Journal of the Marine Biological Association of the United Kingdom, 2017, 97(5), 1143–1153. © Marine Biological Association of the United Kingdom, 2017
doi:10.1017/S0025315417000777

Developmental studies of the enigmatic worm *Caobangia billeti* Giard, 1893 (Annelida; Sabellidae), a symbiont of freshwater snails

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Caobangiids are an aberrant group of annelids with an unusual phoronid-like body plan. The most perceptible anatomical characteristic of caobangiids is the anal trunk, which is recurved outside the body and ends with an anal opening located near the head. So far neither the larval development nor the metamorphosis of these worms have been investigated thoroughly. This study describes the larval development and metamorphosis of Caobangia billeti*, and focuses mainly on the formation of the alimentary tract. The trochophore has eight chaetigers. Embryogenesis includes the development of segmentally arranged bands of cilia and ventral ciliary fields, the development of chaetae and the early formation of anterior radioles. Upon exiting the larval duct, larvae have eyes with lenses, two pairs of rudimentary radioles on the prostomium, a pair of nephridia on the peristomium, and two capillary chaetae in each parapodium of segments 3–9; parapodia of the 10th segment also bear a row of palmate hooks. Metamorphosis takes about 20 h and involves dorso-ventral folding of the body and enlargement of the pygidium along the dorsal side of segments 5–8. Thus, the anus develops on the dorsal side of the 5th segment, the anal trunk forms from the projection of pygidium whereas the prepygidial growth zone appears posterior to the 8th body segment. After the completion of metamorphosis, the prostomial and peristomial segments are fused and reduced, eight full thoracic chaetigers derive from the larva's body. Abdominal chaetigers grow posteriorly from the prepygidial growth zone.*

Keywords: Reproduction, larval development, herpochaeta, freshwater polychaetes, shell-burrowing polychaetes

Submitted 8 February 2017; accepted 12 April 2017

INTRODUCTION

Caobangiids are small shell-boring annelids living on freshwater snails. All seven caobangiid species known to science have been found in the tropics and subtropics of India, Sri Lanka and South-Eastern Asia. The type species, *Caobangia billeti* Giard, 1893 was described from Cao Bang, Vietnam (Jones, 1974a). Jones (1974a) moved *Caobangia* and associated species from the Sabellidae family to a separate family, the Caobangiidae. The phylogenetic analysis by Fitzhugh (1989) suggested that *Caobangia* is a part of the Fabriciinae clade in Sabellidae, while Rouse & Fitzhugh (1994) and Fitzhugh & Rouse (1999) regarded the subfamily placement as *incertae sedis*. Based on observations of living and preserved *Caobangia billeti*, subsequent phylogenetic analyses suggest that the genus is a sister group to the similar looking *Terebrasabella* Fitzhugh & Rouse, 1999 (Fitzhugh, 2003; Nogueira *et al.*, 2010).

Variability of the phylogenetic placement of caobangiids is largely caused by their unusual morphology (Fitzhugh, 1989, 1991, 2003; Rouse & Fitzhugh, 1994; Fitzhugh & Rouse, 1999; Capa, 2007; Nogueira *et al.*, 2010). They do not have the normal annelid segmentation and possess a gut that bends posteriorly and forms an external trunk, running anteriorly around the pear-shaped body, leading to the anus, which opens on the dorsal side of the thoracic region (Jones, 1974a; Fitzhugh, 1989; Rouse, 2004). The route of the trunk follows the faecal groove of other sabellids, which switches from the dorsal position in the thorax to ventral in the abdomen, across the right side on the thoracic-abdominal boundary (Berrill, 1977; Knight-Jones, 1981; Fitzhugh, 1989). The general shape of the caobangiid body is somewhat akin to that of sipunculids and phoronids.

Basic information about the biology and anatomy of caobangiids is summarized in two classic works of M.L. Jones (Jones, 1969, 1974a), primarily based on non-fixed dry material from shells from the malacological collection of the National Museum of Natural History (Smithsonian Institution). Some well-fixed material, however, allowed Jones (1974a) to describe the hermaphroditic mode of reproduction, ovoviviparity, larval (herpochaeta) behaviour, and settlement of *C. brandti* Jones, 1974, and *C. abbotti* Jones, 1974 (Jones, 1969, 1974a). At the end of his monograph, Jones (1974a) summarizes 'some basic questions posed by *Caobangia*', which, in our view, are worthy of future study:

(1) 'What is the mechanism that mediates the internal anterior growth of the ascending gut such that it grows straight,

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beneath the ventral surface, moves dorsally on the right side, then straight to the midthoracic region?
(2) Where is the usual prepygidial growth zone of other polychaetes? Is the caobangiid growth zone just posterior to the posterior-most row of low avicular hooks, or elsewhere?
(3) If the caobangiids are not self-fertilizing hermaphrodites, how are sperm from one worm transferred to another? Are chemotactic behaviorisms implicated or are transfers merely fortuitous, depending on the vagaries of water currents? What is the source and fate of the sperm observed in the short anterior ducts?
(4) Since *Caobangia* burrows are lined with an intact secreted membrane, and the various setae seem not to be unduly worn, how is enlargement of a given burrow accomplished?'

This study on the development of *C. billeti* was conducted in order to detail previously undescribed larval stages and metamorphosis in an effort to shed light on organ development, the formation of the ascending gut, and localization of the prepygidial growth zone. These data may be comparable among different annelid species, or even phoronids and sipunculids in a phylogenetic and morphological context.

MATERIALS AND METHODS

Snails of *Brotia* sp. were collected at night from the Bang Giang River in Cao Bang city, Vietnam (22°39.96′N 106°15.72′W), on 22–26 October 2014. Shells were gently broken with clippers and adults of *C. billeti* were removed from their burrows. Larvae were liberated from the larval duct by bursting the adult, and kept in a plastic box with fresh water or in a cavity slide. Twelve adult worms (9 specimens 4 mm in length and 3 specimens 3 mm in length) were dissected. Liberated embryos (16 spherical and 17 oval), trochophores (8 specimens), metatrochophores (11 specimens), and herpochaetes (11 specimens) were kept in Petri dishes with fresh water. Settlement and metamorphosis were observed for 8 herpochaetes.

For scanning electron microscopy (SEM) larvae were fixed in Bouin solution, dehydrated in an increasing ethanol series, critical point dried with CO_2 (Hitachi HCP–2), mounted on aluminium stubs and sputter-coated with Au–Pd (Ion Coater Eiko IB–3). Examination was carried out with a Cam Scan S–2 scanning electron microscope. For histological investigation (light microscopy), samples were fixed in a mixture of 0.75% glutaraldehyde and 0.75% formaldehyde buffered with 0.05 mol l^{-1} PBS, postfixed in osmium tetroxide ($OsO_4$1%) buffered with 0.05 mol l^{-1} PBS, and dehydrated in successive ethanol and acetone series and embedded in Epone. Semi-thin sections (990 nm) were cut with a Dupont MT 5000 microtome, then stained with a mixture of methylene blue and toluidine blue, and examined with a Leica DM 2500 compound microscope. Digital images were captured with a Leica DFC 290 camera and Leica Application Suite software.

For confocal laser scanning microscopy investigations, specimens were fixed with a 4% paraformaldehyde solution in 0.1 M PBS for 4 h at 4°C with constant mixing, then rinsed in 0.1 M PBS over 20 min at 4°C. Samples were embedded in a gelatin-ovalbumin solution and stored in 10% formaldehyde in 0.1 M PBS overnight at 4°C. Samples were then rinsed in 0.1 M PBS for 4–5 h then stained in the same manner as the whole-mounts, but the PBT contained 2% Triton X-100 for permeabilization. Incubation in primary antibodies was reduced to 24 h. Dilution of antibodies was increased to 1:600 for primary antibodies and 1:800 for secondary antibodies. Animals were mounted in glycerin on glass slides.

All samples were examined using a Nikon A1 CLSM (Nikon Corporation, Tokyo, Japan) confocal microscope. Nikon imaging software was used to generate optical sections with a Z-step size of 0.1–1.5 μm, which were digitally merged to yield maximum and average projection images. Depth-coded Z-stack images, obtained with the Nikon software provided with the confocal microscope, follow the area of the spectral light, with the uppermost structures appearing in blue and the more distant ones in red.

RESULTS

Developmental stages

Caobangia billeti is viviparous; large green-coloured embryos and larvae at different development stages are visible through the transparent body wall of live worms. Spherical embryos are 200–300 μm diameter, oval embryos are about 350 μm long and 170 μm wide; their surface lacks cilia. Larvae from the larval duct are characterized by three consequential morphological stages: trochophore, metatrochophore and herpochaeta (Figure 1). Usually, the larval duct of an adult (4–5 mm length) worm contains 2–3 spherical and 1–2 oval embryos, 1–2 trochophores, 1–2 metatrochophores and 1–2 herpochaetes.

TROCHOPHORE

The trochophore is immotile, about 500 μm long and 200 μm wide. The body consists of the prostomium, peristomium, 8 postoral segments and the pygidium (Figure 1A). The prostomium bears one pair of eyes, two pairs of small ciliary tufts and the prototroch (Figure 1A). The prototroch is 10–12 μm wide, length of cilia is 5–8 μm. The peristomium has a metatroch about 8 μm wide, composed of short (5 μm length) cilia; the oral opening is present. Postoral segments are separated from each other by small (up to 10 μm wide) ciliary bands, formed with cilia 5 μm long (Figure 2A). These bands are located only on the ventral side of larvae; both dorsal and lateral sides of postoral segments lack cilia. Apart from the ciliary band, each postoral segment bears a sparse ciliary field on the ventral side (Figures 1A, 2A).

METATROCHOPHORE

The metatrochophore is immotile, about 470 μm long and 220 μm wide. The body consists of prostomium, peristomium and 8 postoral segments; chaetae are absent (Figure 1B). The prostomium bears two tufts of short cilia on the dorsal side, two c-shaped rows of cilia and two pairs of small ciliary tufts on the ventral side (Figure 1B). On the ventral side prostomium bears a pair of eyes with lenses edged with ciliary bands (Figure 1B). Along the anterior margin of the peristomium two thickened lobes appear. The width of the prototroch is 15 μm, the length of cilia is 20 μm. The peristomium bears a cushion-like ciliary field on the dorsal side and three ciliary fields on the ventral side around the mouth.

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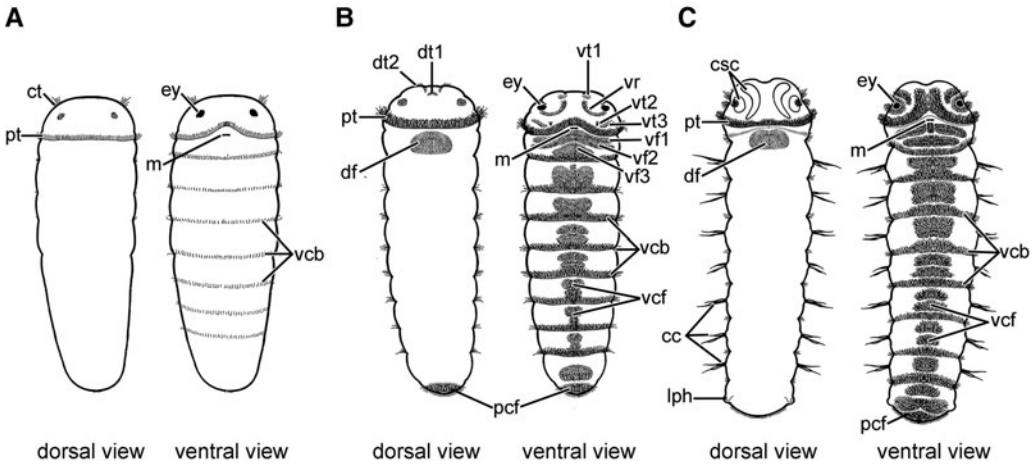

Fig. 1. Larvae from the larval duct of *Caobangia billeti*: (A) trochophore; (B) metatrochophore; (C) herpochaeta. Abbreviations: cc, capillary chaetae; csc, cells of the cartilaginous skeleton; ct, ciliary tuft; df, dorsal ciliary field; dt 1, 2 – dorsal ciliary tufts; ey, eye; lph, larval palmate hook; m, mouth; pcf, pygidial ciliary field; pt, prototroch; vcb, ventral ciliary bands; vcf, ventral ciliary fields; vf 1, 2, 3, ventral ciliary fields; vr, ventral ciliary row; vt 1, 2, 3, ventral ciliary tuft.

The postoral segments have ventral ciliary fields formed by thick and dense cilia which are about 10 μm in length. Together these fields form large and broad ciliary areas along the ventral side of the larva (Figure 1B).

Epidermal cells contain numerous granules of yolk. Dorsal epithelial cells are smaller than ventral cells. On the dorsal side of the first four postoral segments the epithelium forms deep wrinkles; the ventral epithelium is thick and smooth. Glandular cells are rare and distributed predominantly on the ventral side. A histologically distinguishable muscular system is represented by longitudinal muscles. The alimentary canal consists of two parts: the foregut and midgut. The foregut is narrow and short, it is formed by small ciliated epidermal cells. It runs from the ventral mouth transverse to the body axis, and joins the midgut on the dorsal side. The midgut is large and occupies the greater part of the body. The wall of the midgut is formed by large cells containing numerous yolk granules. A hindgut and anus are absent. Histologically distinguishable parts of the circulatory system are indicated by the ventral blood vessel.

The central nervous system consists of two pairs of large cephalic ganglia (prostomial supraoesophageal and peristomal suboesophageal) joined with circumoesophageal commissures and the ventral nerve cord, containing 8 pairs of segmental ganglia, which lie in two broadly placed nerve trunks and are connected by thin commissures.

HERPOCHAETA

Individuals at this stage are motile; about 420–450 μm long and 120–150 μm wide. The body consists of the prostomium, the peristomium, 8 chaetigers and the pygidium (Figure 1C). The large bilobed prostomium bears four tufts of short cilia and two curved ciliary bands on the dorsal side. The cells of the developing cartilaginous skeleton are visible as four pale strips (Figure 1C). On the ventral side, cilia form two fields around the eyes and two U-shaped strips (Figure 1C). The width of the prototroch is 15 μm, the length of cilia is 20–25 μm. The peristomium bears a ciliary field on the dorsal side and three fields on the ventral side (Figures 1C, 2B).

Ventral ciliary bands on postoral segments are up to 15 μm wide. The length of the cilia in the bands is 15–20 μm; ventral ciliary fields bear cilia about 20–25 μm long. The first seven chaetigers have one longer and one shorter limbate capillary chaeta in each fascicle. The eighth chaetiger bears notopodial larval palmate hooks, still embedded in the epithelium and non-visible on the body surface (Figure 1C).

Epidermal cells lack yolk granules but the epithelium is thicker than that of the metatrochophore. The chaetiger 1 has a collar-like bulge of glandular cells on the dorsal side (Figure 3A). Epithelial wrinkles on the dorsal side of segments increase in size and form numerous transverse folds (Figure 3A). The pygidium forms a pillow-like node of cells on the dorsal side of the chaetiger 8; it bears 2–3 paired calcium-dissolving glands on the ventral side, which appear as black spots.

The musculature consists of longitudinal, circular and parapodial muscles. Both the mouth and the foregut cavity are covered with dense cilia. The foregut joins with the midgut on the dorsal side of the first postoral segment (Figure 3A, Figure 4A). The circulatory system is visible as the ventral blood vessel and circumintestinal blood sinus around the midgut. The nephridial system consists of a pair of nephridia, with two nephropores on the dorsal side of the prostomium (Figure 4A). The central nervous system is comprised of 10 pairs of ganglia (prostomial, peristomal and 8 pairs of segmental ganglia in the ventral nerve cord) and the pygidial ring nerve in the pygidium. The prostomial ganglia give rise to eye-innervating nerves. Segmental ganglia of chaetigers give rise to nerves innervating chaetal fascicles (Figure 5A). Ganglia of chaetiger 8 give rise to nerves innervating the notopodial burrowing palmate hooks (Figure 5A).

METAMORPHOSIS STAGE I

The first metamorphosis stage is motile, 420–500 μm long and 120–150 μm wide. The body consists of the prostomium, the peristomium, 8 chaetigers and the pygidium. Two pairs of developing radioles appear on the prostomium dorsolaterally. The anteriormost radioles are 60–65 μm long, the posteriormost are about 80 μm. At the base of radioles there are rudimental skeletal structures, formed by large vacuolated cells. Eyes are present. The pygidium bears 2–3 paired burrowing calcium-dissolving glands on the ventral side.

The first seven chaetigers have two limbate capillary notochaetae in each fascicle. The chaetiger 8 bears one notopodial palmate hook on each parapodium. Parapodial musculature is

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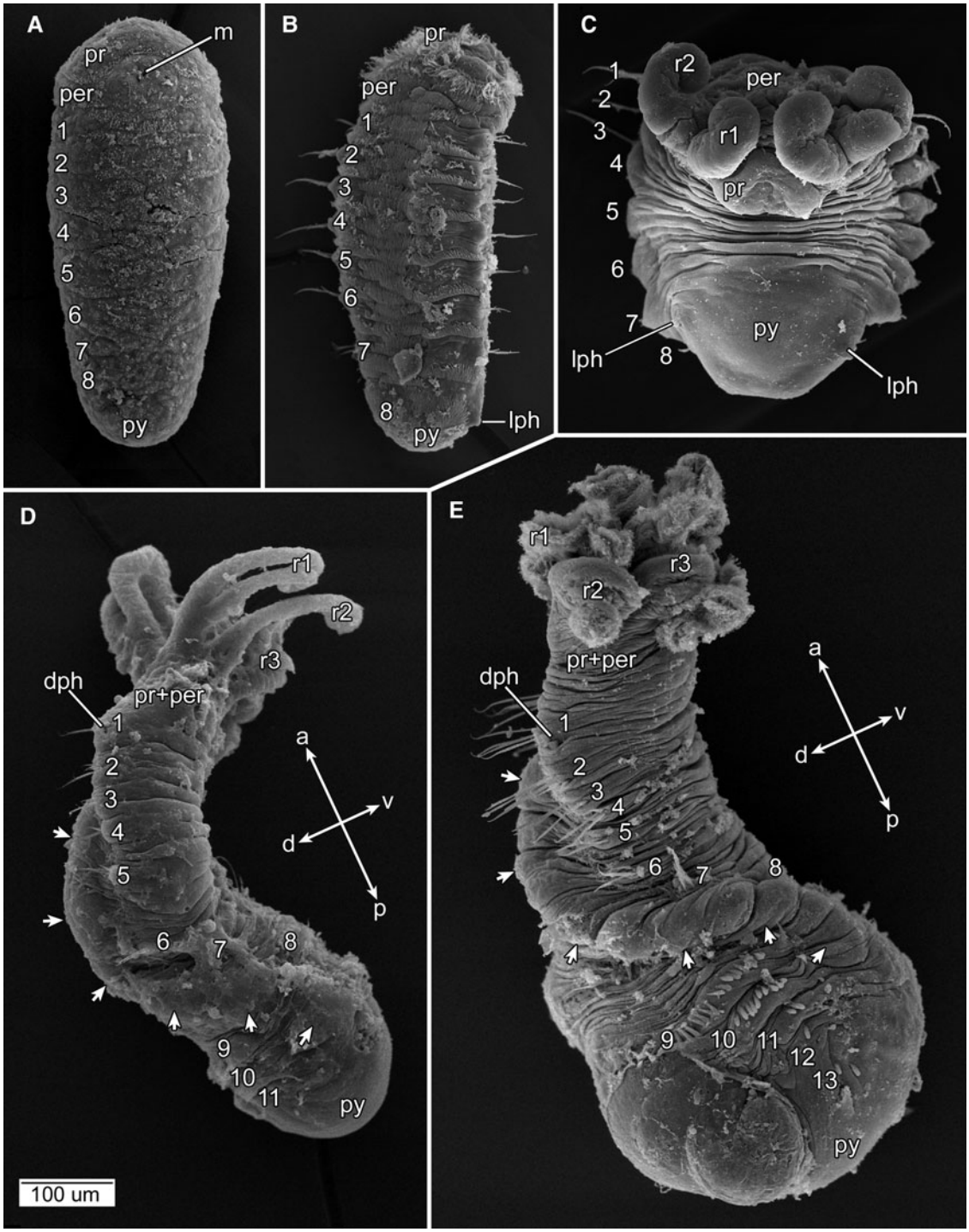

Fig. 2. Main stages of larval development and metamorphosis of *Caobangia billeti* (SEM): (A) trochophore; (B) herpochaeta; (C) metamorphosis stage II, 8.5 h after releasing; (D) postmetamorphosis juvenile, 21 h after releasing from the larval duct; (E) adult worm. Abbreviations: dph, definitive palmate hooks; lph, larval palmate hooks; per, peristomium; pr, prostomium; py, pygidium; r, radioles; arrowheads show the anal trunk.

well developed, the body wall contains longitudinal and circular muscles.

Histologically distinguishable parts of the circulatory system consist of the ventral blood vessel, circumintestinal blood sinus and two pairs of longitudinal branchial blood vessels (one vessel in each radiole). The nephridial system consists of a pair of ducts, each with two nephridia. The central nervous system consists of 10 pairs of ganglia, which innervate the parapodia, and a pygidial ring nerve.

METAMORPHOSIS STAGE II

The fifth developmental stage is immotile, 520–550 μm long and 300–350 μm wide. The body consists of the prostomium, the peristomium, 8 chaetigers and the pygidium. The body bends toward the dorsal side, and the pygidium expands to the dorsal side of chaetigers 5–8 (Figure 2C).

The prostomium bears three pairs of underdeveloped radioles. The first and the second pairs are about 90 μm long. Eyes are present. The peristomium lacks ciliary folds;

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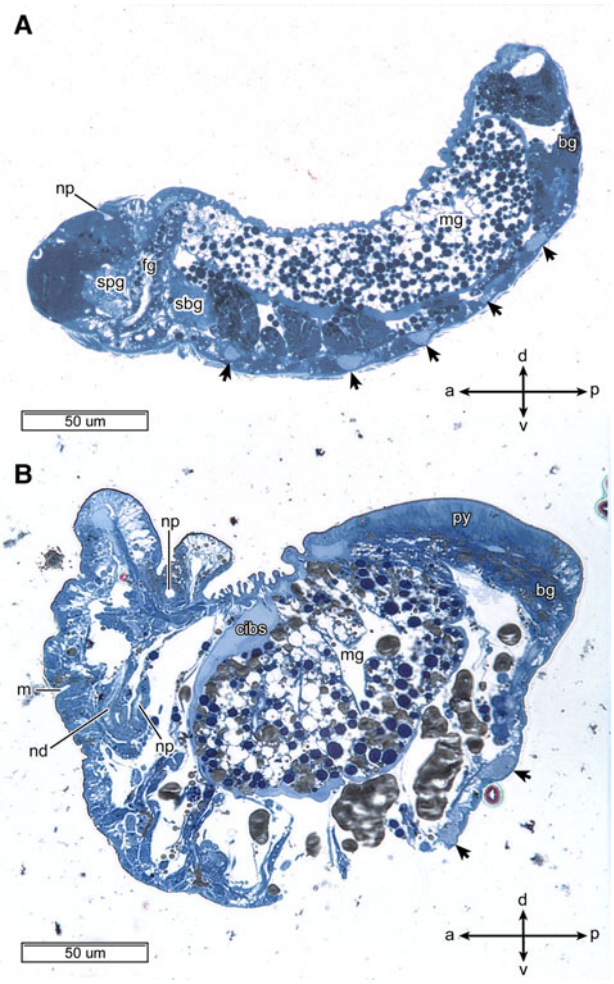

Fig. 3. Metamorphosis stages of *Caobangia billeti*, semithin sections (LM): (A) herpochaeta; (B) metamorphosis stage II. Abbreviations: bg, burrowing glands; cibs, circumintestinal blood sinus; fg, foregut; m, mouth; mg, midgut; np, nephropore; sbg, peristomal suboesophageal ganglia; spg, prostomial supraoesophageal ganglia; py, pygidium; arrows show segmental ganglia of the ventral nerve cord.

however, cilia are present inside the oral opening. Parapodia of the chaetiger 3 arranged with two notopodial limbate capillary chaetae and one notopodial palmate hook, embedded in the epithelium and not visible on the surface (Figure 4B).

Parapodia of chaetigers 4–7 bear two notopodial limbate capillary chaetae in each fascicle. The parapodia of the chaetiger 8 bear one notopodial palmate hook each and a row of 9–10 developing palmate hooks inside the chaetal sac. The pygidium looks like a broad pillow-like node of large cells at the dorsal side of segments 6–10 (Figure 2C). Due to this transformation, the ventral calcium-dissolving glands shift from the ventral side to the lateral sides of the pygidium beneath the chaetiger 8.

The digestive system consists of a foregut, a large midgut, and a hindgut, which appears posteriorly in the pygidial tissue. The hindgut is blind-ended, lies under the midgut and bows anteriorly, making the alimentary canal U-shaped (Figures 3B, 4B). The circulatory system consists of a ventral blood vessel, large circumintestinal blood sinus, two pairs of longitudinal blood vessels (one vessel in each radiole), and a peripharyngeal lacunary system. The dorsal part of the pygidium contains 6–8 parallel intraepithelial blood lacunae, which lie transverse to the body axis (Figure 3B).

The nephridial system consists of two pairs of dorsolateral nephridia, which are located in chaetiger 1 and merging into descending branches of two U-shaped nephridial ducts. Ascending branches join into a single duct on the ventral side of the segment, which bifurcates under the foregut, then joins the single duct again and opens into a nephropore on the dorsal side of the peristomium (Figure 4B). The nervous system, as in the previous stage, contains 10 pairs of ganglia and a pygidial ring nerve.

JUVENILE

Juveniles are 700 μm long, with 7 thoracic and 2–3 abdominal chaetigers (Figure 2D). As in all sabellids, after metamorphosis, the prostomium and peristomium are fused and reduced, thus the larval chaetiger 1 becomes the first (collar) thoracic segment of the juvenile. The radiolar skeleton consists of two rows of large vacuolated cells, located on the base of the branchial crown.

The first thoracic chaetiger bears two larval limbate capillary notochaetae in each fascicle; notopodial palmate hooks are embedded in the chaetal sac and are not visible on the surface. The second chaetiger bears two larval and three new definitive capillary chaetae, the following thoracic chaetigers (chaetigers 3–7) have two larval and 1 definitive chaetae in each fascicle (Figure 2D).

The first and the second abdominal chaetigers are arranged with a row of nine notopodial uncini and one neuropodial capillary chaeta. The third abdominal chaetiger bears one uncinus and one capillary chaeta in each parapodium (Figure 2D).

Calcium-dissolving glands are located on the dorsal side of the pygidium in the posteriormost part of the body. In addition, ventral fields of calcium-dissolving glands are located on thoracic chaetigers 1–4. They appear as large, darkstained triangle spots. As development proceeds, several additional small calcium-dissolving glands develop on the dorsal side of abdomen along the anal trunk.

The body of juveniles is twisted ~90° at the thoracic–abdomen junction (not 180° inversion as in the adult). The ascending gut runs along the mid-ventral line of the abdomen, turns dorsally to the right side of the body posterior to chaetiger 8, and continues along the mid-dorsal line of the thorax (Figure 2D). In abdominal segments 1–2, the midgut is augmented and filled with yolk. The anus opens at the level of the 3–4 thoracic chaetiger.

The nervous system contains 10 pairs of ganglia (supraoesophageal, suboesophageal and 8 pairs of segmental ganglia) as at the earlier stages. The cephalic ganglia extend into the first thoracic chaetiger. In the ventral nerve cord of adults the same 10 pairs of segmental ganglia are present (Figure 5B). The ventral nerve cord is present only in the thorax; in the abdomen the nerve trunks continue as very thin nervous fibres. A pygidial nerve ring is not detected at the post-metamorphosis stage (Figure 5B).

Settlement

Settlement was observed for 11 herpochaetes. Nine of them ejected from adults and two were collected from snails. After release, herpochaetes set out in search of a mollusc

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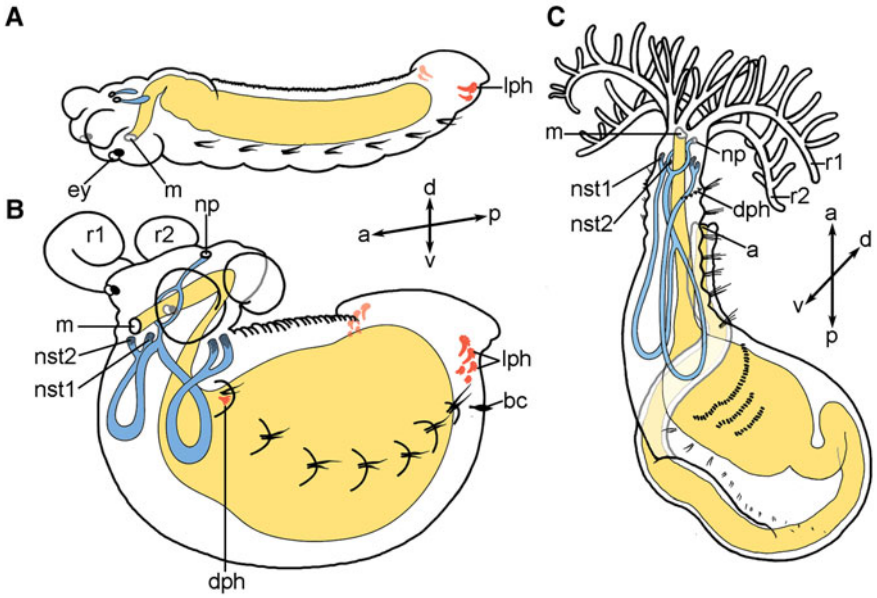

Fig. 4. Developmental stages of *Caobangia billeti*: (A) herpochaeta; (B) metamorphosis stage II; (C) adult worm. Abbreviations: a, anus; bc, bayonet chaetae; dph, definitive palmate hooks; ey, eye; lph, larval palmate hooks; m, mouth; np, nephropore; nst1, the first nephrostome; nst2, the second nephrostome; r, radiole.

host. They glide quickly using the ventral ciliary fields. When we placed herpochaetes into a 10 × 10 cm plastic box with the snail, larvae began to move toward the snail after 1–2 min. When we repeatedly moved the snail, herpochaetes (seven of nine specimens) changed direction of movement toward the presumptive host. One specimen could not locate the snail on its own and crept until the start of metamorphosis; the last remaining specimen settled on a fragment of a broken shell.

At the beginning of the search, herpochaetes move quickly raising the anterior end of the body and sometimes moving from side to side (Figure 6A, B). When the distance to the snail decreased to 3–3.5 cm, herpochaetes begin moving in a straight line without any extra vertical or horizontal motions.

Upon reaching the snail, herpochaetes creep on to the shell and explore it. Larvae quickly move over the external surface of the shell and rub their anterior end against the surface (Figure 6C). Sometimes larvae move to the shell interior or the body of the snail, but in this case, they always returned to the external shell. Among two of the nine larvae observed, if the shell seemed unsuitable, the larvae would leave it and begin searching for another potential host. Exploration of the shell took less than 1 h.

If the shell is suitable for settlement, herpochaetes search for a burrowing location. The manner of creeping changes; herpochaetes creep slower than previously, continuously rubbing the anterior end against the shell surface and from time to time bending the body. After 1–1.5 h, the herpochaeta stops moving. Usually it chooses to settle in a thickened area of the shell, such as the suture in the 1–2 last apical whorls. The herpochaeta then coils and builds a mucoid capsule over itself. At the beginning of this process the capsule is

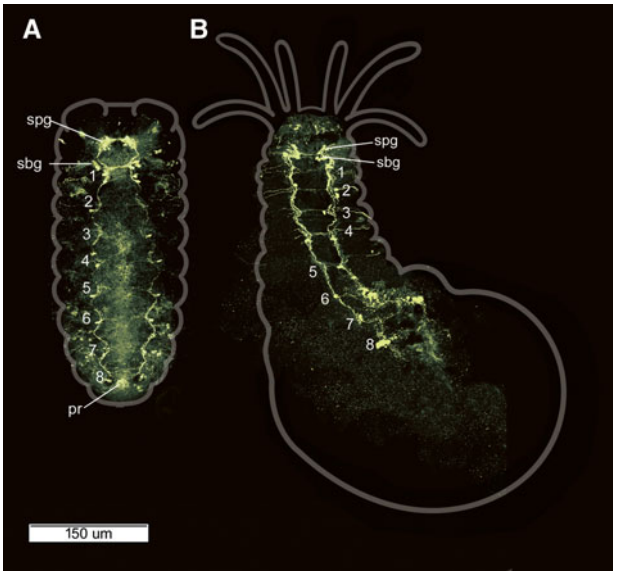

Fig. 5. Ventral nerve cord of *Caobangia billeti* (LSM): (A) herpochaeta; (B) post-metamorphosis juvenile. Abbreviations: pr, pygidial ring sbg, peristomal suboesophageal ganglia; spg, prostomial supraoesophageal ganglia.

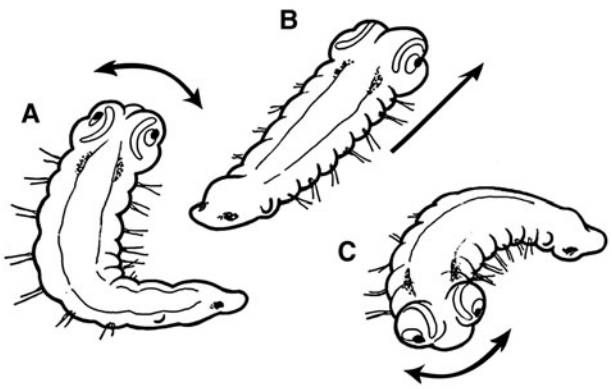

Fig. 6. The main behavioural patterns of herpochaeta during settlement: (A) 'sniffing'; (B) movement towards the snail; (C) exploration of the surface of shell.

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transparent and it is possible to observe that the herpochaeta actively turns around inside. One and a half to 2 h later the larva gradually slows down and starts to burrow into the shell.

According to our observations (two specimens), metamorphosis from the herpochaeta to juvenile takes 18–19 h; during this period the worm makes a burrow 4–4.5 mm long. Burrows have an organic lining and the interior area reflects the pear-like shape of the worm. The capsule covers the opening of the burrow during the first 1–2 days after the settlement. When the yolk runs short and the juvenile begins to feed by filtering, the capsule peels off.

In addition to these nine herpochaetes, two herpochaetes were placed in a box with fragments of a broken shell. The size of fragments was from 2 × 5 mm to 1.5 × 2 cm. The first herpochaeta settled within 40 min of release on the external side of the fragment close to the suture and built a capsule. The second herpochaeta does not settle and died within 6 h at metamorphosis stage II.

Metamorphosis

Metamorphosis was observed on nine herpochaetes. All previous stages, i.e. embryo, trochophore and metatrochophore, that were ejected from the larval duct by piercing of adult, ceased their development and died. Trochophores would lie motionless and died after 48–58 h. Metatrochophores slowly contracted and died 72–96 h after liberation (Figure 7).

After release herpochaetes were pipetted off and placed into a cavity slide with fresh water. Four hours later two pairs of rudimentary radioles of branchial crown appear on the prostomium. Herpochaetes reach about 420–450 μm length and 120 μm wide, and began to make short contractions. Within the next 1.5 h, the body becomes pear-like (metamorphosis stage I). It contracts more and more intensively and loses the creeping ability. During the next 8 h the worm grows dramatically (metamorphosis stage II), the ventral side expands more than the dorsal, at the same time the pygidium increases as a large pillow of cells and expands along the dorsal side of segments 5–8. As a result, the body folds dorso-ventrally (Figures 2C, 3B, 4B). During the next 8 h the prostomium and peristomium fuse together. Larval posterior palmate hooks used for burrowing are reduced; definitive anterior palmate hooks develop in the notopodia of the thoracic chaetiger 1 (Figure 8A, B). The anus appears in the anteriormost part of pygidium under the thoracic chaetiger 4 (Figure 8A). The first three abdominal segments appear simultaneously; they have three pairs of segmental blood lacunae and three pairs of neuropodial chaetal fascicles and 1–5 uncini in each notopodium.

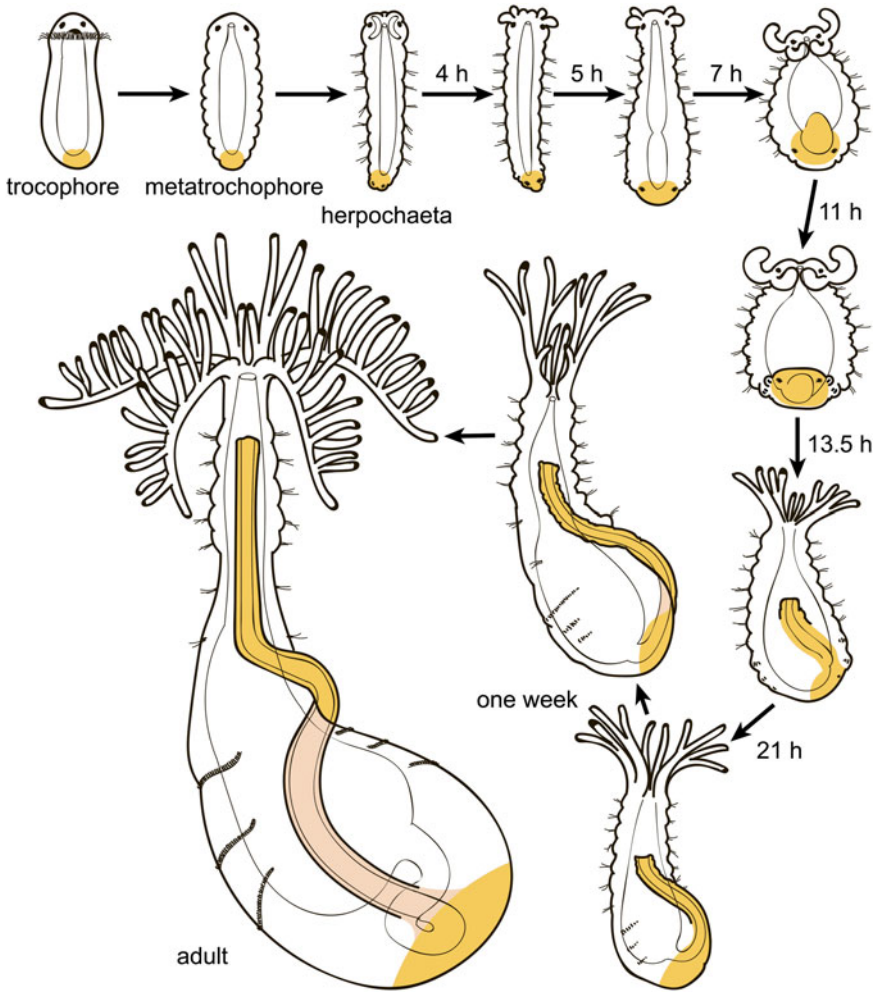

Fig. 7. General scheme of development of *Caobangia billeti*.

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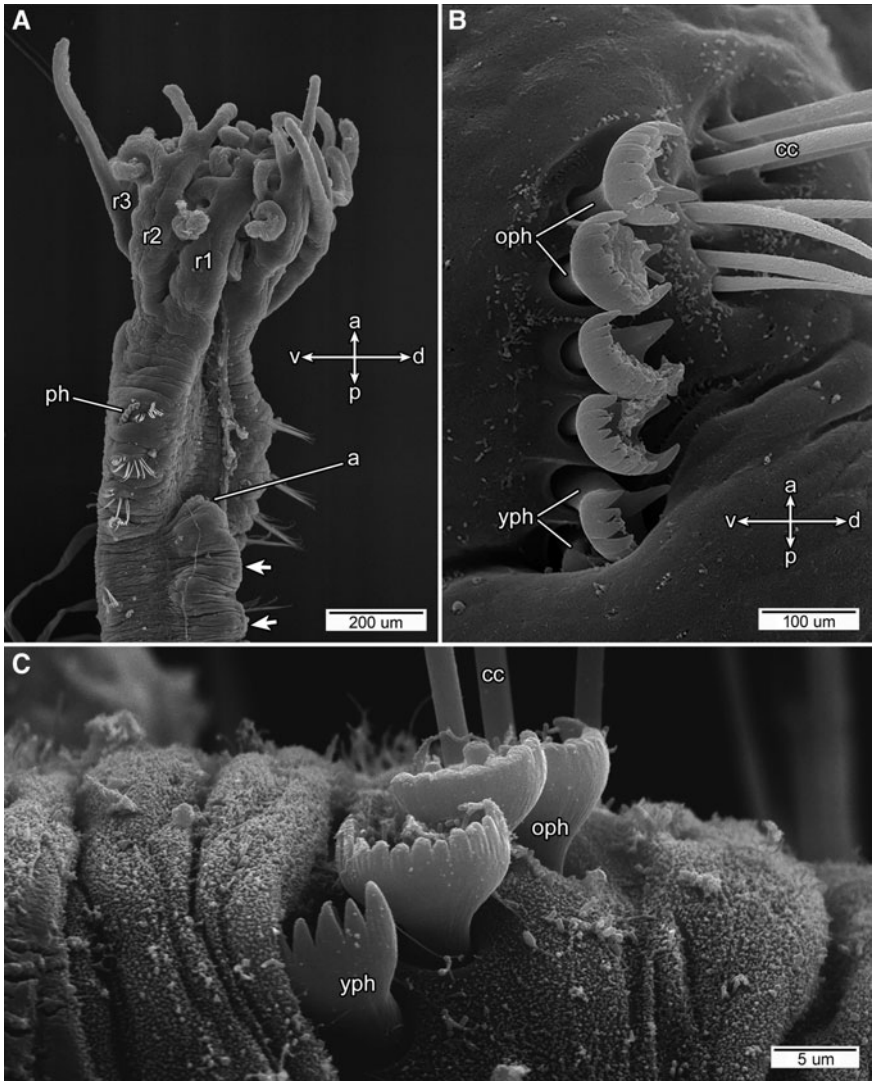

Fig. 8. Adult *Caobangia billeti* (SEM): (A) anterior part; (B) parapodia of the first thoracic chaetiger; (C) the row of palmate hook. Abbreviations: a, anus; cc, capillary chaetae; oph, the oldest palmate hooks in the row (with short worn teeth); ph, palmate hooks; r, radioles; yph, the youngest palmate hooks in the row (teeth are longer than in the oldest hooks).

DISCUSSION

Apart from *Caobangia*, there are three other obligate freshwater sabelliform taxa, *Manayunkia*, *Monroika* and *Brandtika* (Leidy, 1883; Jones, 1974a, b; Fitzhugh, 1989, 1992; Rouse & Fitzhugh, 1994; Rouse, 1996; Rouse & Pleijel, 2001). There are no shell-burrowers among these species, but *Brandtika* attach their tubes to shells of molluscs. Their marine shell-penetrating relative, *Terebrasabella heterounciniata* Fitzhugh & Rouse, 1999, causes the 'sabellid pest' of abalone in maricultures (Fitzhugh & Rouse, 1999; Kuris & Culver, 1999; Chalmers, 2002).

As stated above, the first question raised by Jones (1974a) was: 'Since *Caobangia* burrows are lined with an intact secreted membrane, and the various setae seem not to be unduly worn, how is enlargement of a given burrow accomplished?' We believe we can address this question as follows.

Marine sabellids *Perkinsiana rubra* (Langerhans, 1880) and *Pseudopotamilla reniformis* (O.F. Müller, 1771) penetrate limestone using calcium-dissolving glands, producing acid mucopolysaccharides, which cause decalcification by acting as chelating agents (Blake, 1969; Knight-Jones & Bowden, 1984; Chughtai, 1986; Chughtai & Knight-Jones, 1988). In *P. rubra* and *P. reniformis* both tube-building and calcium-dissolving secretions were found together in parapodial glands and in ventral glandular shields (Chughtai & Knight-Jones, 1988). The localization of calcium-dissolving glands of *C. billeti* has not been determined; perhaps, they are combined with tube-building glands as in *P. rubra* and *P. reniformis*. Despite the fact that *Caobangia* does not build normal tubes, it produces a membrane that lines the burrow (Jones, 1974a). Thus, it has tube-building glands for making a larval capsule and a secreted membrane, lining the burrow.

In addition to glands, some burrowing polychaetes demonstrate modified chaetae, supposedly used for mechanical erosion of the dissolved substrate (Hutchings, 2008). Among the extensively studied limestone-burrowing spionid, *Polydora*, it has been suggested that boring is assisted by the modified chaetae of chaetiger 5 (Haigler, 1969; Hutchings, 2008). This role of the modified chaetae is disputable, but according to our observations, *C. billeti* uses both posterior larval palmate hooks and definitive palmate hooks of the

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chaetiger 1 for burrowing. After settlement, the herpochaeta secretes calcium-dissolving substances to dissolve the shell and scratches out the dissolved substrate using the palmate hooks of the last body chaetiger. The same behaviour was observed by Jones (1969) for *C. abboti* and *C. brandti* larvae. During the metamorphosis, larval palmate hooks are lost simultaneously with the development of definitive palmate hooks in the first chaetiger. Undoubtedly, adult worms are capable of changing direction to deepen their burrows. In parapodia of *C. billeti* older palmate hooks are clearly different from the youngest hooks by the length of teeth (Figure 8B, C). The teeth in older hooks are shorter than those in the youngest hooks in the row. It is possible that the worm's teeth wear down and get shorter due to burrowing.

The second problem formulated by Jones, is the obscurity of how fertilization in *Caobangia* takes place: 'if the caobangiids are not self-fertilizing hermaphrodites, how are sperm from one worm transferred to another? Are chemotactic behaviorisms implicated or are transfers merely fortuitous, depending on the vagaries of water currents? What is the source and fate of the sperm observed in the short anterior ducts?'

Unfortunately, we could not observe fertilization of *C. billeti*. It is supposed that freshwater annelids have to protect their gametes and larvae from osmotic stress (Sato, 1999; Glasby & Timm, 2008). For this reason, they tend to replace the swimming trochophore with brooding in the body of the adult, as in *H. limnicola*, or in the parental tube as in *Manayunkia* spp., and to direct sperm transfer (Bick, 1996; Sato, 1999; Glasby & Timm, 2008). The direct transfer of sperm may be carried out by self-fertilization (*H. limnicola*), copulation, or by the use of spermatophores (e.g. *Manayunkia* spp.) (Glasby & Timm, 2008). At the same time, exceptions are present. For example, the freshwater serpulid, *Marifugia cavatica* Absolon & Hrabě, 1930, and the ampharetid, *Hypania invalida* (Grube, 1860), are broadcast-spawners (Rouse, 2004; Norf *et al.*, 2010).

It seems *Caobangia* form spermatophores, similar to those in *Manayunkia* (Rouse, 1995, 1999). Jones (1974a) described sperm with elongated heads for *C. brandti*. As it was noted by Rouse & Fitzhugh (1994), caobangiids apparently have internal fertilization, because elongate headed sperm are rare among broadcast-spawners (Jones, 1974a; Rouse, 1999; Rouse *et al.*, 2006). Evidently, spermatophores are convenient to concentrate sperm in streaming river water.

Due to their pear-shaped bodies, caobangiids cannot exit their burrows, but the distances between burrow apertures allow them to extend out and transfer spermatophores to the nearest neighbours using the branchial crown. Additional observations of live worms and investigations of sperm ultrastructure are necessary for the continued understanding of *Caobangia* fertilization.

The last basic questions posed by Jones regarding *Caobangia* are: 'where is the usual prepygidial growth zone of other polychaetes? Is the caobangiid growth zone just posterior to the posteriormost row of low avicular hooks, or elsewhere?' and 'what is the mechanism that mediates the internal anterior growth of the ascending gut such that it grows straight, beneath the ventral surface, moves dorsally on the right side, then straight to the midthoracic region?'

These questions are directly related. Segments in annelids originate in two ways: from the larval mesoderm (larval segments), and from the prepygidial growth zone (post-larval segments). Thus, in post-metamorphosis annelida the intestine forms from the prepygidial growth zone – a narrow transverse band of stem cells that delineates one new segment after another and connects the intestine with the anus in the pygidium (Anderson, 1965; Berrill, 1978; Murray, 2010; Licciano *et al.*, 2012).

Caobangia has a posterior pygidium with a posterior growth zone, but the anus is placed anteriorly, near the head. It seems that the formation of the segments and the intestine is implemented in three ways: from the larva's body (pre-metamorphosis), from the posterior meristematic area (during metamorphosis) and from the prepygidial growth zone (post-metamorphosis).

The body of an adult *C. billeti* consists of 8 thoracic chaetigers, which is typical for Sabellidae, and 8–15 abdominal chaetigers. The full number of thoracic chaetigers derives from the larval body. The trochophore has 8 chaetigers; after the completion of the metamorphosis, the prostomial and peristomial segments are fused and the remaining 8 larval segments give rise to 8 thoracic chaetigers. The thoracic region of post-metamorphic *C. billeti* has several segmentation traits, such as parapodia, dissepiments, and a ventral nerve cord with segmentary ganglia.

The abdominal region has no signs of segmentation, except for segmental chaetal fascicles and a metameric blood system. The formation of the abdominal region begins with the metamorphosis, when three blood lacunae appear simultaneously inside the pygidial meristematic area.

The next step of abdominal formation occurs after the metamorphosis, with the engagement of the prepygidial growth zone, as in all annelids. The number of both the chaetal fascicles and blood lacunae gradually increases posteriorly with the age of worm. The formation of segmental lacunae is faster than the formation of abdominal parapodia; worms which have 15 blood lacunae have only 10–12 pairs of chaetal fascicles in the abdomen.

According to our observations, the first (larval) and the third (posterior) modes of segmental formation in *C. billeti* are the same as those in all 'normal' annelids. The second pattern is unusual, because the prepygidial blastema produces several segments not subsequently, but synchronously. This requires more detailed embryological investigation. It seems that this mode of segmental formation is necessary for rapid settlement, metamorphosis and complete formation of the burrow. Such a short process is caused by the unique, unstable conditions of *Caobangia's* life inside the shell of semi-terrestrial snails.

ACKNOWLEDGEMENTS

Firstly, we would like to thank Dr Kirk Fitzhugh for his helpful advice on the sampling of *C. billeti* in Vietnam, and Leslie Harris for six fixed caobangiids that she kindly gifted to us.

We also thank employees of the Joint Russian-Vietnamese Tropical Scientific and Technological Centre, Svetlana and Andrey Kuznetov and Alexey Nemtsov; and, especially, we thank Hoang Thuy Zuong for her invaluable help with the snail-hunting. And last, many thanks to Anatoly Bogdanov and Georgy Davidovich (Laboratory of Electron Microscopy, MSU).

FINANCIAL SUPPORT

This study was funded by grant 14-50-00029 from the Russian Scientific Foundation (sampling expenses), and by grants 15-29-02447, 16-04-00343 and 13-04-00078-a from the Russian Fund for Basic Research (electron and confocal microscopy investigations).

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https://doi.org/10.1017/S0025315417000777 Published online by Cambridge University Press