Techniques in embryological studies of mayflies (Insecta: Ephemeroptera)

KOJI TOJO
Developmental Mechanisms Laboratory, Developmental Biology Department, National Institute of Agrobiological Sciences, Oowashi 1-2, Tsukuba, Ibaraki 305-8634, JAPAN
ktojo@nias.affrc.go.jp

RYUICHIRO MACHIDA
Institute of Biological Sciences, University of Tsukuba, Tennoudai 1-1-1, Tsukuba, Ibaraki 305-8572, JAPAN

Abstract

The embryological studies of Ephemeroptera, which are the closest representatives to the pterygote ancestor, have remained not far-reaching, despite their potential values in analyses of phylogenetic accounts. The reason may be derived mainly from the extremely small sizes of ephemeropteran eggs. By improving techniques, we have succeeded in obtaining more detailed embryological images of mayflies. In this paper, we reviewed the techniques employed in our embryological studies.

Keywords: embryology, time-lapse VTR, external morphology, histology, SEM.

Research Update on Ephemeroptera & Plecoptera
2003, E. Gaino (Ed.), University of Perugia, Perugia, Italy.

Introduction

Ephemeroptera, which represent one of the closest extant taxa to early pterygote ancestors, are the most important group for understanding the groundplan of Pterygota or Insecta as well as for clarifying insect evolution. The comparative embryological approach is one of the most promising and useful methods for clarifying existing phylogenetical arguments such as elucidating the groundplan and its evolutionary transition and reconstructing the phylogeny of the group concerned.

Although, for this reason several comparative embryological studies have been attempted since the late 19th century (Joly, 1876; Heymons, 1896a, b, c; Murphy, 1922; Ando and Kawana, 1956; Wolf, 1960; Bohle, 1969; Tsui and Peters, 1974), embryological knowledge of the group has remained insufficient and rather fragmented. The reason for this may be that their extremely small egg sizes have hindered comprehensive studies. We have been improving techniques and developing some new ones, and have succeeded in accumulating detailed embryological knowledge on ephemeropterans (Tojo and Machida, 1996, 1997, 1998, 2000a, b). In this paper, we review the methods employed in our ephemeropteran embryological studies, expecting further development of studies in the field as well as facilitating the embryological studies of the other insects with small egg sizes.

Time-lapse recording technique

A time-lapse VTR system, which enables viewing a slow process such as embryogenesis in mayflies, as a continuous action, is very useful for understanding the outline of morphogenesis and for documenting its dynamic aspects.

The embryos of most mayflies are externally observable because of the transparencies of the chorion and yolk. We set up a time-lapse VTR system with an inverted microscope with a long-working-distance objective (CK-s or CK-40, Olympus, Tokyo; 20x LWD, Olympus, Tokyo), a video tape recorder (SR-9070 or SR-S990, Victor, Tokyo) and a CCD camera (QB, Kenis, Osaka). This system can record, for example, a one-week-process as 10 minutes at shortest: in recording the embryogenesis of an ephemerid *Ephemera japonica* McLACHLAN, 1875 with an approximately two-week-egg-period, a time-lapse with intervals of eight seconds is recommended. Utilizing this system in our embryological studies on the embryogenesis of *E. japonica*, we

succeeded in 1) clearly demonstrating that the approach of the male pronucleus to the female one during fertilization was driven by a streaming of the yolk (Tojo and Machida, 1998), and 2) determining the blastokinesis types in 27 ephemeropteran species covering 17 genera of 10 families to extend the discussion concerning the implications of pterygote blastokinesis (Fig. 1; Tojo and Machida, 2001a).

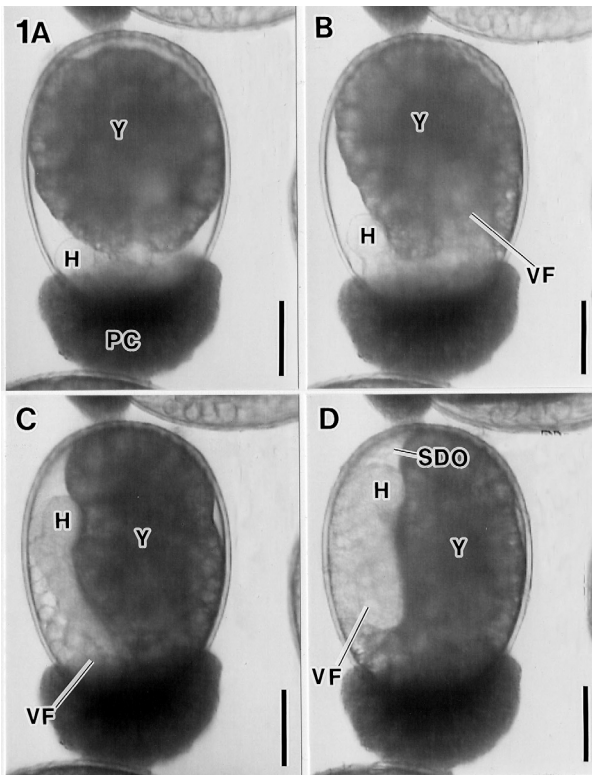


Fig. 1 - A time-lapse VTR observation of *Ephoron eophilum* ISHIWATA, 1996 embryo (lateral views: anterior to the top, ventral to the left). Frames of time-lapse VTR vividly document the dynamic process of blastokinesis. Successive stages of blastokinesis (A-D): katatrepsis (revolution) stage (A-C), and immediately after the katatrepsis stage (D). The egg was oviposited about 9 days before A. Bars = 100 µm. H, head; PC, polar cap; SDO, secondary dorsal organ; VF, ventral flexure; Y, yolk.

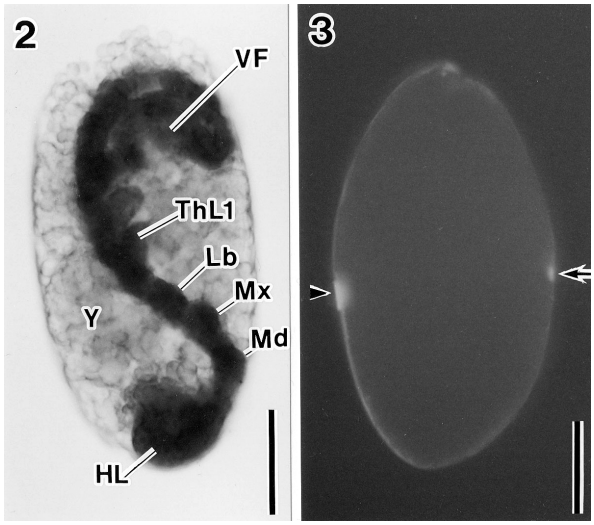
External embryological technique

1. Developmental process in ovo

Total observation of the egg is useful for grasping the localization of the embryo and cellular derivatives *in ovo*. For such an observation, embryo- or cell-specific staining is a prerequisite. For this account, thionine (Thionin, Lauths Violett) and a fluorescent dye Hoechst 33342 (Calbiochem, California) produced good results in the embryological studies of *E. japonica* (Tojo and Machida, 1998).

1-1. Thionine staining

Thionine staining, without any difficulties, produces beautiful and high-contrast images of embryos *in ovo* (Fig. 2). The eggs were fixed with alcoholic Bouin's fluid (saturated alcoholic solution of picric acid : formalin : acetic acid = 15 : 5 : 1) at room temperature for 24 hours, or, after piercing the chorion with a fine needle, with Karnovsky's fluid (2% paraformaldehyde + 2.5% glutaraldehyde HCl-sodium cacodylate solution buffered at pH 7.2) at 4°C for 2 hours. After fixation, the chorion was removed by fine forceps, or a large opening in the chorion (at the extra-embryonic area) was made by the forceps. Eggs fixed and stored in 70% ethyl alcohol were then hydrated through a graded ethyl alcohol series, and stained with 0.1% thionine for several hours. Stained eggs were then dehydrated through a graded ethyl alcohol series (through this process, staining differentiation proceeds), transferred to pure xylene and mounted in Canada balsam.



Figs. 2, 3 – 2) Thionine staining of an *Ephemera japonica* egg (lateral view: anterior to the top). The embryo is beautifully demonstrated in high-contrast. Bar = 50 µm. HL, head lobe; Lb, labium; Md, mandible; Mx, maxilla; ThL1, proleg; VF, ventral flexure; Y, yolk. 3) Hoechst 33342 staining of a newly-laid *E. japonica* egg (lateral view: anterior to the top, ventral to the left). Female (arrowhead) and male (arrow) pronuclei are clearly demonstrated: it is hardly possible to demonstrate these structures in such a high-resolution as this, using any other staining method. Bars = 50 µm.

1-2. Fluorescent dye Hoechst 33342 staining

To observe fine embryonic or cellular structures or demonstrate their localizations *in ovo*, such as pronuclei, cleavage cells and embryonic membrane cells, dyes with higher

specificity to DNA are needed. For this purpose, Hoechst 33342 is one of the most recommended dyes (Fig. 3). In the chorion of eggs fixed with Karnovsky's solution, two small openings were made using fine forceps, and the eggs were washed and stored in the buffer at 4°C. Then the eggs were stained with chilled Hoechst 33342 (1 µg / 1 ml buffer) for several minutes in the dark. Stained eggs were washed with the buffer, then mounted in VECTASHIELD (Vector probes, Burlingame), which is effective against color fading, and observed under a fluorescent microscope.

2. External features of embryos

To examine the morphology of embryos, it is necessary to observe the embryos dissected out of eggs. Embryos prepared from living eggs are more suitable than those processed from fixed eggs: in the latter, aggregates often appear on the embryonic surface and make an accurate examination of important structures difficult.

We squeezed out and dissected embryos from living eggs in physiological saline (Ephrussi-Beadle solution: 0.75% NaCl + 0.035% KCl + 0.021% $CaCl_2$), using forceps sharply ground and fine needles electrolytically made from a tungsten wire (in 15% $NaNO_2$ + 5% NaOH at 6-10V / 5A) or an orthodontic wire Elgiloy (RMO, Denver) (in 50% H_2SO_4 at 6-10V / 5A). Embryos dissected and then washed in saline, were fixed with alcoholic Bouin's fluid or Karnovsky's fluid. The amniotic layer covering the ventral surface of the embryos in the diapause stage was removed with forceps if needed.

For light microscopy, fixed embryos were stained with hematoxylin and observed through an extralong-working-distance objective (20x or 40x Plan ELWD, Nikon, Tokyo) under incident and/or transparent illumination.

For more detailed morphological observation of embryos, a SEM analysis has been employed. Embryos dissected from living eggs and fixed, were sonicated for a few seconds with an ultrasonic cleaner. Then the embryos were dehydrated through a graded ethyl alcohol series and transferred to acetone. The embryos dipped in acetone were dried in an ordinary critical-point drier or transferred to *t*-butyl alcohol and dried in a freeze drier (VFD-21, Shinku Devise, Ibaraki): freeze drying may produce preferable results in less damaged specimens, but it is apt to give rise to extrinsic microaggregates on the embryonic surface, in comparison with that by critical-point drying. Dried embryos were coated with gold and observed under an ordinary high-vacuum SEM (Fig. 4). Specimens without ion-sputtering can be observed under a low-vacuum SEM (SM300 Wet-4, TOPCON, Tokyo): the embryonic surface is observable through thin membranous layers, that hinders accurate observation under usual high-vacuum SEM (cf. Machida, 2000a, b).

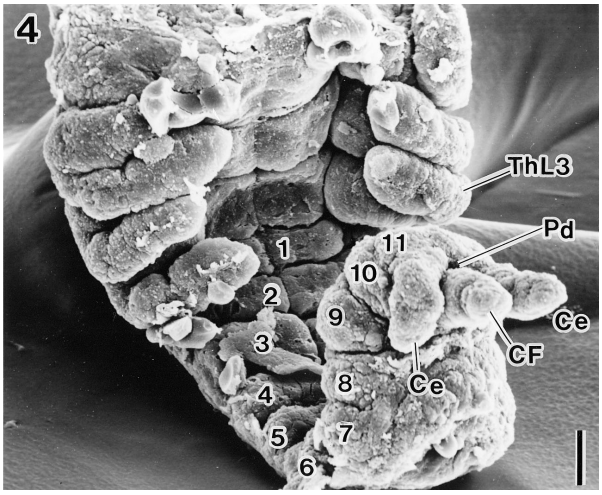


Fig. 4 - A SEM of the posterior half of an advanced *Ephemera japonica* embryo (ventrolateral view). The caudal filament and cerci have different origins: Tojo and Machida (1997) reported that the caudal filament represents the telson whereas the cerci are attributed to the 11th abdominal segment as its appendicular constituents. Bar = 10 µm. 1-11, 1st to 11th abdominal segments; Ce, cercus; CF, caudal filament; Pd, proctodaeum; ThL3, metaleg.

Histological technique

Ephemeropteran small egg size makes it difficult to determine the orientation of eggs or embryos, which is a requisite for the critical histological analysis, and also requires us to minimize artifacts in sectioning as well. Employing the following technique, we obtained satisfactory results in the histological analysis of ephemeropteran embryogenesis (Fig. 5).

For determining the orientation of specimens, we used the agar-embedding method. Eggs or dissected embryos were fixed with alcoholic Bouin's fluid or Karnovsky's fluid, and were stored in 70% ethyl alcohol. The egg or the embryo was hydrated through a graded ethyl alcohol series, then transferred to 3% hot agar solution in a hole slide glass and then oriented. After the agar solution containing the egg or the embryo was coagulated in a refrigerator for a few minutes, the agar block was cut into a small piece (0.5-1.0 cubic mm in volume) containing the egg or embryo, and was stored in 70% ethyl alcohol.

For processing the specimens into sections, we selected the methacrylate resins as embedding media, which yielded sections with fewer artifacts, higher resolution and stainability. Among the methacrylate resins, the Technovit 7100 (Kulzer, Wehrheim) was the most excellent in handling and processing. The agar-embedded specimens were dehydrated through a graded ethyl alcohol series, and transferred to methacrylate resin Technovit 7100 : styrene = 4 : 1 (or = 7 : 3), and then serially sectioned at 1-2 µm thickness using a rotary microtome (JB-4, Polaron, Hertfordshire; HM 325, MICROM, Walldorf) and a tungsten carbide knife (SHK-12 or SHK-20, Meiwa, Tokyo), in accordance with Machida *et al.* (1994a, b). Immersion of eggs or agar blocks with eggs in 10% ammonium mercaptoacetate ethyl alcohol solution for a few hours, before the infiltration on resin, is effective to soften the chorion.

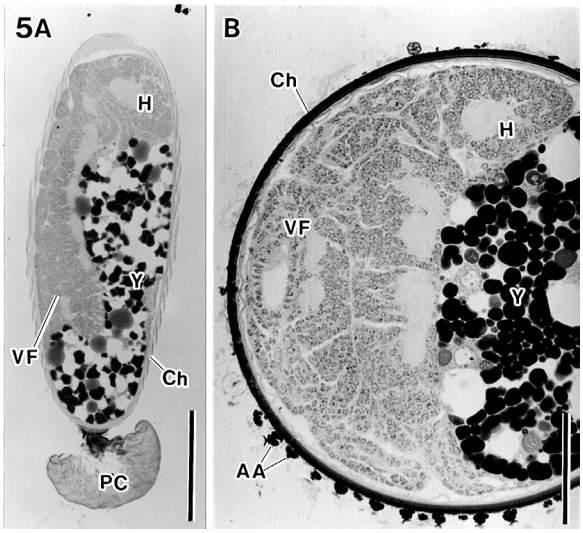


Fig. 5 - Sagittal (a little oblique) sections of *Brachycercus japonicus* GOSE, 1958 (A) and *Isonychia japonica* ULMER, 1919 (B) eggs of post-revolution stages. Methacrylate resin sections yield beautiful sections without structural distortion and histological artifact, despite the tough chorion and serosal cuticle, and provide a high resolution and stainability. Bars = 50 µm. AA, attachment apparatus; Ch, chorion; H, head; PC, polar cap; VF, ventral flexure; Y, yolk.

Sections were stained with Delafield's hematoxylin or Mayer's hematoxylin and eosin (in some cases supplementarily stained with 0.1% fast green FCF), or with Azan (Domagk's modification). When staining with Hoechst 33342 desired, Technovit 8100 is recommended as the embedding resin.

Perspective

We have reviewed the techniques employed in our ephemeropteran embryological studies, with which we have succeeded in obtaining more detailed developmental images of mayflies and reaching the fruitful discussion. Ephemeroptera are the closest representatives to the pterygote ancestor, and embryological studies on them are quite important for elucidating the groundplan of insects and clarifying their evolution. Therefore, we earnestly hope for further investigational developments in this field. The embryological analysis of ephemeropteran wing formation may contribute much to the reconstruction of the insect groundplan.

Recent evolutionary developmental studies analyzed the origin of insect wings from a genetic basis (e.g., Carroll *et al.*, 1995, 2001; Avelof and Cohen, 1997). Their conclusion may indeed support the "gill theory" of insect wing origin (cf. Kukalová-Peck, 1983, 1987, 1991) but results are not conclusive and remain tentative, because these studies came from information only on higher pterygote representatives such as *Drosophila melanogaster* MEIGEN, 1930: information on ancestor pterygotes such as palaeopterans should be seriously searched and taken into account. Ephemeropteran embryological studies combined with the *Hox* genes analysis will break through the problems concerned. We recently found an ephemeropteran that retains and possesses appendages in its abdomen. This has great importance. This implies that we have finally obtained an authentic morphological tool enabling us to analyze the positional homologies between the leg *vs*. wing (bud) (in the thorax) and the appendage *vs*. gill (in the abdomen).

Acknowledgements

We thank Prof. Dr. E. Gaino of Perugia University, and the staff of the 2001 International Joint Meeting Xth International Conference on Ephemeroptera and XIVth International Symposium on Plecoptera for their sincere considerations and cooperation.

References

Ando H., Kawana T., 1956. Embryology of mayfly (*Ephemera strigata* Eaton) as studied by external observation. Kontyû 24:224-232. (in Japanese with English summary).

Avelof M, Cohen S.M., 1997. Evolutionary origin of insect wing from ancestral gills. Nature 385:627-630.

Bohle H.W., 1969. Untersuchungen über die Embryonale Diapause bei *Baëtis vernus* und *Baëtis rhodani*. Zool. Jb. Anat. 86:493-575.

Carroll S.B, Weatherbee S.D., Langeland J.A., 1995. Homeotic genes and the regulation and evolution of insect wing number. Nature 375:58-61.

Carroll S.B, Grenier J.K, Weatherbee S.D., 2001. From DNA to Diversity. Blackwell Science: Wisconsin. pp. 214.

Heymons R., 1896a. Grundzüge der Entwicklung und des Körperbaues von Odonaten und Ephemeriden. Anhang zu den Abhandlungen der Königlichen Akademie der Wissenschaften zu Berlin, Berlin. pp. 66.

Heymons R., 1896b. Mitteilungen über die Lebensweise und Entwicklung von *Ephemera vulagata* L. Sb. Ges. Naturf. Freunde, Berlin 1896: 81-96.

Heymons R., 1896c. Zur Morphologie des Abdominalanhäge bei den Insekten. Morphol. Jb. 24:178-204.

Joly M.N., 1876. On the embryology of the Ephemerae, especially that of *Palingenia virgo*, Oliv. Ann. Mag. Nat. Hist. Lond., Ser. 4. 17:481-484.

Kukalová-Peck J., 1983. Origin of the insect wing and wing articulation from the arthropodan leg. Cand. J. Zool. 61:1618-1669.

Kukalová-Peck J., 1987. New Carboniferous Diplura, Monura, and Thysanura, the hexapod ground plan, and the role of thoracic side lobes in the origin of wings (Insecta). Can. J. Zool. 65:2327-2345.

Kukalová-Peck J., 1991. Fossil history and the evolution of hexapod structures. In: CSIRO, (ed). The Insects Australia 2nd ed. vol. I. Melbourne Univ. Press: Carlton. pp. 141-179.

Machida R., 2000a. Serial homology of the mandible and maxilla in the jumping bristletail *Pedetontus unimaculatus* Machida, based on external embryology (Hexapoda: Archaeognatha, Machilidae). J. Morphol. 245:19-28.

Machida R., 2000b. Usefulness of low-vacuum scanning electron microscopy in descriptive insect embryology. Proc. Arthropod. Embryol. Soc. Jpn. 35:17-19.

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

Machida R, Nagashima T, Yokoyama T., 1994b. Mesoderm segregation of a jumping bristletail, *Pedetontus unimaculatus* Machida (Hexapoda, Microcoryphia), with a note on an automatic vacuum infiltrator. Proc. Arthropod. Embryol. Soc. Jpn. 29:23-24.

Murphy H.E., 1922. Notes on the biology of some of North American species of mayflies. Bull. Lloyd. Lib. Entomol. 2:3-39.

Tojo K., Machida R., 1996. Formation of proctodaeum and terminal abdominal structures of *Ephemera japonica* McLachlan (Insecta: Ephemeroptera). Proc. Arthropod. Embryol. Soc. Jpn. 31:29-32. (in Japanese).

Tojo K., Machida R., 1997. Embryogenesis of the mayfly *Ephemera japonica* McLachlan (Insecta: Ephemeroptera, Ephemeridae), with special reference to abdominal formation. J. Morphol. 234:97-107.

Tojo K., Machida R., 1998. Early embryonic development of the mayfly *Ephemera japonica* McLachlan (Insecta: Ephemeroptera, Ephemeridae). J. Morphol. 238:327-335.

Tojo K., Machida R., 2001a. Katatrepsis of mayflies (Ephemeroptera). Proc. Arthropod. Embryol. Soc. Jpn. 36:11-15. (in Japanese).

Tojo K., Machida R., 2001b. Affinity of Ephemeroptera: a review of the proposed phylogenetic relationships of the major pterygote groups, the Ephemeroptera, Odonata, and Neoptera, based on comparative embryology. In: Bae Y.J., (ed). The 21st Century and Aquatic Entomology in East Asia. Seoul: Jeong Haeng Sa. pp. 85-96.

Tsui P.T.P., Peters W.L., 1974. Embryonic development, early instar morphology, and behavior of *Tortopus inceratus* (Ephemeroptera: Polymitarcyidae). Florida Entomol. 54:349-356.

Wolf B.E., 1960. Zur Karyologie der Eireifung und Furchung bei *Cloeon dipterum* L. (Ephemerida: Baetidae). Biol. Zbl.79:153-198.