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Egg structure and embryonic development of arctoperlarian stoneflies: a comparative embryological study (Plecoptera)

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Abstract. Egg structure and embryonic development of nine arctoperlarian stoneflies from nine families, i.e., Scopuridae, Taeniopterygidae, Leuctridae, Capniidae, and Nemouridae of Euholognatha, and Perlidae, Chloroperlidae, Perlodidae, and Peltoperlidae of Systellognatha were examined and compared with previous studies. The primary aim of this study was to use embryological data to reconstruct the groundplan and phylogeny of Plecoptera and Polyneoptera. Euholognatha has eggs characterized by a thin, transparent chorion, while the eggs of Systellognatha are characterized by a collar and anchor plate at the posterior pole. These features represent an apomorphic groundplan for each group. The embryos form by the concentration of blastoderm cells toward the posterior pole of the egg. Soon after the formation of the embryo, amnioserosal folds form and fuse with each other, resulting in a ball-shaped "embryo-amnion composite" that is a potential autapomorphy of Plecoptera. As an embryological autapomorphy of Polyneoptera, embryo elongation occurs on the egg surface, supporting the affiliation of Plecoptera to Polyneoptera. After its elongation on the egg surface, the embryo sinks into the yolk with its cephalic and caudal ends remaining on the egg surface. This unique embryonic posture may be regarded as an apomorphic groundplan of Plecoptera. Arctoperlarian plecopterans perform three types of katatrepsis: 1) the first type, in which the embryo's anteroposterior and dorsoventral axes change in reverse during katatrepsis, is found in Capniidae, Nemouridae, Perlidae, Chloroperlidae, and Perlodidae, and this sharing is symplesiomorphic; 2) the second one, in which the embryo's axes are not changed during katatrepsis, is found in Scopuridae, Taeniopterygidae, and Leuctridae, and this may be regarded as synapomorphic to them; 3) the third one, in which the embryo rotates around its anteroposterior axis by 90° during katatrepsis as known for Pteronarcyidae, is found in Peltoperlidae, and this type may be synapomorphic to these two families.

Key words. Scopuridae, Euholognatha, Systellognatha, Arctoperlaria, Polyneoptera, comparative embryology, egg structure, blastokinesis.

1. Introduction

Plecoptera or stoneflies are a hemimetabolous, neopteran order, occurring on all continents excluding Antarctica, and more than 3,700 species have been described, including fossil species (DEWALT et al. 2015). Nymphs are almost exclusively aquatic and can be found mainly in cold, well-oxygenated running waters. Stoneflies are important components of clean streams, and they are often used as bioindicators (FOCHETTI & TIERNO DE FIGUEROA 2008).

Plecoptera are divided into the two suborders, Antarctoperlaria and Arctoperlaria. The former is found only in the Southern Hemisphere and contains four families. The Arctoperlaria inhabit mostly the Northern Hemisphere, comprising two subgroups, Systellognatha and Euholognatha, each containing six families (ZWICK 2000; BEUTEL et al. 2014; DEWALT et al. 2017). Recent molecular phylogenetic analyses support the monophyly of each suborder and each arctoperlarian subgroup (TERRY 2004; MCCULLOCH et al. 2016). However, in contrast to Antarctoperlaria, the monophyly of Arctoperlaria is only supported by morphological characters related to the complex mate-finding syndrome "drumming," which is shared by all families of this group (with the exception of the Scopuridae) (ZWICK 1973, 2000).

Polyneoptera comprise ten lower neopteran orders, including the Plecoptera, Dermaptera, Embioptera, Phasmatodea, Orthoptera, Zoraptera, Grylloblattodea, Mantophasmatodea, Mantodea, and Blattodea (= "Blattaria" + Isoptera). However, phylogenetic relationships among these orders have been much disputed over (e.g., Kristensen 1975; Boudreaux 1979; Hennig 1981; Klass 2009; Beutel et al. 2013). In addition, the monophyly of Polyneoptera has been debated over for a long time, but it was recently supported based on morphological and embryological studies (e.g., Yoshizawa 2011; Mashimo et al. 2014; Wipfler et al. 2015) and molecular data (e.g., Ishiwata et al. 2011; Song et al. 2016); Misof et al. (2014) conducted a large-scale phylogenomic analysis based on transcriptomes of 1,478 genes, and provided a strong support for monophyletic Polyneoptera. Phylogenetic positions of Zoraptera and Dermaptera, both of which has long been under debate (see Klass 2003, 2009; Beutel & Weide 2005), were reliably placed in the monophyletic Polyneoptera (e.g., Ishiwata et al. 2011; Yoshizawa 2011; Mashimo et al. 2014; Misof et al. 2014). In spite of recent challenges from various disciplines, phylogenetic relationships within Polyneoptera remain still far apart from consensus (see Beutel et al. 2013).

Plecoptera, of which affiliation to Polyneoptera seems currently established, remain a challenging taxonomic group in the Neoptera (Zwick 2009). Various hypotheses have been proposed for the phylogenetic position of Plecoptera including sistergroup relations to the remaining neopterans (e.g., Kristensen 1975; Beutel & Gorb 2006; Klug & Klass 2007; Zwick 2009), to the remaining polyneopterans (Paurometabola or Pliconeoptera including Zoraptera, cf. Wipfler et al. 2015) (Fausto et al. 2001; Beutel et al. 2014) and even to Paraneoptera s.l. (= Acercaria + Zoraptera) + Holometabola (Ross 1955; Hamilton 1972). Recent comparative morphologies, molecular phylogenetics, and combined analyses have proposed different polyneopteran orders or assemblages as the sister group of Plecoptera, including the "Dermaptera" (Ishiwata et al. 2011), "Embioptera" (Kukalová-Peck 2008), "Orthoptera" (Kômoto et al. 2012), "Zoraptera" (Matsumura et al. 2015), "Zoraptera + Dermaptera" (Terry & Whiting 2005), "Zoraptera + Embioptera" (Grimaldi & Engel 2005), "Chimaeraptera (= Xenonomia = Grylloblattodea + Mantophasmatodea)" (Blanke et al. 2012), "Orthoptera + Chimaeraptera + Eukinolabia (= Embioptera + Phasmatodea) + Dictyoptera (= Mantodea + Blattodea)" (Misof et al. 2014). However, these recent changes were not always based on new evidence for Plecoptera itself, but Plecoptera was only shoved around as other taxa were studied and views of their interrelations changed (Zwick 2009).

A comparative embryological approach can be a potential source of deep phylogenetic information that can help to resolve these debates. However, our knowledge of the embryonic development of Plecoptera is limited. Although several detailed studies exist in the arctoperlarian subgroup Systellognatha, these come from only two of six families: *Pteronarcys proteus* Newman, 1838 of Pteronarcyidae by Miller (1939, 1940) and *Kamimuria tibialis* (Pictet, 1841) of Perlidae by Kishimoto & Ando (1985, 1986) and Kishimoto (1986, 1987). Little data exist on the development of other systellognathan families and Euholognatha (Khoo 1968a,b; Kishimoto 1997), and embryological information on Antarctoperlaria is totally lacking.

On this background, we started a comparative embryological study of Plecoptera. In the present study, we describe an outline of embryogenesis for all the nine Japanese arctoperlarian families (Kawai 1967; Shimizu et al. 2005): five families of the infraorder Euholognatha, i.e., Scopuridae, Taeniopterygidae, Leuctridae, Capniidae, and Nemouridae; four families of the infraorder Systellognatha, i.e., Perlidae, Chloroperlidae, Perlodidae, and Peltoperlidae with special reference to external morphology, and compare the results with previous works, to discuss the groundplan of Plecoptera and the interfamily relationships in Arctoperlaria.

2. Materials and methods

Adults from nine arctoperlarian stoneflies (Table 1) were collected in 2014 to 2016 around the streams in Sugadaira Kogen, Ueda, Nagano, Japan, i.e., Daimyojin-zawa, Kara-sawa, and Naka-no-sawa. As for *Scopura montana*, late instar larvae were reared and raised to adults *en masse* in plastic cases (167 mm × 117 mm × 58 mm) containing stones and a layer of water and fed with fallen leaves.

Females after mating were kept separately in plastic cases (68 mm × 39 mm × 15 mm) containing tissue paper under controlled temperature (Table 1), and fed on fruits (apple or persimmon) and commercial food for insects (Mushi-jelly, Mitani, Ibaraki, Japan) and fish (TetraFin, Spectrum Brands Japan, Yokohama, Japan, or Koi-no-sato, Japan Pet Food, Tokyo, Japan). The eggs deposited by females were incubated in plastic cases (36 mm × 36 mm × 14 mm) filled with water under controlled temperature (Table 1).

Prior to fixation, eggs were soaked in commercial bleach (Kitchen Bleach S, Mitsuei, Fukushima, Japan) for several seconds and cleaned using a small brush to remove the gelatinous layer that covered them. The eggs were rinsed in Ephrussi-Beadle's solution (0.75% NaCl, 0.035% KCl, 0.021% $CaCl_2$) containing detergent (0.1% Triton X-100), punctured with a fine needle, fixed with either Kahle' s fixative (ethyl alcohol : formalin : acetic acid : distilled water = 15 : 6 : 2 : 30) or FAA (ethyl alcohol : formalin : acetic acid = 15 : 5 : 1) for 24 h, and stored in 80% ethyl alcohol at room temperature.

The fixed eggs were stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride) solution diluted to 10 µg/l with PBS (18.6 mM $NaH_2PO_4 : H_2O$, 84.1 mM $NaH_2PO_4 : 2H_2O$, 1.75 M NaCl; pH 7.4) at 4°C for 20 min to several days depending on specimens. The eggs

Table 1. The nine Japanese arctoperlarian stoneflies of which egg structure and embryonic development were examined in the present study.

Materials	Month of oviposition	Temperature for incubation of adults and eggs
Euholognatha		
Scopuridae: *Scopura montana* Maruyama, 1987	October – December	8°C
Taeniopterygidae: *Obipteryx* Okamoto, 1922 sp.	June	12°C
Leuctridae: *Paraleuctra cercia* (Okamoto, 1922)	May – June	12°C
Capniidae: *Apteroperla tikumana* (Uéno, 1938)	February – April	4°C
Nemouridae: *Protonemura towadensis* (Kawai, 1954)	November – December	8°C
Systellognatha		
Perlidae: *Calineuria stigmatica* (Klapálek, 1907)	September – October	12°C
Chloroperlidae: *Sweltsa* Ricker, 1943 sp.	May – June	12°C
Perlodidae: *Ostrovus* Ricker, 1952 sp.	June – July	12°C
Peltoperlidae: *Yoraperla uenoi* (Kohno, 1946)	June – July	12°C

stained with DAPI were observed under a fluorescence stereomicroscope (MZ FL III + FluoCombi, Leica, Heerbrugg, Switzerland) with UV excitation at 360 nm. Systellognatha eggs, which have a tough chorion layer, were dechorionated with a fine needle and fine forceps prior to staining.

For scanning electron microscopy (SEM) observation, eggs were postfixed with 1% OsO_4 for 1 h. Fixed eggs were dehydrated in a graded ethanol series, dried either with a critical point dryer (Samdri-PVT-3D, tousimis, Rockville, Maryland) or naturally dried with HMDS (1,1,1,3,3,3-Hexamethyldisilazane) as described by Faull & Williams (2016), coated with gold, and then observed under an SEM (SM-300, TOPCON, Tokyo, Japan) at 15 kV. Eggs of *Apteroperla tikumana*, which are prone to distortion in the course of processing due to their softness, were observed using the nano-suit method, as described by Takaku et al. (2013) and Fujita et al. (2016). Eggs were soaked in 1% polyoxyethylene sorbitan monolaurate (Tween 20) solution for 1 h, blotted briefly on dry filter paper to remove excess solution, mounted on a stab, and observed with the SEM at 5 kV.

To record blastokinesis, some eggs were observed while alive using a time-lapse VTR system (CK-2 or CK-40, inverted microscope, Olympus, Tokyo, Japan; TSN401A, CCD color camera, Elmo, Nagoya, Japan; Live capture 2, web camera system, downloaded from http://www2.wisnet.ne.jp/~daddy).

3. Results

The orientation of the insect eggs is defined according to the embryo just before hatching (Wheeler 1893). When applying this definition to plecopterans, we encounter a serious problem. As generally found in hemimetabolan insects, i.e., Palaeoptera, Polyneoptera, and Acercaria, usually in plecopterans, 1) the embryo forms at the posterior pole of the egg or the ventral side near the posterior pole; 2) in the course of anatrepsis, the embryo substantially elongates with its posterior end ahead, resulting in its ventral side facing the dorsal side of the egg and the embryo's anteroposterior axis reversed, i.e., both the anteroposterior and dorsoventral axes of the embryo become opposed to those of the egg; 3) katatrepsis then occurs, and the embryo appears again on the egg surface, shifting its position to the ventral side of the egg, and its anteroposterior axis is reversed, i.e., both the anteroposterior and dorsoventral axes of the embryo correspond again to those of the egg (see Anderson 1972; Mashimo et al. 2014). This type of blastokinesis was revealed to occur in a large proportion of the plecopterans examined in the present study such as *Apteroperla tikumana* (Capniidae) (Figs. 9, 16: see also 3.1.4.). However, an aberrant form of katatrepsis, in which the embryo maintained unchanged positions of the anteroposterior and dorsoventral axes, was found to be performed in a small proportion of the plecopterans examined such as *Scopura montana* (Scopuridae) (Figs. 5, 16: see also 3.1.1.). In these plecopterans, the embryo reaches hatching, with its anteroposterior and dorsoventral axes opposed to those of other plecopterans. Simply following to the general definition of the orientation of eggs by Wheeler (1893), we would have to describe, for example, that in these plecopterans the embryo forms at the *anterior* pole of the egg, which would thus differ from other plecopterans. To avoid such a problem in orientation, in the present study we define the orientation of the egg in Plecoptera as follows: 1) the posterior is where the embryo forms, and the anterior is its opposite; 2) the dorsal is where the embryo exists just before katatrepsis, and the ventral is the opposite.

In what follows, we describe the egg structure and an outline of embryonic development in one species from each of nine arctoperlarian families. As for the embryonic development, first we made a detailed description on *S. montana*, dividing it into 12 stages following Kishimoto & Ando (1985), and then we gave descriptions for other species, focusing on the differences from *S. montana* and/or other species.

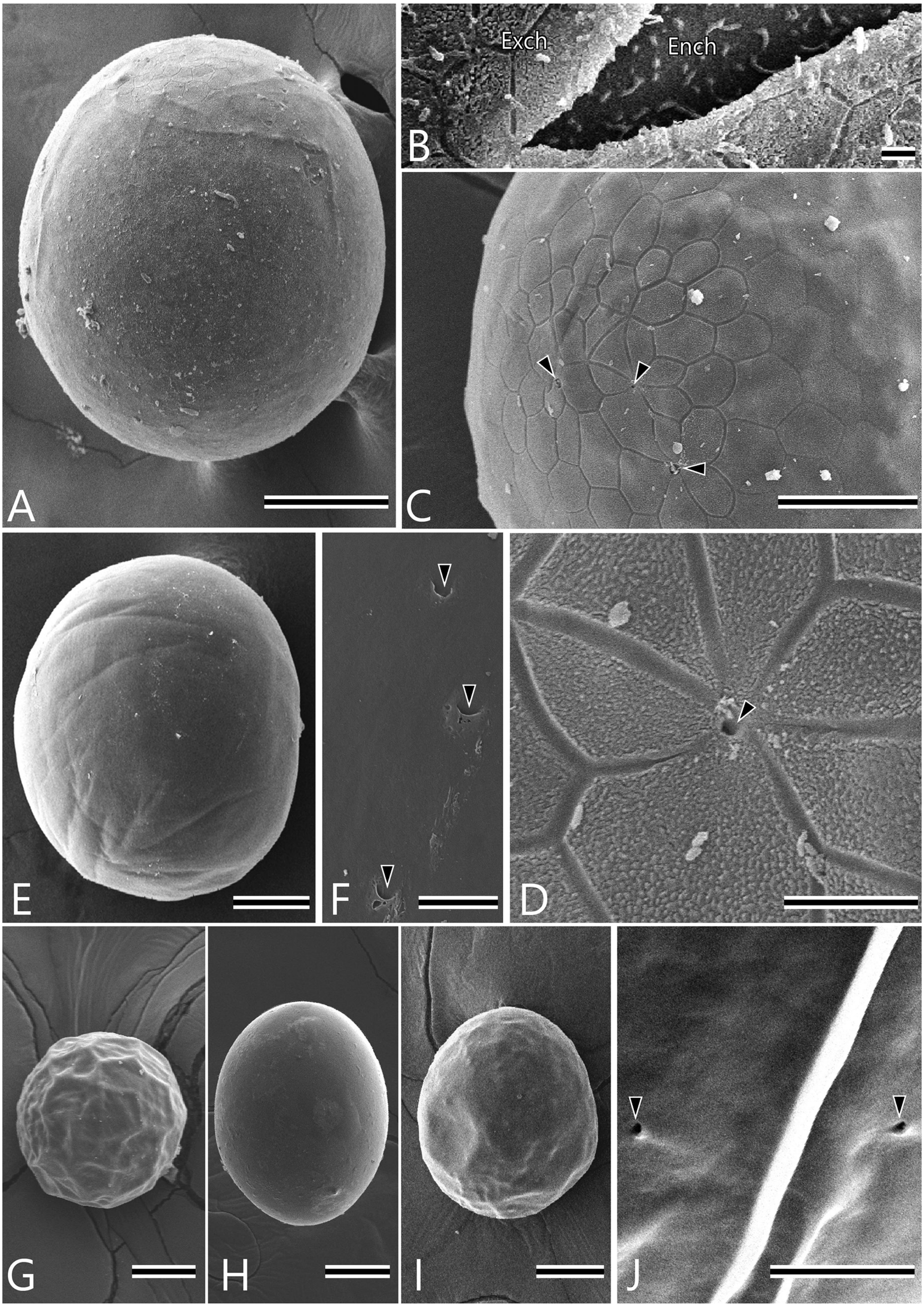

Fig. 1. Eggs of Euholognatha. SEM micrographs. **A–D.** *Scopura montana*; **A**: egg, lateral view, anterior at the top, **B**: enlargement of the egg surface near the anterior pole; endochorion can be seen through a tear of the exochorion, **C**: egg surface of the anterior pole, **D**: enlargement of C, showing a rosette, at the center of which a micropyle is seen. **E,F.** *Obipteryx* sp., lateral view, anterior at the top; **E**: egg (some lines on egg surface are artifacts), **F**: enlargement of a micropylar area. **G**: egg of *Paraleuctra cercia*, which is artificially wrinkled during the drying for processing specimens, lateral view. **H**: egg of *Apteroperla tikumana*, lateral view, anterior at the top. **I,J.** *Protonemura towadensis*; **I**: egg, which is artificially dented during the drying for processing specimens, lateral view, anterior at the top, **J**: micropyles (white slanting line is an artifact). — ***Abbreviations***: Ench, endochorion; Exch, exochorion; arrowhead, micropyle. Scale bars = A, 100 µm; B,D,F,J, 10 µm; C,E,G–I, 50 µm.

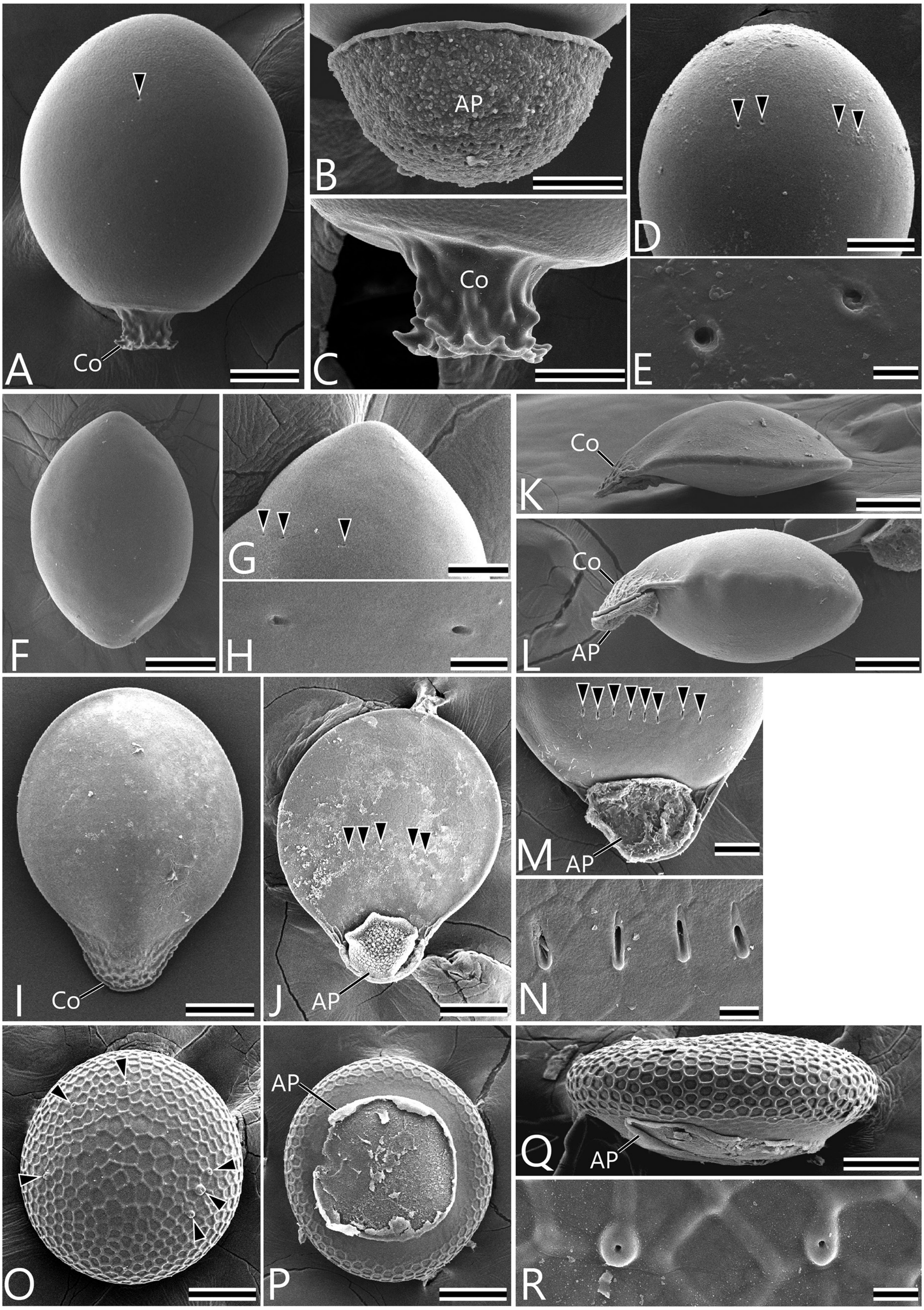

Fig. 2. Eggs of Systellognatha. SEM micrographs. **A–E.** *Calineuria stigmatica*; **A**: egg, with the anchor plate resolved by soaking in bleach for a short time, lateral view, anterior at the top, **B**: enlargement of the posterior pole, **C**: posterior pole, with the anchor plate resolved, **D**: anterior half of egg, showing micropyles, **E**: enlargement of micropyles. **F–H.** *Sweltsa* sp.; **F**: egg, lateral view, anterior at the top, **G**: anterior half of the egg, **H**: micropyles. **I–N.** *Ostrovus* sp.; **I**: right side of the egg, anterior at the top, **J**: left side of the egg, **K**: newly laid egg, ventral view, left side to the top, anterior to the right, **L**: egg just before hatching, ventral view, **M**: posterior half of the left side of the egg, **N**: enlargement of micropyles. **O–R.** *Yoraperla uenoi*; **O**: egg, anterior view, **P**: egg, posterior view, **Q**: egg, lateral view, **R**: enlargement of micropyles. — ***Abbreviations***: AP, anchor plate; Co, collar; arrowhead, micropyle. Scale bars = A,D,F,I–L,O–Q, 100 µm; B,C,G,M, 50 µm; E,H,N,R, 10 µm.

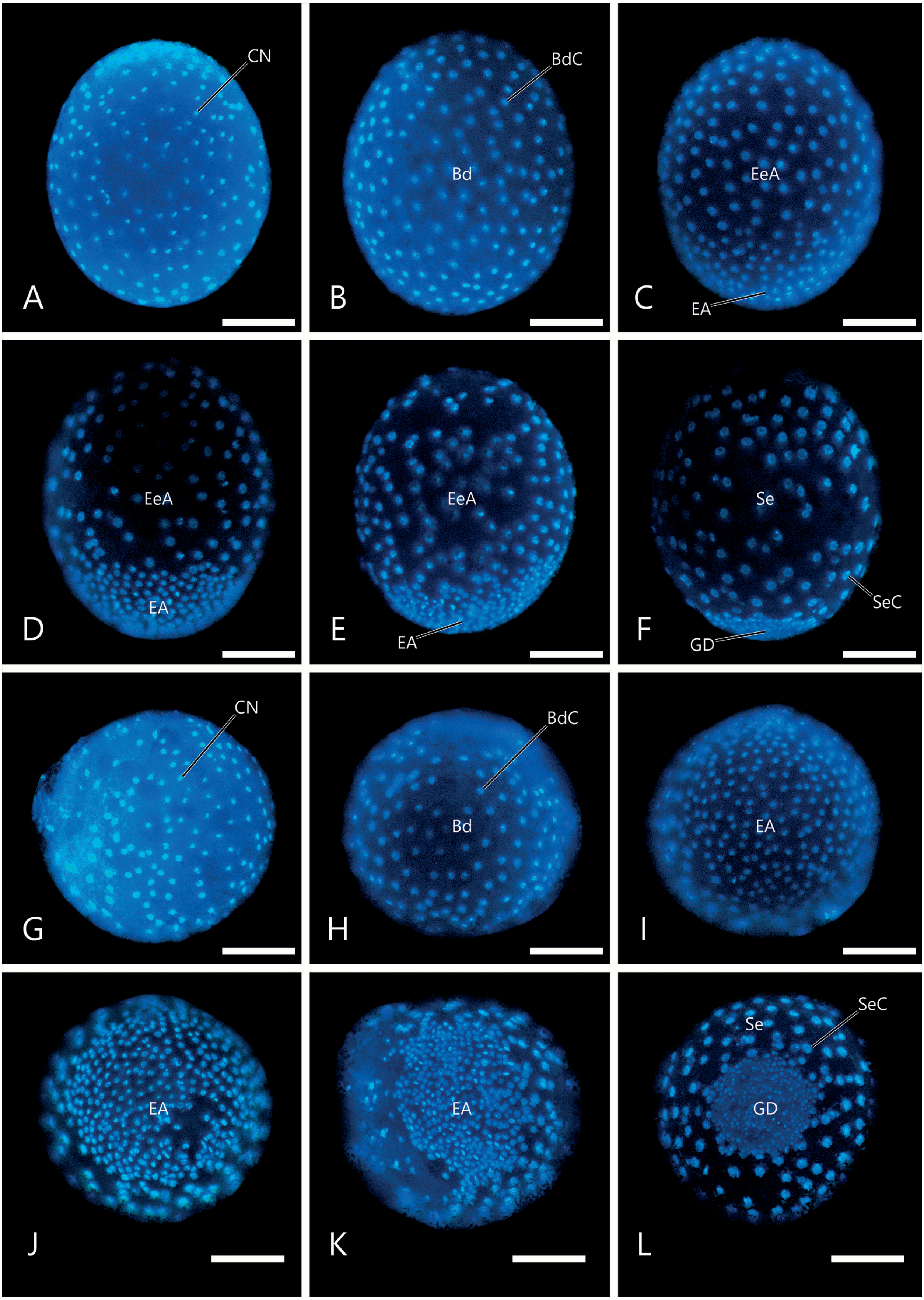

Fig. 3. Embryonic development of *Scopura montana*, Stage 1. DAPI staining, A–F. lateral view, anterior at the top, G–L. posterior view. **A,G**: cleavage, late stage, **B,H**: blastoderm, **C–E,I–K**: differentiation of embryonic and extraembryonic areas, beginning (**C,I**), middle (**D,J**) and late (**E,K**) stages, **F,L**: newly formed germ disc. — ***Abbreviations***: Bd, blastoderm; BdC, blastoderm cell; CN, cleavage nucleus; EA, embryonic area; EeA, extraembryonic area; GD, germ disc; Se, serosa; SeC, serosal cell. Scale bars = 100 μm.

3.1. Egg and embryonic development of Euholognatha

3.1.1. *Scopura montana* (Scopuridae)

Egg. Eggs are spheroidal with long and short diameters of 330 – 400 µm and 300 – 330 µm, respectively (Fig. 1A). They are ivory in color because the yellowish yolk is visible through the transparent egg membranes. The surface is surrounded by a sticky coat. The egg membranes are composed of a two-layered chorion comprising an exochorion and endochorion (Fig. 1B): the former often peels off during embryonic development, and the latter is furnished with numerous rod-like materials about 1 – 2 µm in length on its surface (Fig. 1B). The exochorion of the anterior third of the egg contains a weak, polygonal pattern, which forms several rosettes around the anterior pole of the egg (Fig. 1C): at the center of each rosette, a micropyle of ca. 2 µm in diameter opens, and the micropyles are arranged roughly in circle (Fig. 1C,D).

Embryonic development. The egg period is 75 – 85 days at 8°C.

Stage 1: The cleavage nuclei arrive at the surface of the yolk mass (Fig. 3A,G), and a unicellular blastoderm forms (Fig. 3B,H). Soon after completion of blastoderm formation, the embryonic and extraembryonic areas are differentiated. The former forms at the posterior pole of the egg and is more densely cellulated than the latter (Fig. 3C,I), and the nuclei of the former are smaller and more crowded than those of the latter (Fig. 3D,E,J,K). The embryonic area is differentiated into a discoid germ disc about 100 µm in diameter, and the extraembryonic area is the serosa (Figs. 3F,L, 5A, 6A).

Stage 2: The amnion is produced from the margin of the germ disc or embryo (Fig. 4A). The amnion forms the amnioserosal fold along with the serosa, and anatrepsis starts. The amnioserosal fold, of which formation is more progressive in the posterior region of the embryo than in the anterior, extends beneath the embryo (Fig. 4B – D). Finally, the amnioserosal folds fuse with each other, and the amniotic pore is closed. After the fusion of the amnioserosal folds, the embryo is elliptical, with long and short diameters approximately 120 µm and 85 µm, respectively (Fig. 4D), but then becomes circular about 110 µm in diameter (Figs. 5B, 6B).

Stage 3: The embryo elongates approximately to 185 µm in length along the posterior egg surface (Figs. 5C, 6C). The protocephalon and protocorm differentiate, and the embryo assumes a pear-shape (Fig. 6C). As a result of fusion of amnioserosal folds, the egg surface is entirely covered with the serosa, and the serosal cuticle starts to be secreted beneath the chorion.

Stage 4: The embryo elongates posterior to approximately 350 µm along the egg surface, attaining more than one-third of the egg circumference (Fig. 5D). The protocephalon grows wider into the form of a head lobe (Fig. 6D). Segmentation starts at this stage (not distinctly shown in figures). The serosal cuticle forms a thickening at the posterior pole of the egg (cf. SeCt in Fig. 6D).

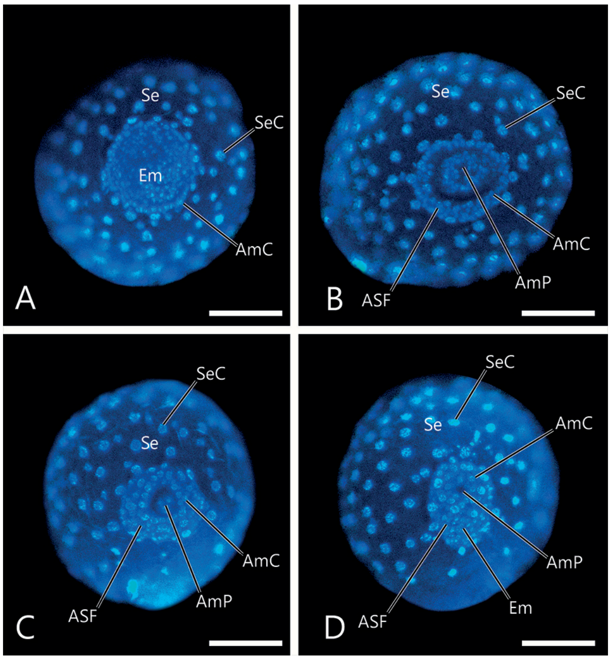

Fig. 4. Embryonic development of *Scopura montana*, Stage 2. DAPI staining, posterior view, anterior of embryo at the top. **A** – **D**: formation of amnioserosal fold, successive stages A to D. — ***Abbreviations***: AmC, amniotic cell; AmP, amniotic pore; ASF, amnioserosal fold; Em, embryo; Se, serosa; SeC, serosal cell. Scale bars = 100 µm.

Stage 5: The embryo elongates posteriorly with its caudal region ahead, and its anteroposterior axis is reversed (Fig. 5E). The thoracic to anterior abdominal region sinks into the yolk with the cephalic and posterior abdominal regions remaining on the egg surface (Figs. 5E, 6E). The embryo then assumes an S-shape. Segmentation proceeds towards the posterior, and appendages develop in the differentiated segments. The stomodaeum appears at the center of the head (Fig. 6E).

Stage 6: The embryo grows and further sinks into the yolk (Fig. 5F). Segmentation and appendage formation continue to proceed. The clypeolabrum appears as a single swelling anterior to the stomodaeum. Antennae and gnathal appendages are clearly visible (Fig. 6F).

Stage 7: The embryo grows in the yolk, attaining its maximum length (Figs. 5G, 6G).

Stage 8: The embryo develops further, with the abdomen enlarged in the yolk, and the head moving close to the egg surface (Figs. 5H, 6H). The appendages develop, and in the maxilla, the palp, coxopodite, galea, and lacinia can be distinguished (Figs. 5H, 6H).

Stage 9: In this stage, katatrepsis occurs. The amnioserosal fold ruptures around the region where the amniotic pore closed, and the embryo appears again on the egg surface (Figs. 5I, 6I). The serosa migrates toward the ventral region of the egg and condenses to form the secondary dorsal organ. The amnion spreads over the area the serosa had occupied, functioning as the provisional dorsal closure (Figs. 5I, 6I). The embryo does not change its position during katatrepsis, and its positioning during intertrepsis on the dorsal side of the egg is maintained,

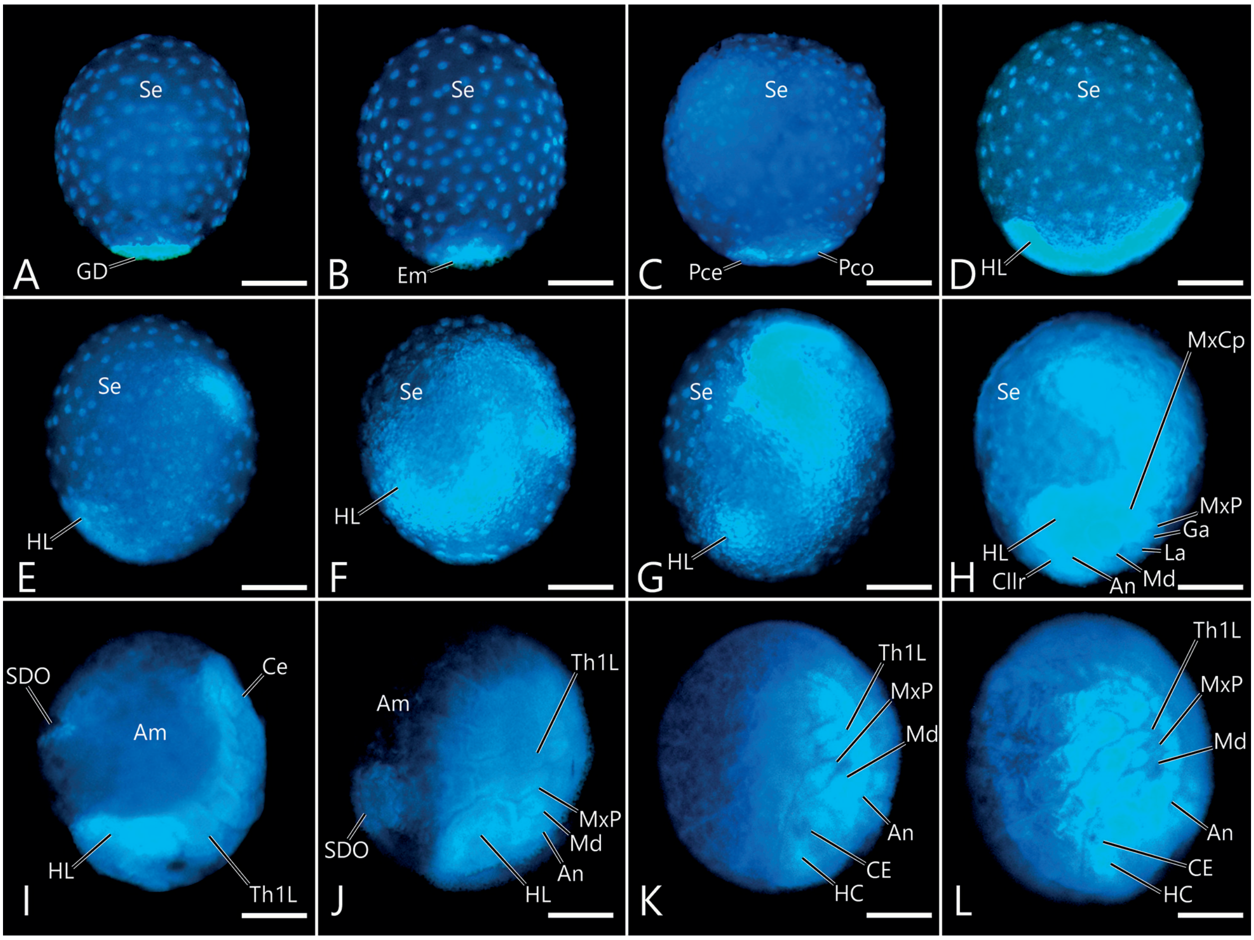

Fig. 5. Embryonic development of *Scopura montana*. DAPI staining, lateral view, anterior at the top, ventral to the left. **A**: Stage 1, **B**: Stage 2, **C**: Stage 3, **D**: Stage 4, **E**: Stage 5, **F**: Stage 6, **G**: Stage 7, **H**: Stage 8, **I**: Stage 9, **J**: Stage 10, **K**: Stage 11, **L**: Stage 12. — ***Abbreviations***: Am, amnion; An, antenna; Ce, cercus; CE, compound eye; Cllr, clypeolabrum; Em, embryo; Ga, galea; GD, germ disc; HC, head capsule; HL, head lobe; La, lacinia; Md, mandible; MxCp, maxillary coxopodite; MxP, maxillary palp; Pce, protocephalon; Pco, protocorm; SDO, secondary dorsal organ; Se, serosa; Th1L, first thoracic leg. Scale bars = 100 µm.

with its head kept at the posterior pole of the egg. As a result, the anteroposterior and dorsoventral axes of the embryo remain opposite to those of the egg, and the embryo reaches hatching keeping this orientation (Figs. 5I–L, 15A–C). The cephalic and thoracic appendages and cerci are well developed: in the labial appendages, the palp and the glossa-paraglossa complex are distinguishable (Figs. 5I, 6I).

Stage 10: The embryo further grows and the definitive dorsal closure proceeds from the posterior (Figs. 5J, 6J). The secondary dorsal organ enlarges.

Stage 11: Definitive dorsal closure is almost complete, and the head capsule acquires its definitive form (Figs. 5K, 6K). The clypeolabrum differentiates into the clypeus and labrum. The frons becomes distinct. A transparent embryonic cuticle is secreted, and the cuticle on the frons forms a sclerotized egg tooth. The compound eyes appear (Fig. 6K).

Stage 12: The larval cuticle is secreted, and the embryo acquires the configuration of the first instar larva (Figs. 5L, 6L). The full-grown embryo tears the chorion and serosal cuticle using the egg tooth and hatches out.

3.1.2. *Obipteryx* sp. (Taeniopterygidae)

Egg. Eggs are spheroidal with long and short diameters approximately 230 µm and 170 µm, respectively (Fig. 1E). The chorion is thin and transparent. A pair of micropylar areas is on both lateral sides at the level of equator, each of which has three to four micropyles ca. 2 µm in diameter with a hood which is the chorionic extension overhanging the micropyle (Fig. 1F).

Embryonic development. The egg period is approximately 125 days, including the diapause period of two months, at 12°C. The embryonic development of *Obipteryx* sp. closely resembles that in *Scopura montana* in general aspects, but in *Obipteryx* sp. the germ disc approximately 20 µm in diameter is much smaller than that of *S. montana*, even when considering its smaller egg size (Stage 1, Fig. 7A). Soon after the amnioserosal folds fuse with each other at the onset of anatrepsis, development enters diapause for approximately 60 days (Stage 2, Fig. 7B). When diapause terminates, the embryo elongates along the posterior egg surface (Stage 3, Fig. 7C). The embryo elongates posterior with its caudal end ahead, attaining more than one-third of the egg circum-

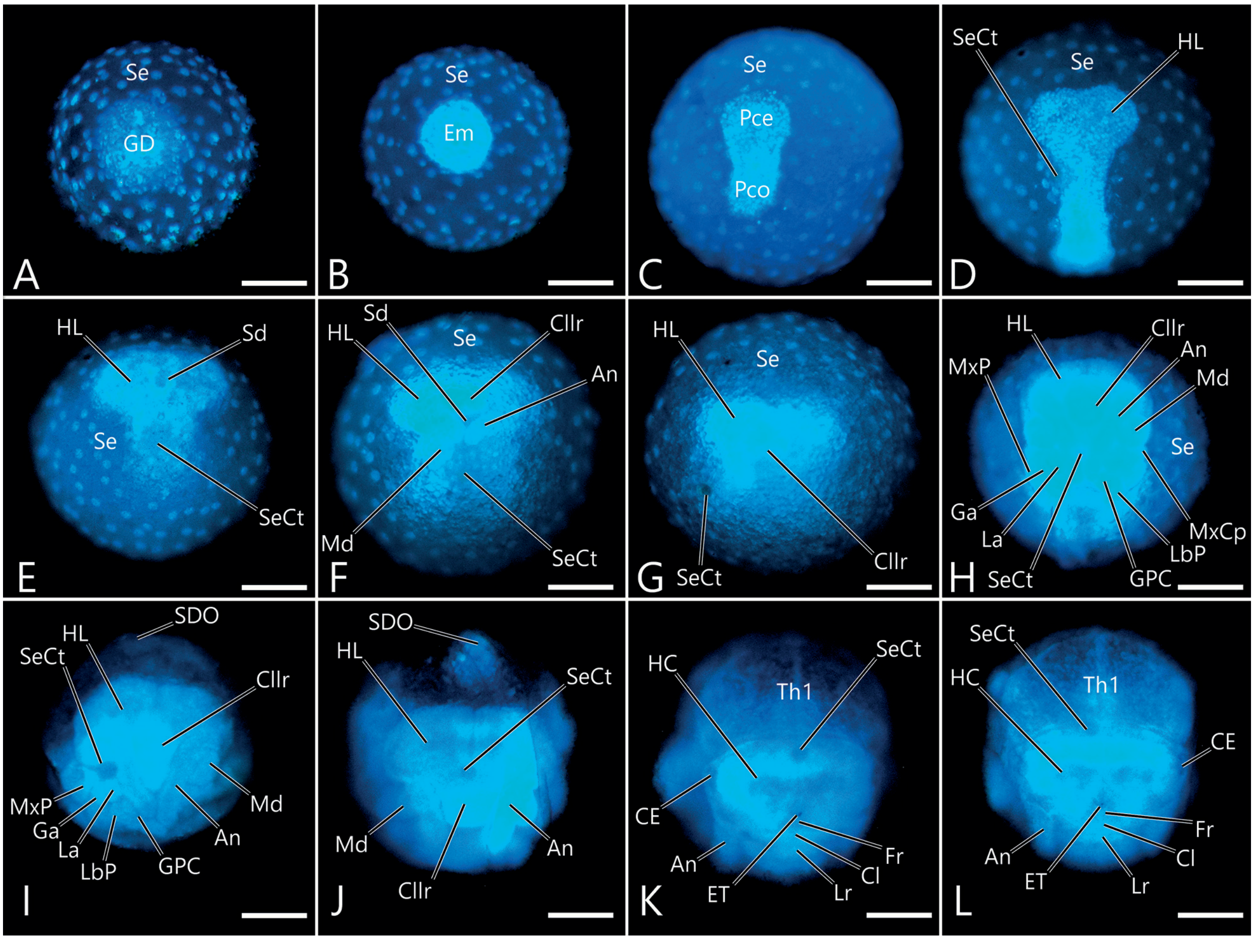

Fig. 6. Embryonic development of *Scopura montana*. DAPI staining, posterior view, ventral at the top. **A**: Stage 1, **B**: Stage 2, **C**: Stage 3, **D**: Stage 4, **E**: Stage 5, **F**: Stage 6, **G**: Stage 7, **H**: Stage 8, **I**: Stage 9, **J**: Stage 10, **K**: Stage 11, **L**: Stage 12. — ***Abbreviations***: An, antenna; CE, compound eye; Cl, clypeus; Cllr, clypeolabrum; Em, embryo; ET, egg teeth; Fr, frons; Ga, galea; GD, germ disc; GPC, glossa-paraglossa complex; HC, head capsule; HL, head lobe; La, lacinia; LbP, labial palp; Lr, labrum; Md, mandible; MxCp, maxillary coxopodite; MxP, maxillary palp; Pce, protocephalon; Pco, protocorm; SDO, secondary dorsal organ; Se, serosa; SeCt, thickening of serosal cuticle; Th1, first thoracic segment. Scale bars = 100 µm.

ference (Stage 4, Fig. 7D). The anterior abdomen (Stage 5, Fig. 7E), followed by the thoracic region, sinks into the yolk with the cephalic region and posterior abdomen remaining on the egg surface, and the embryo assumes an S-shape (Stages 6–8, Fig. 7F–H). The embryo orientation is opposite that of the egg. Katatrepsis (Stage 9, Fig. 7I) and development in post-katatrepsis stages (Stages 10–12, Fig. 7J–L) of *Obipteryx* sp. are similar to those in *S. montana*, and the embryo reaches hatching with its orientation contrary to that of the egg.

3.1.3. *Paraleuctra cercia* (Leuctridae)

Egg. Eggs are spheroidal with long and short diameters approximately 140 µm and 120 µm, respectively (Fig. 1G). The chorion is thin and transparent. No data were obtained on micropyles.

Embryonic development. The egg period is 45–55 days at 12°C. The embryonic development of *Pa. cercia* basically resembles those of the stoneflies described above. The embryo formed (Stages 1, 2, Fig. 8A,B) posteriorly elongates with its caudal end ahead, attaining approximately 40% of the egg circumference (Stages 3, 4, Fig. 8C,D). The following elongation of the embryo and its positioning in the yolk are similar to those shown in the stoneflies described above (Stages 5–8, Fig. 8E–H). The egg and embryo are orientated opposite of each other. Katatrepsis (Stage 9, Fig. 8I) and development in post-katatrepsis stages (Stages 10–12, Fig. 8J–L) are similar to those of the two species described above, and the embryo reaches hatching with its orientation contrary to that of egg.

3.1.4. *Apteroperla tikumana* (Capniidae)

Egg. Eggs are spheroidal with long and short diameters approximately 170 µm and 135 µm, respectively (Fig. 1H). The chorion is thin and transparent. No data were obtained on micropyles.

Embryonic development. The egg period is approximately 55 days at 4°C. Prior to katatrepsis, embryonic development of *A. tikumana* resembles the species shown above (Stages 1–8, Fig. 9A–H), with few minor differences regarding the positioning of the embryo. The early

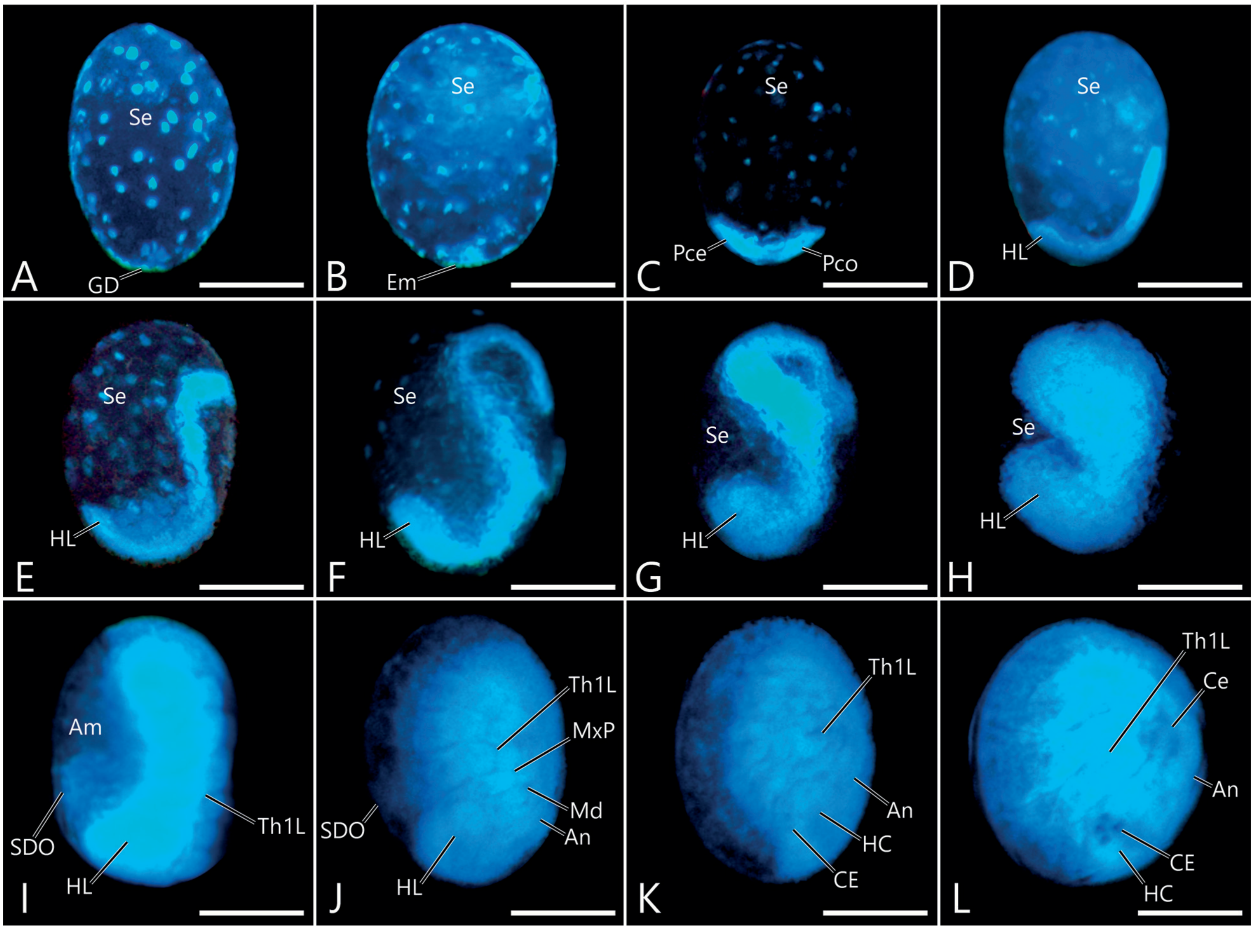

Fig. 7. Embryonic development of *Obipteryx* sp. DAPI staining, lateral view, anterior at the top, ventral to the left. **A**: Stage 1, **B**: Stage 2, **C**: Stage 3, **D**: Stage 4, **E**: Stage 5, **F**: Stage 6, **G**: Stage 7, **H**: Stage 8, **I**: Stage 9, **J**: Stage 10, **K**: Stage 11, **L**: Stage 12. — ***Abbreviations***: Am, amnion; An, antenna; Ce, cercus; CE, compound eye; Em, embryo; GD, germ disc; HC, head capsule; HL, head lobe; Md, mandible; MxP, maxillary palp; Pce, protocephalon; Pco, protocorm; SDO, secondary dorsal organ; Se, serosa; Th1L, first thoracic leg. Scale bars = 100 µm.

embryo grows also anteriorly (Stages 3, 4, Fig. 9C,D), and the cephalic end of the embryo attains approximately the middle of the ventral side of the egg (Stages 6–8, Fig. 9F–H). The immersion of the embryo into the yolk in Stage 5 is restricted to the caudal region of the abdomen (Fig. 9E). In katatrepsis, differently from the three above-mentioned stoneflies, the embryo appeared on the egg surface moves along the egg surface with its head ahead, passing the posterior pole of the egg, then on the egg's ventral side toward the anterior pole. Consequently, the anteroposterior and dorsoventral axes of the embryo, which had been in an opposite orientation to those of the egg during intertrepsis (e.g., Stage 8, Fig. 9H), are reversed and now correspond to those of the egg. The serosa is condensed dorsoposterior to the head, and the secondary dorsal organ is formed on the dorsal side of the egg (Stage 9, Fig. 9I). The embryo maintains its orientation in accord with the egg, and the embryo continues developing and reaches hatching (Stages 10–12, Fig. 9J–L).

3.1.5. *Protonemura towadensis* (Nemouridae)

Egg. Eggs are spheroidal, with long and short diameters approximately 150 µm and 130 µm, respectively (Fig. 1I). The chorion is thin and transparent. In the egg, two micropyles approximately 2 µm in diameter are located on the equator (Fig. 1J).

Embryonic development. The egg period is approximately 45 days at 8°C. The embryonic development of *Pr. towadensis* (Fig. 10A–L) resembles that of *Apteroperla tikumana* in general aspects (Fig. 9A–L), with minor differences regarding the positioning of the embryo. In *Pr. towadensis*, the embryo extends less anteriorly (Fig. 10E–H), and the immersion of the embryo in Stage 5 is more extensive relative to *A. tikumana* (Fig. 9E). Katatrepsis occurs in Stage 9 (Fig. 10I), and as in *A. tikumana*, the anteroposterior and dorsoventral axes of the embryo become to correspond to those of the egg (Stages 9–12, Figs. 10I–L, 15D–F).

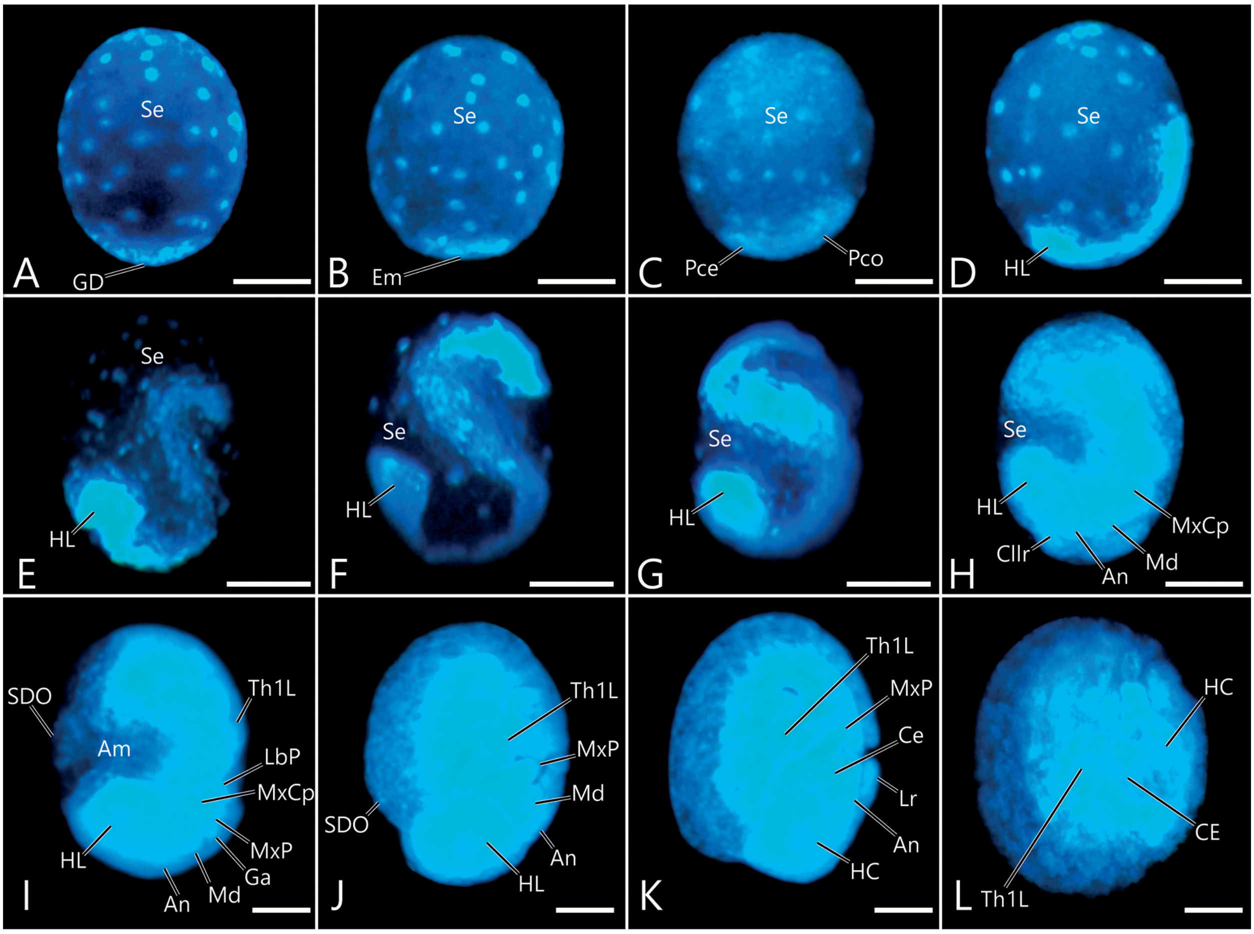

Fig. 8. Embryonic development of *Paraleuctra cercia*. DAPI staining, lateral view, anterior at the top, ventral to the left. **A**: Stage 1, **B**: Stage 2, **C**: Stage 3, **D**: Stage 4, **E**: Stage 5, **F**: Stage 6, **G**: Stage 7, **H**: Stage 8, **I**: Stage 9, **J**: Stage 10, **K**: Stage 11, **L**: Stage 12. — ***Abbreviations***: Am, amnion; An, antenna; Ce, cercus; CE, compound eye; Cllr, clypeolabrum; Em, embryo; Ga, galea; GD, germ disc; HC, head capsule; HL, head lobe; LbP, labial palp; Lr, labrum; Md, mandible; MxCp, maxillary coxopodite; MxP, maxillary palp; Pce, protocephalon; Pco, protocorm; SDO, secondary dorsal organ; Se, serosa; Th1L, first thoracic leg. Scale bars = 50 µm.

3.2. Embryonic development of Systellognatha

Due to the thick, tough chorion of Systellognatha eggs, the chorion was removed prior to observing embryonic development. However, it is very difficult to remove the chorion from Stage 1 eggs because the serosal cuticle is yet to be secreted. Therefore, with the exception of *Yoraperla uenoi* (Peltoperlidae), which has an anteroposteriorly flattened egg, we could not observe Stage 1 in the remaining Systellognatha.

3.2.1. *Calineuria stigmatica* (Perlidae)

Egg. The eggs are spheroidal with long and short diameters approximately 550 µm and 400 µm, respectively (Fig. 2A). The chorion is smooth and fuscous in color. At the posterior pole of the egg, the chorion is modified into a collar-shaped protrusion (Fig. 2A,C), which is covered with an adhesive attachment apparatus, known as the anchor plate (anchor, anchor base, or basal plate) (Fig. 2B). At one third from the anterior pole of the egg, 10–15 micropyles about 5 µm in diameter are arranged in a circle (Fig. 2A,D,E).

Embryonic development. The egg period is 200–250 days, including the diapause period of three months, at 12°C. Embryonic development basically resembles those of the stoneflies described above, especially those stoneflies whose embryos' axes reversed during katatrepsis. When the embryo is about 90 µm in diameter, it forms at the ventral side near the posterior pole (Stage 2, Fig. 11A), and the embryos soon enter diapause for approximately 90 days. When diapause terminates, the embryo starts to elongate (Stage 3, Fig. 11B) with the protocephalon and protocorm differentiated, and the embryo continues to elongate along the egg surface until it extends to approximately 200 µm and covers more than one-third of the egg circumference (Stage 4, Fig. 11C). The anterior abdomen sinks into the yolk in Stage 4, and gnathal and thoracic regions follow in Stages 5–6 (Fig. 11D,E). The embryo develops and acquires an S-shape, with the head and posterior abdomen remaining on the egg surface (Stages 7, 8, Fig. 11F,G). Katatrepsis occurs in Stage 9. The embryo reverses its anteroposterior and dorsoventral

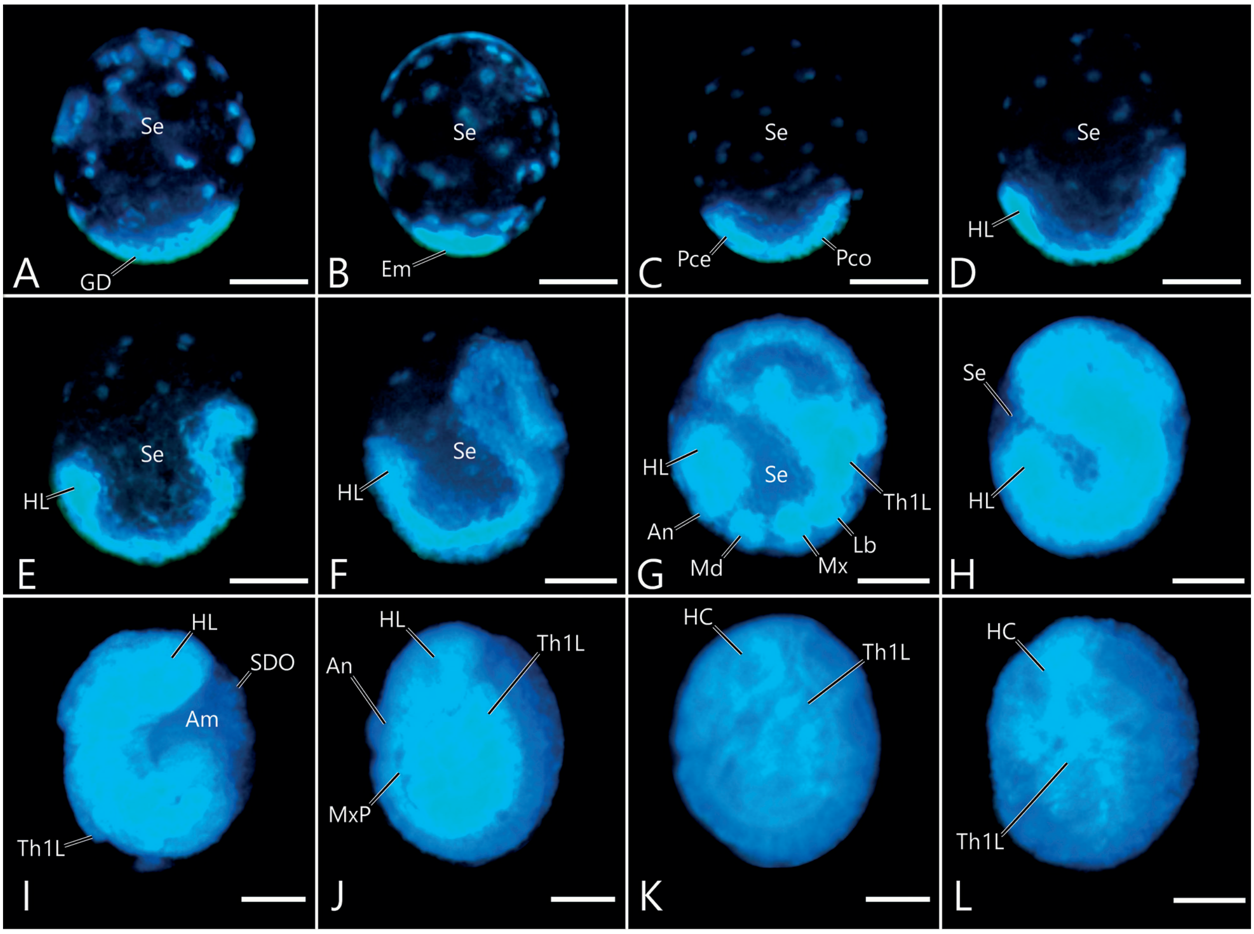

Fig. 9. Embryonic development of *Apteroperla tikumana*. DAPI staining, lateral view, anterior at the top, ventral to the left. **A**: Stage 1, **B**: Stage 2, **C**: Stage 3, **D**: Stage 4, **E**: Stage 5, **F**: Stage 6, **G**: Stage 7, **H**: Stage 8, **I**: Stage 9, **J**: Stage 10, **K**: Stage 11, **L**: Stage 12. — ***Abbreviations***: Am, amnion; An, antenna; Em, embryo; GD, germ disc; HC, head capsule; HL, head lobe; Lb, labium; Md, mandible; Mx, maxilla; MxP, maxillary palp; Pce, protocephalon; Pco, protocorm; SDO, secondary dorsal organ; Se, serosa; Th1L, first thoracic leg. Scale bars = 50 µm.

axes, and the orientation of the embryo corresponds to that of the egg (Fig. 11H). Keeping its orientation according with that of the egg, the embryo continues to develop and then hatches (Stages 10–12, Fig. 11I–K).

3.2.2. *Sweltsa* sp. (Chloroperlidae)

Egg. The eggs are spheroidal with long and short diameters approximately 400 µm and 250 µm, respectively (Fig. 2F). The chorion is smooth and light yellow in color. The specialized structures, including the collar and anchor plate, are lacking. On a third of the anterior part of the egg, six to nine micropyles about 5 µm in diameter are arranged in a circle (Fig. 2G,H).

Embryonic development. The egg period is 50–65 days at 12°C. The embryonic development resembles those of the stoneflies described above, especially those stoneflies whose embryos' axes are reversed in katatrepsis. An embryo of approximately 100 µm in diameter forms at the posterior pole of the egg, which looks thick because of a large curvature around the posterior egg pole (Stage 2, Fig. 12A). The embryo elongates along the egg surface in Stages 3 to 4 (Fig. 12B,C), and the anterior abdomen sinks into the yolk with the cephalic and thoracic regions and posterior abdomen remaining on the egg surface in Stage 5 (Fig. 12D). Development continues through Stages 6–8, with the embryo retaining this posture (Fig. 12E–G). As a result of katatrepsis occurred in Stage 9, the embryo reverses its anteroposterior and dorsoventral axes, and the orientation of the embryo corresponds to that of the egg (Fig. 12H). Maintaining this orientation, the embryo continues to develop and reaches hatching (Stages 10–12, Fig. 12I–K).

3.2.3. *Ostrovus* sp. (Perlodidae)

Egg. The eggs are light yellow in color and unique in shape (Fig. 2I–L). Newly laid eggs are limpet-like in shape and are flattened laterally, i.e., from side to side, their left side being less convex (Fig. 2I–K). As development proceeds, the left side of the egg swells (Fig. 2L). The posterior refers to the direction where the collar and anchor plate exist, and the anterior is opposite: the anchor plate is on the posterior end of the left side (Fig. 2I) and the collar is on the right side (Fig. 2J). When observing the egg from its right side as shown in Fig. 2I, the ventral

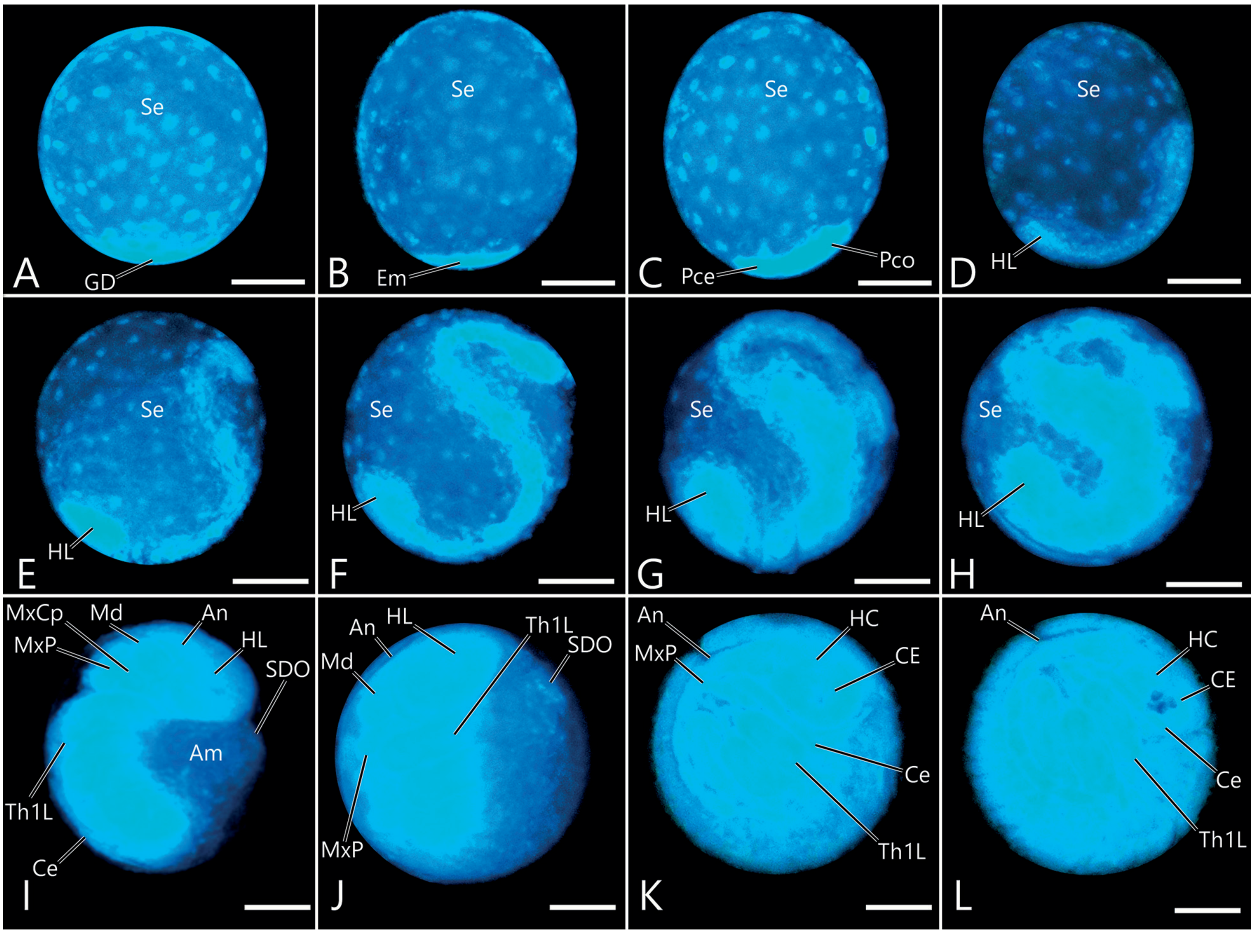

Fig. 10. Embryonic development of *Protonemura towadensis*. DAPI staining, lateral view, anterior at the top, ventral to the left. **A**: Stage 1, **B**: Stage 2, **C**: Stage 3, **D**: Stage 4, **E**: Stage 5, **F**: Stage 6, **G**: Stage 7, **H**: Stage 8, **I**: Stage 9, **J**: Stage 10, **K**: Stage 11, **L**: Stage 12. — ***Abbreviations***: Am, amnion; An, antenna; Ce, cercus; CE, compound eye; Em, embryo; GD, germ disc; HC, head capsule; HL, head lobe; Md, mandible; MxCp, maxillary coxopodite; MxP, maxillary palp; Pce, protocephalon; Pco, protocorm; SDO, secondary dorsal organ; Se, serosa; Th1L, first thoracic leg. Scale bars = 50 µm.

side is to the right hand and the dorsal side to the left. The length (anteroposterior length) of the egg is ca. 400 µm, the width (dorsoventral length) is ca. 320 µm (Fig. 2I,J), and the thickness is ca. 150 µm just after oviposition (Fig. 2K), and ca. 220 µm just before hatching (Fig. 2L). The chorion shows a weak polygonal pattern on its left side and toward the posterior (Fig. 2M,N). Along the equator of the left side of the egg, five to eight longitudinal micropyles are arranged, each with a width of 3 µm (Fig. 2J,M,N).

Embryonic development. The egg period is approximately 80 days at 12°C. In the representations of embryogenesis in the above descriptions, the ventral side is to the left (Figs. 5, 7–12). However, it is difficult to place the eggs with their convex dorsal side down in this species. Therefore, embryogenesis is presented in Fig. 13 with the ventral side of the egg to the right. The embryo ca. 50 µm in diameter forms around the posterior pole (Stage 2, Fig. 13A). However, due to the unique shape of the egg, the newly formed embryo is positioned a little biased to the right side of the egg, as shown in Fig. 13A. The embryo elongates partially twisted (Fig. 13B–F), and the embryos, which have not largely grown within the egg, are seen as if they are sunk in the yolk (Fig. 13B–G). Therefore, while it is difficult to precisely compare embryonic development of *Ostrovus* sp. with the other species, it is clear that it resembles other plecopterans, especially those whose embryonic axes are reversed during katatrepsis. Namely, the formed embryo elongates along the dorsal surface of the egg with its caudal end ahead, and the embryo's anteroposterior and dorsoventral axes become opposed to those of the egg (Fig. 13B–G). Katatrepsis then occurs in Stage 9 (Fig. 13H). The embryo reverses its anteroposterior and dorsoventral axes, and the orientation of the embryo eventually corresponds to that of the egg (Fig. 13H). The embryo grows further and hatches out from the egg, tearing the egg membrane around the anterior third of the right side of the egg (Fig. 13I–K).

3.2.4. *Yoraperla uenoi* (Peltoperlidae)

Egg. The eggs are reddish-brown. Initially the eggs are strongly flattened anteroposteriorly, with a diameter and thickness of approximately 400 µm and 150 µm, respectively (Fig. 2O–R), but as development progresses, they expand to about 240 µm. A transparent anchor plate is on

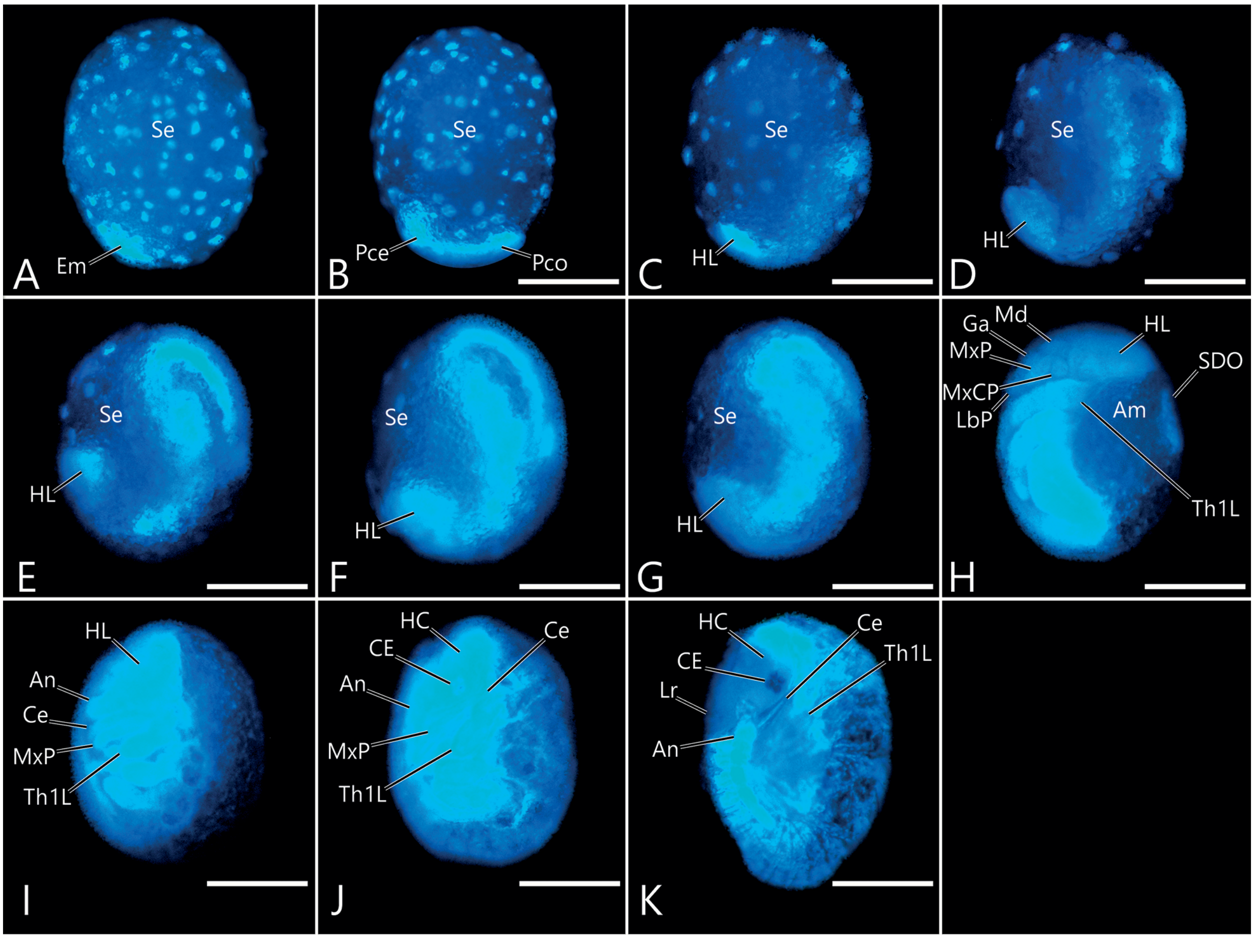

Fig. 11. Embryonic development of *Calineuria stigmatica*. DAPI staining, lateral view, anterior at the top, ventral to the left. **A**: Stage 2, **B**: Stage 3, **C**: Stage 4, **D**: Stage 5, **E**: Stage 6, **F**: Stage 7, **G**: Stage 8, **H**: Stage 9, **I**: Stage 10, **J**: Stage 11, **K**: Stage 12. — ***Abbreviations***: Am, amnion; An, antenna; Ce, cercus; CE, compound eye; Em, embryo; Ga, galea; HC, head capsule; HL, head lobe; LbP, labial palp; Lr, labrum; Md, mandible; MxCp, maxillary coxopodite; MxP, maxillary palp; Pce, protocephalon; Pco, protocorm; SDO, secondary dorsal organ; Se, serosa; Th1L, first thoracic leg. Scale bars = 100 µm.

the posterior side of egg, but the collar is inconspicuous (Fig. 2P,Q). The anterior side of the egg has a honeycomb pattern (Fig. 2O–Q). On the anterior side of the egg five to eight micropylar protuberances of several microns in diameter are roughly arranged in a circle, and a micropyle approximately 1.5 µm in diameter opens at the center of each protuberance (Fig. 2O,R).

Embryonic development. The egg period is approximately 40 days at 12°C. Due to difficultly observing the extremely flattened eggs from the lateral side, we represent embryogenesis in *Y. uenoi* using photos from the posterior side (Fig. 14), which differs from the other species (Figs. 5, 7–13). The germ disc forms at the posterior pole (Stage 1, Fig. 14A). In Stage 2, anatrepsis begins and the amnioserosal folds fuse with each other (Fig. 14B). The embryo begins to elongate in an inverted-triangular shape, with the protocephalon and protocorm differentiated (Stage 3, Fig. 14C), and then forms into a slug-like shape (Stage 4, Fig. 14D). Segmentation and appendage formation commence, and the anterior abdomen sinks into the yolk with the cephalic and thoracic regions and posterior abdomen remaining on the egg surface (Stage 5, Fig. 14E). The embryo then acquires an S-shape (in sections, but not shown here) (Stages 6–8, Fig. 14F–H). Katatrepsis occurs, the embryo slips out of the yolk (Stage 9, Fig. 14I), and is put down sideways with its right side down, changing its posture from warped to ventrally bent (Stage 10, Fig. 14J). Keeping this condition, the embryo further develops (Stage 11, Fig. 14K) and hatches from the egg, tearing the egg membrane at its lateral side (Stage 12, Fig. 14L).

4. Discussion

4.1. Egg

Zwick (1973, 2000) suggested that: 1) the sclerotized hard chorion is a groundplan character of Plecoptera, being universally present in Antarctoperlaria and systellognathan Arctoperlaria; 2) the soft chorion is likely apomorphic to euholognathan Arctoperlaria; whereas 3) systellognathan Arctoperlaria retain the hard chorion, which differentiates into a collar surrounding the adhe-

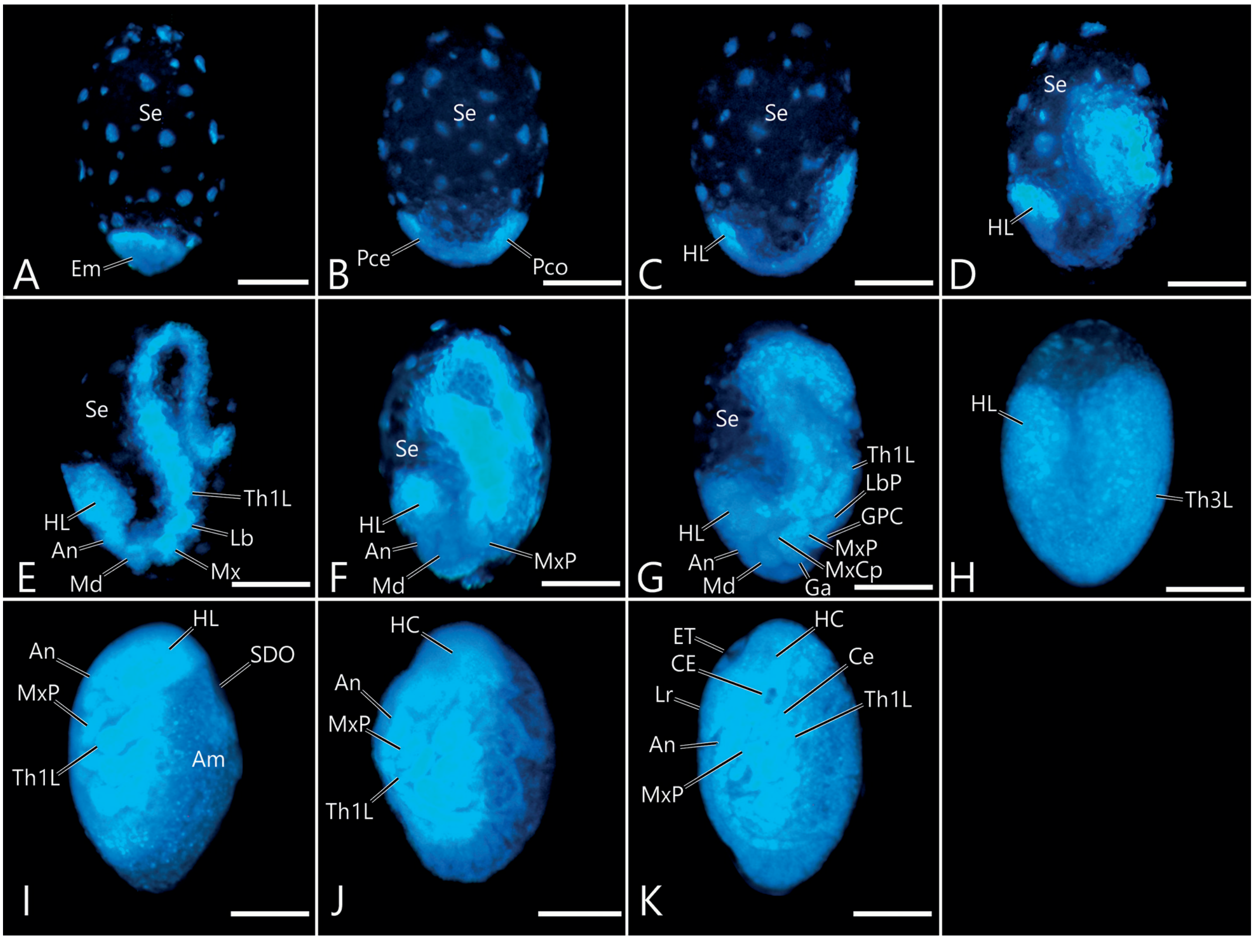

Fig. 12. Embryonic development of *Sweltsa* sp. DAPI staining, lateral view, anterior at the top, ventral to the left. **A**: Stage 2, **B**: Stage 3, **C**: Stage 4, **D**: Stage 5, **E**: Stage 6, **F**: Stage 7, **G**: Stage 8, **H**: Stage 9, **I**: Stage 10, **J**: Stage 11, **K**: Stage 12. — ***Abbreviations***: Am, amnion; An, antenna; Ce, cercus; CE, compound eye; Em, embryo; ET, egg teeth; Ga, galea; GPC, glossa-paraglossa complex; HC, head capsule; HL, head lobe; Lb, labium; LbP, labial palp; Lr, labrum; Md, mandible; Mx, maxilla; MxCp, maxillary coxopodite; MxP, maxillary palp; Pce, protocephalon; Pco, protocorm; SDO, secondary dorsal organ; Se, serosa; Th1,3L, first and third thoracic legs. Scale bars = 100 µm.

sive attachment apparatus (anchor plate) at the posterior pole of the egg. The collar and anchor plate represent the apomorphic groundplan of Systellognatha, because as Hinton (1981) pointed out, these features are not found in other plecopterans, i.e., Antarctoperlaria and Euholognatha, nor in other Neoptera.

Examining the egg structures of nine Japanese arctoperlarians, i.e., five species for Euholognatha – *Scopura montana* (Scopuridae), *Obipteryx* sp. (Taeniopterygidae), *Paraleuctra cercia* (Leuctridae), *Apteroperla tikumana* (Capniidae) and *Protonemura towadensis* (Nemouridae) – and four species for Systellognatha – *Calineuria stigmatica* (Perlidae), *Sweltsa* sp. (Chloroperlidae), *Ostrovus* sp. (Perlodidae) and *Yoraperla uenoi* (Peltoperlidae) – the present study corroborates Zwick's (1973, 2000) understanding of arctoperlarian eggs. We characterize the eggs of Euholognatha and Systellognatha as follows, referring to previous studies as necessary. The eggs of Euholognatha are: 1) spherical or ellipsoid in shape (Fig. 1A,E,G,H,I); 2) without specialized structures such as a collar or anchor plate (Fig. 1A,E,G,H,I); and 3) covered by a thin, transparent chorion, which is smooth and without a conspicuous superficial pattern (Fig. 1E–J), although the exochorion of the anterior third of the egg wears a weak, polygonal network in *S. montana* (Fig. 1B–D). The scopurid egg structure has been described previously with "*Scopura longa* Uéno, 1929" by Kawai & Isobe (1984), but it is likely that the materials examined at the time were in fact *S. montana*, as the scopurids from this sampling site in Mt. Hachibuse, Nagano Prefecture, were more recently identified as *S. montana* (see Uchida & Maruyama 1987).

The eggs of the other arctoperlarian infraorder Systellognatha may be characterized as: 1) spherical or ellipsoidal (Fig. 2A,F), but they sometimes take a specific shape characteristic of each group (Fig. 2I–N,O–Q); 2) equipped with a collar and anchor plate on their posterior pole (Fig. 2A–C,I–N,O–Q); and 3) covered by a thick and hard, colored chorion occasionally containing conspicuous superficial patterns or sculptures (Fig. 2I–N,O–Q) (Knight et al. 1965a,b). The eggs of the chloroperlid species, *Sweltsa* sp., lack the collar and anchor plate. However, because these structures are found predominantly in Systellognatha, and chloroperlid genera are known to include species with and without these structures (Stark et al. 2015), the absence of these struc-

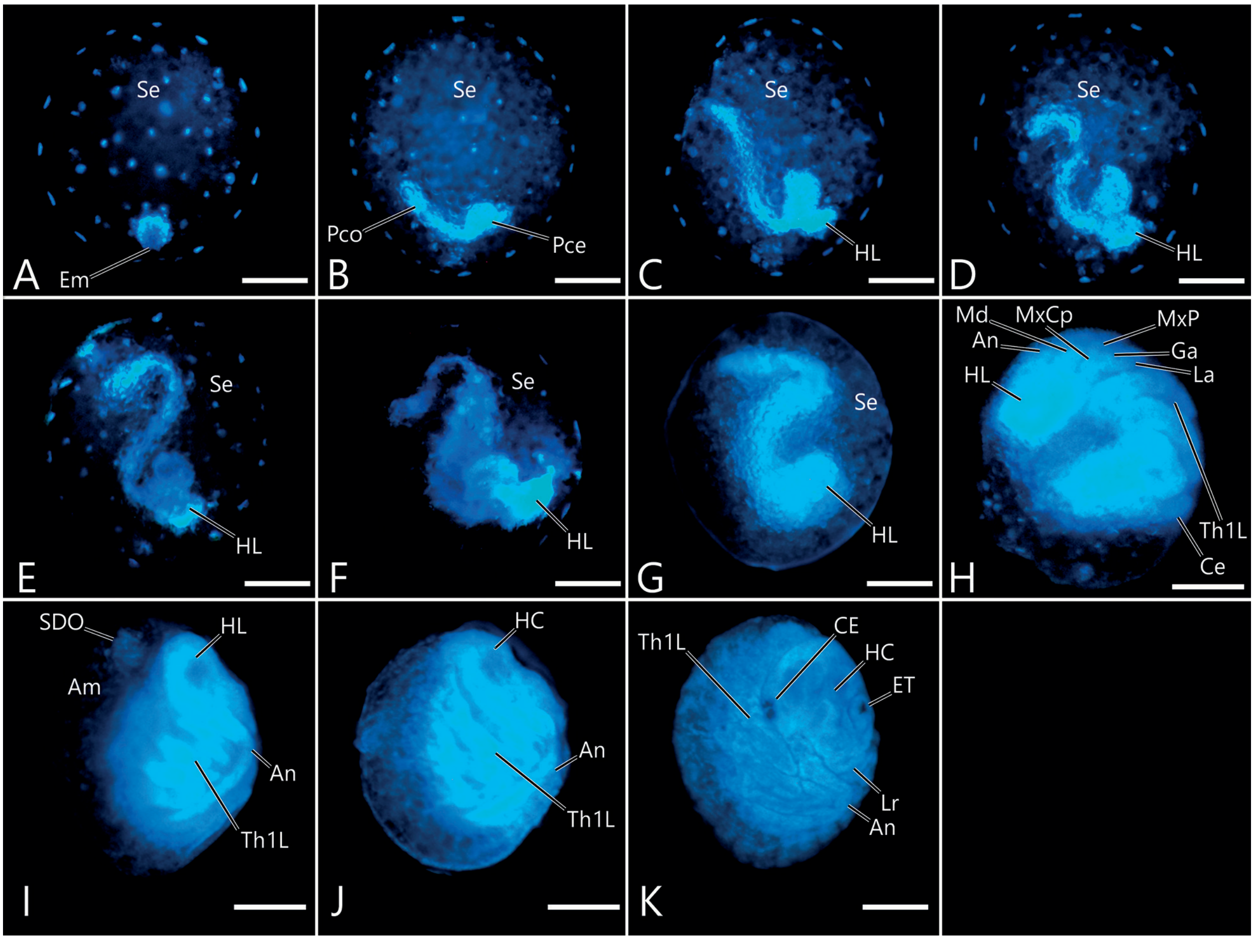

Fig. 13. Embryonic development of *Ostrovus* sp. DAPI staining, lateral view from right side, anterior at the top, ventral to the right. **A**: Stage 2, **B**: Stage 3, **C**: Stage 4, **D**: Stage 5, **E**: Stage 6, **F**: Stage 7, **G**: Stage 8, **H**: Stage 9, **I**: Stage 10, **J**: Stage 11, **K**: Stage 12. — ***Abbreviations***: Am, amnion; An, antenna; Ce, cercus; CE, compound eye; Em, embryo; ET, egg teeth; Ga, galea; HC, head capsule; HL, head lobe; La, lacinia; Lr, labrum; Md, mandible; MxCp, maxillary coxopodite; MxP, maxillary palp; Pce, protocephalon; Pco, protocorm; SDO, secondary dorsal organ; Se, serosa; Th1L, first thoracic leg. Scale bars = 100 µm.

tures in *Sweltsa* sp. may be due to a secondary modification (Fig. 2F).

In the present study, we observed the micropyles of three euholognathan stoneflies: *S. montana*, *Obipteryx* sp., and *Pr. towadensis* (Fig. 1C,D,F,J). In *S. montana*, several micropyles are distributed in a circle in a rosette pattern around the anterior pole of the egg (Fig. 1C,D). In *Obipteryx* sp., micropylar areas with three to four micropyles are located on the equator on both lateral sides of the egg (Fig. 1F), as reported for another taeniopterygid *Brachyptera trifasciata* (Pictet, 1832) (Degrange 1957), and this micropylar arrangement may be characteristic of the Taeniopterygidae. Two micropyles are located on the equator in *Pr. towadensis* (Fig. 1J), while several micropyles were distributed along the equator and in the posterior half of the egg in another *Protonemura*, *Pr. praecox* (Morton, 1894) (Degrange 1957). Although we failed to detect micropyles in *A. tikumana*, Kishimoto (1997; personal comm.) reported two micropyles located on the lateral side of the egg.

With the exception of *Ostrovus* sp., the micropyles of Systellognatha were arranged in a circle (Fig. 2A,D,G,O). A similar pattern of micropyle distribution has been found in *Pteronarcys proteus*, in the Pteronarcyidae (Miller 1939), as well as in other systellognathan representatives (Stark & Stewart 1981; Isobe 1988), with the circular arrangement being a part of the groundplan of Systellognatha. In *Ostrovus* sp., eggs have a laterally-flattened shape and a unique arrangement of micropyles, with several micropyles arranged in a straight line on the left side of the egg (Fig. 2J,M,N). This unusual micropylar arrangement in this species may be due to a secondary modification related to its unique egg shape.

Additional studies in other species, especially the Antarctoperlaria, are required to reconstruct the groundplan of micropylar distributions in the Plecoptera and Arctoperlaria. However, the circular arrangement of micropyles is quite likely a part of the groundplan of Systellognatha. Moreover, given that one of the euholognathan families Scopuridae also shows a circular arrangement of micropyles, this feature may be regarded as a potential groundplan of Arctoperlaria. Potential explanations for the absence of a circular arrangement of micropyles in the remaining Euholognatha species include a partial interruption of the micropylar arrangement or a reduction of the micropyles, i.e., the micro-

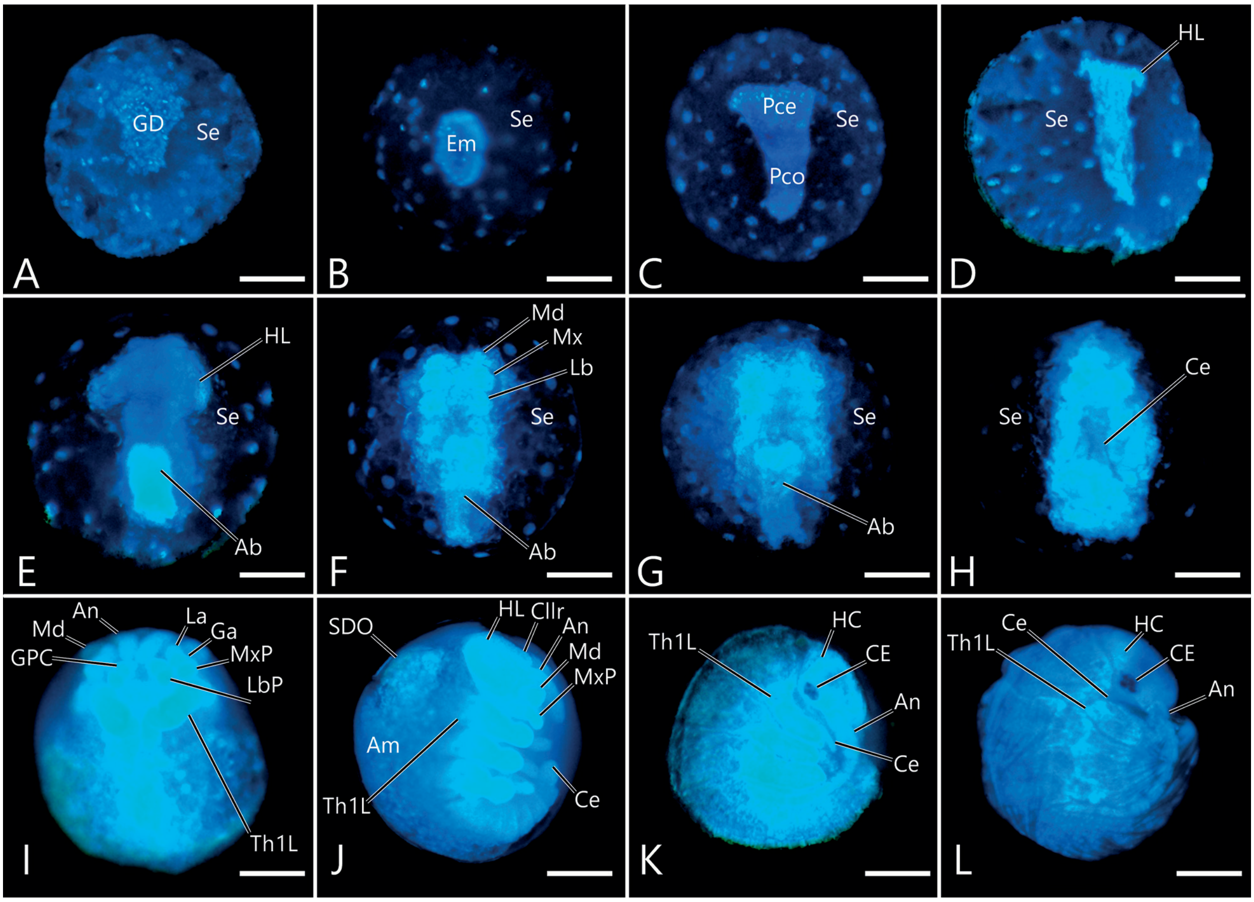

Fig. 14. Embryonic development of *Yoraperla uenoi*. DAPI staining, posterior view. **A**: Stage 1, **B**: Stage 2, **C**: Stage 3, **D**: Stage 4, **E**: Stage 5, **F**: Stage 6, **G**: Stage 7, **H**: Stage 8, **I**: Stage 9, **J**: Stage 10, **K**: Stage 11, **L**: Stage 12. — ***Abbreviations***: Ab, abdomen; Am, amnion; An, antenna; Ce, cercus; CE, compound eye; Cllr, clypeolabrum; Em, embryo; Ga, galea; GD, germ disc; GPC, glossa-palaglossa complex; HC, head capsule; HL, head lobe; La, lacinia; Lb, labium; LbP, labial palp; Md, mandible; Mx, maxilla; MxP, maxillary palp; Pce, protocephalon; Pco, protocorm; SDO, secondary dorsal organ; Se, serosa; Th1L, first thoracic leg. Scale bars = 100 µm.

pyles may have been lost in *Obipteryx* sp. on the dorsal and ventral sides of the egg, and most of those may have been reduced in *Pr. towadensis* and *A. tikumana*. However, explaining the extraordinary arrangement of micropyles reported for *Pr. praecox* will require a reexamination of micropylar arrangement in this and other related species.

4.2. Embryonic development

4.2.1. Formation of the embryo

Mashimo et al. (2014) compared embryogenesis in Hemimetabola, and proposed two embryological autapomorphies of Polyneoptera. One involves elongation of the embryo, as we discuss below in the section "4.2.2.1. Anatrepsis and elongation of the embryo," and the other is on the manner of the embryo's formation. In Polyneoptera, the embryo is formed by the fusion of paired blastoderm regions with higher cellular density: Dermaptera (Shimizu 2013), Embioptera (Jintsu 2010), Phasmatodea (Bedford 1970), Orthoptera (Miyawaki et al. 2004), Zoraptera (Mashimo et al. 2014), Grylloblattodea (Uchifune & Machida 2005), and Blattodea (Fujita & Machida 2017). However, in the Palaeoptera and Acercaria (e.g., Ephemeroptera: Tojo & Machida 1997; Odonata: Ando 1962; Psocodea: Goss 1952; Thysanoptera: Heming 1979), blastoderm cells around the posterior pole concentrate in one area and proliferate to form the embryo. This type of germ disc formation is also known for the apterygote Ectognatha, i.e., Archaeognatha (Machida et al. 1990) and Zygentoma (Masumoto & Machida 2006), clearly suggesting that this is a plesiomorphic condition to Pterygota. Consequently, the formation of the embryo or germ disc by the fusion of paired blastoderm areas with higher cellular density, may be regarded as an apomorphic groundplan of Polyneoptera.

Information on the formation of embryo in Plecoptera is fragmentary. Only two embryological analyses exist for Systellognatha, including Miller (1939) for *Pteronarcys proteus* (Pteronarcyidae) and Kishimoto (1986) for *Kamimuria tibialis* (Perlidae). In *P. proteus*, a small germ disc is formed by the direct migration of a cell group appeared in the yolk on to the blastoderm. In *K. tibialis*, a small germ disc is formed by the simple migration of blastoderm cells. These imply that the embryos form in Plecoptera without involving the fusion of paired blastoderm areas with higher cellular density, which is different from other groups of Polyneoptera. Thus, in the present study, we examined embryo formation in five euholognathan and four systellognathan arctoperlarians, employing DAPI staining, with special reference to the euholognathan *S. montana*. We demonstrated that the embryo is formed by the concentration and proliferation of blastoderm cells around the posterior pole (Fig. 3), as Kishimoto (1986) observed for the systellognathan, *K. tibialis*. Specifically, in Plecoptera the embryo is formed not in the manner involving the fusion of paired blastoderm areas with higher cellular density, which Mashimo et al. (2014) proposed as an apomorphic groundplan feature of Polyneoptera, but in a simple concentration and proliferation of blastoderm cells, as shown in apterygote Ectognatha, Palaeoptera and Acercaria, that may be taken to be plesiomorphic to Pterygota. As described in "1. Introduction," the phylogenetic position of Plecoptera has been debated, but recent comparative morphologies and phylogenomics (e.g., Beutel et al. 2014; Misof et al. 2014) have often bestowed basal positions to Plecoptera within Polyneoptera. The manner of embryo formation in Plecoptera, which seems unique in Polyneoptera, is expected to be critically discussed with respect to phylogenetic reconstruction of Polyneoptera.

In addition, embryo formation in *P. proteus* occurs as a compact cellular aggregation beginning early in development. However, according to Miller (1939), the streaming of a cellular group from inside to the periphery of the egg is involved in embryo formation. Such a centripetal migration of a mass of presumptive embryonic cells is a singular example reported in insects, and critical reexamination is needed.

4.2.2. Blastokinesis

According to Fujita & Machida (2017), we define terms related to blastokinesis as follows. Embryos of Insecta s.str. (Ectognatha: Archaeognatha, Zygentoma, and Pterygota), immerse in the yolk in the early stage of development due to the formation of amnioserosal folds. The embryos then elongate and take their final position in the pre-katatrepsis period. The entire descending process of the embryo from commencement of the amnioserosal fold formation up to this point, is the "anatrepsis." After anatrepsis, the embryos develop until katatrepsis occurs, maintaining this positioning, this phase being the "intertrepsis." The rupture and withdrawal of the amnioserosal folds then occur, which leads to the embryo's reappearance on the egg surface, this ascending process being the "katatrepsis." These processes related to developmental phase are collectively the "blastokinesis." In the present study, we examined blastokinesis in five euholognathan and four systellognathan arctoperlarians, focusing on the euholognathan *Scopura montana*.

4.2.2.1. Anatrepsis and elongation of the embryo. As soon as the germ disc or embryo forms at the posterior pole of the egg, the marginal region begins to extend over the embryo, forming the amnioserosal folds, and anatrepsis starts (Fig. 4A–C). The amnioserosal folds soon fuse with each other (Fig. 4D), and the amniotic pore is completely closed. Thus, in the earliest stage of development, fusion of amnioserosal folds occurs and a compact, ball-shaped "embryo-amnion composite" forms, of which the dorsal and ventral constituents are represented by the embryo proper and amnion, respectively. This process is the same as that reported previously in *Pteronarcys proteus* (Miller 1939) and *Kamimuria tibialis* (Kishimoto & Ando 1985; Kishimoto 1986). The fusion of amnioserosal folds in the earliest stage of development, which leads to the formation of a ball-shaped embryo-amnion composite, is unique to Plecoptera within the Polyneoptera and could be a potential autapomorphy of this group.

The formed embryos elongate along the dorsal side of the egg with their posterior end ahead. After this elongation on the egg surface, the middle part of the embryos curve and sink into the yolk, with their cephalic and caudal ends remaining on the egg periphery (Figs. 5C–E, 6C–E, 7C–E, 8C–E, 9C–E, 10C–E, 11B–D, 12B–D, 13B–D, 14C–E), as described in previous embryological studies on Plecoptera (Miller 1939, 1940; Kishimoto & Ando 1985; Kishimoto 1997). Thus, in Plecoptera, as in the other polyneopteran orders, including Dermaptera (Heymons 1895; Shimizu 2013), Embioptera (e.g., Kershaw 1914), Phasmatodea (e.g., Bedford 1970), Orthoptera (e.g., Roonwal 1937), Zoraptera (Mashimo et al. 2014), Grylloblattodea (Uchifune & Machida 2005), Mantophasmatodea (Machida et al. 2004), Mantodea (Hagan 1917), Blattodea (Heymons 1895; Fujita & Machida 2017), and Isoptera (e.g., Knower 1900), the formation of amnioserosal folds ends at an earlier stage of development and the elongation of the embryo occurring on the egg surface. Mashimo et al. (2014) suggested this feature as another embryological autapomorphy of Polyneoptera, taking it into considerations that in Palaeoptera and Acercaria, the embryo elongates, keeping step with its immersion into the yolk and with the formation of the amnioserosal folds: i.e., Ephemeroptera (Tojo & Machida 1997), Odonata (Ando 1962), Psocoptera (Goss 1952), Phthiraptera (Schölzel 1937), Thysanoptera (Heming 1979), and Hemiptera (Cobben 1968; Heming & Huebner 1994). The present study demonstrates that embryos of Plecoptera elongate in a manner regarded as autapomorphic to Polyneoptera, and the placement of Plecoptera among the Polyneoptera is strongly corroborated. In contrast, there is little support for the phylogenetic hypotheses that places Plecoptera outside of Polyneoptera, i.e., those proposing the sister group relationship of Plecoptera with Neoptera or with "Paraneoptera + Holometabola."

4.2.2.2. Intertrepsis. In most Plecoptera, as a result of anatrepsis (e.g., Fig. 5C–G), the anteroposterior and dorsoventral axes of the embryo become opposed to those of the egg. The exceptions to this pattern are in *Yoraperla uenoi* (Fig. 14) and *Pteronarcys proteus* (Miller 1939), in which free movement of the embryos during blastoki-

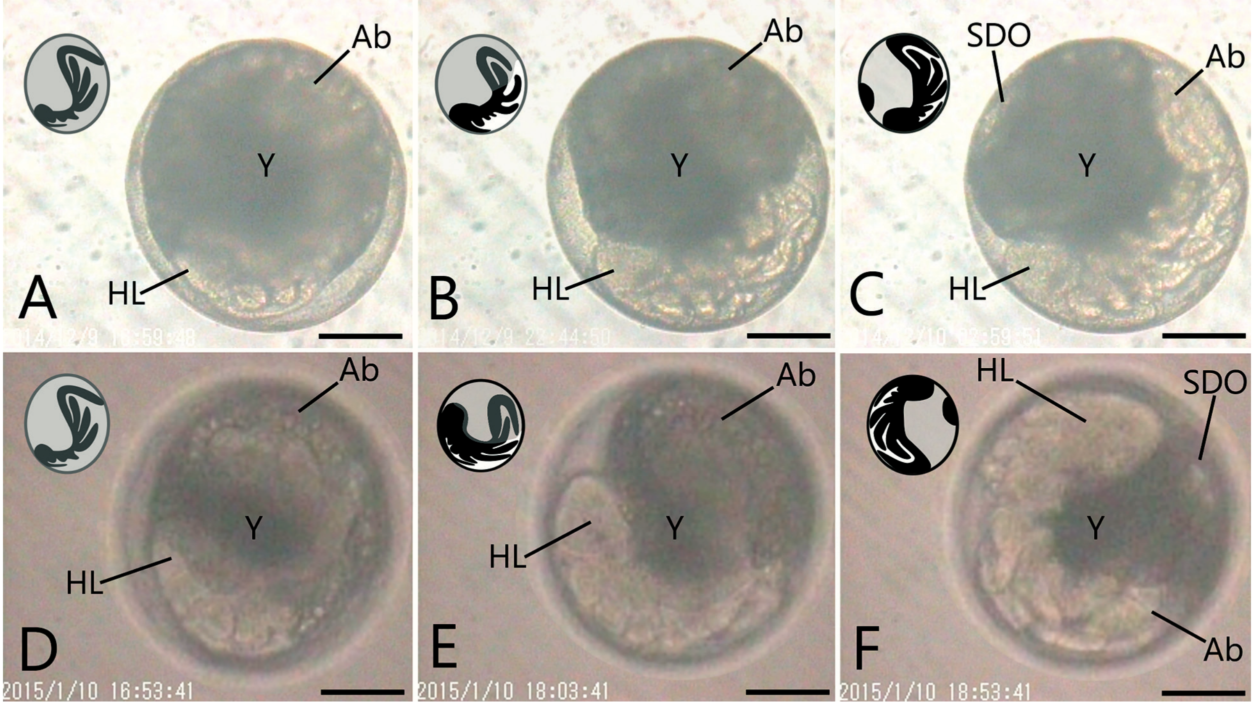

Fig. 15. Time lapse images of katatrepsis in two plecopteran species, lateral view, anterior at the top, ventral to the left. **A**–**C**. *Scopura montana*; **A**: just before katatrepsis, **B**: in katatrepsis, **C**: just after katatrepsis. **D**–**F**. *Protonemura towadensis*; **D**: just before katatrepsis, **E**: in katatrepsis, **F**: just after katatrepsis. — ***Abbreviations***: Ab, abdomen; HL, head lobe; SDO, secondary dorsal organ; Y, yolk. Scale bars A–C = 100 µm; D–F = 50 µm.

nesis may be limited due to their flattened egg shape (cf. "4.2.2.3. Katatrepsis").

As described above, the plecopteran embryos descend into the yolk with their cephalic and caudal ends remaining on the egg periphery, and they keep this posture during intertrepsis (Figs. 5E–G, 7E–G, 8E–G, 9E–G, 10E–G, 11D–F, 12D–F, 13D–F). Such a posture of embryos in intertrepsis may be unique to the Plecoptera within Polyneoptera (see the literature cited in the previous section 4.2.2.1) and may be regarded as a part of the groundplan of Plecoptera.

4.2.2.3. Katatrepsis. In the present study, we examined katatrepsis of nine families of the arctoperlarian Plecoptera, and distinguished three katatrepsis types.

Type 1 – Katatrepsis begins, and the embryo appears on the egg surface. The embryo moves along the egg surface with its head ahead, via the posterior pole of the egg. It then moves to the ventral side of the egg, toward the anterior pole of the egg, and katatrepsis completes. The anteroposterior and dorsoventral axes are reversed to those in intertrepsis. Among the plecopterans examined, the euholognathan Capniidae and Nemouridae, and the systellognathan Perlidae, Chloroperlidae, and Perlodidae fall into this category (Figs. 9I, 10I, 11H, 12H, 13H, 15D–F). Another representative of the Perlidae, *Kamimuria tibialis* also shows Type 1 katatrepsis (Kishimoto & Ando 1985).

Type 2 – Different from Type 1 katatrepsis, the embryo does not change its orientation throughout the course of katatrepsis, and its anteroposterior and dorsoventral axes remain opposed to those of the egg. Among the plecopterans examined, three euholognathan families Scopuridae, Taeniopterygidae, and Leuctridae, are categorized in this type (Figs. 5I, 6I, 7I, 8I, 15A–C). In the study on diapause in the taeniopterygid euholognathan *Brachyptera risi* (Morton, 1896), Khoo (1968b) provided figures showing that this species performs Type 2 katatrepsis.

Type 3 – In the peltoperlid systellognathan *Yoraperla uenoi*, of which eggs are strongly flattened anteroposteriorly (Fig. 2O–Q), the embryo forms at the center of the broad bottom of the egg (Fig. 14A,B), and it grows and elongates there (Fig. 14C–H). Katatrepsis occurs subsequently (Fig. 14I), and the embryo rotates around its anteroposterior axis by 90 degrees, lying sideways on the bottom side of the egg (Fig. 14J). Katatrepsis of this type is also found in the pteronarcyid systellognathan *Pteronarcys proteus*, which has eggs with anterodorsally flattened shape, as in *Y. uenoi* (Miller 1939, 1940).

Katatrepsis involving a reversion of the embryo's axes like Type 1 is predominant in non-holometabolan Pterygota: i.e., in Palaeoptera: Ephemeroptera (Tojo & Machida 1997), and Odonata (Ando 1962); Polyneoptera: Dermaptera (Heymons 1895; Shimizu 2013), Embioptera (e.g., Kershaw 1914), Phasmatodea (e.g., Bedford 1970), Orthoptera (e.g., Roonwal 1937), Zoraptera (Mashimo et al. 2014), Grylloblattodea (Uchifune & Machida 2005), Mantophasmatodea (Machida et al. 2004), Blattodea (Heymons 1895; Fujita & Machida 2017), and Isoptera (e.g., Knower 1900); Acercaria: Psocoptera (Goss 1952), Phthiraptera (Schölzel 1937), Thy-

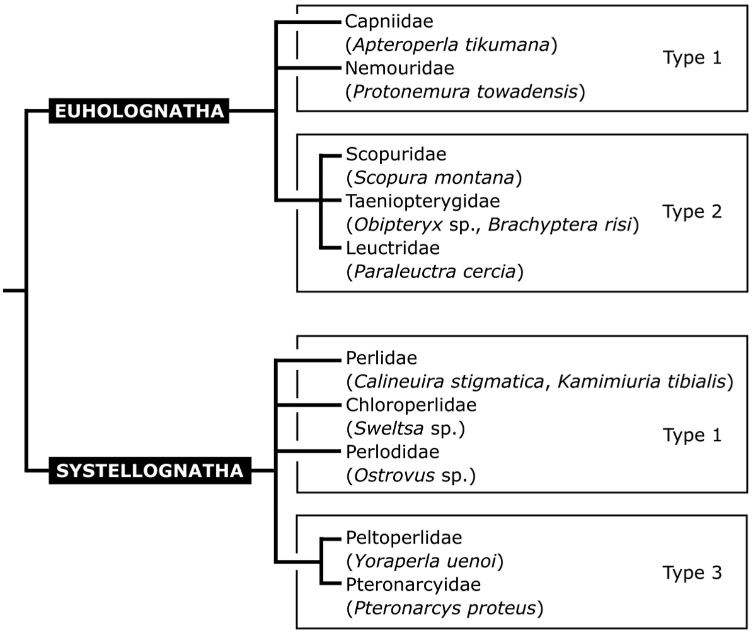

Fig. 16. Phylogenetic reconstruction of 10 arctoperlarian families of which katatrepsis types have been clarified, incorporating the current phylogenetic understanding (e.g., Zwick 2000), i.e., each of Antarctoperlaria, Euholognatha, and Systellognatha is monophyletic (see text).

sanoptera (Heming 1979), and Hemiptera (Cobben 1968). On the other hand, the apterygote Ectognatha, such as the Archaeognatha (Machida et al. 1994) and Zygentoma (Masumoto & Machida 2006), do not follow one of these established types of katatrepsis involving the reversion of the embryo's axes. Therefore, we conclude that this type of katatrepsis is an apomorphic groundplan of Pterygota, and the sharing of Type 1 katatrepsis by some plecopteran lineages, such as the euholognathan Capniidae and Nemouridae, and the systellognathan Perlidae, Chloroperlidae, and Perlodidae, can be referred to as symplesiomorphic.

In contrast, the Type 2 and Type 3 forms of katatrepsis are apparently derived features in Plecoptera. Interfamily relationships in Euholognatha are not well understood (Zwick 2000; Terry 2004; Kjer et al. 2006), but the affinity of the euholognathan Scopuridae, Taeniopterygidae, and Leuctridae is suggested, taking Type 2 katatrepsis for a synapomorphy of them. In Systellognatha, the monophyly of Peltoperlidae, Pteronarcyidae and Styloperlidae is well supported (e.g., Zwick 2000). The sharing of both Type 3 katatrepsis and a flattened egg shape phenotype by the former two families may reflect their phylogenetic affinity.

Figure 16 depicts the distribution of the different katatrepsis types on the phylogeny of the 10 plecopteran families for which katatrepsis type is known. Additional information on blastokinesis from more lineages of Plecoptera, especially the Antarctoperlaria, is strongly desired.

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