Embryonic Development
of *Baculentulus densus* (Imadaté):
An Outline (Hexapoda: Protura, Acerentomidae)

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Abstract

An outline of the embryogenesis of Protura was described using *Baculentulus densus* as materials. Eggs of *Baculentulus densus* are spherical with a long axis of about 120 μm and with numerous, variously sized and shaped protuberances scattered over their surface. The proturan embryogenesis is characterized by 1) holoblastic cleavage, 2) embryogenesis of long germ type, 3) simple blastokinesis, and 4) formation of a primary dorsal organ, all of which represent the ancestral conditions in hexapod embryogenesis.

A noticeable feature of proturan embryogenesis is that the serosa retains the ability to differentiate into the tergum or to participate in the definitive dorsal closure, which in light of the evolutionary transition of the functional specialization between the embryo proper and embryonic membranes among Hexapoda, suggests that Protura may represent a more basal clade in Hexapoda than ever elucidated.

Introduction

Protura, which are minute soil dwellers, are entognathous hexapods. Although generally dealt with as one of the hexapod basal clades (*e. g.*, Kristiansen, 1975; Boudreaux, 1979), Protura have been often regarded as representing a side branch remote from the main line of hexapod evolution (*e. g.*, Sharov, 1966; Manton, 1977).

Henning's 'Entognatha-Ectognatha System' (1969) has been widely accepted with little challenge. However, the reality of the taxon Entognatha is suspect because it is supported principally only by entognathy. Recent comparative paleontological (Kukalová-Peck, 1987), morphological (Koch, 1997), and comparative embryological (Ikeda and Machida, 1998) researches have provided evidence discounting the validity of entognathy as a synapomorphy for members of this group, and some molecular evolutionary evidence (Luan *et al.*, 2003) has emerged, suggesting a closer affinity between Protura and Diplura, and rejecting Ellipura. Furthermore, the internal relationships among Arthropoda still remain furiously debated (*e. g.*, Aguinaldo *et al.*, 1997; Hwang *et al.*, 2001; Sakuma and Machida, 2002). This background makes Protura more significant in the reconstruction of relationships of hexapod basal clades, and elucidation of the origin of hexapods and illustration of the hexapod groundplan, than ever.

The comparative embryological approach is one of the most promising methods for solving such phylogenetic problems. An embryological study of Protura has been desired ever since their discovery by Silvestri (1907) (Richards and Davies, 1977), but attempts at embryology have failed due to difficulties in rearing proturans. In the past few decades, much embryological knowledge concerning hexapod basal clades has been accumulated, and the reconstruction of hexapod basal clades from comparative embryology has progressed (*e. g.*, Machida and Ando, 1998; Machida *et al.*, 2002; Machida, 2006), however, information on proturan embryogenesis has been totally lacking.

Recently, we have succeeded in rearing proturans and collecting their eggs under rearing conditions (Machida and Takahashi, 2003, 2004). To date, we have obtained a good number of the eggs of acerentomid proturan *Baculentulus densus*, and in this paper, we present an outline of the embryogenesis of the species.

Materials and Methods

Adults of *Baculentulus densus* (Imadaté) were extracted from litter of a deciduous broadleaved forest dominated by *Quelcus serrata*, at Shinkoji, Ueda, Nagano Prefecture, with a Tullugren's funnel. The proturans collected were reared and the eggs were collected according to the method of Machida and Takahashi (2004).

The eggs obtained were fixed with FAA (formalin 5 : ethanol 15 : acetic acid 1), Carl's fixative (formalin 6 : ethanol 15 : acetic acid 2 : DW 30) or Karnovsky's fixative (2% paraformaldehyde + 2.5 glutaraldehyde; in some cases, 1% tannic acid was added). The fixed eggs were stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride diluted to about 5 μg/ml with DW), and observed under a fluorescence stereomicroscope (LEICA MZ FL III + FLUOCOMBI, UV-excitation). Some eggs were processed into methacrylate resin sections 2 μm thick according to the methods of Machida *et al.* (1990, 1994a, b).

Results

Eggs of *Baculentulus densus* are spherical to spheroidal with a long axis of about 120 μm and with numerous, variously sized and shaped protuberances scattered over their surface (Fig. 1). Major protuberances are distributed in a restricted area of the egg, where the cephalic lobe of the embryo is located in later developmental stages [Machida and Takahashi (2003) misleadingly identified the orientation of the embryo as being opposite]. We designated the orientation of the egg as follows: the area with major protuberances is the anterior, opposite it is the posterior, the area with the middle part of the future embryo is the ventral area, and opposite it is the dorsal area. The egg period of *Baculentulus densus* has not been precisely determined, but may be approximately one month at room temperature [Machida and Takahashi (2003) speculated it to be about two months].

Figure 2 shows an egg in the 4-cell stage. Two cleavage nuclei are visible, between which a cell boundary runs along the equator. Cleavage in proturans is holoblastic or total. Cleavage nuclei proliferate and migrate to the egg surface to form a cellular layer, the blastoderm (Fig. 3), which secretes a cuticular egg envelope or the blastoderm cuticle on the egg surface soon after its differentiation (cf. Fig. 12B).

The blastoderm becomes more densely cellulated, followed by regional differentiation into a long, broad embryonic area and a restricted extraembryonic area or serosa (Fig. 4). The embryo then begins to segment, and the appendages soon appear in the segments newly differentiated. In the embryo shown in Figure 5, the mandibular, maxillary, labial, thoracic and first abdominal segments are differentiated. It should be noted that the serosal area next to the embryo differentiates into a body element or the tergum (Fig. 5A). In the ventral view, the embryo is found to be very wide, and this is another remarkable feature of proturan early embryos (Fig. 5B).

With progressive segmentation, the embryo elongates until its cephalic and caudal ends almost meet (Fig. 6A). Then, blastokinesis occurs. The embryo shown in Figure 6 has started to bend upward between the thorax and abdomen as shown by the arrow in Figure 6A: in Figure 6B, the bending point is shown by an asterisk. As blastokinesis continues, the thoracic and abdominal regions further bend dorsally, and the caudal end of the embryo recedes from its original location (Fig. 7A). The cephalic region is also involved in the blastokinesis, and in the embryo shown in Figure 7 the maxillary and abdominal segments are adjacent to each other (Fig. 7B). The anterior and posterior halves of the embryo are further involved in the progressive blastokinesis, and finally they completely face each other (Figs. 7A, 8,

Figs. 1–6   Development of a proturan *Baculentulus densus* I. Anterior to the left. Figs. 2–6 are fluorescence micrographs (DAPI staining, UV excitation). Black and white arrowheads respectively show the cephalic and caudal extremities of embryos.

Fig. 1   Egg, SEM.

Fig. 2   Egg at the 4-cell stage. Arrow shows a cell boundary.

Fig. 3   Egg during the formation of the blastoderm.

Fig. 4   Egg undergoing differentiation of the embryonic and extraembryonic areas.
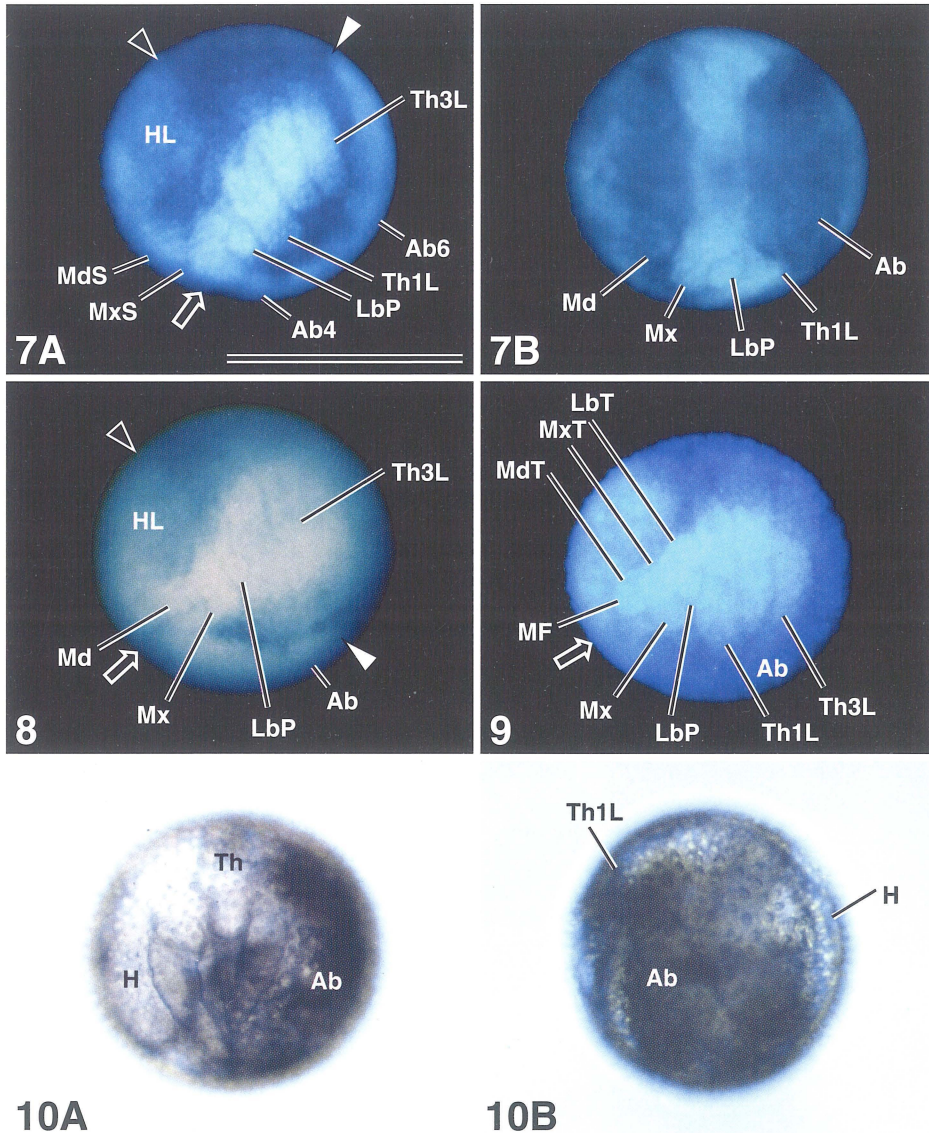
Fig. 5   Egg with an embryo in the initial stage of segmentation. A. Lateral view. B. Ventral view of the egg in A.

Fig. 6   Egg with an embryo in the initial stage of blastokinesis. A. Lateral view. The embryo has started to bend upward between the thorax and abdomen (arrow). B. Ventral view of the egg in A. An asterisk shows the bending point of the embryo.

Ab1: first abdominal segment, Ap: appendage, Bd: blastoderm, CN: cleavage nucleus, EemA: extraembryonic area, EmA: embryonic area, HL: head lobe, LbS: labial segment, MdS: mandibular segment, MxS: maxillary segment, Se: serosa, Tg: tergum, Th1–3: first to third thoracic segments, Th1L–3L: first to third thoracic appendages. Scale = 50 μm.
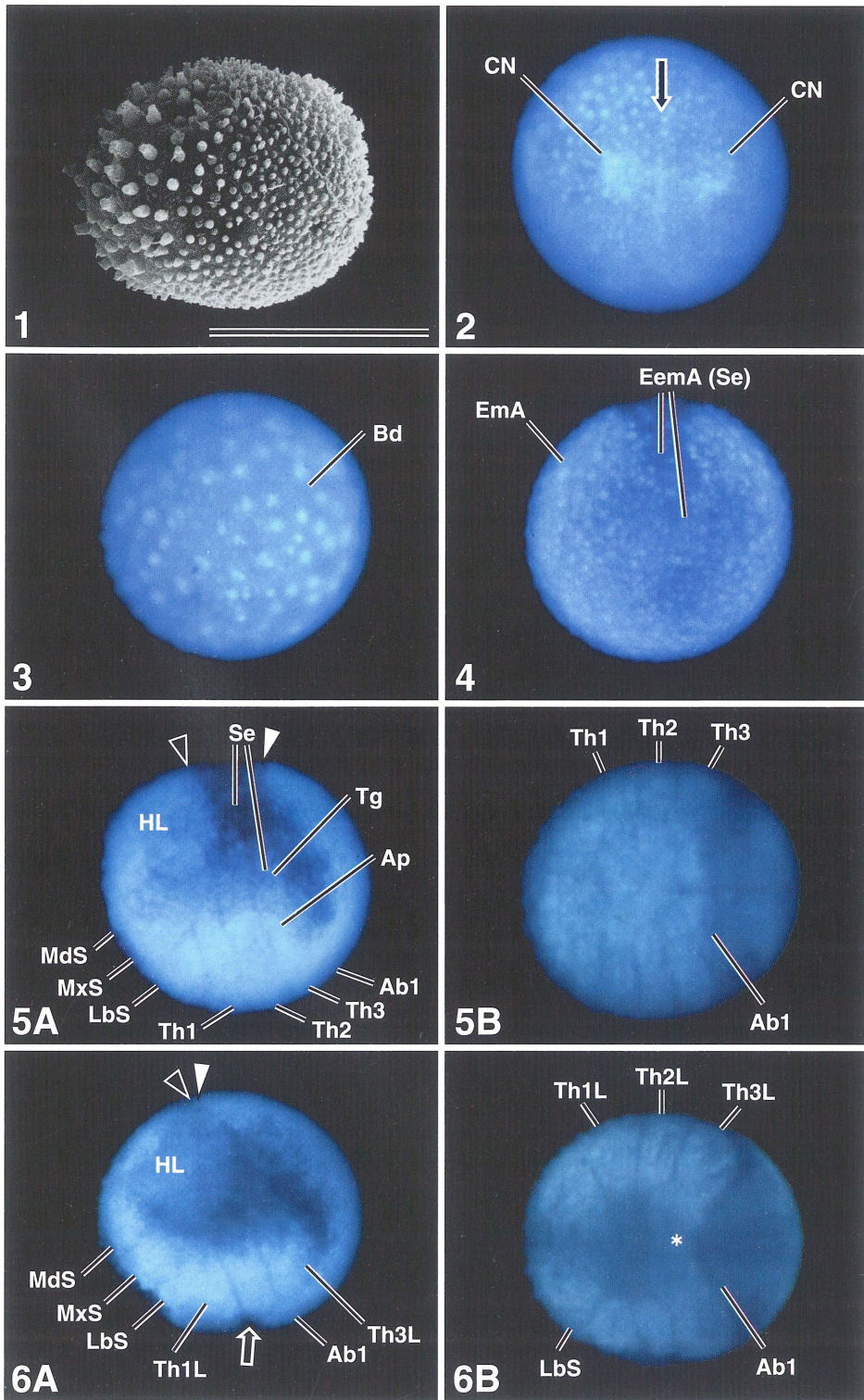
Figures 1-6

Figs. 7–10   Development of a proturan *Baculentulus densus* II. Anterior to the left, with the exception of Fig. 10B. Figs. 7–9 are fluorescence micrographs (DAPI staining, UV excitation). Black and white arrowheads respectively show the cephalic and caudal extremities of embryos. Arrows show the bending of embryos.

Fig. 7   Egg with an embryo in the mid stage of blastokinesis. A. Lateral view. B. Ventral view of the egg in A.

Fig. 8   Egg with an embryo in the last stage of blastokinesis.

Fig. 9   Egg with an embryo which has completed blastokinesis.

Fig. 10   Egg with an embryo just before hatching. A. Lateral view. B. Frontal view of the egg in A.

Ab: abdomen, Ab4,6: fourth and sixth abdominal segments, H: head, HL: head lobe, LbP: labial palp, LbT: labial tergum, Md: mandible, MdS: mandibular segment, MdT: mandibular tergum, MF: mouth fold, Mx: maxilla, MxS: maxillary segment, MxT: maxillary tergum, Th: thorax, Th1L, 3L: first and third thoracic appendages. Scale = 50 μm.

9). Initially, the plane of facing of anterior and posterior halves of the embryo is nearly upright (Fig. 7A), but it gradually inclines (Figs. 8, 9), since the cephalic end retains its original position.

Simultaneously with the progression of blastokinesis, the embryo continues to grow, its appendages developing markedly (Figs. 7–9). The formation of the entognathy starts, and in grown embryos, mandibular, maxillary, and labial terga are observed to participate in the development of the mouth fold (Fig. 9). As the embryo develops further and elongates, it assumes a twisted posture (Fig. 10), and after a little while, the prelarva hatches (Fig. 11). The abdomen of prelarva is segmented into nine, with its midgut filled with yolk (Fig. 11B), and its entognathy complete (Fig. 11A). Prelarva, which vigorously moves at hatching, is inactive.

Figure 12A is a sagittal section of an embryo at approximately the same stage as that shown in Figure 7. A clump of cells is found on the dorsum of the embryo next to its cephalic end. The cells, with much accumulated cytoplasm, are radially arranged, and their nuclei are located near the periphery of the structure (Fig. 12B). This is identified as the primary dorsal organ. It degenerates soon after blastokinesis (cf. Fig. 13), to disappear.

Discussion

Cleavage

In *Baculentulus densus*, cleavage is holoblastic or total at least in the initial stage. Cleavage in Diplura (Uzel, 1898; Ikeda, 2001) and Dicondylia including Pterygota is widely accepted as superficial (cf. Johannsen and Butt, 1941). On the other hand, in the Collembola (Jura, 1972) and Archaeognatha (Machida *et al.*, 1990), the cleavage starts with the total pattern as proven here for Protura. Machida *et al.* (1990) suggested total cleavage to be ancestral among Hexapoda.

Germ type

In *Baculentulus densus*, there forms a long and broad germ band occupying nearly the entire circumference of the egg. The embryo undergoes the embryogenesis, without elongation, and may be categorized as an embryo of long germ band type, although the segmentation of the posterior regions of the embryo is less progressive. In this respect, the embryos of proturans resemble those of collembolans (Jura, 1972) and diplurans (Uzel, 1898; Ikeda and Machida, 1998). Ikeda and Machida (1998) suggested the long germ band of Entognatha to be ancestral among Hexapoda.

The early embryo of *Baculentulus densus* is, however, characterized by its extraordinary width. It reminds us of the wide germ bands found in myriapods (Symphyla: Tiegs, 1940; Pauropoda: Tiegs, 1947; Diplopoda: Dohle, 1964), but there is no evidence with which to correlate the embryos of proturans and myriapods.

Embryonic membrane

The embryonic membrane is represented only by the serosa in *Baculentulus densus*. The serosa of *Baculentulus densus* was revealed to retain an ability to differentiate into the tergum or participate in the definitive dorsal closure. Machida and Ando (1998) and Machida *et al.* (2002) demonstrated the evolutionary changes in embryonic membranes and their developmental potential in different hexapod embryos. According to them, the embryonic membrane system only with the serosa is the most ancestral among Hexapoda, and thus, Protura represent the most primitive condition among Hexapoda as well as Collembola: Diplura, the embryonic membranes of which had been previously recognized as being represented only by the serosa, develop a second embryonic membrane or amnion (see Ikeda and Machida, 2001).

The serosa of Hexapoda other than Protura has lost the ability to participate in the definitive dorsal closure (Machida and Ando, 1998; Machida *et al.*, 2002). In this respect, it should be noted that the proturan serosa retains this ability. Namely, this condition is a primitive feature shown in the potential sister group to Hexapoda, *i. e.*, Myriapoda or Crustacea (Machida *et al.*, 2002). Provided that the loss of the ability to participate in the definitive dorsal closure in the serosa could be an autapomorphy for Hexapoda as Machida and Ando (1998) and Machida *et al.* (2002) deduced, Protura might represent a primitive clade other than Hexapoda (see Machida, 2006).

Blastokinesis

Blastokinesis of *Baculentulus densus* is merely a simple flexion of the embryo, being utterly different from the blastokinesis of Ectognatha, which the formation and regression of amnioserosal folds are involved in and is often accompanied by a substantial reversal of the embryo's direction. Blastokinesis in Protura is similar to that in
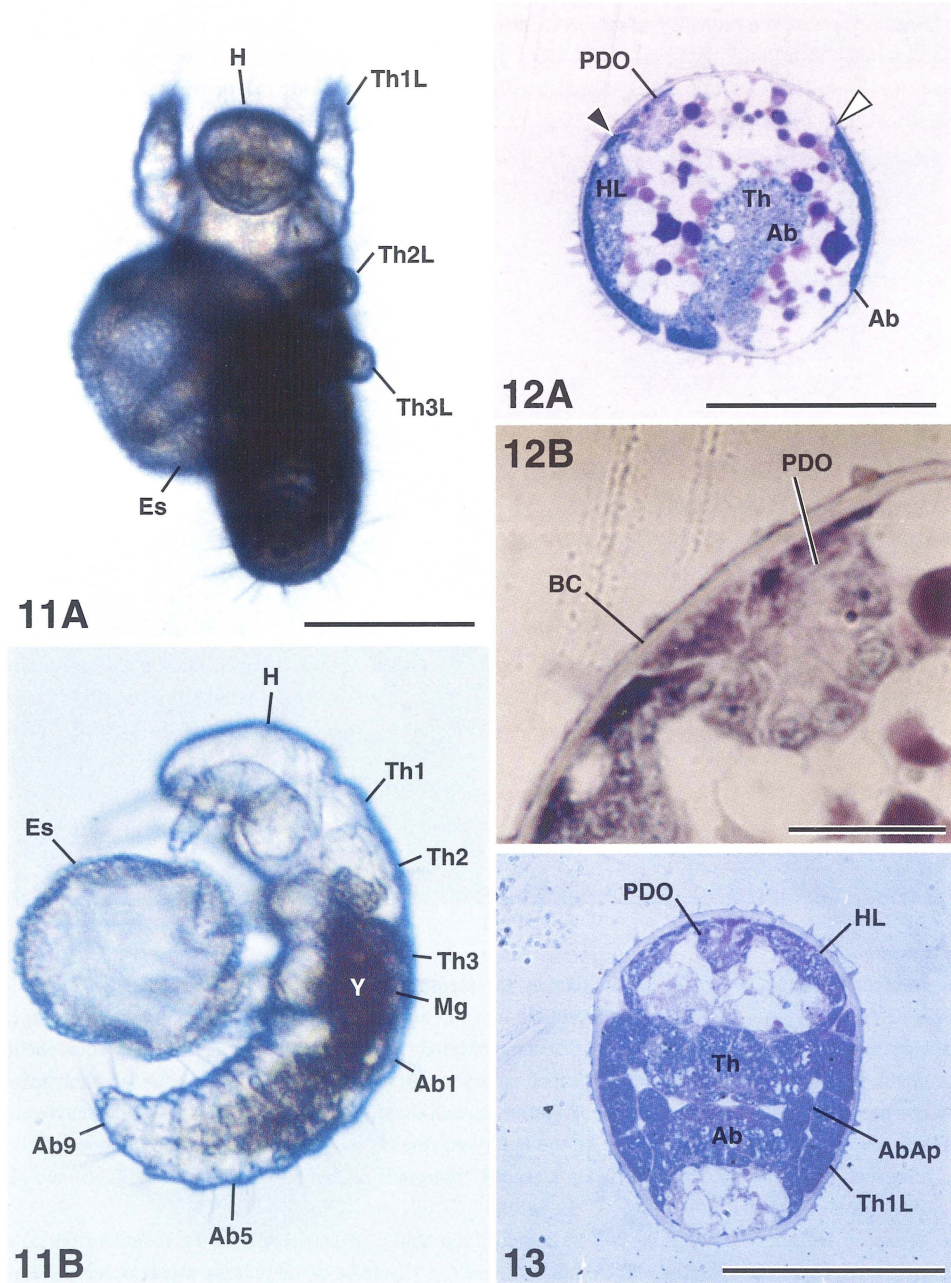
Figs. 11–13   Development of a proturan *Baculentulus densus* III.

Fig. 11   Hatching prelarvae. A. Ventral view. B. Lateral view.

Fig. 12   A. Sagittal section of an egg at approximately the same stage as that shown in Fig. 7. Black and white arrowheads respectively show the cephalic and caudal extremities of the embryo. B. Enlargement of the primary dorsal organ.

Fig. 13   Transverse section of an egg at approximately the same stage as that shown in Fig. 8. The primary dorsal organ shows signs of degeneration.

Ab: abdomen, Ab1,5,9: first, fifth, and ninth abdominal segments, AbAp: abdominal appendage, BC: Blastoderm cuticle, Es: eggshell, H: head, HL: head lobe, Mg: midgut, PDO: primary dorsal organ, Th: thorax, Th1–3: first to third thoracic segments, Th1–3L: first to third thoracic appendages, Y: yolk. Scales = 11, 12A, 13: 50 μm; 12B: 10 μm.

Collembola and Diplura (Jura, 1972; Ikeda and Machida, 1998), and can be regarded as being of the ancestral type within Hexapoda as Ikeda and Machida (1998) suggested.

Primary dorsal organ

The primary dorsal organ is formed in some apterygote hexapods (Collembola: Tiegs, 1942a; Jura, 1972; Diplura: Uzel, 1898; Tiegs, 1942b; Ikeda and Machida, 2001), myriapods (Symphyla: Tiegs, 1940; Pauropoda: Tiegs, 1947), and several crustaceans (Anderson, 1973). *Baculentulus densus* also develops a primary dorsal organ, although, as in Pauropoda (Tiegs, 1947), it seems to be less developed than the well-developed ones in Collembola, Diplura, and Symphyla.

Due to this finding, we know that all of the non-ectognathous hexapod groups develop this organ, and taking it account that it is common to some myriapod and crustacean groups, the primary dorsal organ might be regarded as one of the features comprising the groundplan of Mandibulata, although no information is available at present concerning whether it is plesiomorphic or apomorphic: any way, the absence of this organ in higher lineages could be regarded as being derived. A critical survey and comprehensive examination of primary dorsal organs are desired throughout Arthropoda.

Formation of entognathy

Although details are still sketchy, the mandibular, maxillary, and labial terga are revealed to be involved in the formation of the entognathy as constituents of mouth folds in Protura. An examination of the homology of the entognathy is especially important to test the validity of 'Entognatha' (see 'Introduction'). Full documentation of the formation of the proturan entognathy is earnestly desired.

Conclusion

An outline of the embryogenesis of Protura was presented. The proturan embryogenesis is characterized by the following ancestral features: 1) holoblastic cleavage, 2) embryogenesis of long germ type, 3) simple blastokinesis, and 4) the formation of a primary dorsal organ. It should be noted that the serosa retains the ability to participate in the definitive dorsal closure, suggesting that Protura represent a more basal clade in Hexapoda than previously believed. A comprehensive elucidation of proturan embryology will provide a sound basis for arguments on the origin of hexapods, phylogeny in higher ranks, and reconstruction of the hexapod groundplan.

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