

Structural Features of Eggs of the Basal Phasmatodean *Timema monikensis* Vickery & Sandoval, 1998 (Insecta: Phasmatodea: Timematidae)

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> Abstract

Structural features of the eggs of a basal phasmatodean, *Timema monikensis* Vickery & Sandoval, 1998 (Timematidae) were examined. The eggs of this species are soft and deposited coated with soil and/or other extraneous particles. The chorion, which is transparent and weakly sclerotized, is composed of an endochorion and an exochorion. The non-inclined operculum is located at the anterior pole of the egg. The chorion in the marginal region of the operculum is thinned to form an opercular collar together with the chorion of the egg body. An inverted triangular micropylar plate is on the ventral side of the egg attached to the opercular collar. The micropylar plate is without external differentiations but is specialized inside the chorion. A single micropyle, with a simple funnel-shaped chorionic opening, occurs on either side of the micropylar plate. The posterior mound, located at the posterior pole, is a thickened chorion rich in fine vertical striations, and the serosal cuticle beneath is thickened and highly specialized. The eggs of Timematidae were characterized and compared with those of Euphasmatodea and Embioptera. A phylogenetic discussion is presented, strongly supporting the assemblage of Timematodea, Euphasmatodea and Embioptera as monophyletic.

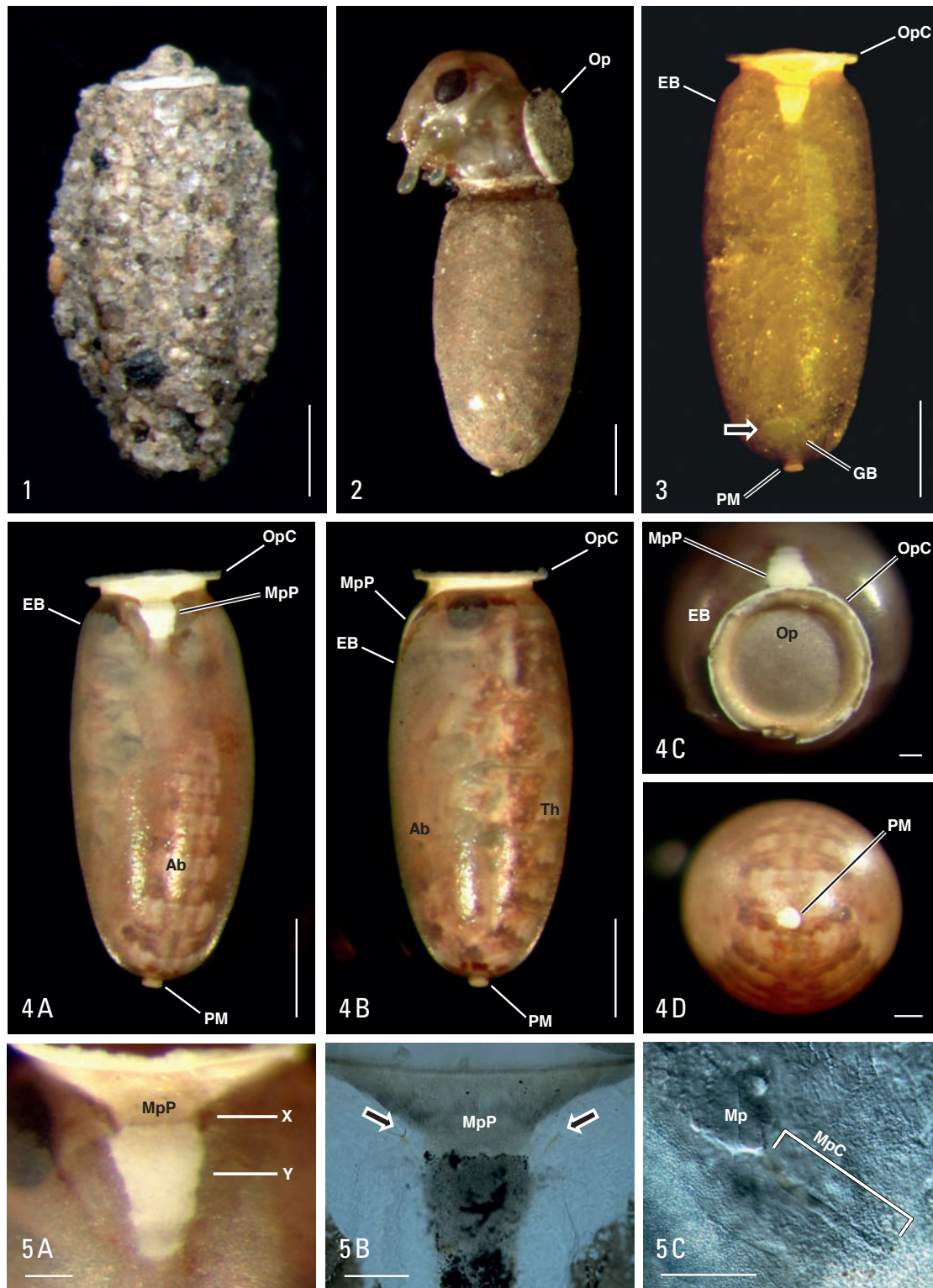
> Key words

Timema, Timematidae, Phasmatodea, Embioptera, egg structures.

1. Introduction

The genus *Timema* Scudder, 1895 (Timematidae), endemic to western North America, is usually considered the basalmost clade of Phasmatodea, i.e., the sister group of the remaining Phasmatodea (cf. BRADLEY & GALIL 1977; WHITING et al. 2003; BRADLER et al. 2003; BRADLER 2009). ZOMPRO (2004, 2005), in contrast, suggested a close affinity between *Timema* and Embioptera, based on several features including egg structure. However, information on *Timema* eggs remains sketchy, although there have been studies by CLARK SELICK (1997, 1998) and SELICK (1997) on the eggs of *Timema cristinae* Vickery, 1993, *T. douglasi* Sandoval & Vickery, 1996, *T. chumash* Hebard, 1920

and *T. podura* Strohecker, 1936 and by ZOMPRO (2004, 2005) on the eggs of *T. podura*. JINTSU et al. (2007) and JINTSU & MACHIDA (2009) examined in detail the eggs of an embiopteran, *Aposthonia japonica* (Okajima, 1926), and pointed out a close structural resemblance between the eggs of Embioptera and those of Phasmatodea, suggesting a close affinity between these two orders. However, their view should be tested by a detailed examination of the eggs of *Timema*, representing the proposed basalmost clade of Phasmatodea. Recently, we obtained a number of eggs of *Timema monikensis* Vickery & Sandoval, 1998, and examined their structural features.



Figs. 1–5. Eggs of *Timema monikensis* Vickery & Sandoval, 1998. **1:** An egg with its coating. Scale bar = 500 μ m. **2:** An egg just hatching, lateral view, ventral to the left. Scale bar = 500 μ m. **3:** An egg with the coating removed, containing an early germ band, ventral view. One can see the protocephalon (arrow) of the embryo in the posterior region of the egg: the embryo takes its position at the posterior pole of the egg, a little biased to the ventral side. Scale bar = 500 μ m. **4:** Eggs with the coating removed, containing an embryo that has acquired a definitive form. **4A:** Ventral view. Scale bar = 500 μ m. **4B:** Lateral view, ventral to the left. Scale bar = 500 μ m. **4C:** Anterior view, showing the operculum, ventral to the top. Scale bar = 100 μ m. **4D:** Posterior view, showing the posterior mound, ventral to the top. Scale bar = 100 μ m. **5:** Microcylar plate and micropyles. Scale bars = 100 μ m. **5A:** Microcylar plate. Lines X and Y represent levels of sections shown in Fig. 8. **5B:** Microcylar plate with paired micropyles, light field microscopy with transmission illumination. Arrows point to the micropyles. **5C:** Enlargement of a micropyle (left one in **5B**), differential interference contrast microscopy: two images with different levels in focus are merged into one frame. Scale bar = 50 μ m. Ab = abdomen; EB = egg body; GB = germ band; Mp = micropyle; MpC = microcylar canal; MpP = microcylar plate; Op = operculum; OpC = opercular collar; PM = posterior mound; Th = thorax.

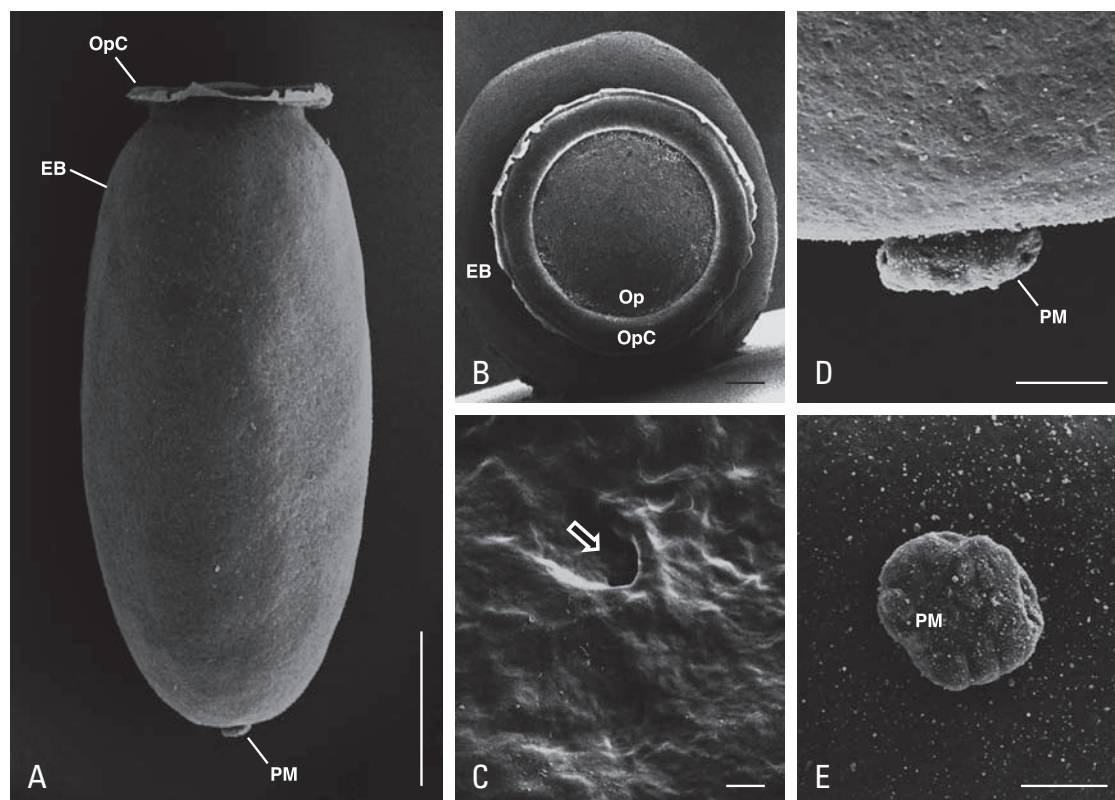


Fig. 6. SEM micrographs of eggs of *Timema monikensis* Vickery & Sandoval, 1998. **A:** Ventral view. The micropylar plate is not revealed, implying that it is devoid of external structural differentiation. Scale bar = 500 μm . **B:** Anterior view, showing the operculum, ventral to the top. Scale bar = 100 μm . **C:** Micropyle (arrow). This specimen was completely cleaned by treatment with antiformine for 30 min and 5% KOH for 30 min prior to the processing for SEM, so as to reveal the micropyles; the micropyles are not detected in the specimen shown in **A**, which was cleaned only in 30% antiformine for 5 min. Scale bar = 10 μm . **D, E:** Posterior mound, ventral (**D**) and posterior (**E**) views. Scale bars = 50 μm . EB = egg body; Op = operculum; OpC = opercular collar; PM = posterior mound.

2. Material and methods

Second or third instar juveniles of *Timema monikensis* (basically a parthenogenetic species) were collected in March and April 2009 on *Ceanothus* and *Cercocarpus* leaves in Los Angeles County, California. They were reared on *Ceanothus* cuttings until they were adults. Adults were kept individually in petri-dishes with soil brought back from the *T. monikensis* habitat, and fed on *Ceanothus* cuttings and a mixture of wheat gluten, sugar and vitamins, enabling females to produce and deposit their eggs. The eggs were incubated at 20°C without humidity control and entered diapause in several months. Eggs in diapause were used in this study.

After being dipped in water for a while, the eggs were transferred to a 10% solution of dishwashing detergent ("Joy" by Procter & Gamble) for 1 h, to remove material coating the eggs. Then, the eggs were immersed in 30% antiformine (4% hypochlorite solution) for 5 min and cleaned in water by means of forceps and a small brush. To observe the micropyles under SEM, the eggs were further treated in antiform-

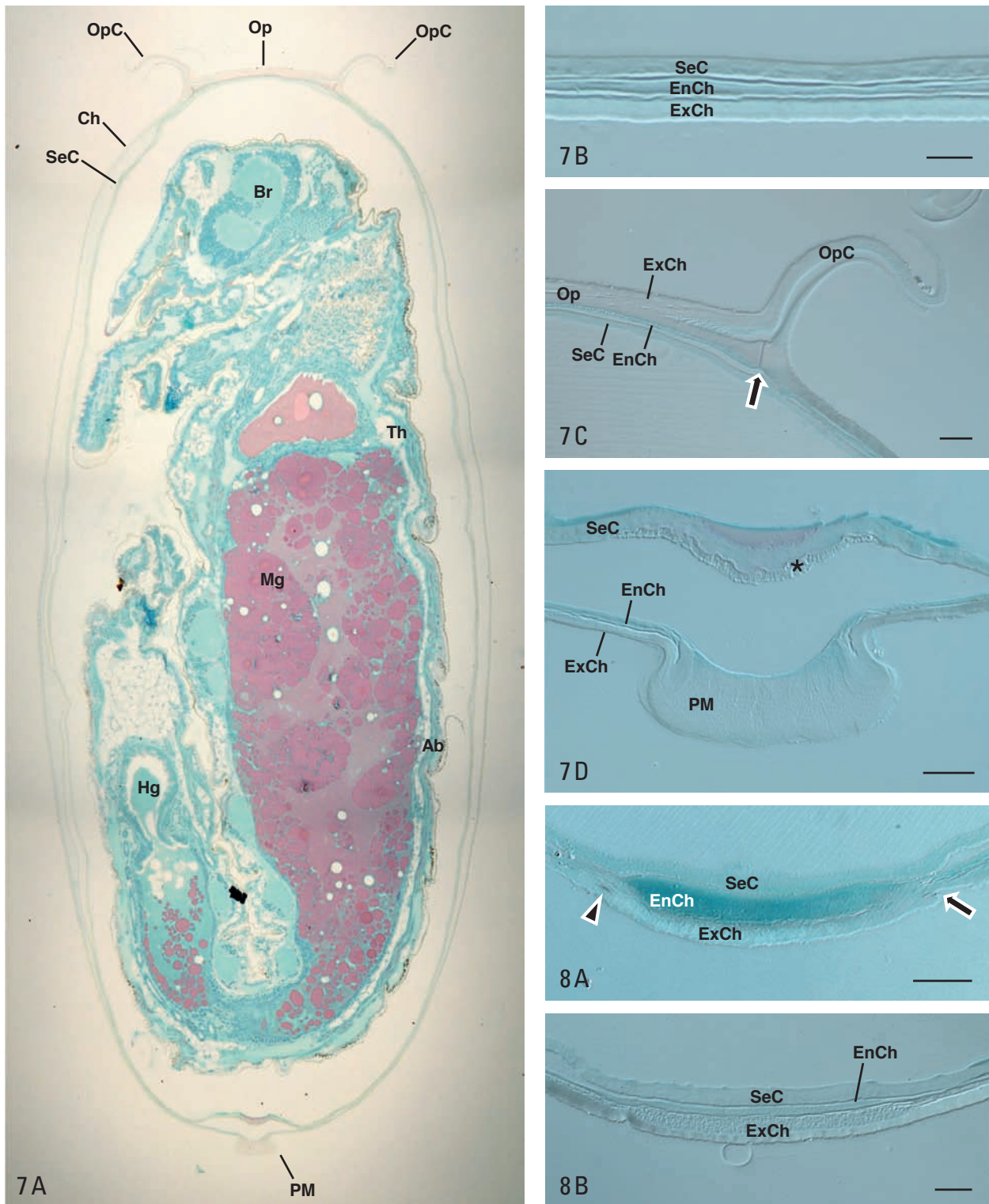
ine for 30 min and then in 5% KOH for 30 min, to completely remove the coating. The cleaned eggs were fixed either in Karnovsky's fixative according to MACHIDA et al. (1994a,b), or in Bouin's fixative.

General features of the eggs were observed under a stereomicroscope, Leica MZ12. To observe micropyles by light microscopy, torn egg membranes were mounted in a lactic acid solution, Heinz liquid, and examined with a Nikon OPTIPHOTO for light field images or a Leica DM6000B for differential interference contrast images.

For SEM, the fixed eggs were dehydrated in a graded ethanol series, dried with a critical point dryer, and coated with gold, and the specimens were observed under an SEM TOPCON SM-300.

Some of the fixed eggs were processed into 2- μm -thick methacrylate sections according to MACHIDA et al. (1994a,b). Sections were stained with 10% Mayer's acid haemalum at 60°C, 0.5% eosin G for 1 h and a 0.5% fast green FCF 80% ethyl alcohol solution for 5 min.

Diapause was easily broken by placing the eggs in wet conditions. Forty to 50 days later, the juveniles hatched.



Figs. 7, 8. Sections of eggs of *Timema monikensis* Vickery & Sandoval, 1998. **7A:** Parasagittal section of an egg, ventral to the left. Scale bar = 500 μm . **7B:** Enlargement of the egg membranes of the egg body, differential interference contrast microscopy. Scale bar = 10 μm . **7C:** Enlargement of the area around the opercular collar, differential interference contrast microscopy. An arrow shows the line of detachment that borders the part of the opercular collar derived from the operculum and that derived from the egg body. Scale bar = 20 μm . **7D:** Enlargement of the posterior mound, differential interference contrast microscopy. An asterisk shows the serration in the outer surface of the serosal cuticle. Scale bar = 20 μm . **8:** Transverse sections of the micropylar plate through the approximate levels indicated by lines X (A) and Y (B) in Fig. 5A, differential interference contrast microscopy. Scale bars = 20 μm . **8A:** Transverse section of the anterior half of the micropylar plate. The endochorion is thickened. The arrow and arrowhead show the micropyle and micropylar canal, respectively. **8B:** Transverse section of the posterior half of the micropylar plate. The exochorion is thickened and porous in structure. Ab = abdomen; Br = brain; Ch = chorion; EnCh = endochorion; ExCh = exochorion; Hg = hindgut; Mg = midgut; Op = operculum; OpC = opercular collar; PM = posterior mound; SeC = serosal cuticle; Th = thorax.

3. Results

The eggs of *Timema monikensis* are coated with soil and/or other particles ingested by females (Fig. 1). Figs. 3, 4, 6 show the eggs with this coating removed. The eggs are ellipsoidal, about 1 mm wide and about 2 mm long, and are soft, and light brown because the egg's inside is seen through the thin, transparent chorion (Figs. 3, 4A,B).

The operculum (Op) is located at the anterior pole (Figs. 3, 4A–C, 6B). It is a low dome-shaped disc (Figs. 4C, 6B, 7A) and not inclined, and its marginal region forms the opercular collar (OpC; Figs. 3, 4A–C, 6A,B, 7A,C) together with the chorion of the egg body. The operculum is devoid of a knob or capitulum.

By light microscopy, a whitish, inverted-triangular area is found attached to the opercular collar on the ventral side of the egg: the micropylar plate (MpP; Figs. 3, 4A,B, 5A,B). The micropylar plate does not show any structural differentiation externally, and is not recognizable under the SEM (Fig. 6A). Lateral to each margin of the micropylar plate is a single micropyle (Mp; arrows in Fig. 5B). The two micropyles are funnel-shaped openings through the chorion, without any specializations such as a micropylar hood or tube (Figs. 6C, 8A). From the micropyle, the micropylar canal (MpC) runs inwards medioposteriorly, across the chorion (Fig. 5B,C).

In this study, we examined about 80 eggs of *T. monikensis*, all of which were in diapause. Approximately 90% were at a stage where the embryos had acquired a definitive form (Fig. 4A,B). In the remaining eggs (10%), the embryos were at the early germ band stage (GB; Fig. 3). Although diapause occurs at a single, species-specific stage in most insect eggs (cf. DENLINGER 2002), it is known that one or two diapauses may occur in Euphasmatodea, depending on species or environmental condition (BEDFORD 1978). Since the venter of embryos in the final stage of development faces that side of the egg where the micropylar plate is located (Fig. 4A,B), this plate of *T. monikensis* can be designated as being on the ventral side of the egg. Early germ bands were located around the posterior pole of the egg, slightly toward the ventral side (Fig. 3). Thus, it is likely that the embryos of this species do not rotate longitudinally about the anteroposterior axis of the egg, unlike those of other phasmatodeans (FOURNIER 1967; BEDFORD 1970, 1978).

A knob-like chorionic projection (PM) approximately 100 μ m in diameter occurs at the posterior pole of the egg (Figs. 3, 4A,B,D, 6A,D,E, 7A,D), which is comparable to the structure CLARK SELICK (1997, 1998) named the “posterior mound” in eggs of other *Timema* spp.

Figs. 7, 8 show sections of eggs of *T. monikensis*. The chorion is composed of an exochorion (ExCh) and endochorion (EnCh), and in areas other than the operculum, opercular collar, micropylar plate and posterior mound, is 8–10 μ m thick, of which about 70% is exochorion. We could not find a vitelline membrane and it may be too fragile to observe under a light microscope. In eggs containing fully developed embryos, a thick serosal cuticle (SeC) is found beneath the chorion that is almost equal to the latter in thickness (Fig. 7B). The chorion of the operculum is about twice as thick as that of the egg body (Fig. 7C), but is thinned at its margin to form the opercular collar together with chorion of the egg body (Fig. 7C). That part of the opercular collar derived from the operculum and that derived from the egg body are clearly delimited by a detachment line (Fig. 7C): the operculum opens as the opercular collar ruptures along this detachment line, and the juvenile hatches (Fig. 2). The chorion in the area of the micropylar plate is thickened about 20 μ m and is specialized inside (Fig. 8): in the anterior half the endochorion is thickened (Fig. 8A), while in the posterior half the exochorion is thickened and porous in structure (Fig. 8B). In the posterior mound, the chorion is heavily thickened (about 20–30 μ m), with the exochorion and endochorion fusing; the chorion of the posterior mound has numerous vertical striations inside (Fig. 7D). Beneath the posterior mound, the serosal cuticle is strongly thickened and specialized with a serrated outer surface (Fig. 7D).

4. Discussion

Our observations of the structural features of *T. monikensis* eggs are consistent with previous findings made on eggs of *T. cristinae*, *T. douglasi*, *T. chumash* and *T. podura* (SANDOVAL & VICKERY 1996; CLARK SELICK 1997, 1998; SELICK 1997; ZOMPRO 2004, 2005). Based on that information and including the new findings presented here (indicated by italics), the eggs of *Timema* can be characterized as: 1) ellipsoidal, about 2 mm long, soft and with a coating of soil and/or other extraneous particles when deposited; 2) having a transparent chorion which is weakly sclerotized and composed of an endochorion and exochorion; 3) having a low dome-shaped operculum, which is not inclined, is devoid of a capitulum, and located at the anterior pole of the egg; *the chorion of the marginal region of the operculum is thinned to form an opercular collar together with the chorion of the egg body; there is a line of detachment between the two in the opercular*

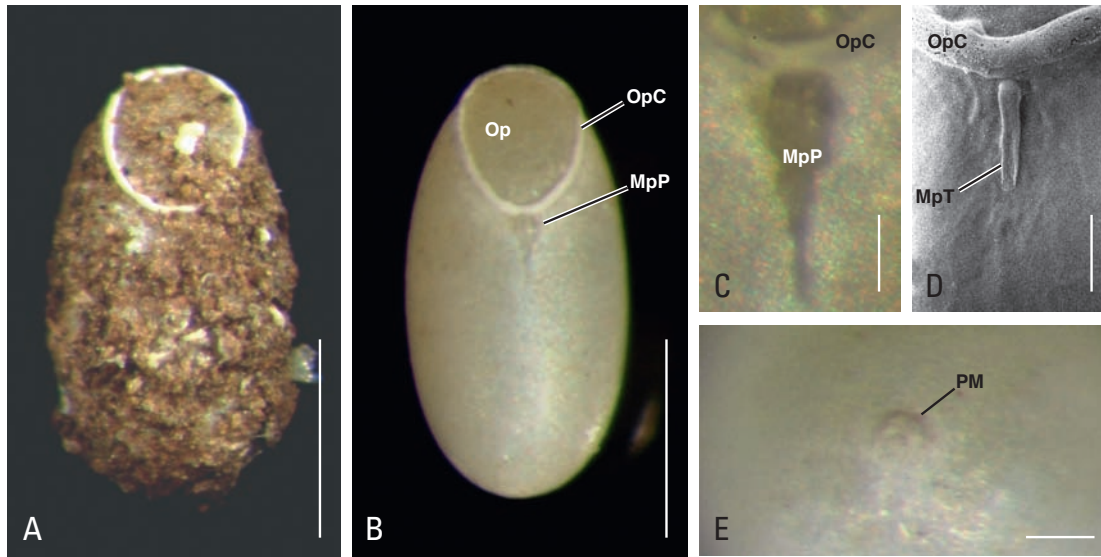


Fig. 9. Eggs of the embiopteran *Aposthonia japonica* (Okajima, 1926). **A:** An egg coated with extrinsic substances such as silk, plant tissues and excrements, ventral view. Scale bar = 500 μm . **B:** An egg with the coating removed, ventral view. The operculum surrounded by the opercular collar is at the anterior pole of the egg. The micropylar plate is attached to the opercular collar on the ventral side of the egg. The micropylar tube cannot be distinguished in this photo. Scale bar = 500 μm . **C:** Enlargement of the micropylar plate. The micropylar tube cannot be distinguished in this photo. Scale bar = 50 μm . **D:** SEM of the micropylar plate, accompanied by the micropylar tube. The micropylar plate cannot be distinguished in this photo. Scale bar = 50 μm . **E:** Posterior view, showing the polar mound. Scale bar = 100 μm . MpP = micropylar plate; MpT = micropylar tube; Op = operculum; OpC = opercular collar; PM = polar mound.

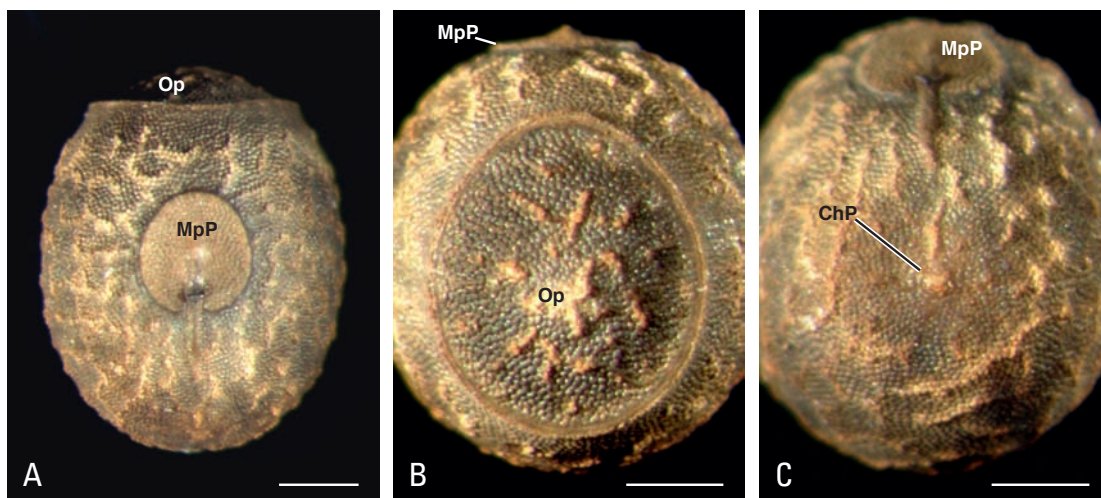


Fig. 10. Egg of the phasmatodean *Neohirasea japonica* (de Haan, 1842). Scale bars = 500 μm . **A:** Ventral view. The operculum is at the anterior pole of the egg, and the micropylar plate is in the center of the ventral side of the egg. **B:** Anterior view, showing the operculum, ventral to the top. **C:** Posterior view, showing the chorionic projection at the posterior pole of the egg, which is often named the “posterior area”, “pseudoplate” etc., ventral to the top. ChP = chorionic projection at posterior pole of egg; MpP = micropylar plate; Op = operculum.

collar, and the opercular collar ruptures along this line on hatching; 4) having an inverted triangular micropylar plate on the ventral side attached to the opercular collar; the plate is not externally differentiated but is specialized within the chorion; 5) possessing a pair of micropyles, each a simple funnel-shaped chorionic opening, on either side of the micropylar plate; a micropylar canal runs medioposteriorly from each micropyle and penetrates the chorion; and 6) having

a knob-shaped structure or posterior mound at the posterior pole; the posterior mound consists of thickened chorion rich in fine vertical striations, where the exochorion and endochorion fuse; the serosal cuticle beneath the posterior mound is thickened and highly specialized.

Orientation of the egg is defined by the orientation of the embryo in the final stage of development. According to this definition, the micropylar plate is on

the ventral side of the eggs of *Timema*, while in those of other Phasmatodea (Euphasmatodea) it is on the dorsal side (e.g., CLARK SELICK 1998; ZOMPRO 2004). As JINTSU et al. (2007) pointed out, the dorsal position in Euphasmatodea results from longitudinal rotation of the embryo along the anteroposterior axis of the egg during embryogenesis (e.g., FOURNIER 1967; BEDFORD 1970). In *T. monikensis* eggs the embryo does not rotate during embryogenesis. Consequently, the micropylar plates are most likely on homologous sides of the eggs in both *Timema* and euphasmatodean eggs, and the different positions only indicate a shortcoming in the definition; the actual difference between *Timema* and euphasmatodean eggs concerns the presence or absence of embryonic rotation.

ZOMPRO (2004) suggested a close relationship between Timematidae and Embioptera based on several characteristics including egg features such as 1) a prominent opercular collar, 2) a micropylar plate that externally is not specialized and 3) a micropyle close to the opercular collar; as a result, he considered Phasmatodea to be polyphyletic. Although ZOMPRO's idea is interesting, it requires further consideration. Euphasmatodeans have tough eggs mimicking plant seeds with a heavily sclerotized chorion and elaborate micropylar plate (cf. CLARK SELICK 1997, 1998; ZOMPRO 2004). These features of Euphasmatodea are apparently apomorphic. However, more plesiomorphic is probably the condition seen in Timematidae and Embioptera, whose eggs have a weakly sclerotized chorion that must be protected with a coating of extrinsic materials at time of deposition (for Timematidae, Fig. 1; CLARK SELICK 1997, 1998; ZOMPRO 2004; for Embioptera, Fig. 9A; KERSHAW 1914; ROSS 2000). At least two of the features proposed by ZOMPRO (2004) as evidence for a close affinity between Timematidae and Embioptera (1 and 2 above) may be correlated with the likely plesiomorphic condition the eggs of these two groups share: that is, because the chorion is only weakly sclerotized, the micropylar plate is not so elaborate and the opercular collar is prominent. (As for character 3, see below: the number of micropyle(s) is revealed to differ between these two groups!)

Our study revealed the posterior mound in *T. monikensis* to closely resemble the polar mound of embiopteran eggs in having: 1) a thickened chorion where the exochorion and endochorion fuse, 2) numerous striations inside, and 3) a thickened and highly specialized serosal cuticle beneath it. These features clearly play a significant role in eggs of Timematidae and Embioptera, perhaps, as a hypopyle or aeropyle. The congruence may reflect homology between these mounds, suggestive of a closer affinity between Timematidae and Embioptera. However, eggs of Euphasmatodea often have specialized chorionic structures at the posterior pole, known as the "polar area" or "pseudoplate"

(e.g., ZOMPRO 2004), which are likely homologous to the posterior mound or polar mound in timematid and embiopteran eggs. Indeed, there are phasmatodeans in which such polar structures are seemingly lacking (e.g., CLARK SELICK 1997, 1998; ZOMPRO 2004), but even if the chorion is not specialized externally, it may have special features inside. More study is needed, and there is no clear support for a clade Timematidae + Embioptera based on egg structure.

Attention needs to be paid to the fact that *T. monikensis* eggs have a pair of micropyles associated with the micropylar plate, whereas the eggs of Euphasmatodea (e.g., CLARK SELICK 1997, 1998; ZOMPRO 2004) and Embioptera (JINTSU et al. 2007; JINTSU & MACHIDA 2009) have only one. Taking into account that possession of a single micropyle on the ventral side of the egg is unique to euphasmatodeans and embiopterans among Polyneoptera (cf. HINTON 1981; JINTSU et al. 2007), this feature could be regarded as a potential "synapomorphy" of these two groups. However, the exclusive phylogenetic correlation of Embioptera and Euphasmatodea may be incorrect, according to recent morphological (BRADLER 2009) and molecular (WHITING et al. 2003; TERRY & WHITING 2005) data where monophyly of Timematidae + Euphasmatodea is well-supported.

Whatever the interrelationships among Timematodea, Euphasmatodea and Embioptera, it should be emphasized that these three groups share several features: 1) a detachable operculum, 2) a micropylar plate on the "ventral side" of the egg (as in the phasmatodeans discussed above), 3) a small number of micropyles (1 or 2) associated with the micropylar plate, and 4) a specialized chorionic structure at the posterior pole (as in the case of the phasmatodeans discussed above) (for Embioptera and Phasmatodea, see Figs. 9 and 10, respectively; cf. HINTON 1981; CLARK SELICK 1997, 1998; ZOMPRO 2004; JINTSU et al. 2007; JINTSU & MACHIDA 2009). Only eggs of these three groups bear such features (cf. HINTON 1981; CLARK SELICK 1998; JINTSU et al. 2007; JINTSU & MACHIDA 2009), and the monophyly of a clade comprising these three taxa would appear well-supported based on egg structure. To clarify the interrelationships within this clade, determination of the states of the relevant egg characters in the stem lineage is necessary. A comprehensive structural analysis of eggs throughout the Polyneoptera is desired, including detailed comparisons within Embioptera and of the chorionic thickening at the posterior pole of the egg in various Phasmatodea, together with a careful survey of the number and positioning of the micropyles in the eggs of other polyneopterans.

A clade comprising Timematidae, Euphasmatodea, and Embioptera has found support in several recent phylogenetic studies (e.g., WHITING et al. 2003; TERRY & WHITING 2005; KJER et al. 2006; BRADLER 2009);

however, there is also much evidence in conflict with this hypothesis (summary in KLASS 2009). Furthermore, a monophyletic Phasmatodea including *Timema* appears to be well-supported by both morphological (BRADLER 2009) and molecular data (TERRY & WHITING 2005, who also included morphological data); yet, in the combined analyses of KJER et al. (2006) the relationships between *Timema*, Euphasmatodea, and Embioptera are ambiguous.

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