Appearance and development of skeletal structures in *Holothuria scabra* larvae and epibiont juveniles

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Abstract

Various types of calcareous structures have been observed in *Holothuria scabra* sea cucumber larvae and epibiont juveniles at various ages and stages of development. An unpaired calcareous body can be observed as early as the dipleurula stage (less than two days after fertilisation); it is located in the posterior part of young larvae (36 h), grows, changes appearance (from a star, it becomes a half-sphere) and can still be found in epibiont juveniles. The madreporite appears in older auricularia (10 days) in the form of a curved stalk located at the base of the water ring canal; it develops to form a screened sphere in pentactulae and juveniles. Tables are recognisable in doliolaria larvae (15 days) and the initial formation of the peripharyngeal calcareous ring can been observed in pentactulae (17 days). The first podia rods and perforated disks appear in 20-day-old juveniles and anal plates from day 22 onwards, but button spicule only develop at the end of the epibiont life stage (45 days).

Introduction

The calcareous parts of a sea cucumber's tegument, oral tentacles and podia are the most important taxonomic features. However, these parts change during growth and can be very different in juveniles and adults of the same species (Féral 1980; Cutress 1996; Massin et al. 2000). In particular, these differences involve size or architecture of the structures (e.g. bigger or smaller, more complex or simpler), but they can also be linked to the early disappearance or late appearance of some structures. Massin et al. (2000) described in detail the wide range of spicules found in *Holothuria scabra* juveniles. However, apart from what can be found in Mortesen's general and fairly dated works (1921, 1937, 1938), there is almost no information available on calcareous structures in sea cucumber larvae (see Hamel et al. 2002; Sewell and McEuen 2002). This paper looks into such structures in the pre- and immediately post-metamorphic phase of *H. scabra*. Its purpose is to identify the exact time they appear, describe them and follow the growth of young larvae to the epibiont juvenile stage.

Materials and methods

The larvae and juveniles analysed came from fertilisation and breeding efforts carried out at the "Aqua-lab" in Toliara (southwest coast of Madagascar) (Jangoux et al. 2001; Rasolofonirina 2004). The formation and development of calcareous structures were monitored on a daily basis, from the gastrula stage through to the end of the juveniles' epibiont stage (i.e. about 50 days after fertilisation, when the juveniles began burrowing [endobionts]). To do this, batches of several dozen larvae were set in 70% ethanol, placed in water glasses and soaked for 10 minutes in a 10% bleach solution. The digesta were then rinsed six times in distilled water, dried, coated with gold and examined with a scanning electron microscope (JEOL JSM-6100). The same procedure was used for juveniles except that they were left longer in the bleach solution, and were monitored under a microscope (followed by digestion of the fleshy tissues). Calcareous structures in the larvae were located using a photon microscope with a polarizing filter. Locating them in whole juveniles was facilitated by soaking the juveniles in distilled water. The specimens then became turgid and the calcareous structures could be easily seen using a binocular or photon microscope.

Results

Various calcareous structures observed in larvae and epibiont juveniles and the exact stage of development they appeared are shown in Table 1.

Calcareous structures in larvae

Calcareous body

The calcareous body is the first structural element to differentiate (Fig. 1A). It appears just 36 hours after fertilisation, in the posterior part of the larva blastocoel. It takes the form of a five-arm star about 13 µm in diameter (Fig. 2A). The star grows by multiplying and branching off its arms (Fig. 2B) and takes the form, in the late auricularia stage (10 days), of a half-sphere about 50 µm in di-

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Table 1. *Holothuria scabra*: **type and order of appearance of calcareous structures in larvae and epibiont juveniles. (B = button; BT = tentacle rods; BP = podia rods; CC = Calcareous body; CCP = peripharyngeal calcareous ring; DTP = podia terminal disk; M = madreporite; PPA = perforated anal plate; PPP = perforated podia plate; T = table, + = this structure exists).**

Age (days)	Stage of development	Type of calcareous structure									
		CC	M	CCP	Spicules						
					T	BT	BP	DTP	PPA	PPP	B
1.5	Dipleurula	+									
3	Auricularia	+									
10	Auricularia	+	+								
15	Doliolaria	+	+	+	+						
17	Pentactula	+	+	+	+						
20	Juvenile	+	+	+	+	+	+	+	+		
22	Juvenile	+	+	+	+	+	+	+	+	+	
45	Juvenile	+	+	+	+	+	+	+	+	+	+

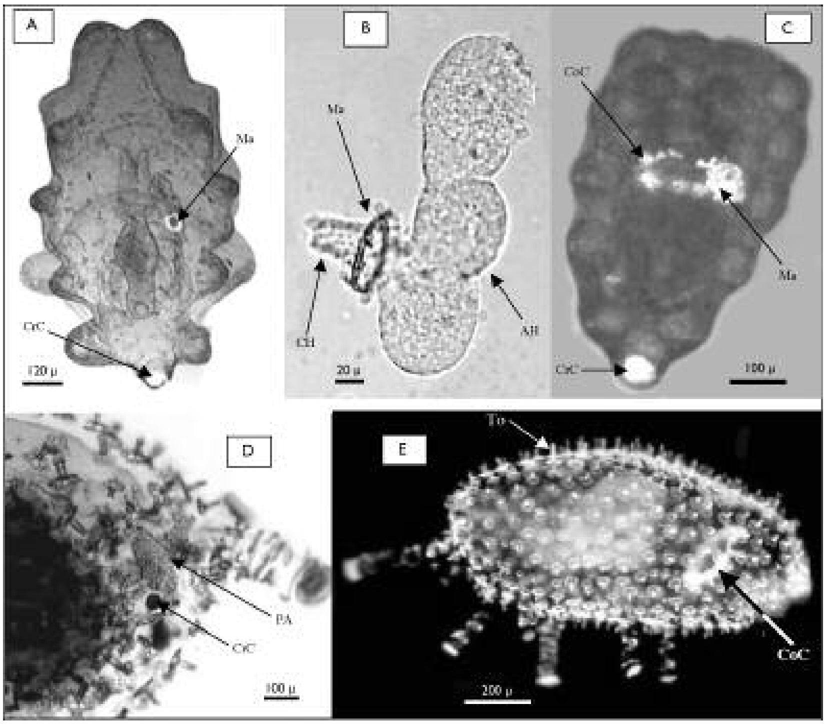

Figure 1. *Holothuria scabra.*
Position of calcareous structures in auricularia (A,B) and doliolaria (C) larvae and in epibiont juveniles (D,E); (AH = Axohydrocoel, CH = water-ring canal, CoC = Calcareous ring, CrC = Calcareous body, Ma = madreporite, PA = anal plate, To = table).

ameter whose flat side is facing forward. The calcareous body in auricularia larvae takes the form of relatively smooth but spiny protuberances that gradually appear on its rounded side (posterior side) (Fig. 2C), while the flat side maintains a fairly even aspect (Fig. 2D). Throughout development, the calcareous body stays in the same place (i.e. in the anterior lobe of larvae and the posterior part of the bodies of juveniles) (Fig. 1A and 1C).

Madreporite

The madreporite is the second calcareous formation to differentiate in larvae. It appears in the late auricularia stage (10 days), when it can be observed in the left dorsal-lateral part of the larval body, in the form of a curved stalk about 40 µm long. This stalk is located at the base of the water ring canal (Fig. 2E). It lengthens during development to form a ring surrounding that canal (Fig. 1B). The ring then branches off to form a sort of hollow sphere with screened walls measuring from 45 µm (doliolaria stage) to 50 µm (25-day-old juveniles) in diameter (Fig. 2F).

Peripharyngeal calcareous ring

The peripharyngeal calcareous ring appears beginning with the doliolaria stage (Fig. 1C). It surrounds the larva's oesophagus and is made up of 10 side-by-side pieces — five radial and five interradial.

Each part forms from a main rod that stretches out and branches off at both ends. In pentactulae the interradial pieces are not compact but rather formed from an unpaired median stem, from the ends of which joined stems grow (Fig. 3A). In juveniles, the stems become more numerous and massive. In older juveniles, these stem fuse and the ends of each piece become compact. The calcareous ring increases in size as specimens grow.

Tables

Tables are made up of a base plate and spire. These are the first type of intra-tegument spicules to form in *H. scabra*. They appear in the doliolaria stage in the form of small bobbin-like structures 7 µm long (Fig. 3B). These bobbins grow longer and their free ends branch out to form a cross with four arms (primary arms) (Fig. 3C). The ends of the primary arms then also branch out to make secondary (Fig. 3D), and then tertiary arms. The latter get closer to each other as they grow and end up touching and fusing into pairs to form closed rings and a perforated disk (Fig. 3E). Four protuberances then grow out in the middle of each of the primary arms. They develop the same number of stalks that lengthen vertically to then touch laterally with short traversal junctions laid out like the rungs of a ladder, and the overall structure forms the spicule's spire (Fig 3F). Complete formation of this type of spicule takes about four days; it then reaches a height of about 70 µm. The tables of doliolaria and pentactulae always appear in groups, with the first complete tables found only in juvenile specimens. In these juvenile specimens, they can be seen along the entire length of the tegument and in the walls of the podia, papillae and the oral tentacles (Fig. 1E). The table's spires are always facing outside, forming a row of small knobs on the tegument.

Calcareous structures in juveniles

All the calcified structures that appear during the larval stage continue to exist in epibiont juveniles. Still others begin to differentiate at the very outset of the juvenile stage.

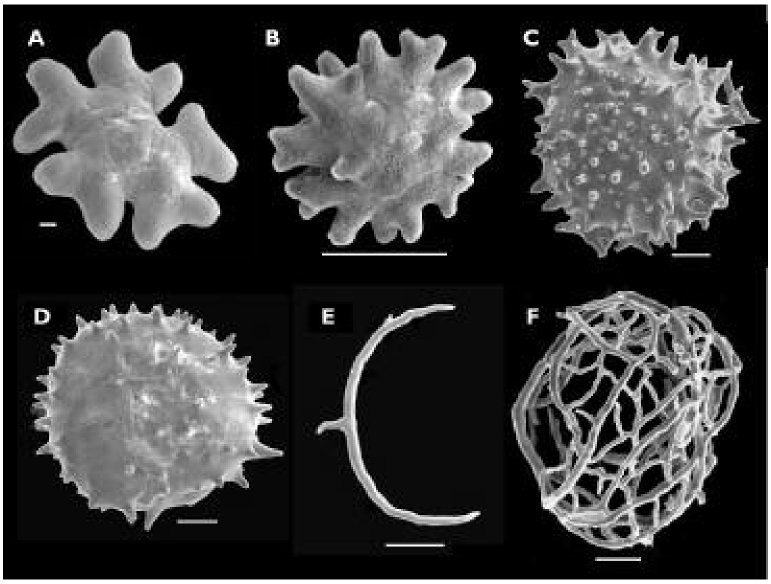

Figure 2. *Holothuria scabra*: Calcareous body (2A–D) and madreporite (2E–F) in dipleurula (2A; age 36 h) and auricularia (2B; age 45 h et 2E; age 11 days) larvae and epibiont juveniles (2C,D,F; age 30 days). Scale: A (Bar = 1 µm) ; B–F (Bar = 10 µm).

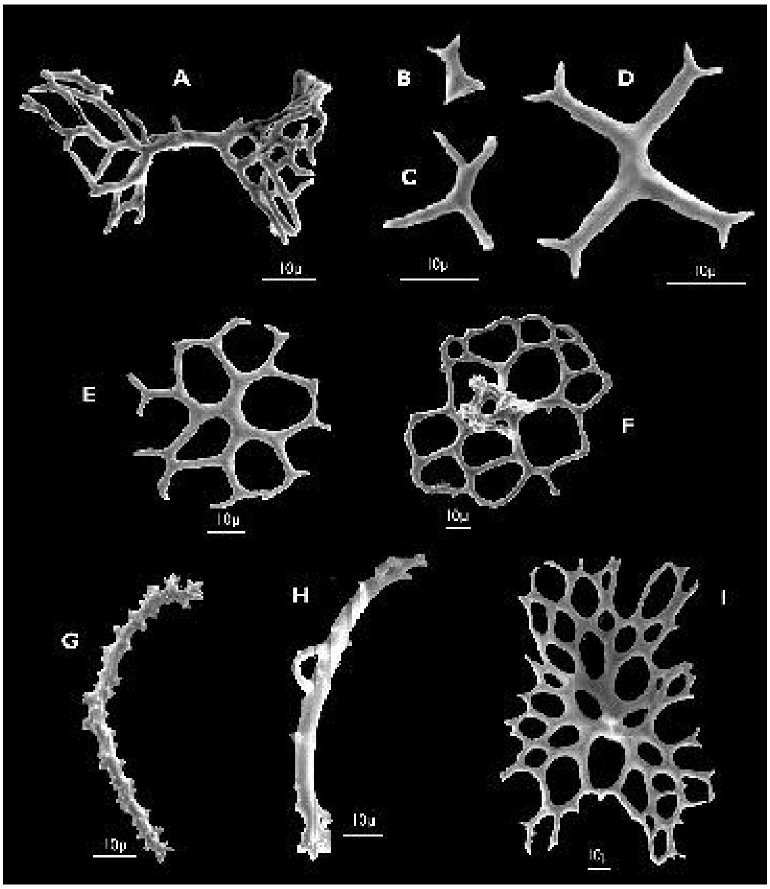

Figure 3. *Holothuria scabra*: Interradial piece of the peripharyngeal calcareous ring of a 17-day-old pentactula larva (3A); table development stages (3B–F), tentacle rod spicule (3G) and podia rod spicule (3H); perforated anal plate (3I). Scale bar = 10 µm (A–I).

Podia and tentacle rods, podia terminal disks and anal plates

All these structures appear at the same time (i.e. on day 20 after fertilisation. The podia and oral tentacles rods are curved. They are slightly (podia) to highly (tentacles) spiny, but podia rods are shorter (about 75 µm as compared with 95 µm for the tentacle rods) (Fig. 3G,H).

The podia terminal disks are pierced circular spicules made up of a web of joined trabeculae. They form at the apex of each podium and reach some 110 µm in diameter in 27-day-old juveniles.

The anal plates are formed from two large perforated spicules found in the posterior part of juveniles near the anus (Fig. 1D). They appear in 22-day-old juveniles. They do not have the same size or the same shape: on day 27, the right anal plate is about 220 µm long and 160 µm wide, as compared with 170 µm and 115 µm for the left plate (Fig. 1D,3I). The anal plates develop in the same way as the table base plates, i.e. from a main structure that branches out, forms primary and then tertiary arms that join and fuse to form closed rings (Fig. 3I).

Button-type spicules

The buttons appear in juveniles at the end of the epibiont stage (40 to 50-day-old juveniles that measure 10– 15 mm in length). Appearance of these buttons seems to depend more on the specimen's size than its age; they are observed in higher densities in the largest specimens.

Discussion

In *Holothuria scabra*, most intra-tegument spicules differentiate after metamorphosis as tables, and are the only type of spicule observed in an initial form at the end of the larval stage (doliolaria larvae). In contrast, everything suggests that the unpaired calcareous body, which appears very early (36-hour-old larvae), is a truly larval formation. It does, in fact, disappear in juveniles some time after metamorphosis (Mortensen 1937, Massin et al. 2000). Other calcified structures, that are not spicules, develop early on (madreporite in the auricularia; the calcareous ring in doliolaria) and continue to develop and exist in post-metamorphic specimens.

The existence of a calcareous structure in the posterior part of larvae, known as the calcareous body, in many different Holothuriidae species, including *Holothuria scabra,* was mentioned by Mortensen (1921, 1937, 1938; also see Ramofafia et al. 2003). Depending on the species and stage of development, the calcareous body takes on a variety of shapes (e.g. rounded, flat, star-shaped or with several lobes) (Mortensen 1937, 1938). In *H. scabra*, the shape differentiates at the very outset of the larval stage and appears as a star-shaped structure that gradually becomes a half-sphere. The calcareous body can be found throughout the larval stage and can still be seen in epibiont juveniles (also see Massin et al. 2000). Although this calcareous body does exist in the larvae of many species, its role remains a mystery. (It may help to stabilise the larvae during movement.) Auricularia, doliolaria and pentactula larvae do, in fact, swim by rotating around their anterior-posterior axis (vertical axis), which goes through the calcareous body. Moving larvae always keep their posterior part (where the calcareous body is located) down when they are moving vertically up or down, as if they were carrying ballast (Rasolofonirina 2004).

The development of spicules in *H. scabra* juveniles was studied by Massin et al. (2000). The authors reported that in juveniles measuring 0.9 mm in length, the peripharyngeal calcareous ring is made up of 10 unfused pieces (5 big pieces that are presumed to be radial and 5 smaller, so-called interradial pieces). They also mentioned the existence of large perforated plates in juveniles measuring less than 6 mm in length but they did not indicate their position or numbers; these were probably anal plates. According to them, these plates disappear in specimens measuring more than 6 mm in length, whereas we observed them in 45-day-old juveniles (about 18 mm in length). The existence of such anal plates in juvenile of the species *Holothuria floridana* was reported by Edwards (1909). Edwards did not talk about what happens to them but theorized that these plates were the remnants of the anal teeth that can be observed in *Actinopyga* species.

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