

Musculature and innervation of the pygidium in Eunicida (Annelida: Errantia)

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Abstract

The pygidium is a terminal part of the annelid body that is considered non-homologous to body segments. Despite the high level of morphological diversity, the internal morphology of the pygidial region is very poorly studied. Recent research revealed that in some errant annelids the pygidium possesses complex musculature and innervation. To provide new data for the comparative analysis of pygidial organization, the musculature and innervation in the pygidial region in five annelid species belonging to the order Eunicida were studied using phalloidin labeling, immunohistochemistry and confocal scanning microscopy. In all studied species the pygidial musculature consists of a circular or horseshoe-shaped muscle. The pygidial innervation comprises two pairs of main longitudinal nerves and paired circumpygidial nerves. The single pair of longitudinal nerves in *Ophryotrocha irinae* may be regarded as a secondary loss. In *Schistomeringos japonica* a small terminal commissure between longitudinal nerves was found. The finding of numerous receptor cell endings in the surface of the pygidium suggests its important sensory function. Comparison with Phyllodo-cida demonstrates the high level of similarities in the pygidial organization and the loss of the terminal commissure in Eunicida.

Keywords: Annelida, Eunicida, nervous system, musculature, confocal microscopy.

Introduction

The phenomenon of metameric body organization is a common feature of different groups of metazoan animals (Schmidt-Rhaesa, 2007). Annelida is a group that almost ideally matches the definition of metameric body organization (Bala-voine, 2014). The annelid body is constructed of serially arranged units, called segments. In the most ideal way, each segment comprises an outer annulus, one pair of mesodermal hollow spaces, one pair of ventral ganglia, one pair of nephridia, a set of muscles and one pair of appendages (Scholtz, 2002). However, the anteriormost and posteriormost metameres differ significantly from the body segments and are mentioned to be of non-segmental origin (Anderson, 1966). It is believed that they lack many segmental features, such as coelomic cavities, segmental organs and appendages.

The morphology of the annelid body has been studied in detail using different microscopy techniques (Harrison, 1992; Bartolomaeus and Purschke, 2005). Nevertheless, the pygidium remains one of the “white spots” in our knowledge. The external morphology of the pygidium is extremely diverse, and it can be used in taxonomic purposes in several annelid families (Jirkov, 2001). However, until recently very little was known about pygidium internal morphology and possible functions. Our recent studies revealed that in Nereidids the structure of the pygidium quite different from how it was previously considered. In *Alitta virens* and

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Platynereis dumerilii complicated musculature and innervation were found (Starunov and Lavrova, 2013; Starunov et al., 2015). To determine whether this organization is characteristic of other annelids we examined the musculature and innervation of the pygidium in other families of the order Phyllodocida (Starunov, 2018; Starunov and Zaitseva, 2018). We found that despite the highly variable character of pygidial musculature, the pattern of pygidial innervation is surprisingly stable. However, it remains unknown whether the pygidial organization found in Phyllodocida has basal characters expressing in other annelids.

According to the modern phylogenomic concept on annelid phylogeny, the annelids can be subdivided into Pleistoannelida and some small basally branching groups (Struck, 2011; Struck et al., 2011; Weigert et al., 2014; Weigert and Bleidorn, 2016). Pleistoannelida comprises two major clades: Errantia and Sedentaria. Since Clitellata falls deeply in the Sedentaria clade, the taxon Polychaeta becomes synonymous to Annelida. Errant annelids are subdivided into two main orders: Phyllodocida and Eunicida, which are also well supported by both morphological and molecular analyses (Rouse and Fauchald, 1997; Rousset et al., 2007; Struck, Schult, and Kusen, 2007; Kvist and Siddall, 2013; Weigert and Bleidorn, 2016). Eunicid annelids comprise almost 1400 species (Horton et al., 2018). Most of the described species belong to four families: Eunicidae, Onuphidae, Lumbrineridae, and Dorvilleidae.

In this paper I describe the innervation pattern and the muscular system organization of the pygidial region in five species belonging to the order Eunicida using confocal laser scanning microscopy in combination with fluorescent immunohistochemical labeling. The goal of the given study is to provide a comparative analysis of this region of the metameric annelid body, and to try to reconstruct its possible ancestral condition in errant annelids.

Material and methods

The specimens of *Lysidice ninetta* Audouin & H Milne Edwards, 1833 were collected from the intertidal zone near the Concarneau Marine Biological Station (Bay of Biscay, France). *Onuphis holobranchiata* Marenzeller, 1879, *Lumbrineris inflata* Moore, 1911, and *Schistomeringos japonica* (Annenkova, 1937) were collected from the trawl samples near the marine biological station Vostok (Sea of Japan, Russia). Specimens of *Ophryotrocha irinae* Tsetlin, 1980 were collected at the intertidal zone near the marine biological station Dalnie Zelentsi (Barents Sea, Russia). Species identification was performed using common identification keys (Ushakov, 1955; Hartmann-Schröder, 1996) and original descriptions (Tsetlin, 1980).

For immunohistochemical study the specimens were relaxed in 7.5% MgCl_2 and fixed in 4% Paraformaldehyde at 0.1M phosphate buffered saline (PBS, pH = 7.4) for 8–12 h at +4°C or 3–6 h at room temperature. After fixation the specimens were washed 3 times for 30' in PBS containing 0.1% Triton X-100 (PBT) and stored in PBS with 0.1–0.05% NaN_3 at +4°C. The immunolabellings were performed according to the standard protocol (Starunov et al., 2015). Monoclonal mouse primary antibodies against acetylated α -tubulin (T-6793, Sigma-Aldrich, St. Louis, USA) diluted 1:1000–1:2000 and fluorescent anti-mouse secondary antibodies (AlexaFluor 633, AlexaFluor 647, Thermo Fisher Scientific, Waltham, USA or CF 633, Sigma-Aldrich) diluted 1:500–1:800 were used. After immunolabelling the specimens were stained with 1 $\mu\text{g}/\text{ml}$ TRITC-conjugated phalloidin (Sigma, P1951) in PBT for 2 h, mounted in Mowiol and examined using Leica TCS SPE or Leica TCS SP5 laser confocal microscopes (Leica Microsystems, Wetzlar, Germany). The z-projections were made in Fiji (Schindelin et al., 2012), and the 3D reconstructions were built using Bitplane Imaris (Bitplane, Zurich, Switzerland). The schemes were drawn using Inkscape vector graphics editor.

For scanning electron microscopy, the animals that had been relaxed in 7.5% MgCl_2 were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (PB, pH = 7.2) with 0.3M NaCl. After fixation, the samples were rinsed 1–3 times in PB with 0.3M NaCl and incubated in 1% OsO_4 in the same buffer. The dehydration was performed in an acetone series of increasing concentration. The specimens were critical point dried, coated with platinum, and examined under a FEI Quanta 250 scanning electron microscope (FEI Company, Netherlands).

Results

The pygidium in all studied species is well developed (Fig. 1). The anus is situated terminally (Figs. 1E, 2B) or shifted slightly to the dorsal side (Fig. 1D, F). All species except *Ophryotrocha irinae* bear two pairs of cirri at the ventral side of the pygidium. In *Onuphis holobranchiata* the cirri are long and situated close to each other (Fig. 1A, D). The ventro-lateral pair of cirri is longer than the dorso-medial one. The dorso-lateral cirri of *Lysidice ninetta* are longer than the ventral. In *Schistomeringos japonica* the ventral pygidial cirri are shorter than the dorsal pair and are directed laterally (Fig. 1C, F). The whole surface of the pygidium bears numerous scattered cilia. There is also a tiny ciliary band around the anus and the pygidial cirri (Fig. 1B, E, *pcb*). *Ophryotrocha irinae* has only one pair of papilliform cirri situated laterally (Fig. 3D) and a small unpaired papilla at the ventral side of the pygidium (Fig. 3G). *Lumbrineris inflata* possesses shortened cirri, of which the dorsal pair is slightly longer than the ventral (Fig. 1C, F).

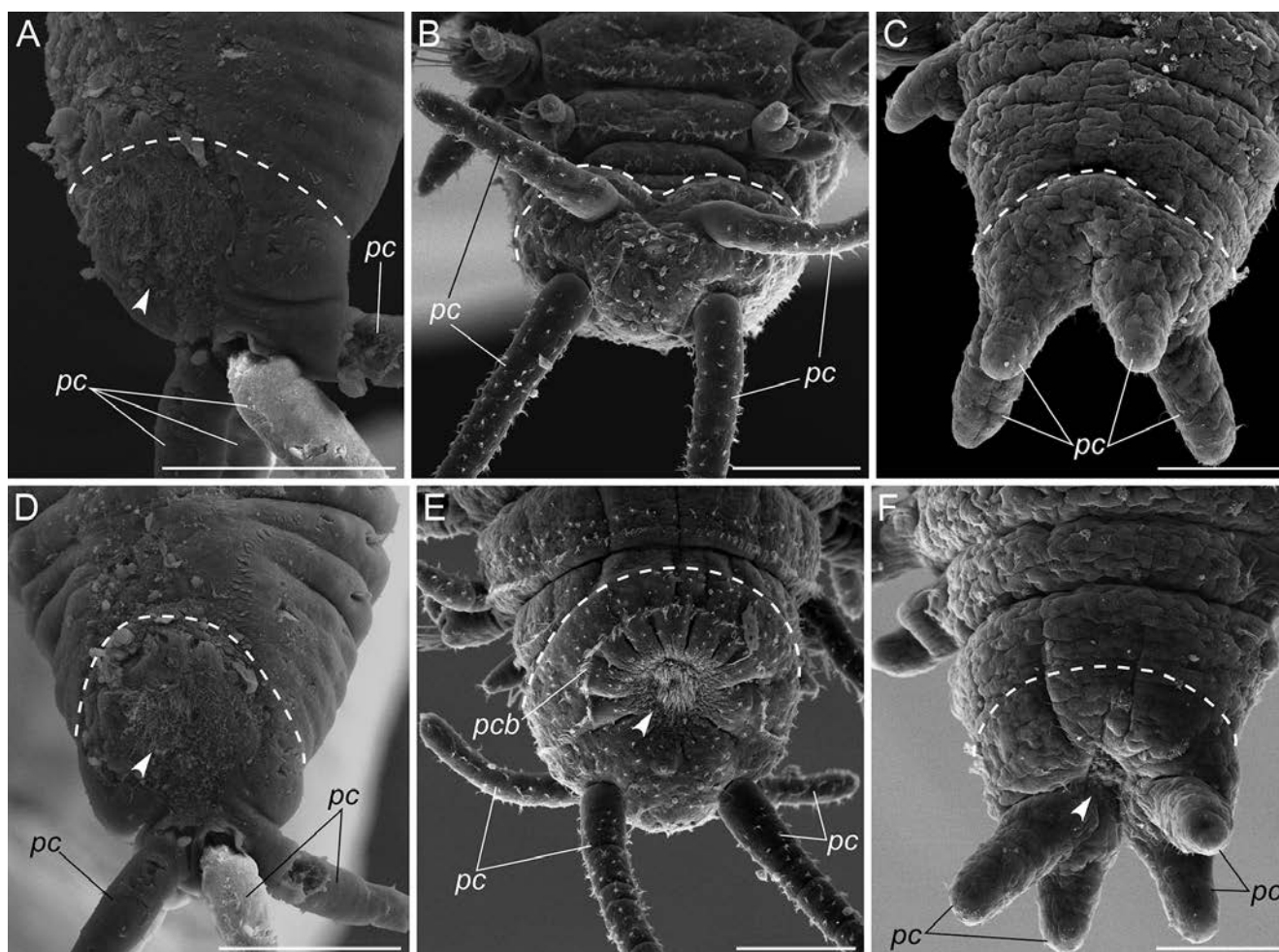


Fig. 1. Scanning electron micrographs of the pygidium of *Onuphis holobranchiata* (A, D), *Schistomerings japonica* (B, E), and *Lumbrineris inflata* (C, F)

A — lateral view, B, C — ventral view, D–F — dorsal view. Dotted line demarcates the border between the pygidium and body segments. Arrowheads indicate the position of the anus. *pc* — pygidial cirri, *pcb* — pygidial ciliary band. Scale bar — 50 microns.

MUSCULATURE OF THE PYGIDIUM

All studied species possess muscular elements surrounding the hindgut. In *L. ninneta* a single but well-developed circular muscle surrounds the hindgut (*cpm*, Fig. 2 A, B). At the dorsal side the circular muscle is thicker than at the ventral. The longitudinal muscular bands of the body segments attach directly to the circular pygidial muscle.

In *O. holobranchiata* the hindgut is surrounded by a horseshoe-shaped muscle with the gap at the ventral side (*hpm*, Fig. 2C, D). The endings of this muscle extend slightly anteriorly and meet the terminal part of the thin medial ventral longitudinal muscle of the body (*mvm*).

Dorvilleids possess the most prominent and complex pygidial musculature of the studied species. The single pygidial muscle of *S. japonica* is relatively huge and bears a pair of “horns” at the ventral side (Fig. 2E, F, arrowheads). These “horns” attach to the basements of the dorsal pygidial cirri. The ventral longitudinal muscu-

lar bands of the body (*vlm*) enter the pygidium and can be followed up to the dorsal pygidial muscular “horns”.

The characteristic feature of *Ophryotrocha irinae* is the presence of muscular fibers inside the pygidial cirri (Fig. 2G, H, I). These muscular fibers are in fact the extensions of the ventral longitudinal muscular bands of the body. The medial ventral longitudinal muscle also extends to the pygidium and terminates even behind the circular pygidial muscle. In contrast to *S. japonica*, the circular pygidial muscle do not form “horns” at the ventral side.

The pygidial musculature of *L. inflata* is presented by a thin muscular ring, which is poorly seen in some preparations (Fig. 2J, K).

INNERVATION OF THE PYGIDIUM

In *L. ninneta* the paired nerves of the ventral nerve cord enter the pygidium and give rise to paired medial (*mln*) and lateral (*lln*) longitudinal pygidial nerves that supply the corresponding pygidial cirri (Fig. 3A, inset). From

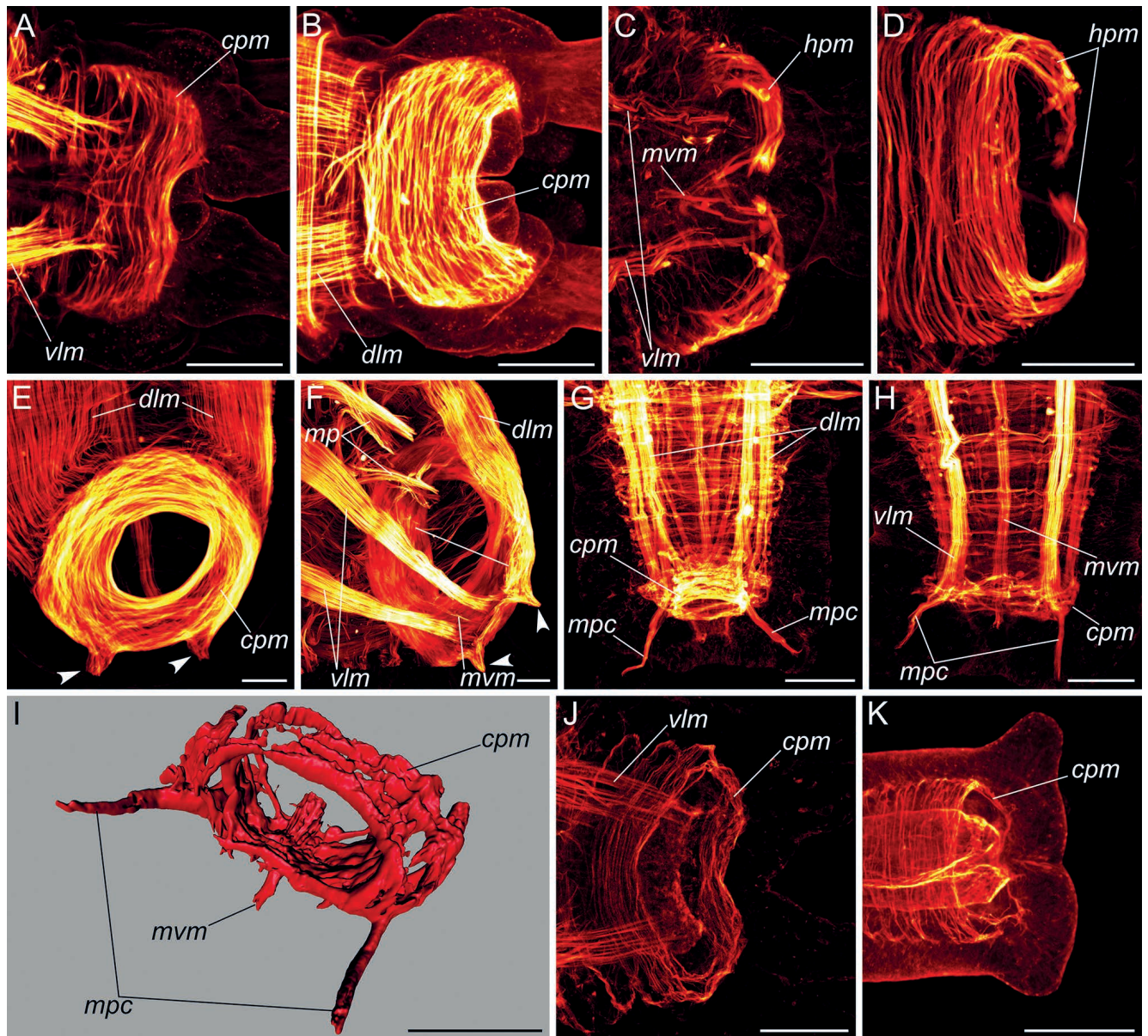


Fig. 2. Organization of the musculature in the pygidium in Eunicida

A, B — *Lysidice ninneta*, C, D — *Onuphis holobranchiata*, E, F — *Schistomerings japonica*, G–I — *Ophryotrocha irinae*, J, K — *Lumbrineris inflata*. A, C, F, H, J — ventral view, B, D, E, G, K — dorsal views. I — 3D-reconstruction of the pygidial musculature in *Ophryotrocha irinae*. Arrowheads in E and F indicate the ventral muscular “horns” of *S. japonica*. cpm — circular pygidial muscle, dlm — dorsal longitudinal muscular band, hpm — horseshoe-shaped pygidial muscle, mp — parapodial muscles, mpc — muscles of the pygidial cirri, mvm — medial ventral longitudinal muscle, vlm — ventral longitudinal muscular band. Scale bar — 50 microns.

the branching points of longitudinal pygidial nerves, paired circumpygidial nerves (cpn) run to the dorsal side of pygidium. These nerves provide the innervation of lateral and dorsal pygidial walls. The unpaired medial nerve of the ventral nerve cord can be followed only to the anterior margin of the pygidium.

The innervation of the pygidium in *O. holobranchiata* (Fig. 3 B) is in general similar to *L. ninneta*. The only difference is in relative dimensions of the corresponding nerves. The circumpygidial nerves are thin, and the pygidial longitudinal nerves are more prominent.

S. japonica demonstrates the most comprehensive pygidial innervation among the studied species (Fig. 3C–E). Two pairs of lateral nerves are prominent and connected by a thin terminal commissure (Fig. 3D, tc). At the same place several small nerves branch off from longitudinal nerves and go medially to innervate the ventral surface of the pygidium (Fig. 3C, D, arrows). Usually these nerves are paired (Fig. 3C), but in some animals an additional asymmetrical nerve can be detected (Fig. 3D). The circumpygidial nerves lie underneath the pygidial ciliary band (Fig. 3E, pcb), and have connec-

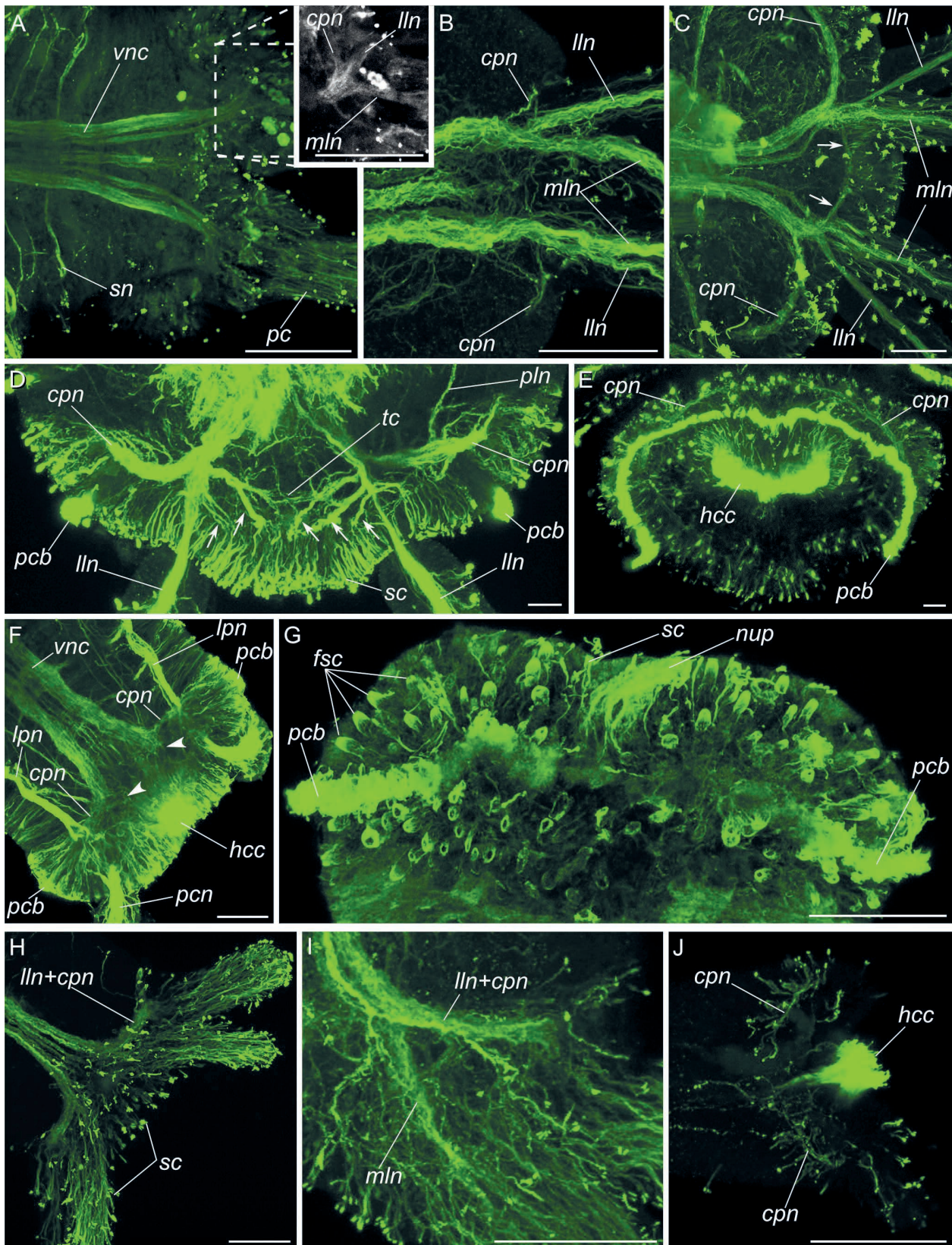


Fig. 3. Innervation of the pygidium in Eunicida

A — *Lysidice ninneta*, inset shows the branching point of the longitudinal pygidial nerves B — *Onuphis holobranchiata*, C–E — *Schistomeringos japonica*, F, G — *Ophryotrocha irinae*, H–J — *Lumbrineris inflata*. Partial maximum intensity projections. E, J — dorsal view, all other — ventral view. Arrows in C and D label additional medial nerves. Arrowheads in F indicate the longitudinal pygidial nerves branching points. *cpn* — circumpygidial nerves, *fsc* — flask-shaped cells, *hcc* — hindgut ciliated cells, *llm* — lateral longitudinal nerves, *lpn* — longitudinal peripheral nerves, *mln* — medial longitudinal nerves, *nup* — nerves of the unpaired cirrus, *pc* — pygidial cirri, *pcb* — pygidial ciliary band, *pcn* — pygidial cirri nerves, *pln* — peripheral longitudinal nerve, *sc* — receptor cell endings, *sn* — segmental nerves, *tc* — terminal commissure, *vnc* — ventral nerve cord. Scale bar — 50 microns.

tions with peripheral longitudinal nerves (Fig. 3D, *pln*). The whole pygidial surface, as well as the pygidial cirri, bear numerous receptor cell endings with short cilia (*sc*).

The pygidium of *Ophryotrocha irinae* express a high level of background fluorescence. The terminal parts of the ventral nerve cord enter the pygidium, give rise to paired circumpygidial nerves and split into numerous nerve fibers that innervate the pygidial surface (Fig. 3F, *arrowheads*). Some of these fibers innervate the unpaired medial papilla (Fig. 3G, *nup*). In addition to receptor cell endings, the surface of pygidium possesses flask-shaped cells with a high level of immunoreactivity to acetylated α -tubulin (*fsc*). Neither longitudinal pygidial nerves, nor a terminal commissure, were found. The circumpygidial nerves have connections with huge peripheral longitudinal nerves (Fig. 3F, *lpn*). At the same place they branch off small nerves that contribute to the highly immunoreactive pygidial cirri nerves (*pcn*).

In *L. inflata* the pygidial longitudinal nerves are shortened (Fig. 3 H–J). They split into numerous thin nerve fibers as they enter the pygidial cirri. The circumpygidial nerves are thin and branch from the lateral longitudinal nerves only at the ventro-lateral sides of the pygidium. Numerous receptor cell endings are scattered through the pygidium surface. The terminal commissure was not found.

Discussion

This study presents a detailed description of the musculature organization and innervation pattern of the pygidium in a variety of eunicid annelids. The summarized comparative results are present in Figures 4 and 5. The obtained data, together with my recent findings on the pygidial musculature and innervation in Phyllodocida — another main order of errant annelids (Starunov, 2018) — provide a background for the comparative analysis and reconstruction of the annelid pygidial region ground plan.

All studied species have prominent musculature in the pygidial region (Figs. 2, 4). The pygidial musculature of *O. holobranchiata* is represented by a horseshoe-shaped muscle; no circular fibers were found (Fig. 4B). All other studied animals possess a more or less developed circular muscle surrounding the hindgut. In *S. japonica* this circular muscle demonstrates further complication by the presence of the small muscular “horns” at the ventral side (Fig. 4D). Nevertheless, no other pygidial muscles were found in studied species, which indicates the overall simplicity of pygidial musculature in Eunicida. This muscular organization corresponds to those found in other annelids. The circular pygidial muscle was found in different families of the order Phyllodocida (Filippova, Purschke, Tzetlin and Müller, 2010; Starunov, 2018), in Polygordiidae (Rota and Carchini,

1999; Lehmacher, Fiege and Purschke, 2014), and Spirogonidae (Filippova, Purschke, Tzetlin, and Müller, 2005). The horseshoe-shaped muscle or muscle fibers were described in different members of the order Phyllodocida (Starunov, 2018). Among them, the pygidial musculature, found in the family Hesionidae, is strikingly similar to those found in *O. holobranchiata*. Namely, in *Oxydromus pugettensis* the terminal part of the medial ventral longitudinal muscle bifurcates in the pygidium, extends laterally, and attaches to the horseshoe-shaped pygidial muscle. Since Onuphidae and Hesionidae belong to different annelid orders and their closely related families exhibit different patterns of pygidial musculature, I can assume that the similarities found may be a result of parallel evolution.

In all studied species the ventral longitudinal muscles of the body extend posteriorly, enter the pygidium and can be followed up to the pygidial cirri basements. These extensions may contribute to the pygidial cirri motility. Similar organization was found in different annelid families belonging to the order Phyllodocida (Starunov, 2018), nerillids (Müller and Worsaae, 2006), and *Polygordius appendiculatus* (Lehmacher, Fiege and Purschke, 2014). In *Ophryotrocha irinae* the longitudinal muscles extend even in parapodial cirri (Fig. 2G–I, 4E). Similarly, muscle fibers in pygidial cirri were also reported for nerillids and syllids (Müller and Worsaae, 2006; Starunov, 2018). Nevertheless, this similarity is superficial. In syllids the muscular fibers of the pygidial cirri are separate muscular elements and do not relate to the longitudinal muscular bands of the body segments. In *Nerilla antennata* and *Nerillidium sp.* the authors describe in the pygidium two “muscular commissures” from which muscles stretch into the pygidial cirri (Müller and Worsaae, 2006). It is not clear whether these muscles constitute the extensions of ventral longitudinal muscular bands. Moreover, according to the recent phylogenomic data, nerillids, syllids and dorvilleids belong to different annelid clades (Struck et al., 2015; Weigert and Bleidorn, 2016). The members of closely related families or even of the same family (compare for example *S. japonica* and *O. irinae*, Figs. 2E–H, 4D, E) do not possess such muscular elements. Therefore, I can suggest that the muscular fibers inside the pygidial cirri in these annelid families emerged independently.

Comparison of the obtained results with other annelid species reveals that the circular muscle is the most common element of pygidial musculature (Filippova, Purschke, Tzetlin and Müller, 2005; Müller and Worsaae, 2006; Filippova, Purschke, Tzetlin and Müller, 2010; Starunov, 2018). Probably it constituted the main element of pygidial musculature in the hypothetical ancestral annelid. Unfortunately, the information on pygidial musculature in sedentary species is fragmentary and does not allow any final conclusion to be made. On

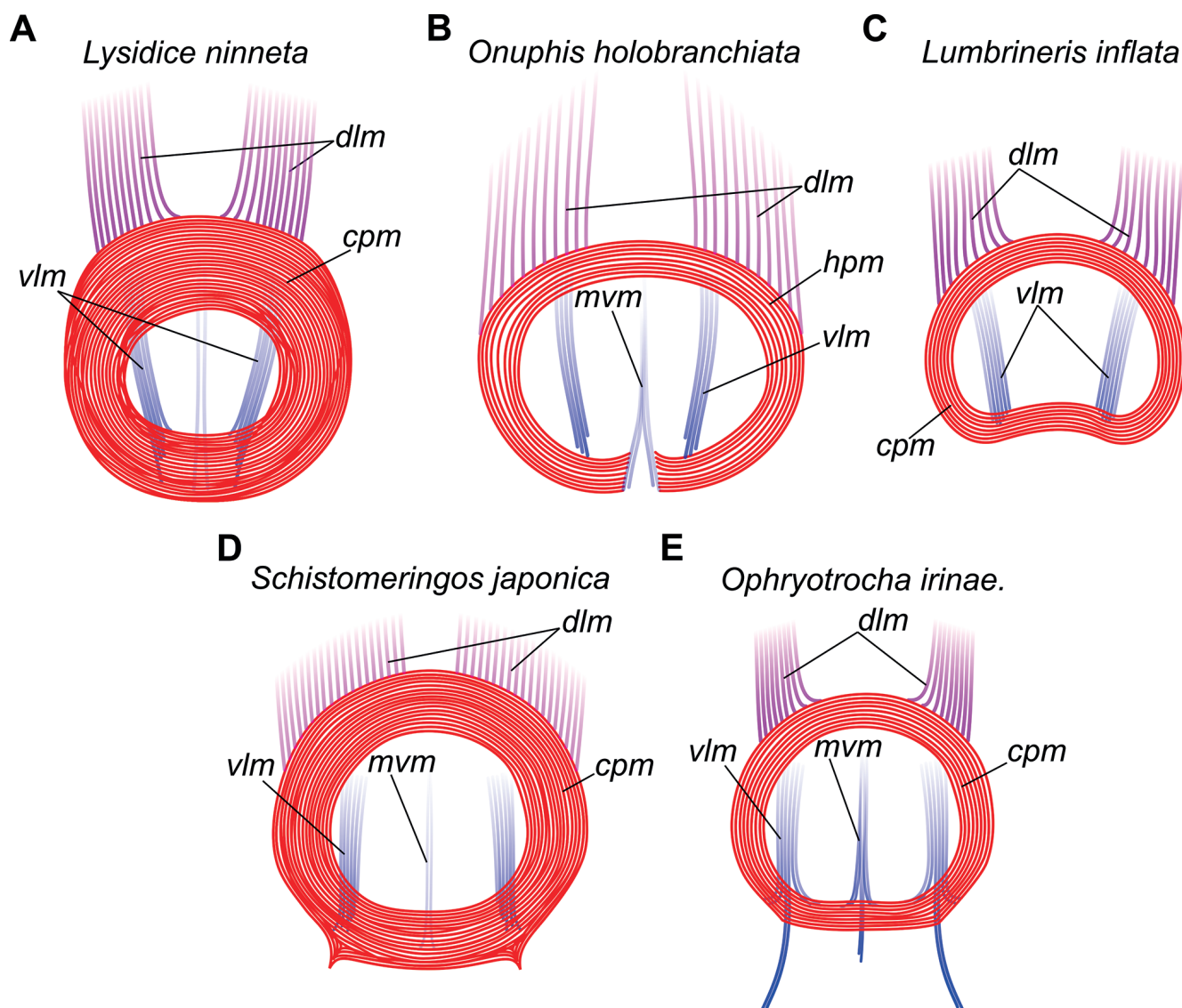


Fig. 4. Schemes of the pygidial musculature in Eunicida

A — *Lysidice ninneta*, B — *Onuphis holobranchiata*, C — *Lumbrineris inflata*, D — *Schistomeringos japonica*, E — *Ophryotrocha irinae*. cpm — circular pygidial muscle, dlm — dorsal longitudinal muscular band, hpm — horseshoe-shaped pygidial muscle, mvm — medial ventral longitudinal muscle, vlm — ventral longitudinal muscular band. Relative dimensions are not kept.

the other hand, in *Magelona* cf. *mirabilis* and *Eurythoe complanata*, belonging to the basally branching annelids, circular muscles in the pygidium were not found (Filippova, Purschke, Tzvetlin and Müller, 2005; Weidhase, Bleidorn, Beckers and Helm, 2016). This may suggest that the circular pygidial muscle may either be a synapomorphy of Pleistoannelida, or it may be lost in studied basal branching species. Further studies on the pygidial organization in sedentary annelids as well as in basally branching groups are highly needed to shed light on this problem.

The innervation of the pygidial region in all studied families is represented by two pairs of longitudinal nerves and paired circumpygidial nerves (Fig. 5). In *S. japonica* additional medial nerves and a terminal com-

missure were also detected. The longitudinal nerves correspond to two pairs of pygidial cirri. Only *O. irinae* possesses a single pair of cirri. Other members of the genus *Ophryotrocha* in general also possess one pair of pygidial cirri. However, in several species, for example *O. obtusa*, both pairs of cirri can be found (Hilbig and Blake, 1991). Moreover, members of the genus *Ophryotrocha* are considered to be neotenic (Westheide, 1987). Therefore, I can suggest that the pygidium of *O. irinae* demonstrates the derived condition and its innervation does not correspond to the ground plan for the order Eunicida and even for the family Dorvilleidae.

In another main clade of errant annelids, the Phyllocladocida, only one pair of pygidial cirri was found (Starunov et al., 2015; Starunov, 2018). Nevertheless, some

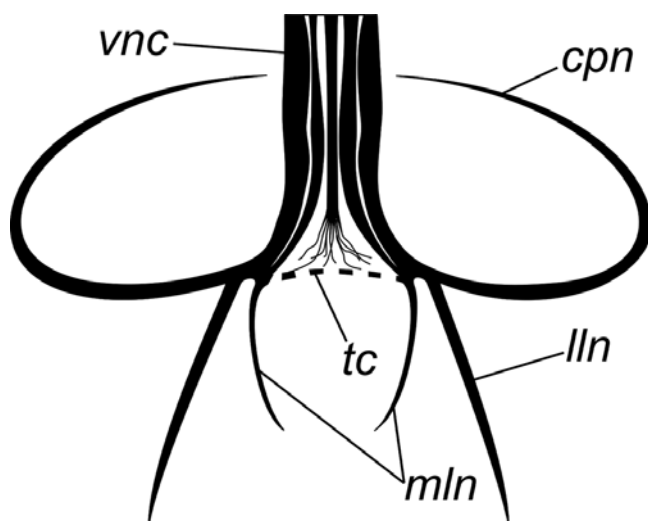


Fig. 5. Generalized scheme of the pygidial innervation in Eunicida
cpn — circumpygidial nerves, *lln* lateral longitudinal nerves,
mln — medial longitudinal nerves, *tc* — terminal commissure,
vnc — ventral nerve cord.

phyllocid species possess a pair of small additional medial longitudinal nerves besides the main longitudinal pygidial nerves. It would be very natural to homologize these additional nerves to medial longitudinal pygidial nerves of Eunicida. However, *S. japonica* also demonstrates additional medial nerves together with two pairs of main longitudinal nerves, arguing the independent formation of additional nerves in order to innervate the ventral side of the pygidium. Thus, I can suggest the presence of two pairs of pygidial longitudinal nerves and the corresponding pygidial cirri as a synapomorphy of the order Eunicida.

The terminal commissure was found in different annelid clades (Orrhage and Müller, 2005; Kerbl, Bekkouche, Sterrer and Worsaae, 2015; Weidhase, Helm and Bleidorn, 2015; Starunov, 2018;). However, in the given study it was found only in *S. japonica*. In other studied dorvilleids the terminal commissure was reported for *Dorvillea bermudensis* (Müller and Henning, 2004) and *Parapodrilus psammophilus* (Müller and Westheide, 2002). In *Ophryotrocha puerilis* the commissure-like structure is also recognizable (Schlawny, Hamann, Müller and Pfannenstiel, 1991: Fig. 19). Therefore, I can assume the initial presence of the terminal commissure at least in dorvilleids. In other families of Eunicida order the absence of terminal commissure may also be a secondary loss.

Among other annelids, the terminal commissure was also not found in *Polygordius apendiculatus*, nephtyids, and *Galathowenia oculata* (Lehmacher, Fiege and Purschke, 2014; Rimskaya-Korsakova, Kristof, Malakhov and Wanninger, 2016; Starunov and Zaitseva, 2018). In *Polygordius* the commissure may be lost in the

course of overall miniaturization and pygidium specialization during adaptation to interstitial life conditions such as development of adhesive glands (Struck et al., 2015). Nephtyids possess only one unpaired pygidial cirrus, innervated by paired longitudinal nerves. Nephtyidae belong to the order Phyllodocida, and according to recent phylogenomic results are closer to Nereididae (Struck et al., 2015; Weigert and Bleidorn, 2016; Helm et al., 2018), which possess paired pygidial cirri and a prominent terminal commissure. Moreover, in several syllid specimens the similar pygidial organization with a single cirrus innervated by paired nerves was also found (Starunov, 2018; Starunov and Zaitseva, 2018). However, these unusual syllid specimens did possess the normally developed terminal commissure. Thus, the nephtyid pygidial organization may be achieved by the fusion of initially paired pygidial cirri, and the absence of the terminal commissure may be related to a further modification of the innervation pattern.

On the contrary, *G. oculata* is a basally branching annelid and the absence of the terminal commissure may therefore represent the ancestral state. Nevertheless, oweniids possess many specific characters that hardly can be assumed to be ancestral (Bubko and Minichev, 1972; Harrison, 1992). Thus, the lack of comparative data does not allow a final conclusion to be made.

The surface of the pygidium and the pygidial cirri bear numerous receptor cell endings. They are more prominent in dorvilleids and lumbrinerids (Fig. 3D–J). The number of receptor cells in the pygidium is much higher than in adjacent body segments. The cells may be mechanoreceptors, suggesting the important sensory function of the pygidium in backward movement.

The comparison of pygidial musculature and innervation in Eunicida and Phyllodocida allows us to reconstruct its hypothetical ancestral state for the clade Errantia. I speculate that ancestral pygidial musculature should be represented by a circular or a horseshoe-shaped muscle. The ancestral innervation of the pygidium may comprise one pair of longitudinal nerves, paired circumpygidial nerves and a terminal commissure between them. The organization most similar to this hypothetical plan was found in members of Syllidae family. Nevertheless, due to the lack of comparative data, it is still not clear whether this pattern is characteristic for other annelids. Further comparative studies on pygidial morphology are highly needed, especially in another main annelid clade — the Sedentaria — as well as in basal branching groups.

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