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ORIGINAL RESEARCH

Expression of the *engrailed* Homologue in Larvae and Juveniles of the Annelid *Alitta virens* Characterizes the Formation of Segments from the Growth Zone

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Abstract—The evolutionary origin of segmentation remains a mystery. In arthropods, gene *engrailed* is recognized as one of the most important and conservative members of the segmentation developmental program. Orthologues of this gene have been identified in annelids, but their role is interpreted as contradictory, because their expression in some species precedes subdivision of the body into segments but it does not in others. The expression of *engrailed* in the nereid polychaete *Alitta virens* during metamorphosis and development of the first postlarval segments was studied herein. Our data support the possible involvement of this gene in the process of segment formation from the growth zone in *A. virens*. At the larval stages, *engrailed* is expressed in neuroectodermal cells, in the growth zone, and in metameric epidermal cell rows at the anterior boundary of each segment. Upon transition from the metatrochophore to the nectochaete stage, the circular expression domain in the growth zone expands and then resolves into two serial domains. Over time, the distance between these circular domains increases, indicating the growth of the first postlarval segment anlage. Formation of subsequent postlarval segments occurs in a similar way. Analyzing our results and literature data, we compared *engrailed* expression patterns in annelids and arthropods. Our work indicates an absence of conservation in patterning of sequentially developing segments from the growth zone in protostomes. We suggest that the anteroposterior axis elongation in *A. virens* occurs simultaneously with the specification of a new segment. These features differ from the known models of the growth zone and indicate the possibility that nereids have a specific mechanism of segmentation.

Keywords: segmentation, Annelida, Nereididae, larval development, metamorphosis, axis elongation, segment polarity gene *engrailed*, expression pattern

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INTRODUCTION

Three groups of bilaterians are considered to be segmented animals: arthropods, chordates, and annelids. However, there is still no consensus on the origin of segmentation, which could either arise from a common ancestor of all bilaterians, or evolve in different taxa independently (Chipman, 2018, 2020a).

Among arthropods, the process of segmentation establishment is most studied in *Drosophila melanogaster* (Chipman, 2020b). Complex interactions between genes with maternal effect and zygotic genes result in simultaneous patterning of the blastoderm into metameric regions, at the boundaries of which the expression of "segment polarity genes" *wingless* (*wnt1*) and *engrailed* (*en*) is activated. They are responsible for creating a coordinate system inside the segment and are the "markers" of segmentation. However, *Drosophila* has a highly modified development: it has a long germ-band, devoid of a growth zone. These specific features significantly complicate the comparative study of the mechanisms of development of a segmented body plan.

Insects with a short germ-band, one of the representatives of which is the beetle *Tribolium castaneum*, are recognized as more suitable objects for the evolutionary analysis of the phenomenon of segmentation. Despite the differences in the molecular genetic mechanisms of segmentation in insects, *wingless* and *engrailed* genes in *Tribolium* perform the same function as in *Drosophila*. Moreover, "segment polarity genes" are the most conservative regulators of body metamerization in all arthropods: patterns of their expression with a periodicity of one segment are noted in insects, crustaceans, chelicerates, and millipedes (Patel et al., 1989a; Damen, 2002; Hughes and Kaufman, 2002).

Homologues of the "segment polarity genes" have also been identified in representatives of annelids. The expression of *wingless* and *engrailed* was characterized in Nereididae polychaetes (Prud'homme et al., 2003;

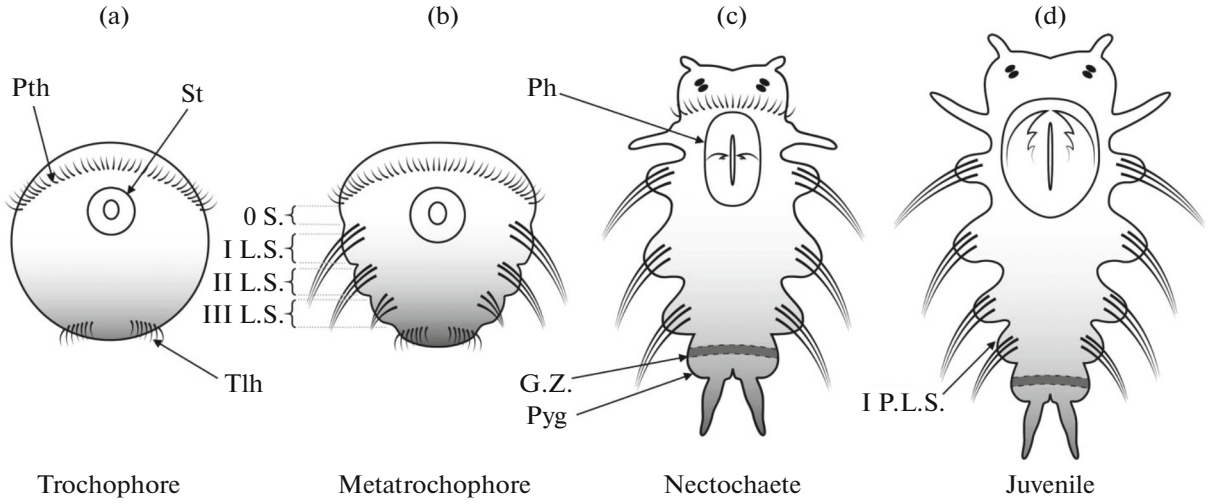

Fig. 1. Stages of postembryonic development of *Alitta virens*. The picture represents the relative size of the body; the anterior end is directed upwards. (a) Trochophore. (b) Metatrochophore. (c) Nectochaete. (d) A juvenile with four bristle-bearing segments. Pth—prototroch; St—stomodeum; Tlh—telotroch; Ph—pharynx; G.Z.—growth zone; Pyg—pygidium; 0, I, II, III L.S.—zero, first, second, and third larval segment; I P.L.S.—first postlarval segment.

Steinmetz et al., 2011; Kozin et al., 2019a) as well as in *Capitella teleta*, *Hydroides elegans*, *Chaetopterus* sp., *Pristina leidyi*, and *Helobdella* sp. (Patel et al., 1989b; Bely and Wray, 2001; Seaver et al., 2001; Seaver and Kaneshige, 2006). The expression patterns of these genes in the Nereididae polychaetes *Platynereis dumerilii* and *Alitta virens* suggest their participation in the creation of a metameric body plan: *engrailed* is expressed in a row of cells at the anterior border of the segment, and *wingless* is expressed at the posterior border, which corresponds to the distribution of orthologous gene products in parasegments in insects. However, other annelids lack coupling of *wingless* and *engrailed* expression to intersegmental boundaries, that casts doubt on their participation in patterning the entire body into metameric regions (Seaver and Kaneshige, 2006).

In addition, the expression of *engrailed* in annelids was described either at the larval stages or during regeneration or anamorphic growth (i.e., the sequential appearance of segments from the growth zone) of adult worms, but was not characterized during the transition from larval to postlarval development (i.e., at the end of metamorphosis and the appearance of the first postlarval segments). To clarify the molecular genetic basis of the sequential formation of segments from the growth zone and to determine evolutionarily conservation of the expression pattern of *engrailed*, we studied the dynamics of the mRNA distribution of this gene during metamorphosis and in young juvenile individuals of the White Sea polychaete *A. virens*.

MATERIALS AND METHODS

Mature individuals of *A. virens* were caught in the vicinity of the Marine Biological Station (MBS) of St. Petersburg State University (Chupa Inlet, Kandalaksha Bay of the White Sea). Artificial insemination and maintenance of the embryonic culture were performed according to the previously described method (Dondua, 1975). Embryos and early larval stages were cultured at 14°C, and the temperature was raised up to 20°C at the stage of early nectochaete (180 h post fertilization, hpf).

The development of the White Sea *A. virens* in natural and laboratory conditions was described by Dondua (1975) and Sveshnikov (1978). Embryogenesis is characterized by heteroquadrant spiral cleavage and epibolic gastrulation. At the end of the second day after fertilization, a rounded floating lecithotrophic trochophore larva appears (Fig. 1a). The ciliary band of the prototroch encircles the larva's body along the equator, separating the episphere (anterior half) from the hyposphere (posterior half). The hyposphere contains the rudiment of the foregut (stomodeum) and the discontinuous ciliary band of the telotroch, which delimits the area of the future pygidium. Under the temperature conditions we used, the trochophore stage lasts from approximately 44 to 90 hpf. The trochophore gradually transforms into a metatrochophore (Fig. 1b); the hyposphere elongates and metamerically arranged structures, i.e., bundles of bristles and ciliary bands (paratrochs), appeare. At the posterior end of the late metatrochophore, two lobes of the pygidium develop, with the rudiment of the hindgut, invaginated between the lobes. The metatrochophore stage lasts from approximately 91 to 170 hpf. After the appearance of functioning appendages (three pairs of parapodia) and the formation of head appendages (antennae and peristomial cirri), the nectochaete stage begins (Fig. 1c). The nectochaete larva consists of an

anterior terminal region (prostomium), a criptic (number zero) segment bearing peristomial cirri, three bristle-bearing (chaetigerous) segments, a growth zone, and a pygidium. Nectochaetes initially live as plankton and then settle to the bottom. Gradually, the area of the pygidium increases in the nectochaete, and, due to the cells of the growth zone, the first postlarval segment is formed in its anterior part. The transition from a larva to a juvenile individual (Fig. 1d) occurs asynchronously but not earlier than 300 hpf. Juvenile worms with four bristle-bearing segments begin to feed, and new postlarval segments are formed from the prepygidial growth zone.

Larvae and juveniles were fixed in 4% formaldehyde at 1.75× PBS/0.1% Tween-20 overnight at 4°C, followed by washing in PBS/0.1% Tween-20 and dehydration in 100% methanol. The following stages were selected for the work: late metatrochophore (156 hpf), early nectochaete (180 hpf), late nectochaete (210 hpf), juvenile with a forming fourth segment (300 hpf), juvenile with a fully formed fourth segment (330 hpf), and five-segment juvenile (420 hpf). The fixed samples were stored at –20°C.

From 50 to 100 objects of each stage were used for in situ hybridization. In situ hybridization was performed according to the protocol described earlier (Shalaeva et al., 2021). Incubation with digoxygenin-labeled RNA probes lasted 48 h; staining with BCIP/NBT lasted from 20 to 40 h.

To visualize the results of chromogenic hybridization in situ by differential interference contrast (DIC), we used an Axio Imager D1 (Carl Zeiss) microscope equipped with an AxioCam ICs5 (Carl Zeiss) digital camera. Image analysis was carried out using the ImageJ program. The diagrams were created in Adobe Illustrator and Microsoft PowerPoint programs.

RESULTS

At the stage of late metatrochophore, *engrailed* mRNA is expressed on the ventral side of the larva's body: in individual cells of the neuroectoderm and in superficial epidermal cells closer to the lateral sides. The epidermal expression domains form short, metamerically arranged strips adjacent to the anterior border of the segments and do not reach the middle part of the body (Figs. 2a, 2a", 3a). Metameric expression strips are present in all segments: both in the three bristle-bearing and in the zero segment (directly posterior to the prototroch). Also, a pointed signal is detected in the surface and deep cells of the neuroectoderm; the expression pattern is bilaterally symmetrical (Figs. 2a, 3a). In the pygidium region, *engrailed* mRNA is expressed in a ring of cells located between the posterior furrow of the third segment and the telotroch. This posterior ring domain of expression includes both superficial and internal cells, which together correspond to the localization of the future growth zone (see Discussion). In addition, *engrailed* mRNA is detected in four groups of closely spaced cells on the episphere closer to the dorsal side of the body as well as in pharyngeal cells (Figs. 2a, 2a').

At the stage of the early nectochaete, the expression pattern remains similar to that of the late metatrochophore: metameric expression domains on the ventrolateral sides of the body, as well as expression in the episphere and neuroectoderm, are retained (Fig. 2b). The most important change in the pattern occurs in the pygidium: the circular domain of expression expands in the antero-posterior direction (Figs. 2b', 3b) and covers at least two rows of cells on the surface (instead of one superficial row of cells at the stage of late metatrochophore). The molecular boundary of the future first postlarval segment is forming at this moment.

At the stage of late nectochaete, the expression of *engrailed* mRNA in the pygidium region is represented by two ring domains: one is located posteriorly from the furrow of the third segment and the other is at a more caudal position (Figs. 2c, 2c', 3c). At the same time, metameric expression domains on the territory of larval segments disappear. After the separation of the fourth segment, when a furrow separating it from the pygidium has already been formed, one expression domain is located on the anterior border of the fourth segment, and the other is located on the anterior edge of the pygidium, i.e., behind the last furrow (Figs. 2d, 3d). At later stages, the *engrailed* mRNA is expressed in a group of superficial ventrolateral cells on the anterior morphological border of the pygidium and on the territory of the pygidium in a more posterior position relative to the first group of cells (Figs. 2e, 3e). These metameric domains seem to mark the rudiment of the emerging fifth (second postlarval) segment. A similar pattern is observed in case of formation of the sixth segment, whose territory is marked in front and behind by continuous transverse bands of *engrailed*-positive cells (Fig. 2f). In four-segmented worms, the expression (which was practically absent at the stage of late nectochaete) is again detected in the cells of the neuroectoderm of larval segments. In addition, the expression of *engrailed* mRNA in the cells of the anterior edge of the pharynx appears in juveniles (Fig. 2d').

DISCUSSION

In this paper, the expression of gene *engrailed* during the transition to postlarval development in polychaetes was described for the first time. The data obtained suggest the participation of the product of this gene in the patterning of the rudiments of segments in *A. virens*. The analysis of information on the expression of regulatory genes, the dynamics of cell proliferation and differentiation of segmental organs in *A. virens* and in the closely related species *P. dumerilii* allows us to characterize the features of segment development in representatives of the family Nereididae (Fig. 1). Such features include the following:

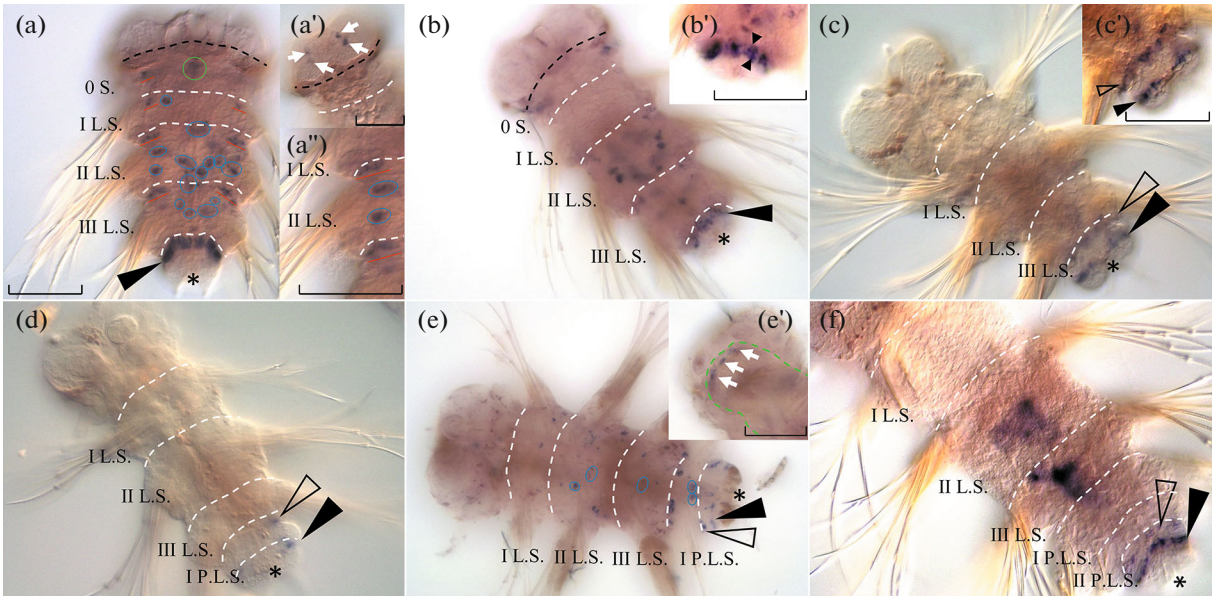

Fig. 2. Expression of *engrailed* in larval and postlarval development of *A. virens*. In all photos, the view is from the ventral side, unless otherwise is indicated. The asterisk marks the posterior end of the body and the pygidium, the black arrowhead marks the anterior border of the pygidium and the expression domain associated with it, the contour arrowhead is at the anterior border of the emerging postlarval segment, the white dotted lines indicate the boundaries of the segments, the black dotted line indicates the position of the prototroch. Metameric expression bands confined to the anterior border of the segment are underlined with a red line. I, II, III L. S.—bristle-bearing larval segments; P.L.S.—postlarval segments; 0 S.—zero segment. (a) Late metatrochophore, 156 hpf. The expression of *engrailed* was detected in individual neuroectodermal cells (outlined in blue), metameric ventrolateral rows of epidermal cells (red lines), and on the anterior edge of the pharynx (outlined in green). (a') Anterior end of the body, view from the dorsal side. As part of the episphere (anterior to the black dotted line), four clusters of cells close to each other (white arrows) expressing *engrailed* are visible. (a") Close-up view of the ventrolateral part of the body, on which two metameric expression domains are visible (red contour). (b) Early nectochaete, 180 hpf. The ring domain of *engrailed* expression at the pygidium border consists of two rows of superficial cells. (b') Close-up view of the posterior end of the body, the arrowheads point to two adjacent rows of *engrailed*+ cells. (c) Late nectochaete, 200 hpf. Expression in neuroectodermal cells disappears; on the territory of the pygidium there are two separate rows of cells expressing *engrailed* (arrowheads). (c') Close-up view of the posterior end of the body. (d) The stage of separation of the first postlarval segment, 300 hpf. (e) Juvenile with a fully formed fourth segment, 330 hpf. The image is obtained by combining a series of photographs of one object in different foci. (e') Close-up view of the anterior end of the body. *Engrailed*+ cells (white arrows) are visible on the anterior edge of the pharynx (green dotted line). (f) Juvenile with five segments, 420 hpf. View from the dorsal side. *Engrailed* mRNA is detected in two transverse bands of cells in the pygidium: the border of the sixth segment is formed. Dark blue staining in the center of the body is a nonspecific background in food particles in the intestinal cavity. The scale bar is 50 μm.

almost simultaneous and relatively early specification of all larval segments in embryogenesis (Prud'homme et al., 2003; Steinmetz et al., 2011; Kozin et al., 2016, 2019a); formation of metameric somatic muscles and parapodia in planktonic larvae (Fischer et al., 2010; Balavoine, 2014; Kozin and Kostyuchenko, 2016); the appearance of a local area of cell proliferation in the prepygidial growth zone at the nectochaete stage, i.e., long before the larva settles (Gazave et al., 2013; Kozin et al., 2019b); the beginning of the formation of the postlarval segment in the form of a single row of *engrailed*-positive cells that correspond to the anterior border of the segment; the subsequent growth of the segment rudiment anteriorly from the pygidium. In Nereididae, *engrailed* is expressed at the posterior end of the body, starting from the protrochophore stage (Prud'homme et al., 2003; Steinmetz et al., 2011). We assume that this posterior transverse domain of *engrailed* activity, located anteriorly from the telotroch, marks the molecular boundary, which positions the future growth zone formation. This region spatially corresponds to the expression of multipotency markers, such as *Vasa* and *Nanos* (Kozin and Kostyuchenko, 2015; Kozin et al., 2019b; Kostyuchenko, 2022). With the expansion and subsequent division of this domain into two bands of *engrailed* expression, an active growth of pygidium occurs in *A. virens* nectochaetes (Figs. 3a–3d). However, the anterior boundary of the future fourth segment has already been determined within its limits, and it is likely that the formation of posterior part of the segment continues due to the proliferation of *engrailed*-negative cells (Kozin et al., 2019b). This indicates that the accumulation of cellular material of the future segment and its specification occur in *A. virens* not sequentially, but simultaneously.

Since the nature of *engrailed* expression differs greatly in different annelids (Fig. 3), the features of

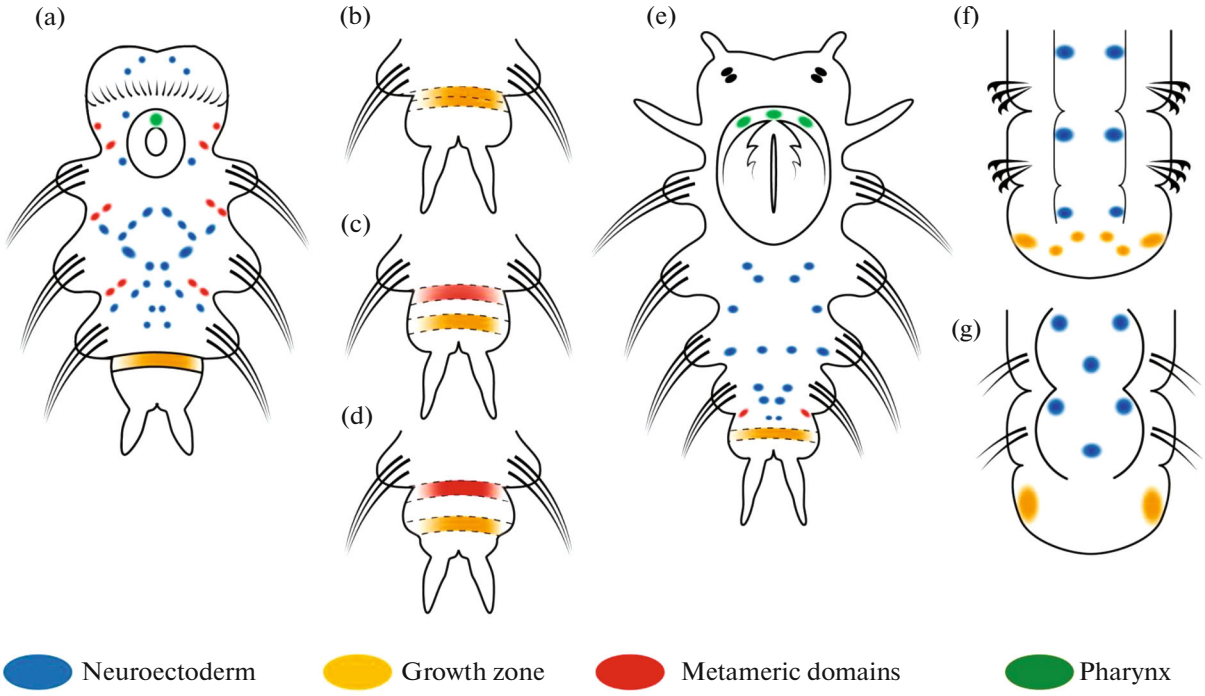

Fig. 3. Patterns of *engrailed* expression during anamorphic growth in annelids. The expression domains are indicated by a color fill: red is for metameric epidermal domains, blue is for neuroectodermal cells, yellow is for the area of the growth zone, green is for pharyngeal cells. The anterior end of the body is directed upwards. (a–e) *A. virens*: (a) late metatrochophore, (b–d) posterior end of the nectochaete body during the formation of the primordium of the first postlarval segment, (e) juvenile. (f) The posterior end of the body of the freshwater oligochaete *Pristina leidyi* (according to Bely and Wray, 2001). (g) The posterior end of the body of sedentary polychaete *Capitella teleta* (according to Seaver and Kaneshige, 2006).

patterning of nereid segments that we have identified may have a different evolutionary interpretation. Hypothetically, early patterning of the segments' primordia involving *engrailed* may be an ancestral trait of annelids. This is indicated by a metameric (with a periodicity of one segment) expression pattern of *engrailed*, which appears even before the morphological separation of the segments, as well as the expression of this gene in the growth zone of most of the annelids studied (Fig. 3). However, *engrailed* does not form extended expression domains in the form of bands or rings, which are located metamerically and reflect the same positional value (i.e., mark the anterior boundary along the circumference of the entire segment: ventrally, dorsally, and laterally) in any annelid taxon other than Nereididae. Basically, the expression of *engrailed* in annelids was detected in serial organs: nephridia, CNS ganglia, chaetal sacs (Seaver et al., 2001; Prud'homme et al., 2003; Seaver and Kaneshige, 2006) (Fig. 3). Thus, it can be assumed that *engrailed* took part in the specification of various metamerically arranged structures initially in the ancestor of annelids. In the phylogenetic lineage leading to the nereids, this gene could be coopted into a gene regulatory network that controls the creation and marking of the morphogenetic field of the entire segment. In this case, specific features of nereid segmentation, such as early specification of all larval segments in embryogenesis and accelerated formation of segmental organs in a planktonic larva, arose in evolution secondarily.

It is likely that the accumulation of significant yolk reserves in the egg, which ensured the transition to lecithotrophy, is the prerequisite for the improvement of segmentation mechanisms, which led to acceleration of the development of segmented nereid larvae. The evolutionary transition from planktotrophy to lecithotrophy is associated with heterochronous changes consisting in later differentiation of digestive organs and earlier appearance of definitive (juvenile) features, which include segmentation. In the extreme case of such heterochrony, the free-living larva disappears, and all segments differentiate during embryogenesis, as it happens in leeches (Kuo, 2017). Future studies of the molecular genetic regulation of the formation of annelid segments will help understand which of scenarios is correct.

The presence of *engrailed* mRNA in the cells of the neuroectoderm is another interesting feature of nereids. In many animals, *engrailed* is necessary for the proper formation of the nervous system. In vertebrates, the cooperative participation of *engrailed* and *FGF8* in the functioning of the isthmus organizer at the midbrain-hindbrain boundary (Omi and Nakamura, 2015), as well as in the specification of

some groups of neurons (Ekker et al., 1992), was shown. In insects and crustaceans, *engrailed* is expressed in the ganglia of the ventral nerve cord (Patel et al., 1989a). For the grasshopper *Schistocerca americana,* the role of this gene in determining the fate of neuroblasts of the median line is shown: after the beginning of *engrailed* transcription, neuroblasts begin to produce glial precursors instead of neural ones (Condron et al., 1994). In echinoderms, *engrailed* transcripts are detected in the radial nervous system in juvenile animals (Byrne et al., 2005). In annelids *A. virens, P. dumerilii, Chaetopterus* sp., *C. teleta*, and *P. leidyi, engrailed* is also expressed in neural and neuroectodermal cells (Bely and Wray, 2001; Seaver et al., 2001; Seaver and Kaneshige, 2006; Steinmetz et al., 2011). Thus, one of the possible ancestral functions of *engrailed* can be involved in neural differentiation.

A large-scale comparative analysis of the patterns of expression of *engrailed* (Vellutini and Hejnol, 2016) showed that its activity in many animals is confined to areas where constrictions between various organs and structures are formed, such as the isthmus (*isthmus rhombencephali*) between the midbrain and hindbrain in vertebrates, the boundary between the body parts of brachiopod and hemichordate larvae, and the edge of the shell gland of mollusks. Summarizing the known hypotheses, we can conclude that the marking of border territories between morphologically separate parts of the body could also be an ancestral function of *engrailed*. Apparently, the expression of *engrailed* was not initially associated with the formation of segments. We assume that *engrailed* was repeatedly involved in the development of a segmented body plan in the course of evolution. When this evolutionary event occurred in annelids remains unclear. This could have happened in the common ancestor of all annelids or in the phylogenetic lineage leading to the Nereididae family.

Our original and literary data indicate that the nature of the development of postlarval segments in *A. virens* does not correspond to either existing models of the functioning of the growth zone (segment addition zone, SAZ), either teloblastic or diffuse. This circumstance raises the question of whether nereids have a unique mechanism of anamorphic growth. According to the available data, it is not yet possible to propose a new model for the formation of segments from the growth zone, but it is worth conducting a comparative analysis of the already established patterns of segmentation of different animals.

The difference from teloblastic SAZ, characteristic of the embryos of clitellate annelids and some crustaceans from the class Malacostraca, is the absence of both teloblasts and the stereotypical pattern of division of their descendants in the postlarval development of Aclitellata (Zattara, 2020). In addition, considering teloblasts as multipotent stem cells (Balavoine, 2014; Chipman, 2020a) does not allow us to interpret SAZ of nereids in this way (Niwa et al., 2013) since the expression of "segment polarity genes" in the growth zone (*engrailed* in *A. virens* and other annelids (Fig. 3), *wnt1* and *hh* in *Perinereis nuntia*) suggests much more limited potencies of its cells. The diffuse SAZ model is applicable to the growth zone of most arthropods and vertebrate embryos (implying the presomitic mesoderm and tail bud). This model assumes cyclic changes in gene activity (oscillations of the expression of the "pair-rule" and Notch signaling genes) in an extensive cell population (Williams and Nagy, 2017; Diaz-Cuadros et al., 2021) (Fig. 4), which is not observed in annelids. In both arthropods and vertebrates, molecular markers of segment/somite polarity begin to be expressed along the new boundaries only after the specification of the entire metamere territory, whereas the expression of *engrailed* in *A. virens* marks the anterior border of the segment anlage long before the complete production of the cell material of the segment and its specification. Thus, we come to the conclusion that the models described above are inapplicable to the postembryonic period of annelid ontogenesis.

Since the question of the unity of the evolutionary origin of the segments of annelids and arthropods has not been fully resolved, it is necessary to consider their developmental patterns taking into account the presented data. The patterns of expression of *engrailed* in nereids and arthropods are similar in the way that the sequential appearance of expression bands occurs before the morphological separation of segments. Functional experiments proved the participation of *engrailed* in the processes of patterning and morphogenesis of segments in arthropods (Gustavson et al., 1996; Lim and Choe, 2020). It can be assumed that *engrailed* performs similar functions in *A. virens*. However, the interrelation between the processes of axis elongation (creation of cellular material), specification and polarization of segments, as well as the genes involved in these processes, apparently differ greatly in annelids and arthropods (Balavoine, 2014).

In arthropods with terminal growth, the cellular material of a parasegment is formed firstly (Fig. 4a), which is subjected to specification (Fig. 4b), and then the boundaries marked with expression bands of "segment polarity genes" are finally established (Fig. 4c) (Williams and Nagy, 2017). At the same time, there is no expression of *engrailed* in the growth zone but transcripts of this gene are detected in cells at the anterior boundaries of parasegments corresponding to the posterior boundaries of definitive segments. In addition to insects, the expression of *engrailed* is localized at the posterior border of segments in millipedes, crustaceans, chelicerates, onychophores, and tardigrades (Patel et al., 1989a; Damen, 2002; Hughes and Kaufman, 2002; Gabriel and Goldstein, 2007; Janssen, 2017; Lim and Choe, 2020). Thus, unlike in arthropods, the conservative "borderline" pattern of *engrailed* occurs in *A. virens* even before the complete determination of the primordium of the segment

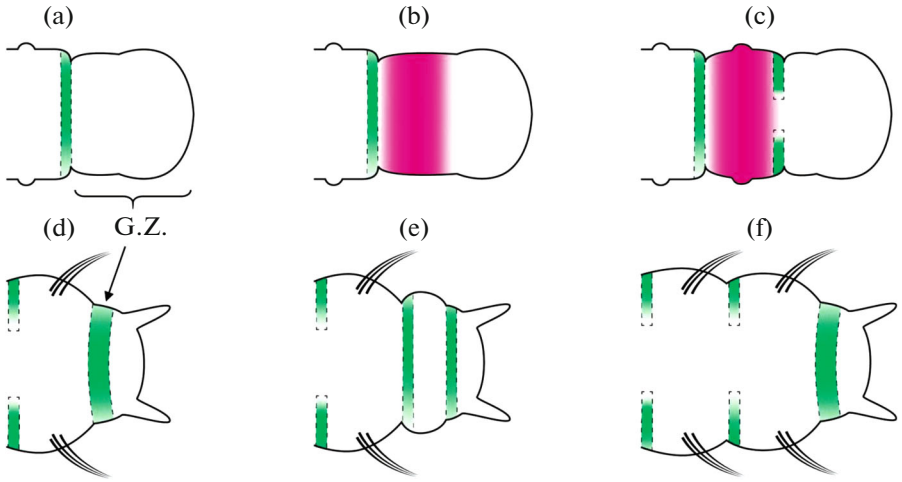

Fig. 4. Scheme of sequential formation of segments (a–c) in arthropods (according to Williams and Nagy, 2017) and (d–f) in the annelid *A. virens*. Green fill is for areas of *engrailed* expression, purple is for the area of specification of a new segment, G. Z.—growth zone. (a) Accumulation of cellular material in the growth zone (the area posterior to the last expression band of *engrailed*). (b) Specification of the parasegment in the anterior part of the growth zone due to the oscillation of the expression of the "pair-rule" and Notch signaling genes. (c) The separation of the segment associated with the final determination of the boundaries (the formation of a new expression band of *engrailed*), and the beginning of morphogenesis (the formation of rudiments of limbs and intersegmental furrows). (d) The expansion of the ring domain of *engrailed* expression in the growth zone is the first stage of formation of a new segment, when, apparently, the molecular profile of cells is established at its anterior border. (e) The accumulation of cellular material via proliferation leads to the isolation of two *engrailed* expression domains that separate the segmental primordium from the preceding segment (infront) and from the pygidium (behind). The mechanisms of specification of the segmental primordium of annelids remain unknown. (f) Morphogenesis, growth, and differentiation of a new segment are accompanied by a gradual reduction of the *engrailed* expression domain along the intersegmental furrow.

(Fig. 4). We assume that the specification of the anterior segment boundary occurs first in *A. virens*, while the posterior specification, which is accompanied by the development of the cellular material of the corresponding segment, occurs after that. This indicates cardinal differences in the segmentation mechanisms of nereids and metameric Ecdysozoa.

CONCLUSIONS

The evolutionary origin of such a complex feature as segmentation is a fundamentally important and unresolved issue of biology. Currently, the segmented body plan is analyzed at the level of comparison of molecular genetics and cellular mechanisms of segment development in different taxa. This analysis is significantly limited by the lack of detailed descriptive and functional studies of segmentation in aclitellate annelids. Having studied one of the most conservative segmentation marker genes, *engrailed*, we compared the expression patterns of its orthologues in annelids and arthropods and also analyzed the applicability of existing SAZ models to annelids. This allowed us to conclude that the formation of segments from the growth zone in the postlarval development of nereids has a number of specific features. We assume that the specification of *A. virens*'s segment goes in parallel with the elongation of the anterior-posterior axis, since the anterior boundary of the segment is first determined (the marker of which is the expression of *engrailed* mRNA), followed by the completion of its posterior territories, and then the final posterior boundary appears (the furrow between the segment and the pygidium). Analysis of the patterns of expression of *engrailed* in bilaterians suggests that the regulation of neural differentiation and the creation of border territories should be considered the ancestral role of this gene. An involvement of *engrailed* in the processes of patterning the primordia of segments in nereids (and possibly in all annelids), arthropods, and chordates, apparently, occurred independently. Further research should determine which functions of *engrailed* and other segments-patterning genes were inherited by nereids from the common ancestor of annelids and which arose later in evolution. This will allow for a full-fledged comparison of the functioning of the genetic repertoire of segmented animals and reconstruct the evolutionary history of segmentation more reliably.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. Applicable ethical standards were implemented considering the objects of research.

REFERENCES

Balavoine, G., Segment formation in annelids: patterns, processes and evolution, *Int. J. Dev. Biol.*, 2014, vol. 58, pp. 469–483.

Bely, A.E. and Wray, G.A., Evolution of regeneration and fission in annelids: insights from engrailed- and orthodenticle-class gene expression, *Development*, 2001, vol. 128, no. 14, pp. 2781–2791.

Byrne, M., et al., Engrailed is expressed in larval development and in the radial nervous system of *Patiriella* sea stars, *Dev. Genes Evol.*, 2005, vol. 215, no. 12, pp. 608–617.

Cellular Processes in Segmentation, Chipman, A.D., Ed., CRC Press, 2020a.

Chipman, A.D., The evolution and development of segmented body plans, in *Evolutionary Developmental Biology*, Nuno de la Rosa, L. and Muller, G., Eds., Cham: Springer, 2018, pp. 1–10.

Chipman, A.D., The evolution of the gene regulatory networks patterning the *Drosophila* blastoderm, *Curr. Topics Dev. Biol.*, 2020b, vol. 139, pp. 297–324.

Condron, B.C., Patel, N.H., and Zinn, K., Engrailed controls glial/neuronal cell fate decisions at the midline of the central nervous system, *Neuron*, 1994, vol. 13, no. 3, pp. 541–554.

Damen, W.G.M., Parasegmental organization of the spider, *Development*, 2002, vol. 129, no. 5, pp. 1239–1250.

Diaz-Cuadros, M., Pourquié, O., and El-Sherif, E., Patterning with clocks and genetic cascades: segmentation and regionalization of vertebrate versus insect body plans, *PLoS Genet.*, 2021, vol. 17, no. 10, p. e1009812.

Dondua, A.K., Effect of actinomycin D and sibiromycin on the embryonic and larval development of *Nereis virens* (Sars.), *Ontogenez*, 1975, vol. 6, no. 5, pp. 475–484.

Ekker, M., et al., Coordinate embryonic expression of three zebrafish engrailed genes, *Development*, 1992, vol. 116, no. 4, pp. 1001–1010.

Fischer, A.H., Henrich, T., and Arendt, D., The normal development of *Platynereis dumerilii* (Nereididae, Annelida), *Front. Zool.*, 2010, vol. 7, no. 1, p. 31.

Gabriel, W.N. and Goldstein, B., Segmental expression of Pax3/7 and engrailed homologs in tardigrade development, *Dev. Genes Evol.*, 2007, vol. 217, no. 6, pp. 421–433.

Gazave, E., Behague, J., Laplane, L., Guillou, A., Préau, L., Demilly, A., Balavoine, G., and Vervoort, M., Posterior elongation in the annelid *Platynereis dumerilii* involves stem cells molecularly related to primordial germ cells, *Dev. Biol.*, 2013, vol. 382, no. 1, pp. 246–267.

Gustavson, E., et al., The *Drosophila engrailed* and *invected* genes: partners in regulation, expression and function, *Genetics*, 1996, vol. 142, no. 3, pp. 893–906.

Hughes, C.L. and Kaufman, T.C., Exploring myriapod segmentation: the expression patterns of *even-skipped, engrailed*, and *wingless* in a centipede, *Dev. Biol.*, 2002, vol. 247, no. 1, pp. 47–61.

Janssen, R., A molecular view of onychophoran segmentation, *Arthropod Struct. Dev.*, 2017, vol. 46, no. 3, pp. 341–353.

Kostyuchenko, R.P., Nanos is expressed in somatic and germline tissue during larval and post-larval development of the annelid *Alitta virens, Genes*, 2022, vol. 13, no. 2, p. 270.

Kozin, V.V. and Kostyuchenko, R.P., Vasa, PL10, and Piwi gene expression during caudal regeneration of the polychaete annelid *Alitta virens, Dev. Genes Evol.*, 2015, vol. 225, no. 3, pp. 129–138.

Kozin, V.V. and Kostyuchenko, R.P., Evolutionary conservation and variability of the mesoderm development in Spiralia: a peculiar pattern of nereid polychaetes, *Biol. Bull.* (Moscow), 2016, vol. 43, no 3, p. 216–225.

Kozin, V.V., Filimonova, D.A., Kupriashova, E.E., and Kostyuchenko, R.P., Mesoderm patterning and morphogenesis in the polychaete *Alitta virens* (Spiralia, Annelida): expression of mesodermal markers *Twist*, *Mox*, *Evx* and functional role for MAP kinase signaling, *Mech. Dev.*, 2016, vol. 140, p. 1–11.

Kozin, V.V., Borisenko, I.E., and Kostyuchenko, R.P., Establishment of the axial polarity and cell fate in Metazoa via canonical Wnt signaling: new insights from sponges and annelids, *Biol. Bull.* (Moscow), 2019a, vol. 46, no. 1, pp. 14–25.

Kozin, V.V., Shalaeva, A.Yu., Filippova, N.A., and Kostyuchenko, R.P., How to build a polychaete larva? Natural history of the development and metamorphosis of *Alitta virens* (Annelida: Nereididae), *Proceedings of the VII International Scientific and Practical Conference "Marine Research and Education (MARESEDU-2018),"* Tver: PoliPRESS LLC, 2019b, vol. IV (IV), p. 81–85.

Kuo, D.-H., The polychaete-to-clitellate transition: an EvoDevo perspective, *Dev. Biol.*, 2017, vol. 427, no. 2, pp. 230–240.

Lim, J. and Choe, C.P., Functional analysis of engrailed in *Tribolium* segmentation, *Mech. Dev.*, 2020, vol. 161, p. 103594.

Omi, M. and Nakamura, H., Engrailed and tectum development, *Dev. Growth Differ.*, 2015, vol. 57, no. 2, pp. 135–145.

Patel, N.H., Kornberg, T.B., and Goodman, C.S., Expression of engrailed during segmentation in grasshopper and crayfish, *Trends Genet.*, 1989a, vol. 107, no. 2, pp. 201–212.

Patel, N.H., et al., Expression of engrailed proteins in arthropods, annelids, and chordates, *Cell*, 1989b, vol. 58, no. 5, pp. 955–968.

Prud'homme, B., et al., Arthropod-like expression patterns of engrailed and wingless in the annelid *Platynereis dumerilii* suggest a role in segment formation, *Curr. Biol.*, 2003, vol. 13, no. 21, pp. 1876–1881.

Seaver, E.C. and Kaneshige, L.M., Expression of 'segmentation' genes during larval and juvenile development in the polychaetes *Capitella* sp. I and *H. elegans, Dev. Biol.*, 2006, vol. 289, no. 1, pp. 179–194.

Seaver, E.C. et al., The spatial and temporal expression of Ch-en, the engrailed gene in the polychaete *Chaetopterus*, does not support a role in body axis segmentation, *Dev. Biol.*, 2001, vol. 236, no. 1, pp. 195–209.

Shalaeva, A.Y., Kostyuchenko, R.P., and Kozin, V.V., Structural and functional characterization of the FGF signaling pathway in regeneration of the polychaete worm *Alitta virens* (Annelida, Errantia), *Genes*, 2021, vol. 12, no. 6, p. 788.

Steinmetz, P.R.H., Kostyuchenko, R.P., Fischer, A., and Arendt, D., The segmental pattern of *otx, gbx*, and *Hox* genes in the annelid *Platynereis dumerilii, Evol. Dev.*, 2011, vol. 13, no. 1, pp. 72–79.

Sveshnikov, V.A., *Morfologiya lichinok polikhet* (Morphology of Polychaete Larvae), Moscow: Nauka, 1978.

Vellutini, B.C. and Hejnol., A., Expression of segment polarity genes in brachiopods supports a non-segmental ancestral role of engrailed for bilaterians, *Sci. Rep.*, 2016, vol. 6, no. 1, p. 32387.

Williams, T.A. and Nagy, L.M., Linking gene regulation to cell behaviors in the posterior growth zone of sequentially segmenting arthropods, *Arthropod Struct. Dev.*, 2017, vol. 46, no. 3, pp. 380–394.

Zattara, E., Axial regeneration in segmented animals a post-embryonic reboot of the segmentation process, *Cell. Proc. Segment.*, 2020, pp. 255–292.

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