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Gastrulation in Calcareous Sponges: In Search of Haeckel's Gastraea[1]

SALLY P. LEYS[2,*] AND DAFNE EERKES-MEDRANO†

*Department of Biological Sciences, CW405, University of Alberta, Edmonton, Alberta T6G 2E9, Canada
†Deparment of Biology, University of Victoria, Victoria, British Columbia V8W 3N5, Canada

SYNOPSIS. Haeckel's studies of development in calcareous sponges (1872) led him to develop the "Gastraea Theory," which proposes that the ancestral mode of germ layer formation, or gastrulation, was by invagination to produce a functional gut. His observations that gastrulation in the Calcarea occurs by invagination of a ciliated larva upon settlement and metamorphosis were supported by remarkable photomicrographs of the stage by Hammer in 1908. Although no later work found the same stage, these concepts are repeated in texts today. We have re-examined embryogenesis and metamorphosis in *Sycon* sp. *cf. S. raphanus* in order to understand when gastrulation occurs. Almost all larvae settle on their ciliated anterior pole and metamorphose into a bilayered juvenile whose interior cells rapidly differentiate into choanocytes and other cells of the young sponge. After a four-year search we have found the transitory stage shown by Hammer in which the anterior cells invaginate into the posterior half of the larva. The hole closes and it is not until some days later that the sponge forms an osculum at its apical pole. To understand whether invagination comprises gastrulation and if the hole can be considered to be a blastopore we have carried out a review of the literature dealing with this brief moment in calcaronean sponge development. Despite the intrigue of this type of metamorphosis, we conclude that gastrulation occurs earlier, during formation of the two cellular regions of the larva, and that metamorphosis involves the reorganization of these already differentiated regions. Considering the pivotal position occupied by the Calcarea as the possible sister-group to all other Metazoa, these results call for a reassessment of germ layer formation and of the relationships of the primary germ layers among basal metazoan phyla.

INTRODUCTION

One of the principal features that distinguishes multicellular animals from colonial protists is development through embryogenesis to form multiple cell layers. The process by which this occurs is gastrulation. The term is derived from the name given by Ernst Haeckel to a stage in the development of calcareous sponges, the gastrula, a ciliated egg-shaped larva with a mouth and a gut (Haeckel, 1872). According to Haeckel, the gastrula stage can be found in the development of all animals, and represents the recapitulation of the ancestral metazoan, the Gastraea, a diploblastic animal with a ciliated gut (Haeckel, 1874). The Metazoa, he argued, was therefore monophyletic. Although Haeckel's proposal incited many other studies (*e.g.,* Metschnikoff, 1874; Schulze, 1875, 1878; Minchin, 1896; Hammer, 1908), only Hammer was able to capture a stage that represented Haeckel's gastrula, a larva invaginating to form a ciliated gut. It is on these remarkable photomicrographs that we base our understanding of the origin of gastrulation by invagination.

Even though over a century of research on animal development has shown that embryogenesis is not so tidy (Richardson, 1995), and that modes of gastrulation are highly varied throughout the animal kingdom (Gilbert and Raunio, 1997), gastrulation by invagination to form the endoderm, or gut, is still widely conceived to be the ancestral method of germ layer formation (Wolpert, 1992; Denis, 1997; Nielsen, 2001; Arendt, 2004). However, it has also long been argued that ingression, rather than invagination, is the more common mode of germ layer formation among basal metazoans (Metschnikoff, 1874; Lankester, 1877) and that the ancestral metazoan did not necessarily possess a gut (Price and Patel, 2004). We believe that the association of the ancestral mode of gastrulation with gut formation by invagination is largely due to Haeckel's hypothesis and Hammer's images of invagination in the calcaronean larva.

Gastrulation in its broadest sense is the reorganization of the cells of the blastula to form a multilayered embryo, the gastrula (Brusca *et al.,* 1997). The remarkable consistency in the fate of these embryonic germ layers during the development of animals is, as Haeckel implied, one of the principal unifying feature of the Metazoa (Price and Patel, 2004). In many animals formation of the germ layers is concomitant with formation of the gut. But sponges are not generally considered to possess a gut in either the larval or adult stage. They are unusual among metazoans in that their tissues surround a series of canals and chambers through which water is filtered to feed. Nevertheless reorganization of the tissues by ingression or delamination to form multilayered larvae does occur during embryogenesis in many sponge groups, and is considered to represent gastrulation (Efremova, 1997; Boury-Esnault *et al.,* 1999; Leys and Degnan, 2002; reviewed in Leys, 2004).

Two new developments have brought us to re-examine development in calcareous sponges. First, while molecular phylogenies agree that the Metazoa is monophyletic, recent comparison of rRNA sequences and of sequences of protein coding genes suggest that

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[2] E-mail: sleys@ualberta.ca

in fact calcareous sponges might be more closely related to cnidarians, ctenophores, and other metazoans, than they are to other sponges (Kruse *et al.*, 1998; Borchiellini *et al.*, 2001; Medina *et al.*, 2001). The prospect of a paraphyletic Porifera indicates that, rather than sponges being a dead-end phylum, a sponge-like animal was indeed ancestral to all metazoans. Second, the finding that expression patterns for gene markers of germ layers (*e.g.*, *Brachyury, twist, snail*, Endo 16, β-*catenin*) are highly conserved across disparate phyla means that we can now use these techniques to re-examine germ layer formation in basal metazoans; essentially, we can try to re-evaluate Haeckel's hypothesis.

Despite the recent proposals of poriferan paraphyly, the Calcarea have long been considered the most primitive sponges because of their supposedly simple body forms. As a result, these sponges have featured in introductory invertebrate courses providing the 'primer' to sponge biology and presenting the general idea that they are well-studied and well-understood (Wallace and Taylor, 1997). But even a cursory look at what is known of the morphology of calcareous sponges quickly reveals only a smattering of published photomicrographs–most of various developmental stages, none presenting a complete series, and none of the tissues of adult sponges. Text and review material largely stems from drawings from a suite of papers by Duboscq and Tuzet in the 1930s and '40s (Duboscq and Tuzet, 1933, 1935, 1937; Tuzet, 1947). Reviews on the Calcarea were compiled by Tuzet (1963, 1973), Brien (1967) and Borojevic (1969, 1970).

The Calcarea is divided into two subclasses, the Calcinea and the Calcaronea (Manuel *et al.*, 2002). In the former, cleavage gives rise to a hollow blastula, which is filled in to a greater or lesser extent by the unipolar immigration of cells, much as occurs in hydrozoan cnidarians. The free-swimming larva has many similarities to the parenchymella of demosponges: it has a ciliated pseudostratified epithelium, most have some non-ciliated cells at the posterior pole, and most have a central cavity containing few to many cells (Fell, 1997; Amano and Hori, 2001). There are few studies of embryogenesis and metamorphosis in this group (Borojevic, 1969; Johnson, 1979).

The abundance of sponges from the subclass Calcaronea in littoral waters partially explains the bias of research on their development. Embryogenesis is unusual: cleavage leads to a hollow blastula with internally facing cilia; this turns inside out to form the amphiblastula larva, which has ciliated columnar cells on the anterior half, granular, globular cells making up the posterior half and center, except for a small inner cavity at the base of the anterior ciliated cells that contains extracellular matrix and bacteria (Amano and Hori, 1992; Leys and Eerkes-Medrano, submitted). It is this group that is for the most part represented in texts today, and this group that has historically been at the focus of the question of gastrulation in the Porifera.

In an attempt to understand gastrulation in calcareous sponges and determine whether there might be homology of the germ layers between the Calcarea and other metazoans, we have studied the development, metamorphosis, structure and function of the calcareous sponge *Sycon*, a member of the Calcaronea, the sponges studied by Haeckel and by his followers.

After four years we have finally found the transitory stage shown by Hammer (1908). Metamorphosis in calcaronean sponges takes place very rapidly so the events are difficult to capture. Because all of the work that addresses this vital point in the development of calcareous sponges was published between 1866 and 1908, we feel it is necessary to first re-examine this work. This paper, therefore, presents an historical review of the concepts of gastrulation that arose from research spawned by the publication of Haeckel's monograph, together with a précis of our current findings. We feel a review is also necessary because most of the articles from that period, and many of the reviews on the subject (Brien, 1967; Borojevic, 1969), are in German or French. Full details of our work appear in three other papers on the structure and function, and embryogenesis and metamorphosis of calcaronean sponges (Leys and Eerkes-Medrano, submitted; Eerkes-Medrano and Leys, submitted; Leys *et al.*, in preparation).

METHODS

Specimens of the calcareous sponge *Sycon* sp. cf. *S. raphanus*[3] were collected at 10m depths from dock pilings and from ropes suspended off the docks at the Bamfield Marine Sciences Center, Bamfield, B.C., Canada, from May–August in each of 2001–4. The sponge was identified using keys by Manuel *et al.* (2002) and Austin and Ott (1987); a detailed description of the soft tissues of the sponge is given elsewhere (Eerkes-Medrano and Leys, submitted) and a specimen has been deposited at the Royal British Columbia Museum (RBCM 004-049-001). Briefly, the sponge is a cream coloured tube 5–10 cm long and 0.5 to1 cm in diameter, that arises from a short stalk (0.2–0.5 cm long). Choanocyte chambers radiate out from a central cavity and are coalescent for most of their length. The mesohyl of the atrial cavity wall contains tetracts whose long rays are 150–250 μm and whose short ray (35 μm) projects into the atrial cavity. The walls of the choanocyte chambers are lined by triacts whose rays are 20–250 μm. Diactines (oxeas) 600–1,000 μm long form a fringe of spicules at the osculum and tufts at the distal tips of the choanocyte chambers.

Sponges were placed in bowls of sea water which was allowed to warm slightly on the counter top (from 9–13°C). After 2–3 hr numerous larvae swam out through the oscula of adult sponges to the surface of the water; these were collected by pipette and transferred to petri dishes containing one new glass or plastic coverslip on the bottom and a glass coverslip floating on the surface. Live larvae and juveniles were ob-

[3] Past and current species terminology is provided in Appendix.

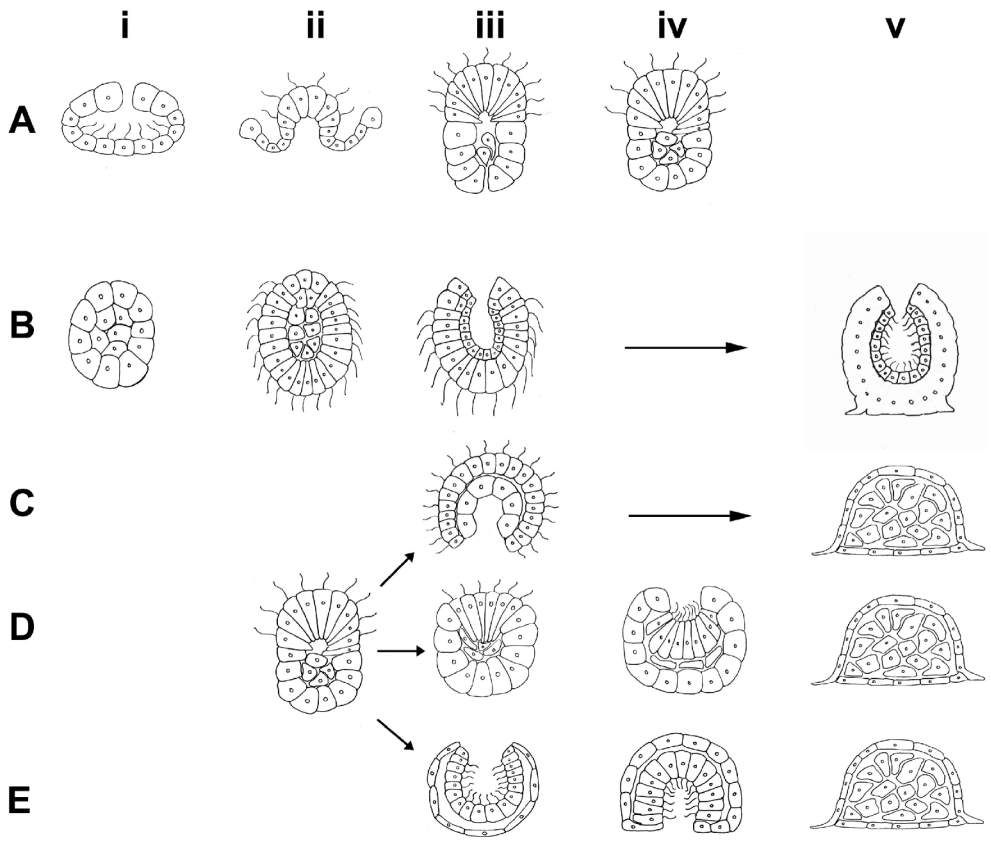

FIG. 1. Schematic of the possible gastrulation stages in the Calcaronea. A, Embryogenesis: (i–ii) eversion of the ''stomoblastula,'' considered by all to be pseudogastrulation; (iii) ingression of cells into the larval cavity to form the posterior half of the amphiblastula larva (iv); (iii) is the stage considered by Barrois (1876) and by Metschnikoff (1874) to be gastrulation. B–E Metamorphosis: B, Haeckel's (i) morula, (ii) planula, (iii) gastrula, and (v) ascula (juvenile) with a syncytial outer layer. C, Schulze's (1875) first study: gastrulation by invagination of the posterior cells (iii) to form a bilayered juvenile (v). D, Ingression (iii) and invagination (iv) of the anterior cells at metamorphosis according to Metschnikoff (1874). E, (iii) Invagination of the anterior cells, considered by Schulze (1878), Hammer (1908) and Duboscq and Tuzet (1937) to be gastrulation. (iv) Settlement of the invaginating larva according to Schulze (1878) and Hammer (1908).

served with an Olympus SZX12 stereomicroscope with a 1.6× objective. Free-swimming and settled larvae were fixed at 0, 3, 6, 12, 24, and 48 hr after release from the parent in a cocktail fixative of 1% OsO_4, 2% glutaraldehyde in 0.45 M sodium acetate buffer, pH 6.4, with 10 % sucrose in the final volume (see Leys and Degnan, 2002). As much sea water as possible was removed from the samples and 4–5 volumes of cocktail fixative were added. Samples were fixed at 4°C for 2 hr. Specimens were then rinsed three times in filtered sea water, dehydrated in ethanol and transferred to the University of Alberta. Coverslips were either cut or broken into smaller pieces and both individual larvae and coverslip pieces with settled larvae were critical point dried and mounted on electron microscope stubs using silver paint or nail polish; stubs were coated with gold and viewed in a JEOL 6301F field emission scanning electron microscope. Other specimens were embedded in epoxy (Taab 812) for thick and thin sections as described previously (Leys and Degnan, 2002). In 2004, large numbers of fixed adherent postlarvae were gently pried off the coverslips using a microscalpel while still in 70% ethanol, dehydrated and prepared for scanning electron microscopy and thick and thin sections as described above.

EXISTING INFORMATION: GASTRULATION IN THE CALCARONEA

During the development of calcaronean sponges there are three moments when radical cell movements cause a dramatic change in the morphology of the embryo or larva; the first two occur during embryogenesis (Gatenby, 1920; Duboscq and Tuzet, 1933; Gallissian, 1983; Franzen, 1988), the third, at metamorphosis (Metschnikoff, 1874; Schulze, 1875; Barrois, 1876; Schulze, 1878; Maas, 1894; Minchin, 1896; Hammer, 1908) (Fig. 1). The most difficult to observe are those at metamorphosis, and these cell movements are what has long been considered gastrulation.

Embryogenesis

Schulze (1875) provides an excellent description of early development (Fig. 2). Cleavage in *Sycon* (*Sycandra*) *raphanus* is total and regular; the first three cleavages are meridional, producing a ring of 8 cells with a hole in the center. The fourth cleavage is equatorial

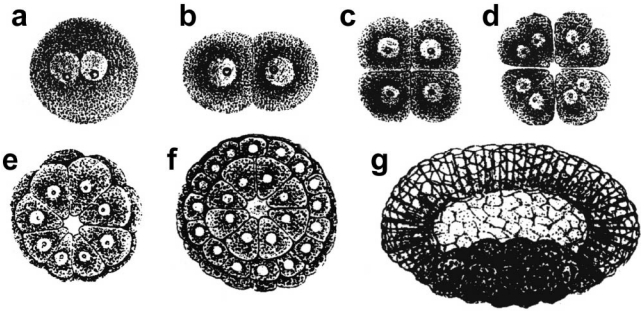

FIG. 2. Early embryogenesis in *Sycon raphanus* according to Schulze (1875). Images from plate 20. (a–f) 1–32 cell cleavage stages; (g) 64-cell stage blastula.

producing two layers of 8 cells and by the fifth cleavage, a single layered blastula with a central cavity is formed. The cells are all the same size. Cleavage then becomes unequal and at the 64-cell stage an opening appears among 8 of the large cells.

Descriptions of what the embryo does after this point are highly variable and probably reflect in part differences between species, and in part the difficulty of catching all the stages in paraffin sections. This stage was clearly difficult to interpret, and it was not until a series of studies by Duboscq and Tuzet (Duboscq and Tuzet, 1933, 1935, 1937), and later by Lufty (1957) and Franzen (1988), that the next steps were resolved. Schulze's (1875) description most clearly illustrates what early observers saw. According to him, the embryo does not change directly into a round amphiblastula simply by reducing the cavity and increasing the size of the cells. Rather there is a shallow to deep invagination of the dark round cells into the lighter cylindrical cells to form a convex-shaped embryo in the mesohyl against the parent's choanocyte layer. This bowl-shaped larva undergoes an abrupt change to become the amphiblastula larva, which breaks through into the choanocyte chamber.

We now know that in all species studied the blastula has inwardly directed cilia and forms an opening at one side through which it will evert, and is thus called a stomoblastula. The eversion of the embryo, in a manner similar to that seen in the Volvocales, is very rapid. In some species it results in the embryo being pulled through into the choanocyte chamber (Franzen, 1988), and in others, the eversion occurs within a so-called placental membrane within the mesohyl and is then released to the choanocyte chamber (Lufty, 1957). The eversion results in the formation of the amphiblastula larva, which has a columnar ciliated epithelium on the anterior hemisphere, and a posterior hemisphere of granular, globlular cells (Fig. 1A i–iii). This is the first rapid morphological change that could be interpreted as gastrulation. The second involves the ingression of granular cells into the center of the larva and differentiation of the anterior and posterior cells, which occurs from the moment of eversion until release of the larva (Fig. 1A iii–iv).

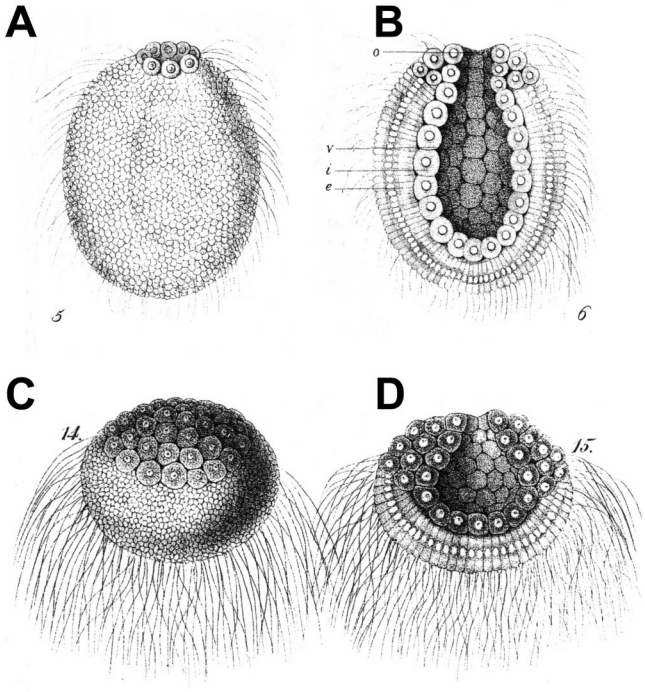

FIG. 3. Images from plates 13 (A,B) and 44 (C,D) from Haeckel's (1872) monograph on calcareous sponges. *Asculmis armata* gastrula, whole larva (A) and longitudinal section (B) showing mouth opening (o), stomach (v), entoderm (i) and exoderm (e). *Sycyssa huxleyi* gastrula, whole larva (C) and longitudinal section (D).

Metamorphosis

The free-swimming larva settles and undergoes metamorphosis into a juvenile sponge. This is the stage that Haeckel focused upon as the root of gastrulation. Haeckel's description, however, differs significantly from all others. For example, Haeckel (1872) was the only author who thought that during embryogenesis cleavage gave rise to a solid morula, which then differentiated into an outer layer of ciliated cylindrical cells and an inner mass of non-ciliated cells (Fig. 1B i, ii). He calls this stage the ''flimmerlarva'' or ''planula.''

According to Haeckel (1872), an opening is formed at one end of the planula, thus forming the ''gastrula,'' the stage that Haeckel claimed has great phylogenetic significance (Fig. 1B iii). The gastrula is ''a spherical, egg-like or elongated body which has an inner cavity with an opening (the primordial mouth). The wall of this cavity is made of two different cell layers–an outer, lighter, ciliated layer, and an inner, darker, unciliated layer; the first is the ectoderm, or outer layer (animal, sensory or dermal) and the second is the entoderm or inner (vegetative, nutritive or gastral) layer in higher animals'' (Haeckel, 1872, p. 333) (Fig. 3). Larvae from all the sponges he examined (asconoid, syconoid and leuconoid) are depicted the same way, with two cell layers (the outer ciliated, the inner not), a hollow center, and an opening at one end. (One species, *Ascetta clathrus*, has no opening and is called by Haeckel a ''planogastrula.'') Haeckel does not say whether the

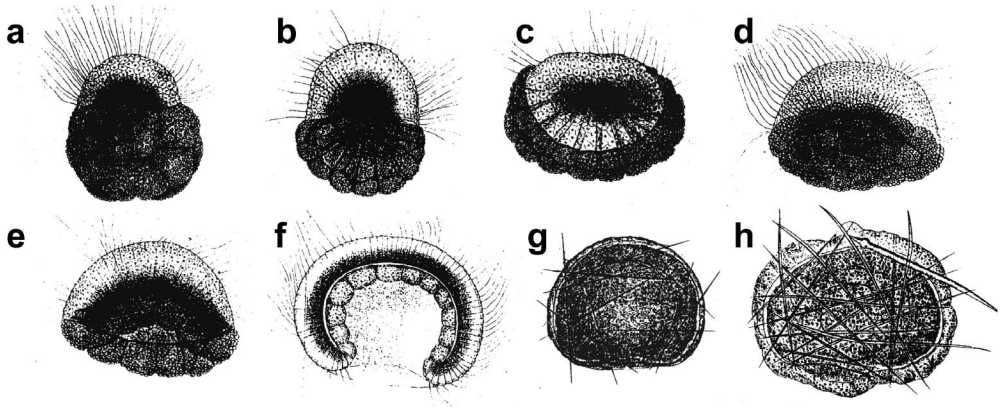

FIG. 4. Images from plate 21 from Schulze (1875) showing (a, b) two views of the larva, (c) an older larva viewed from the anterior pole, (d) a later stage in which the 'entoderm' (the large granular cells) is flattened, (e) larva with the endoderm beginning to pull in, (f) side view of the gastrula, (g) larva after loss of the cilia and with the first spicules, (h) an older larva with thicker spicules.

gastrula arises by the ingression or invagination of the non-ciliated cells but the result is the formation of the endoderm and future gut. At metamorphosis, he says, the larva settles on the side opposite the opening, the ciliated cells absorb their cilia, fuse to become a syncytium, and begin to produce the spicules (Fig. 1B v). The endodermal cells become ciliated, and the gut is formed. Nevertheless, he admits he has imagined this description of metamorphosis to be the case, because he did not actually observe it. He says that the events that take place at metamorphosis are very quick, but what occurs can be inferred from a comparison between the ascula (juvenile) and the gastrula.

Schulze's (1875) first work offers a perspective of the cell movements at metamorphosis that is not unlike Haeckel's. According to Schulze, however, the larva is not a bi-layered planula, but rather an amphiblastula with a ciliated anterior pole and non-ciliated posterior pole. Prior to metamorphosis the dark granular posterior cells invaginate into a cup formed by the ciliated cells (Fig. 4). No other authors confirm this to be the case.

The third viewpoint on metamorphosis is provided first by Schmidt (1866) and elaborated on by Metschnikoff (1874). In this description, the ciliated cells are engulfed by the non-ciliated cells. According to Metschnikoff (1874), the central cavity of the amphiblastula is lost while the ciliated half becomes much reduced and the granular cells of the posterior half fuse into a mass. The posterior cells may start to produce spicules even prior to settlement of the larva. The central issue at metamorphosis, he says, is that the non-ciliated, skeletal building half becomes larger, while the ciliated half pulls in to the middle of the larval body (Fig. 1D iv). He stresses that the ciliated half becomes covered by the increase in size of the non-ciliated half. ''To understand how the ciliated cells are taken in, you must find a larva at a comparatively early stage (before the spicules have started to form). In these one can see that while the posterior half is little changed, the previously ciliated portion pulls into the body, leaving an opening to the outside at the upper pole'' (Metschnikoff, 1874, p. 3). This opening, the ''Einstühlpungsöffnung,'' does not become the definitive osculum, but rather changes, until the fully attached sponge is a solid body with two layers. On the outside is the skeleton forming layer in which spicules may already be formed and on the inside are the cells that represent the endoderm. The true osculum, he says, does not arise until at least 6 days after settlement. From this description we understand that the opening referred to is at the apical side of the settled juvenile.

Schulze (1878) re-examined metamorphosis in *Sycon* (*Sycandra*) *raphanus*, and observed the following changes to the larva in a drop of water hanging from a coverslip (Fig. 5). One half of the larva flattens or invaginates 'into' the other half; often only the ciliated half flattens but sometimes there are depressions all over. He depicts an extreme case of the flattening (Fig. 5b), but says that this is unusual. What normally happens, he says, is that the long axis shortens and the larva gets wider at the equator. The ciliated half then pulls into the larva, while the granular cells from the posterior half maintain their cup shape, so that the larva ''assumes the form of a plano-convex lens, whose flat surface is formed by the delicate mosaic of the ciliated cell ends, whose convex side is formed by the free surface of the extremely flattened granular cells, but whose slightly rounded edge is formed by the 15 or 16 cells of the outermost granular cell ring.'' (Schulze, 1878, p. 267) (Fig. 5d). In this way a two-layered sack-like gastrula is formed, where the inner cells are ciliated. Gradually as the ciliated layer pulls in more and more, the ''hole'' closes and a dome-shaped two-layered sack is formed, whose layers lie adjacent to one another. All this, he claims, occurs within half an hour, and the speed of the process is probably the reason it is difficult to observe. According to Schulze, the larva settles on the newly closed hole, and the granular cells lose their opaqueness al-

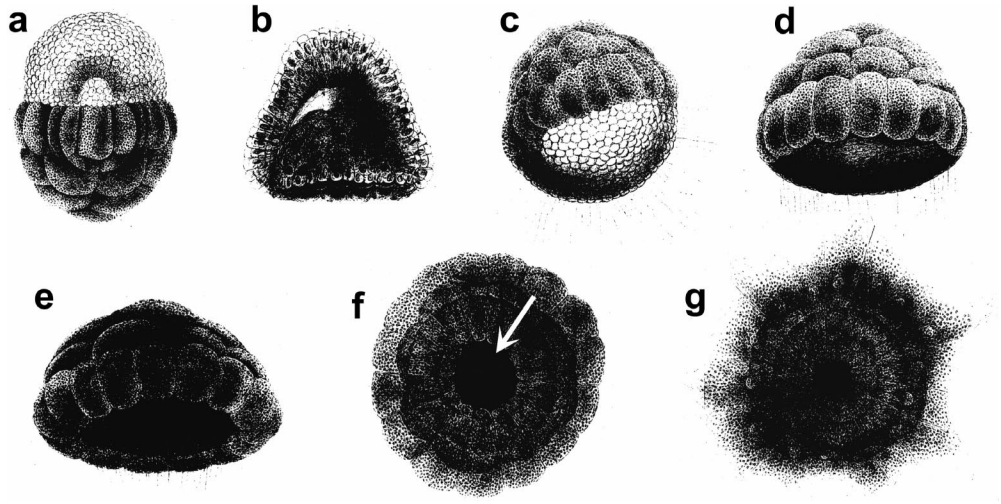

FIG. 5. Images from plate 18 from Schulze (1878). (a) A live 'flimmerlarva'; (b) Abnormal appearance of a ''flimmerlarva'' in which the granular cells are invaginating into the center of the larva; (c) Normal development of a ''flimmerlarva,'' flattened at the poles, and wider at the equator; (d) Larva whose ciliated cell layer has pulled in to the granular cells so that the ciliated layer appears flat; (e) Larva whose ciliated cell layer is fully withdrawn into the granular cells; (f) View of narrowing invagination furrow of a larva (an arrow has been added to the image to indicate the invagination furrow); (g) View from the basal side of a larva that has settled.

lowing a view to the inside of the inner cylindrical cell layer and the remnants of the gastral hole.

Thirty years later, Hammer (1908), a student of Schulze, revisited the question of metamorphosis, again studying *Sycon raphanus* in Naples, Italy. He found stages in which the blastula appeared to invaginate while still in the mesohyl of the adult sponge, and like previous authors, he interpreted this as pseudogastrulation. The real gastrula, he wrote, ''comes about simply and only through the invagination of the ciliated columnar cells'' (Hammer, 1908, p. 315), in the free-swimming larva prior to metamorphosis (Fig. 1E iii). He shows two photomicrographs of amphiblastula larvae, and two photomicrographs of free-swimming larvae in which the ciliated cells form a concave surface against the cup-like granular cells (Fig. 6). Cilia are present on all of the columnar cells, and the granular cells are flattened against a small space between the two layers. There are no images of the next stages, but he says that in larvae just prior to

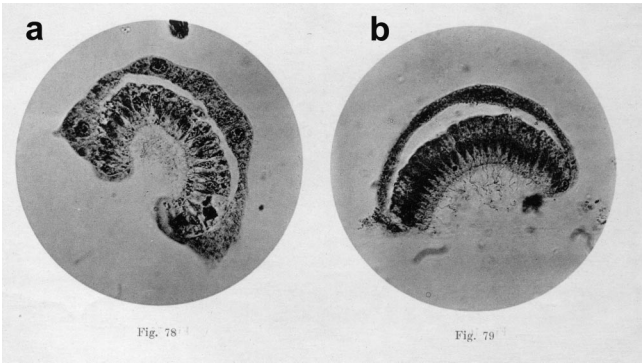

FIG. 6. Photomicrographs from Hammer (1908) showing free-swimming larvae in which the the ciliated half has invaginated into the non-ciliated half.

or actually undergoing settlement, the ciliated cells are enveloped by the large cells at the rim of the ''invagination furrow,'' and the furrow itself is no longer visible. This furrow or hole, he agrees with Metschnikoff, is not related to the future osculum of the juvenile sponge.

According to Hammer, after gastrulation the larva settles on the gastral mouth with the edge of the ''invagination furrow'' directed inwards. The settled larva has two layers, an outer layer of flattened cells—the granular cells which have lost much of their dark granular appearance—and an inner mass of the former ciliated cells. Unlike Schulze (1878), Hammer was not able to see the gastral hole through the now translucent granular cells.

No subsequent research has managed to capture the very early stages of metamorphosis between the free-swimming amphiblastula larva and the dome-shaped, bi-layered juvenile (Duboscq and Tuzet, 1937; Amano and Hori, 1993). According to Amano and Hori, larvae of *Sycon* and *Leucandra* settle 12 hr after release from the parent, and appear to be a flattened mass of cells. At this stage, they have already resorbed their cilia and consist of an inner cell mass derived from the ciliated cells, enveloped by a layer of pinacocytes, derived presumably from the granular non-ciliated cells.

NEW RESULTS

We have studied embryogenesis and metamorphosis in *Sycon* sp. *cf. S. raphanus* using scanning electron microscopy (SEM) and thick and thin plastic sections with light and electron microscopy. The larvae are 30–50 μm long with ciliated columnar cells on the anterior pole and large globular granular cells on the posterior pole and in the centre (Fig. 7a, b). There are four indented cells around the equator of the anterior hemi-

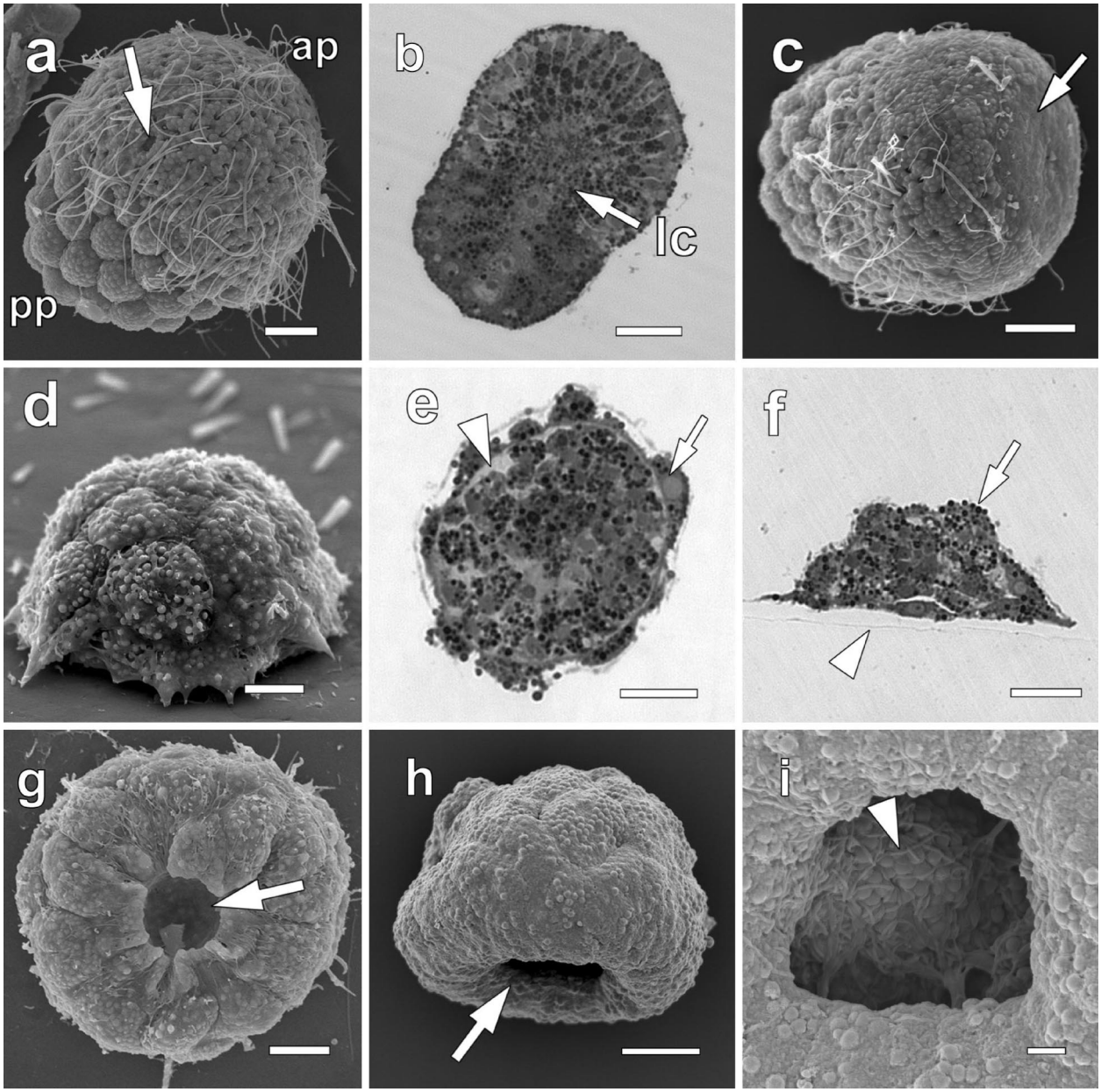

FIG. 7. Light and scanning electron micrographs of *Sycon* sp. cf. *S. raphanus*. (a) A 2-hr-old free-swimming larva showing the anterior ciliated hemisphere (ap) and large globular, granular cells of the posterior hemisphere (pp). One of the four ''cross cells'' on the anterior hemisphere is shown by the arrow. (b) A longitudinal section through a plastic embedded free-swimming larva in the same orientation as (a); lc, larval cavity. (c) A scanning electron micrograph (SEM) of a 12-hr old larva that has lost the cilia and flattened (arrow) on the anterior pole as though it had been attached to the substrate. (d) SEM of a newly adherent postlarva. (e) Light micrograph of a section horizontally through a plastic embedded newly adherent and metamorphosing larva (between 12 hours and 2 days after release from the parent). The postlarva has an outer layer of granular cells (arrow) surrounding a group of variously shaped cells in a collagenous extracellular matrix (arrowhead). (f) Longitudinal (vertical) section through a newly adherent postlarva at the same stage as (e). The outer epithelium is continuous around the entire postlarva from apical (arrow) to basal (arrowhead) surfaces. (g) SEM of one of two postlarvae that settled with a depression or 'hole' (arrow) in the apical side. (h) SEM of a postlarva that was detached from the substrate after fixation with osmium. The anterior ciliated cells have invaginated into the posterior cells forming a 'hole' in the basal side (arrow). (i) SEM providing a view into the invagination hole of the adherent larva in (h) showing cilia (arrowhead). Scale bars: a–h:10 μm; i: 2 μm.

sphere that correspond to the cross cells of Duboscq and Tuzet (1941). The larvae swim by rotating in a right hand direction (clockwise as seen from the posterior pole) as described previously (Elliott *et al.*, 2004). Several thousand larvae were observed live and of these, some 300 were observed as they underwent metamorphosis in petri dishes. Larvae and postlarvae collected during four different years (2001–4) were fixed for electron microscopy; we examined over a thousand individual larvae and postlarvae at different stages of settlement and metamorphosis and captured images of 380 individuals.

While in the parent sponge the posterior half of the larva is small relative to the anterior ciliated half, but the relative sizes of ciliated and non-ciliated halves are highly variable in all ages of free swimming larvae (Fig. 7a), as is the size of the central cavity. Most larvae have a very small cavity (5 μm in diameter) at the base of the anterior ciliated cells (Fig. 7b); in a few larvae the cavity is much larger (10–20 μm in diameter). The larvae settle within 12 hours of release from the parent, usually on their anterior pole but occasionally on their side or posterior pole, and attach by their cilia, which seem to be adhesive. Many larvae lose their cilia and flatten at the anterior pole as though they have been attached (Fig. 7c).

All settled postlarvae (12–48 hr old) have a central mass of cells that is enveloped by a single layer of the former granular cells (Fig. 7d–f). Thick sections show an outer layer of granular cells enveloping a region of dense collagen and a mass of variously shaped cells (Fig. 7e, f). In some 1–2 day old metamorphosed postlarvae choanocytes have already differentiated.

Early in our study two postlarvae were found with a large depression in the apical side of the settled juvenile (Fig. 7g). No other settled larvae (of over 300 examined) had an apical depression, and no free-swimming larve were found with an invagination of the anterior end. But, by carefully removing newly settled larvae from the substrate after they were fixed, we eventually discovered four settled larvae with a clear invagination of the anterior cells into the posterior half, such that a hole was formed at the basal side of the postlarva (=former anterior pole of the larva) (Fig. 7h). The hole was quickly filled in by the formation of basal epithelium. Though there were no cilia in the hole on the apical side of the two settled postlarvae, cilia were quite evident in the hole on the basal side of attaching larvae (Fig. 7i); it is assumed these larvae had settled on their posterior swimming (unciliated) poles, so the hole was still in the former anterior pole of the larva. Spicules first appeared 2 days after release from the parent and an osculum was first seen in 9-day old settled juveniles.

DISCUSSION

The idea that the ancestral mode of gastrulation was by invagination to form a gut clearly stems from Haeckel's (1872) description of metamorphosis in calcareous sponges and is supported by Hammer's (1908) remarkable photomicrographs. In revisiting the question of gastrulation and the formation of the germ layers in *Sycon* we were surprised not to find any invagination stage in free swimming larvae as described by Hammer, and were nearly convinced of the absence of this stage until we discovered it in four recently settled specimens that were pried off the substrate after fixation in osmium. Nearly all the postlarvae we studied over the course of four reproductive seasons appeared to change instantly from the amphiblastula to a bilayered postlarva. The anterior cells lost their cilia and migrated in to form the inner cell mass; some of these cells rapidly differentiated into choanocytes, while others remained amoeboid. Two postlarvae of all those studied had a depression on the apical surface of the juvenile and had apparently settled on the posterior pole.

Our results confirm Hammer's micrographs, and, considering how many larvae we had to process to find the invagination stage, reaffirm the excellence of this early work. All authors but Haeckel were correct as to the mode of metamorphosis. Typically the larvae settle on their anterior end, on the hole left by the invagination of the anterior ciliated cells as described by Schulze (1878) and Hammer (1908) (Figs. 1e, 7h). Some settle on their posterior pole, leaving an invagination hole on the apical side of the settled postlarva, as described by Metschnikoff (1874) (Figs. 1d, 7g). In neither case is the hole related to the future osculum; the hole is engulfed by the newly forming basal epithelium and the ciliated cells dedifferentiate to form an inner cell mass. Thus the transient cavity that is formed by invagination is not the future gut of the sponge, unlike the case in anthozoans such as *Nematostella* (Byrum and Martindale, 2004) or the echinoderm larva (Wray, 1997). Though the ciliated cells transdifferentiate into the future choanocytes, they also form other cells of the juvenile, as is the case during the metamorphosis of several demosponge larvae; modern authors do not consider the process to represent gastrulation (Amano and Hori, 1996; Efremova, 1997; Leys and Degnan, 2002; reviewed in Leys, 2004).

The early workers were divided as to the interpretation of the ''invagination hole.'' Schulze (1878) and Hammer (1908) said that it was the mouth of the gastrula, sensu Haeckel (1874), which implied that the larva is a blastula. This interpretation has lasted sufficiently for the larva to still be called a blastula stage–the Amphiblastula. A different view was espoused by Barrois (1876) and Metschnikoff (1874) who thought that the larva had already attained its germ layers—the anterior ciliated half and the posterior, skeleton building half—and thus was a gastrula prior to settlement. Barrois even suggested it could be called an ''Amphigastrula.''

Though the invagination stage is very compelling, we feel that since considerable differentiation and reorganization occurs through embryogenesis to form a fully differentiated larva that has at least two cell layers and is responsive to environmental stimuli (see Elliott *et al.*, 2004; Leys and Eerkes-Medrano, submitted), gastrulation must occur during embryogenesis, as is the case for all other metazoans. The reorganization of the germ layers at metamorphosis can be compared to the radical reorganization of cells of many invertebrate larvae (*e.g.*, Chia and Burke, 1978). Just as many hydrozoan cnidarians with non-feeding, gutless larvae are nevertheless considered to have undergone gastrulation and already have two cell layers separated by a collagenous mesohyl (*e.g.*, Thomas *et al.*, 1987), we would interpret the moment of gastrulation as the in-

gression of cells into the larval cavity after eversion and prior to release from the parent, that is during embryogenesis, not at metamorphosis. This is in accordance with other modern interpretations of gastrulation in the Porifera (Efremova, 1997; Boury-Esnault *et al.*, 1999; reviewed in Leys, 2004).

The problem with interpretation of the 'invagination hole' today comes from the association of gastrulation with gut formation, which stems directly from Haeckel's definition and descriptions of this pivotal stage in sponge development. Though in many animals gastrulation results in formation of the endoderm, in basal groups it is frequently a two step process, first resulting in the formation of germ layers, and only later in the formation of the gut. Within our current understanding of sponge structure, few authors would homologize the choanocyte epithelium of sponges with the lining of the gut in other animals.

Thus we suggest that the principal function of gastrulation is formation of the germ layers and that formation of the gut has secondarily become part of the process. As a corollary we suggest that the primitive mode of gastrulation was by ingression or delamination, not invagination. If gastrulation is not primarily to do with gut formation, this may explain why we find expression of conserved endomesodermal genes in animals which are not otherwise considered to have a third germ layer (Gröger *et al.*, 1999; Adell *et al.*, 2003; Manuel *et al.*, 2004). This said, the image of invagination of *Sycon* at metamorphosis shows a process intriguingly similar to the invagination that occurs during echinoderm gastrulation and begs interpretation. A re-evaluation of the relationships between germ layers of basal and higher metazoan phyla including a comparison of gene expression patterns in poriferan models—when possible—may shed light on the primary function of gastrulation.

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APPENDIX

A note on species identifications:

The taxonomy of the Calcarea has a long and convoluted history. Haeckel proposed the first taxonomy choosing names for genera that reflected body architecture; his system was not followed. Metschnikoff (1874) identified the species he studied, *Sycon ciliatum*, as equivalent to Haeckel's *Sycandra raphanus*. The species referred to as *Sycandra raphanus* by Haeckel and Schulze has been synonomized under *Sycon* (see Manuel *et al.*, 2002), and the species referred to as *Sycon ciliatum* by Duboscq and Tuzet (1937) was later identified as *Sycon raphanus*. *Scypha* is now also included in *Sycon*. Gatenby (1920) also equated *Grantia* with *Sycon*. According to Manuel *et al.* (2002), all genera but *Scypha* are now valid. Hence, for the purposes of this paper, all workers investigated a syconoid sponge identified at some point as *Sycon*. The genus *Sycon* is currently considered to be polyphyletic (Manuel *et al.* 2003). No revision of the Pacific species has been carried out, but the sponge studied here was identified as *Sycon* according to the keys and descriptions of Manuel *et al.* (2002), and as most similar to *S. raphanus* according to the Keys of Austin and Ott (1987).

Table of current and past species names:

Current name*	Past name	Author
Sycon raphanus	*Sycandra raphanus*	Schmidt, 1866 Haeckel, 1872 Schulze, 1875, 1878 Jorgensen, 1910
Sycon raphanus	*Sycon raphanus*	Hammer, 1908
Sycon raphanus	*Sycon ciliatum*	Metschnikoff, 1874 Duboscq and Tuzet, 1937
Sycon ciliatum	*Scypha ciliata*	Franzen, 1988
Grantia compressa	*Grantia compressa* *Sycon compressa*	Gatenby, 1920 Barrois, 1876

* From Manuel *et al.* (2002).