

## [ORIGINAL PAPER]

# Embryonic Development of a Diving Beetle, *Hydaticus pacificus* Aubé (Insecta: Coleoptera; Dytiscidae): External Morphology and Phylogenetic Implications\*

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## Abstract

The external features of the egg and developing embryo of the diving beetle *Hydaticus pacificus* Aubé are described from observations based on light and scanning electron microscopy. The egg period is about 50 h at 28°C. The newly laid egg is nearly long ellipsoid, about 2.2 mm long and 0.7 mm wide, and develops to a maximum of 2.7 mm long and 0.9 mm wide. The chorion is thin (about 2 µm thick) and fragile, and has a homogeneous internal structure with a smooth surface. The vitelline membrane beneath the chorion is tough, and appears to have a potential role of an egg-shell in protecting the egg contents when the chorion is damaged during the egg period. On the anterior pole, about 35 micropyles have a circular arrangement in the chorion, and the vitelline membrane just beneath the micropyles forms a large disk-shaped micropylar bulge. The early embryo forms on the ventral side of the egg and is wide and long, occupying about three quarters of the egg length. The embryo elongates and widens, retaining this superficial position throughout development. Based on our embryological observations of this species, the following features of the egg and embryogenesis are presumed to be unique to the Dytiscidae (diving beetles): 1) The presence of many micropyles with a circular arrangement on the anterior pole of the egg; 2) The temporal appearance of a long intercalary segment in the early embryo; 3) The formation of thick longitudinal tracheal trunks accompanied by the closure and disappearance of segmental tracheal openings, except those of the 8th abdominal segment; 4) Fusion of the 9th and 10th abdominal segments accompanied with extreme diminution of their size, and their incorporation into the 8th segment during embryogenesis. The phylogeny of Adephaga is discussed by comparing of the features of eggs and developing embryos among three adephagan families, the Gyrinidae, Carabidae, and Dytiscidae, and the discussion suggests the non-monophyly of aquatic adephagan families, or Hydradephaga.

## Introduction

The Coleoptera, the largest order of Insecta, is composed of four suborders, Archostemata, Myxophaga, Adephaga, and Polyphaga. The second largest suborder, Adephaga, comprises over 36,000 species in 10 families (Maddison *et al.*, 2009). Based on their habitats, the families are often categorized into the terrestrial Geadephaga (Carabidae and Trachypachidae) and the aquatic Hydradephaga [Amphizoidae, Aspidytidae,

Dytiscidae, Gyrinidae, Haliplidae, Hygrobiidae (=Paelobiidae), Meruidae, and Noteridae] (Crowson, 1960), but each monophyly of Geadephaga and Hydradephaga is highly controversial (*e.g.*, Kavanaugh, 1986; Beutel and Roughley, 1988; Maddison *et al.*, 2009).

Despite numerous studies on the embryology of the largest coleopteran suborder, Polyphaga, that comprises about 90% species of Coleoptera, embryological information

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is entirely lacking for Archostemata and Myxophaga, and is scarce for Adephaga, where embryological studies have been undertaken in only three of the 10 families, Gyrinidae, Carabidae, and Dytiscidae. In our recent series of comparative embryological studies of this suborder, the external features of the eggs and developing embryos were described in the whirligig beetle, *Dineutus mellyi* (Gyrinidae), and the carabid ground beetle, *Carabus insulicola* (Carabidae) (Komatsu and Kobayashi, 2012; Kobayashi *et al.*, 2013). In the carabid subfamily Cicindelinae (tiger beetles), the gross embryonic development of *Cicindela togata* was reported by Willis (1967). In the Dytiscidae, however, there are only two old, fragmental studies on the embryonic development of *Dytiscus marginalis* (Korschelt, 1912; Blunk, 1914), and thus our embryological knowledge of the Dytiscidae is insufficient to understand the features of this family from the standpoint of comparative embryology.

The present paper is the third report of our comparative embryological study of Adephaga. It focuses on the external embryonic features of the diving beetle *Hydaticus pacificus* Aubé, a member of the aquatic family Dytiscidae. Based on the features of eggs and developing embryos of this species, the phylogenetic relationships of three adephagan families, the Gyrinidae, Carabidae, and Dytiscidae, are discussed.

## Materials and Methods

The eggs of *Hydaticus pacificus* Aubé, 1838, were deposited singly on the surface of the floating aquatic fern *Salvinia molesta* Mitchell by breeding females in an aquarium from July to September of 2007. A total of about 100 eggs were obtained during this period. Progenitors of the females were captured at several bogs in Fukushima Prefecture, Japan. The egg period was about 50 h at a water temperature of 28°C. The eggs were removed from *S. molesta* by fine brushes, and fixed with alcoholic Bouin's fluid every 1 to 3 h after oviposition.

For observations of whole embryos with a stereomicroscope, the egg-shells (chorion and vitelline membrane) were removed with fine forceps and tungsten needles. For observations of the egg surface by scanning electron microscopy (SEM), fixed eggs were punctured with tungsten needles, dehydrated by passing through an ethyl alcohol/isoamyl acetate series and then air dried. For observations of embryos by SEM, the egg-shells and embryonic membranes (amnion and serosa) were first removed from the fixed eggs immersed in 70% ethyl alcohol using fine forceps, and the fixed embryos were dehydrated and dried as described for the SEM observations of the egg surface. Specimens for SEM were coated with gold and observed using an SEM (JSM-5610, JEOL, Japan).

## Results

### Egg

The newly laid egg is white and long ellipsoid with a slight convexity at the ventral side on which the prospective embryo is formed (Fig. 1A). The anterior pole of the egg is

slightly more roundish than the posterior pole. The egg is about 2.2 mm long and 0.7 mm wide, and enlarges to a maximum of about 2.7 mm long and 0.9 mm wide.

The chorion is thin (about 2  $\mu$ m thick), soft, and transparent, and has a homogeneous internal structure with a smooth surface (Fig. 1A, F, ch). The chorion often teared and peeled away from the egg surface during dehydration for preparing SEM specimens, exposing the vitelline membrane from beneath the chorion onto the egg surface (Fig. 1F, vit). On the anterior pole of the egg, micropyles of about 35 minute holes are arranged in a circular of about 40  $\mu$ m in diameter (Fig. 1D, arrows). Just beneath these micropyles, the micropylar area is distinguished as a disk-shaped bulge of about 45  $\mu$ m in diameter and about 10  $\mu$ m in height (Fig. 1E, mpa). This area is also observed in the eggs with advanced embryonic development (Fig. 4, mpa).

### Embryonic development

Changes in external embryonic features indicate that the egg period (50 h) of *H. pacificus* is divided into 9 stages, which can be expressed as a percentage of total developmental time (DT), with 0% at oviposition and 100% at hatching (Bentley *et al.*, 1979). The major developmental events in these stages are shown in Table 1.

0–8% DT: Maturation of meiotic divisions, fertilization, and cleavage are assumed to occur in the early half of this stage, but their processes were not observed in the present study. At about 6% DT, numerous nuclei of blastoderm cells are observed on the whole egg surface (Fig. 1B, blc).

8–12% DT: A long and wide early embryo, occupying about three quarters of the egg length, is formed on the ventral side of the egg (Fig. 1C). The amnion and serosa are formed by the fusion of amnioserosal folds on the ventral side of the embryo (Fig. 1C, asf). Towards the end of this stage, the embryo divides into a bi-lobed protocephalon and a long protocorm (Fig. 1C, pce, pco).

12–24% DT: The posterior end of the embryo elongates towards the dorsal side of the egg, and thus the embryo is nearly J-shaped in lateral view (Fig. 2A, B). At the posterior end of the protocephalon, antennary rudiments appear as a pair of minute projections (Fig. 2A, at). At the anterior half of the protocorm, segmentation of future gnathal and thoracic regions occurs; *i.e.*, mandibular, maxillary, and labial segments appear in the former region, and three thoracic segments appear in the latter (Fig. 2A, mds, mxs, lbs, th1). In the latter half of this stage, segmentation also begins in the future abdominal region (Fig. 2C, D). Near the anterior end of the protocephalon, the stomodaeum appears as a shallow invagination (Fig. 2C, stom). Although the formation of the primitive groove is assumed to occur in the previous stage, the vestige of the groove is observed along the ventral midline of the protocorm at the beginning of this stage (Fig. 2A, prg), but it disappears by the end of this stage. Yolk segmentation occurs in the latter half of this stage.

24–38% DT: The embryo widens, but its length

somewhat contracts, so that the abdominal end situates at the posterior pole of the egg (Fig. 2E, F). Abdominal segmentation completes and this region becomes 10-segmented. The clypeolabral rudiment appears as a large bi-lobed projection just anterior to the stomodaeum (Fig. 2E, F, cllr). In the gnathal segments, mandibular appendages appear as a pair of globose lobes, but maxillary and labial appendages are formed as a pair of three- and two-segmented projections, respectively. Late in this stage, the rudiments of galeae, or

maxillary endites, appear as globose swellings extending medially in the proximal part of the maxillary appendages (Fig. 2G, ga). In the thoracic segments, the appendages are formed as thick projections developing medio-posteriorly (Fig. 2G, H, th1.l). In the first abdominal segment, pleuropodia first develop into globose projections (Fig. 2E, F, pp), but later become flat and cup-shaped (Fig. 2G, H, pp). In the mesothoracic and metathoracic segments and the first eight abdominal segments, tracheal invaginations appear on both

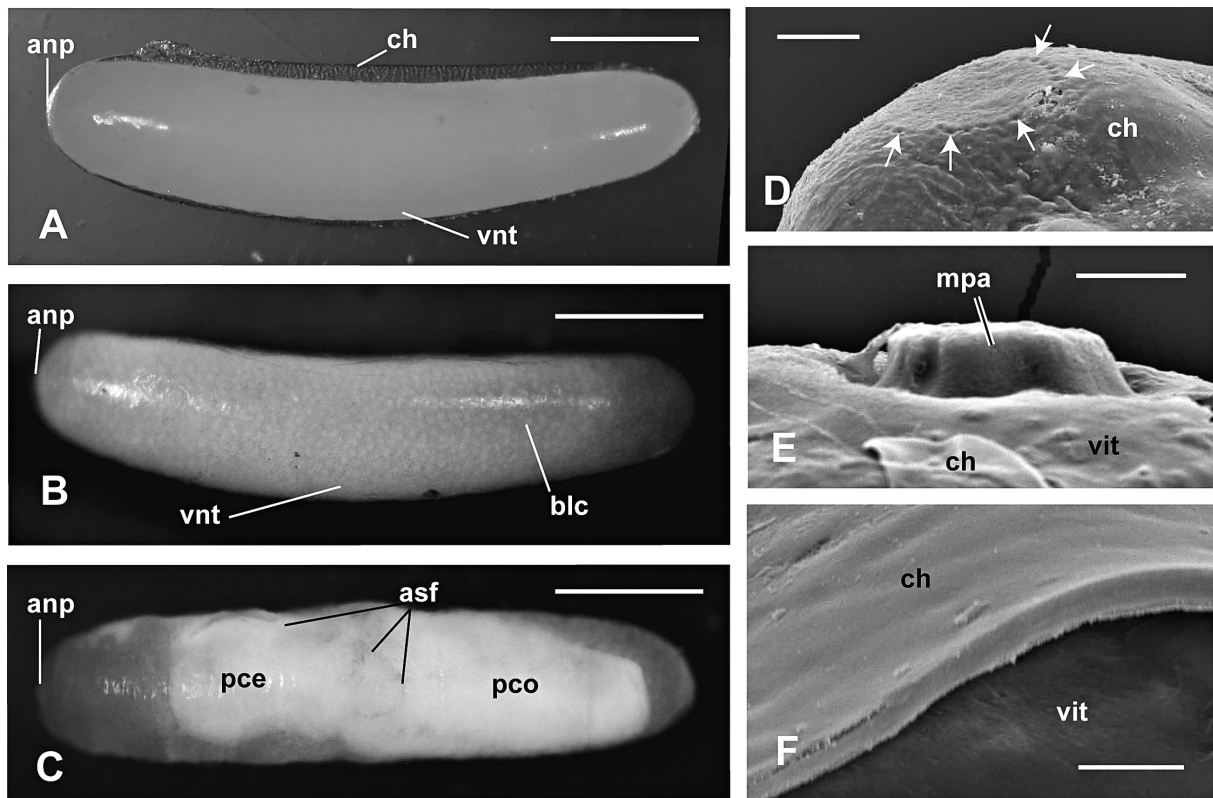


Fig. 1 Light micrographs (A–C) and scanning electron micrographs (D–F) of fixed eggs of *Hydaticus pacificus*. A. Lateral view of a newly laid egg. B. Lateral view of the egg at 6% DT. C. Ventral view of the egg at 12% DT. D. Anterior pole of a newly laid egg, showing micropyles (arrows) arranged in a circle. E. Anterior pole of a newly laid egg, showing the micropylar area (mpa) on the vitelline membrane (vit), and the chorion (ch) peeling away from the micropylar area. F. Egg surface of a newly laid egg, showing the chorion and vitelline membrane; the chorion is partly peeled away from the vitelline membrane. anp: anterior pole, asf: amnioserosal fold, blc: blastoderm cell, pce: protocephalon, pco: protocorm, vnt: ventral side of egg. Scales = A–C: 0.5 mm; D, E: 20  $\mu$ m; F: 5  $\mu$ m.

Table 1 Major developmental events according to successive embryonic stages in *Hydaticus pacificus*. Each stage is expressed as a percentage of total developmental time (DT), with 0% at oviposition and 100% at hatching, which is taken as 50 h after oviposition.

Stage (DT) (%)	Major developmental event
0–8	Formation of blastoderm
8–12	Formation of embryo with protocephalon and protocorm. Formation of amnioserosal fold and completion of embryonic membranes
12–24	Elongation of embryo accompanied by commencement of segmentation. Formation of stomodaeum
24–38	Completion of segmentation. Differentiation of labrum. Appearance of gnathal and thoracic appendages. Formation of proctodaeal invagination. Formation of tracheal invaginations
38–52	Elongation of gnathal and thoracic appendages. Closure of openings of tracheal invaginations except those of the 8th abdominal segment. Fusion of the 9th and 10th abdominal segments
52–66	Rupture of embryonic membranes and progress of provisional dorsal closure. Formation of secondary dorsal organ. Katatrepsis or postural change with ventrally flexed abdominal end. Appearance of urogomphi
66–80	Completion of definitive dorsal closure. Establishment of basic form of the first instar larva. Embryonic molt
80–92	Completion of thick longitudinal tracheal trunks
92–100	Establishment of definitive first instar larval form with egg teeth in head capsule

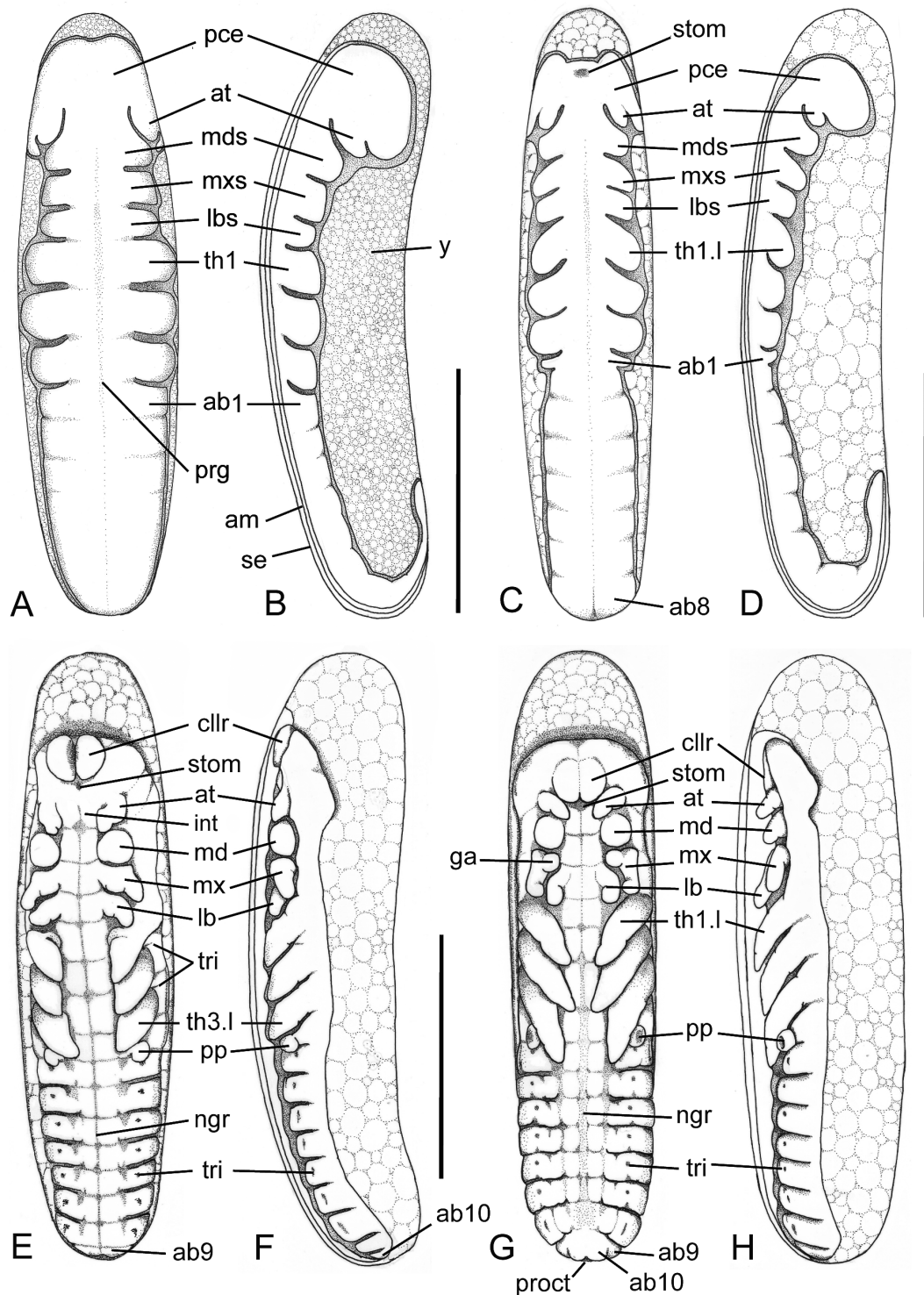


Fig. 2 Successive stages of embryonic development of *Hydaticus pacificus*. The chorion is omitted in all figures. A. About 14% DT (ventral view). B. About 14% DT (lateral view). C. About 20% DT (ventral view). D. About 20% DT (lateral view). E. 30% DT (ventral view). F. 30% DT (lateral view). G. 38% DT (ventral view). H. 38% DT (lateral view). ab1,8,9,10: 1st, 8th, 9th, and 10th abdominal segment. am: amnion, at: antenna, cllr: clypeolabrum, ga: galea, int: intercalary segment, lb: labium, lbs: labial segment, md: mandible, mds: mandibular segment, mx: maxilla, mxs: maxillary segment, ngr: neural groove, pce: protocephalon, pp: pleuropodium, prg: primitive groove, proct: proctodaeum, se: serosa, stom: stomodaeum, th1: prothoracic segment, th1.l: prothoracic leg, th3.l: metathoracic leg, tri: tracheal invagination. y: yolk. Scales = 1 mm.

sides of each segment (Fig. 2E, F; 4, tri). The neural groove appears along the ventral midline of the germ band (Fig. 2E–H, ngr). The small proctodaeal invagination is observed at the abdominal end (Fig. 2G, proct).

In the SEM micrograph showing the embryo at about

30% DT, the intercalary segment is clearly observed as a long (about 110  $\mu\text{m}$ ) segment without appendages, just anterior to the mandibular segment (Fig. 4, mds, int). In the anterior half of the intercalary segment, a pair of low swellings is observed (Fig. 4, arrow). These swellings are assumed to represent to

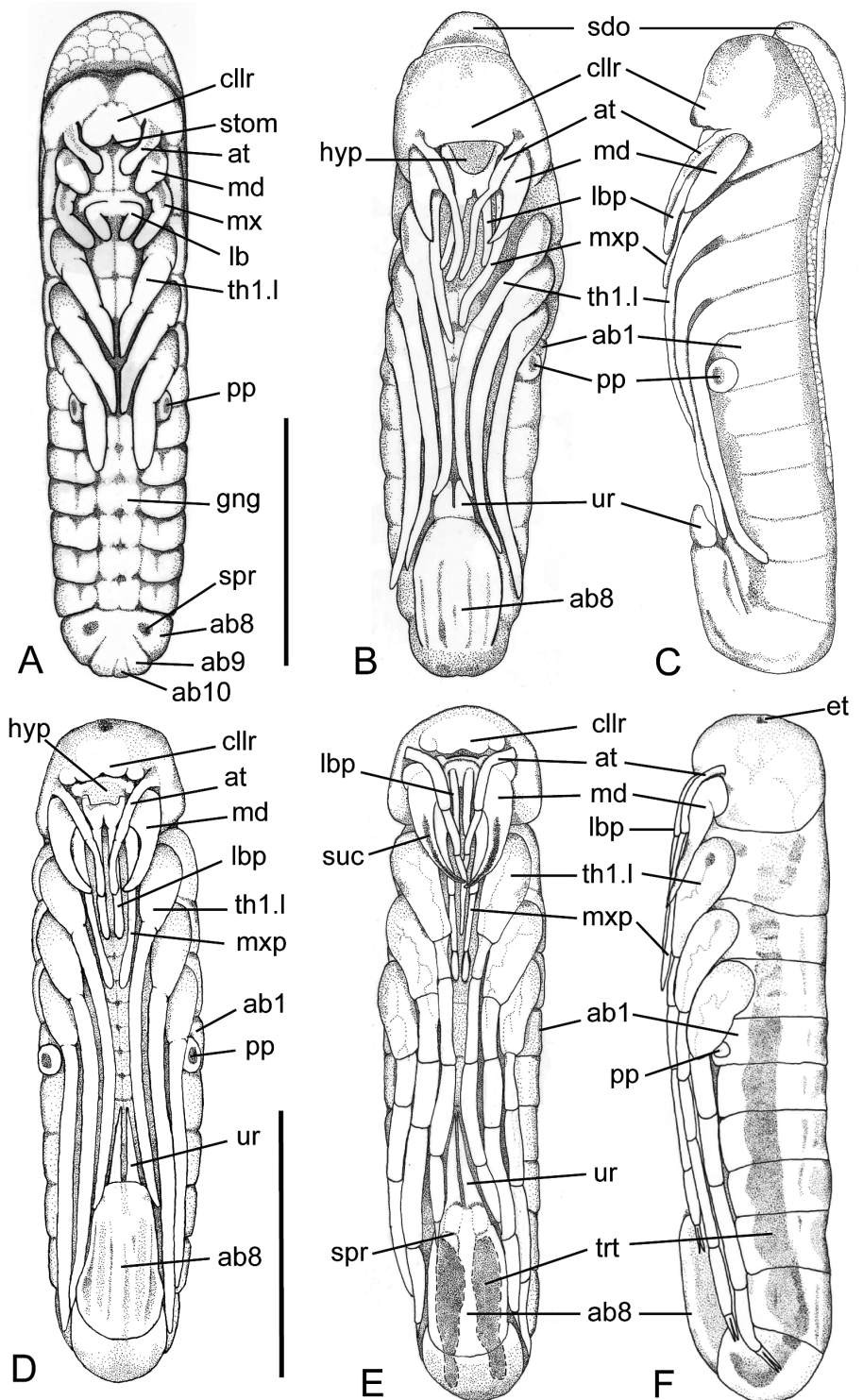


Fig. 3 Successive stages of embryonic development of *Hydaticus pacificus*. The chorion is omitted in all figures. A. 48% DT (ventral view). B. 60% DT (ventral view). C. 60% DT (lateral view). D. 72% DT (ventral view). E. 96% DT (ventral view). F. 96% DT (lateral view). ab1,8,9,10: 1st, 8th, 9th, and 10th abdominal segment, at: antenna, cllr: clypeolabrum, et: egg tooth, gng: ganglion, hyp: hypopharynx, lb: labium, lbp: labial palp, md: mandible, mx: maxilla, mxp: maxillary palp, pp: pleuropodium, sdo: secondary dorsal organ, spr: spiracle, stom: stomodaeum, suc: sucking channel, th1.l: prothoracic leg, trt: tracheal trunk, ur: urogomphus. Scales = 1 mm.

the ganglion mother cells. In the abdominal segments, the openings of the tracheal invaginations of the 7th and 8th segments assume an L-shape and are larger than those of other segments (Fig. 4, tri).

38–52% DT: Early in this stage, the integration of the

cephalognathal region advances (Fig. 3A), *i.e.*, the clypeolabral rudiment becomes flat and extends posteriorly, and the labial appendages on both sides shift medially and fuse to form a labium. The mandibular appendages slightly extend medio-posteriorly, and the maxillary and labial appendages also

extend medio-posteriorly and differentiate their palpi. The thoracic appendages further elongate and their tips attain to the ventral midline. By the end of this stage, the openings of tracheal invaginations close and disappear in the mesothorax, metathorax and the first seven abdominal segments, but the openings in the 8th abdominal segment remain open as spiracles (Fig. 3A, spr). The neural groove is obliterated, and the newly formed ganglia of the ventral nerve cord are observed through a thin ectodermal layer (Fig. 3A, gng). At the abdominal end, the 9th and 10th abdominal segments are fused into one segment accompanied with diminution of size (Fig. 3A, ab9, ab10), and thus the abdomen seemingly consists of 9 segments.

52–66% DT: Katatrepsis or the postural changes of the embryo proceeds (Fig. 3B, C). In this process, the posterior abdominal segments turn ventrally, directing the abdominal end anteriorly. At the abdominal end, the fused 9th and 10th segments are further reduced and integrated into the ventro-posterior side of the large 8th segment so that the abdomen seemingly consists of 8 segments, and urogomphi appear as a pair of stout projections on the ventral side of the abdominal end (Fig. 3B, C, ur). The integration of the cephalognathal region further proceeds and the basic form of the larval head is completed; the antennae, maxillary and labial palpi elongate posteriorly, and the mandibles elongate and become sickle-shaped. The basal region of the fused labium forms the hypopharynx (Fig. 3B, hyp), shifts anteriorly between the maxillae and almost touches the clypeolabrum. The thoracic appendages extremely elongate posteriorly and the tips of the meso- and metathoracic appendages almost reach the posterior pole of the egg.

Accompanying katatrepsis, the embryonic membranes (amnion and serosa) fuse at the ventral side of the embryo, and rupture at this point to form an amnioserosal fold. The embryonic membranes then regress on the dorsal surface of the yolk mass where the concentrated serosa forms a large secondary dorsal organ (Figs. 3B, C; 5A, sdo, asf), and the inverted amnion provisionally covers the dorsal surface of the yolk mass (provisional dorsal closure) (Fig. 5A, am). The anterior region of the secondary dorsal organ temporarily assumes a cap-like structure on the dorsal side of the embryonic head (Fig. 3B, C, sdo).

66–80% DT: Early in this stage, the lateral walls of the embryo extend dorsally, causing the yolk mass to be completely enclosed by the dorsolateral walls so that the definitive dorsal closure is completed. After dorsal closure, the basic form of the first instar larva is established (Fig. 3D); the antennae, maxillary and labial palpi are three-segmented, and the thoracic appendages become five-segmented. The clypeolabrum becomes flat and wide. Urogomphi elongate and become slender (Figs. 3D; 5B, C, ur). At the ventral side of the abdominal end, the fused 9th and 10th abdominal segments assume as a minute swelling between the basal parts of urogomphi (Fig. 5C, ab9+10).

At about 70% DT, a delicate membrane, or embryonic

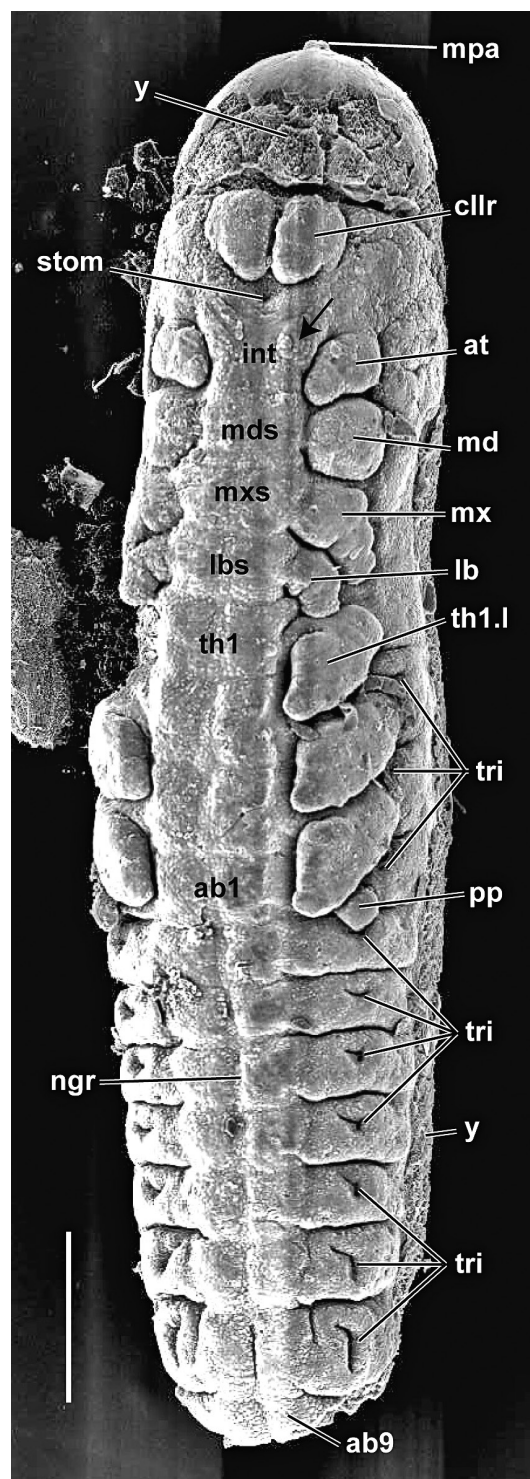


Fig. 4 Scanning electron micrograph of the *Hydaticus pacificus* embryo (ventral view) at 30% DT. The amnion and serosa are removed, and the right prothoracic leg accidentally destroyed during preparation of the specimen for SEM. ab1,9: 1st and 9th abdominal segment, at: antenna, cllr: clypeolabrum, int: intercalary segment, lb: labium, lbs: labial segment, md: mandible, mds: mandibular segment, mx: maxilla, mxs: maxillary segment, mpa: micropylar area on vitelline membrane, ngr: neural groove, pp: pleuropodium, stom: stomodaeum, th1: prothoracic segment, th1.l: prothoracic leg, tri: tracheal invagination, y: yolk, arrow: ganglion mother cells. Scale = 200  $\mu$ m.

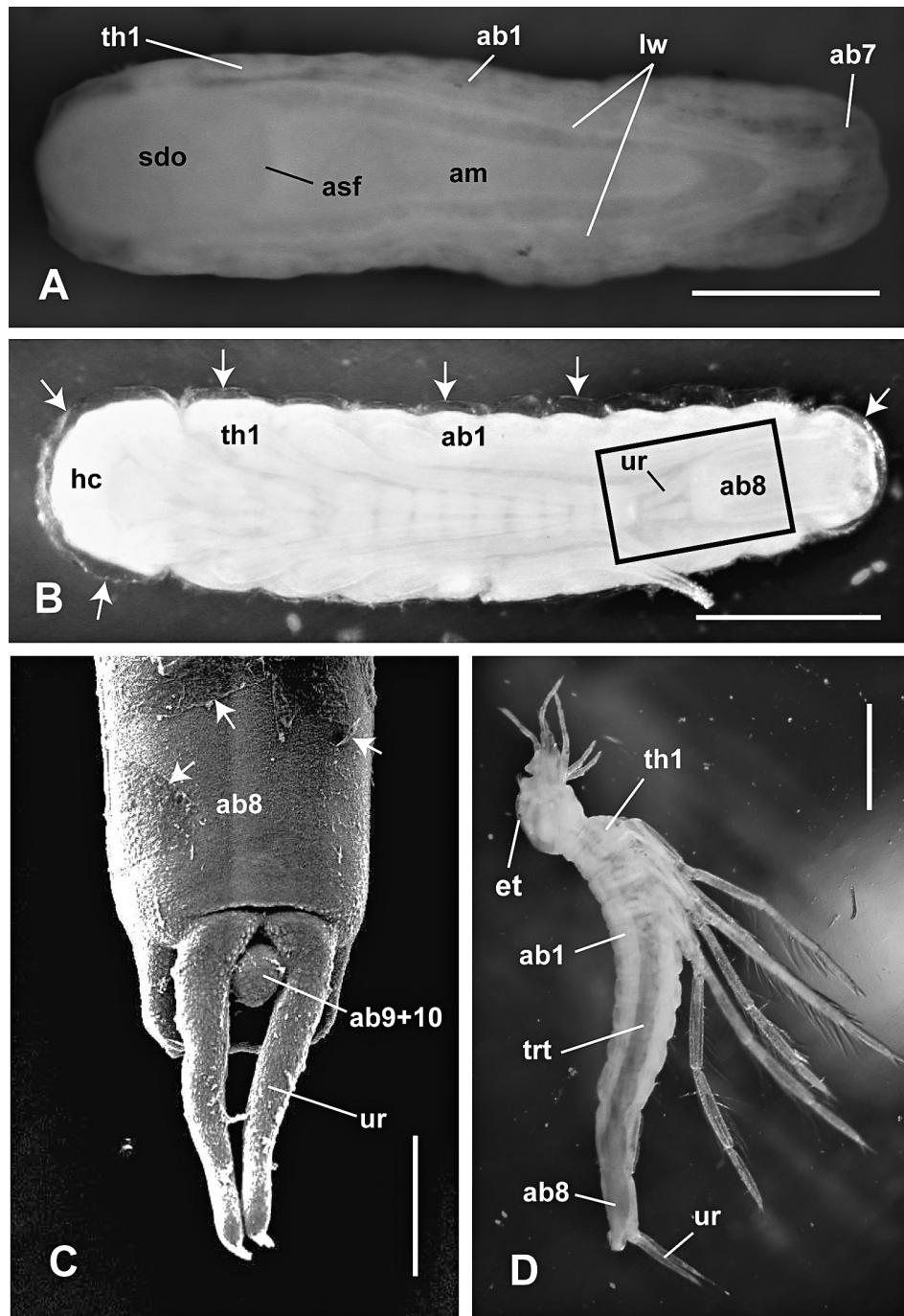


Fig. 5 A. Light micrograph of the *Hydaticus pacificus* egg (dorsal view) at 60% DT, showing the secondary dorsal organ (sdo) and provisional dorsal closure by the inverted amnion (am). The chorion is removed. B. Light micrograph of the *Hydaticus pacificus* egg (ventral view) at 72% DT, showing the embryonic cuticle (arrows) which detaches from the whole body wall. C. Scanning electron micrograph of the abdominal end (ventral view) of the embryo at 72% DT, showing the developing urogomphi (ur) and the embryonic cuticle (arrows) which peels away from the body wall. The micrograph shows the ventral view of the abdominal ends which corresponds to the area of rectangle in B. D. Light micrograph of the newly hatched larva (lateral view) of *Hydaticus pacificus*. ab1,7,8,10: 1st, 7th, 8th, 9th, and 10th abdominal segment, et: egg tooth, hc: head capsule, lw: lateral wall, th1: prothoracic segment, trt: tracheal trunk. Scales = A, B: 0.5 mm; C: 100  $\mu$ m; D: 1 mm.

cuticle, is often observed to slightly detach from the whole body wall (Fig. 5B, arrows). The SEM micrograph of this stage also shows fragments of the embryonic cuticle peeling away from the larval cuticle (Fig. 5C, arrows). These observations suggest that the embryonic molt occurs around this stage.

80–92% DT: The egg rapidly enlarges to about 2.7 mm long and 0.9 mm wide. A pair of thick longitudinal tracheal

trunks appears as brown tubes through the semi-transparent integument on both sides of the embryo (cf. Figs. 3E, F; 5D, trt). The two tracheal trunks run from the prothorax to the posterior end of the 8th abdominal segment, where the tracheal trunks open as a pair of large spiracles.

92–100% DT: The definitive form of the first instar larva completes and hatching occurs at about 50 h after oviposition

(Fig. 3E, F). In the medial part of the mandibular distal half, the sucking channel is observed as a thin dark-brown tube (Fig. 3E, suc). On the frons, a pair of brown sclerotized spines, or egg teeth, appears at about 95% DT (Fig. 3F, et). The egg teeth are retained on the head of the hatched larva (Fig. 5D, et). The pleuropodia gradually degenerate and disappear by the time of hatching. At hatching, the larval head first ruptures the anterodorsal part of the egg-shells (chorion and vitelline membrane), and the larval thorax and abdomen throw off the egg envelopes. The embryonic cuticle, which has already detached from the embryonic body wall at the time of the embryonic molt (about 70% DT), remains on the inner surface of the vitelline membrane.

## Discussion

### Egg

The chorion of *Hydaticus pacificus* is thin and soft (about  $2\text{ }\mu\text{m}$  thick), and has a homogeneous internal structure with a smooth surface (Fig. 1A, F, ch). Although the chorion does not tear under natural conditions, it often peels away from the egg surface during dehydration for preparing SEM specimens. In such a case, the vitelline membrane beneath the chorion is exposed on the egg surface, even in newly laid eggs (Fig. 1E, F, vit). Thus, the vitelline membrane appears to have a potential role in protecting the egg throughout the egg period. In the newly laid eggs of another dytiscid species, *Dytiscus marginalis*, Blunck (1914) observed two discrete membranes, the chorion and vitelline membrane, with almost the same thickness, although the author did not specify the thickness. As the egg enlarges long before hatching, the chorion on both the dorsal and ventral sides of the egg splits longitudinally (Blunck, 1914; Jackson, 1959) in contrast to *H. pacificus*. Moreover, the thick serosal cuticle is formed beneath the vitelline membrane by the secretion of the serosal cells, and thus both the vitelline membrane and serosal cuticle practically protect the egg contents (Blunck, 1914; Jackson, 1959). The presence of a thin and fragile chorion and a tough vitelline membrane is also reported in the eggs of another dytiscid species, *Agabus bipustulatus*, although the split of the chorion does not occur in this species in natural conditions (Jackson, 1958).

In the newly laid egg of the carabid ground beetle *Carabus insulicola*, the chorion is also thin and soft, but has a mesh-like chorionic structure on its surface (Kobayashi *et al.*, 2013). This is also the case for other carabid beetles (Luff, 1981; Kaupp *et al.*, 2000). Conversely, in the aquatic family Gyrinidae the eggs have a relatively thick chorion (*e.g.*, about  $20\text{ }\mu\text{m}$  thick in *Dineutus mellyi*, Komatsu and Kobayashi, 2012), and their surface is either covered in blunt conical projections (*Dineutus horni*, Baker and Ma, 1987; *Di. mellyi*, Komatsu and Kobayashi, 2012) or has a sculptured, ribbed, or honeycombed structure (*Gyrinus mirinus* and *Gyrinus. sp.*, Hinton, 1981). Moreover, in both *C. insulicola* and *Di. mellyi*, the hard serosal cuticle (*e.g.*, 4 to  $5\text{ }\mu\text{m}$  thick in *C. insulicola*) completes beneath the chorion at about 20% DT. In *C.*

*insulicola*, enlargement of the egg during development causes the soft chorion to stretch and, occasionally, to peel away from the serosal cuticle (Kobayashi *et al.*, 2013). In *Di. mellyi*, as the size of the egg increases, the tough thick chorion splits longitudinally along the ventral midline well before hatching (Komatsu and Kobayashi, 2012). Therefore, as in other gyrinid species, the newly formed serosal cuticle beneath the chorion actually plays the role of an egg-shell protecting the egg contents after the splitting of the chorion.

In summary, the fragile character of the chorion and the major role played by the vitelline membrane in protecting the egg contents must be features that are phylogenetically constrained for all eggs of the Dytiscidae. In *H. pacificus*, a hard and tough serosal cuticle like that of *C. insulicola*, *Di. mellyi*, and *Dy. marginalis* is not detected by external observations throughout the egg period, although the possibility of the formation of a very thin serosal cuticle undetectable by external observations is not denied. Thus, the degree of development of the serosal cuticle may vary according to the taxa within the Dytiscidae.

In *H. pacificus*, about 35 micropyles (Fig. 1D, arrows) are arranged in a circle of about  $40\text{ }\mu\text{m}$  in diameter around the anterior pole of the egg. In the eggs of other dytiscid species, many micropyles are also arranged in a circle, such as about 60 in *Dy. marginalis* (Blunck, 1914) and about 17 in *A. bipustulatus* (Jackson, 1958). On the other hand, in the eggs of all gyrinid species examined to date, the micropyles are present at the top of the stalk-like projection situated at the anterior pole of the egg (Saxod, 1964; Hinton, 1981; Baker and Ma, 1987; Komatsu and Kobayashi, 2012). Thus, the Dytiscidae and Gyrinidae are strikingly different, not only in the thickness of the chorion but also in the structures of the micropylar region.

In the eggs of the suborder Polyphaga, information about the detailed structures of micropyles is scarce (Hinton, 1981). However, in the several species whose micropyles have been examined, many micropyles are arranged almost in a circle; *e.g.*, the numbers of micropyles are 16 to 24 in *Lytta viridana* (Meloidae) (Sweeny *et al.*, 1968), about 10 in *Rhagophthalmus ohbai* (Rhagophthalmidae) (Kobayashi *et al.*, 2002), about 15 in *Prionotolytta binotata* (Meloidae) (Bologna and Di Giulio, 2003), and about 20 in *Hermonia axyridis* (Osawa and Yoshinaga, 2009). While there are considerable variations in the number of micropyles, their circular arrangement near the anterior pole seems to be a character common to Polyphaga and Adephaga excluding the Gyrinidae.

In *H. pacificus*, just beneath the circularly arranged micropyles in the chorion, the micropylar area on the vitelline membrane is present as a disk-shaped bulge (Fig. 1E, mpa). A similar disk-shaped area is also detected on the vitelline membrane in the anterior pole of the egg of *Dy. marginalis* (Jackson, 1959). It is presumed that this area functions as the passages through which spermatozoa enter the egg, but the detailed internal structure of this area remains unknown.

### Growth pattern of embryo

The early embryo of *H. pacificus*, like that of *Dy. marginalis* (Korschelt, 1912; Blunck, 1914), is formed on the ventral side of the egg, and it subsequently develops on the egg surface throughout the egg period. Thus, the embryo belongs to the superficial type found in many holometabolous insects. The embryos of the gyrinid *Di. mellyi*, and the carabids *Carabus insulicola* and *Cicindela togata* also belong to the superficial type (Komatsu and Kobayashi, 2012; Kobayashi *et al.*, 2013; Willis, 1967). Therefore, it is presumed that the embryos of adephagan families probably belong to the superficial type. On the other hand, although most embryos of the polyphagan Coleoptera belong to the superficial type (*e.g.*, *Lytta viridana*, Rempel and Church, 1969), in some families such as the Lampyridae (fireflies), Rhagophthalmidae (glowworms), and some Chrysomelidae (leaf beetles), the early embryos are formed by deep invagination of the germ disks into the yolk (Williams, 1916; Miya, 1965; Zakhvatkin, 1967, 1968; Ando and Kobayashi, 1975; Krysan, 1976; Kobayashi and Ando, 1985; Kobayashi *et al.*, 2002, 2006). In particular, embryos of the Lampyridae and Rhagophthalmidae are differentiated from the spherical germ rudiments, which are formed by deep invagination of the small circular germ disks into the yolk, and the embryos then develop within the yolk in the submerged condition until close to katarépsis (Ando and Kobayashi, 1975; Kobayashi and Ando, 1985; Kobayashi *et al.*, 2002, 2006). Thus, the growth pattern of the polyphagan embryos is specialized or diversified in some groups, whereas that of the adephagan embryo is probably uniform throughout the suborder.

### Cephalognathal region

In the *H. pacificus* embryo at about 30% DT, the intercalary segment is clearly observed as a long segment just anterior to the mandibular segment (Figs. 2E; 4, int). The intercalary segment is somewhat longer than the mandibular segment in this stage, although the former becomes undetectable in the next stage (Fig. 2G, H). In insect embryogenesis generally, the intercalary segment is short and appears temporarily in the early stage when the segmentation of the cephalognathal region commences, and the segment soon disappears because it merges into the cephalic lobe. Thus, the presence of the intercalary segment is often overlooked by external observations, particularly in holometabolous insect embryos, and is detected only by histological observations (*e.g.*, *Lytta viridana* in Coleoptera, Rempel and Church, 1969). However, in a non-holometabolous insect, such as *Galloisiana yuasai* (Grylloblattodea), the large intercalary segment is formed in the young embryo (Uchifune and Machida, 2005). In Adephaga, the intercalary segment is also temporarily observed in the young embryos of *Di. mellyi* (Gyrinidae) and *C. insulicola* (Carabidae) (Komatsu and Kobayashi, 2012; Kobayashi *et al.*, 2013). In both species, the intercalary segment appears at 10 to 20% DT, or before the appearance of pleuropodia, and becomes undetectable

thereafter. In *H. pacificus*, however, the intercalary segment remains clearly observable externally, even at around 30% DT when the pleuropodia appear (Fig. 4). Thus, the period of appearance of this segment is exceptionally long in the *H. pacificus* embryo, and this character is unique to this species.

In the *H. pacificus* embryo, the galea is formed as a small, medially extended endite lobe at the proximal part of the maxillary appendage, and another endite corresponding to the lacinia is not formed (Fig. 2G, H, ga). In the embryos of *Di. mellyi* and *C. insulicola*, both the lacinia and galea arise as separate endite lobes at the proximal part of the maxillary appendages, and are retained in the larval stages, although the lacinia in the *C. insulicola* larva is highly vestigial (Komatsu and Kobayashi, 2012; Kobayashi *et al.*, 2013). Beutel (1993) postulated that the absence of the lacinia from larvae of the Trachypachidae and the superfamily Dytiscoidea (Noteridae, Amphizoidae, Hygrobiidae, and Dytiscidae) is a synapomorphy of these groups, suggesting the sister group relationship of them. Alarie and Bilton (2005) also confirmed the absence of the lacinia in the larvae of another dytiscoid family Aspidytidae. In most non-holometabolous insects, both the lacinia and galea arise as endites of the maxillary appendage in the embryonic stage, but in some groups of Holometabola (*e.g.*, Trichoptera, Kobayashi and Ando, 1990) the lacinia is not formed in the embryos. The appearance of both the lacinia and galea in *Di. mellyi* and *C. insulicola* in their embryonic stages is thus regarded as the primitive state, and the absence of lacinia in *H. pacificus* as the derived one. These embryological results support Beutel's postulation that the loss of lacinia should be an apomorphic feature.

### Abdominal segmentation

Among adephagan families, the larvae of the Gyrinidae, Carabidae, and Trachypachidae have a 10-segmented abdomen (Arndt *et al.*, 2005; Beutel, 2005; Beutel and Arndt, 2005; Beutel and Roughley, 2005). Embryological observations revealed that the larval 10th abdominal segment in the Gyrinidae and Carabidae is formed by the fusion of the embryonic 10th and 11th segments and non-segmental telson in which the proctodaeal invagination is formed (Komatsu and Kobayashi, 2012; Kobayashi *et al.*, 2013).

In other adephagan families, excluding the Halipidae, and in the superfamily Dytiscoidea, the larval 9th and 10th abdominal segments are strongly reduced or entirely lacking (Vondel, 2005; Dettner, 2005a, b, c; Balke, 2005). Extreme reduction of abdominal segments is found in dytiscid larvae, where the abdomen of the fully grown larva consists of 8 segments with paired urogomphi on the abdominal end, although urogomphi are absent in *Cybister* (Kamite, 2008). In *Dy. marginalis*, however, both Korschelt (1912) and Blunck (1914) showed that the number of abdominal segments in the old embryo is 9, instead of 11 (excluding the telson), in the young embryo. Korschelt (1912) attributed the fewer abdominal segments in the old embryo to reduction (or loss) of the posterior segments. The present study revealed that

the 9th and 10th abdominal segments form in the young embryo of *H. pacificus*, but did not detect the 11th segment by external observations (Fig. 2E–H, ab9, ab10). In this species, as katatrepsis comes close, the 9th and 10th segments are fused into one segment with the latter being diminutive in size (Fig. 3A, ab9, ab10). After katatrepsis, the fused 9th and 10th segments are further reduced and incorporated into the ventro-posterior part of the 8th segment, so that the abdomen becomes seemingly composed of 8 segments with paired urogomphi (Fig. 3B–F, ab8, ur). The fused 9th and 10th segments are observed at the ventro-posterior end of the 8th segment, or between the bases of urogomphi (Fig. 5C, ab9 + 10). Thus, the larval 8th abdominal segment is formed by incorporation of the embryonic 9th and 10th segments into the 8th segment, not by the complete loss of the 9th and 10th segments.

The present study could not trace the detailed process of the formation of urogomphi of *H. pacificus*. Snodgrass (1931) thought that the urogomphi of coleopteran larvae are merely an integumentary outgrowth of the 9th tergum. Conversely, Matsuda (1976) assumed that coleopteran larval urogomphi are homologous with cerci often arising at the 11th abdominal segment in primitive insects such as Archaeognatha. He further considered that the urogomphi of dytiscid larvae arise from the posterolateral angles of the 8th segment where the embryonic 11th segment has been incorporated. In the carabid ground beetle *C. insulicola*, however, detailed embryological observations revealed that the urogomphi originate in the 9th tergum (Kobayashi *et al.*, 2013), thus supporting Snodgrass' view. As mentioned before, the present study on *H. pacificus*, like the studies on *Dy. marginalis* by Korschelt (1912) and Blunck (1914), could not clarify the exact positions from which the urogomphi arise. However, it is unlikely that the urogomphi of *H. pacificus* originate in the 11th segment as presumed by Matsuda (1976), because the segment cannot be found during embryogenesis, or if the segment is temporarily present, it must be extremely vestigial and undetectable from external observations. Instead, in the light of the 9th tergal origin of the urogomphi in *C. insulicola*, it may be possible to consider the origin of urogomphi of *H. pacificus* being also in the 9th segment, which is incorporated into the 8th segment during embryogenesis.

#### *Tracheal invaginations and longitudinal tracheal trunks*

The first instar larva of *H. pacificus*, like that of other dytiscid species, has a pair of spiracles near the posterior end of the 8th abdominal segment; thus, despite its aquatic habitat, the larva breathes air through the spiracles by raising the abdominal end to the water surface periodically. In the embryonic period, however, tracheal invaginations are formed in the meso- and metathoraxes and the first eight abdominal segments at about 30% DT (Figs. 2E, F; 4, tri). In insect embryogenesis in general, the bottom of each tracheal invagination forks, and the posterior branch of one fork fuses with the anterior branch of the next one, thereby forming two

major longitudinal tracheal trunks. It is thus presumed that the thick longitudinal tracheal trunks in the fully grown embryo or the first instar larva of *H. pacificus* (Figs. 3E, F; 5D, trt) likewise complete by fusion of the anterior and posterior branches of tracheal invaginations. In this species, however, the tracheal openings, excluding those of the 8th abdominal segment, close and disappear at about 50% DT, or before katatrepsis (Fig. 3A). The completed tracheal trunks of this species, probably as well as those of other dytiscid larvae, are much thicker than those of terrestrial insect larvae. This may be relevant to that dytiscid larvae, which submerge in the water for a long time, need to keep a large amount of air in the tracheal trunks. The closure of tracheal openings, excluding those of the 8th segment, is also regarded as an adaptation to the aquatic life style of the Dytiscidae, and probably occurs during embryogenesis of other Dytiscoidea (Hygrobiidae, Noteridae, Aspidytidae, and Amphizoidae) whose first instar larvae have functional spiracles only in the 8th abdominal segment. In contrast to these Dytiscoidea, the first and second instar larvae of the Gyrinidae have no spiracle; instead, they have 10 pairs of long tracheal gills in the abdomen (Beutel and Roughley, 2005). In the embryonic period of the gyrid *Di. mellyi*, however, 10 pairs of tracheal invaginations (two thoracic and eight abdominal) are formed, but these openings close as the tracheal gills grow before katatrepsis, and so the newly hatched larva has no spiracle and breathes through the gills (Komatsu and Kobayashi, 2012). It has been known that the closure and disappearance of all tracheal openings in the embryonic period occurs in the following orders, all of which have larvae that are completely aquatic: Odonata (Ando, 1962); Megaloptera (Miyakawa, 1979; Ando *et al.*, 1985); and Trichoptera (Kobayashi and Ando, 1990). In this aspect, the first and second instar larvae of *Di. mellyi*, whose all embryonic tracheal openings close before katatrepsis, are completely aquatic as those of the aquatic orders mentioned above. Therefore, from an embryological standpoint it can be interpreted that the degree of adaptation to the aquatic life style in the Gyrinidae is far stronger than in the Dytiscidae (or the Dytiscoidea).

#### *Embryonic molt and egg teeth*

In the *H. pacificus* embryo, embryonic molt occurs at around 70% DT, or several hours after the definitive dorsal closure completes. The embryonic cuticle detaches from the whole body surface (Fig. 5B, arrows), and at hatching the shed embryonic cuticle remains on the inner surface of the vitelline membrane. In the eggs of other dytiscid species, *Dy. marginalis* and *A. bipustulatus*, Jackson (1957) observed the shed embryonic cuticle on the surface of fully grown embryos near hatching, but the exact timing of the embryonic molt was not specified. An embryonic molt was also suggested in two other adepagan species, *Di. mellyi* and *C. insulicola*, but the exact timing and fate of the shed embryonic cuticle were obscure (Komatsu and Kobayashi, 2012; Kobayashi *et al.*, 2013). Thus, the present study provides the first examples in

which both the exact time of embryonic molt and the fate of the shed embryonic cuticle have been shown in Adephaga.

In *H. pacificus*, egg teeth appear as a pair of minute sclerotized projections on the frons of the head of the fully grown embryo at 95% DT (Fig. 3F, et), or at the stage well after embryonic molt. The egg teeth are retained during the first instar larval stage (Fig. 5D, et). The presence of paired egg teeth (egg-bursters) in newly hatched larvae has been reported in many other dytiscid species (e.g., as in Emden, 1925, 1946; Jackson, 1957, 1958) and other dytiscoid families Amphizoidae (Xie, 2000), Hygrobiidae (Alarie *et al.*, 2004), and Aspidytidae (Alarie and Bilton, 2005). In the carabid ground beetle *C. insulicola*, a pair of egg teeth also arises on the head of the fully grown embryo, and they are retained in the newly hatched larva (Kobayashi *et al.*, 2013). In the gyrinid *Di. mellyi*, however, neither the embryo nor larva has egg teeth (Komatsu and Kobayashi, 2012).

In insects, the presence of teeth has been reported in 22 of 29 dicondylarian orders including the apterygote Zygentoma, and egg teeth are classified into several types according to the locations where they are formed and the period when they exist (Kobayashi, 2016). In most orders, other than Coleoptera, a single egg tooth is formed as a minute sclerotized projection in the serosal cuticle covering the frontal region of the head at the last embryonic stage. At hatching, when the serosal cuticle is shed off from the larval cuticle, the egg tooth also is peeled from the larval cuticle of the head, and consequently the hatched larva has no egg tooth. In some of the adephagan Coleoptera, however, as mentioned before, egg teeth appear as a pair of projections on both sides of the frons near the frontal sutures. Furthermore, the adephagan egg teeth exist in the first instar larval stage, or persist even in the second instar stage in the Aspidytidae (Alarie and Bilton, 2005). Thus the paired egg teeth of Adephaga are probably not homologous with a single egg tooth in other orders, which suggests the paired egg teeth being autapomorphic structures acquired within a lineage of Adephaga. Beutel (1993) assumed that the presence of egg teeth in first instar larvae is an autapomorphy of Adephaga, excluding the Gyrinidae and Haliplidae. Thus, the embryological data obtained to date, though scarce, are not contradictory to Beutel's assumption.

#### Phylogenetic considerations

Crowson (1960) kept the traditional classification system in which Adephaga are divided into two main sections, Geadephaga and Hydradephaga. However, Crowson's approach was a pre-cladistic method, and most of the subsequent cladistics approaches by many authors questioned whether Geadephaga and Hydradephaga are each monophyletic. For example, based on various larval and adult characters, Beutel and Roughley (1988) first proposed that the aquatic family Gyrinidae is the sister group of the remaining adephagan families, in which the Rhysodidae (the carabid subfamily Rhysodinae) is the sister group of the remaining

families; the result suggested that Geadephaga and Hydradephaga are each non-monophyletic. In another analysis by Beutel (1993) based on larval head characters, the Gyrinidae also occupied the basal position of Adephaga, but of the remaining families the aquatic family Haliplidae occupied the basal position. In both studies, the aquatic families Dytiscidae, Hygrobiidae, Amphizoidae, and Noteridae form a monophyletic unit, or the Dytiscoidea, forming the sister group of the terrestrial family Trachypachidae. On the other hand, in recent years, several molecular studies designed to infer the phylogenetic relationships among the adephagan families recovered a monophyletic Geadephaga and a monophyletic Hydradephaga (e.g., Ribera *et al.*, 2002; Hunt *et al.*, 2007). However, the most recent molecular phylogenetic analysis by Maddison *et al.* (2009) supported only the monophyletic Geadephaga, but not the monophyly of Hydradephaga, because the analysis suggested that only the Gyrinidae is the sister group of Geadephaga. As a result, the validity of Geadephaga and Hydradephaga in the adephagan phylogenetic system remains debatable.

As mentioned in the 'Introduction', embryological information has been accumulated in only three of the 10 adephagan families (Gyrinidae, Carabidae, and Dytiscidae), and thus is not yet sufficient for discussing the phylogeny of the whole Adephaga. However, through comparisons of the external features of the eggs and developing embryos among these three families (as partly discussed above), the following phylogenetic considerations can be applied to part of Adephaga. Although the superficial-typed embryos are common in these three families, the aquatic families Gyrinidae and Dytiscidae are entirely different in the following characters. Character 1: The presence of a stalk-like micropylar region in the eggs of the Gyrinidae (autapomorphic), whereas the presence of many micropyles in a circular arrangement in the eggs of the Dytiscidae (probably synapomorphic, shared with Polyphaga). Character 2: A thick chorion with a complicated internal structure and a sculptured surface in the Gyrinidae (autapomorphic), whereas a thin and fragile chorion with a smooth surface in the Dytiscidae (probably synapomorphic, shared with the Carabidae). Character 3: The temporary appearance of a short intercalary segment in the Gyrinidae (plesiomorphic, observed in many other insects including the Carabidae), whereas the appearance of a long intercalary segment whose period of existence also is long in the Dytiscidae (autapomorphic). Character 4: The fusion of the embryonic 10th and 11th abdominal segments to form the larval terminal abdominal segment in the Gyrinidae (symplesiomorphic, shared with the Carabidae), whereas the fusion of the 8th to 10th segments to form the terminal segment in the Dytiscidae (probably autapomorphic). Character 5: The formation of both the lacinia and galea in the maxillary appendage in the Gyrinidae (plesiomorphic, also observed in the Carabidae and many non-holometabolous insects), whereas the absence of lacinia in the Dytiscidae (apomorphic, often observed in many

other holometabolous insects). Character 6: The absence of urogomphi on the 9th abdominal segment in the Gyrinidae (plesiomorphic), whereas the formation of urogomphi probably originating in the 9th abdominal tergum in the Dytiscidae (synapomorphic, shared with the Carabidae and probably other adephagan families excluding the Gyrinidae). Character 7: The formation of 10 pairs of abdominal tracheal gills accompanied with the closure of all tracheal openings in the Gyrinidae (autapomorphic), whereas the formation of thick longitudinal tracheal trunks accompanied with the closure of tracheal openings except those of the 8th abdominal segment on which large spiracles open in the Dytiscidae (autapomorphic). Character 8: The absence of the egg tooth in the Gyrinidae (plesiomorphic), whereas the formation of paired egg teeth on the frons in the Dytiscidae (synapomorphic, shared with the Carabidae and probably all other adephagan families excluding the Gyrinidae).

In summary, there is no embryonic synapomorphy between the Gyrinidae and Dytiscidae, which means that there is no positive embryological data supporting the monophyly of Hydradeephaga to date. Conversely, three apomorphic characters (Characters 2, 6, and 8) are shared between the Carabidae and Dytiscidae, suggesting an affinity of these families, which means the non-monophyly of Hydradeephaga, although the Carabidae retains some plesiomorphic characters (Characters 3, 4, and 5) shared with the Gyrinidae. To discuss the phylogeny of the whole Adephaga from embryological standpoints, further embryological studies on other adephagan families are needed, particularly on the aquatic family Haliplidae and the terrestrial family Trachypachidae.

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