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CONTRIBUTION TO THE PRELARVA STATUS: THE MOLTING CYCLE OF THE CALYPTOSTASIC PRELARVA OF THE TROMBICULID MITE
LEPTOTROMBIDIUM ORIENTALE (ACARIFORMES: TROMBICULIDAE)

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(accepted June 1999)

SUMMARY: The calyptostasic prelarva of the trombiculid mite Leptotrombidium orientale (Schluger, 1948) and its moulting were investigated by means of transmission and scanning electron microscopy. Even before the formation of the prelarva, a particular embryonic cuticle above ectoderm cells is secreted; this soon detaches, leaving an exuvial space where haemocytes migrate. Prelarval cuticle is deposited above short microvilli of hypodermal cells and, in the completely developed state, is very similar to the cuticle of quiescent proto- and tritonymphs. It is composed of a thick, clear procuticle with curved pore canals and a narrow epicuticular layer, completely lacking setae. The hypodermal layer of the prelarva consists of flattened epithelial cells, which undergo mitotic divisions. The prelarva, as an individual instar with cuticle closely applied to the hypodermis, exists for a very short period and is completely concealed within the eggshell, being only a pharate instar. Within the eggshell, the prelarva undergoes the next moult, expressed again by detachment of cuticle, migration of haemocytes into the exuvial space, reorganization of hypodermis and, finally, secretion of an essentially new, strongly-folded, cuticle of the active larva. At this time, the eggshell splits and the prelarval exuvium with the pharate larva inside appears. After some time, the latter hatches actively from the prelarval cuticle. Since the egg at the later stages is not an embryo, but only a cover for the pharate prelarva as well as for the larva, the processes considered cannot be attributed to an embryonization and the prelarva cannot be regarded as an embryonic instar.

RéSUMÉ: La prélarve calyptostasique du trombiculide Leptotrombidium orientale (Schluger, 1948) et les processus de mues sont examinés en microscopie à transmission et à balayage. Avant formation de la prélarve, une cuticule embryonnaire est sécrétée par les cellules de l’ectoderme. Peu après elle s’en sépare en menageant un espace exuvial où les hémocytes migrent. La cuticule de la prélarve se dépose au-dessus des courtes microvillosités hypodermiques des cellules et à son plein développement, elle ressemble à la cuticule des proto- et tritonymphes immobiles. Elle se compose de la grosse procuticule claire avec des canaux poreux sinueux et de la couche épicuticulaire mince ; elle est d’épouvurvue de poils. La couche hypodermale de la prélarve se compose de cellules épithélia
Les aplaties qui subissent des mitoses. Une prélave fugace avec la cuticule étroitement liée à l'hypoderme, est renfermée dans l'enveloppe de l'œuf et constitue la calyptostase. La prélave subit la mue à l'intérieur de l'œuf : la cuticule se détache, les hémocytes migrent dans l'espace exuvial, l'hypoderme est réorganisé, et enfin, la nouvelle cuticule, très plissée, de la prélave active est sécrétée. Ensuite, l'enveloppe de l'œuf se fend et celle de la prélave apparaît. Ce n'est pas la prélave elle-même avec la prélave pharate à l'intérieur. Celle-ci éclot rapidement de la cuticule prélave. Puisqu'un œuf au stade final n'est plus l'embryon mais l'enveloppe de la prélave, les processus examinés ne peuvent pas être considérés comme faisant partie du développement embryonnaire.

INTRODUCTION

As is known, there are one to several provisional embryonic cuticles in insects deposited one after another during late embryonic development (SHARAN, 1958; SHARAN & SAHNI, 1960; SBRENNA MICCIARELLI & SBRENNA, 1972; LOUVET, 1974; DORN, 1976; DORN & HOFFMANN, 1981; MACHIDA et al., 1994). The main protective role of the embryonic cuticles was assumed and some functional and developmental reasons concerning their deposition associated, in particular, with muscle rearrangement were proposed (SHARAN, 1958). However, no comprehensive ontogenetic analysis of their real significance has been performed.

In mite ontogenesis, the first developmental instar, a regressive prélav, possesses its own cuticle and is usually concealed within the egg. Correspondingly, detection of this instar is only possible after careful examination of eggs, whereas only the apoderma (REUTER, 1909; GRANDJEAN, 1938), or secondary cuticle (deutovum) (WHARTON 1946), may be recognized after splitting of the eggshell. A rapid changing of prélav larval cuticle, which always occurs within the chorion, is a widespread phenomenon among insects (see, for instance, DORN & HOFFMANN, 1981), termed the "embryonic moult". Is the mite prélav cuticle a true embryonic cuticle which changes owing to the embryonic moult?

The main aim of this paper is to provide an objective view of the processes of early postembryonic development, occurring under the eggshell in the majority of mites. As was mentioned above, this developmental period is typically occupied by a strongly reduced, endostastic prélav (KETHLEY, 1990), concealed within the egg and, moreover, frequently considered as a completely embryonic "foetal" instar (CANNARD & STOCKMANN, 1993). To solve this problem I conducted ultrastructural (TEM and SEM) investigations of the eggs and prélavlarvae of Leptotrombidium orientale (Schluger, 1948) as a representative of the family Trombiculidae, the group of Acariform mites possessing the most generalized type of prélavlarval reduction.

MATERIAL AND METHODS

Eggs and prélavlarvae of L. orientale were obtained from the laboratory culture maintained at the Laboratory of Parasitology, Zoological Institute, Russian Academy of Sciences, according to procedures described in detail previously (SHATROV, 1993). In the laboratory, females lay eggs gradually from one to about four eggs per day, up to a total number of 100–200 eggs per female during the first cycle of deposition, lasting for one to three months in general. Eggs and prélavlarvae of the fourth laboratory generation, at different ages from three days up to three weeks after deposition, were used. It should be noted, however, that all laid eggs started to develop very asynchronously, even under constant laboratory conditions. Some eggs started development immediately after deposition, whereas others were inactive for several months. As a result, the exact age of an egg cannot be determined with accuracy and the degree of development of an egg can only be determined by internal examination. However, after the beginning of development, all processes proceed very rapidly in eggs and the average time from deposition (or activation) of eggs to emergence of the active larvae is...
about two to three weeks in the laboratory (Shatrov, 1993).

For transmission electron microscopy (TEM), eggs and prelarvae were fixed in 2.0% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.2–7.4) for 2 h. They were fixed either intact or carefully pierced with sharp entomological pins for better penetration of the fixing fluid without loss of the contents. The specimens were then washed in several changes of 0.2 M phosphate buffer, postfixed in 2.0% osmium tetroxide solution in 0.1 M phosphate buffer with 8.56% sucrose for 2 h, dehydrated in alcohol and acetone, and embedded in an Araldite mixture. Serial ultrathin sections were cut on an LKB-III ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Tesla BS-500 transmission electron microscope at 60–90 kV.

For scanning electron microscopy (SEM), eggs and developing prelarvae were fixed in alcohol, dried in a Hitachi HCP-2 critical point dryer, covered with platinum in an Eiko-5 vacuum evaporator and examined in a Hitachi S-570 electron microscope at 20 kV.

RESULTS

After complete formation of the ectoderm layer of the embryo, which occupies a position under the secondary vitelline envelope (chorion, or eggshell) (Shatrov, 1997b), a very thin apical membrane is produced above, as may be sometimes distinguished, the tiny, short, scarce microvilli of ectoderm cells (Figs. 1, 2). This membrane, which has not been detected previously in mites, corresponds, presumably, to the first provisional embryonic cuticle (membrane or envelope) of various insect groups (Sharan, 1958; Sharan & Sahni, 1960; Dorn & Hoffmann, 1981, Machida et al., 1994). The embryonic cuticle in L. orientale consists of a flocculent material of moderate electron density, with an irregular inner border, and seems to have no apparent layers (Fig. 2) such as are found in epi-, exo- and endocuticle. The embryonic cuticle is always tightly appressed to the chorion at its outer border, and at the moment of splitting of the eggshell it is found shed together with the latter.

The period during which the embryonic cuticle is closely applied to ectoderm cells appears to be very short. In almost all eggs examined, in which the embryo had just started to develop, the cuticle was found to be detached already from cells, leaving a relatively large evuvial space (Figs. 3, 4). Haemocytes of two types (Shatrov, 1997a) actively migrate from the body cavity (haemocoelic space) into this space, either through rupture of the epithelial layer or directly through the intact epithelium (Fig. 4). In the euvial space, haemocytes are found to be gradually broken and frequently only their debris may be seen.

At the time of detachment of the embryonic cuticle, or later, initial ectoderm cells transform into the definitive hypodermis of the prelarva.

The initial ectoderm cells contain an oval nucleus with large chromatin globules and an eccentrically located nucleolus, free ribosomes and polysomes scattered throughout the cytoplasm, small scarce mitochondria and some oval lipid inclusions (Figs. 1, 3). The cells join each other via desmosomes and gap junctions in their apical parts. In contrast to the ectoderm cells, the hypodermal cells additionally possess long, single cisterns of a rough endoplasmic reticulum in the cell periphery, as well as long, isolated microtubules orientated freely in the cytoplasm or directed from its base towards the apical portion (Figs. 5, 6). Hypodermal cells also contain a nucleus, sometimes with two nucleoli, rarely found Golgi complexes and some lysosomal bodies (Fig. 6). The completely formed hypodermal layer of the prelarva is composed of flattened or sometimes prismatic epithelial cells, which undergo mitotic divisions. Although cells of the hypodermal layer lie close to one another, they have no immediate contacts and leave extracellular spaces almost to their apical parts (Figs. 6, 7).

Soon after apolysis of the embryonic cuticle, a true prelarval cuticle begins to cover the epithelium. Deposition of this cuticle occurs in the classical arthropod way of cuticle formation above short tiny microvilli of the apical plasma membrane (Figs. 5, 6). An electron-dense cuticulin layer appears first during this process. The moment when the cuticulin layer becomes continuous may be considered as the starting point of the prelarval instar. During the subsequent formation of the cuticle, the simultaneously
Figs. 1–4: 1. — Peripheral part of egg with eggshell (ESH) and yolk (Y). Note ectoderm cell with nucleus (N) depositing embryonic cuticle (EC) ($\times$ 12700).
2. — Flocculent embryonic cuticle (EC), without clear lamination, deposited above apical plasma membrane of ectoderm cell. ESH: eggshell ($\times$ 31200).
3. — Embryonic cuticle (EC) detached from ectoderm cells, with nuclei (N) leaving relatively large exuvial space (ES) with the haemocytes debris. Note eggshell (ESH) tightly appressed to embryonic cuticle. Y: yolk ($\times$ 9300).
4. — Haemocytes (HC) invading exuvial space (ES) between hypodermis (H) and embryonic cuticle (EC) tightly adjoined the eggshell (ESH). N: nucleus of haemocyte ($\times$ 6700).
Figs. 5–8. 5. — Newly formed cuticulin layer (CL) of prelarva above short microvilli of hypodermal cell (H) with nucleus (N). Note embryonic cuticle (EC) and eggshell (ESH), which remain closely adjoined. HC: haemocyte in the haemocoelic space (×16000). 6. — Prismatic hypodermal cells (H) with nuclei (N) and slightly folded cuticulin layer (CL) of prelarval cuticle deposited above microvilli of apical plasma membrane. Note small Golgi complex (G) in hypodermal cell (×16000). 7. — Part of developing prelarva within eggshell (ESH) with strongly folded integument, consisting of hypodermis (H) and cuticulin layer (CL). Note broken haemocyte (HC) in exuvial space (ES) (×3100). 8. — Peripheral part of a more advanced prelarva with its own folded cuticle (C), hypodermis (H) and haemocyte (HC) in the haemocoelic space. Note eggshell (ESH) and embryonic cuticle beneath (×4000).
Figs. 9-12: 12. — Completely developed, slightly laminated, clear cuticle of prelarva (C), with curved pore canals (PC) and hardly distinguished epicuticle. Note that hypodermal cells (H) with nuclei (N) are joined by desmosome (D) (× 40000). 10. — Part of prelarva in more developed condition, with detached cuticle (C) and exuvial space (ES) beneath it. H: hypodermis of prelarva (× 4000). 11. — Developing larva, with strongly folded epicuticle (E) within old cuticle of prelarva (C) flattened due to pressure of exuvial fluid. Note haemocyte (HC) in exuvial space (ES) and an electron-dense eggshell, which remains intact during this period (× 9300). 12. — Prelarval cuticle (C) with small tubercles appearing from under split eggshell (ESH), as seen with SEM (× 4700).
developing prelarva, with its strongly reduced mouthparts and legs and somewhat folded integument, is strongly retracted from the eggshell which, however, stays intact during this period (Figs. 7, 8). The completely developed cuticle is composed of a thick, clear, slightly laminated procuticle with curved pore canals and a narrow, hardly visible, epicuticular layer (Fig. 9). The total thickness of the cuticle is about 0.8 μm. The cuticle of the prelarva completely lacks setae on both the body and the appendages, but it possesses some small tubercles (Fig. 12).

Just after its complete formation, the prelarval cuticle undergoes its own apolysis, marking the beginning of the prelarval moult (Fig. 10). Thus, it appears that the period of existence of prelarva, with its own cuticle closely applied to hypodermis, is very short, lasting only a few hours, within the eggshell. During this time, however, the prelarva is an independent instar with its own internal organs and structures. A new molting process is also accompanied by an intensive migration of haemocytes into the wide exuvial space through the hypodermal layer (Fig. 11).

Within the exuvial space, gradually broken haemocytes are found taking part in partial destruction of old prelarval cuticle from the inner side, as, probably, occurs in acaridid nymphs (KANUNGO, 1969). Old prelarval cuticle is flattened due to pressure of the exuvial fluid.

Some time after apolysis of prelarval cuticle, the hypodermal cells begin to deposit a cuticulin layer of the active larva, which stays for some days within both the eggshell and the old prelarval cover, as a pharate phase. The newly-deposited epicuticle of the larva is found to be strongly folded (Fig. 11), as can also be seen in active unfed larvae in the laboratory after hatching from the old prelarval cast. The hypodermal layer undergoes some reorganization to the time of deposition of the larval cuticle. In particular, its cells become more flattened, possess no marked spaces between themselves and may contain some small, residual bodies.

At the time of formation of the active larva, the eggshell splits into two halves, approximately along the vertical and middle cleft-line. The old prelarval cuticle, which looks like the true prelarval instar but contains only the developing larva, emerges from the split eggshell. Small tubercles on some parts of the prelarval body may be seen on the otherwise smooth prelarval cuticle (Fig. 12).

**Discussion**

It is not the purpose of the present paper to provide a detailed description of embryogenesis in mites. Nevertheless, it is evident that the process of deposition of embryonic cuticle(s) differs greatly in insects (see LOUVET, 1974; MACHIDA et al., 1994) and Acari (see HAFIZ, 1935; HUGHES, 1950; AESCHLIMANN, 1958; LANGENSCHEIDT, 1958; AKIMOV & YASTREBSOV, 1990). In some insects, for instance, primary blastoderm cells are transformed into serosa, a particular extraembryonic area, which secretes a serosal, or blastoderm cuticle over the entire egg surface (MACHIDA et al., 1994). This serosal cuticle is not a true embryonic cuticle deposited by ectoderm cells of the embryo proper (SHARAN & SAHNI, 1960; DORN & HOFFMAN, 1981). In mites (HUGHES, 1950; AESCHLIMANN, 1958; AKIMOV & YASTREBSOV, 1990), blastoderm cells may be directly transformed into an ectoderm layer and later into mature epidermis, as, apparently, is found in *L. orientale*. For this reason, the cuticle, which first appears above the ectoderm cells in the *L. orientale* embryo, may obviously be considered the true embryonic cuticle of trombiculid mites. In this sense, it is a homologue to that found in some insects (SHARAN & SAHNI, 1960). Embryonic cuticle, as mentioned above, had not been detected previously in mites, apparently due to its close association with the eggshell after its apolysis, which makes it very hard to identify.

Haemocytes, as the apparent derivatives of mesoderm elements, migrate into the exuvial space and provoke a rapid apolysis of the embryonic cuticle. The early differentiation of haemocytes clearly shows that the cellular layer depositing embryonic cuticle is already represented by ectoderm cells.

The ontogenetic significance and functional role of the embryonic cuticle in trombiculid mites remain unclear. The changing of the embryonic cuticle into the prelarval one proceeds quite rapidly during formation of the prelarva and may be interpreted as the true embryonic moult. In insects, various functions have been ascribed to provisional embryonic cuti-
cle(s), such as protective envelopes, egg bursters or a mould for the developing organs and tissues (Shar
ran, 1958; Sharan & Sahni, 1960). Such secondarily acquired adaptations are certainly plausible for mites, but another, simple, ontogenetic explanation may also be proposed for the production of this cuticle. In particular, it is thought to represent the vestigial cover of the completely lost hypothetical pre-prelarval instar. This instar seems to be strongly embryonized and at present cannot be detected with certainty as an individual stage in any group of mites. Thus, the morphogenetic processes of development of this hypothetical instar are totally concealed within the embryo and have no apparent signs in mite ontogene
sis. Since embryonic cuticle differs strongly from the prelarval one, further morphogenetic processes take place during the formation of the prelarva and replacement of provisional cuticle. These processes are, in particular, mediated by reprogramming of the epidermis and by development of both prelarval appendages and tissues. As in some insects (Sharan & Sahni, 1960; Sbrenna Micciarelli & Sbrenna, 1972), the embryonic moult in trombiculid mites appears not to be controlled by ecdysial glands or hormones.

The embryonic moult leads to the formation of the prelarva, which, in this sense, may not be considered as a proper embryo, but only as the first postembryonic, in particular calyptostastic instar, although existing within the egg. The finding of the active prelarvae both in different groups of the primitive Endeostigmata (Cornet, 1977, 1979; Schuster & Potsch, 1988) and in the derived Anystoidea (Otto & Olomski, 1994; Otto, 1997) appears to confirm the fact that the prelarva is a postembryonic state as Grandjean (1957) suggested. This statement contradicts the interpretation of Canard & Stockmann (1993) of the embryonic status of the prelarva in mites, which, according to them, “can be compared with foetal instars of other arachnids, but it is not postembryonic” (Canard & Stockmann, 1993: 466). As shown here, the eggshell is only a cover for early stages of mite development and cannot be classified as the embryo, which is also true for the prelarva. However, since the prelarva contains large masses of yolk that will be digested in the giant mid-gut (entoderm) cells during both prelarval and larval lives, a foetal aspect of the former appears to be quite legitimate. In trombiculid mites, in particular, no special vitellophagous cells are found and entoderm cells, initially spread throughout the yolk, taking part in its digestion. The epithelization of the mid-gut proceeds only some days after the emergence of the unfed larvae (post-moult development). The absence of special vitellophagous cells was also shown in cheyletid mites (Hafiz, 1935).

The prelarva, with its own cuticle closely applied to the hypodermis, evidently lives for a very short period and undergoes moult ing just after the complete formation of cuticle. It may be supposed, therefore, that the complete formation of prelarval cuticle may be the endogenic signal for the next moult, occurring partly or entirely within the eggshell. Thus, the latter may typically contain the embryo, the prelarva, and the developing larva as well. The same situation is found during the formation and moult ing of quiescent nymphs of trombiculid mites, namely the calypto
stastic proto- and tritonymph. Like the prelarva, proto- and tritonymphs live for a very short period, entirely within the old cuticle of the previous active instars (larva and deutonymph). Moreover, they possess cuticle structurally very similar to that of the prelarva. It is clearly seen from this example that there is strong morphogenetic correlation during the on
togenesis of trombiculid mites.

Another problem concerning the prelarval ontogenetic status arose only recently. According to the assumption of Otto (1997), the mite prelarva is not a regressive instar, but an embryonic stage with some larval characters already expressed (Otto, 1997). Motile prelarvae in some groups are consequently thought to be embryos developed to a greater extent than immobile prelarvae. Nevertheless, from the data obtained here, it may be reasonably concluded that the prelarva has its own cuticle and morphology, which had been expressed much more strongly as the signs of the first larval instar in the earlier historic periods. Therefore, the life and morphology of the prelarva are affected by morphogenetic processes already belonging to postembryonic ontogenesis, and it must only be attributed to the first postembryonic instar, as is still admitted by many acarologists (see Grandjean, 1957; etc.). It should be noted that the eggshell has no immediate morphological contacts.
with the embryo and prelarva and seems to be a cover for the strongly reduced prelarva, which only exists in a pharate condition as a quiescent calyptostasic instar.

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