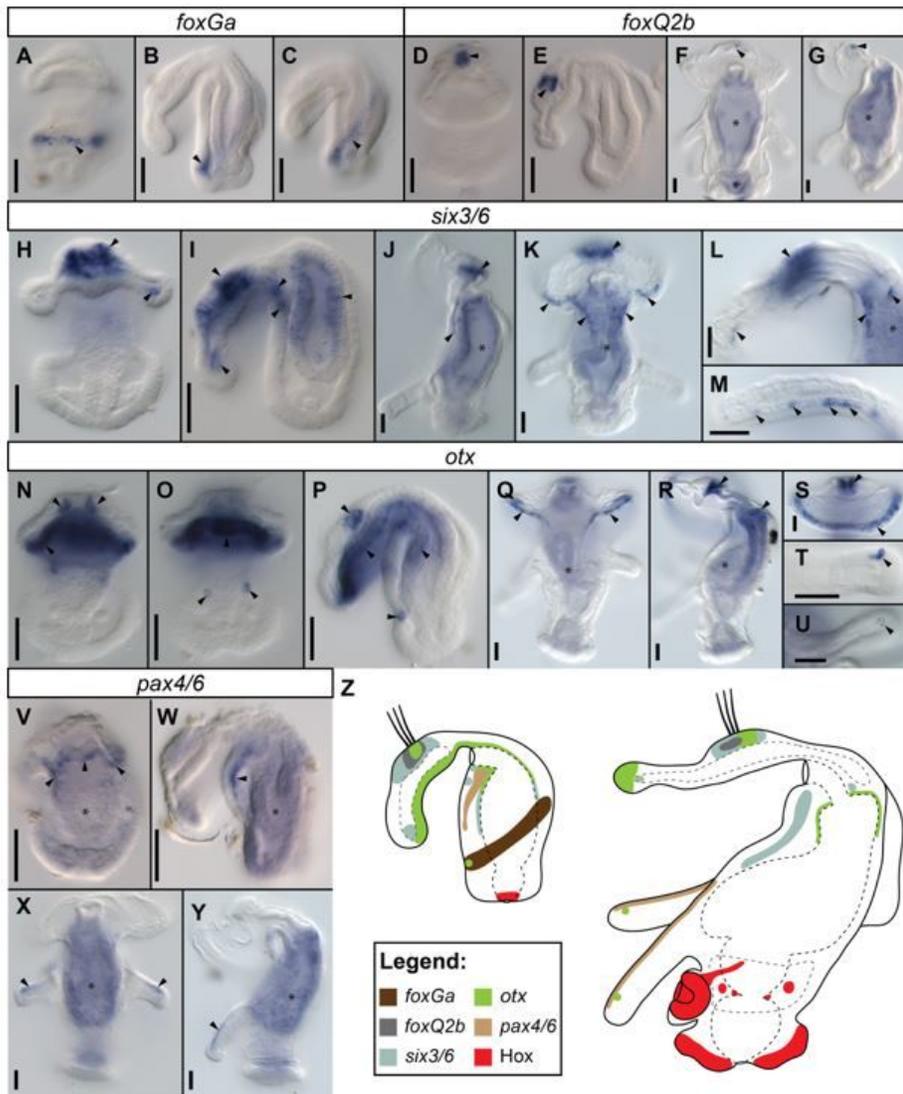
**Figure 4**

Whole-mount in situ hybridization of each Hox gene during larval development of *Phoronopsis harmeri*. Name of each hybridized gene is shown on the left, while developmental stages are indicated on the top. All the stages are presented with anterior to the top. Larvae on panels a, c, e, g and i are in dorso-ventral view, whereas larvae on panels b, d, f, h and j in lateral view with ventral to the left. The black line indicates the onset of expression of each Hox gene based on in situ hybridization data. Black arrowheads indicate expression in the metasomal sac, red arrowheads expression in the mesoderm, green arrowheads expression in the telotroch and blue arrowheads expression in the endoderm. The detailed expression patterns are described in the text. Photographs are not to scale.



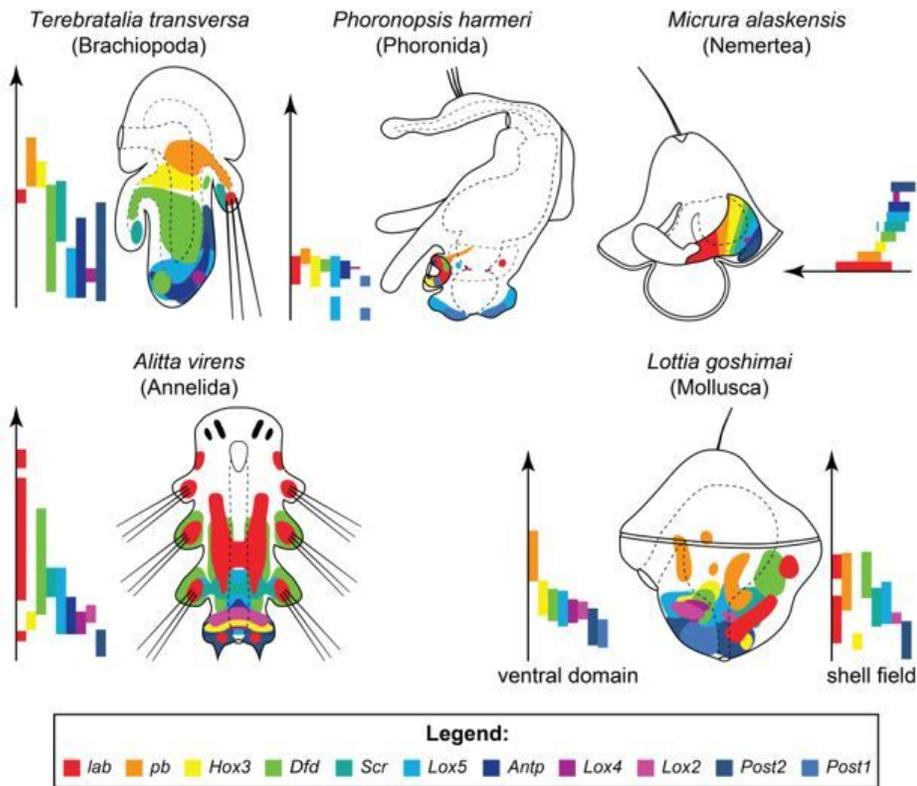
**Figure 5**

Details of the expression of some of the Hox genes in the actinotrocha larvae of *Phoronopsis harmeri*. Expression of the Hox genes in the metasomal sac of 8-tentacle actinotrochae (A–F) and schematic interpretation of those expression patterns (A'–F'). Expression of *pb* in the telotroch flexors of 8-tentacle actinotrocha (G). Expression of *lox5* in the left mesoderm of 6-tentacle (H) and 8-tentacle actinotrocha (I). Expression of *lox4* in the endoderm of 8-tentacle actinotrocha (J). Scale bars 25µm. Abbreviations: ms metasomal sac, iw intestinal wall.



**Figure 6**

. Expression of head-specific genes in early larva (A–E, H, I, N–P, V, W) and 8-tentacle stage actinotrocha (F, G, J–M, Q–U, X, Y) of *Phoronopsis harmeri* and comparison of expression of head-specific and Hox genes in both larval stages (Z). For each panel the name of hybridized gene is shown in the white box above micrographs. Entire larvae in the dorso-ventral (A, D, F, H, J, N, O, Q, V, X) and lateral (B, C, E, G, I, K, P, R, W, Y) views. Details of expression in 8-tentacle stage larvae in oral hood and anterior body region (L), hood musculature (M), apical organ and rim of the hood (S) and tips of the tentacles (T, U). Black arrowheads point to the particular expression domains (see text for details), while asterisks indicate unspecific staining in the digestive system. Scalebars 25µm.



**Figure 7**

Comparison of Hox gene expression in selected spiralian larvae. For each larva the antero-posterior Hox gene expression gradients along body axis is indicated. All larvae are shown from the left side (with exception of *Alitta virens*, which is shown in the dorso-ventral view) with the anterior (apical) side oriented to the top. Note, that in brachiopod larva pb, hox3 and dfd exhibit staggered A-P expression. In *Micrura alaskensis*, the hox genes are not expressed in the larval tissue but only in the worm rudiment (note that larval and adult body axes are not aligned). In *Lottia goshimai* Hox genes show spatial collinearity in the ventral neuroectoderm but not in dorsal shell field. Drawings based on this study as well as Schiemann et al.(16), Hiebert and Maslakova(37), Kulakova et al.(27) and Huan et al.(41).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1.docx](#)