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The muscular system of *Nemertoderma westbladi* and *Meara stichopi* (Nemertodermatida, Acoelomorpha)

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Abstract

Nemertodermatida is a small taxon of marine worm-like animals; its position in the tree of life is highly contested. The musculature of *Nemertoderma westbladi* and *Meara stichopi* is studied here in detail using fluorescent phalloidin and confocal microscopy.

In both species the musculature is composed of an outer layer of circular and an inner layer of longitudinal musculature, diagonal muscles form a distinct layer in *N. westbladi* but in *M. stichopi* these fibres connect to both other layers. The supraterrminally opening male pore and antrum are formed by invagination of the whole body-wall in both species and the seminal vesicle is lined by a thin net of musculature only in full male maturity. Modifications of the ventral body-wall adjacent to the mouth are small and transient in *N. westbladi* including no extra musculature whereas it consists of additional strong U-shaped musculature in *M. stichopi*. Myogenesis in *N. westbladi* is not finished in hatchlings and will be completed dorsally in juvenile specimens and ventrally in male mature ones, after the loss of the mouth.

Musculature between the two species differs considerably and might give insights into the internal relationships of Nemertodermatida and might prove to be useful in studies investigating their phylogenetic position. More data of other species and developmental changes are needed.

37 Introduction

38 Nemertodermatida is a small taxon of marine worms with eight nominal, but rather broadly described
39 species in two families (Sterrer 1998). The animals are found in marine sediments, or in the case of
40 Meara spp. in the foregut of holothurians, all around the world. Nemertodermatids are readily
41 identifiable by the presence of two statoliths in the statocyst as compared to only one in acoels and
42 proseriate flatworms. Species identification is relatively difficult due to their very simple body plan and
43 species delimitations mainly relying on the structure of the sexual organs.

44 The phylogenetic position of Nemertodermatida, along with that of Acoela and *Xenoturbella*, is still
45 highly contested (Carranza et al. 1997; Telford et al. 2003; Ruiz-Trillo et al. 2004; Jimenez-Guri et al.
46 2006; Philippe et al. 2007; Wallberg et al. 2007; Egger et al. 2009; Mwinyi et al. 2010; Philippe et al.
47 2011). Nemertodermatids were first described as a genus within Acoela (Steinböck 1930) but were
48 subsequently revised and classified in a separate order (Karling 1940). Later, Ehlers (1985) recognized
49 the taxon Acoelomorpha, combining Nemertodermatida and Acoela as sistergroups within
50 Platyhelminthes, based on the ultrastructural characters of epidermal cilia and ciliary rootlets. Acoela
51 (Ruiz-Trillo 1999), followed by Nemertodermatida (Jondelius et al. 2002), have been repositioned out
52 of the Platyhelminthes and to the base of Bilateria but with separate origins. Baguna and Riutort (2004)
53 reinstated the taxon Acoelomorpha and assigned it the rank of a new phylum based on results of
54 several studies. Since then, Acoelomorpha has been dismissed as paraphyletic based on large and small
55 ribosomal subunit genes (Wallberg et al. 2007), several protein coding genes (Paps et al. 2009) and
56 Hox gene clusters (Sempere et al. 2007). However, a monophyletic taxon Acoelomorpha was
57 recovered in a phylogenomic study by Hejnol et al. (2009), which, together with the elusive
58 *Xenoturbella*, formed a sister group to the remaining Bilateria. Recently, Philippe et al. (2011)
59 retrieved Acoelomorpha plus *Xenoturbella*, named Xenacoelomorpha, nested within Deuterostomia as
60 the sister group of Ambulacraria. The two competing hypotheses, Nemertodermatida as primarily
61 simple early bilaterians or as secondarily reduced deuterostomes that have lost e.g. gill slits, dorsal
62 nervous system, anus and excretory organs, obviously entail vastly divergent predictions about
63 nemertodermatid character evolution. If Nemertodermatida were an early bilaterian lineage (outside
64 Nephrozoa) we would expect to observe a basic subset of bilaterian characters lacking the more
65 complex structures found in Deuterostomia. A position within Deuterostomia would be supported if a
66 more complex muscular system were found, perhaps similar to that observed in larval stages of several
67 echinoderm lineages (Dyachuka 2013).

68 There are two conflicting studies of the phylogenetic internal relationships of Nemertodermatida.
69 Lundin (2000) used 72 morphological characters that were analysed in a parsimony framework, which
70 yielded a basal split of Nemertodermatida into Ascopariidae and Nemertodermatidae. Wallberg et al.
71 (2007) used SSU and LSU rRNA genes analysed under both Bayesian and parsimony frameworks and
72 found a strongly supported phylogeny where the genus *Nemertoderma* formed a sister group to the
73 remaining Nemertodermatida, and Ascopariidae were nested within Nemertodermatidae.

74 Changes in the original pattern of the muscular system have been shown to be phylogenetically
75 informative in Acoela (Hooge 2001; Tyler & Hooge 2004; Jondelius et al. 2011). So far the body-wall

76 musculature of only one species of Nemertodermatida, *Sterreria psammicola* (Sterrer, 1970), has been
77 briefly described from three specimens found in the north-western Atlantic (Hooge 2001).

78

79 The aims of this study are i) to describe the body-wall musculature of two nemertodermatid species,
80 *Nemertoderma westbladi* (Westblad) Steinböck, 1938 and *Meara stichopi* (Bock) Westblad, 1949, ii)
81 to further support Westblad's (1937) hypothesis that the mouth in *N. westbladi* is a transient feature
82 lost with male maturity and iii) discuss the ancestral states of body-wall musculature within
83 Nemertodermatida in the context of current phylogenetic hypotheses (Lundin 2000; Wallberg et al.
84 2007).

85

86

87 **Material and methods**

88 **Taxa studied**

89 About 30 specimens have been examined. Specimens of various developmental stages of
90 *Nemertoderma westbladi* (Fig. 1a) were collected at the depth of 30–50 m from muddy bottoms in the
91 vicinity of the Sven Lovén Centre for Marine Sciences in Kristineberg at the Gullmar Fjord on the
92 Swedish West coast. We examined about 50 specimens in different life stages of *N. westbladi*.
93 Specimens of *Meara stichopi* (Fig. 1b) were obtained from the intestine of the holothurian
94 *Parastichopus tremulus* (Gunnerus, 1767) sampled at 400 m depth in the Raunefjord near Bergen
95 (Norway).

96

97 **Phalloidin staining of muscles**

98 Worms were fixed in Stefanini's fixative (2 % paraformaldehyde and 15 % picric acid in 0.1 M Na-
99 phosphate buffer) at pH 7.6 or 4 % Paraformaldehyde in filtered seawater for 2 to 4 hours, prior MgCl₂
100 relaxation was not applied consistently. Specimens were stored for several weeks in Stefanini or
101 0.01 M phosphate buffered saline (PBS) with 0.1 M NaN₃ and then rinsed for 1.5–48 h in 0.1 M PBS
102 (pH 7.6) containing 10 % sucrose. Prior to staining, the animals were immersed in PBS containing
103 0.2 % or 0.4 % Triton X-100 (PBS-T). Staining of F-actin fibres with TRITC-labelled phalloidin
104 (Sigma) (1:100 - 1:200) was performed for 1 h to 2 h at room temperature (Wahlberg 1998), in some
105 experiments a shaker was used. After rinsing in PBS the animals were mounted in 50 % - 60 %
106 glycerol in PBS. The preparations were examined with a confocal scanning laser microscope (CSLM)
107 LEICA TCS 4D (Leica Microsystems, Mannheim, Germany) or Zeiss LSM510meta and LSM780.
108 Specimens were viewed either from the dorsal or the ventral side. Usually 20–30 optical sections 0.5–
109 2 µm thick were obtained while scanning through the specimen. The maximum projection option was
110 used to make reconstructions from all or from several adjacent optical sections in a series. The files
111 obtained were processed with the programs LSM Image Browser v. 4.2, Zen 2011, Graphic Converter
112 v. 7.6 and Adobe PhotoShop v. 7.0.

113

114

115 **Results**

116 *Nemertoderma westbladi*

117 *General pattern.* Three morphological stages of *N. westbladi* can be distinguished in this study. First
118 there are very small, nearly completely round specimens that lack a mouth and male copulatory organ
119 (mco). These are about 250 μm long and 200 μm wide and will be referred to as hatchlings. Animals
120 then grow larger usually have a mouth but no fully developed mco. These we call juveniles. These
121 specimens are longer, somewhat bottle-shaped with a blunt posterior end and reach lengths up to
122 950 μm with a width of about 550 μm . The third group is comprised of specimens with mco, which
123 have a small pointed tip in the posterior formed by a supraternal male pore, here called mature
124 specimens. The size of these animals is highly variable, the average size being 450 μm long and
125 400 μm wide. Live larger specimens contract into ball-shape when kept undisturbed but resume their
126 elongated body shape when fixed. Hatchlings are near-round when alive and active. There appears to
127 be little distortion of size and shape through fixing in this species.

128 The body-wall musculature of *N. westbladi* is composed of a three-layered subepidermal muscle grid
129 (Figs. 2, 3a). Circular fibres form the outer layer while longitudinal fibres form the innermost one
130 (Fig. 4g). In between those lies a layer of diagonal fibres. The body-wall musculature forms a closed
131 “integumental muscle sac” (Westblad 1949) underneath the thick epidermis (Fig. 3a). The anterior end
132 is almost cylindrical and widens gradually to the level just posterior of the statocyst, where it widens
133 abruptly to the maximum width (Fig. 3a). In very young animals the “muscle-sac” immediately
134 narrows again therefore appearing near-round in shape (Figs. 3d, e). With maturity, the worms elongate
135 with the maximum width kept over most of the body length. The posterior end in mature specimens is
136 sharper due to the supraternal male pore (Fig. 3a); in immature specimens it is more rounded
137 (Figs. 3d, 4b).

138 Within the epidermis F-actin positive thin fibres can be observed to emanate from the body-wall
139 musculature (Fig. 3b). In the outer layer of the epidermis there are also F-actin positive “rings”
140 (arrowheads in Figs. 3 and 4), which, however, appear not to be specifically connected to the thin
141 fibres. These “rings” differ in diameter (1.5 to 4.2 μm) and are distributed all over the body. Over the
142 anterior pole there is a concentration of smaller rings (Fig. 4f). At the same level two fine layers of
143 phalloidin positive tissue can be observed, the outer line being microvilli of the epidermal surface
144 (Fig. 4g wide indented arrowheads), the lower one the terminal web of the epidermis (Fig. 4g narrow
145 indented arrowheads).

146

147 *Circular musculature* forms a dense layer from anterior to posterior (Figs. 2, 3a). In all specimens
148 investigated in this study the individual fibres are well separated and evenly distributed. Only in the
149 anterior region until roughly the level of the statocyst the fibres appear to be generally denser than in

the rest of the body (Fig. 3b). No difference could be distinguished between the ventral and dorsal sides, apart from modifications around the mouth (Figs. 3a, 4a). In very young specimens the circular fibres appear relatively weaker (Figs. 3d, e) compared to longitudinal and diagonal fibres than in later developmental stages (Figs. 3a, 4a).

Longitudinal musculature is composed of strong fibres running along the length of the body (Fig. 3a, b, 4a). Individual fibres cross the anterior pole (Fig. 3c) but not the posterior pole in immature specimens (not shown). On the dorsal side in very young specimens there is a set of strong, often bifurcating median muscle fibres running from pole to pole, while no longitudinal muscles could be detected laterally (Fig. 3d). At the same stage the ventral longitudinal musculature is built out of lateral fibres, which leave an oval shaped space without fibres along the midline of the body, in which the mouth will develop (Fig. 3e). In slightly bigger animals the dorsal fibres lose their relative strength and lateral fibres appear. A complete and even distribution of longitudinal musculature dorsally and ventrally could be seen in animals in early male maturity lacking the mouth. Anastomoses are frequent in all stages.

Diagonal musculature is composed of opposing strands crossing each other at approximately right angles (Figs. 2, 3a). This grid is rather strong and regular on the dorsal side of the animal and less regular ventrally. Dorsally, the diagonal fibres are present in all developmental stages (Figs. 3a, d) whereas ventrally the pattern changes with development. In the smaller specimens the diagonal fibres don't cross over the body, but can only be observed laterally (Fig. 3e). Only in mature specimens do these fibres cross the main body axis and form a tight and regular grid from pole to pole (not shown).

Mouth musculature. A mouth could be observed in some specimens in this study but only in two specimens in top view (Fig. 4a). It is situated ventrally, about mid-body, and not equipped with any extra muscles but rather appears as a space in the body-wall musculature (Figs. 2a, 4a). The longitudinal muscles bend from pole to pole, leaving a roughly oval space; they do not follow the outline of the mouth (Figs. 3e, 4a). The circular musculature follows the outline of the mouth opening more directly; those fibres that meet the mouth in its middle bend rather sharply (Fig. 4a). Diagonal muscle fibres form a complete grid but where fibres can't cross because of the mouth they merge with or stop at the last fibre of the opposing diagonal strand (Figs. 3e, 4a).

Male pore musculature. The male pore opens supraternally at the posterior end (Figs. 2b, 3a). In hatchlings the integumental muscle sac at the posterior pole is completely rounded (Fig. 3d) and only becomes sharp with the formation of the male pore (Fig. 3a). The male antrum is formed by invagination of the whole body-wall (Figs. 4b, d), its lumen is lined by epidermis with the "rings" in the outer layer (Figs. 4c, d). The epidermis forming male antrum does not connect bluntly to the seminal vesicle but shows a small rim-like bulge reminiscent of a plug (Fig. 4d). In mature male

specimens the seminal vesicle itself is lined by a very fine and irregular net of muscle fibres (Figs. 4c, d, comp. 4b).

Parenchymal muscles are present essentially in the anterior region in all developmental stages. They compose a three-dimensional network (Fig. 4e). The statocyst is suspended by sets of strong muscles in about the middle of the cross-section of the body (Fig. 4e). The strongest muscles are connected to the statocyst postero-dorsally and draw laterally towards the body-wall musculature. Another pair of muscles is connecting the statocyst dorsally, one attaching to the statocyst above each statolith. Other muscles are attached to the statocyst capsule anteriorly and posteriorly.

Meara stichopi

General pattern. The body-wall musculature of *M. stichopi* appears very strong (Figs. 5, 6). The grid is composed of an outer layer of circular and an inner layer of longitudinal muscles (Figs. 5, 6a, b). Diagonal muscles form a less-defined layer with fibres connecting to mainly longitudinal but also circular muscle fibres (Fig. 5b).

Circular musculature comprises the weakest individual fibres in this species. They surround the body in a pattern of irregularly dense fibres (Figs. 5, 6b).

Longitudinal musculature is composed of thicker fibres than the circular musculature (Figs. 5, 6b, d). The fibres are evenly distributed and the distance between them widens with the broadening of the animal. Median on the dorsal side there are some stronger and straighter longitudinal fibres (Figs. 5b, 6e).

Diagonal musculature consists of two opposing sets crossing each other at approximately right angles; individual fibres are as thick as longitudinal ones (Figs. 5, 6b). Many of the dorsal fibres and the anterior ventral fibres, however, do not cross the width of the body completely. After crossing the main body axis they bend medially and connect to longitudinal fibres (Fig. 6b). Fibres close to the poles loop around it like circular muscles with both ends attaching to longitudinal muscles.

Mouth musculature. The mouth lies on the ventral side and is easily observed in all specimens studied (Figs. 5a, 6a, c). Ventro-lateral muscles bend around the posterior rim of the mouth, forming a thick and wide lip of U-shaped musculature (Figs. 6a, c). Longitudinal muscle fibres lying closer to the body midline form weaker U-shaped muscles (Fig. 6c arrowheads), continue posteriorly curving around the sides of the mouth or connect to the circular muscles forming the anterior rim of the mouth opening (Fig. 6c arrows). No sphincter around the mouth opening or pharynx musculature could be observed.

224

225 *Male pore musculature.* The male pore opens supraternally in the posterior (Figs. 5b, 6d) and is built
226 through invagination of the whole integument. The male antrum has inner circular and outer
227 longitudinal musculature and connects to a seminal vesicle with thin muscular walls.

228

229 *Parenchymal musculature* in this species is rather strong and is composed mostly of dorso-ventral
230 muscles distributed rather evenly in the whole body but for the areas of the mouth and intestine
231 (Fig. 6f).

232

233 **Character matrix**

234 In order to allow for better comparability between taxonomic groups in morphological as well as
235 molecular frameworks we coded the body-wall musculature of the two here studied species according
236 to the characters defined in Jondelius et al. 2011. We also coded the same character for *S. psammicola*
237 from literature (Hooge 2001, Todt 2009).

238

239 Table 1: Coding of musculature characters 39-44 after Jondelius et al. (2011) for *Meara stichopi* and
240 *Nemertoderma westbladi* (here presented data) and *Sterreria psammicola* (from Hooge et al. 2001,
241 Todt 2009). 0 = absent, 1 = present.

	<i>Nemertoderma westbladi</i>	<i>Meara stichopi</i>	<i>Sterreria psammicola</i>
39 Longitudinal muscles enwrap mouth	0	1	1
40 Ventral diagonal muscles	1	1	1
41 Ventral crossover muscles	0	0	0
42 Dorsal diagonal musculature	1	1	1
43 Longitudinal muscles outside circular muscles	0	0	0
44 Longitudinal muscles between mouth and frontal pore	0	1	0

242

243 **Discussion**

244 Westblad (1937; 1949) gave rather precise descriptions of the general pattern of musculature in both
245 *Nemertoderma westbladi* and *Meara stichopi* from sections stained with traditional histological dyes.

Both species were reported to have an outer layer of circular and an inner layer of longitudinal muscles and our observations correspond very well with that. There is a difference between the two here described species in the arrangement of the diagonal muscles, which do not form a distinct layer in *M. stichopi* but rather branch off and interweave with both circular and longitudinal fibres. In *N. westbladi* the diagonal fibres are separate from longitudinal and circular fibres, as is the case in *Sterreria psammicola* (Hooge 2001).

Functional morphology and myogenesis

We hypothesize, based on the size of the egg, about 120 µm in diameter (Jondelius et al. 2004), and the size of the smallest specimen, about 220 µm in length and 180 µm in width, that the smallest specimens in this study are hatchlings. The whole development between egg laying and hatching in *N. westbladi* is slow, 10 days (Jondelius et al. 2004), compared to that of two acoel species, 1.5 days for *Isodiametra pulchra* (Smith & Bush, 1991) (Ladurner & Rieger 2000) and 5 days for *Symsagittifera roscoffensis* (Graff, 1891) (Semmler et al. 2008); the difference may depend on the breeding temperature. In both acoel species the general pattern of musculature is established when hatching, but the number of fibres increases several times after hatching. In contrast, hatchlings of *N. westbladi* show a complete circular musculature but not yet fully developed longitudinal and diagonal musculature. The set of fibres along the dorsal midline of the body that can be observed in these represents the only dorsal longitudinal musculature at this stage. Ladurner & Rieger (2000) observed that after the initial establishment of the orthogonal grid a second phase of myogenesis follows. This second stage multiplies the existent number of fibres via different modes. One of them they called “double fibres”, secondary fibres that develop along the primary fibres but eventually become independent fibres. The dorsal midline fibres in the hatchlings of *N. westbladi* are forking towards the poles and could therefore be interpreted as double fibres in the second phase of myogenesis developing fibres that will eventually surround the rest of the dorsal side of the animal.

In *M. stichopi*, some dorsal fibres in mature animals are rather straighter and stronger than more lateral ones. This stronger set could not be seen in *N. westbladi* (Steinböck 1930, Westblad 1937) or in *Sterreria psammicola* (Hooge 2001). Westblad (1949) attributes the peculiar way in which fixed *M. stichopi* curl up with the ventral side facing outwards to the contraction of these dorsal fibres. Given the apparent apomorphy of this character, attempts to find a connection with the commensal lifestyle seem reasonable, yet little is known about the natural behaviour of this species. Copulation has not been described, although Westblad postulates that it might be a rather primitive form during which the worms press their posteriors together “a sufficiently long time to allow the spermatozooids to get through (the) epithelium” (Westblad 1949). As the male pore is positioned slightly supraternal in the posterior, the sperm-delivering animal may either have to be beneath the receiving animal or bend its posterior up and to the side to impregnate the dorsal side of the receiving animal; this upward bending could be aided by the dorsal midline muscles. Given the presence of the muscles of the seminal vesicle, the act may be less passive than Westblad supposed. The copulatory behaviour has not been described

284 in any nemertodermatid species and is extremely variable among acoels (Hyman 1937; Costello &
285 Costello 1938; Apelt 1969).

286 Sterrer (1998) frequently observed egg-laying behaviour in which the animals “first bend into a
287 dorsally-convex shape, then protrude the circumoral body surface (like puckered lips) to disgorge, via
288 the mouth, an egg” (Sterrer 1998). The strong dorsal musculature may also help in this mode of egg
289 laying. It would be interesting to see whether this strong dorsal set of fibres in *M. stichopi* is retained
290 from an early developmental stage or whether it develops later, secondarily. This might also help to
291 answer the question of its function.

292 The egg laying mechanism in *N. westbladi* has not been described, even though animals laying eggs
293 were kept over several days. Animals in a sorting dish can often be seen to protrude a tissue ball
294 through the mouth. This appears to be normal behaviour, as animals stayed alive for several days
295 afterwards in the dish. The cone mostly stays protruded when animals are fixed, which also leads to
296 most specimens being mounted lying on their sides. In some of these animals the protruding tissue can
297 be seen to contain eggs in the context of the surrounding dorsal parenchyma when stained. The cone
298 can also be released, leaving the worm considerably slimmer. Westblad (1937) describes from his
299 sections that animals with a mouth have larger eggs, and no well-developed testes or mco, whereas
300 animals without a mouth but a functional mco and large testes showed “an utter degradation of the
301 ovary” with “a notable quantity of eggs in states of high dissolution in the intestinal syncytium”. This
302 further supports the view that female maturity precedes male maturity with eggs being laid via the
303 mouth, like in *M. stichopi*. After the loss of the mouth remaining eggs could be resorbed in order to
304 sustain life, which also explains the overall smaller size in male mature animals. This agrees with our
305 observation and life stages might be roughly outlined as follows with smooth changes between them 1)
306 mouth absent, mco absent, ovary small, 2) mouth present, mco absent, ovary mature, and 3) mouth
307 absent, mco mature, ovary in dissolution. Whether the protruding of the cone constitutes natural egg
308 laying behaviour is however not clear.

309

310 **Mouth musculature**

311 *M. stichopi* has strong U-shaped musculature associated with the mouth which is lacking completely in
312 *N. westbladi* but is described as weak but present in *S. psammicola* (Todt 2009). In many apharyngeal
313 species of the Acoela ventral U-shaped muscles are associated with feeding behaviour (Tyler & Rieger
314 1999; Jondelius et al. 2011). The ventral diagonal muscles are also useful as a phylogenetic marker in
315 Acoela (Hooge 2001). Jondelius et al. (2011) grouped Acoela with ventral crossover muscle and no
316 pharynx into the taxon Crucimusculata, as opposed to the sister taxon Prosopharyngida, which includes
317 acoels with a muscular pharynx in the anterior part of the body. The hypothesis is that in acoels ventral
318 diagonal and U-shaped muscles appear with the loss of the plesiomorphic posterior pharynx and take
319 over its function. No known nemertodermatid species has a muscular pharynx of any kind, but
320 *M. stichopi* and *N. westbladi* both show ventral diagonal fibres. In *S. psammicola*, however, the latter
321 are present but not associated with the mouth, being restricted to the most anterior part of the body
322 (Hooge 2001). The ventral musculature of *M. stichopi* with its strong U-shaped fibres, however,

323 corresponds well with that of Crucimusculata and its hypothesized function in feeding; these
324 apparently evolved convergently in Crucimusculata and *M. stichopi*, being therefore uninformative
325 regarding the Acoelomorpha hypothesis.

326 *N. westbladi* displays ventral diagonal musculature, but no U-shaped or sphincter musculature. This
327 species lives freely on very fine, muddy sediments and its food and feeding behaviour is unknown.
328 Westblad (1937) argues that the above mentioned cone could be protruded like a “sticky tongue”,
329 which could then be retracted with adhering food particles, effecting a swallowing movement. The gut
330 has been described as “well-defined” from the parenchyma “although a membrana propria is missing”
331 (Westblad 1937). Slightly ventro-posteriorly of the statocyst a few strong parenchymal fibres can be
332 observed that originate in the body-wall musculature, but no posterior attachment points could be
333 identified. These fibres draw towards the gut and could be involved in the ingestion of food. It can be
334 hypothesized that *N. westbladi* is not a predatory species but rather grazes the surface of its habitat. The
335 lack of any kind of pharyngeal or mouth-specific muscles supports this view. After the mouth has been
336 lost, the ventral body-wall musculature closes the gap and the three-layered grid is regular and
337 complete. In *S. psammicola* a mouth can also be found only in some specimens and appears to become
338 lost with maturity (Faubel 1976, Hooze 2001). In no species of the family Ascopariidae a mouth has
339 been reported yet. Instead the broom organ everted at the anterior pole in *Flagellophora* has been
340 associated with feeding (Faubel et al. 1978, Tyler 1986, Sterrer 1998), which however does not appear
341 to be connected directly to the gut (Faubel et al. 1978, Tyler, 1986). The mouth in these species may
342 also be a transient feature that has simply never been documented in the rather low number of studied
343 specimens. Only in *M. stichopi*, the arguably most derived species of Nemertodermatida, the mouth
344 seems to be present in most animals and built by strong specialized musculature. This supports the
345 hypothesis of the presence of the mouth only during a limited part of the post-embryonic life cycle or
346 its absence as the ancestral condition in Nemertodermatida.

347

348 **Male copulatory organ musculature**

349 The male pore in both species opens supraternally in the posterior. Westblad (1937; 1949) describes
350 for both species a muscular male antrum (“ductus ejaculatorius” in Westblad 1937) being formed by
351 invagination of the whole integument. Westblad (1937) suggested that there “apparently appears an
352 opening in the integumental muscle sack”, which in fact is present in all developmental stages because
353 the longitudinal fibres never cross the last ring of circular musculature. In Figures 4 b-d the
354 invagination of the whole body-wall can be seen with circular and longitudinal musculature forming a
355 wide tube lined by thick epidermis, including “rings”, surrounding a narrow canal. This then opens into
356 the *vesicula seminalis*. The seminal vesicle is described by Westblad (1937; 1949) as having a
357 “*membrana propria*” in fully mature stages but no muscular lining in *N. westbladi*. In *M. stichopi* he
358 observed “a very thin membrane with a net of fine, crossing muscle fibres” (Westblad 1949). Our data
359 suggest that in both cases the fully mature specimens do have a muscular seminal vesicle, however
360 with muscles not originating in the body-wall musculature. The epidermis folding into the seminal
361 vesicle forms a lip in its lumen but does not line the inside of the seminal vesicle.

362

363 **Epidermal “rings”**

364 The “rings” can be seen on the outer surface of the epidermis all over the body (Figs. 2, 3) and in the
365 invagination of the body-wall forming the mco (Figs. 3c, d). Similar rings have been shown in *I.*
366 *pulchra* by Pfistermueller et al. (2001) and Ladurner et al. (2000), interpreted as zonulae adherentes
367 between epidermal cells and gland necks, and *Sterreria psammicola* (Hooge 2001), where they were
368 not discussed. *N. westbladi*, (Fig. 1b) and some populations of *S. psammicola*, have many, sometimes
369 large epidermal glands therefore we interpret these rings as cell junctions of zonulae adherentes type
370 between epidermal cells and gland necks. The main component of this type of junction are actin fibres
371 underlying the membranes of both contacting cells, it stands to reason that they stain strongly with
372 phalloidin. Thus the “rings” described here surround the gland ducts opening on the surface of the
373 epidermis. The “rings” are situated at the level of the terminal web, also stained by phalloidin (Fig. 4g)

374 There is an accumulation of gland necks of the big glands of the frontal complex at the anterior pole
375 (Fig. 4f). The only other nemertodermatid in which the structure of the anterior pole was studied, by
376 EM (Smith et al. 1986), is *Flagellophora*, a member of the second family in Nemertodermatida,
377 Ascopariidae. This species shows one anterior pore through which a number of frontal glands open.
378 However, this species also possesses a unique broom organ, which is everted through the anterior pore;
379 the presence of this special feature makes comparisons difficult. In this paper, several acoel species
380 were studied, including the early branching acoels *Diopisthoporus gymnopharyngeus* Smith & Tyler,
381 1985, Diopisthoporidae being the sister group to all other Acoela (Jondelius et al. 2011), and
382 *Paratomella* sp. All of these species but *Paratomella* showed a frontal pore combining several gland
383 openings similar to that seen in *Flagellophora*. *Paratomella* has a complex of tight but separated gland
384 necks at its frontal pole, thus not a frontal pore, similar to that observed in *N. westbladi* here.

385

386 **Phylogenetic considerations**

387 Most Cnidaria have so called epitheliomuscular cells, which can form longitudinal musculature in the
388 epithelium and circular musculature in the gastrodermis, However, these cells cannot be homologized
389 with mesodermal musculature of triploblastic animals (Westheide & Rieger, 2004) and muscle cells
390 may have developed independently in Cnidaria and Bilateria (Steinmetz et al. 1012). A simple
391 orthogonal grid of body-wall musculature without diagonal musculature is believed to be the ancestral
392 state of a probably worm-shaped bilaterian ancestor (Westheide & Rieger, 2004). This simple grid with
393 only partial, mainly ventral diagonal musculature can be found only in some early branching species of
394 Acoela outside Crucimusculata and is considered to be, with a posterior pharynx, their ancestral state
395 by Jondelius et al. (2011). Higher acoels all display a more complex muscular pattern with one or two
396 additional layers of diagonal muscles. Both nemertodermatid species studied here show not only a
397 simple orthogonal grid but well developed diagonal musculature as well. Additionally, neither species
398 shows a posterior pharynx. This observation does not support the taxon Acoelomorpha. However,
399 whether complete diagonal musculature is the ancestral state in in Nemertodermatida cannot be

400 decided especially given the less complex body wall musculature observed in *S. psammicola* (Hooge
401 2001) The morphology of the frontal gland openings in *N. westbladi* is similar to that of the acoel
402 *Paratomella* sp. (Smith et al. 1986), which however is a derived state (Jondelius et al. 2011) indicating
403 convergent evolution in this character. More information is needed to evaluate the pattern of
404 musculature and its evolutionary implications and to infer its ancestral state within Nemertodermatida.
405 However, no results have been found to indicate complex or secondarily reduced muscle patterns,
406 which would support a position of Nemertodermatida within Deuterostomia rather than as an early
407 bilaterian lineage.

408 Three species of the Nemertodermatida have been described so far by means of fluorescent-labelled
409 phalloidin. Observed patterns in body-wall musculature and myogenesis, although not as simple as the
410 hypothesized earliest bilaterian ancestor, are not complex enough to allow phylogenetic placement on a
411 larger scale, i.e. they cannot help to associate the taxon with Protostomes or Deuterostomia. The
412 monophyly of Acoelomorpha is not supported by our results. Some important questions have still to be
413 answered: 1. How does the muscular pattern and the mode of myogenesis in the Ascopariidae look
414 like? 2. How does the body-wall musculature change with maturity, with the loss of the mouth and the
415 development of the sexual organs in the other species? Clearly more data, especially for the less well-
416 studied genera *Ascoparia*, *Flagellophora* and *Nemertinoides*, are needed before the ancestral condition
417 of the Nemertodermatida can be inferred.

418

419

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426

427

428 **List of abbreviations**

429 bwm: body-wall musculature

430 cl: cilia

431 cm: circular musculature (blue in sketches)

432 cw: cell web

433 db: dorsal bands of stronger longitudinal fibres in *M. stichopi*

434 dm: diagonal musculature (green in sketches)

435 eg: epidermal glands
436 ef: epidermal fibres
437 ep: epidermis
438 int: intestine
439 lm: longitudinal musculature (red in sketches)
440 m: mouth
441 ma: male antrum
442 mo: male opening
443 mco: male copulatory organ
444 mv: microvilli
445 ov: ovaries
446 pm: parenchymal musculature
447 st: statocyst with two statoliths
448 stm: statocyst musculature
449 sv: seminal vesicle
450 U: U-shaped musculature (orange in sketches)
451

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 545

546 **Figure Captions**

547

548 **Fig. 1** Light microscopic images of live specimens of *Nemertoderma westbladi* (**a**) and *Meara stichopi*
 549 (**b**) in squeeze preparation. In **a** note the dark outline of the intestine (int) along the central antero-
 550 posterior axis, the posterior ovaries (ov) and the supraterminal male opening (asterisk) in the posterior.
 551 In **b** the abundance and size of epidermal glands (arrowheads) is clearly visible as well as the statocyst
 552 with the double statoliths (st).

553

554 **Fig. 2** *Nemertoderma westbladi*, schematic drawing of the ventral (**a**) and dorsal (**b**) musculature with
 555 outer circular muscles (cm, shown in blue), inner longitudinal muscles (lm, shown in red) and diagonal
 556 muscles (dm, shown in green) in between them. Note denser but thinner circular musculatur in anterior
 557 region, mouth (m) on ventral side and supraterminal male opening (mco); two black rings indicate
 558 statocyst position.

559

560 **Fig. 3** *Nemertoderma westbladi*, phalloidin-stained whole mount preparations, anterior end up. **a**, **b**, **d**
 561 and **e** are projections of merged optical sections, **c** is one single section. Arrowheads indicate “rings”
 562 around the gland necks in distal part of epidermis. **a** Dorsal musculature with circular (cm),
 563 longitudinal (lm) and diagonal musculature (dm) of a male mature specimen with the supraterminal
 564 male pore (mco) in the posterior and clearly visible circular gland necks; **b** Detail of the anterior region
 565 with denser but thinner circular musculature (cm), stonger longitudinal musculature (lm) and epidermis
 566 with thin fibres (ef) emanating from the body-wall musculature and circular gland necks (arrowheads)
 567 in the distal part of the epidermis; **c** Frontal pole with crisscrossing musculature in an immature
 568 specimen; **d** Dorsal view of a very young specimen with incomplete muscular grid. Circular
 569 musculature is fully developed while diagonal muscles are missing in the anterior and longitudinal
 570 fibres laterally. Note the absence of a mco; **e** Ventral view of the same specimen. Circular musculature
 571 is fully developed while diagonal and longitudinal musculature is missing along the body midline.

572

573 **Fig. 4** *Nemertoderma westbladi*, merged optical sections of phalloidin-stained whole mount
 574 preparations, anterior end up. **a** Ventral view with mouth (m). Circular fibres curve sharply around
 575 mouth opening (indented arrowheads), longitudinal fibres bend smoother around the mouth (arrows)
 576 and diagonal grid (dm) is interrupted for the mouth opening; **b** Posterior pole with beginning
 577 invagination of the body-wall to form the male antrum (ma); the seminal vesicle (sv) is visible already.
 578 Arrowheads indicate gland necks in distal part of epidermis; **c** Male pore of a fully male mature
 579 specimen with a weak net of muscle fibres lining the seminal vesicle. Arrowheads indicate gland necks
 580 in distal part of epidermis; **d** Male copulatory organ with male antrum built by invagination of the
 581 complete body-wall. In the lumen of the male antrum weakly stained epidermis with its characteristic
 582 circular gland necks (arrowheads) at the outer rim can be seen. The body-wall musculature forms a
 583 sphincter. The male antrum connects to the sv showing a muscular anterior wall, forming a small lip-
 584 like a plug in its inside; **e** Anterior end (outline of the animals visible as arch of body-wall musculature
 585 (bwm) with statocyst (with two dark statoliths, st). The statocyst is held by thicker bundles of muscles
 586 (stm) connecting eventually to the body-wall musculature. Parenchymal musculature is mainly present
 587 in form of single fibres (pf), which criss-cross the anterior region of the animal emanating from the
 588 body-wall musculature; **f** Outer layer of the epidermis over the anterior pole. Note circular gland necks
 589 of different size and the concentration of small gland necks of frontal glands directly over the pole; **g**
 590 Detail of the epidermis. At the bottom, the longitudinal fibres can be seen underneath circular ones.
 591 Above this two thin stained layers can be seen, the lower one corresponding to the cell web (narrow
 592 indented arrowheads) and the upper one to the microvilli (wide indented arrowheads) of the epidermal
 593 surface. The zonulae adhaerentes of the gland necks (arrowheads) can be seen as bright stained areas
 594 on the same level, “rings” seen from the side. On the surface unstained cilia (cl) are visible.

595

596 **Fig. 5** *Meara stichopi*, schematic drawing of the ventral (**a**) and dorsal (**b**) musculature with outer
597 circular muscles (cm, shown in blue), inner longitudinal muscles (lm, shown in red), diagonal muscles
598 (dm, shown in green) and U-shaped muscles (U, shown in orange). Note mouth (m) and associated
599 muscles on ventral side and male opening (mco) and the stronger concentrated longitudinal fibres along
600 the body midline on dorsal side; two black rings show statocyst position

601

602 **Fig. 6** *Meara stichopi*, merged optical sections of phalloidin-stained whole mount preparations;
603 anterior end up. **a** Muscular grid of the ventral body-wall with circular (cm), longitudinal (lm) and
604 diagonal musculature (dm) and mouth (m) with associated U-shaped musculature (U); **b** Muscular grid
605 with circular, longitudinal and diagonal musculature in the anterior. Note the change in angles of
606 diagonal muscles and the variance in relative thickness of the different kinds of fibres; **c** Mouth (m)
607 with wide (U) and narrow (arrowheads) U-shaped musculature posterior to the mouth opening and
608 anterior longitudinal muscles attaching to the circular fibres anterior of the mouth (arrows); **d** Male
609 copulatory organ with seminal vesicle (sv) and its thin lining of irregular muscle fibres and the male
610 antrum lined by circular muscles; **e** Muscular grid of the dorsal body-wall with circular, longitudinal
611 and diagonal musculature and stronger longitudinal fibres of the dorsal band; **f** Sections of the same
612 specimen with focus on the parenchymal musculature (pm) with body-wall musculature visible as
613 glowing outline of the body and intestine (int) as dark area.

614

a

b