

Comparative Embryogenesis of Mecoptera and Lepidoptera with Special Reference to the Abdominal Prolegs

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ABSTRACT The eruciform larvae of holometabolous insects are primarily characterized by bearing a varying number of abdominal prolegs in addition to three pairs of thoracic legs. However, whether the prolegs are evolutionarily homologous among different insect orders is still a disputable issue. We examined the embryonic features and histological structure of the prolegs of the scorpionfly *Panorpa byersi* Hua and Huang (Mecoptera: Panorpidae) and the Oriental armyworm *Mythimna separata* (Walker) (Lepidoptera: Noctuidae) to investigate whether the prolegs are homologous between these two holometabolous insect orders. In the scorpionfly, paired lateral process primordia arise on abdominal segments I–VIII (A1–A8) in line with the thoracic legs in early embryonic stages, but degenerate into triangular protuberances in later stages, and paired medial processes appear along the midventral line before dorsal closure and eventually develop into unjointed, cone-shaped prolegs. Histological observation showed that the lumina of the prolegs are not continuous with the hemocoel, differing distinctly from that of the basic appendicular plan of thoracic legs. These results suggest that the prolegs are likely secondary outgrowths in Mecoptera. In the armyworm, lateral process primordia appear on A1–A10 in alignment with the thoracic legs in the early embryonic stages, although only the rudiments on A3–A6 and A10 develop into segmented prolegs with the lumina continuous with the hemocoel and others degenerate eventually, suggesting that the prolegs are true segmental appendages serially homologous with the thoracic legs in Lepidoptera. Therefore, we conclude that the larval prolegs are likely not evolutionarily homologous between Mecoptera and Lepidoptera. *J. Morphol.* 277:585–593, 2016. © 2016 Wiley Periodicals, Inc.

KEY WORDS: eruciform larva; proleg; homology; embryology

INTRODUCTION

The eruciform larvae of holometabolous insects are primarily characterized by bearing a varying number of prolegs on abdominal segments besides three pairs of well-developed thoracic legs. Abdominal prolegs commonly occur on the larvae of Lepidoptera, Mecoptera and symphytan Hymenoptera (Stehr, 2003), and some basal groups of Diptera

(Courtney, 1994; Schneeberg et al., 2012), and are divergent in segmental arrangement and number among different insect groups (Nagy and Grbic, 1999). The larvae of glossatan Lepidoptera (moths and butterflies) usually have lobe-like prolegs on abdominal segments III to VI (A3–A6) and A10 (Scoble, 1995), whereas the larvae of Mecoptera (or strictly Pistillifera, Willmann, 1987) usually bear conical prolegs on A1–A8 (Byers and Thornhill, 1983; Tan and Hua, 2008; Du et al., 2009). Considering morphological differences of appendages, comparative anatomy and embryology can contribute evidence to reveal homology relationship of them (Richter, 2005; Scholtz, 2010).

The larval prolegs in different insect groups are regarded to be of appendicular origin by some authors (Snodgrass, 1931, 1935; Matsuda, 1976; Barlet, 1981; Bitsch, 2012). However, Hinton (1955, 1958) regarded the abdominal prolegs in panorpoid insects as secondary adaptive structures. Hinton's viewpoint was accepted by Snodgrass (1961). Based on embryological data, the prolegs were found to be secondary outgrowths and were considered not serially homologous with the thoracic legs in mecopteran larvae by Suzuki (1990). This conclusion was confirmed by Du et al. (2009) and Yue and Hua (2010), but not congruent with Bitsch's (2012) view that the prolegs represent coxal endites in Mecoptera, and thus, are of appendicular origin. In Lepidoptera, early studies

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of the embryonic development concluded that the prolegs are serially homologous with the thoracic legs (Eastham, 1930; Friedmann, 1934). This argument was confirmed by recent molecular evidence, which showed similar mechanisms underlying the development between prolegs and thoracic legs, suggesting that the prolegs are true segmental appendages in Lepidoptera and Hymenoptera (Panganiban et al., 1994; Warren et al., 1994; Suzuki and Palopoli, 2001). The common pattern detected by the musculature and innervation of prolegs and thoracic legs in lepidopteran and sawfly larvae also indicated that they are serially homologous organs (Birket-Smith, 1984). However, whether the abdominal prolegs are evolutionarily homologous between the Lepidoptera and Mecoptera is still a disputable issue.

In this study, we trace the position, developmental process and muscular pattern of the abdominal prolegs in comparison with those of the thoracic legs between the scorpionfly *Panorpa byersi* Hua and Huang, 2007 (Mecoptera: Panorpidae) and the Oriental armyworm *Mythimna separata* (Walker, 1865) (Lepidoptera: Noctuidae), in an attempt to elucidate if the prolegs are evolutionarily homologous between the Mecoptera and Lepidoptera.

MATERIAL AND METHODS

Insects and Eggs

Adults of *P. byersi* Hua and Huang, 2007 were captured at the Daguanzi Forest Farm in the Qinling Mountains, 18 km south of Taibai County, Shaanxi Province in central China in late July 2014. They were reared in a net cage and fed chopped mealworms. Gravid females were transferred into a jar filled with humid soil of 4–5 cm in depth. Newly laid eggs were maintained in the soil and incubated at approximately 20°C (Du et al., 2009; Yue and Hua, 2010).

Adults of *M. separata* (Walker, 1865) were obtained from a laboratory population and fed on 10% honey. Eggs of the armyworm were deposited on wheat straw and kept at 20°C until dissection.

Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to obtain detailed images of the external morphology of the embryos every two to four hours for 90 h until hatching for the scorpionfly and every 2 h for 38 h until hatching for the armyworm. The eggs were fixed in 2.5% glutaraldehyde at 4°C for 12 h, rinsed with phosphate buffer, and then the embryos were dissected out of the chorion under a Motic SMZ-168 stereo microscope. They were dehydrated through a graded acetone series, then replaced by tertiary butanol, freeze-dried for 3 h, coated with gold and observed under a Hitachi S-3400N scanning electron microscope at 15 kV (Jiang and Hua, 2015).

Light Microscopy

Light microscopy was used to observe the embryonic structure of different developmental stages by serial sections at the same time intervals as for SEM. After fixation in Bouin's solution, the eggs were dissected in 70% ethanol solution. The embryos were then dehydrated in a series of graded acetone solutions (from 30% to 100%), infiltrated in a serial mixture of Epon 812 resin and acetone, and finally embedded in Epon 812

(Yue and Hua, 2010). Sections were cut at 5- μ m thickness using a LKB2088 microtome, stained with 0.5% toluidine blue, and examined under a light microscope with a digital camera.

Anatomy of Muscles of the Larvae

After fixation in Bouin's solution, full-grown larvae of *P. byersi* (fourth-instar) and *M. separata* (sixth-instar) were cut at the sagittal plane of mid-dorsal line, their viscera and loose fat bodies were removed, and their external structure and shallow grooves of the cuticle in the metathorax and abdominal segments were drawn under a microscope (Tsujimura, 1983). All muscles and their attachment sites inside the metathorax and A1 of the scorpionfly, metathorax, and A4 of the armyworm were drawn from internal to external muscles using a camera lucida drawing tube, with a focus on the leg muscles. Draft drawings were improved with Adobe Illustrator CS4.

RESULTS

Embryonic Development of Thoracic and Abdominal Appendages of the Scorpionfly

The developmental duration of the scorpionfly eggs from oviposition to hatching is approximately 160 h at 20°C. We mainly focused on the morphogenesis of thoracic and abdominal appendages.

At 90 h, shortly after the body segmentation, paired processes were observed on the head, thorax, and to a lesser extent on the abdomen, marking the beginning of formation of antennae, mouthparts, and thoracic and abdominal appendages (Fig. 1A). The abdominal process primordia represent lateral processes in line with the thoracic legs on A1–A8, grow in size without any subdivisions (Fig. 1A,B), but then degenerate into triangular processes persisting into the larval stage (Fig. 1B–D). At 110 h, eight pairs of new small processes appear medially of the lateral processes on A1–A8 (Fig. 1B). These medial processes extend to form conical prolegs without segmentation characteristic of the thoracic legs that show distinct segmentation—the coxa, femur, tibia, tarsus, and apical pretarsus in the first-instar larvae (Fig. 1B–F). The setae SV1, SV2, and MSV1 are present on the thoracic coxae, SV1, SV2, and SV3 on the lateral processes (Fig. 1D–F).

At 110 h, the anlagen of the thoracic appendages appear as limb-like structures (Fig. 1B). They are evidently ectodermal evaginations with the lumen continuous with the hemocoel (Fig. 2A), as is also the case for the lateral processes (Fig. 2B–D). Some muscles are connected to the base of the lateral process in the first-instar larva (Fig. 2F). In the event of proleg formation, small paired medial processes, which consist of ectodermal cell masses in sections, first become evident below each ganglion of the first eight abdominal segments; these are anlagen of abdominal prolegs (Fig. 2B,C). As embryonic development proceeds, the peripheral regions of the proleg primordia swell to become cone-shaped prolegs while the cell masses move from the proximal part to the middle of the developing abdominal prolegs and separate the prolegs

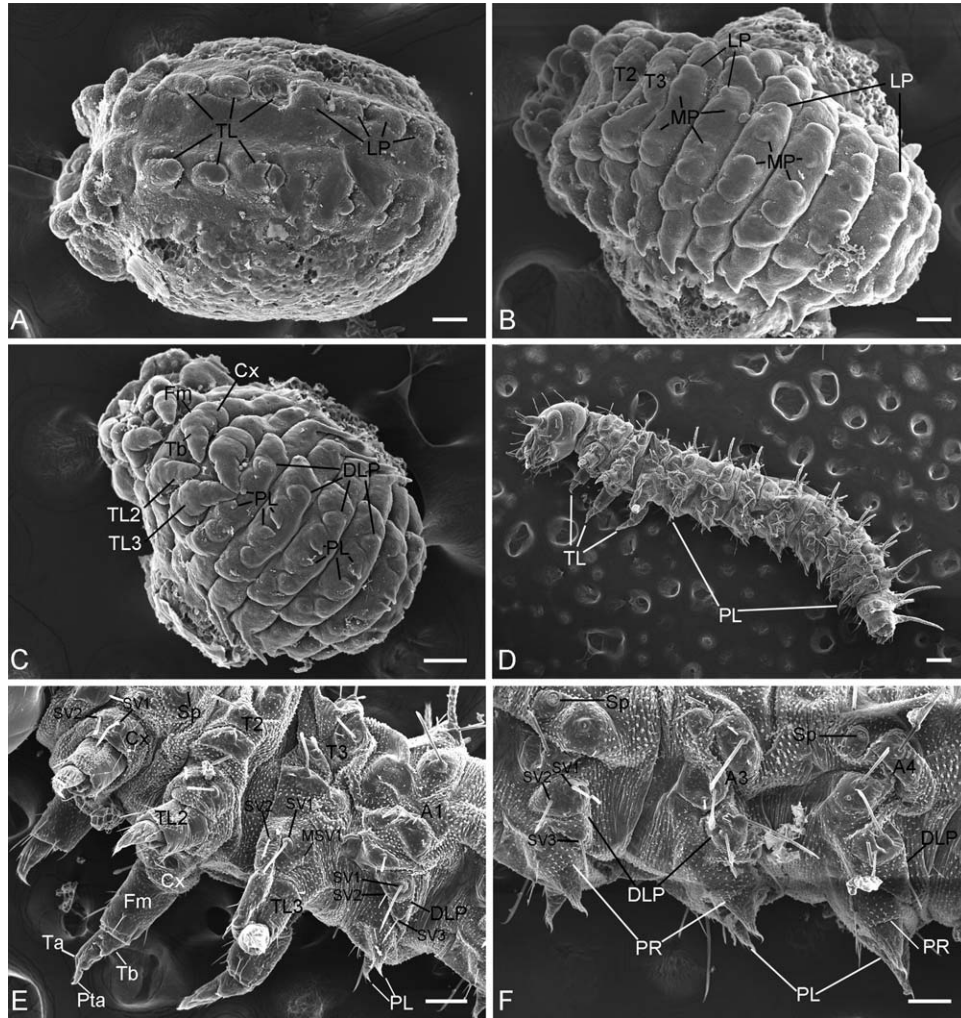


Fig. 1. *Panorpa byersi*; SEM of embryos and the first-instar larvae. Ventral view of the embryo at 90 h (A), 110 h (B), and 120 h (C). (D) Lateral view of the larva. High magnification of T1–T3 (E), and A2–A4 (F) of the larva. A1–A8, abdominal segments 1–8; Cx, coxa; DLP, degraded lateral process; Fm, femur; LP, lateral process; MP, medial process; MSV, minute subventral seta; PL, proleg; PR, rim of proleg; Pta, pretarsus; Sp, spiracle; SV, subventral seta; T1–T3, thoracic segments 1–3; Ta, tarsus; Tb, tibia; TL1–3, thoracic legs 1–3. Scale bars: A and B = 60 μ m; C and F = 80 μ m, D = 200 μ m; E = 100 μ m.

from the hemocoel; therefore, a cavity appears within the proleg (Fig. 2D–F).

Embryonic Development of Thoracic and Abdominal Appendages of the Armyworm

In *Mythimna separata*, appendage formation in body segments begins at 40 h after oviposition and the dorsal closure completes at 54 h (Wang et al., 1997). We focused on the developmental stages from 38 to 54 h.

At 38 h, the thoracic and abdominal leg primordia are distinctly in the same row (Fig. 3A). The abdominal swellings on A3–A6 and A10 enlarge to become prolegs. In contrast, the other swellings, which were initially prominent, gradually degenerate and disappear before larval hatching (Fig. 3A–D). The proleg consists of a basal

segment and a distal planta, which bears numerous crochets on the distal end (Fig. 3F). The thoracic leg primordia slightly elongate with obscure joints and do not differentiate until hatching into five joints—a coxa, a femur, a tibia, a tarsus, and an apical pretarsus (Fig. 3A–E). One lateral seta appears on the sub-basal area in the full-grown larvae, but not in the first-instar larvae, corresponding in position to that on the subcoxa of thoracic legs (Figs. 3D–F and 5B,C).

In sections, the thoracic legs and abdominal prolegs are recognizable as ectodermal evaginations (Fig. 4A,B), and resemble hollow cylindrical outgrowths of the body wall, the lumina being continuous with the hemocoel (Fig. 4C,D). Some muscles are attached on the base of thoracic legs and abdominal prolegs in the first-instar larva (Fig. 4C,D).

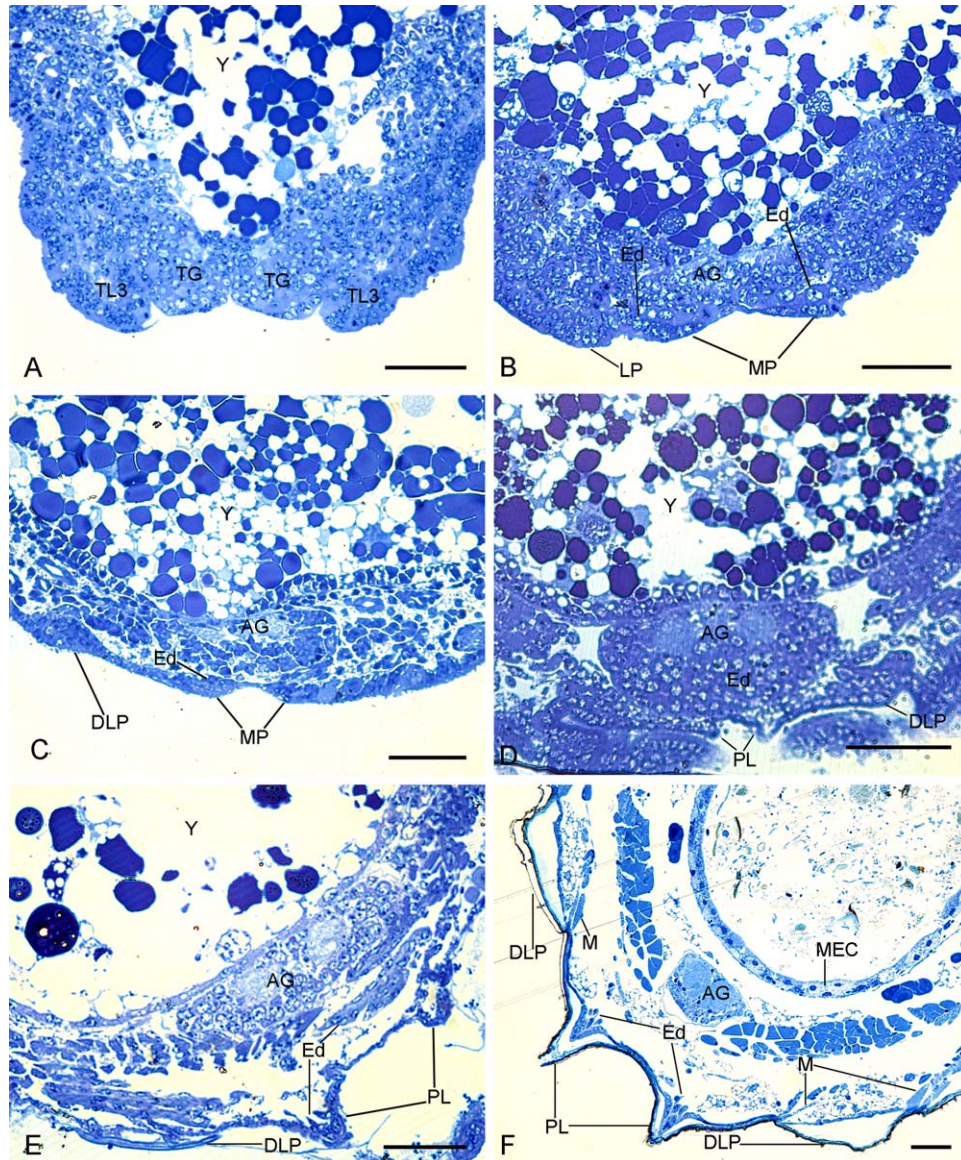


Fig. 2. *Panorpa byersi*, micrographs of semithin section of embryos and the first-instar larvae. Transverse sections of T3 at 112 h (A) and of A3 at 112 h (B), 116 h (C), 124 h (D), 132 h (E), and the larva (F). AG, abdominal ganglion; DLP, degraded lateral process; Ed, ectoderm; LP, lateral process; M, muscle; MEC, midgut epithelium cell; MP, medial process; PL, proleg; TG, thoracic ganglion; TL3, third thoracic leg; Y, yolk. Scale bars = 50 μ m.

Musculature of Thoracic and Abdominal Segments in the Larvae

For convenience, we only record the main leg muscles relevant for the homology assessment of prolegs on abdominal segments mentioned. The thorax and abdomen of panorpid larvae are well characterized by small setiferous sclerites in a generally membranous cuticle (Byers and Thornhill, 1983). Here, the sclerite and chaetotaxy pattern descriptions of the body of scorpionflies refer to Chen and Hua (2011) and Ma et al. (2014). It is common for apodemes to furnish attachment points for muscles, and the bases of sclerites are

also provided with muscles. Lateral abdominal extrinsic muscles (m1 and m2) arising on the lateral wall are connected to the base of lateral processes. These muscle groups are comparable in similar arrangement with lateral thoracic extrinsic muscle groups (M1 and M2), which have also their origin on the lateral wall and are associated with the thoracic legs (Fig. 5A). It is remarkable that some ventral external medial muscles originating from the post intersegmental fold are inserted on the rims of the prolegs.

The muscles attached on the subbasal (m3–m7) and basal (m8–m10) rims of the prolegs also

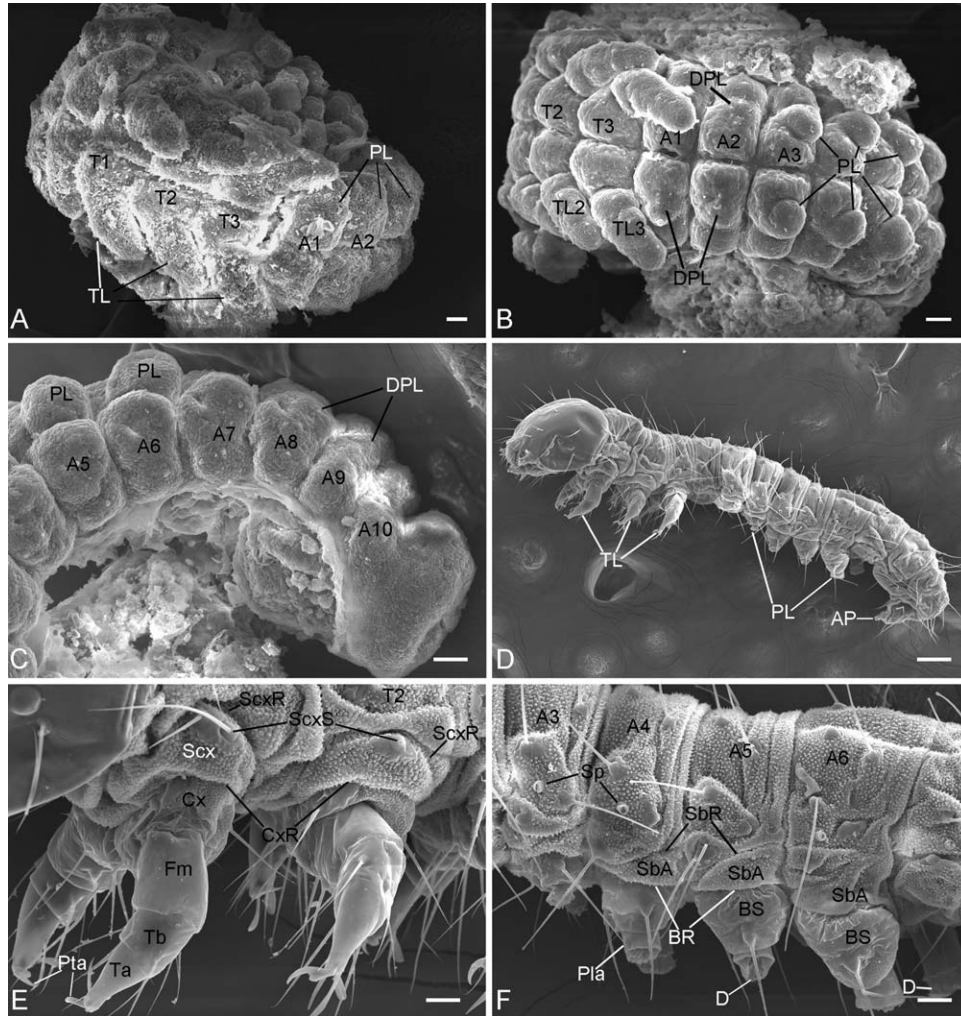


Fig. 3. *Mythemna separata*, SEM of embryos and the first-instar larvae. Ventral view of the embryo at 38 h (A) and 46 h (B). Lateral view of post embryonic abdomen at 46 h (C) and of the larva (D). High magnification of T1–T2 (E), and A4–A6 (F) of the larva. A1–A10, abdominal segments 1–10; AP, anal proleg; BR, basal rim; BS, basal segment; CxR, coxal rim; Cx, crochet; DLP, degraded lateral process; Fm, femur; PL, proleg; Pla, planta; Pta, pretarsus; SbA, subbasal area; SbR, subbasal rim; Scx, subcoxa; ScxR, subcoxal rim; ScxS, subcoxal seta; T1–T3, thoracic segments 1–3; Ta, tarsus; Tb, tibia; TL2–3, thoracic legs 2–3. Scale bars: A–C, E, F = 20 μ m; D = 100 μ m.

appear to have a similar distribution pattern with those attached on the subcoxal (M3–M7) and coxal (M8–M10) rims of the thoracic legs of armyworms (Fig. 5B,C).

DISCUSSION

The evolutionary homology of larval abdominal prolegs between insect orders has long been controversial (Chen, 1946; Erezylmaz, 2006; Giribet, 2009; Oka et al., 2010; Bitsch, 2012). Noting similarities of the musculature and innervation of prolegs in lepidopteran and sawfly larvae, Birket-Smith (1984) suggested that the prolegs are homologous structures between these two taxonomic groups. Based on the phylogenetic distribution of prolegs among holometabolous larvae, Nagy and Grbic (1999) hypothesized that larval

prolegs evolved independently in different lineages. Our research data support the argument that the abdominal prolegs are true appendages in Lepidoptera, but are secondary outgrowths in Mecoptera, thus, suggesting that prolegs are not evolutionarily homologous between these two holometabolous insect orders.

Based on embryological evidence, Suzuki (1990) and Yue and Hua (2010) thought that the scorpionfly prolegs are not of appendicular origin, but merely secondary outgrowths of the body wall. In a review of different types of abdominal appendage-like processes of immature insects, Bitsch (2012) regarded the medial prolegs as derived from coxal endites. Two pairs of similar appendage-like swellings appear on the abdominal segments during a very short period of embryogenesis of the coleopteran *Carabus insulicola*, and

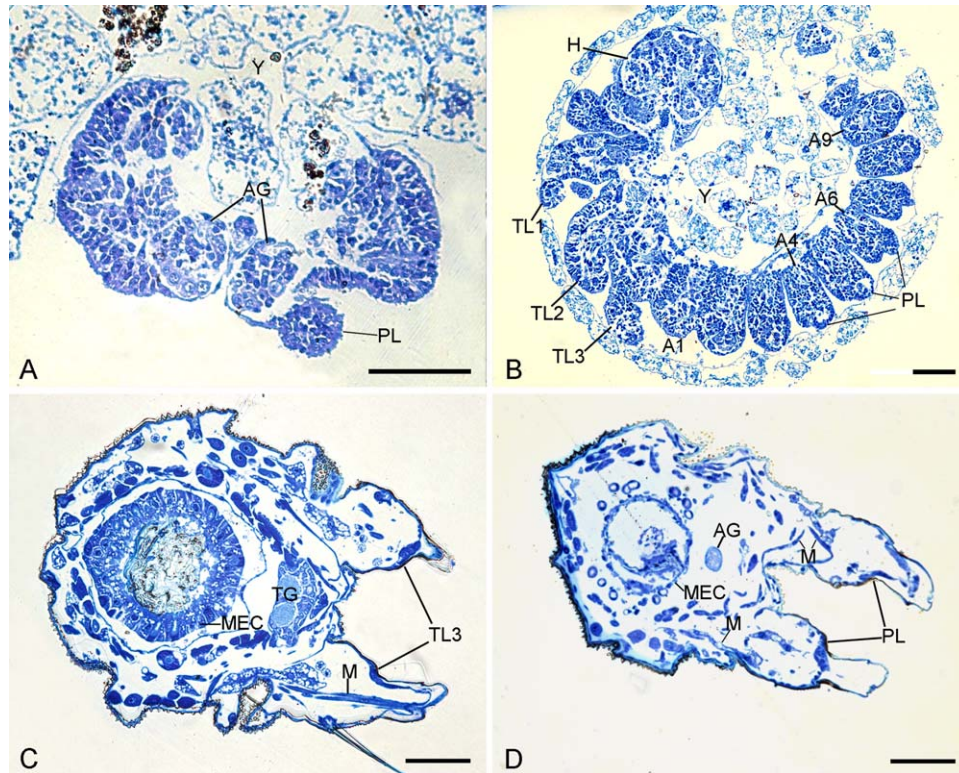


Fig. 4. *Mythemna separata*, micrographs of the semithin section of embryos and the first-instar larvae. Transverse sections of A4 at 44 h (A), and of T3 (C) and A4 (D) in the larva. (B) Longitudinal section of the embryo at 48 h. A1–A10, abdominal segments 1–10; AG, abdominal ganglion; H, head; M, muscle; MEC, midgut epithelial cell; PL, proleg; TG, thoracic ganglion; TL1–3, thoracic legs 1–3; Y, yolk. Scale bars = 50 μ m.

was also considered coxal origin (Kobayashi et al., 2013). Our study on the prolegs of scorpionflies is largely in agreement with Suzuki (1990) and Yue and Hua (2010). The medial prolegs are fleshy cone-shaped structures without any subdivisions, suggesting that they are not the main appendage axis as in sawfly prolegs (Oka et al., 2010). Moreover, the lumina of prolegs are not continuous with the hemocoel, lacking the characters of true appendages. The remnants of the lateral processes were still visible in the early larval stage, further confirming that the prolegs are not serially homologous with the thoracic legs (Yue and Hua, 2010). These lateral processes are similar to the ventral swellings of the primitive moth *Neornicropteryx nipponensis* (Kobayashi and Ando, 1981) and the trichopteran *Nemotaulius admorsus* (Kobayashi and Ando, 1990) during embryogenesis, and they all gradually cease to grow and eventually degenerate as indicated by Yue and Hua (2010). Judging from their positions corresponding to those of the thoracic legs, the lateral processes may resemble the coxopodites of appendages, as Kobayashi and Ando (1990) inferred in light of the supposition that the pleuron is supported by the subcoxa, coxa, and trochanter (Kukalová-Peck, 1983).

Previous embryological studies found that the abdominal processes fall in line with the thoracic

appendages in Lepidoptera. Both have a similar histological structure, and are thus regarded as serially homologous structures (Eastham, 1930; Friedmann, 1934). Matsuda (1976) noted that the embryonic abdominal appendages in most insects tend to persist into the larval stage, therefore, strongly supported the appendicular nature of the larval abdominal legs. Our embryological observations of *M. separata* show that the prolegs are similar to the thoracic legs in development and position, indicating the prolegs are derived from segmental appendages as in other lepidopterans (Suzuki, 1990). The basal segment of the proleg resembles the coxa and the planta a rudimentary telopodite, consistent with Snodgrass (1931).

Recent Evo-Devo studies of the appendage-regulated Hox genes also supported the appendicular origin of the prolegs in Lepidoptera and Hymenoptera (Vachon et al., 1992; Warren et al., 1994; Suzuki and Palopoli, 2001; Tomita and Kikuchi, 2009). Suzuki and Palopoli (2001) suggested that sawfly prolegs are limb bases, and that lepidopteran prolegs consist of both proximal and distal regions. Based on morphogenetic observations and the expression pattern of *decapentaplegic (dpp)* and *Distal-less (Dll)* genes, Oka et al. (2010) also suggested that the sawfly prolegs are appendicular in origin but represent coxopodial endites

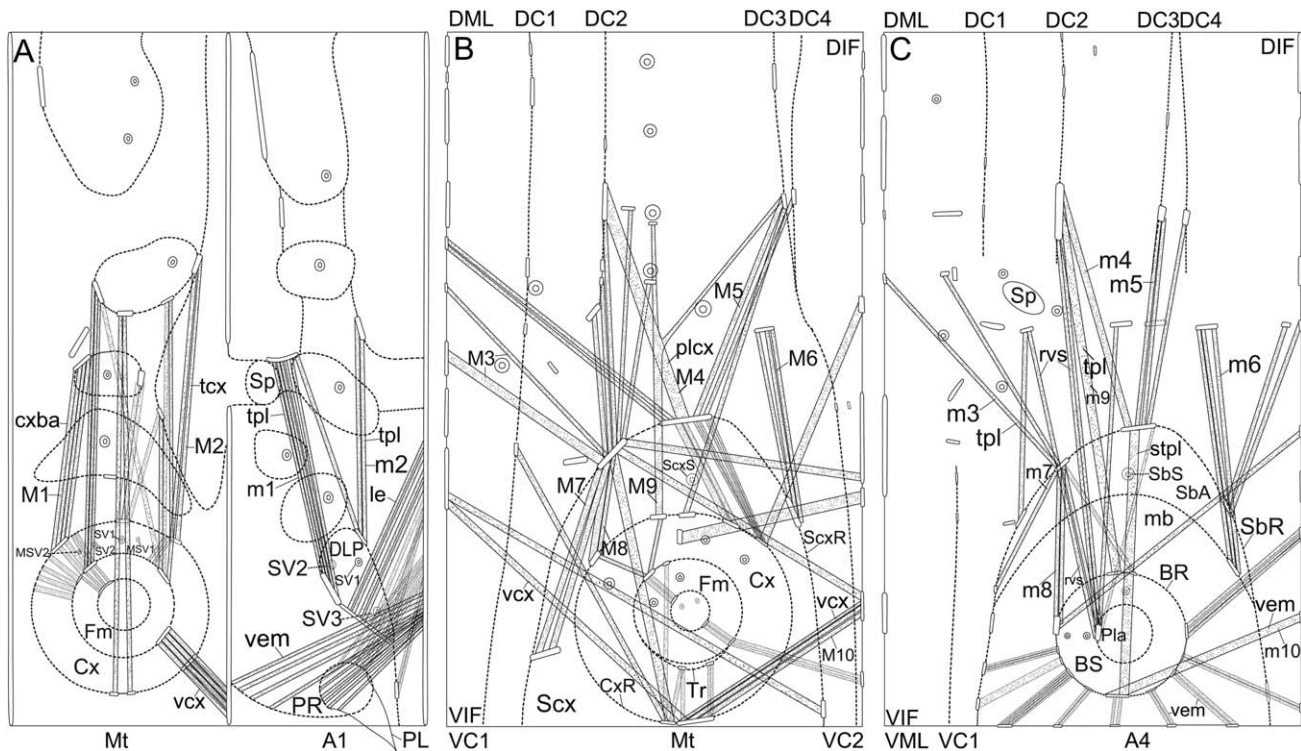


Fig. 5. Internal view of leg musculature in the right half of the full-grown larvae. Muscles in similar arrangement of metathorax and A1 (A) of *Panorpa byersi* are indicated by M (m)1 and M (m)2, and of metathorax (B) and A4 (C) of *Mythimna separata* by M (m)3–M (m)10. cxba, coxobasalar muscle; le, lateral external muscle; plcx, pleurocoxal muscle (abductor); rvs, retractor muscle fibers; stpl, sternopleural muscle; tcx, tergoxal muscle; tpl, tergoleural muscle (compressor); vcx, ventral coxal muscle; vel, ventral external lateral muscle; vem, ventral external median muscle (Nomenclature after Snodgrass, 1935). A1 and A4, abdominal segments 1 and 4; BR, basal rim; BS, basal segment; Cx, coxa; CxR, coxal rim; DC, dorsal costa; DIF, dorsal intersegmental fold; DLP, degraded lateral process; DML, mid-dorsal line; Fm, femur; M (m), muscle; mb, flexible integument; MEC, midgut epithelial cell; MSV, minute subventral seta; Mt, metathorax; Pla, planta; PR, rim of proleg; SbA, subbasal area; SbR, subbasal rim; SbS, subbasal seta; Scx, subcoxa; ScxR, subcoxal rim; ScxS, subcoxal seta; Sp, spiracle; SV, subventral seta; Tr, trochanter (not shown in SEMs); VC, ventral costa; VIF, ventral intersegmental fold; VML, mid-ventral line.

of appendages. This viewpoint was agreed by Bitsch (2012). However, the Hox genes controlling the proleg development have not been reported in Mecoptera to date.

Muscle origins and insertions can provide evidence in homological analyses of insect structures, including larvae (Mutuura, 1956; Hinton, 1958; Birket-Smith, 1984; Suzuki, 1990). Based on the general parallelism of muscle arrangement between prolegs and thoracic legs of the caterpillar, Snodgrass (1931) interpreted that the basal ring represents a subcoxal section of the appendage, the cylindrical section a coxa, and the planta either a rudimentary telopodite or as a specialized retractile vesicle of the coxa. The homologous sclerotized areas of the thorax and abdomen are distinguished by the homologous muscles (Mutuura, 1956). However, the larval trunk morphology is to a large extent influenced by the environment in Lepidoptera (Dupont, 2012, 2013, 2014), indicating that the abdominal muscles are almost inevitable to be modified in adaptation to functional specializations during evolution. To assess the homology of parts of the body wall based on muscles in

holometabolous larvae is beset with difficulties, especially in the abdominal region. Our observations on muscle connections support Snodgrass (1931, 1935) and Suzuki (1990) that prolegs are serially homologous with thoracic legs in Lepidoptera, but are not homologous in Mecoptera.

It is noteworthy that no muscles arise in mecopteran prolegs (Hinton, 1955; Suzuki, 1990; Yue and Hua, 2010). Barlet (1981) showed that an extrinsic muscle arose from an endosternal apodeme to each proleg of *Panorpa*, regarding them as telopodite remnants based on a comparison with the musculature of a caterpillar proleg. In contrast, our findings show that the prolegs are provided with extrinsic muscles taking their origin from the intersegmental fold.

The abdominal prolegs originally served as an aid in locomotion (Snodgrass, 1935; Birket-Smith, 1984; Headrick and Gordh, 2003). In primitive Nannochoristidae and Boreidae, the larvae have no prolegs (Byers and Thornhill, 1983), suggesting these structures must be de novo formations in Pistillifera. The fact that the abdominal prolegs are degenerated with limited movement living in

soil is likely compatible with the interpretation that they evolved secondarily in Mecoptera (Yue and Hua, 2010; Jiang et al., 2014). Accordingly, the prolegs are likely secondary auxiliary organs in the evolutionary course of Mecoptera based on functional morphology. Conversely, the larvae of glossatan lepidopterans and sawflies bear typical caterpillar-like prolegs as locomotory organs assisting their movement on the surface of plants (Snodgrass, 1935; Voigt and Gorb, 2012). The prolegs were regarded as an autapomorphic feature ascribed to the Neolepidoptera because they do not appear in the basal lineages of Lepidoptera (Dupont, 2012, 2013, 2014), suggesting nonhomology between the hymenopteran and lepidopteran prolegs.

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