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Adhesive Papillae of *Phallusia mamillata* Larvae: Morphology and Innervation

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ABSTRACT—The swimming larvae of most solitary ascidians belonging to the Ascidiidae family bear three anterior, simple conic adhesive papillae. They secrete adhesive substances that are used to effect transitory settlement at the beginning of the metamorphosis.

The adhesive papillae of newly hatched *Phallusia mamillata* larvae examined by the SEM are covered by the tunic. When the larvae are about to settle, the tunic becomes fenestrated over the central part of the papilla and bulb-ended microvilli protrude through the holes. These papillae have two types of elongated cells: many peripheral cells and few larger central cells with microvilli and bundles of microtubules oriented along the major axis of the cells.

We have done immunofluorescence experiments with an anti-β-tubulin monoclonal antibody (clone 2-28-33) reacting with axonal microtubules. Only the central cells of the papillae were stained and the axons appeared to arise from the proximal ends of these cells. These axons form a long nerve that reaches the brain vesicle. Branches of the same nerve appear to connect to the basal ends of the peripheral cells. By confocal laser microscopy we were able to follow the course of the papillary nerve. The two nerves connecting the dorsal papillae fuse together into a single nerve that runs posteriorly. The nerve connecting the ventral papilla runs posteriorly for a long tract before fusing with the nerve of the dorsal papillae just near the brain.

The reported observations raise the hypothesis that the central cells of the adhesive papillae might be primary sensory neurons and that they may have chemosensory function.

INTRODUCTION

Most solitary and compound ascidian larvae have adhesive papillae at the anterior end of the trunk, the main function of which is secretion of a sticky substance that enables the larvae to adhere to the substrate when they begin metamorphosis.

There is great variability among different species of ascidians in the morphology and function of the papillae, but in all ascidians they are located anteriorly, in the best position for a sensory organ to receive information about the substrate upon which the larvae are going to settle and adhere by means of the secretion. However there is evidence that adhesive papillae participate in substrate selection only in a few species (Burighel and Cloney, 1997). Some Styelidae species have larvae in which the attachment involves the entire larval wall, and in these cases the papillae probably have only sensory function (Grave and Riley, 1935) but in all other ascidians the papillae are secretory and sensory. In the genera *Molgula* and *Eugyra* tadpoles are apapillate, but larvae of *Molgula occidentalis* have primary sensory cells at the anterior end of the trunk and glandular epidermal cells secrete adhesives at the onset of metamorphosis (Torrence and Cloney, 1983; Burighel and Cloney, 1997).

Adhesive papillae are formed by the anterior epidermal cells and are induced together with the nervous system by the A4.1 cells and by their progeny (Ortolani, 1954). Both in their way of induction and in their way of differentiation, the adhesive papillae may be considered homologous to the embryonic adhesive organs of some vertebrates (De Bernardi and Fascio, 1994; Sive and Bradley, 1996).

Adhesive papillae begin to organize just before hatching. Usually three ectodermal areas at the anterior end of the trunk thicken as their cells elongate. In solitary ascidians papillae are anterior, disposed in a triangular field, but the number and the spatial disposition can vary among different ascidian species, and the cellular organization of the papillae is also very different, as shown by light and electron microscopy studies of many authors.

Adhesive papillae have been divided by Cloney (1978) into two main categories, everting and non-everting, depending on the capacity for rapid eversion and exposure of the secreted sticky substances. In most compound ascidians are present complex everting papillae, they are formed of many kinds of cells with different functions, as demonstrated by

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the ultrastructure of *Polysyncraton lacazei*, *Diplosoma spongiforme* and *Ecteinascidia turbinata* (Turon, 1991), *Diplosoma macdonaldi* (Torrence and Cloney, 1983) and *Distaplia occidentalis* (Cloney, 1978). Several kinds of different cells have been described: glandular cells with the cytoplasm filled with rough endoplasmic reticulum and vesicles containing electrondense granules, sensory cells bearing cilia and microvilli with bulbous apical terminals and other cells recognized as primary sensory neurons, the basal processes of which are axons that join together to form a papillary nerve. The mechanism of eversion may be different, but myoepithelial cells with myofilaments in their cytoplasm play an important role. Non-everting papillae, usually present in solitary ascidians, are simpler. In *Clavelina lepadiformis* (Turon, 1991), there is a single kind of secretory cell. In many other solitary ascidians the papillae are conic and are formed of two kinds of cells: elongated peripheral cells, probably secretory, and a few tall central cells thought to be sensory. TEM studies of *Ascidia malaca* (Gianguzza and Dolcemascolo, 1994, 1997) showed some differences in the papillae between the peripheral and the central cells which are thought to be sensory cells. The connection between the cells and the papillary nerve was found in the sections examined, but without serial sections the authors could not follow the course of the nerve. Histological differences were found within each group and Cloney (1978) tried to classify them into eight groups, but these groups are not representative for all kinds of papillae. Probably each species of ascidians has papillae that are more or less different from all other.

The morphology and consequently the function of the attachment organs are strictly related to the stage of larval development and metamorphosis, and for this reason we considered it of interest to study the adhesive papillae throughout the period of larval life, from hatching to settling. In a previous paper (Sotgia *et al*., 1993) we reported some events that occur during the metamorphosis of *Phallusia mamillata*, a solitary ascidian belonging to the order of Phlebobranchiata. We have now studied the modifications of the shape and of the function of the adhesive papillae during the stages of metamorphosis and we have also studied the nerve connection between the papillae and the brain vesicle.

MATERIALS AND METHODS

Phallusia mamillata eggs were obtained from oviducts of dissected animals. Eggs were collected in Millipore-filtered sea water and fertilized with a suspension of self and non-self sperm. One hr after fertilization they were rinsed in filtered sea water and allowed to develop at 20°C. Larvae at different stages of development were fixed in cold 0.37% formaldehyde in absolute methanol or in 5% paraformaldehyde-PBS. The stages chosen were: a) newly hatched larvae, b) swimming larvae, c) larvae at the beginning of tail retraction and d) larvae with half the tail retracted.

Immunofluorescence

Methanol-fixed larvae were processed for whole-mount immunofluorescence staining with anti-β-tubulin or anti-α-actin antibody by the method of Elinson and Rowning (1988) as modified by E. Houliston (personal communication). Briefly, after two hr fixation, larvae were gradually rehydrated to PBS, extracted for 20 minutes with 0.25% Triton X-100 in PBS, rinsed in PBS and then incubated in mouse anti-β-tubulin monoclonal antibody (clone no. N 375 - Amersham) diluted 1:50 or in anti-β-tubulin (clone no. 2-28-33 Sigma) (Crowther and Whittaker, 1990) diluted 1:100 or in anti-α-actin (clone asm-1 Boehringer) diluted 1:100. After repeated rinsing in PBS, larvae were incubated with FITC-conjugated anti-mouse IgG diluted 1:50. They were then rinsed in PBS and mounted in Citifluor (Sigma). The larvae were observed either by epifluorescence microscopy or with a Leica TCS NT confocal laser scanning microscope equipped with laser Argon/Krypton, 75mW multiline.

SEM microscopy

Living larvae were fixed in PAF (picric acid-formaldehyde) 1200 mOsm pH 7.5 (Stefanini *et al*., 1967), as modified by G. Melone. The fixed larvae were dehydrated in a graded ethanol series, critical point dried and gold sputtered. They were then observed and photographed in a Cambridge Stereoscan S 250 Mk2 scanning electron microscope.

Histology

Paraformaldehyde-fixed larvae, dehydrated in graded ethanol series, were embedded in JB-4 plastic medium (Polyscience, Inc.) and 2 μm serial sections were stained with 10% methylene blue and mounted in Neu-Entellan (Merck).

RESULTS

Phallusia mamillata larvae have three simple conic papillae at the anterior end of the trunk, positioned at the vertices of a triangular field; two are dorsal and one is ventral. The shapes of the papillae are different during larval development, and data concerning four larval stages are reported.

Newly hatched larvae

The SEM image of the frontal region of the trunk (Fig. 1A) shows the thick coat of the tunic to be continuous, without openings (many irregularly scattered ruptures in the tunic are preparation artefacts); papillae are not externally evident. Larvae at the same stage, observed by interference contrast microscopy reveal papillae with slightly elongated cells (Fig. 2A). Immunofluorescence with anti-β-tubulin antibody shows a strong reaction localized in the papillary nerves. The cells of the papillae show only a diffuse fluorescence, probably from the few microtubles scattered in the cytoplasm and not yet organized into bundles (Fig. 2B,C), whereas the epidermal cells around the papillae are not fluorescent.

Swimming larvae

In larvae with fully developed tails (Fig. 1B) the adhesive papillae are well developed, although they are still completely covered by the tunic (Fig. 1C). The papillary cells are elongated, more than twice as high as the ectodermal ones (Fig. 3A). In sections it is possible to recognize two kinds of cells: many elongated peripheral cells surrounding a few larger and elongated ones in the center of the papilla (Fig. 3C). Peripheral cells form a columnar simple epithelium continuous with the epidermis of the trunk. Between the peripheral and the central cells there is a lumen that is an extension, inside the papilla, of the hemocoel (Fig. 3C). Anti-β-tubulin antibody im-

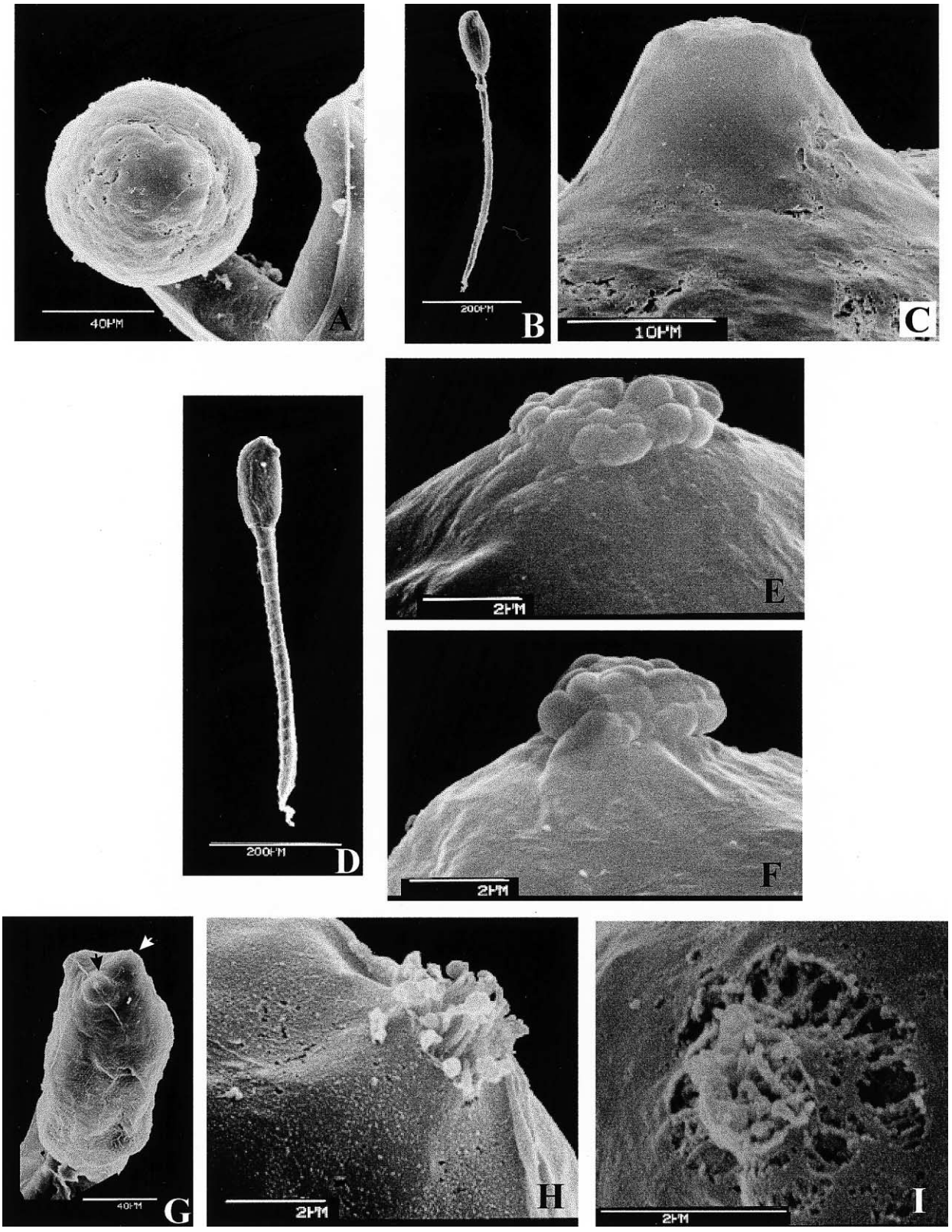

Fig. 1. SEM microscopy of *Phallusia mamillata* larvae. Newly hatched larva. (**A**) Image of the anterior region of the trunk. Swimming larva. (**B**) Image of the entire larva. (**C**) Magnification of a papilla covered by a continuous layer of tunic. Larva at the beginning of metamorphosis. (**D**) Entire larva showing the tip of the tail retracted. (**E, F**) High magnification of the region at the top of the papilla, showing bubbles in the tunic. See explanation in the text. Larva with retracted tail. (**G**) Trunk showing the dorsal papillae (arrows). (**H**) High magnification of one papilla: microvilli with bulbous terminations emerging from the tunic fenestration. (**I**) Higher magnification and frontal vision of (**H**) showing the holes around the central fenestration.

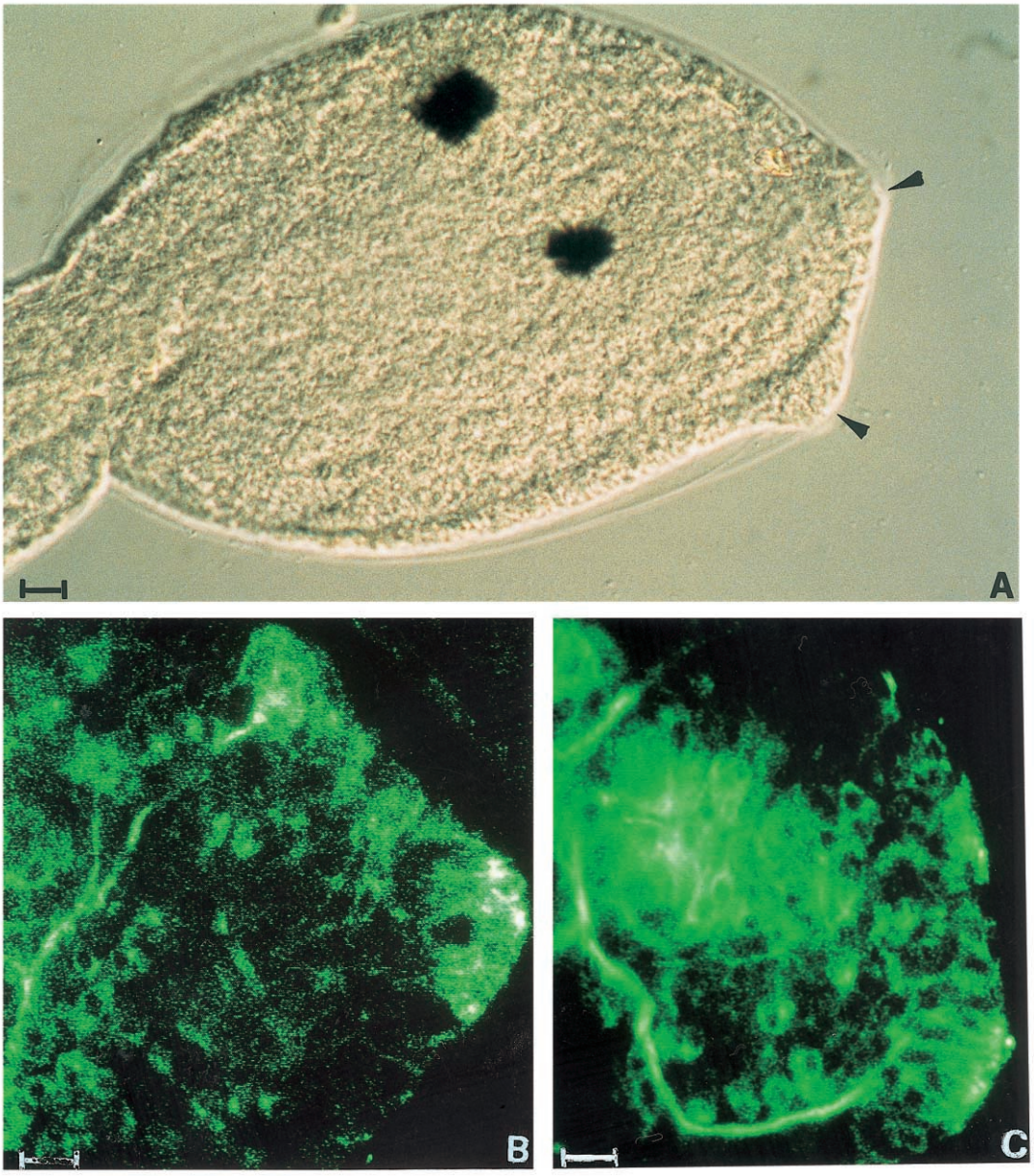

Fig. 2. Newly hatched larvae of *Phallusia mamillata*. (**A**) Interference contrast microscope image showing the adhesive papillae (arrows) whose cells are not fully elongated. (**B, C**) FITC-conjugated anti-β-tubulin antibody immunofluorescence showing diffuse fluorescence in the cells of the papillae and bright fluorescence in the papillary nerves. (**B**) Nerve of the two dorsal papillae. (**C**) Nerve of the ventral papilla. Scale bars equal 10 μm.

munofluorescence shows a bright stain in the brain, in the papillary nerves and in the papillae themselves (Fig. 3B). Two optical sections of the papillae at different levels, obtained by laser scan confocal microscopy show the same intensity of fluorescence in the peripheral and the central cells (Fig. 3D,E), but with the antibody 2-28-33, which specifically reacts with neuronal β-tubulin, fluorescence was positive in the nerves (Fig. 3F) and was also present in a prismatic area of the papilla corresponding to that of the central cells. The peripheral cells were not stained. Two contributions of the papillary nerve can be seen, one branch arising from the central part of the base of the papilla, the other coming from the base of the papilla, laterally (Fig. 3G).

Confocal microscopy was used to obtain images resulting from the superimposition of many optical sections and this allowed us to follow the course of the papillary nerve (Fig. 3B). Each papilla is connected with two thin branches to the papillary nerve, one branch connected to the central cells, the second to the peripheral cells (Fig. 3B,D,E). The two branches fuse into a single nerve that runs posteriorly and dorsally (Fig. 3B,D). The nerves of the two dorsal papillae penetrate into the trunk and fuse behind the papillae at a short distance from their bases (Fig. 3D), while the nerve of the ventral papilla fuses to the nerve arising from the other two papillae more

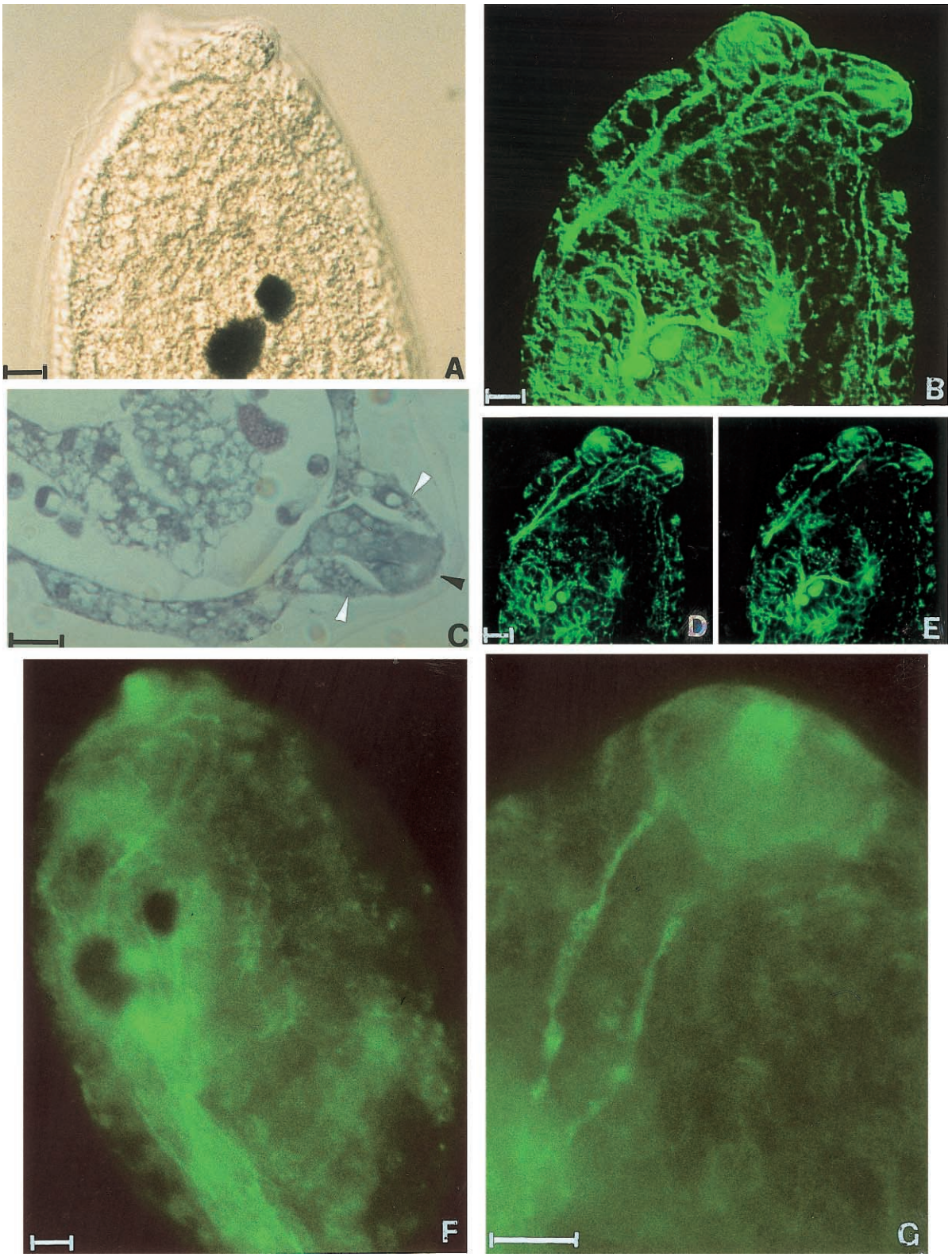

Fig. 3. Swimming *Phallusia mamillata* larva. (**A**) Interference contrast microscope image of adhesive papilla. (**B**) Swimming larva stained by FITC-conjugated anti-β-tubulin antibody, viewed by confocal microscopy, showing bright fluorescence in the cells of the papillae and in the nerves arising from the dorsal and the ventral papillae. (**C**) Longitudinal section of the papilla stained by methylene blue showing the fully elongated peripheral (white arrows) and central cells (black arrow). (**D, E**) Two optical sections of the papilla in (**C**) obtained by confocal microscopy showing fluorescence in both central (**D**) and peripheral (**E**) cells. Swimming larva, stained by FITC-conjugated anti-β-tubulin clone 2-28-33 antibody immunofluorescence, viewed by epifluorescence microscopy. (**F**) Fluorescence is evident in the papillae, in the brain and in the nerves. (**G**) Magnification of a papilla showing staining in central cells and in nerves. Scale bars equal 10 μm.

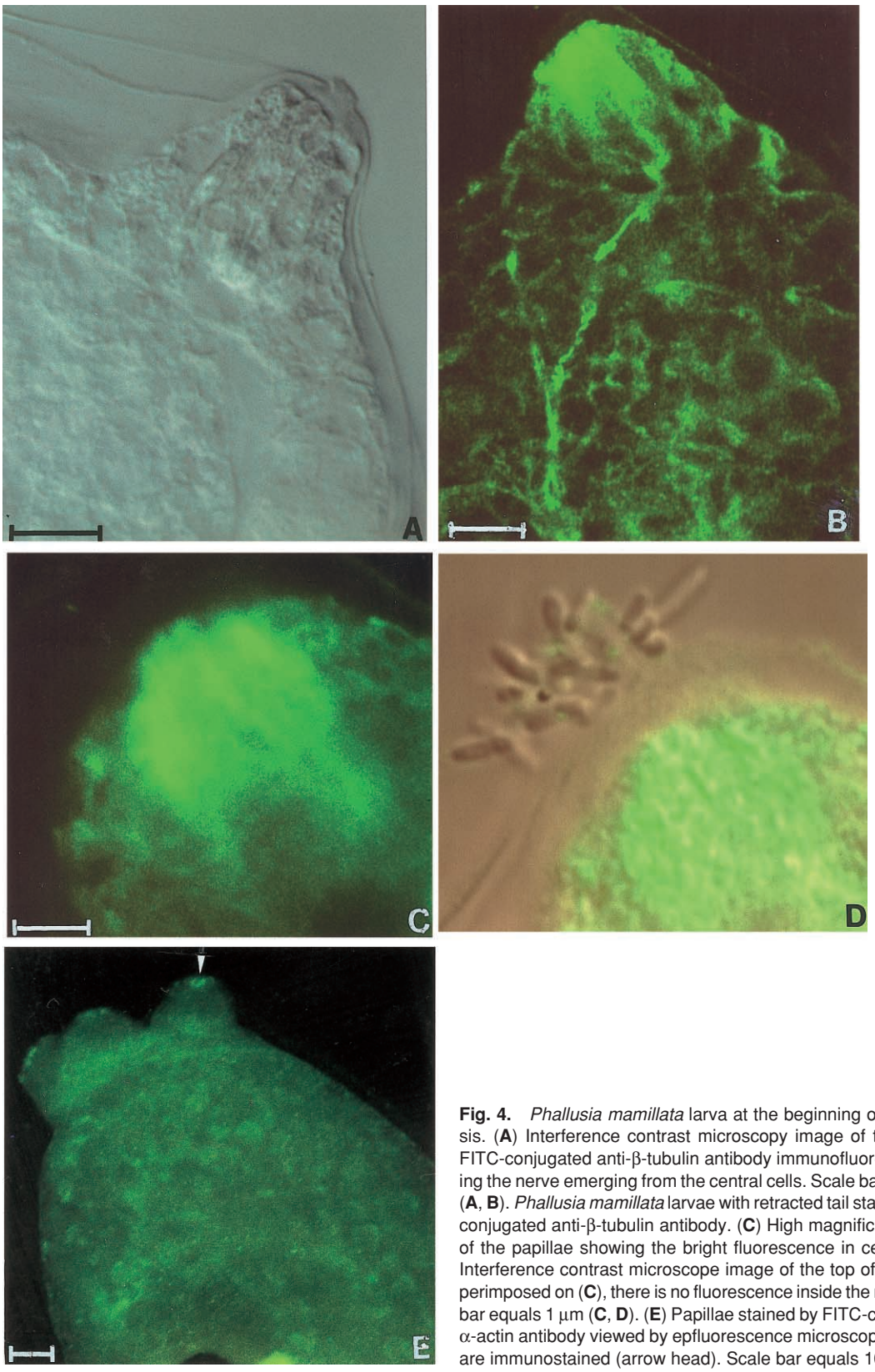

Fig. 4. *Phallusia mamillata* larva at the beginning of metamorphosis. (**A**) Interference contrast microscopy image of the papilla. (**B**) FITC-conjugated anti-β-tubulin antibody immunofluorescence showing the nerve emerging from the central cells. Scale bars equal 10 μm (**A**, **B**). *Phallusia mamillata* larvae with retracted tail stained with FITC-conjugated anti-β-tubulin antibody. (**C**) High magnification of the top of the papillae showing the bright fluorescence in central cells. (**D**) Interference contrast microscope image of the top of the papilla superimposed on (**C**), there is no fluorescence inside the microvilli. Scale bar equals 1 μm (**C**, **D**). (**E**) Papillae stained by FITC-conjugated anti-α-actin antibody viewed by epfluorescence microscopy: the microvilli are immunostained (arrow head). Scale bar equals 10 μm (**E**).

posteriorly just before the brain, after which a single nerve, the common papillary nerve, runs posteriorly and connects the three papillae to the brain, probably at the level of the ventral ganglion.

Larvae at the beginning of metamorphosis

These larvae were defined as being at the beginning of metamorphosis, when the caudal half of the tail had retracted. SEM images of these larvae show an empty tunic of the tail tip (Fig. 1D), the papillae are high, still covered by the tunic (Fig. 4A), which at the very top of the papilla gives rise to bulbous outgrowths, similar to bubbles (Fig. 1E) that appear to swell (Fig. 1F) as the tail retracts. After anti-β-tubulin antibody staining, optical sections reveal fluorescence in both the peripheral and the central cells, but the last are more brightly fluorescent, with an evident nerve connection to the brain (Fig. 4B).

Larvae with retracted tails

The larvae at this stage have tails almost completely retracted, with the tunic in the tail region empty, but the extension of the ampullae, the three organs that mark metamorphosis and by which the larvae definitively attach to the substrate, has not yet occurred. In SEM images the tunic is broken above the apex of the papillae, and many microvilli with bulbous apical terminals emerge from a central fenestration, around which there are many little holes (Fig. 1G,H,I). The anti-β-tubulin fluorescence is evident in the central cells but there is none in the peripheral cells and in the microvilli (Fig. 4C,D). At this stage too, the nerve connecting the central cells of the papillae to the brain is present, as already clearly shown by specific anti-neuronal β-tubulin antibody and by confocal microscopy (Figs. 3G, 4B). As expected, the microvilli (arrow) were fluorescent after immunostaining with monoclonal anti-α-actin antibody (Fig. 4E). By means of the images obtained we tried to draw the course of the papillary nerve (Fig. 5).

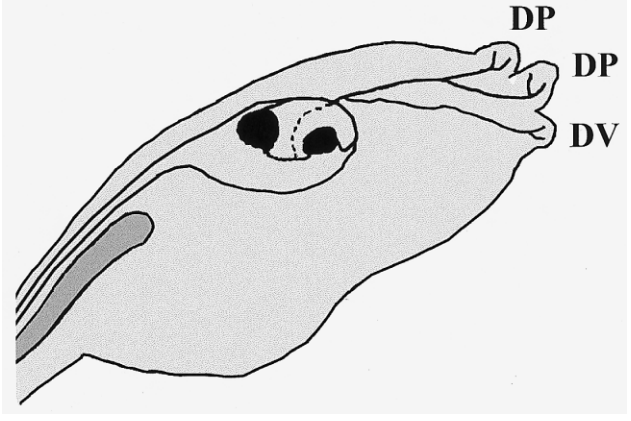

Fig. 5. Diagram showing schematic draw of the papillary nerve course in the larval trunk of *Phallusia mamillata* in dorso-lateral view. DP, dorsal papillae; DV ventral papilla. Papillary nerve is drawn solid black (the parts hidden behind the dorsal papillae and the brain are drown dash-line).

DISCUSSION

SEM and light microscopy images combined with the immunofluorescence results are useful for understanding the morphology and function of the adhesive papillae at different periods of larval life. The anti-β-tubulin immunofluorescence observations provide evidence that in the cytoplasm of the cells of the papillae there are many microtubules organized in bundles and aligned in parallel with the long axis of the cells. After hatching and during the period in which the larvae are actively swimming, the papillary cells change their shape becoming fully elongated. It is well known that microtubules oriented and organized into bundles are responsible for change of the cell shape. In the papillae, as soon as the cells begin to elongate, there is an evident increase of bundles of microtubules, as demonstrated by the intensity of the anti-β-tubulin immunofluorescence in the cytoplasm of both peripheral and central cells. The papillae of the swimming larvae are not yet sticky and are covered by a double layer of tunic, but we think that the secretory activity occurs during this period in the peripheral cells which are rich in bundles of microtubules. The antibody 2-28-33 has been used by Crowther and Whittaker (1990) to stain neurons in *Ciona* larvae. The same authors reported that staining of the peripheral nervous system was possible only in older larvae. In our experiments, the central cells of the papillae were already stained in younger larvae (newly-hatched larvae), but the axons coming from these cells, and the papillary nerves showed intense fluorescence only in older larvae (Fig. 3F,G). We never observed bright fluorescence with this antibody in the peripheral cells. This observation suggests to us that the central cells are primary sensory neurons. The presence of a nerve arising from the central cells of the papillae of the *Ascidia malaca*, a species closely related to *Phallusia mamillata*, has been demonstrated by TEM (Gianguzza *et al*., 1996). In addition, the presence of primary sensory neurons in the papillae of many ascidian species representative of the three suborders: Phlebobranchiata, Aplousobranchiata and Stolidobranchiata has been demonstrated (Torrence and Cloney, 1983).

At the beginning of tail retraction, the number of microtubules in the peripheral cells starts to decrease, in larvae with a fully retracted tail anti-β-tubulin immunofluorescence is faint (Fig. 4B). The main function of the microtubule bundles in the peripheral cells seems to be linked to glandular activity, because the microtubules were never stained by anti-neuronal β-tubulin specific antibody, which excludes any correlation with sensory activity. The papillae of the larvae at this stage probably secrete and accumulate adhesives that will be used afterwards at settlement. In *Ascidia malaca*, TEM studies showed a mass of finely granular material, probably adhesives, secreted by peripheral cells and accumulated in the hyaline cap, which is an expansion of the larval tunic above the papilla (Gianguzza and Dolcemascolo, 1994, 1997). The adhesive secretion of the papillae in *Clavelina lepadiformis* larvae also seems to occur in the same way (Turon,1991).

In swimming larvae of *Phallusia mamillata*, the periph-

eral cells of the papillae have a nerve connection to the brain by means of a branch of the papillary nerve, we may hypothesize that this branch of the papillary nerve may act as regulator of the secretory activity at the onset of metamorphosis. Crisp and Ghobashy (1971) reported that the larvae of *Diplosoma listerianum* are positively phototactic as soon as they emerge from the parent family, but just before settlement they avoid light and prefer to settle in the dark; larvae of *Diplosoma macdonaldi* behave similarly (Cloney, 1982). The same author (Cloney, 1978) observed that in the last species settlement can occur immediately after the first adhesive papillary contact with a settlement-inducing substrate. In this species, the papillae have cells recognized as primary sensory neurons that bear microvilli. These cells are assumed to be chemoreceptors that are stimulated by contact with the substrate (Cloney and Torrence, 1984).

With anti-α-actin immunofluorescence we were able to recognize the microvilli at the apex of the central cells of the swimming larva, long before the moment of settlement, when they were still covered by the double layer of the tunic. The papillae remain completely covered by the tunic until the beginning of settlement, when the tail starts to retract. At this moment, the tunic above the papillae swells, giving raise to the bubbles shown by SEM, then breaks and microvilli appear on the surface. The breaking may be due to chemical digestion of the tunic, by substances produced and stored in the hyaline cap. We could hypothesize that the substances accumulated in the hyaline cap of *Ascidia malaca* are not only adhesive (Gianguzza and Dolcemascolo, 1994, 1997) but also substances with enzymatic activity able to digest the tunic.

The behaviour of the larvae changes at this moment, they stop swimming around horizontally, and start to swim towards the bottom of the glass vessel, and when the tips of their papillae contact with the glass, tail movements cease immediately and many larvae settle if they are not disturbed. When the bottom of the glass vessel is crowded with larvae, the larvae ready to settle change their direction and move up, attaching themselves by the papillae to the air-water interface. In laboratory conditions, *Phallusia mamillata* larvae seem to prefer a glass substrate, since they avoid settling on pieces of plastic or on pieces of conspecific adult tunic or on small stones. Experiments carried out with various substrates indicate that larvae are able to select the more suitable one, but the inducing-settlement substrate may be artificial or different from the natural one (Cloney, 1978; Svane and Young, 1989; Railkin and Dysina, 1997).

The morphology of proximal portion of the papillary nerve we found in *Phallusia mamillata* is similar to that described by Torrence and Cloney (1983) for the larvae of *Diplosoma macdonaldi*. The distal portion differs in the two species for the different position of the papillae (aligned in sagittal plane in *D. macdonaldi*, disposed at the vertices of a triangular field in *P. mamillata*) and for the different morphology of the papillae (scyphate and everting in *D. macdonaldi*, conic and non-everting in *P. mamillata*).

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