Proc. Arthropod. Embryol. Soc. Jpn. 53, 9–13 (2021)

ISSN 1341–1527


## [ORIGINAL PAPER]

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

**Shodo MTOW[1, 2] and Ryuichiro MACHIDA[1]**

[1] *Sugadaira Research Station, Mountain Science Center, University of Tsukuba, Sugadaira Kogen 1278–294, Ueda, Nagano 386–2204, Japan*

[2] *Current affiliation: Faculty of Symbiotic Systems Science and Technology, Fukushima University, Kanayagawa 1, Fukushima, Fukushima 960–1296, Japan*

*E-mail: impulse610@gmail.com (SM)*

**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., 2011; 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 *Pteronarcys 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 tikumana* of Capniidae, *Protonemura towadensis* of Nemouridae and Systellognatha: *Calineuria stigmatica* of Perlidae, *Sweltsa* sp.

of Chloroperlidae, *Ostrovus* 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 Karnovsky'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 methacrylate 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-1500, Bio-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 Karnovsky'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 (Optiphoto-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 scopurid stonefly *Scopura montana* (Table 1).

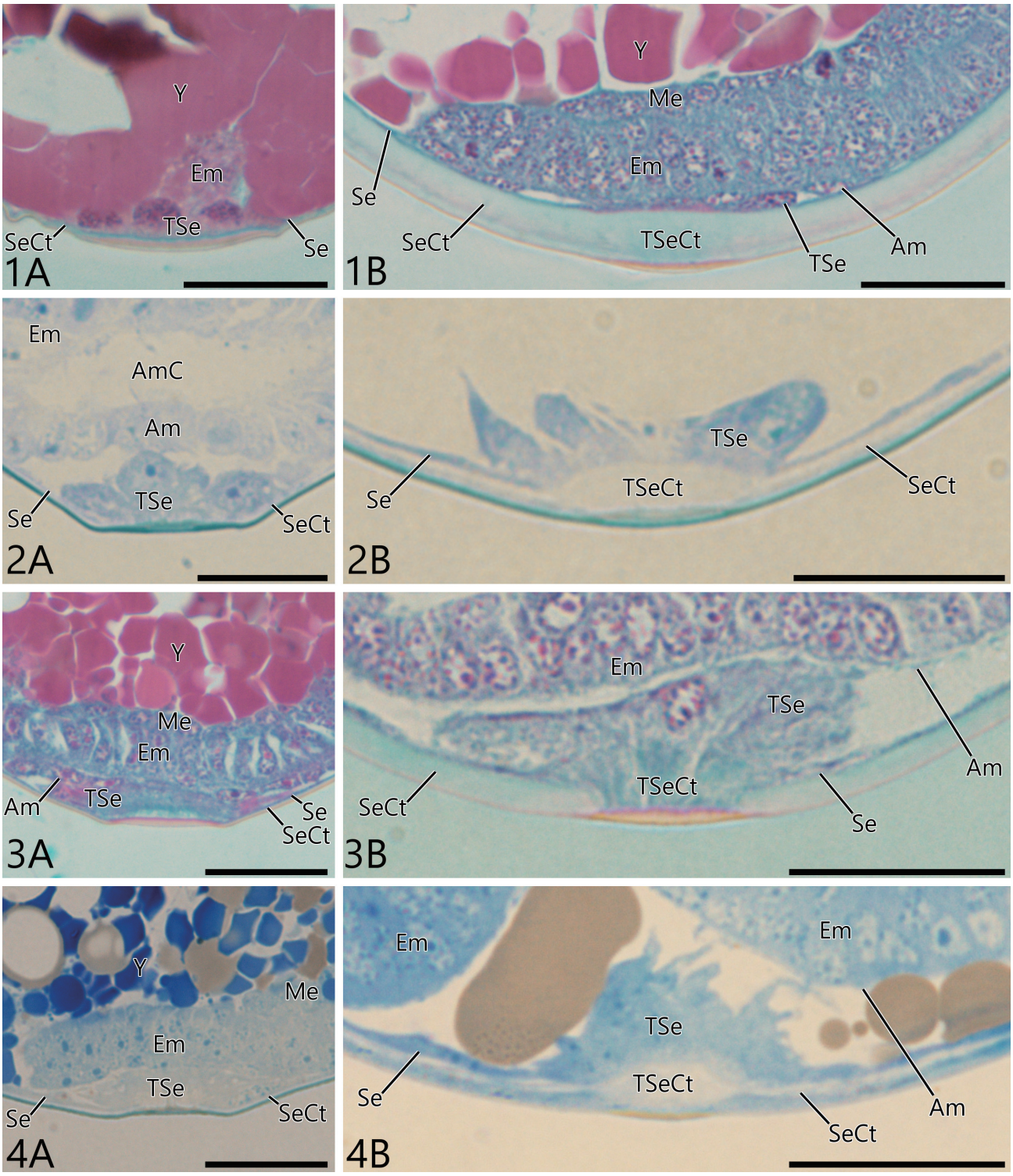
In the case of the Euholognatha, in *Obipteryx* sp. of Taeniopterygidae, in Stage 2, the small thickened serosa forms beneath the embryo as in *S. montana* (Mtow and Machida, 2018b, 2019) and *Pteronarcys proteus* (Millar, 1939, 1940) (Fig. 1A). Almost simultaneously, the serosa starts to secrete the 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 intertrepsis (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 towadensis* of Nemouridae forms beneath the embryo in the same manner as in *Obipteryx* sp. and *Pa. 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, *Sweltsa* sp. of Chloroperlidae, *Ostrovus* 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 (Miller, 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 1 Developmental stages used in the present study based on Mtow and Machida's staging (2018a).

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



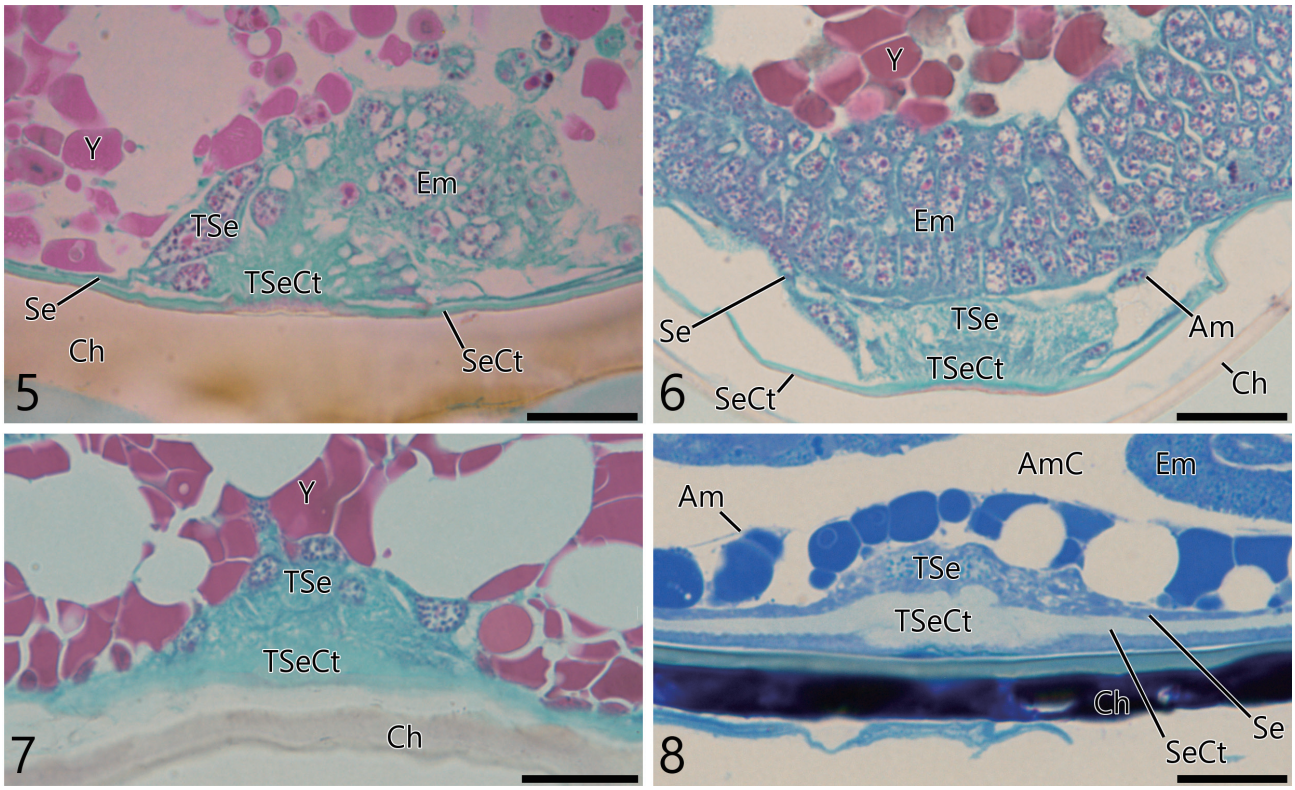

Figs. 1–4 Thickened serosa and serosal cuticle beneath the embryo of four euholognathan stoneflies, chorion removed, anterior of the egg to the top.

Fig. 1 Sections of embryos of *Obipteryx* sp. (Taeniopterygidae), 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% $OsO_4$ for 1 h. A. A section of an embryo in Stage 3. B. A section of an embryo in Stage 7.
Am: amnion, AmC: amniotic cavity, Em: embryo, Me: mesoderm, Se: serosa, SeCt: serosal cuticle, TSe: thickened serosa, TSeCt: thickened serosal cuticle, Y: yolk. Scales = 20 μm.




Figs. 5–8 Thickened serosa and serosal cuticle beneath the embryo of four systellognathan stoneflies, anterior of the egg to the top.
Fig. 5 A vertical section of an embryo of *Calineuria stigmatica* (Perlidae) in Stage 2, methacrylate resin.
Fig. 6 A sagittal section of an embryo of *Sweltsa* sp. (Chloroperlidae) in Stage 4, methacrylate resin, anterior of the embryo to the left.
Fig. 7 A vertical section of an embryo of *Ostrovus* sp. (Perlodidae) in Stage 4, methacrylate resin.
Fig. 8 A sagittal section of an embryo of *Yoraperla uenoi* (Peltoperlidae) in Stage 8, epoxy resin, anterior of the embryo to the left.
Am: amnion, AmC: amniotic cavity, Ch: chorion, Em: embryo, Se: serosa, SeCt: serosal cuticle, TSe: thickened serosa, TSeCt: thickened serosal cuticle, Y: yolk. Scales = 20 μm.

*Acknowledgments*: The present study was supported by a Grant-in-Aid from the JSPS, Research Fellow (JP18J10360) to SM; Scientific Research B (JP16H04825); and Scientific Research C (JP19K06821) to RM. This is a contribution from the Sugadaira Research Station, Mountain Science Center, University of Tsukuba.

## References

Beutel, R.G., F. Friedrich, S.-Q. Ge and X.-K. Yang (2014) Insect Morphology and Phylogeny. Walter de Gruyter, Berlin.

Blumer, M.J.F., P. Gahleitner, T. Narzt, C. Handl and B. Ruthensteiner (2002) Ribbons of semithin sections: An advanced method with a new type of diamond knife. Journal of Neuroscience Methods, **120**, 11–16.

Ishiwata, K., G. Sasaki, J. Ogawa, T. Miyata and Z.-H. Su (2011) Phylogenetic relationships among insect orders based on three nuclear protein-coding gene sequences. Molecular Phylogenetics and Evolution, **58**, 169–180.

Kishimoto, T. and H. Ando (1985) External features of the developing embryo of the stonefly, *Kamimuria tibialis* (Pictet) (Plecoptera, Perlidae). Journal of Morphology, **183**, 311–326.

Kristensen, N.P. (1975) The phylogeny of hexapod "orders". A critical review of recent accounts. Journal of Zoological Systematics and Evolutionary Research, **13**, 1–44.

Machida, R., T. Nagashima and H. Ando (1994a) Embryonic development of the jumping bristletail *Pedetontus unimaculatus* Machida, with special reference to embryonic membranes (Hexapoda: Microcoryphia, Machilidae). Journal of Morphology, **220**, 147–165.

Machida, R., T. Nagashima and T. Yokoyama (1994b) Mesoderm segregation of a jumping bristletail, *Pedetontus unimaculatus* Machida (Hexapoda, Microcoryphia), with a note on an automatic vacuum infiltrator. Proceedings of Arthropodan Embryological Society of Japan, **29**, 23–24. (in Japanese with English figure legend).

Mashimo, Y., R.G. Beutel, R. Dallai, C.-Y. Lee and R. Machida (2014) Embryonic development of Zoraptera with special reference to external morphology, and its phylogenetic implications (Insecta). Journal of Morphology, **275**, 295–312.

McCulloch, G.A., G.P. Wallis and J.M. Waters (2016) A time-calibrated phylogeny of southern hemisphere stoneflies: Testing for Gondwanan origins. Molecular Phylogenetics and Evolution, **96**, 150–160.

Miller, A. (1939) The egg and early development of the stonefly, *Pteronarcys proteus* Newman (Plecoptera). Journal of Morphology, **64**, 555–609.

Miller, A. (1940) Embryonic membranes, yolk cells, and morphogenesis of the stonefly *Pteronarcys proteus* Newman (Plecoptera: Pteronarcidae). Annals of the Entomological Society of America,

**33**, 437–477.

Misof, B., S. Liu, K. Meusemann, R.S. Peters, A. Donath, C. Mayer, P.B. Frandsen, J. Ware, T. Flouri, R.G. Beutel, O. Niehuis, M. Petersen, F. Izquierdo-Carrasco, T. Wappler, J. Rust, A.J. Aberer, U. Aspöck, H. Aspöck, D. Bartel, A. Blanke, S. Berger, A. Böhm, T.R. Buckley, B. Calcott, J. Chen, F. Friedrich, M. Fukui, M. Fujita, C. Greve, P. Grobe, S. Gu, Y. Huang, L.S. Jermiin, A.Y. Kawahara, L. Krogmann, M. Kubiak, R. Lanfear, H. Letsch, Y. Li, Z. Li, J. Li, H. Lu, R. Machida, Y. Mashimo, P. Kapli, D.D. McKenna, G. Meng, Y. Nakagaki, J.L. Navarrete-Heredia, M. Ott, Y. Ou, G. Pass, L. Podsiadlowski, H. Pohl, B.M. von Reumont, K. Schütte, K. Sekiya, S. Shimizu, A. Slipinski, A. Stamatakis, W. Song, X. Su, N.U. Szucsich, M. Tan, X. Tan, M. Tang, J. Tang, G. Timelthaler, S. Tomizuka, M. Trautwein, X. Tong, T. Uchifune, M.G. Walzl, B.M. Wiegmann, J. Wilbrandt, B. Wipfler, T.K.F. Wong, Q. Wu, G. Wu, Y. Xie, S. Yang, Q. Yang, D.K. Yeates, K. Yoshizawa, Q. Zhang, R. Zhang, W. Zhang, Y. Zhang, J. Zhao, C. Zhou, L. Zhou, T. Ziesmann, S. Zou, Y. Li, X. Xu, Y. Zhang, H. Yang, J. Wang, J. Wang, K.M. Kjer and X. Zhou (2014) Phylogenomics resolves timing and pattern of insect evolution. Science, **346**, 763–767.

Mtow, S. and R. Machida (2018a) Egg structure and embryonic development of arctoperlarian stoneflies: A comparative embryological study (Plecoptera). Arthropod Systematics and Phylogeny, **76**, 65–86.

Mtow, S. and R. Machida (2018b) Development and ultrastructure of the thickened serosa and serosal cuticle formed beneath the embryo in the stonefly *Scopura montana* Maruyama, 1987 (Insecta, Plecoptera, Scopuridae). Arthropod Structure and Development, **47**, 643–654.

Mtow, S. and R. Machida (2019) Note on the thickened serosa and serosal cuticle formed beneath the embryo in *Scopura montana* Maruyama, 1987 (Insecta, Plecoptera, Scopuridae). Proceedings of the Arthropodan Embryological Society of Japan, **52**, 11–14.

Song, N., H. Li, F. Song and W. Cai (2016) Molecular phylogeny of Polyneoptera (Insecta) inferred from expanded mitogenomic data. Scientific Reports, **6**, 36175.

Wipfler, B., R. Klug, S.-Q. Ge, M. Bai, J. Göbbels, X.-K. Yang and T. Hörnschemeyer (2015) The thorax of Mantophasmatodea, the morphology of flightlessness, and the evolution of the neopteran insects. Cladistics, **31**, 50–70.

Wipfler, B., H. Letsch, P.B. Frandsen, P. Kapli, C. Mayer, D. Bartel, T.R. Buckley, A. Donath, J.S. Edgerly-Rooks, M. Fujita, S. Liu, R. Machida, Y. Mashimo, B. Misof, O. Niehuis, R.S. Peters, M. Petersen, L. Podsiadlowski, K. Schütte, S. Shimizu, T. Uchifune, J. Wilbrandt, E. Yan, X. Zhou and S. Simon (2019) Evolutionary history of Polyneoptera and its implications for our understanding of early winged insects. Proceedings of the National Academy of Science of the United States of America, **116**, 3024–3029.

Yoshizawa, K. (2011) Monophyletic Polyneoptera recovered by wing base structure. Systematic Entomology, **36**, 377–394.

Zwick, P. (2000) Phylogenetic system and zoogeography of the Plecoptera. Annual Review of Entomology, **45**, 709–746.

Zwick, P. (2009) The Plecoptera – who are they? The problematic placement of stoneflies in the phylogenetic system of insects. Aquatic Insects, **31**, 181–194.