Thickened Serosa and Serosal Cuticle Formed beneath the Embryo in Eight Arctoperlarian Stoneflies (Insecta, Plecoptera)

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Abstract
We examined the embryological development of arctoperlarian stoneflies belonging to the eight families, i.e., Taeniopterygidae, Leuctridae, Capniidae, and Nemouridae of Euholognatha as well as Perlidae, Chloroperlidae, Perlodidae, and Peltoperlidae of Systellognatha, all of which developed a thickened serosa and serosal cuticle beneath the embryo. By combining these results with those of existing studies, it was revealed that the major lineages of Arctoperlaria share these structures and that they can be regarded as embryological groundplan features of Arctoperlaria.

Introduction
Plecoptera represent a polyneopteran order, comprising two suborders, Arctoperlaria, primarily from the Northern Hemisphere, and Antarctoperlaria, exclusively from the Southern Hemisphere. The monophyly of each suborder and two arctoperlarian subgroups, Euholognatha and Systellognatha, is supported by both morphological (Zwick, 2000) and molecular phylogenetic evidence (McCulloch et al., 2016). The phylogenetic position of Plecoptera has been debated for a long time (e.g., Kristensen, 1975; Zwick, 2009; Beutel et al., 2014), but recent morphological (Yoshizawa, 2011; Wipfler et al., 2015) and molecular evidence, including a large-scale phylogenomic analysis based on transcriptomes of protein-coding genes (Ishiwata et al., 2021; Misof et al., 2014; Song et al., 2016; Wipfler et al., 2019) revealed that Plecoptera is a member of Polyneoptera. Our comparative embryological study of Plecoptera (Mtow and Machida, 2018a) also corroborated the placement of Plecoptera in Polyneoptera based on the finding that the plecopteran embryos elongate along the egg surface, and this feature represents one of the polyneopteran embryological autapomorphies (Mashimo et al., 2014).

We have been conducting comparative embryological studies of Plecoptera, aiming at the reconstruction of the groundplan of Plecoptera and subsequently of Polyneoptera (Mtow and Machida, 2018a, b, 2019). The embryos of some stoneflies show thickening of the serosa beneath the embryo. Miller (1939, 1940) described the thickened serosa and thickened cuticular layer secreted beneath it in the systellognathan stonefly Pteronarcyis proteus of Pteronarcyidae. These thickened serosa and serosal cuticle being named the “grumulus” and “grumorium,” respectively. Similarly, serosal structures were reported in another systellognathan, Kamimuria tibialis of Perlidae, by Kishimoto and Ando (1985), who named the thickened serosa “columnar serosal cells.” In the case of Euholognatha, we recently found that the thickened serosa and serosal cuticle also develop beneath the embryo in Scopura montana of Scopuridae and gave a detailed description of their structure and development (Mtow and Machida, 2018b, 2019). In the current study, we examined the embryological development of the arctoperlarian stoneflies belonging to the seven major families of which embryogenesis has not hitherto been examined, specifically, Taeniopterygidae, Leuctridae, Capniidae, and Nemouridae of Euholognatha as well as Chloroperlidae, Perlodidae, and Peltoperlidae of Systellognatha, also including one representative species of Perlidae, to discuss whether the thickened serosa and serosal cuticle formed beneath the embryo are groundplan features of Arctoperlaria.

Materials and Methods
Adults of eight arctoperlarian stoneflies from the eight families, i.e., Euholognatha: Obipteryx sp. of Taeniopterygidae, Paraleuctra cercia of Leuctridae, Apteroperla tikumanai of Capniidae, Protonemura iwadesensis of Nemouridae and Systellognatha: Calineuria stagnatica of Perlidae, Sweltsa sp.
of Chloroperlidae, Ostronus sp. of Perlodidae, and Yoraperla uenoi of Peltoperlidae, were collected between 2014 and 2017 from the streams in Sugadaira Kogen, Ueda, Nagano, Japan.

The deposited eggs were either fixed with Kahle’s fixative (ethyl alcohol : formalin : acetic acid : distilled water = 15 : 6 : 2 : 30) for 24 h and stored in 80% ethyl alcohol at room temperature or fixed with Kornovsky’s fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M HCl-sodium cacodylate buffer, pH 7.2) for 24 h and stored in the buffer at 4°C.

The eggs fixed with Kahle’s fixative were dehydrated in a graded ethanol series, immersed in acetone, and embedded in a methacylate resin (Technovit 7100, Kulzer, Wehrheim, Germany) in accordance with the protocol described by Machida et al. (1994a, b). Semi-thin sections at a thickness of 2 μm were cut using a semi-thin microtome (H-μı, Rad, Hercules, USA), equipped with a tungsten carbide knife (Superhard Knife, Meiwafosis, Tokyo, Japan). Sections were stained with 0.5% Delafield’s hematoxylin for 12 h, 0.5% eosin G for 1 h, and 0.5% fast green FCF 100% ethanol solution for 1 min.

The eggs fixed with Kornovsky’s fixative were dehydrated in a graded ethanol series, immersed in methyl oxirane, and embedded in an epoxy resin (Agar Low Viscosity Resin Kit, Agar Scientific, Essex, UK). Serial, semi-thin sectioning at a thickness of 0.75 μm was performed using an ultramicrotome (MT-XL, RMC, Arizona, USA) equipped with a diamond knife (Histo Jumbo, DiATOME, Nidau, Switzerland) according to the methods described by Blumer et al. (2002). The sections were then stained using 0.1% toluidine blue O solution for 1 h.

The stained sections were observed under a biological microscope (Optiphot-2, Nikon, Tokyo, Japan) and captured with a CCD camera (DS-Fi2, Nikon, Tokyo, Japan).

Results and Discussion

Staging used in the description is based on Mtow and Machida’s staging (2018a) for the embryogenesis of the scoporid stonefly Scopora montana (Table 1).

In the case of Euholognatha, in Obipteryx sp. of Taeniopterygidae, in Stage 2, the small thickened serosa forms beneath the embryo as in S. montana (Mtow and Machida, 2018b, 2019) and Pteronarcyx proteus (Millar, 1939, 1940) (Fig. 1A). Almost simultaneously, the serosa starts to secrete the serosal cuticle over the entire surface of the egg, and with the progression of embryonic development, the serosal cuticle is thickened (Fig. 1B). The serosal cuticle is a slightly thicker in the region beneath the thickened serosa (Fig. 1A, B). In Paraleuctra cercia of Leuctridae, the thickened serosa is also formed beneath the embryo at the stage of amnioserosal fold formation (Stage 2) and shows a radial cellular arrangement in sections, as in S. montana and Pt. proteus (Fig. 2A). Soon, the serosal cuticle starts to be secreted, and with progressive embryogenesis, it is gradually thickened up to the middle stage of intertripsis (around Stage 6). The serosal cuticle is extremely thickened beneath the thickened serosa (Fig. 2B).

The thickened serosa of Apteroperla tikumana of Capniidae and Protonemura tovaidensis of Nemouridae forms beneath the embryo in the same manner as in Obipteryx sp. and Pt. cercia (Figs. 3A, 4A), with the serosal cuticle beneath the thickened serosa bloated as the thickened serosal cuticle (Figs. 3B, 4B).

In Systellognatha, the thickened serosa and serosal cuticle are formed beneath the embryo, as in Euholognatha. Figures 5–8 present these structures of the definitive form in Calineuria stigmatica of Perlidae, Sveltsa sp. of Chloroperlidae, Ostronus sp. of Perlodidae, and Yoraperla uenoi of Peltoperlidae, respectively.

The current study revealed that all the eight arctoperlarian families examined developed thickened serosa and thickened serosal cuticle beneath the embryo. Hence, it was ascertained that the major lineages of Arctoperlaria, i.e., Euholognatha: Taeniopterygidae, Leuctridae, Capniidae, Nemouridae, and Scopuridae (Mtow and Machida, 2018b, 2019) and Systellognatha: Perlidae (Kishimoto and Anto, 1985; herein), Chloroperlidae, Perlodidae, Peltoperlidae, and Pteronarcyidae (Müller, 1939, 1940) possess thickened serosa and serosal cuticle. The lineages still lacking information are only two families, specifically Notonemouridae, which is a close relative of Nemouridae, and Styloperlidae, which is regarded as the sister group of Peltoperlidae by Zwick (2000). Therefore, it can be concluded that the thickened serosa and thickened serosal cuticle should be counted as embryological groundplan features of Arctoperlaria. Embryological information on Antarctoperlaria, which is totally lacking, is needed.

<table>
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<tr>
<th>Stages</th>
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<tr>
<td>Stage 1</td>
<td>Cleavage; formation of blastoderm; differentiation of embryo</td>
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<tr>
<td>Stage 2</td>
<td>Formation of amnioserosal fold</td>
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<td>Stage 3</td>
<td>Differentiation of protocephalon and protocorm</td>
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<td>Stage 4</td>
<td>Elongation of embryo along the egg surface; commencement of segmentation</td>
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<td>Stage 5</td>
<td>Immersion of embryo into the yolk; formation of appendages</td>
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<td>Stage 6</td>
<td>Embryo assuming an S shape in the yolk</td>
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<td>Stage 7</td>
<td>Embryo attaining its maximum length in the yolk</td>
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<td>Stage 8</td>
<td>Head of embryo moving close to the egg surface</td>
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<td>Stage 9</td>
<td>Regression of amnioserosal fold (lateral); embryo appearing on the egg surface; formation of secondary dorsal organ</td>
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<td>Stage 10</td>
<td>Progressive definitive dorsal closure</td>
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<td>Stage 11</td>
<td>Completion of definitive dorsal closure; secretion of embryonic cuticle; formation of compound eye and egg tooth</td>
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<tr>
<td>Stage 12</td>
<td>Acquisition of definitive form by embryo; secretion of larval cuticle</td>
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SEROSAL STRUCTURES BENEATH ARCTOPERLARIAN EMBRYOS

Figs. 1–4 Thickened serosa and serosal cuticle beneath the embryo of four euholognathan stoneflies, chorion removed, anterior of the egg to the top.

Fig. 1 Sections of embryos of Obipteryx sp. (Theniopterygidae), methacrylate resin. A. A vertical section of an embryo in Stage 2. B. A sagittal section of an embryo in Stage 4, anterior of the embryo to the left.

Fig. 2 Sections of embryos of Paraleuctra cercia (Leuctridae), epoxy resin. A. A vertical section of an embryo in Stage 3. B. A sagittal section of a thickened serosa beneath the embryo in Stage 6, anterior of the embryo to the left.

Fig. 3 Sagittal sections of embryos of Apteroperla tikumana (Capniidae), methacrylate resin, anterior of the embryo to the left. A. A section of an embryo in Stage 3. B. A section of an embryo in Stage 6.

Fig. 4 Sagittal sections of embryos of Protonemura towadensis (Nemouridae), epoxy resin. Eggs, which were fixed with Karnovsky’s fixative, were postfixed with 1% OsO₄ for 1 h. A. A section of an embryo in Stage 3. B. A section of an embryo in Stage 7.

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