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ORIGINAL ARTICLE

Hox gene expression in larval development of the polychaetes *Nereis virens* and *Platynereis dumerilii* (Annelida, Lophotrochozoa)

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Abstract The bilaterian animals are divided into three great branches: the Deuterostomia, Ecdysozoa, and Lophotrochozoa. The evolution of developmental mechanisms is less studied in the Lophotrochozoa than in the other two clades. We have studied the expression of Hox genes during larval development of two lophotrochozoans, the polychaete annelids *Nereis virens* and *Platynereis dumerilii*. As reported previously, the Hox cluster of *N. virens* consists of at least 11 genes (de Rosa R, Grenier JK, Andreeva T, Cook CE, Adoutte A, Akam M, Carroll SB, Balavoine G, *Nature*, 399:772–776, 1999; Andreeva TF, Cook C, Korchagina NM, Akam M, Dondua AK, *Ontogenez* 32:225–233, 2001); we have also cloned nine Hox genes of *P. dumerilii*. Hox genes are mainly expressed in the descendants of the 2d blastomere, which form the integument of segments, ventral neural ganglia, pre-pygidial growth zone, and the pygidial lobe. Patterns of expression are similar for orthologous genes of both nereids. In *Nereis*, *Hox2*, and *Hox3* are activated before the blastopore closure, while *Hox1* and *Hox4* are activated just after this. *Hox5* and *Post2* are first active during the metatrochophore stage, and *Hox7*, *Lox4*, and *Lox2* at the late nectochaete stage only. During larval stages, *Hox* genes are expressed in staggered domains in the developing segments and pygidial lobe. The pattern of expression of Hox cluster genes suggests their involvement in the vectorial regionalization of the larval body along the antero-posterior axis. Hox gene expression in nereids conforms to the canonical patterns postulated for the two other evolutionary branches of the Bilateria, the Ecdysozoa and the Deuterostomia, thus supporting the evolutionary conservatism of the function of Hox genes in development.

Keywords Hox gene expression · Larval development · *Nereis virens* · *Platynereis dumerilii* · Annelida · Polychaeta · Lophotrochozoa

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Introduction

The Hox genes encode a family of transcription factors that are key determinants of vectorial positional information in bilaterians (Akam 1998; Peterson and Davidson 2000;

Peterson et al. 2000a; Carroll et al. 2001). The Bilateria are divided into three great clades: two large groups of protostomes, the Ecdysozoa (nematodes, arthropods, kinorhynchs, priapulids, nematomorphs, etc.) and the Lophotrochozoa (platyhelminths, annelids, molluscs, brachiopods, bryozoans, phoronids, etc.), and the Deuterostomia (echinoderms, hemichordates, chordates, etc.) (Aguinaldo et al. 1997; de Rosa et al. 1999; Philippe et al. 2005). Hox gene function has been studied extensively in a number of ecdysozoans and deuterostomes, particularly in model organisms, but relatively little work has been carried out with lophotrochozoans (Kmita-Cunisse et al. 1998; Irvine and Martindale 2000; Peterson et al. 2000b; Shankland and Seaver 2000; Andreeva et al. 2001; Callaerts et al. 2002; Lee et al. 2003; Hinman et al. 2003).

The Lophotrochozoa include taxa with both segmented and unsegmented bodies and both direct and indirect development. Understanding the functions of Hox genes in the Lophotrochozoa could help elucidate the formation of morphological variety among this great clade, and could also shed light on the evolutionary history of this group.

Published data on Hox gene function in larval development of the Lophotrochozoa are limited to two molluscs and two groups of annelids. Hinman et al. (2003) examined the expression of Hox genes in the mollusc, *Haliotis asinina*. This gastropod has homoquadrant spiral cleavage, trochophore larvae with the rudiments of some adult structures, a shell gland, and a foot. The larval and adult bodies of the gastropods are unsegmented. The authors studied the expression of the five "anterior" Hox genes. They observed spatiotemporal colinearity during gangliogenesis in pre-torsional veliger larva for *Has–Hox2*, *Has–Hox3*, *Has–Hox4*, and *Has–Hox5*, but *Has–Hox1* was expressed in the larval shell gland. Later, *Has–Hox1* and *Has–Hox4* take part in shell morphogenesis of the adult mollusc. The morphogenetic programs of larval and adult shell formation are evolutionary novelties, suggesting that the Hox genes may have been co-opted into a shell patterning role during the evolution of the molluscs.

Another group of molluscs, the cephalopods, is characterized by morphological modifications of the shell, mantle, and foot. The oocytes of these molluscs are very yolk-rich, and even early development has no "spiral" period: the cleavage is discoidal. These animals are direct-developers. Fragments of nine Hox genes of the squid, *Euprymna scolopes*, were cloned (Callaerts et al. 2002) and expression patterns of eight of them were examined in early development (Lee et al. 2003). These authors showed that Hox genes have kept their functions of specification of the neural ganglia using overlapping colinear domains of expression. The Hox genes in cephalopods have also been co-opted into the morphogenetic programs of development of two evolutionary novelties in this group, the brachial crown and the funnel tube. In these structures, one of the fundamental features of the Hox cluster, colinear expression, is not used for their patterning.

Among the annelids (segmented worms), Hox gene expression has been mostly studied in the leeches. For this group, spatial colinearity of Hox gene expression has been shown during specification of the ventral neural ganglia (Nardelli-Haefliger and Shankland 1992; Nardelli-Haefliger et al. 1994; Kourakis et al. 1997). However, the leeches have derived features: direct development and a complex reproductive system that are not representative of most annelids and which make extrapolation of studies of leeches to other annelids problematic.

Expression of five Hox genes was also analyzed in the larvae of *Chaetopterus* sp., a sedentary polychaete with a tagmatized body (Irvine and Martindale 2000). This worm has an atypical trochophore-like larva, which develops into a secondary larva with 15 segments that later form the heteronomous segments of the adult body. A homonomously segmented part of the adult trunk is formed by postlarval segmentation. Larval expression patterns of five Hox genes, *CH–Hox1*, *CH–Hox2*, *CH–Hox3*, *CH–Hox4*, and *CH–Hox5*, demonstrate both spatial and temporal colinearity. Expression was observed mostly in tissues derived from the ectoderm, including the neuroectoderm and, sometimes, in mesodermal tissues as well. The boundaries of expression appeared to correspond to the segment boundaries. The posterior boundaries of expression of some Hox genes correlate with tagmatic boundaries in the adult body. Persistent expression of all five anterior Hox genes was shown in the posterior part of the larvae, in the putative growth zone.

In this work, we focus on two species of errant nereidid polychaetes: *Nereis virens* from the White Sea and *Platynereis dumerilii* from the Mediterranean Sea. The larvae of these nereids form a peristomial segment and three parapodial segments, whereas the adult animals have dozens of morphologically similar segments. Morphological, developmental, and genome characteristics of nereids make us believe that these worms retain many primitive characteristics of the annelids (Arendt 2003; Tessmar-Raible and Arendt 2003; Raible et al. 2005), the molecular phylogeny of which remains problematic (Bleidorn et al. 2003; Hall et al. 2004; Seaver et al. 2005). Using RT-PCR and WMISH, we have examined patterns of Hox gene expression during development in these animals, from early trochophore to nectochaete larva.

Materials and methods

Animals

Adult *N. virens* were collected near the Marine Biological Station of St. Petersburg State University, at the White Sea,

Chupa Inlet. Mature animals were caught with a hand net at the water surface during their spawning period (June and July). Artificial fertilization and cultivation of the embryos were carried out at 10.5°C (Dondua 1975). Cultures of *P. dumerilii* were kept in the laboratory under artificial food and light conditions at 18°C (Hauenschild and Fischer 1969).

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from *N. virens* at various stages of development using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. In every case, RNA was isolated from 5,000 eggs, embryos or larvae. The quality and concentration of the RNA were checked by agarose gel electrophoresis. Contaminating DNA was removed from RNA preparations using a DNA-free kit (Ambion) according to the manufacturer's instructions.

RT-PCR reactions were carried out using the One-Step RT-PCR kit (Ambion), according to the manufacturer's instructions. Total RNA was used as a template with gene-specific primers. All reactions included a control using untreated RNA as a template to ensure that there was no contamination with DNA. A detailed protocol is available upon request.

Cloning of *N. virens* and *P. dumerilii* Hox genes

N. virens DNA was isolated from sperm obtained from males during spawning. A PCR survey using degenerate primers designed to match the most conserved regions of the homeodomain was performed and 103 clones sequenced. Inverse PCR with gene specific primers was used to obtain longer sequences of each gene (Andreeva et al. 2001). Partial sequences of 11 Hox genes from *N. virens* were reported previously (GenBank AF151663-AF151673; de Rosa et al. 1999). Additional sequences for eight of these genes, AF151663, AF151664, AF151666, AF151668, AF151670, AF151671, AF151673, and DQ366682, were obtained by inverse PCR, and the GenBank accessions have been updated.

A *P. dumerilii* cDNA library (Invitrogen Sport 6 vector) was created using total RNA isolated from the metatrochophore stage (48 h) of development. We used degenerate primers corresponding to the first and third α helixes of the homeodomain to amplify *Pdu–Hox1*, *Pdu–Hox2*, *Pdu–Hox3*, *Pdu–Hox4*, *Pdu–Hox5*, *Pdu–Lox5*, *Pdu–Lox2*, *Pdu–Post1*, and *Pdu–Post2* genes from the library. Multiple gene-specific primers were designed from these short fragments and used in nested PCR reactions with vector-specific primers to amplify longer fragments of genes from the library. Sequences of primers are available on request. Amplified products were cloned into the T-easy vector (Promega) and sequenced. Genes were unambiguously identified by comparison with previously identified *N. virens* Hox genes and submitted to GenBank (DQ366676-DQ366681, DQ366683-DQ366686). Sequences of all primers are available upon request.

Whole mount in situ hybridization (WMISH)

Embryos were fixed with 4% PFA in 1.75× PBS. WMISH was performed in *Nereis* as described in Irvine et al. (1999) and in *Platynereis* following established protocols with some modifications for various stages (Arendt et al. 2004). For intensification of alkaline phosphatase activity, 10% polyvinyl alcohol was sometimes added to the substrate (Fig. 3, brown color). A detailed protocol is available upon request. Digoxigenin-labeled RNA probes were prepared according to the manufacturer's protocol (Roche). Hybridization was carried out at 65°C. In *Nereis*, BM-purple (Roche) was used as a chromogenic substrate to localize the hybridized probe (Kulakova et al. 2002). The results were imaged on DMRXA microscope (Leica) with a Leica DC500 digital camera under Nomarski optics.

Results

Brief description of *N. virens* development

The cleavage is typically spiral. The largest blastomere at the four-cell stage is called D, and this cell receives the greatest part of the cytoplasm. The blastomeres of the 16-cell stage are already much diversified with respect to their cytoplasmic volume and composition. The 2d cell, called the first somatoblast, gives rise to a large part of the trunk tissue of the larva. The second somatoblast, 4d, gives rise to large parts of the mesoderm. Gastrulation involves epiboly in that the micromeres at the animal pole divide and cover the vegetal macromeres. The daughter cells of the second and the third quartet of micromeres, including stomatoblasts and some descendants of the second somatoblast, form the blastopore. The blastopore closes by convergent movement of blastoporal cells towards a central point, which is somewhat anterior to the dorsal lip. The 2d blastomere undergoes a series of mitotic divisions and gives rise to ectoteloblasts (as defined by Wilson 1892) located in the dorsal region; these are surrounded by earlier 2d daughter cells. The descendants of all these cells proliferate actively and move from dorsolateral positions to the ventral side towards the location of blastopore closure to form the ventral somatic plate. During blastopore closure, the stomatoblasts divide rapidly and form first the stomodaeal arch, which enlarges by divisions of its cells, and then the

stomodaeal plate. The stomodaeal arch transforms later into a circle with a small opening in the center (the mouth), and the stomodaeal plate forms the stomodaeal cavity by invagination. The trochoblasts become ciliated before the blastopore is fully closed and the mouth is formed. Very similar embryonic development was described by Wilson (1892) for another nereid, *Nereis limbata*, (now *Neanthes*, cit. by Nielsen 2004).

Embryonic development of *N. virens* is finished with the formation of a free-swimming larva, the trochophore. The trochophore displays no external signs of segmentation. An equatorially located ciliated ring, the prototroch, divides the larva into the episphere and the hyposphere.

We distinguish three different stages of the trochophore (Fig. 1, Table 1). Stage *ET*, *the early trochophore*, is characterized by an open blastopore; a stomodaeum that has not yet formed, and two large stomatoblasts surrounding the blastopore (Fig. 1, ET). On the dorsal side, there is an intensive proliferation of 2d descendants resulting in formation of material for the future somatic ventral plate and dorsal ectoderm. At the *MT* stage, *the middle trochophore*, the larva is spherical and has a fully formed stomodaeum which lies close to the future anal region. The ventral plate is poorly developed and larval eyes appear (Fig. 1, MT). At the *LT* stage, *the late trochophore*, the larva is characterized by some elongation of the hyposphere, at the posterior end of which the ciliated telotroch can be seen. The stomodaeum and future anal region are further apart, while the ventral plate extends by divisions of its cells and some convergent cell movement from lateral sides. The chaetal sacs become morphologically apparent, the first two pairs invaginating somewhat earlier then the third one (Fig. 1, LT).

Metamorphosis of the trochophore into a segmented metatrochophore occurs by external segmentation of the hyposphere. At stage *EM*, the *early metatrochophore*, the larva shows the first signs of external metamerization. The chaetae begin to develop in the two anterior pairs of chaetal sacs, but they do not yet protrude from the larval body (Fig. 1, EM). At the *MM* stage, the *middle metatrochophore*, the chaetae of the two anterior pairs of chaetal sacs protrude from the larval body, and the chaetae of the third chaetal sac start to form; adult eyes appear (Fig. 1, MM). At stage *LM*, the *late metatrochophore*, the larva is characterized by the progressive formation of the parapodial anlage and the pygidial (anal) lobe (Fig. 1, LM).

The metatrochophore develops into the nectochaete, the larval body of which acquires definitive features (Fig. 1, N). The nectochaete is pelagic and has functional parapodia and a distinct head with some head appendages. By this time, the midgut has formed, and definitive ventral neural ganglia have developed. The nectochaete has two split adult eyes, head protrusion, four segments (one peristomial and three parapodial), a pygidial lobe, and a pre-pygidial posterior growth zone from which new postlarval segments will be formed.

Development of *Platynereis* is very similar (Fischer and Dorresteijn 2004), but there are some minor differences in larval morphology and in the relative timing of events. For example, the embryos of *N. virens* are more yolk-rich than those of P. *dumerilii*, and as a consequence, the *N. virens* larvae develop some organs with some delay, or even do not form them at all in comparison to *P. dumerilii* larvae.

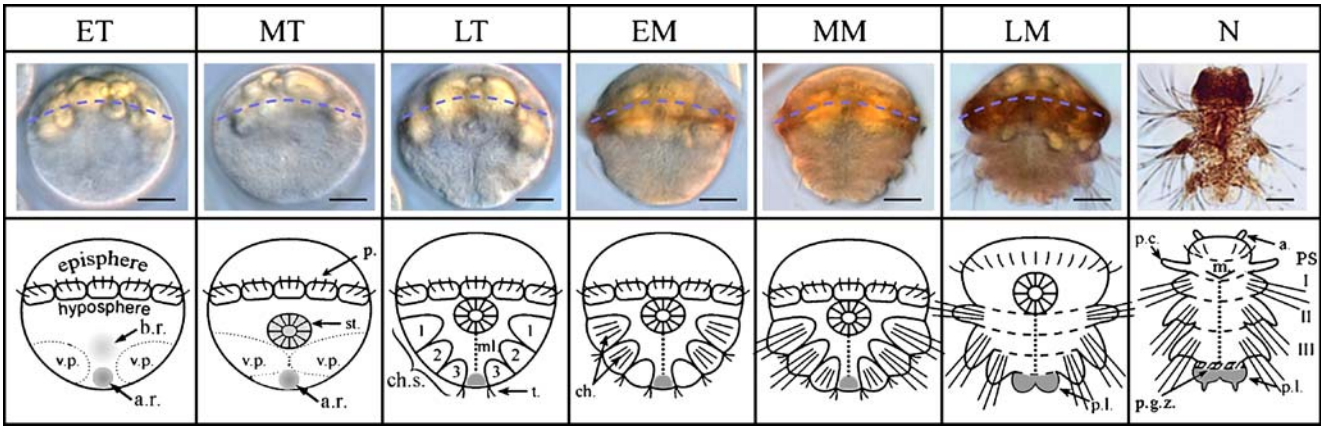


Fig. 1 Larval development of *Nereis virens*. ET, MT, LT, EM, MM, LM, N stages of larval development of *Nereis virens*. *Upper row*—microphotographs, *ventral view*; a *dashed line* marks the position of the prototroch. *Lower row*—schematic drawings. At ET and MT a *dotted line* delineates the ventral plate. *Grey zone* marks position of the future stomodaeum, *dark gray zone*—position of the anal region, which includes future proctodaeum and pygidial lobe. At LT, EM, MM, LM, and N a *dotted line* marks the position of the ventral midline. At LM 1, 2, 3 mark positions of chaetal sacs of the first, the second, and the third parapodial segments. At LM and N, *dashed lines* mark positions of segments, prepygidial growth zone, and pygidial lobe. *PS*—the peristomial segment; I, II, III—the first, the second, and the third parapodial segments. *a.* antennae, *a.r.* anal region, *b.r.* blastoporal region, *ch.* chaetae, *ch.s.* chaetal sacs, *m.* mouth, *ml.* midline, *p.* prototroch, *p.c.* peristomial cirri, *p.g.z.* prepygidial growth zone, *p.l.* pygidial lobe, *st.* stomodaeum, *t.* telotroch, *v.p.* ventral plate. *Scale bar*—50 μm

Table 1 Timing of developmental stages in the larvae of *Nereis virens* (at 10.5°C) and *Platynereis dumerilii* (at 18°C)

Name of stage	Brief description of main features	Approximate time of development, hours after fertilization	
		N. virens	*P. dumerilii*
ET, early trochophore	Trochoblasts are ciliated; stomodaeum is not formed	44–62	12–16
MT, middle trochophore	Larva is perfectly spherical; stomodaeum is fully formed; stomodaeum lies close to the future anal region; ventral plate is developed weakly	63–85	17–23
LT, late trochophore	Hyposphere is slightly elongated, ciliated telotroch is formed at its posterior end; stomodaeum and anal region lie more widely apart from each other; chaetal sacs become morphologically apparent	86–105	24–29
EM, early metatrochophore	Larva shows the first signs of external metamery; chaetae begin to develop in the two anterior pairs of chaetal sacs, but do not yet protrude from the larval body	106–122	30–40
MM, middle metatrochophore	Chaetae of the two anterior pairs of chaetal sacs protrude from the larval body, chaetae of the third chaetal sac start to form	123–152	41–52
LM, late metatrochophore	Larval body gradually elongates; parapodia anlage appear; beginning of the pygidial (anal) lobe formation	153–180	53–72
N, nectochaete	Functional parapodia; distinct head with some head appendages (two antennae and two peristomial cirri)	181–390	73–120
Juvenile worm	Fourth trunk segment (first postlarval) begins to form	16–17 days of development	5 days of development

Also, the apical ciliated tuft does not form in *N. virens*, although the apical rosette does form just as in *P. dumerilii* embryos. In general, development of *Platynereis* proceeds more quickly (Table 1). For the *Hox* gene expression comparisons presented here, the best matching *Platynereis* stages have been chosen.

Nereidid Hox gene complement and nomenclature

Previous work has identified 11 Hox genes in *N. virens* (Andreeva et al. 2001) and in two other lophotrochozoans, the earthworm, *Perionyx excavatus* (Cho et al. 2003), and the scallop, *Mizuhopecten yessoensis* (unpublished, GenBank accessions AB206313–AB206232). We believe that these 11 genes represent the ancestral complement of Hox genes for the annelids and, perhaps, the Lophotrochozoa. Figure 2 shows the Hox genes of *N. virens* and *P. dumerilii* as a reference for the discussion below. The five "anterior" nereid genes are clearly orthologous to the five anterior-most Hox genes in the Ecdysozoa and the Deuterostomia (de Rosa et al. 1999; Telford 2000). Similarly, the two lophotrochozoan "posterior" genes, *Post1* and *Post2*, are orthologous to the ecdysozoan *AbdB* genes and the deuterostome, *Hox9–Hox14* genes, and are derived from a single ancestral gene that has duplicated once in the lophotrochozoan lineage and multiple times within the deuterostome lineage. The four remaining lophotrochozoan Hox genes, represented by the nereidid, *Lox5*, *Hox7*, *Lox2*, and *Lox4* genes, are orthologous to three ecdysozoan genes represented by *Drosophila Antp*, *Ubx*, and *AbdA*, but the exact relationship between the two sets of genes is still unclear. It seems likely that a lophotrochozoan/ecdysozoan ancestor had at least two of these genes and that there have been independent gene duplications in one or both lineages, but neither the sequence data nor the expression data allow us to resolve the relationships at present. The *Lox5*, *Lox2*, and *Lox4* genes are named after previously identified homologues in leeches. For each of the 11 genes, we have assigned a generic label (Fig. 2) which we use to refer to results that pertain to that gene generally. For results that pertain to only a single taxon we use the gene name for that taxon.

Expression of Hox genes in larval development of *N. virens* and *P. dumerilii*

The expression of some Hox genes is activated during the early ET stage in descendants of two blastomeres, the first (2d) and the second (4d) somatoblasts which form the somatic plate and mesodermal bands, respectively. Mesodermal expression starts earlier than expression in the forming somatic plate and ends at the ET stage. In this paper, we focus on ectodermal Hox gene expression in descendants of the first somatoblast only, in the forming somatic plate. Mesodermal expression will be described by our subsequent investigations.

Hox1 in situ hybridization results

Nvi–Hox1 is first expressed at the ET stage (47 h). Initial expression of *Nvi–Hox1* (probe 548 nt) is two lateral spots in the hyposphere (Fig. 3, *Nvi–Hox1*). Expression spreads

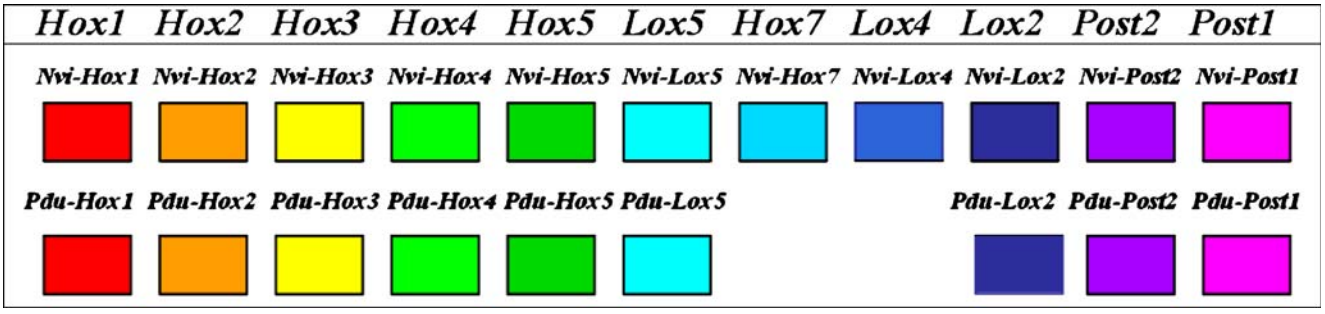


Fig. 2 Known Hox genes from the polychaete annelids, *Nereis virens* (*Nvi*) and *Platynereis dumerilii* (*Pdu*). Top row shows generic names for orthologue groups used in the text. *Lox2* and *Lox4* are related to the ecdysozoan *abdA* and *Ubx* genes and are thus placed immediately adjacent to the two *Posterior* genes in the figure. *Lox5*, by default, occupies the last remaining position

ventrally at MT and forms a strip in the area of the future first parapodial segment. It becomes stronger at LT. At this stage, weak expression is also observed in the future second parapodial segment. It becomes robust at EM, MM, and LM and remains localized. At MM, weak expression is observed in the future third parapodial segment. During nectochaete development, the expression of *Nvi–Hox1* becomes weaker in the larval ectoderm, except the ectoderm of the parapodia, and stronger in the ventral neuroectoderm which forms the ventral ganglia of the first, second, and third parapodial segments. Patterns of *Nvi–Hox1* and *Pdu–Hox1* (probe 457 nt) expression in the ventral plate are identical (Fig. 3, *Nvi–Hox1*, *Pdu–Hox1*).

Additional expression domains of *Hox1* are detected in both species of polychaetes, but the patterns are dissimilar. In apical tuft cells, expression of *Hox1* is seen only in *P. dumerilii*, at LT (not shown), where this gene seems to be involved in differentiation of these tuft cells. It will be recalled that *N. virens* larvae do not develop the apical ciliated tuft at all. *Nvi–Hox1* (not shown) and *Pdu–Hox1* (Fig. 4a) are expressed in stomodaeal cells in the metatrochophore. *Nvi–Hox1* (Fig. 4b) marks the regions of the forming and formed peristomial cirri. This expression starts at the MM stage and persists during all postlarval development. One more additional domain of *Hox1* expression is seen in the anal cirri (not shown).

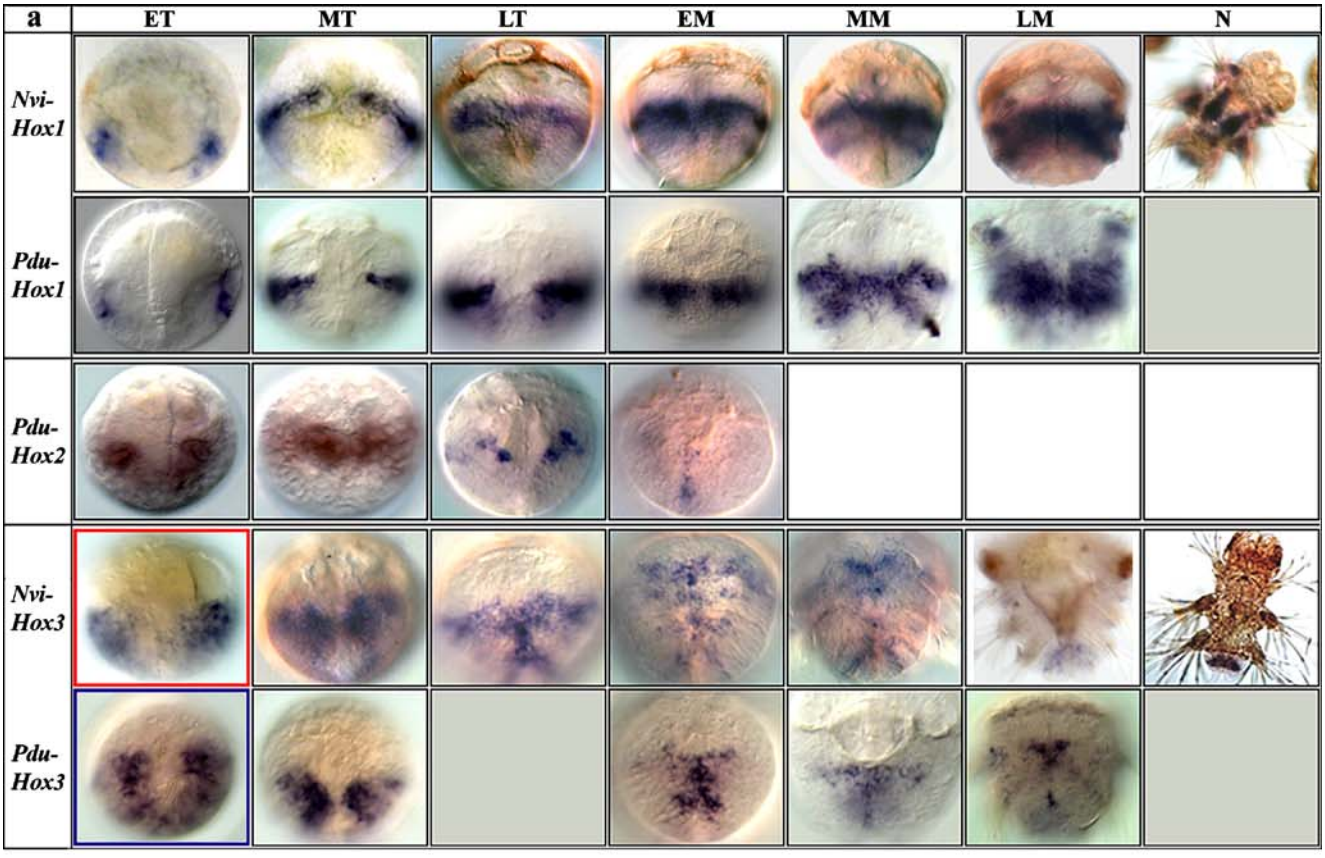
Hox2 in situ hybridization RT-PCR results

The RT-PCR data show that *Nvi–Hox2* expression starts at 34 h during embryonic stages (Fig. 5). We were not able to detect *Nvi–Hox2* expression at any stage of development by WMISH (probe 580 nt), but in situ probing with *Pdu–Hox2* was successful (probe 1400 nt). We do not know yet when *Pdu–Hox2* expression begins, but at the ET stage, expression already exists (Fig. 3, *Pdu–Hox2*). During stages ET and MT, two broad domains of *Pdu–Hox2* expression are localized under the stomodaeal region in the most anterior part of the forming ventral plate. Expression is superficial. The domains of expression considerably narrow at LT and become restricted to two narrow zones on both sides of the stomodaeum, which perhaps correspond to the forming peristomium, the first, and probably, the second and the third parapodial segments. Thus, within the ventral plate, the anterior boundary of *Pdu–Hox2* expression lies more anterior than that of *Pdu–Hox1* expression. Expression in the ventral plate persists into the early metatrochophore and is then downregulated, remaining weakly only in a few cells (Fig. 3a). Note that at the ET and MT stages, the signal was detected using a more sensitive in situ hybridization method (brown staining), while the usual method was used at the LT and EM stages (blue staining). These results suggest that the level of *Hox2* expression is very low in both worms.

A new domain of *Pdu–Hox2* expression appears at the EM stage in prismatic cells at the surface of the posterior pole of the metatrochophore (Fig. 4c). Additional domains of *Pdu–Hox2* expression are also observed at the LT stage in deep internal zones of the chaetal sacs at the location of chaetoblast determination of each parapodial segment. Expression in the third parapodial segment begins slightly later than in the first and the second. Expression persists to the MM stage (Fig. 4d) and then gradually disappears in the first, second, and then the third parapodial segments when larval chaetae are completely formed (not shown).

Hox3 in situ hybridization results

Nvi–Hox3 expression (probe 454 nt) is first detectable by in situ at 39 h before the end of blastopore closure. Expression begins as two very large dorsolateral patches in the hyposphere (not shown). Later expression occurs also in the dorsocaudal cells of the larva. During stages ET and MT these cells, descendants of the first somatoblast (2d), divide extensively, move laterally to the ventral side, and then meet in the midline. These cells, except those in the dorsal ectoderm, form the greater part of the ventral plate, and all express *Nvi–Hox3* (Fig. 3, *Nvi-Hox3*). During stage MT, the domain of expression narrows very quickly towards the midline and extends again at LT (Fig. 6). The most robust expression is observed in the ventral midline and in strips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ET
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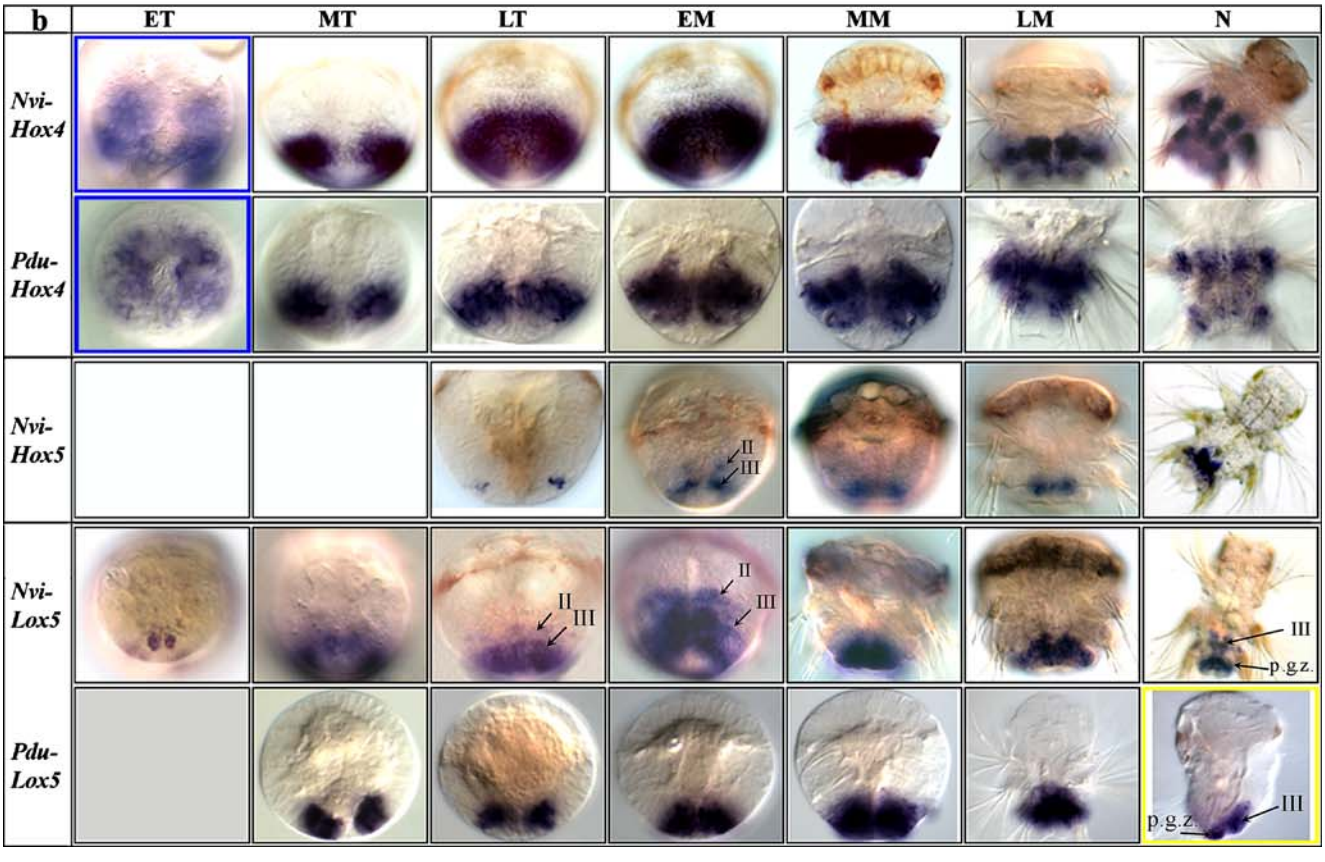
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III

Fig. 3 (continued)

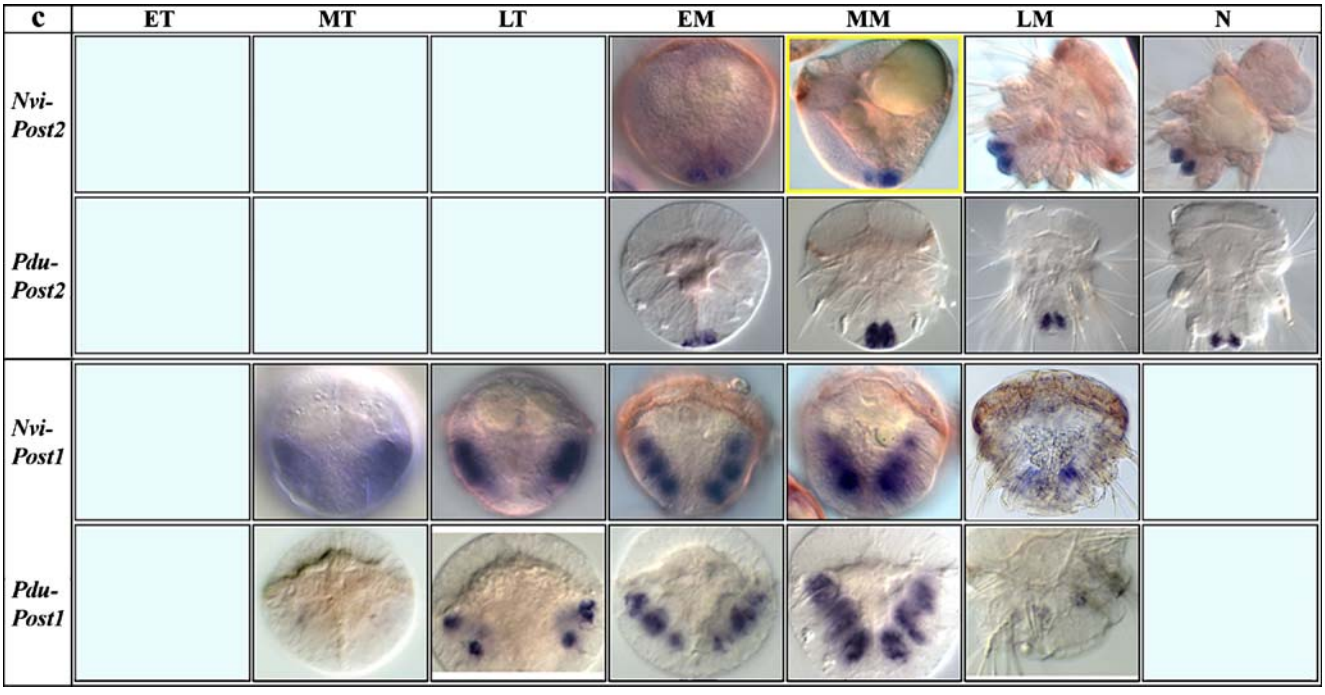

Fig. 3 Expression of Hox genes defines vectorial regionalization of the larval body. Pattern of Hox gene expression in larvae of *Nereis virens* and *Platynereis dumerilii* during different stages of development. Different frame colors determine different views of larvae: *ventral—black*, *dorsal—red*, *posterior—blue*, *lateral—yellow*. Different color of *empty squares* mark: *gray*—data is absent, *clear blue*—expression is absent. Comments are in the text. The abbreviations are as in Fig. 1

of cells extending laterally from the midline. During the metatrochophore stage, the intensity of *Nvi–Hox3* expression decreases, but the pattern of expression does not change. During the nectochaete stage expression is observed only in the pre-pygidial zone (Fig. 3, *Nvi-Hox3*). Patterns of expression of *Nvi–Hox3* and *Pdu–Hox3* (probe 619 nt) are largely similar (Fig. 3, *Pdu–Hox3*).

Hox4 in situ hybridization results

Nvi–Hox4 (probe 453 nt) is first expressed at 50 h as two bilateral domains in the posterior portion of the somatic plate (Fig. 3, *Nvi-Hox4*). This expression strengthens very quickly until a broad zone of expression covers the ventral and lateral parts of the future second and third parapodial segments (Fig. 3, *Nvi–Hox4*). This pattern of expression continues throughout stages EM to LM, then expression gradually decreases during the nectochaete stage, when *Nvi–Hox4* is expressed mostly in the ectoderm of the parapodia and in the neuroectoderm of the forming ventral neural ganglia of the second and third parapodial segments (Fig. 3, *Nvi–Hox4*). Patterns of *Nvi–Hox4* and *Pdu–Hox4* are identical.

Hox5 in situ hybridization results

Nvi–Hox5 (probe 1,010 nt) is first expressed much later (96 h) than *Nvi–Hox1* (47 h) and *Nvi–Hox4* (50 h), at the

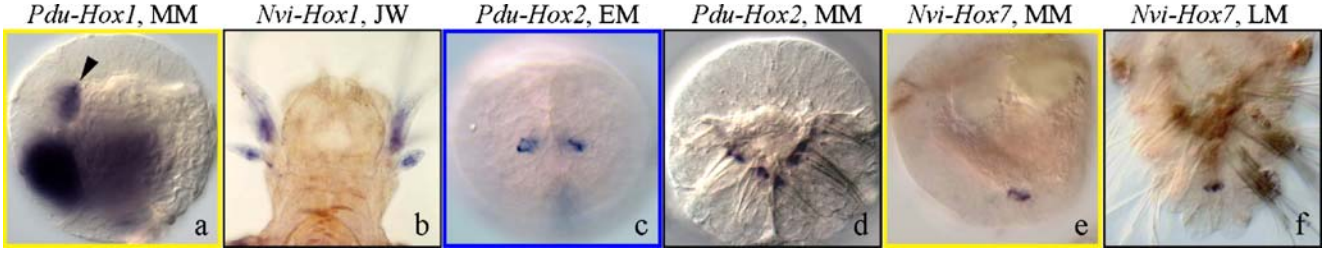

Fig. 4 Expression of Hox genes in domains not obviously related to vectorial patterning of the larval body. **a** *Pdu–Hox1* in the cells of the stomodaeal plate, *arrow*; **b** *Nvi–Hox1*, in peristomial cirri; **c** *Pdu–Hox2* in the cells at the posterior pole; **d** *Pdu–Hox2* at the base of the chaetae; **e** *Nvi–Hox7* in the deep cells of the posterior pole; **f** *Nvi–Hox7* later in the same place at the border of the midgut and hindgut. *Frame color* determines different views of larvae as in Fig. 3

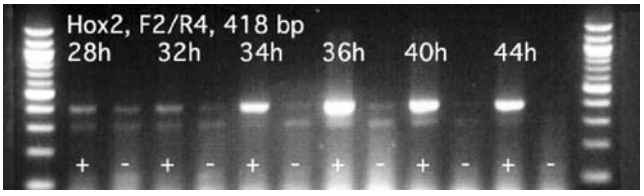

Fig. 5 *Nvi–Hox2* expression during early development of *N. virens*, RT-PCR data. RT-PCR(+) results (RT reaction and RCR reaction) were compared with RT-PCR(−) results (only PCR reaction)

beginning of stage LT in two pairs of small bilateral spots at the ventral and dorsal sides of the larva in the area of the future third parapodial segment (Fig. 3, *Nvi–Hox5*; Fig. 7g). At the EM stage, a transitory expression domain appears in the second parapodial segment (Fig. 3, *Nvi–Hox5*). The domain in the third parapodial segment forms a semi-ring with a gap on the dorsal side at MM. At LM, intensive expression of this gene is observed in the region of the forming ventral ganglia (Fig. 7h and i). During the development of the nectochaete, expression of this gene is located mainly in the neuroectoderm cells of the forming ganglia of the third parapodial segment and in the ectoderm of the parapodia (Fig. 3, *Nvi–Hox5*). We do not yet have in situ hybridization results for *Pdu–Hox5*.

Lox5 in situ hybridization results

Activation of *Nvi–Lox5* occurs during the ET stage, 8–10 h later than *Nvi–Hox4*, but 25–30 h before *Nvi–Hox5* expression begins. The *Nvi–Lox5* expression domain (probe 573 nt) first appears as two cells on the ventral side (Fig. 3, *Nvi–Lox5*). Several hours later, two bilateral domains of expression appear on the dorsal side of the larva (Fig. 7a). *Nvi–Hox5* and *Nvi–Lox5* expression overlap in the third parapodial segment but initial domains of *Nvi–Hox5* appear much later, at the LT stage, in a population of cells that already express *Nvi–Lox5* (Fig. 3, *Nvi–Hox5*; *Nvi–Lox5*; Fig. 7a–i). During the MT stage, domains of *Nvi–Lox5* expression enlarge and spread around the dorsal side of the larva (Fig. 7b). Eventually, the ventrolateral edges of the expression draw together, and a full ring is formed (Fig. 7c). Later, the level of *Nvi–Lox5* expression weakens on the dorsal side and becomes stronger on the ventral side (Fig. 7d,e, and f). During stage LT, weak expression of *Nvi–Lox5* appears on the ventral side of the second parapodial segment (Fig. 3, *Nvi–Lox5*). At EM, expression in the second and third parapodial segments becomes very strong. However, at MM and LM stages, expression is restricted only to the ventral side of the third parapodial segment (Fig. 3, *Nvi–Lox5*). In the nectochaete, expression of this gene in the third parapodial segment is restricted mostly to the developing definitive ventral neural ganglia and to the ectoderm of the parapodia. At this stage, *Nvi–Lox5* expression is also activated in the pre-pygidial growth zone (Fig. 3, *Nvi–Lox5*; Fig. 8b). Similar patterns of expression (probe 690 nt) are observed for *Pdu–Lox5* (Fig. 3, *Pdu–Lox5*).

Hox7, Lox4, and Lox2 in situ hybridization results

Hox7 and *Lox4* have been cloned only from *N. virens*, and we do not yet have *Pdu–Lox2* in situ results. In *N. virens*, expression of all three genes (probes for *Nvi–Hox7*, *Nvi–Lox4*, and *Nvi–Lox2* are 522, 302, and 498 nt, correspondingly) appears in the pre-pygidial growth zone beginning from the nectochaete stage. Their domains probably overlap with the *Nvi–Hox3* and the *Nvi–Lox5* pre-pygidial domains at this stage (Fig. 8b–e). *Nvi–Hox7* also shows additional earlier expression at the metatrochophore stage, where it is expressed strongly in a group of deep cells which lie at the boundary between the third larval segment and the pygidial area. This domain of expression disappears in the early nectochaete when the midgut joins with the hindgut (Fig. 4e and f).

Post2 in situ hybridization results

Expression of *Nvi–Post2* (probe 380 nt) begins as two bilateral spots in the most dorsocaudal region of the future pygidial lobe during stage EM (Fig. 3, *Nvi–Post2*; Fig. 7j). Later, two small transient additional spots appear in the ventrocaudal part of this region (Fig. 7k). Expression

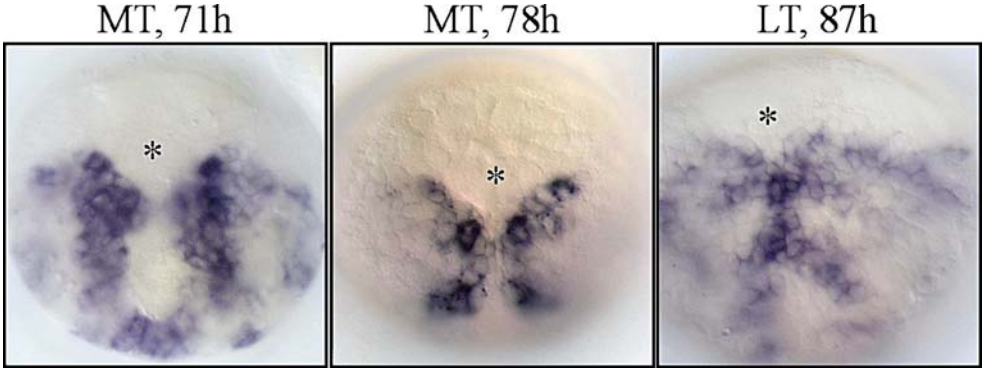

Fig. 6 Expression of *Nvi–Hox3* during joining of the lateral domains of the ventral plate and formation of the ventral midline: **a** strong expression before midline formation; **b** considerable decreasing of expression at the time of joining; **c** increasing expression along the midline and in the long loose rows of cells along lateral and caudal domains of ventral plate. *Asterisk* marks position of stomodaeum

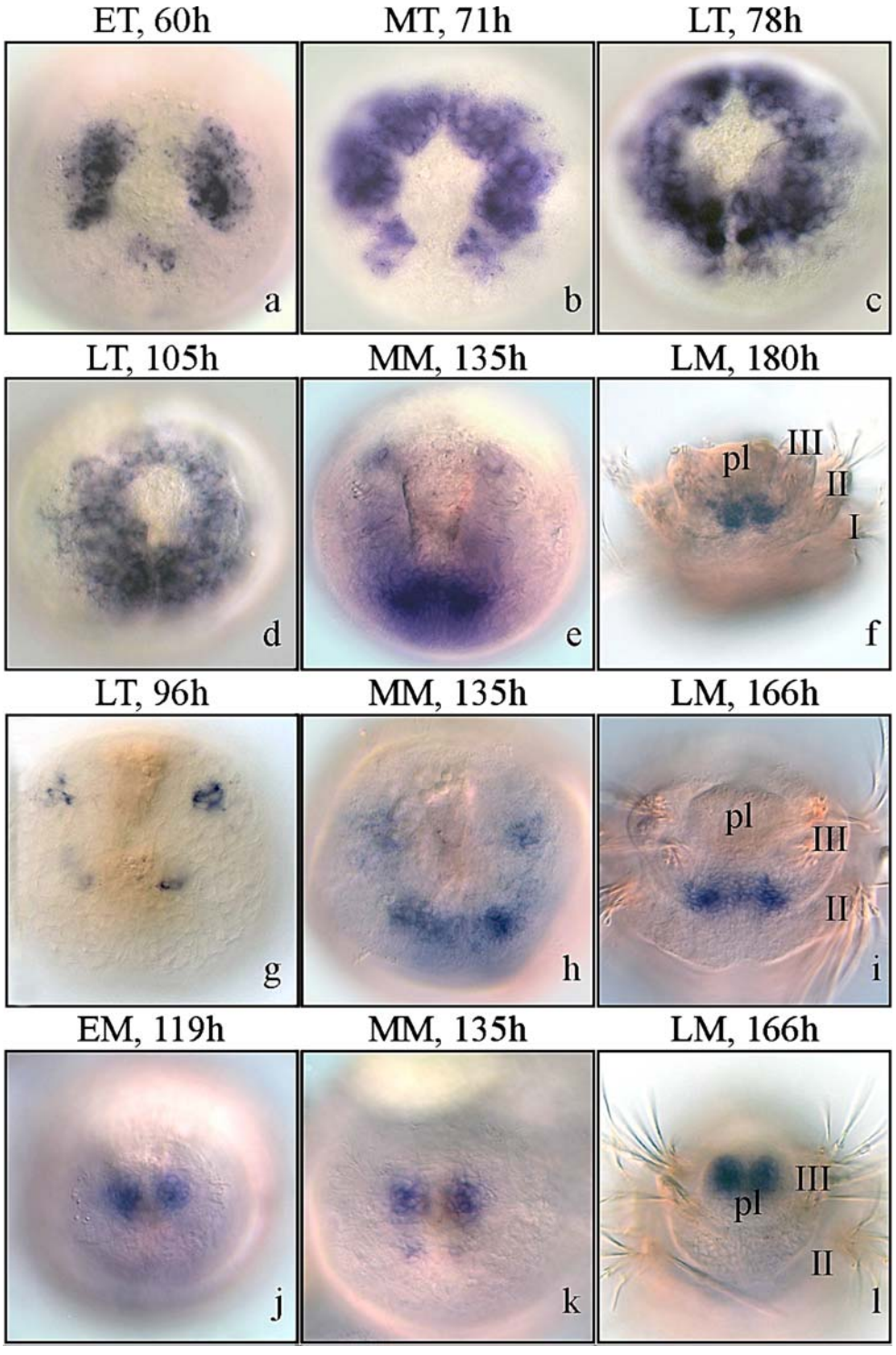

Fig. 7 Expression of *Nvi–Lox5* (**a**–**f**), *Nvi–Hox5* (**g**–**i**), and *Nvi–Post2* (**j**–**l**). View from posterior pole. Comments are in the text. The abbreviations are as in Fig. 1

remains localized in the pygidial lobe while pygidial cirri form during metatrochophore and nectochaete development (Fig. 3, *Nvi–Post2*; Fig. 7l). At this time, there is no expression in the pre-pygidial growth zone (Fig. 8f). The pattern of *Pdu–Post2* expression (probe 291 nt) is similar to that of *Nvi–Post2* (Fig. 3, *Pdu-Post2*).

Post1 in situ hybridization results

The pattern of *Nvi–Post1* expression (probe 316 nt) was described previously (Kulakova et al. 2002). Expression of this gene begins during stage MT, much earlier then *Nvi–Post2* expression begins. *Nvi–Post1* expression starts in the

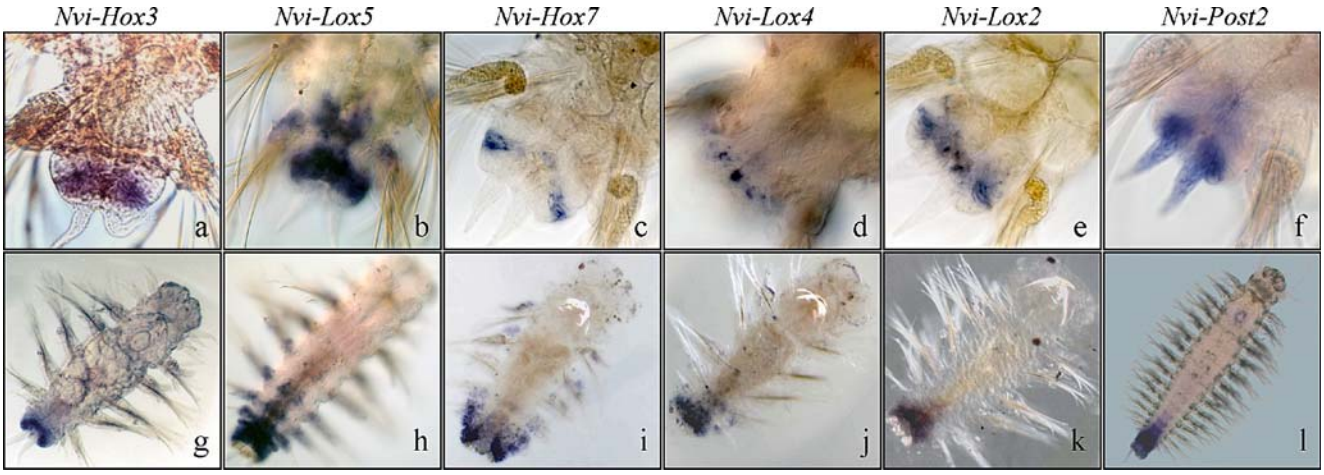

Fig. 8 Expression of six *Nvi–Hox* genes during nectochaete stage (**a**–**f**) and in juvenile worm (**g**–**l**)

territories of the forming chaetal sacs of the first and second parapodial segments, then expression spreads into the chaetal sac anlagen of the third parapodial segment during the EM stage. Expression then gradually disappears from the first, second, and third parapodial segments. This gradual disappearance of expression correlates with the formation of the chaetae. The pattern of expression of *Pdu–Post1* (probe 333 nt) is practically identical to that of *Nvi–Post1* (Fig. 3, *Nvi–Post1*, *Pdu–Post1*).

Discussion

This paper describes expression patterns of a complete set of Hox genes during larval development in polychaete annelids of the family Nereidae, *N. virens* and *P. dumerilii*. This is the first time that this has been done for any lophotrochozoan taxon.

Polychaete annelids (Lophotrochozoa) show great diversity in morphology as a result of adaptation to different ecological niches (Anderson 1973; Brusca and Brusca 2002). However, these errant nereids are thought to retain many ancestral characteristics of the annelids, including life history, homonomy of segmentation, simple reproductive system, gradual type of metamorphosis, absence of highly specialized larval structures, and high fecundity (Ushakov 1972; Arendt 2003; Tessmar-Raible and Arendt 2003; Bleidorn et al. 2003; Hall et al. 2004; Nielsen 2004). Also, the *Platynereis* gene inventory and gene structure have derived relatively little from ancestral conditions, especially if compared to conventional protostome models (Raible et al. 2005).

The patterns of expression of orthologous Hox cluster genes of *N. virens* and *P. dumerilii* are very similar. Expression patterns suggest that the functions of the Hox genes may be divided into two categories; those that relate to vectorial regionalization of the larval body and those that do not. In the first case, expression is observed during formation and development of a somatic plate, which forms most of the larval trunk integument, the definitive ventral neural ganglia, the pre-pygidial posterior growth zone, and the pygidial lobe. In the second case, Hox gene expression is associated with the patterning or the specification of definite morphological structures at different stages of development: tuft (*Pdu–Hox1*), peristomial, and pygidial cirri (*Hox1*), parapodial territories (*Post1*), parapodial sacs (*Pdu–Hox2*), and the border between different parts of the gut (*Hox1*). Further comparative studies will reveal the evolutionary origin of this second category of functions.

No evidence for temporal colinearity

We have observed that *Nvi–Hox1* and *Nvi–Hox4* are activated almost simultaneously (47 and 50 h, respectively) and the expression of *Nvi–Hox5* starts considerably later (86 h) than that of *Nvi–Lox5* (57 h). Note that morphological determination of the first and the second parapodial segments occur much earlier than that of the third one. *Nvi–Hox2* and *Nvi–Hox3* are activated earlier than *Nvi–Hox1* (Fig. 3, Fig. 5). *Nvi–Hox7*, *Nvi–Lox4*, and *Nvi–Lox2* are expressed only after 250 h, much later than *Nvi–Post2* (105 h) as shown in Fig. 3 and Fig. 8. Ferrier and Minguillon (2003) have noted a correlation between violations of the rule of temporal colinearity and disruption to the structural organization of the Hox cluster. The prediction made by these authors is that loss of the mechanisms generating temporal colinearity can allow Hox clusters to disintegrate. This is currently being tested for *Nereis*. (Andreeva et al. 2001; Korchagina and Andreeva, unpublished data). Even after the loss of

temporal colinearity and disintegration of the entire Hox cluster, spatial colinearity may be preserved (Seo et al. 2004).

Expression of Hox genes and the regionalization of the larval body of nereidids

The main function of Hox cluster genes, and one that is probably ancestral to all bilaterians, is to determine the vectorial regionalization of the body of bilaterian animals along the antero-posterior axis (Slack et al. 1993; Akam 1995, 1998; Davidson et al. 1995; Davidson 2001; Erwin and Davidson 2002). Our results show that the nereidid larval body plan is characterized by staggered domains of Hox gene expression, suggesting that Hox genes retain this function in nereids (Fig. 3, Fig. 9). All Hox cluster genes (with the exception of *Post1*) participate in the subdivision of the larval body into defined territories along the antero-posterior axis. Accordingly, the dynamics of spatial and temporal expression of the different Hox genes make it possible to divide them into three groups: (1) genes with stable and prolonged expression in the larval segments and pygidial lobe whose function is likely related to the regionalization of larval body; (2) genes with very early and dynamic expression whose function is not so clear; and (3) genes which are expressed in the forming pre-pygidial posterior growth zone and probably in some parts of the pygidium in the late stages of nectochaete development.

Stable expression of *Hox1*, *Hox4*, *Hox5*, *Lox5*, and *Post2*

These five genes are expressed in accordance with the rule of spatial colinearity in ectodermal cells of the forming segments and pygidial zone, and later, in the forming definitive ventral neural ganglia (Fig. 3, Fig. 9), if one extrapolates the 3′–5′ order of genes in the putative nereidid cluster from the cluster of other bilaterians.

Hox1 marks the territories of the first, the second, and the third parapodial segments, *Hox4* marks the second and the third parapodial segments, and *Hox5*, the third segment. *Lox5* marks the third parapodial segment and the pre-pygidial zone, and *Post2* marks the pygidial lobe (Fig. 3, Fig. 9). At the nectochaete stage, *Hox1* is expressed in the forming ventral ganglia of the first, the second, and the third parapodial segments, *Hox4* in those of the second and the third segments, and *Hox5* and *Lox5* in those of the third segment (Fig. 3, N; Fig. 9).

Dynamic expression of *Hox2* and *Hox3*

The patterns of expression of *Hox2* and *Hox3* differ considerably from those of the five genes discussed above. Expression domains of these genes at the ET stage are very

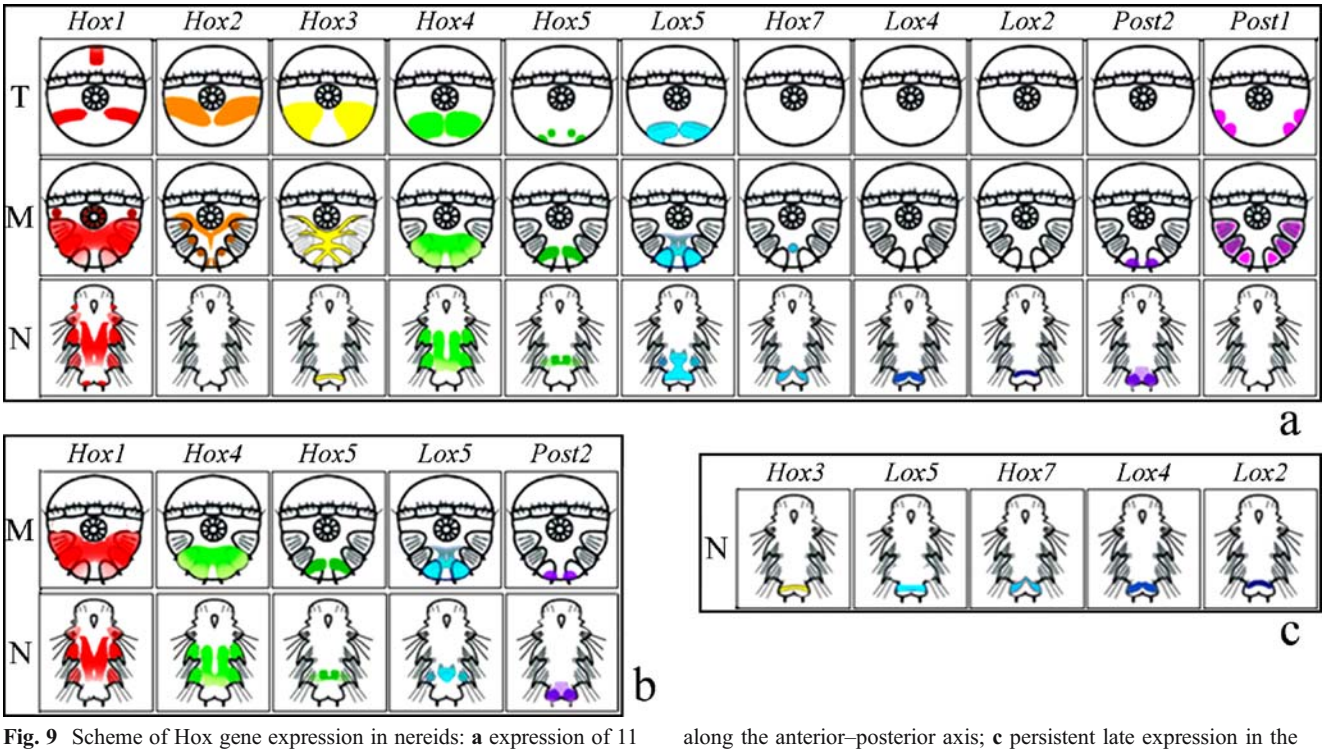

Fig. 9 Scheme of Hox gene expression in nereids: **a** expression of 11 Hox genes at different stages of larval development; **b** Hox genes of the first group take part in vectorial regionalization of the larval body along the anterior–posterior axis; **c** persistent late expression in the prepygidial zone

wide and include most of the forming ventral somatic plate (Fig. 3, *Hox3*). Shortly thereafter, the ventrolateral parts of the ventral plate contact each other, the midline forms, and the territories of the future pygidial and stomodaeal areas are separated. These morphological changes correlate with a very rapid alteration of the *Hox3* expression domain around the time of the midline formation (Fig. 6). The *Nvi–Hox3* expression domain, at first, increases as the ventral plate enlarges (Fig. 3, Fig. 6, 71 h) and then narrows just at the moment of formation of the midline (Fig. 6, 78 h). After midline formation, expression of the *Nvi–Hox3* domain increases again but the expression pattern changes: *Hox3* is expressed only in cells along the midline and in separate loose cell rows near the midline along each segment and the pre-pygidial zone (Fig. 6, 87 h). During this period, there are no gross changes of expression levels of *Hox1*, *Hox4*, and *Lox5* (Fig. 3).

We suggest that this biphasic *Hox3* expression may be related to this gene having two different functions. The wide initial expression pattern may be connected with the participation of the *Hox3* gene in vectorial regionalization of the larval somatic plate together with genes of the first group. The pattern of the second phase of expression may rather be associated with the determination of certain cell types in each segment, but the nature of these cells is unknown. It may be that they are sensory nervous cells commonly associated with mesotrochal ciliated bands forming in every segment, and with midline neurotrochal and telotrochal bands (Irvine and Martindale 2000).

At the nectochaete stage, we do not observe *Hox3* expression in the cells of the forming neural ganglia. The pre-pygidial expression domain of *Hox3* persists up to the end of larval development and in the juvenile worm (Fig. 3, Fig. 8).

The expression of *Pdu–Hox2* is strong in the ET. The domain of superficial expression is, like that of *Hox3*, very wide and includes all larval segments (Fig. 3, *Pdu–Hox2*). The expression domain quickly diminishes in a ventromedial direction at the LT stage, and we do not detect any superficial expression later.

RT-PCR shows that the expression of *Nvi–Hox2* starts at 34 hours of development, during blastopore closure (Fig. 5). In situ hybridization data does not detect *Nvi–Hox2* expression at any stage of *N. virens* larval development (the probe size is 580 bp). This suggests that the level of expression of this gene is probably very low.

Thus, some particular features of *Hox2* and *Hox3* expression differentiate them from the other genes of the Hox cluster. Their expression starts much earlier than the expression of other genes; the initial domains of expression are broad, covering a large part of the forming ventral somatic plate; the expression level rapidly reduces as the lateral parts of the ventral plate join; and the second phase of *Hox3* expression is associated with the formation of the lateral rows and ventral midline of the somatic plate.

Expression of Hox genes in the pre-pygidial zone

Three central group genes in *N. virens*, *Nvi–Hox7*, *Nvi–Lox4*, and *Nvi-Lox2*, participate little in regionalization of the larval body. Their expression starts late in the nectochaete stage and only in the pre-pygidial zone (Fig. 8, Fig. 9; we do not have data for *P. dumerilii*). *Nvi–Hox3* and *Nvi–Lox5* are also expressed in the pre-pygidial zone. *Nvi–Hox3* marks the cells of this zone at the early metatrochophore stage (Fig. 3, *Nvi–Hox3*), probably at the moment of the formation of this territory. Later, at the end of the metatrochophore stage, *Nvi–Lox5* expression begins in the same domain (Fig. 3, *Nvi–Lox5*). All five of these genes are actively transcribed during postlarval segmentation of the juvenile worm in the pre-pygidial posterior zone from which the segments form. As segment numbers increase, expression in previously formed segments gradually decreases so that the expression of each gene forms a gradient, but the pattern of this gradient is different for each gene (Fig. 8g–l). This will be described in more detail elsewhere.

Hox1, *Hox2*, and *Hox7* are involved in nonvectorial patterning

Some nereidid Hox genes not only participate in patterning the larval body plan but also regulate other morphogenetic processes (Fig. 4). In these cases, the Hox genes are involved in new regulatory programs. Davidson termed this phenomenon "co-option," and defined it as "the evolutionary process by which regulatory genes are reassigned to different functions in a pattern formation network (Davidson 2001 p. 158).

Pdu–Hox1 is expressed in the cells that form the apical ciliated tuft (data not shown). *Hox1* in both nereids is expressed in the cells of the stomodaeal plate at MM (Fig. 4a). This expression persists during postlarval development and probably marks a foregut–midgut border (data not shown). Irvine and Martindale (2000) have also shown an additional expression domain of *CH–Hox1* in the stomodaeal plate of the sedentary polychaete *Chaetopterus* sp., and they believe that this expression marks the border between the foregut and midgut. Note that the foregut forms from stomatoblasts, but not from descendants of the 2d blastomere (Wilson 1892; Ackermann et al. 2005). The expression of *Hox1* in both nereids also marks the peristomial and anal cirri (Fig. 4b).

Pdu–Hox2 is expressed in the region of the forming chaetal sacs, possibly in the chaetoblast cells. The role of

Pdu–Hox2 expression in several caudal cells is unclear (Fig. 4c and d).

Nvi–Hox7 is expressed probably at the border of the midgut and hindgut. This expression is found only in metatrochophore larva and ceases at the early nectochaete stage, by which time a hindgut has just formed (Fig. 4e and f). We do not know yet whether these cells are mesoderm or endoderm.

Post1 does not participate in vectorial patterning

In neither *N. virens* nor *P. dumerilii* does the *Post1* gene participate in vectorial patterning. The function of this gene probably lies in the specification of prospective chaetal sac territories of larvae (Fig. 3, *Post1*, and Kulakova et al. 2002). Nonvectorial patterning has also been described in one of the two *Post2* orthologues of planaria, *DjAbdBb* (Nogi and Watanabe 2001).

Hox genes and construction of nereid larval body plans

Our analysis shows that all of the nereid Hox genes, except *Post1*, are involved in the process of vectorial regionalization of the larval body (Fig. 3, Fig. 9). This process starts at very early stages of development before the end of gastrulation (expression of *Hox2* and *Hox3*) and ends at the nectochaete stage (expression of *Hox7*, *Lox4*, and *Lox2*) when all regions of the larval body are already determined. All of these events occur sequentially in a single spatial domain created by the descendants of the 2d blastomere that form the greater part of the trunk of the larva (Wilson 1892; Anderson 1973; Ackermann et al. 2005). However, in nereidids only *Hox1*, *Hox4*, *Hox5*, *Lox5*, and *Post2* are expressed in the canonical pattern of spatial colinearity, with some violation of temporal colinearity. These genes have intensive and stable expression domains, whose boundaries are clearly determined (Fig. 9). By contrast, *Hox2* and *Hox3* have very dynamic patterns of expression. The most intensive phase of their expression occurs during the period of somatic plate formation, when the majority of cells in this territory express these genes (Fig. 3).

Expression of the three central genes, *Hox7*, *Lox4*, and *Lox2*, begins only in the nectochaete stage when all the main parts of the nectochaete body have already been determined. Their expression domains are largely overlapping and coincide with the formation of the growth zone of postlarval segments. We plan to characterize the temporal patterns of expression and the extent of coincident expression of these genes in future work. Only comparative analysis of Hox gene expression in other polychaetes will clarify whether the peculiarities of Hox gene function in nereidids are typical of all polychaetes.

Hox genes and construction of larval body plan of polychaetes

Hitherto, Hox gene expression in polychaetes was only analyzed in the sedentary tagmatized worm *Chaetopterus* sp. (Irvine and Martindale 2000; Peterson et al. 2000b). It was shown that *CH–Hox1* and *CH–Hox2* expression begins at the first larval stage, L1. Sequential activation of the genes, *CH–Hox3*, *CH–Hox4*, and *CH–Hox5* occurs at the L2 stage. For all five genes, expression initially occurs in a largely overlapping domain which is restricted to the most posterior part of the larvae, particularly to a deep zone where teloblasts are thought to exist (Peterson et al. 2000b). During larval growth, the early zones of expression expand from the growth zone as the larval body is formed. By late larval stages, each of the genes has a defined anterior boundary of expression, with *CH–Hox2* expressed most rostrally. The level of expression for each gene is highest in the central nervous system (Irvine and Martindale 2001).

Larval development of *Chaetopterus* differs from nereid development (Wilson 1892; Irvine et al. 1999; this study). *Chaetopterus* has an atypical trochophore that develops 15 larval segments. Morphological manifestation of these larval segments occurs late in development and in inverted order, whereby segments appear first in the posterior zone of the larva. Postlarval segmentation takes place in an antero-posterior direction in a manner typical for most polychaetes. According to Anderson (1973), larval and postlarval segments are both formed from the posterior growth zone. By contrast, Iwanoff (1928), in his theory of primary heteronomy of segments, states that development of larval and postlarval segments is fundamentally different. This would suggest that the morphogenetic programs for larval and postlarval body formation should be different. Different cellular mechanisms of larval and postlarval segmentation were recently shown for other polychaetes (Seaver et al. 2005; Seaver and Kanashige 2006). Regardless of these differences, however, both larval and postlarval segments contribute to the definitive adult body plan.

In conclusion, in nereids, Hox genes appear to pattern the early forming larval segments, and may also be involved in the regionalization of the later forming postlarval segments in juvenile worms. At least for the larval segments, the colinearity rules of Hox genes appear to be the same as those patterning the body plans of the other two bilaterian clades, the Ecdysozoa and the Deuterostomia, suggesting that this mode of patterning originated in the ancestor of all bilaterian animals.

Our future work aims at the detailed analysis of Hox gene expression in postlarval development.

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