Hox gene expression in postmetamorphic juveniles of the brachiopod Terebratalia transversa

Ludwik Gąsiorowski¹ and Andreas Hejnol¹*

¹ Sars International Centre for Marine Molecular Biology, University of Bergen, Bergen, Norway

* Correspondence: andreas.hejnol@uib.no
Abstract

Background: *Hox* genes encode a family of homeodomain containing transcription factors that are clustered together on chromosomes of many Bilateria. Some bilaterian lineages express these genes during embryogenesis in spatial and/or temporal order according to their arrangement in the cluster, a phenomenon referred to as collinearity. Expression of *Hox* genes is well studied during embryonic and larval development of numerous species; however, relatively few studies focus on the comparison of pre- and postmetamorphic expression of *Hox* genes in animals with biphasic life cycle. Recently, the expression of *Hox* genes was described for embryos and larvae of *Terebratalia transversa*, a rhyynchonelliformean brachiopod, which possesses distinct metamorphosis from planktonic larvae to sessile juvenile. During premetamorphic development, *T. transversa* does not exhibit spatial collinearity and several of its *Hox* genes are recruited for the morphogenesis of novel structures. In our study we determined expression of *Hox* genes in post-metamorphic juveniles of *T. transversa* in order to examine metamorphosis-related changes of expression patterns and to test if *Hox* genes are expressed in the spatially collinear way in the postmetamorphic juveniles.

Results:

*Hox* genes are expressed in a spatially non-collinear manner in juveniles, generally showing similar patterns as ones observed in competent larvae: genes *labial* and *Post1* are expressed in chaetae-related structures, *sex comb reduced* in the shell forming epithelium, whereas *Lox5* and *Lox4* in dorso-posterior epidermis. After metamorphosis expression of genes *proboscipedia*, *Hox3*, *Deformed* and
Antennapedia becomes restricted to, respectively, shell musculature, prospective hinge rudiments, and pedicle musculature and epidermis.

**Conclusions:**

All developmental stages of *T. transversa*, including postmetamorphic juveniles, exhibit a spatial non-collinear *Hox* genes expression with only minor changes observed between pre- and postmetamorphic stages. Our results are concordant with morphological observation that metamorphosis in rhynchonelliformean brachiopods, despite being rapid, is rather gradual. The most drastic changes in *Hox* genes expression patterns observed during metamorphosis could be explained by the inversion of the mantle lobe, which relocates some of the more posterior larval structures into the anterior edge of the juveniles. Cooption of *Hox* genes for the morphogenesis of novel structures is even more pronounced in post-metamorphic brachiopods when compared to larvae.

**Key words:** metamorphosis, *Hox* gene collinearity, indirect development, morphology, Spiralia, Lophophorata, biphasic life cycle
Background

Hox genes encode a family of conserved homeodomain transcription factors from the ANTP class, which by binding to regulatory DNA sequences can activate or suppress transcription of downstream genes (e.g. [1, 2]). Hox genes are present in genomes of almost all investigated animals (with exception of Porifera, Ctenophora and Placozoa [3-7]) and are hypothesized to represent a synapomorphy of the clade consisting of Cnidaria and Bilateria [4, 8-10]. In most of Bilaterians Hox genes are expressed during embryogenesis, being involved in antero-posterior (A-P) patterning of either the whole embryo or at least some of its developing organ systems (e.g. [1, 2, 11]). Interestingly, in the genomes of some animals, the Hox genes are clustered along the chromosomes in the same order as they are expressed along A-P axis, a phenomenon referred to as spatial collinearity [2, 11-13]. The clustering of Hox genes in the genome is hypothesized as a plesiomorphic feature of Bilateria (e.g. [13]), which however, went through extensive remodeling in some evolutionary lineages (e.g. [12, 14-22]. Yet, spatial collinearity can be preserved despite a disorganization or split of the ancestral Hox cluster (e.g. [14]), the situation for which the term trans-collinearity was coined by Duboule [12].

Initially the role of Hox genes has been studied in the developing embryo of Drosophila melanogaster [23], later supplemented by the data from other insects, vertebrates and nematodes [24-26]. Recent advance of molecular and bioinformatic techniques allowed the investigation of Hox gene expression in the embryos and larvae of several non-model species, including e.g. xenacoelomorphs [16, 27, 28], hemichordates [29], onychophorans [30], tardigrades [31], rotifers [32], annelids [33-35], mollusks [36-40], nemerteans [41] and brachiopods [19], essentially
increasing knowledge on diversity of Hox genes based patterning systems in Bilateria.

Many animals are characterized by an indirect life cycle in which embryos develop through a larval stage and subsequent metamorphosis, during which the larval body is reshaped into the adult one (e.g. [42, 43]). As larvae and adults can significantly differ in their morphology, the transition process might be quite dramatic and hence attracted attention of many researchers as one of the pivotal moments of the animal development [44-46]. Although the process of metamorphosis has puzzled numerous developmental biologists, there are relatively few studies regarding shifts of Hox gene expression accompanying it [15, 47-51]. In some animals both larvae and adults show canonical spatial collinearity, which often correlates with gradual type of metamorphosis. This can be exemplified by investigated annelid species, in which both life stages exhibit spatial collinearity of most of the Hox genes, yet there are shifts in the combinations of genes defining particular body regions before and after metamorphosis [47, 48]. On the other hand, in the other animals (especially those with the more pronounced metamorphosis) only one of the developmental stages exhibits canonical spatial collinearity of Hox genes expression, whereas the remaining stage shows either a non-collinear expression or does not express Hox genes at all. For instance, in the tunicate Ciona intestinalis Hox genes exhibit spatially collinear expression in the nervous system of larvae, whereas in juveniles only three posterior genes are expressed in the intestine [15]. Conversely in pilidiophoran nemertean Micrura alaskensis and in indirectly developing enteropneust Schizocardium californicum the specialized larvae develop without expressing any of the Hox genes, which, in turn, are expressed in the canonical collinear way only in
the rudiments of juvenile worms developing either inside larval body (pilidiophorans) or as the posterior extension of late larva (enteropneusts) [49, 50]. A somehow similar situation is found in the indirectly developing sea urchin *Strongylocentrotus purpuratus*, in which only two *Hox* genes (*Hox7* and *Hox 11/13b*) take part in the larva formation, whereas the rudiments of adult animal, developing inside the larval body, show collinear expression of five *Hox* genes (*Hox7, Hox8, Hox9/10, Hox11/13a and Hox11/13b*) in the extra-axial mesoderm [51-54]. It is therefore evident, that metamorphosis-related shifts in *Hox* gene expression and function vary a lot from one animal clade to another, as a result of diverse evolutionary and developmental processes which shape the ontogeny of each particular group [55].

![Fig. 1. Phylogenetic position of Brachiopoda (A, based on Laumer et al 2015[57]) and metamorphosis of *Terebratalia transversa* (B, based on Freeman 1993 [67]). S stands for Spiralia, L for Lophophorata. 1. Competent planktonic larva, anterior to the right; 2. Larva settles on the substrate; 3. Inversion of the mantle lobe in the settled larva; 4 Juvenile; note that over the course of metamorphosis the internal surface of the larval mantle lobe becomes](image-url)
external, shell covered surface of juvenile animal, external surface of the mantle lobe becomes inner surface of the mantle, whereas anterior lobe contributes to the lophophore rudiment developing inside mantle cavity. Abbreviations: *al* larval anterior lobe, *ds* dorsal shell, *lr* lophophore rudiment, *m* mantle, *ml* larval mantle lobe, *pe* pedicle, *pl* larval pedicle lobe, *vs* ventral shell.

One of the animal groups with a distinct metamorphosis event are rhynchonelliformean brachiopods, represented by *Terebratalia transversa* for which Schiemann et al. recently described *Hox* genes expression in embryos and larvae [19]. Brachiopods, along with phoronsids and possibly ectoprocts, constitute the clade Lophophorata (Fig. 1 A, [56, 57]), which, together with e.g. annelids, mollusks, flatworms, nemerteans and rotifers, belongs to a large clade of protostome animals called Spiralia (Fig. 1A, [57-60]). Extant brachiopods are traditionally divided into three groups: Rhynchonelliformea, Craniiformea and Linguliiformea, the two latter forming sister clades [18, 57, 60, 61], historically united into group Inarticulata. As all brachiopods, adults of *T. transversa* are filter feeding animals with external anatomy superficially similar to bivalves – most of the body, including lophophore, a filtering organ, is enclosed in the two-valved shell, which covers the dorsal and ventral surfaces of the body. The clade Rhynchonelliformea is further characterized by the set of morphological features, including: posterior soft-tissued pedicle (by which animal attaches to the substrate), blind gut devoid of anus and articulated valve-hinge [62]. Additionally, rhynchonelliformean larvae differ from those found in other brachiopods by possessing three distinct body regions (Fig. 1B1) – anterior lobe, mantle lobe (bearing four chaetal sacs) and the most posterior pedicle lobe [62-66]. The rhynchonelliformean larva settles by adhering to the substrate with the
posterior tip of the pedicle lobe (Fig. 1B2) and undergoes a specific metamorphosis, which in case of *T. transversa* is relatively rapid (few hours to one day [63]) and involves inversion of the mantle lobe (Fig. 1B2–3, [63, 67]). The latter results in profound relocation of some larval tissues – in competent larvae the mantle lobe partially covers the pedicle one and its chaetae projects posteriorly, after metamorphosis mantle lobe with chaetae projects anteriorly, its former interior surface becomes exposed and produce protogulum (the first rudiment of the shell), whereas its former exterior surface constitute walls of the mantle cavity of juvenile (Fig. 1B2–4) [63, 67, 68]. Therefore, the rapid transition from larvae to the juvenile involves profound reshaping of the entire body, which poses a question to which extent are those two stages are continuous [69].

Schiemann et al. investigated genomic order of *Hox* genes of *T. transversa* and *Hox* genes expression in embryos and larvae of *T. transversa* and craniiform *Novocrania anomala* [19]. *T. transversa* has a split *Hox* cluster comprising of 10 *Hox* genes in three independent parts. One scaffold contains two anterior *Hox* genes, *labial* (*lab*) and *proboscipedia* (*pb*). Second, longest part, contains genes *Hox3, deformed* (*Dfd*), *sex combs reduced* (*Scr*), *Lox5, antennapedia* (*Antp*), *Lox4* and *Post2*, whereas the most posterior gene *Post1* is located in the third independent scaffold [19]. A disorganization of the *Hox* cluster has also been reported for linguliform brachiopod, *Lingula anatina*, in which although all *Hox* genes are in the single cluster *Post1, Post2, Lox4* and *Antp* has been translocated upstream to the *lab* [18]. In embryos and larvae of *T. transversa*, but also of craniiform *N. anomala*, detected expression pattern of *Hox* genes does not show the canonical spatial collinearity [19]. However, as stated before, in some indirectly developing animals, larvae and
juveniles can show collinear expression of Hox genes in patterning of one of life stages while other develops without evident Hox expression collinearity.

Therefore, in this study, we supplemented findings of Schiemann et al. (2017) by examination of the postmetamorphic Hox gene expression in Terebratalia transversa juveniles 2 days after metamorphosis. The main questions, which we were aiming to answer, were: 1) If and how is Hox gene expression pattern shifted during metamorphosis in rhyynchonelliformean brachiopods? 2) Is there any staggered Hox gene expression along the A-P axis emerging after metamorphosis as a result of displacement and development of certain larval Anlagen?

**Results**

**Description of T. transversa juvenile morphology**

The existing knowledge on the detailed morphology of juvenile T. transversa is based mostly on confocal laser scanning microscopy (CLSM) investigation of musculature [69, 70], as well as transmission electron microscope (TEM) sections [63, 68, 71] and scanning electron microscopy (SEM) [68, 71, 72] of different developmental stages, including juveniles 1 day after metamorphosis [68, 69, 72], 4-5 days after metamorphosis [68, 70-72] and older than one week after metamorphosis [68, 69, 71, 72]. Therefore, to facilitate interpretation of our gene expression results [73], we examined morphology of the juveniles two days after metamorphosis utilizing light microscopy (LM) and CLSM combined with DAPI, phalloidin and immunohistochemical stainings (with primary antibodies against tyrosinated and acetylated tubulin).
Fig. 2. Morphology of the juvenile of *Terebratalia transversa* two days after metamorphosis, visualized with light microscopy (A) and CLSM (B, D-I). A Micrograph of the entire animal. B Frontal section through the median part of animal, cell nuclei visualized with DAPI staining. C Schematic drawing of the anatomy of juvenile. D, G Musculature visualized with F-actin phalloidin staining, arrowheads in D points to the ventral mantle margin muscles. E, J tyrosinated tubulin-like immunoreactivity. F, I acetylated-tubulin-like immunoreactivity. H Transverse section through the nephrostome, cell nuclei visualized with DAPI in cyan, acetylated tubulin-like immunoreactivity in yellow. A-F Dorso-ventral view, anterior to the top. G Lateral view, anterior to the left, dorsal to the top. I Virtual transverse section through the middle part of animal, dorsal to the top. J Ventral, magnified view of the commissural region from E. Abbreviations: *aam* anterior shell adductor muscle, *asc* anterior supraoesophageal commissure, *ch* chaeta, *cr* coelom rudiment, *cs* chaetal sac, *csm* chaetal sac musculature, *dc* dorsal commissure, *dms* shell diductor muscle, *dt* digestive tract, *lr* lophophore rudiment, *lrf* fibers in the lophophore rudiment, *mc* mantle cavity, *mm* mantle margin musculature, *mmc*...
mantle margin ciliation, mr mantle margin, nd nephroduct, ns nephrostome, pam posterior shell adductor muscle, pe pedicle, psc posterior supraoesophageal commissure, vmc ventral mantle cavity, vpa ventral pedicle adjustor muscle.

Two days after metamorphosis juveniles of *T. transversa* already resemble the adult animal in their general shape (Fig. 2A–C). The body is clearly divided into main part covered by the two-valved juvenile shell and a posterior pedicle (pe, Fig. 2A–C), by which juvenile is attached to the substrate.

Anteriorly, the shell is lined with mantle margin (mr, Fig. 2B, C), where tissues responsible for secretion of prospective adult shell are localized (Stricker and Reed 1985a). Phalloidin staining revealed presence of developing mantle margin muscles (arrowheads Fig. 2D; mm, Figs. 2G and 3C, C'), which have been already described for the older juveniles [69, 70]. Additionally, four chaetal sacs (cs, Fig. 2A, B), associated with degenerating larval musculature (csm, Figs. 2D, G; 3D, D') [69], are embedded in the mantle margin, one pair dorso-medially and another in the more lateral position, which respectively protrudes numerous chaetae (ch, Fig. 2A, C) anteriorly and laterally.

Optical sections through the animal show the narrow mantle cavity (mc, Figs. 2B, C; 3C–G'), which expands ventro-medially to about two-thirds the length of animal body and is lined with ciliated cells (vmc, Fig. 2C, F, I). The remnant of the larval anterior lobe, from which the prospective lophophore will develop [65, 74], is situated inside the mantle cavity (lr, Figs. 2B; 3B, B', C, C'). Posteriorly the lobe is connected with the dorsal mantle and ventrally it faces the extension of the mantle cavity (Fig. 3D, D', E, E'). Medially the lobe is divided by the ciliated slit (cs, Fig. 2F, I),
which anteriorly communicates with the mantle cavity through the ventral infold, a stomodeum (Fig. 3 C–E, C′–E′) and posteriorly continues as the tubular rudiment of digestive tract (dt, Figs. 2A–C; 3F, F′).
Fig. 3. Transverse sections through *Terebratalia transversa* juvenile (two days after metamorphosis), showing detailed morphology of the animal. Each panel consists of CLSM image (left) and schematic representation (right). Cell nuclei visualized with DAPI in cyan,
acetylated-tubulin like immunoreactivity (F and G) and tyrosinated tubulin-like immunoreactivity (E) in yellow, F-actin phalloidin staining in green. A Juvenile in dorso-ventral view, anterior to the top, dashed red lines indicates sections shown on the subsequent panels. B-H Virtual transverse sections through the juvenile, as indicated on A, dorsal to the top on all panels, on the right panels outline of the body is depicted in light blue, musculature in green, body cavities in grey, nervous system in red and excretory organs in orange. Abbreviations: aam anterior shell adductor muscle, asc anterior supraoesophageal commissure, cr coelom rudiment, csm chaetal sac muscle, dc dorsal commissure, dm dorsal mantle, dms shell diductor muscle, dt digestive tract, lr lophophore rudiment, mc mantle cavity, mm mantle margin muscle, np nephropore, ns nephrostome, pam posterior shell adductor muscle, psc posterior supraoesophageal commissure, vm ventral mantle, vpa ventral pedicle adjustor muscle.

At this stage the lophophore rudiment is poorly developed and consists of two, scarcely ciliated lobes without tentacles (lr, Figs. 2B; 3B, B', C, C'). The lobes are penetrated by numerous, fine tyrosinated-tubulin-like immunoreactive (tTLIR) fibers (lrf, Fig. 2E), some of which communicate with the nervous system and probably represent the developing innervation of the prospective lophophore.

The nervous system is inconspicuous. Its most prominent structures are two tTLIR and acetylated-tubulin like immunoreactive (aTLIR) commissures (anterior and posterior supraoesophageal commissures, respectively: asc and psc, Figs. 2E, I, J; 3E, E', F, F'), positioned dorsally to the ciliated slit, more or less one third from its most posterior extremity. Some tTLIR and aTLIR fine fibers extend laterally from those commissures to lophophore rudiments and mantle tissues (including mantle margin and chaetal sacs). Few tTLIR fine neurites extends from posterior supraoesophageal commissure to the intestinal tissue (arrowheads, Fig. 2J; Fig. 3F'). Additional dorsal
tTLIR commissure (dc, Figs. 2E, J; 3E, E′) connects with the anterior supraoesophageal commissure. The similar arrangement of nervous system in early juveniles of *T. transversa* has been reported based on immunostaining against serotonin [28].

Two prominent tTLIR and aTLIR longitudinal structures are present in the ventral part of the animal (nd, Fig. 2F, I), extending along the ventral surface from the mid-posterior region to the ventral part of the mantle cavity. Dorsally those structures have numerous finger-like projections (ns, Figs. 2H, I, 3G, G′), which contact nucle-free regions (as revealed by DAPI staining, Fig. 2H). We suggest, that those structures represent metanephridia (composed of nephrostome and nephridial duct) of juveniles, which connects the developing coelom with the mantle cavity. Their form and position is similar to what has been described for the metanephridia of relatively closely related *Terebratulina retusa* [75]. Although metanephridia in brachiopods are considered to be responsible only for release of gametes and not for excretion [75], they are present (albeit initially as non-functional rudiments) already in the early juveniles of *N. anomala* [76]. It is possible that aTLIR structures described by Santagata [69] as larval protonephridia in *T. transversa* actually represent rudiments of the metanephridial ducts or nephrostomes which acquire their final form during or soon after metamorphosis.

The DAPI staining revealed an empty cavity inside the body of the juvenile with two pairs of anterior and posterior branches (cr, Figs. 2B, C; 3), which most probably represents the developing coelom in which some of the forming muscles are freely positioned (Fig. 3E, E′, H, H′) [77]. Its two anterior branches extend along digestive tract and penetrate the lophophore rudiment (Fig. 2B; 3C–F, C′–F′). A similar
arrangement of the coelom in the lophophore rudiment of postmetamorphic juveniles has been described for relatively closely related rhyynchonelliformean Calloria inconspicua [78].

In addition to the already mentioned musculature related with the mantle margin, we identified rudiments of all the muscle groups (pedicle adjustors, shell diductors as well as anterior and posterior shell adductors; respectively: vpa, dms, aam, pam, Figs. 2D, G; 3D–H, D’–H’) described for the older juveniles of T. transversa [70] with the only exception of lophophore-related tentacle muscles (which correlates with lack of lophophore tentacles 2 days after metamorphosis). In different specimens the particular groups of muscles were developed to different degree corroborating the observation of the extensive and rapid remodeling of muscular tissue in the postmetamorphic juveniles [69].

**In situ hybridization of Hox genes**

The expression of the Hox genes in juvenile Terebratalia transversa (2 days after metamorphosis) was examined with colorimetric (CISH; Fig. 4) and fluorescent (FISH, Fig. 5) *in situ* hybridization. Generally, Hox genes in the juveniles of Terebratalia transversa are not expressed in a collinear way (Figs. 4–6).

The most anterior Hox gene *lab* is expressed in two bilaterally paired domains at the mantle margin, which correspond to the larval chaetal sacs (arrowheads, Figs. 4A; 5A, B; also compare with Fig. 2B and D).
Fig. 4. Whole-mount colorimetric in situ hybridization of the Hox genes (A–J) and schematic representation of expression patterns (K) in Terebratalia transversa postmetamorphic juveniles (two days after metamorphosis). All micrographs and drawings in dorso-ventral view, anterior to the top. For each plate (A–J) name of the hybridized gene is provided in the lower right corner. Particular structures in which each of the genes is expressed are indicated with arrowheads, asterisks and double arrowheads (see text for detailed explanation). Note that signal on I (asterisk) represents unspecific background (see text for details).
The gene *pb* has bilaterally paired strong expression domains (arrowheads, Figs. 4B, 5C), which correspond to the position in which shell adductor muscles are developing (compare Figs. 4B, 5C and 2D). The FISH and CLSM investigation of juveniles further reveals that those two domains extend obliquely from the more anterior point on the dorsal shell to the more posterior point on the ventral shell, in the same orientation as the anterior shell adductors (compare Figs. 5D and 2G).

*Hox3* is expressed in two paired domains posteriorly to the most lateral projections of the shell (arrowheads, Figs. 4C, 5E), where prospective hinge rudiments will form in older juveniles [68]. CISH investigation showed additional broad weak staining in the posterior part of the body (Fig. 4C), which was not reproduced with FISH (Fig. 5E) and which might result from unspecific probe binding in the posterior shell as shown by sense probe staining (Additional file 1, Fig. S1B and C).

The gene *Dfd* is expressed in the ventro-posterior domain (Figs. 4D, 5F–H) composed of extensive lateral elements (arrowheads, Figs. 4D, 5F–H) and a narrow median stripe, detectable only with CISH. Fibrous appearance of those structures in LM imaging (Fig. 4D) as well as their position revealed with FISH (Fig. 5F-H) indicates that *Dfd* is expressed in the ventral pedicle adjustor muscles (compare Figs. 5F–H and 2D, G).

Expression of the gene *Scr* is restricted to the mantle margin (Figs. 4E, 5I). Signal from probes against *Scr* in CISH seems to be diversified into smaller domains with strong signal interspaced by wide regions of relatively weaker expression (respectively: arrowheads and asterisks, Fig. 4E), not visible in FISH examination (compare Figs. 4E and 5I).
Fig. 5. Whole-mount fluorescent *in situ* hybridization of the *Hox* genes (red) combined with DAPI staining of cell nuclei (cyan, with exception of E and N) in *Terebratalia transversa* postmetamorphic juveniles (two days after metamorphosis). For each plate name of the hybridized gene is provided in the right corner. Particular structures in which each of the genes is expressed are indicated with arrowheads, asterisks and double arrowheads (see...
Expression of lab in chaetal sacs in dorso-ventral view (A) and virtual cross section (B); note autofluorescence of chaetae. Expression of pb in anterior shell adductor muscles in dorso-ventral view (C) and virtual parasagittal section (D). Expression of Hox3 in dorso-ventral view (E). Expression of Dld in ventral pedicle adjustor muscles in dorso-ventral view (F), virtual cross section (G) and parasagittal section (H). Expression of Scr in mantle margin (I). Expression of Lox5 in dorso-posterior epidermis in dorso-ventral view (J) and virtual sagittal section (K). Expression of Lox4 in dorso-posterior epidermis in dorso-ventral view (L) and virtual cross section (M). Expression of Antp in pedicle tissue in dorsoventral view (N), and virtual cross (O) and longitudinal (P) sections through pedicle. Expression of Post 2 in mantle margin, dorso-ventral view (R). Dashed lines with letters on A, C, F, J, L and O indicate section planes shown on respective plates. Scale bars on all images represent 20μm. Anterior to the top on A, C, E, F, I, J, L, N and R; dorsal to the top on B, D, G, H, K and M; anterior to the left on D, H and K.

The gene Lox5 is expressed in the continuous dorso-posterior domain, which extend from posterior region of shell-covered body (asterisks, Figs. 4F, 5J) to the pedicle tissues (arrowheads, Figs. 4F, 5J) and its expression is restricted to the dorsal epidermal cells as revealed by FISH (double arrowheads, Fig. 5K).

Antp has a distinct expression domain only in the epidermis of the pedicle, as revealed by both CISH (arrowhead, Fig. 4G) and FISH (arrowhead, Fig. 5N; Fig. 5O, P). The signal in the CISH staining developed for the long time (33h in 4°C plus 16h in room temperature) and before it became evident the strong staining had appeared in some specimens also in the dorso-posterior part of the shell-covered body. However, the control with sense probe showed that this staining results from unspecific binding of the probe in larval dorsal protegulum (asterisks, Additional file 1, Figure S1B) and on the borders between dorsal protegulum and remaining parts of the shell (arrowheads, Additional file 1: Figure S1B). The strong dorsal band was also
visible in FISH staining (double arrowheads, Fig. 5N) but combined staining with DAPI showed, that it is restricted to the surface area and does not penetrate the epidermis (arrowhead, Fig. S1D, E), supporting our finding that it represents an unspecific probe binding by shell components. The central-class Hox gene Lox4 is expressed only in the small area of epidermal tissues in the dorso-posterior part of the shell-covered body (arrowheads, Figs. 4H; 5L, M) and its expression domain is not extending to the pedicle tissues. We did not manage to detect expression of Post2 with in situ hybridization, which corresponds to the reported overall low level of Post2 transcription in postmetamorphic juveniles of T. transversa [19]. After long developmental time (33h in 4°C plus 20h at room temperature) CISH stainings yielded signal in the dorso-posterior part of the shell covered body (asterisk, Fig. 4I), however, the control with the sense probe showed that this results from unspecific binding of the probe in the larval dorsal protégulum (Additional file 1: Figure S1C). The FISH stainings only revealed a signal at the borders of the larval protégulum and remaining parts of the shell (arrows, Fig. S1F) and, similarly as in case of Antp, FISH combined with DAPI staining revealed that this signal is restricted to the surface (shell components) and does not penetrate to the cellular epidermal layer (arrowhead, Additional file 1: Figure S1G). The unspecific binding of some probes by the larval protégulum has been already reported for T. transversa larvae [79] and apparently this phenomenon can also pose a problem in investigation of postmetamorphic animals. Expression of the most posterior Hox gene Post1 is detected along mantle margin (arrowheads, Figs. 4J, 5R), showing a relatively equal strength of signal with both CISH and FISH.
Discussion

Metamorphosis and Hox gene expression in Rhynchonelliformea

Comparison of the expression of Hox genes between late, competent larva and postmetamorphic juvenile of Terebratalia transversa (Fig. 6) shows that in both stages almost all Hox genes (with the exception of Hox3, Post2 and Post1) are expressed in corresponding organs and body regions: lab in chaetal sacs, pb and Dfd in, respectively, anterior and posterior mesoderm, Scr in the shell growth zone, whereas Lox5, Antp and Lox4 are expressed in the dorso-posterior ectoderm. Most of the observed differences and shifts in the expression domains can be explained by the inversion of the mantle lobe, which constitutes the most profound process during the whole metamorphosis in Rhynchonelliformea (Fig. 1B, [63]). Another factor, which contributes to the observed changes, is the restriction of the expression of some Hox genes from broad, less specific larval domains to particular structures of the juvenile, which emerge during or after metamorphosis. For example, pb is generally expressed in the anterior mesoderm in late larvae but in juveniles its expression becomes restricted only to particular mesodermal structures, i.e. newly formed anterior shell adductors muscles.

The comparison of the Hox gene expression between larvae and juveniles allows to identify Anlagen of adult structures in the larva. For example, the expression patterns of the Hox genes before and after metamorphosis suggest that only the posterior half of the larval pedicle lobe contributes to the pedicle of the adult, whereas the more anterior part becomes the posterior region of the shell-covered body, as it has been proposed by Stricker and Reed [63, 71]. Among six Hox genes
expressed in the pedicle lobe of the late larvae of *T. transversa*, only *Lox5* and *Antp* are expressed in the pedicle of the postmetamorphic juvenile (Fig. 6), both of them being expressed in the most posterior part of the larval pedicle lobe [19].

![Fig. 6. Comparison of the Hox genes expression between the late competent larva (A, based on Schiemann et al. 2017[19]) and juvenile (B) of *Terebratalia transversa*. Animals are shown in the dorso-ventral view (right panels) and in the lateral view with dorsal to the right (left panels). Anterior to the top on all panels. Bars on the right show antero-posterior Hox gene expression gradients in each developmental stage.](https://doi.org/10.1101/449488)

Next to Rhynchonelliformea, two inarticulate clades belong to the Brachiopoda: Craniiformea and Linguliformea [80], both possessing a planktonic larvae, which undergoes more or less pronounced metamorphosis [62, 69, 81-85]. In Linguliformea
the metamorphosis itself is extended over time with some of the juvenile traits present already in planktotrophic larvae [66, 69, 81, 86] and the most advanced larval stages are even commonly considered as representing planktonic juveniles or paralarvae [62, 69, 86]. One can therefore speculate, that as larval and adult body plans in Linguliformea are continuous, their patterning by Hox genes should be similar as is a case in T. transversa. On the other hand, there are two competing hypotheses about nature of the rearrangement of the larval body plan during metamorphosis of craniiformean brachiopods [62, 82, 83, 87, 88]. The main controversy regards whether N. anomala larva, which lacks the distinct pedicle lobe, attaches to the substrate with its dorso-posterior side [82] or with the posterior tip of the posterior lobe [83]. Expression of the Hox genes is relatively similar between embryos and larvae of N. anomala and corresponding stages of T. transversa [19], indicating a conserved nature of Hox genes patterning between Craniiformea and Rhynchonelliformea. Lox5 and Antp, which after metamorphosis are expressed in the pedicle of T. transversa juveniles are expressed in the posterior tip of the posterior lobe of N. anomala larvae [19] favoring interpretation that posterior tip of N. anomala larvae corresponds to the pedicle of Rhynchonelliformea [83, 88]. Further investigation of the postmetamorphic expression of Hox genes, especially Lox4 and Antp, in N. anomala could support this hypothesis.

Unlike some bilaterians in which metamorphosis seems to be related with highly different Hox gene expression between larvae and adults (e.g. tunicates [15], Bryozoa [89]) or in which Hox genes are not expressed in the larvae and only pattern adult body (pilidiophoran nemerteans [49], indirectly developing Hemichordates [50], sea urchins [51-53]), rhynchonelliformean brachiopods exhibit continuity in the
patterning of larval and adult body plans. Consequently, in regards of Hox gene expression, metamorphosis in T. transversa is similar to the condition found in another spiralian clade, Annelida. Although there are some shifts in expression patterns of particular Hox genes between annelid larvae and juvenile worms [47, 48], those differences are mostly related with restriction of some of the genes from broader larval to more specific adult domains [47]. This similarity can be explained if one assumes that, same as in Annelida, the metamorphosis of rhynchonelliformean larvae is not as drastic as it might seem and instead represents a relatively gradual process [66]. In T. transversa several of the adult structures, including shell secreting epithelium [63, 67] or pedicle muscles [69, 71], are already present in the competent larvae as the Anlagen. Thus, even though transition from larva to juvenile poses large ecological change, from the morphological point of view the mantle lobe inversion is related mostly with tissue relocation and not with the degeneration or formation of entire body regions, as is the case in pilidiophoran nemertans, indirectly developing hemichordates, sea urchins or ascidians.

From the phylogenetic and developmental point of view, it would be interesting to compare shifts of Hox genes expression observed during metamorphosis between T. transversa and Phoronida. Phoronids are closely related to brachiopods [56, 57, 90, 91] (in past even proposed as specialized clade belonging to Brachiopoda [61, 80]) and their rapid metamorphosis involves drastic rearrangements of the larval body plan [66, 92-95], which is much more complicated than the transition found in Rhynchonelliformea and sometimes referred to as catastrophic or cataclysmic metamorphosis [92, 94, 96]. The recent analysis of the body region specific transcriptomes revealed that in adults of Phoronis australis, which possesses an
organized Hox cluster, Hox gene expression does not exhibit spatial collinearity [18].

Unfortunately, data on the spatial expression of Hox genes in early developmental stages of any phoronid species are still lacking [96], preventing analysis of metamorphosis-related Hox genes expression shifts. Nevertheless, it is possible that in phoronids the larvae and juveniles exhibits pronounced differences in the Hox genes expression as is a case in some other animals with catastrophic and extensive metamorphosis [15, 49, 50].

Expression of Hox genes during the morphogenesis of brachiopod specific structures in T. transversa

Although Hox genes are believed to originally be responsible for antero-posterior patterning [1, 2, 11], in certain animal lineages some of them were coopted for morphogenesis of evolutionary novel structures [97-100]. Among Spiralia such phenomenon has been reported in e.g. conchiferan molluscs [36-38] and annelids [34, 35, 47, 48], whereas recently Schiemann et al. suggested that in brachiopod larvae 4 out of 10 Hox genes have been recruited for patterning of chaetae (lab and Post1) and shell fields (Scr and Antp) [19]. Our results generally support findings of Schiemann et al. – although we did not find evidence for the expression of Antp in the shell field – and show that cooption of Hox genes for morphogenesis of novel structures is even more pronounced in juveniles of T. transversa than it is in the larvae.

lab and Post1 are recruited for the morphogenesis of chaetae in the embryos and larvae of T. transversa [19]. The lab is constantly expressed in the chaetal sacs from formation of their early Anlagen up to the latest larval stage, whereas Post1 is only
briefly expressed during short time window, when the Anlagen are formed. In our study we detect expression of lab in the chaetal sacs of juveniles as well, but surprisingly we found that Post1 is also expressed in postmetamorphic juveniles. Moreover, its expression is not only restricted to the chaetal sacs but instead could be detected in the whole marginal zone of the mantle. This finding, however, makes sense when one takes into consideration that as adults T. transversa, as most of the rhynchonelliformean brachiopods, possess numerous chaetae along the whole mantle margin [86, 101]. We therefore propose, that although both lab and Post1 are involved in the chaetae formation in Rhynchonelliformea, they play different roles: Post1 is expressed in the regions where prospective chaetae will develop, possibly stimulating epidermal cells to differentiate into chaetal sacs before its expression decays. A similar role has been suggested for Post1 in annelids, whose chaetae are considered homologous to brachiopod ones based on morphological [102] and molecular [19] similarities. In annelids Post1 is expressed in the cells of developing chaetae-bearing parapodia but the expression faints over the time of development and is not detectable in the already formed parapodia [34, 35, 47]. lab, on the other hand, is possibly involved in the patterning of the growth of the chaetae itself, remaining expressed long after onset of chaetal sac formation. This hypothesis needs to be tested in future by functional gene inference and the examination of older juveniles or adults, in which, accordingly, we would expect lack of Post1 expression and broad expression of lab along the whole mantle margin.

The two-valved shell and posterior pedicle represent two distinct apomorphies of brachiopods and we found four out of ten Hox genes expressed in the structures related with those morphological novelties. Our results indicates that Scr is likely co-
opted for the juvenile shell formation, as the gene is expressed in the mantle margin in the region specialized for shell secretion [68]. This finding corresponds to the results of Schiemann et al. [19], who found expression of Scr in the epithelial cells forming larval shell rudiment. Both shell and pedicle require sets of specialized muscles, which constitute an important part of the brachiopod body. In late larvae of *T. transversa* the genes *Pb* and *Dfd* are likely responsible for A-P patterning of mesoderm [19], yet during post-embryonic development they seem to be recruited into morphogenesis of specific muscular structures that drive the biomechanics of respectively shell and pedicle. Additionally, *Hox3*, another gene that seems to play a role in mesoderm patterning during earlier developmental stages [19], is expressed in the regions where future hinge rudiments will develop [68], suggesting that it could be involved in the morphogenesis of this autapomorphic rhychoelliformian feature.

**Conclusions**

All developmental stages of *Terebratalia transversa*, including juveniles, express *Hox* genes in a spatially non-collinear manner [19]. Most of the patterns observed in the late larvae seem to persist throughout metamorphosis and are retained in juveniles, corroborating morphological observations that metamorphosis, despite being rapid, is of gradual type and most of the adult organs are present as Anlagen in the competent larvae. The most drastic shifts in *Hox* gene expression patterns observed during metamorphosis can be explained by: 1) the inversion of the mantle lobe which relocates some of the more posterior larval structures into the anterior edge
of the juveniles and 2) restriction of broad expression domains, present in larvae, to specific structures in juveniles.

Concordantly to the previous study on larvae of *T. transversa* we found that certain *Hox* genes have been evolutionary coopted for morphogenesis of specialized structures in brachiopods. In both larvae and juveniles *lab* is expressed in the chaetal sacs whereas *Post1* marks the area where prospective chaetae will develop. In juveniles four out of the ten *Hox* genes are expressed in the epidermal (*Scr, Hox3*) and muscular (*pb, Dfd*) tissues related with shell and pedicle, two autapomorphic features of Brachiopoda.

**Methods**

**Animal collection and fixation**

Gravid adults of *Terebratalia transversa* (Sowerby, 1846) were collected near San Juan Island, Washington, USA. Eggs obtained from the animals were fertilized and developing larvae were cultured following previously published protocols (e.g. [19, 63, 67]) up to the metamorphosis. Two days after metamorphosis, juvenile animals were gently scraped from the bottom of the dish with a razor blade, relaxed with MgCl₂, fixed in 3.7% formaldehyde and washed in phosphate buffer. Fixed animals were stored in 100% methanol.

**In situ hybridization**

Probes against *Hox* genes were synthesized using the same plasmid clones as used in Schiemann et al. 2017, where the gene orthology assessment has been performed[19]. Single whole-mount *in situ* hybridization was performed following an
dUTP-digoxigenin-labelled probes were hybridized at a concentration of 1 ng/μl at 67°C for 48 hours, 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 (in colorimetric in situ hybridization) or detected with anti-digoxigenin-POD antibody in 1:200 concentration in blocking buffer and visualized with TSA-Cy5-Plus (in fluorescent in situ hybridization). Additionally, animals prepared for FISH where stained for 30 minutes in DAPI to visualize cell nuclei. Stained juveniles where mounted in 70% glycerol and examined with Zeiss Axiocam HRc connected to a Zeiss Axioscope Ax10 using bright-field Nomarski optics (CISH) or scanned in Leica SP5 confocal laser-scanning microscope (FISH).

**Immunohistochemistry**

For investigation of juvenile morphology, mouse primary antibodies against anti-tyrosinated tubulin (Sigma, T9028) and anti-acetylated tubulin (Sigma, T6793) were used in 1:500 concentration. To visualize the primary antibodies, secondary goat anti-mouse antibodies (Life Technologies) conjugated with fluorochrome (AlexaFluor647) were applied in 1:50 concentration. F-actin was visualized with AlexaFluor555-labeled phalloidin and cell nuclei were stained with DAPI. Stained juveniles where mounted in 80% glycerol and scanned in Leica SP5 confocal laser-scanning microscope.

**Image processing and figure preparation**
Z-stacks of confocal scans were projected into 2D images and 3D reconstructions in IMARIS 9.1.2. Both light micrographs and CLSM images were adjusted in Adobe Photoshop CS6 and assembled in Adobe Illustrator CS6. All the schematic drawings were done with Adobe Illustrator CS6.
Declaration

Ethics approval and consent to participate

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

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

AH designed the study, collected samples and contributed to writing; LG conducted the in situ hybridizations and the confocal studies, arranged figures and drafted the manuscript.

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References

1. McGinnis W, Krumlauf R: Homeobox genes and axial patterning. *Cell* 1992, 68(2):283-302.
2. Pearson JC, Lemons D, McGinnis W: Modulating Hox gene functions during animal body patterning. *Nat Rev Genet* 2005, 6(12):893-904.
3. Larroux C, Fahey B, Degnan SM, Adamski M, Rokhsar DS, Degnan BM: The NK homeobox gene cluster predates the origin of Hox genes. *Curr Biol* 2007, 17(8):706-710.
4. Ryan JF, Pang K, Program NCS, Mullikin JC, Martinez MQ, Baxevanis AD: The homeodomain complement of the ctenophore Mnemiopsis leidyi suggests that Ctenophora and Porifera diverged prior to the ParaHoxozoa. *Evodevo* 2010, 1(1):9.
5. Jakob W, Sagasser S, Dellaporta S, Holland P, Kuhn K, Schierwater B: The Trox-2 Hox/ParaHox gene of *Trichoplax* (Placozoa) marks an epithelial boundary. *Dev Genes Evol* 2004, 214(4):170-175.
6. Monteiro AS, Schierwater B, Dellaporta SL, Holland PW: A low diversity of ANTP class homeobox genes in Placozoa. *Evol Dev* 2006, 8(2):174-182.
7. Srivastava M, Begovic E, Chapman J, Putnam NH, Hellsten U, Kawashima T, Kuo A, Mitros T, Salamov A, Carpenter ML et al: The *Trichoplax* genome and the nature of placozoans. *Nature* 2008, 454(7207):955-960.
8. Yanze N, Spring J, Schmidli C, Schmid V: Conservation of Hox/ParaHox-related genes in the early development of a cnidarian. *Developmental Biology* 2001, 236(1):89-98.
9. Finnerty JR: Cnidarians reveal intermediate stages in the evolution of Hox clusters and axial complexity. *Am Zool* 2001, 41(3):608-620.
10. Wallberg A, Thollesson M, Farris JS, Jondelius U: The phylogenetic position of the comb jellies (Ctenophora) and the importance of taxonomic sampling. *Cladistics* 2004, 20(6):558-578.
11. Garcia-Fernández J: The genesis and evolution of homeobox gene clusters. *Nat Rev Genet* 2005, 6(12):881-892.
12. Duboule D: The rise and fall of Hox gene clusters. *Development* 2007, 134(14):2549-2560.
13. Ferrier DEK, Minguillon C: Evolution of the Hox/ParaHox gene clusters. *Int J Dev Biol* 2003, 47(7-8):605-611.
14. Seo HC, Edvardsen RB, Maeland AD, Bjordal M, Jensen MF, Hansen A, Flaat M, Weissenbach J, Lehrach H, Wincker P et al: Hox cluster disintegration with persistent anteroposterior order of expression in *Oikopleura dioica*. *Nature* 2004, 431(7004):67-71.
15. 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):15118-15123.
16. Moreno E, Nadal M, Baguna J, Martinez P: Tracking the origins of the bilaterian Hox patterning system: insights from the acelo flatworm *Symosagittifera roscoffensis*. *Evol Dev* 2009, 11(5):574-581.
17. Cameron RA, Rowen L, Nesbitt R, Bloom S, Rast JP, Berney K, Arenas-Mena C, Martinez P, Lucas S, Richardson PM et al: Unusual gene order and
organization of the sea urchin hox cluster. J Exp Zool B Mol Dev Evol 2006, 306(1):45-58.

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

19. Schiemann SM, Martín-Durán JM, Børve A, Vellutini BC, Passamanek C, 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.

20. Aboobaker AA, Blaxter ML: Hox Gene Loss during Dynamic Evolution of the Nematode Cluster. Curr Biol 2003, 13(1):37-40.

21. Albertin CB, Simakov O, Mitros T, Wang ZY, Eddsinger-Gonzales E, Brenner S, Ragsdale CW, Rokhsar DS: The octopus genome and the evolution of cephalopod neural and morphological novelties. Nature 2015, 524(7564):220-4.

22. Simakov O, Marletaz F, Cho SJ, Eddsinger-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.

23. Lewis EB: A gene complex controlling segmentation in Drosophila. Nature 1978, 276(5688):565-570.

24. Beeman RW: A Homeotic Gene-Cluster in the Red Flour Beetle. Nature 1987, 327(6119):247-249.

25. Graham A, Papalopulu N, Krumlauf R: The murine and Drosophila homeobox gene complexes have common features of organization and expression. Cell 1989, 57(3):367-378.

26. Kenyon C, Wang B: A cluster of Antennapedia-class homeobox genes in a nonsegmented animal. Science 1991, 253(5019):516-517.

27. Hejnol A, Martindale MQ: Coordinated spatial and temporal expression of Hox genes during embryogenesis in the acoel Convolutriloba longifissura. BMC Biol 2009, 7:65.

28. Martín-Durán JM, Pang K, Børve A, Le HS, Furu A, Cannon JT, Jondelius U, Hejnol A: Convergent evolution of bilaterian nerve cords. Nature 2018, 553(7686):45-50.

29. Aronowicz J, Lowe CI: Hox gene expression in the hemichordate Saccoglossus kowalevskii and the evolution of deuterostome nervous systems. Integr Comp Biol 2006, 46(6):890-901.

30. Janssen R, Eriksson BJ, Tait NN, Budd GE: Onychophoran Hox genes and the evolution of arthropod Hox gene expression. Front Zool 2014, 11(1):22.

31. 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.

32. Frohbius AC, Funch P: Rotiferan Hox genes give new insights into the evolution of metazoan bodyplans. Nat Commun 2017, 8(1):9.

33. 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.
34. Kulakova MA, Kostyuchenko RP, Andreeva TF, Dondua AK: The abdominal-B-like gene expression during larval development of Nereis virens (polychaeta). Mech Dev 2002, 115(1-2):177-179.

35. 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.

36. 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.

37. Lee PN, Callaert P, De Coet HG, Martindale MQ: Cephalopod Hox genes and the origin of morphological novelties. Nature 2003, 424(6952):1061-1065.

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

39. 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.

40. Fritsch M, Wollesen T, de Oliveira AL, Wanninger A: Unexpected co-linearity of Hox gene expression in an aculiferan mollusk. BMC Evol Biol 2015, 15:151.

41. 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, 6:26.

42. Sly BJ, Snoke MS, Raff RA: Who came first--larvae or adults? origins of bilaterian metazoan larvae. Int J Dev Biol 2003, 47(7-8):623-632.

43. Raff RA: Origins of the other metazoan body plans: the evolution of larval forms. Philos Trans R Soc Lond B Biol Sci 2008, 363(1496):1473-1479.

44. Wald G: Metamorphosis: An Overview. In: Metamorphosis: A Problem in Developmental Biology. Edited by Gilbert LF, E., 2nd edn. New Yourk and London: Plenum Press; 1981: 1-39.

45. Tata JR: Gene expression during metamorphosis: an ideal model for post-embryonic development. Bioessays 1993, 15(4):239-248.

46. Atkinson BG: Metamorphosis - Model Systems for Studying Gene-Expression in Postembryonic Development. Dev Genet 1994, 15(4):313-319.

47. Frobius 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.

48. Bakalenko NI, Novikova EL, Nesterenko AY, Kulakova MA: Hox gene expression during postlarval development of the polychaete Alitta virens. Evodevo 2013, 4(1):13.

49. 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, 13:23.
50. 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.

51. 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 U S A* 1998, 95(22):13062-13067.

52. Arenas-Mena C, Cameron AR, Davidson EH: Spatial expression of Hox cluster genes in the ontogeny of a sea urchin. *Development* 2000, 127(21):4631-4643.

53. Swalla BJ: Building divergent body plans with similar genetic pathways. *Heredity (Edinb)* 2006, 97(3):235-243.

54. Mooi R, David B: Radial Symmetry, the Anterior/Posterior Axis, and Echinoderm Hox Genes. *Annu Rev Ecol Evol S* 2008, 39:43-62.

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

56. 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 Evolutionary Biology* 2013, 13.

57. Laumer CE, Bekkouche N, Kerb A, Goetz F, Neves RC, Sorensen MV, Kristensen RM, Hejno A, Dunn CW, Giri et al: Spiralian Phylogeny Informs the Evolution of Microscopic Lineages. *Curr Biol* 2015, 25(15):2000-2006.

58. 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.

59. Hejnol A: A twist in time--the evolution of spiral cleavage in the light of animal phylogeny. *Integr Comp Biol* 2010, 50(5):695-706.

60. 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.

61. Cohen BL, Weydmann A: Molecular evidence that phoronids are a subtaxon of brachiopods (Brachiopoda : Phoronata) and that genetic divergence of metazoan phyla began long before the early Cambrian. *Org Divers Evol* 2005, 8(4):253-273.

62. Santagata S: Brachiopoda. In: *Evolutionary Developmental Biology of Invertebrates, Vol2 Lophotrochozoa (Spiralia).* Edited by Wanninger A. Wien: Springer; 2015: 263-277.

63. Stricker SA, Reed CG: The ontogeny of shell secretion in *Terebratalia transversa* (Brachiopoda, Articulata). I. Development of the mantle. *J Morphol* 1985, 183(3):233-250.

64. Vellutini BC, Hejnol A: Expression of segment polarity genes in brachiopods supports a non-segmental ancestral role of engrailed for bilaterians. *Sci Rep* 2016, 6:32387.
65. Kuzmina TV, Malakhov VV: Structure of the brachiopod lophophore. *Paleontol J* 2007, 41(5):520-536.

66. Freeman G: A developmental basis for the Cambrian radiation. *Zool Sci* 2007, 24(2):113-122.

67. Freeman G: Metamorphosis in the Brachiopod Terebratulina: Evidence for a Role of Calcium Channel Function and the Dissociation of Shell Formation from Settlement. *Biol Bull* 1993, 184(1):15-24.

68. Stricker SA, Reed CG: The Ontogeny of Shell Secretion in Terebratulina transversa (Brachiopoda, Articulata) 2: Formation of the Protegulum and Juvenile Shell. *J Morphol* 1985, 183(3):251-271.

69. Santagata S: Evaluating neurophylogenetic patterns in the larval nervous systems of brachiopods and their evolutionary significance to other bilaterian phyla. *J Morphol* 2011, 272(10):1153-1169.

70. Altenburger A, Wanninger A: Comparative larval myogenesis and adult myoanatomy of the rhynchonelliform (articulate) brachiopods Argyrotheca cordata, A. cistellula, and Terebratulina transversa. *Frontiers in Zoology* 2009, 6.

71. Stricker SA, Reed CG: Development of the Pedicle in the Articulate Brachiopod Terebratulina transversa (Brachiopoda, Terebratulida). *Zoomorphology* 1985, 105(4):253-264.

72. Stricker SA, Reed CG: The Protegulum and Juvenile Shell of a Recent Articulate Brachiopod - Patterns of Growth and Chemical-Composition. *Lethaia* 1985, 18(4):295-303.

73. Wanninger A: Morphology is dead – long live morphology! Integrating MorphoEvoDevo into molecular EvoDevo and phylogenomics. *Frontiers in Ecology and Evolution* 2015, 3:1 - 9.

74. Long JAS, S.A.: 2. Brachiopoda. In: *Reproduction of Marine Invertebrates*. Edited by Giese AGP, J. S.,; Pearson, V. B., vol. VI Echinoderms and Lophophorates. Pacific Grove, California: The Boxwood Press; 1991: 47-84.

75. Lüter C: Ultrastructure of the Metanephridia of Terebratulina retusa and Crania anomala (Brachiopoda). *Zoomorphology* 1995, 115(2):99-107.

76. Rowell AJ: Some early stages in the development of the brachiopod Crania anomala (O.F. Müller). *Annals and Magazine of Natural History* 1960, 13:35-52.

77. Percival E: A Contribution to the Life-History of the Brachiopod Tegularrhynchia nigricans. *Q J Microsc Sci* 1960, 101(4):439-457.

78. Lüter C: The origin of the coelom in Brachiopoda and its phylogenetic significance. *Zoomorphology* 2000, 120(1):15-28.

79. Sinigaglia C, Thiel D, Hejnol A, Houlston E, Leclerc L: A safer, urea-based in situ hybridization method improves detection of gene expression in diverse animal species. *Dev Biol* 2018, 434(1):15-23.

80. Cohen BL: Monophyly of brachiopods and phoronids: reconciliation of molecular evidence with Linnaean classification (the subphylum Phoroniformea nov.). *Proc Roy Soc B-Biol Sci* 2000, 267(1440):225-231.

81. Chuang SH: Larval Development in Discinisca (Inarticulate-Brachiopod). *Am Zool* 1977, 17(1):39-53.
82. Nielsen C: **The Development of the Brachiopod Crania (Neocrania) anomala (Muller, O.F.) and Its Phylogenetic Significance.** *Acta Zool - Stockholm* 1991, 72(1):7-28.

83. Altenburger A, Wanninger A, Holmer LE: **Metamorphosis in Craniformea revisited: Novocrania anomala shows delayed development of the ventral valve.** *Zoomorphology* 2013, 132(4):379-387.

84. Freeman G, Lundelius JW: **Changes in the timing of mantle formation and larval life history traits in linguliform and craniform brachiopods.** *Lethaia* 1999, 32(3):197-217.

85. Altenburger A, Wanninger A: **Neuromuscular development in Novocrania anomala: evidence for the presence of serotonin and a spiralian-like apical organ in lecithotrophic brachiopod larvae.** *Evol Dev* 2010, 12(1):16-24.

86. Lüter C: **Ultrastructure of larval and adult setae of Brachiopoda.** *Zool Anz* 2000, 239(1):75-90.

87. Cohen BL, Holmer LE, Lüter C: **The brachiopod fold: A neglected body plan hypothesis.** *Palaeontology* 2003, 46:59-65.

88. Altenburger A, Martinez P, Budd GE, Holmer LE: **Gene Expression Patterns in Brachiopod Larvae Refute the "Brachiopod-Fold" Hypothesis.** *Front Cell Dev Biol* 2017, 5:74.

89. 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.

90. Helmkampf M, Bruchhaus I, Hausdorf B: **Phylogenomic analyses of lophophorates (brachiopods, phoronids and bryozoans) confirm the Lophotrochozoa concept.** *P Roy Soc B-Biol Sci* 2008, 275(1645):1927-1933.

91. Hausdorf B, Helmkampf M, Nesnidal MP, Bruchhaus I: **Phylogenetic relationships within the lophophorate lineages (Ectoprocta, Brachiopoda and Phoronida).** *Mol Phylogenet Evol* 2010, 55(3):1121-1127.

92. Santagata S: **Structure and metamorphic remodeling of the larval nervous system and musculature of Phoronis pallida (Phoronida).** *Evol Dev* 2002, 4(1):28-42.

93. Temereva EN, Malakhov VV: **Development of excretory organs in Phoronopsis harmeri (Phoronida): From protonephridium to nephromixium.** *Zool Zh* 2006, 85(8):915-924.

94. 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 Evolutionary Biology* 2015, 15.

95. Temereva EN, Tsitrin EB: **Development, organization, and remodeling of phoronid muscles from embryo to metamorphosis (Lophotrochozoa: Phoronida).** *BMC Dev Biol* 2013, 13:14.

96. Santagata S: **Phoronida.** In: *Evolutionary Developmental Biology of Invertebrates, Vol2 Lophotrochozoa (Spiralia).* Edited by Wanninger A. Wien: Springer; 2015: 231-246.

97. True JR, Carroll SB: **Gene co-option in physiological and morphological evolution.** *Annu Rev Cell Dev Biol* 2002, 18:53-80.
98. Wasik BR, Rose DJ, Moczek AP: Beetle horns are regulated by the Hox gene, Sex combs reduced, in a species- and sex-specific manner. *Evol Dev* 2010, 12(4):353-362.

99. Tsuchimoto J, Yamaguchi M: Hox expression in the direct-type developing sand dollar *Peronella japonica*. *Dev Dyn* 2014, 243(8):1020-1029.

100. Glassford WJ, Johnson WC, Dall NR, Smith SJ, Liu Y, Boll W, Noll M, Rebeiz M: Co-option of an Ancestral Hox-Regulated Network Underlies a Recently Evolved Morphological Novelty. *Dev Cell* 2015, 34(5):520-531.

101. Williams A: Brachiopoda: introduction and integumentary system. In: *Microscopic Anatomy of Invertebrates* Edited by Harrison FW, R., vol. Volume 13. Lophophorates, Entoprocta, and Cyclophora. New York: Wiley-Liss Inc.; 1997.

102. Lüter C, Bartolomaeus T: The phylogenetic position of Brachiopoda - a comparison of morphological and molecular data. *Zool Scr* 1997, 26(3):245-253.

103. Hejnol A: In situ protocol for embryos and juveniles of *Convolutriloba longifissura*. *Protocole Exchange* 2008, 201.
Fig. S1. Background signal resulting from unspecific binding of probes by surface of larval dorsal protegulum (asterisks) and borders between protegulum and remaining shell (arrowheads). The control without probes (A). Colorimetric *in situ* hybridization with sense probe of *Antp* (B) and *Post2* (C) genes, signal developed for the same time as for antisense probes. Fluorescent *in situ* hybridization with antisense probes of *Antp* (D, E) and *Post2* (F, G) genes, on E and G combined with DAPI staining of cell nuclei. Dorso-ventral view with anterior to the top (A–D, F) and virtual cross section with dorsal to the top (E, G). Dashed lines with letters on D and F indicate section planes shown on respective plates.