Journal of
Molluscan Studies

The Malacological Society of London

Journal of Molluscan Studies (2017) **83**: 422–433. doi:10.1093/mollus/eyx030
Advance Access publication date: 18 July 2017

Distribution and morphology of defensive acid-secreting glands in Nudipleura (Gastropoda: Heterobranchia), with an emphasis on Pleurobranchomorpha

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(Received 10 January 2017; editorial decision 6 June 2017)

ABSTRACT

Secretion of acidic substances with defence and repellent properties is known in several metazoan taxa, including Gastropoda. Here we investigate and compare defensive acid-secreting cell types of various genera within the heterobranch taxon Pleurobranchomorpha by analysing the sizes and distribution of the secretory epithelial cells and subepithelial glands of the epidermis. Additionally, we investigate the median buccal gland (MBG), which is only known from pleurobranchs and one nudibranch species, *Plocamopherus ceylonicus*. The present data indicate a high similarity among the epidermal acid glands (EAGs), which consist of highly elongate cells containing a large vacuole with nonstaining contents. When acid-gland cells are concentrated into larger subepidermal acid glands (SAGs), the cells are of cuboidal or globular form, again containing a large nonstaining vacuole. This is also the case for the internal MBGs, although here the epithelial cells are considerably larger. In the latter, overall cell size seems to be related to body size, because specimens of similar size possess acid cells of equal size, whereas larger specimens (e.g. adult *Bathyberthella*) exhibit much larger cells. In contrast to SAGs, in which cells are often fused, the cells in MBGs are always distinct and fusion was hardly observed. Preliminary results indicate a uniform distribution of EAGs all over the body, whereas SAGs (only present in *Berthellina* spp.) are more densely distributed along the lateral sides than along the mid-part of the notum. The evolution of acid-gland types within Pleurobranchomorpha is discussed. The MBG has probably evolved twice in Heterobranchia, once within the Pleurobranchomorpha and independently in *P. ceylonicus*, a member of the nudibranch Euctenidiacea.

INTRODUCTION

Secretion of acidic substances as a repellent or defensive strategy has been documented in a wide range of metazoans, including polycladid flatworms (Thompson, 1965), echinoderms (Fontaine, 1964; Rideout & Sutherland, 1981), sponges (Yonge, 1964), polychaetes (Dorsett, 1961), tunicates (Henze, 1911; Goodbody, 1975) and even from some marine algae (*Desmarestia*; Thompson, 1988; Pelletreau & Muller-Parker, 2002; Molis *et al.*, 2009).

According to Thompson (1986), the ability to secrete acids from epidermal cells has arisen at least three times within Gastropoda, although the currently known taxonomic distribution suggests this is an underestimate. Within Caenogastropoda it is known in the cypraeoidean families Cypraeidae and Triviidae, as well as in the related velutinoidean family Velutinidae (Thompson, 1960a, b, 1961, 1969; Edmunds, 1968; Kniffen, 1968). Within Heterobranchia, it probably occurs in all members of the taxon Pleurobranchomorpha (Marbach &Tsurnamal, 1973; Thompson & Colman, 1984; Thompson, 1988; Gillette, Saeki & Huang, 1991), in some members of the nudibranch taxon Euctenidiacea (Edmunds, 1968; Thompson, 1969) and in some cephalaspids of the genus *Philine* (Thompson, 1983). The histology, anatomy and distribution of acid glands in Pleurobranchomorpha, as well as the chemical composition of their secretions, have been analysed in various studies. Garstang (1890) and Schulz (1905) first mentioned sulphuric acid in the mucus of these slugs, as was later confirmed by Thompson & Slinn (1959) and the subsequent analyses of Thompson (1961, 1983, 1986). Additionally, high chloride content within the epidermal cells indicated the presence of hydrochloric acid (Thompson, 1983, 1986), contributing to the acidic pH of 1 to 2 on the epidermis of the slugs. Traces of organic acids were mentioned by Marbach & Tsurnamal (1973) and studied in more detail by Moustafa, Wägele & El Behairi (2014), who found a high amount of taurine in the subepidermal cells of *Berthellina citrina*.

Acid glands, as described in the literature, can be classified into three types. Glandular epithelial cells in the epidermal monolayer are termed epidermal acid glands (EAGs). Agglomerations of glandular cells in the subepidermal layers, and of ectodermal origin, are called subepidermal acid glands (SAGs). Both EAGs and SAGs secrete directly into the external environment. Additionally, epithelial acid-secreting cells can be arranged in tubules lying in the body cavity, forming a gland that discharges via the anterior oral tube; this type is called a median buccal gland (MBG) (Thompson, 1988; Wägele, Ballesteros & Avila, 2006). The

epithelial cells of all three types are characterized by large, non-staining vacuoles. It is usually inferred that cells of similar appearance in other gastropods also produce acids and have the same defensive function (Edmunds, 1968; Wägele & Klussmann-Kolb, 2005; Wägele *et al.*, 2006).

The mechanism of acid discharge varies according to the glandular type (Thompson, 1988). In the single cells of EAGs the vacuoles discharge by means of holocrine cell bursting. In multicellular SAGs the cells are discharged through acid pores by muscular contraction or discharge passively after the trauma of a predator's attack. In the MBG, discharge is triggered by contraction of a muscular sheath, without tissue damage (Thompson, 1988).

The secretion of high amounts of acid requires energy and is potentially a danger to the animals themselves. Therefore, the acidic secretion must function very effectively as a potent allomone against predators. This assumption is supported by the results of aquarium experiments. Acid-secreting gastropods were invariably rejected as food when offered to a variety of fish, crustaceans, cephalopods and even anemones (e.g. Bateson, 1890; Thompson & Slinn, 1959; Thompson, 1960a, b, 1988; Marbach & Tsurnamal, 1973; Gillette *et al.*, 1991). It has been suggested that external acid secretions might also act as antifouling agents against epibionts (Thompson, 1988).

Acid production within MBGs connected with the oral tube of the digestive system is unusual and only known from members of the Pleurobranchomorpha and a single species of the nudibranch group Euctenidiacea, *Plocamopherus ceylonicus* (Kelaart, 1858). The purpose of this organ, sometimes occupying large areas of the visceral body, is not understood, but a defensive role has been assumed (Thompson, 1988; Wägele *et al.*, 2006).

Since the extensive studies on acid glands by Thompson in the 1980s, only a short summary has been presented (Wägele *et al.*, 2006), discussing these structures in comparison with other glandular structures that have been described or mentioned in relation to defence in marine heterobranchs. Our aim in this study is to make quantitative comparisons of the acid-producing glandular cells and whole glands, by recording their size, shape, location and distribution in the various nudipleuran groups known to possess them. This will help to identify morphological constraints and/or ecological adaptations. We investigated members of the genera *Pleurobranchus*, *Berthella*, *Berthellina*, *Bathyberthella* and *Tomthompsonia* (Nudipleura: Pleurobranchomorpha: Pleurobranchidae), as well as *P. ceylonicus* (Nudipleura: Nudibranchia: Euctenidiacea: Polyceridae), in detail. Additionally, *Berthellina edwardsi* from the Mediterranean Sea and *B. citrina* from the Red Sea were selected to compare two congeneric pleurobranchomorph species known to possess SAGs. We also gathered literature data and mapped the combined results on a published phylogeny of Pleurobranchomorpha to reveal evolutionary trends. The evolution of chemical defence strategies has been of particular significance to heterobranchs, which in many cases have internalized the protective shell or lost it completely.

MATERIAL AND METHODS

Table 1 summarizes specimen data. Specimens were preserved in 5–10% formaldehyde in seawater for several days or weeks and subsequently stored in 70% ethanol until final processing. Specimens were dehydrated in ascending ethanol series and embedded in Methacrylate (Technovit®7100, Heraeus Kulzer). Serial sections (2.5 µm) were stained with toluidine blue and investigated under a Zeiss Axio Imager Z2M with an AxioCam camera for digital imaging. Using the provided Zeiss ZEN software, morphometric measurements were made of the three different types of acid glands present in the material, as described below.

Morphometric measurements of three-dimensional irregular structures in a two-dimensional section can be biased according to preservation methods and selection of the regions of interest. Therefore, we used standard fixation and histological techniques, and similar regions of the body. All cells were measured, irrespective of size, in the selected areas, with no preference for large cells.

EAGs: Height and width of 60–80 randomly selected cells were measured in sections from each of frontal, mid and posterior area of the dorsal notum, thus measuring around 200 cells throughout the body. This was not possible for *Bathyberthella antarctica* and *Tomthompsonia antarctica* due to fragmentary conservation of the epidermis.

SAGs: These were only present in *Berthellina* species, represented by one specimen each of *B. citrina* and *B. edwardsi*. To analyse their frequency and distribution, the total area of SAGs was measured in a range of histological sections from near the head region to the posterior part of each animal. The total number of slides for these two species was 143 and 170, respectively, with 3–6 sections per slide; one section on every 5th slide (*B. citrina*) and on every 6th to 10th slide (*B. edwardsi*) was measured, giving 23 and 19 sections, respectively. The notum was divided into a lateral and middle part and occurrence of SAGs was compared in both, as the relative area of the cross section of the mantle occupied by the glands. A comparison of the size of the cells (as was intended in the first place) was not possible, because membranes between cells were often not visible and vacuoles with acidic contents were fused into larger areas.

MBGs: Five pleurobranchids (*Berthellina citrina*, *B. edwardsi*, *Pleurobranchus albiguttatus*, *B. antarctica*, *Berthella stellata*) and one nudibranch (*Plocamopherus ceylonicus*) were investigated by measuring the areas of the individual acid cells in sections. For each specimen between 150 and 200 cells were analysed.

Additional specimens were investigated for comparative purposes, but not measured in detail (see Table 1).

Table 1. List of heterobranch species and specimens investigated in this study.

Taxon	Locality and date	Number of specimens
Pleurobranchomorpha: Pleurobranchidae		
Berthella stellata (Risso, 1826)	Magnetic Island, Australia. 1999	1
Berthellina citrina (Rüppell & Leuckart, 1828)	Al-Quseir and Safaga, Red Sea, Egypt. 2012–2014	3
Berthellina edwardsi (Vayssière, 1896)	Rosas, Mediterranean Sea, Spain. 1998	3
Pleurobranchus albiguttatus (Bergh, 1905)	Safaga, Red Sea, Egypt. 2014	1
Bathyberthella antarctica Willan & Bertsch, 1987	Gould Bay, Antarctica. 1985	1 adult
B. antarctica	Signy Island, Antarctica. 1985	1 juvenile
Tomthompsonia antarctica (Thiele, 1912)	Weddell Sea, Antarctica. 1985–1990.	2
Nudibranchia: Euctenidiacea: Polyceridae		
Plocamopherus ceylonicus (Kelaart, 1858)	Rowes Bay, Townsville, Australia. 1999	1

RESULTS

Epidermal acid glands

EAG cells of similar shape were found on the dorsal notum surface of all examined pleurobranchid genera (*Berthellina*, *Berthella*, *Pleurobranchus*, *Bathyberthella* and *Tomthompsonia*; Table 1; Fig. 1). EAGs were apparently absent from the foot, tentacles and gills. Results for *Bathyberthella* (Fig. 1F) and *Tomthompsonia* (Fig. 1D) were not clear for all body parts because part of the epidermis was missing in available specimens. Therefore, these two taxa were not included in the subsequent morphometric analyses. *Plocamopherus ceylonicus* lacked EAGs in the notum epithelium (Table 1).

The average width of the EAGs (Fig. 2) was similar in *Berthellina citrina*, *Berthellina edwardsi*, *Berthella stellata* and *Pleurobranchus albiguttatus*. With regard to height, *P. albiguttatus* had more elongated columnar cells with an average height of 52 µm, compared with the other species with values around 30 µm. There were no differences in height and width of EAG cells in the front, mid and the hind areas of the body in these three species.

Subepidermal acid glands

Of the investigated species, only *B. citrina* and *B. edwardsi* exhibited SAGs in the notum (Fig. 3). These glands are multicellular sac-like structures and occur at higher density in the lateral parts compared with the dorsal parts of the notum (Fig. 4). Some of the SAGs could be seen to open to the exterior, the epithelium of the glands merging with the epidermal layer to form a pore (Fig. 3C, E). Ciliated cells were not detected interspersed with the gland cells. The vacuoles of the SAG cells usually coalesced into larger areas and individual cells were difficult to demarcate. Therefore measurements were made of the areas of whole glands in the sections. The acid glands of *B. citrina* were smaller (mean area 5.8 µm^2) and situated further apart than those of *B. edwardsi* (mean area 70 µm^2). In both species muscle fibres surrounded the SAGs and the muscle

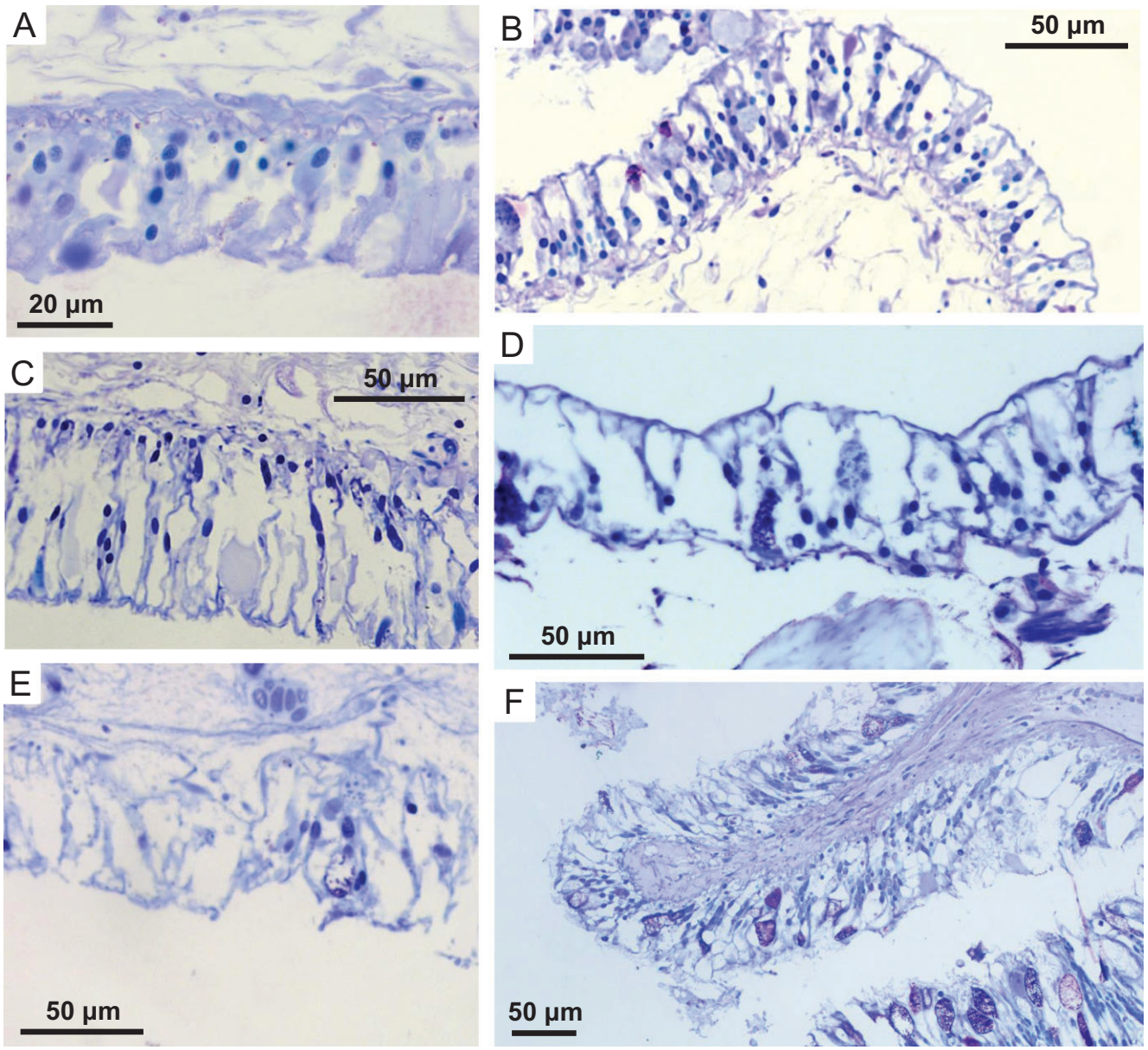

Figure 1. Histology of epidermal acid glands (EAGs) of Pleurobranchidae. **A.** *Berthellina citrina*. **B.** *Berthellina edwardsi*. **C.** *Pleurobranchus albiguttatus*. **D.** *Tomthompsonia antarctica*. **E.** *Berthella stellata*. **F.** *Bathyberthella antarctica* adult.

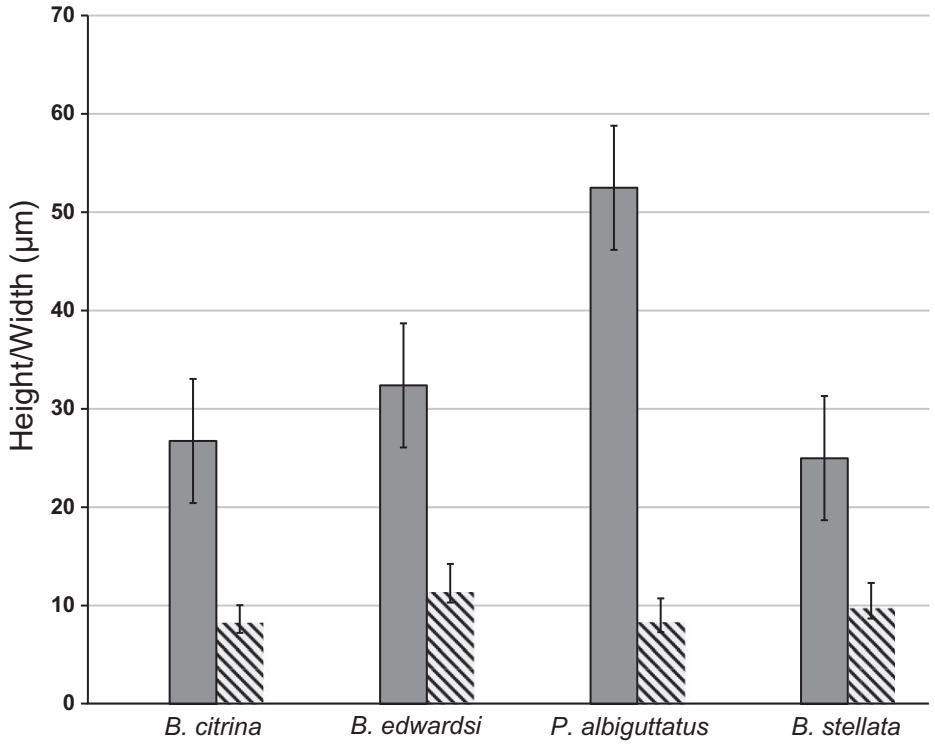

Figure 2. Comparison of the average height (filled bars) and width (hatched bars) of the epidermal acid glands (EAGs) of *Berthellina citrina*, *Berthellina edwardsi*, *Pleurobranchus albiguttatus* and *Berthella stellata*. Vertical bars are standard deviation.

layer appeared thicker when the content of the glands was partly extruded (Fig. 3E, F). These results were confirmed by examining a few sections of two other specimens from each species. In the measured *B. citrina* specimen, a 'spongy' appearance of the cytoplasm at the base of the gland cells suggested the formation of new vacuoles (Fig. 3B).

To quantify the distribution of SAGs, the lateral and dorsal part of the notum (defined by a kink in the mantle profile, Fig. 4 inset) were analysed separately. The average percentage area of SAGs in the notum sections of *B. edwardsi* was 46% (but cf. 28% in five sections of another specimen) and thus higher than in *B. citrina* with 11%. In both species SAG area was higher in the lateral notum than in the mid-part (Fig. 4). This contrast was more evident in *B. citrina* (14% in the lateral part *vs* 8% in the dorsal part) than in *B. edwardsi* (49% *vs* 41%, respectively).

Median buccal gland

A MBG was found in all five examined pleurobranchid species, opening between the jaws at the transition from oral tube into pharynx. In *B. citrina* and *B. edwardsi* this gland ramified among the viscera, occupying a large part of the anterior body cavity and foot. In *Bathyberthella antarctica*, *B. stellata* and *P. albiguttatus* the gland was of simple (not ramifying) tubular form. Whereas the MBG of *B. antarctica* was as extensive as in the two *Berthellina* species, the gland of *B. stellata* and *P. albiguttatus* was smaller than in all other investigated pleurobranchids. The MBG of the polycerid *P. ceylonicus* was similar in distribution and shape to that of the *Berthellina* species, i.e. ramifying among the viscera as far as the posterior part of the body, although (in contrast to both *Berthellina* species) it did not penetrate into the foot.

The MBG cells of all six species were arranged in a single layer around the central lumen of the tubule and surrounding each tubule was a thin layer of muscle fibres (or single fibres) (Fig. 5B–D, G). The cytoplasm of the MBG cells contained clear, nonstaining vacuoles, sometimes coalescing to form a single vacuole (Fig. 5). In contrast to the similar SAG cells of *Berthellina*, MBG cells were interspersed with ciliated cells that had smaller nuclei than the MBG cells and lay adjacent to the lumen (Fig. 5C, H). The basal cytoplasm of the MBG cells often appeared spongy and contained the cell nucleus (Fig. 5B, G). In *P. albiguttatus*, cross sections of the MBG were seldom of this typical circular shape and the gland cells were more elongate and irregularly shaped (Fig. 5D). The histology of *P. ceylonicus* was similar to that of the five pleurobranchid species (Fig. 5I).

The areas of the MBG cells in the sections of all six species are shown in Figure 6. Since *B. antarctica* adults are much larger in size than the others, a juvenile of similar size to the other species was included for comparison. The cells were far larger in *B. antarctica* than in the others, up to 130 μm^2 in cross section, with a median size of 14 μm^2. Even the juvenile *B. antarctica* showed larger cells than the other species. Median values for the other pleurobranchid species were around 2 μm^2 and that of *P. ceylonicus* 3 μm^2. The high variance of the measurements indicated in Figure 6 is likely because we measured all cells visible in cross section, with no preference for large cells.

Distribution of acid glands in nudipleuran heterobranchs

Table 2 summarizes the known occurrence and location within the body of the three types of acid glands in heterobranch species.

DISCUSSION

Epidermal acid glands

We confirm here the presence of EAGs in several members of the Pleurobranchidae and describe them for the first time in *Berthellina edwardsi*, *Pleurobranchus albiguttatus*, *Berthella stellata*, *Bathyberthella antarctica* and *Tomthompsonia antarctica* (Table 2).

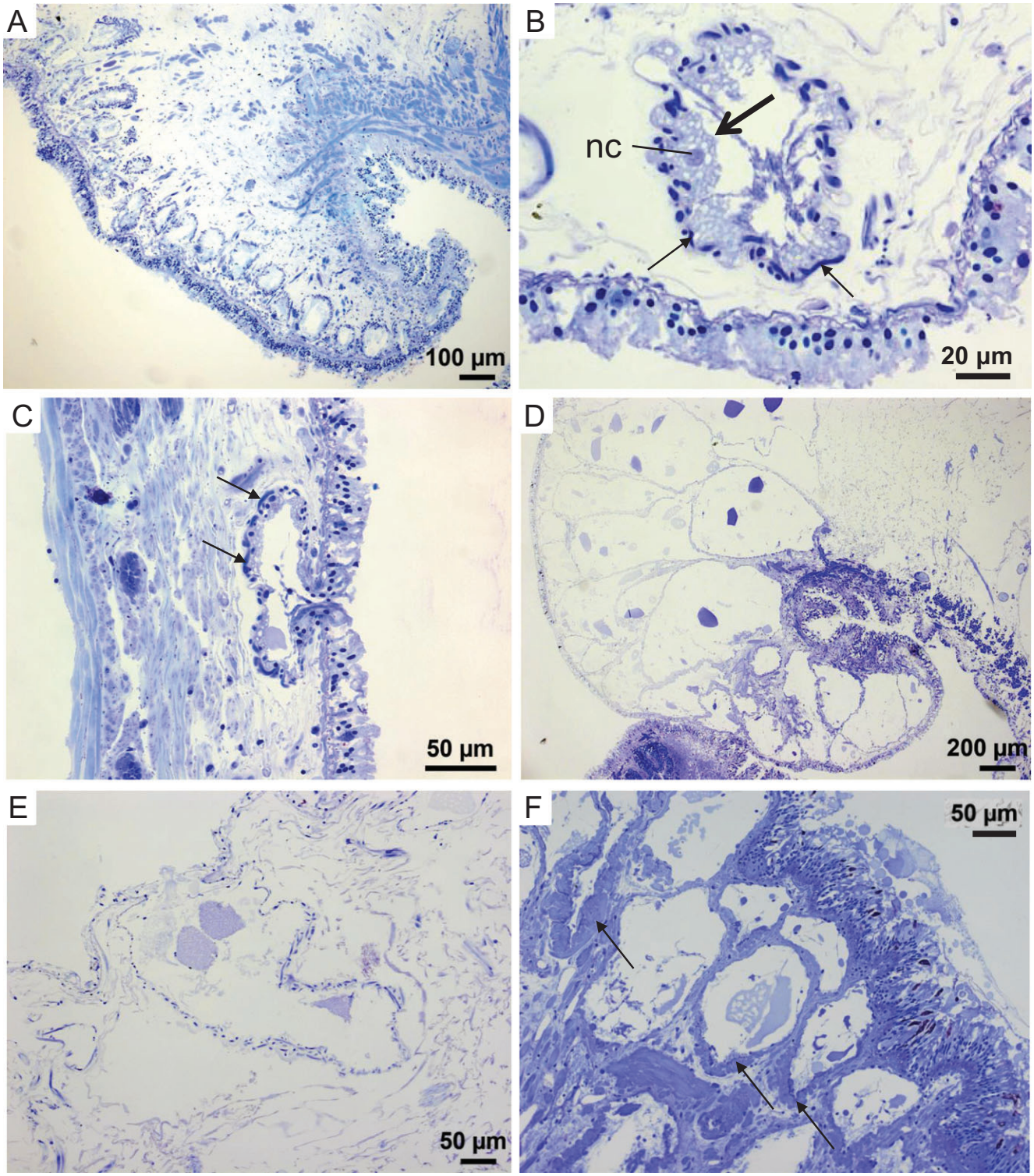

Figure 3. Histology of subepidermal acid glands (SAGs) in the genus *Berthellina*. **A–C.** *B. citrina*. **D–F.** *B. edwardsi*. Thick arrow indicates 'spongy' cytoplasm; thin arrows indicate muscle fibres and muscles layers (darker staining). Abbreviations: nc, nucleus of SAG cell.

The investigated species exhibited an epidermis of similar appearance and were all provided with epithelial acid cells of columnar shape with large clear vacuoles. These vacuoles have been identified as the storage location for acids (Thompson, 1983, 1986; Thompson & Gathercole, 1986). *Pleurobranchus albiguttatus* exhibited the largest EAG cells. Lacking even an internal shell, as well as SAGs, and with only a small MBG, we suggest that this species relies more on the EAGs for defence than do the other investigated pleurobranchids. The shell-less nudibranch *Plocamopherus ceylonicus* has neither EAGs nor SAGs; however, several other glandular structures along the mantle probably provides the necessary protection (Wägele *et al.*, 2006).

Thompson (1988) showed that the discharge of acid from EAGs is triggered by the direct attack of a predator. This is consistent

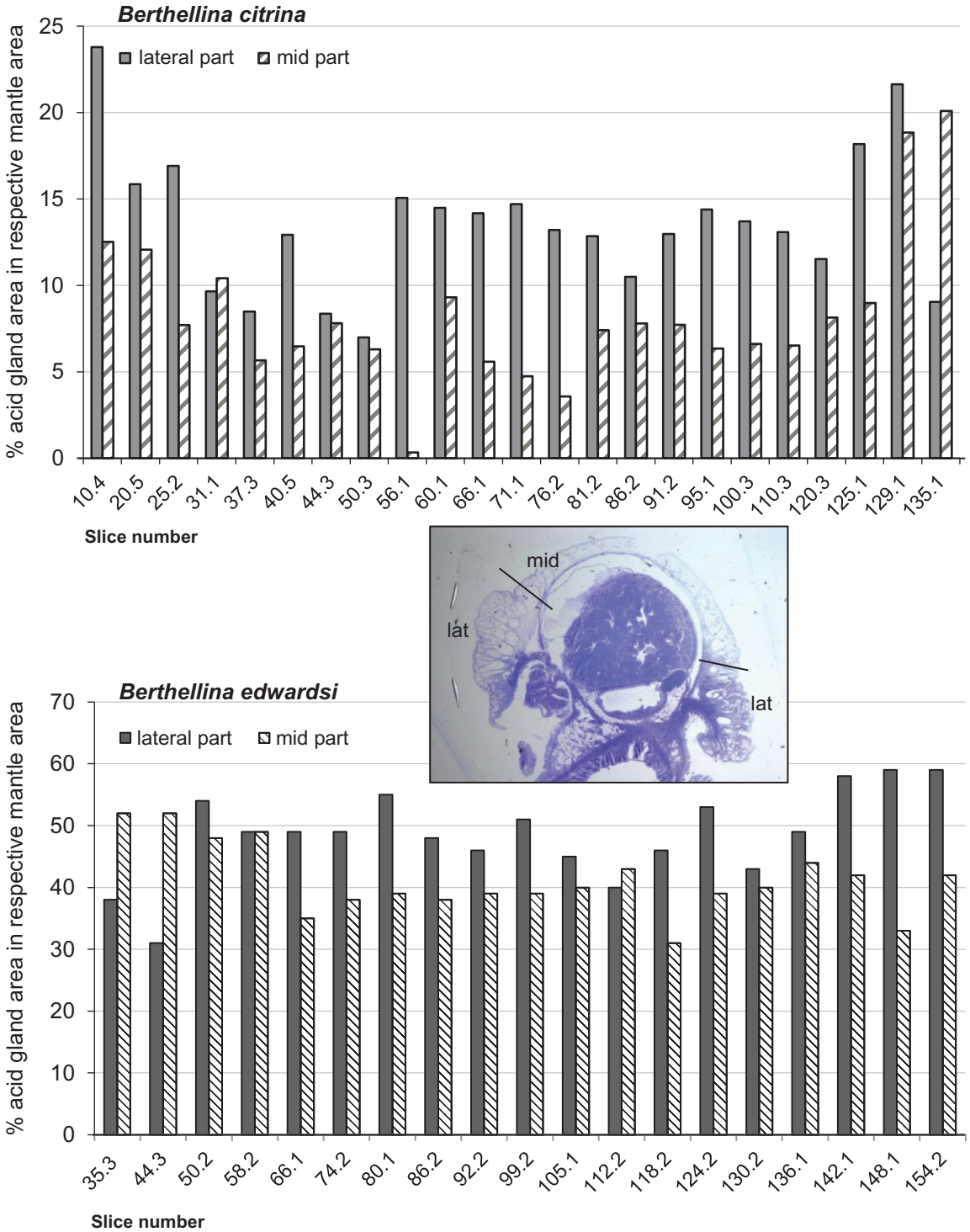

Figure 4. Proportion (by area, in cross section of mantle) of subepidermal acid glands (SAGs) in the lateral and mid-part of the notum of *Berthellina citrina* and *Berthellina edwardsi* (see inset). Section numbers start in head region (10.4 and 35.3, respectively) and end in region above tail (135.1 and 154.2, respectively). Insert shows cross section of *B. edwardsi* with distinction of lateral (lat) and dorsal mid-part (mid) of notum.

with the observations by Marbach & Tsurnamal (1973) on *Berthellina citrina* specimens, which secreted more acid when stimulated. The release of the acid fluid is probably achieved by holocrine cell bursting, since no underlying muscle layers can be seen in histological sections (e.g. Fig. 1D).

No change in the size of EAG cells with regard to body location was observed in the investigated species. Therefore, we assume that an all-over protection is of advantage against predators and parasites that might attack from all sides. Marbach & Tsurnamal (1973) mentioned a much lower number of EAG cells in the gill and rhinophore epidermis of *B. citrina*, despite the observation of acid exudation on these organs in live animals. We confirm their results, since all investigated pleurobranchids exhibited few acid-gland cells on these structures.

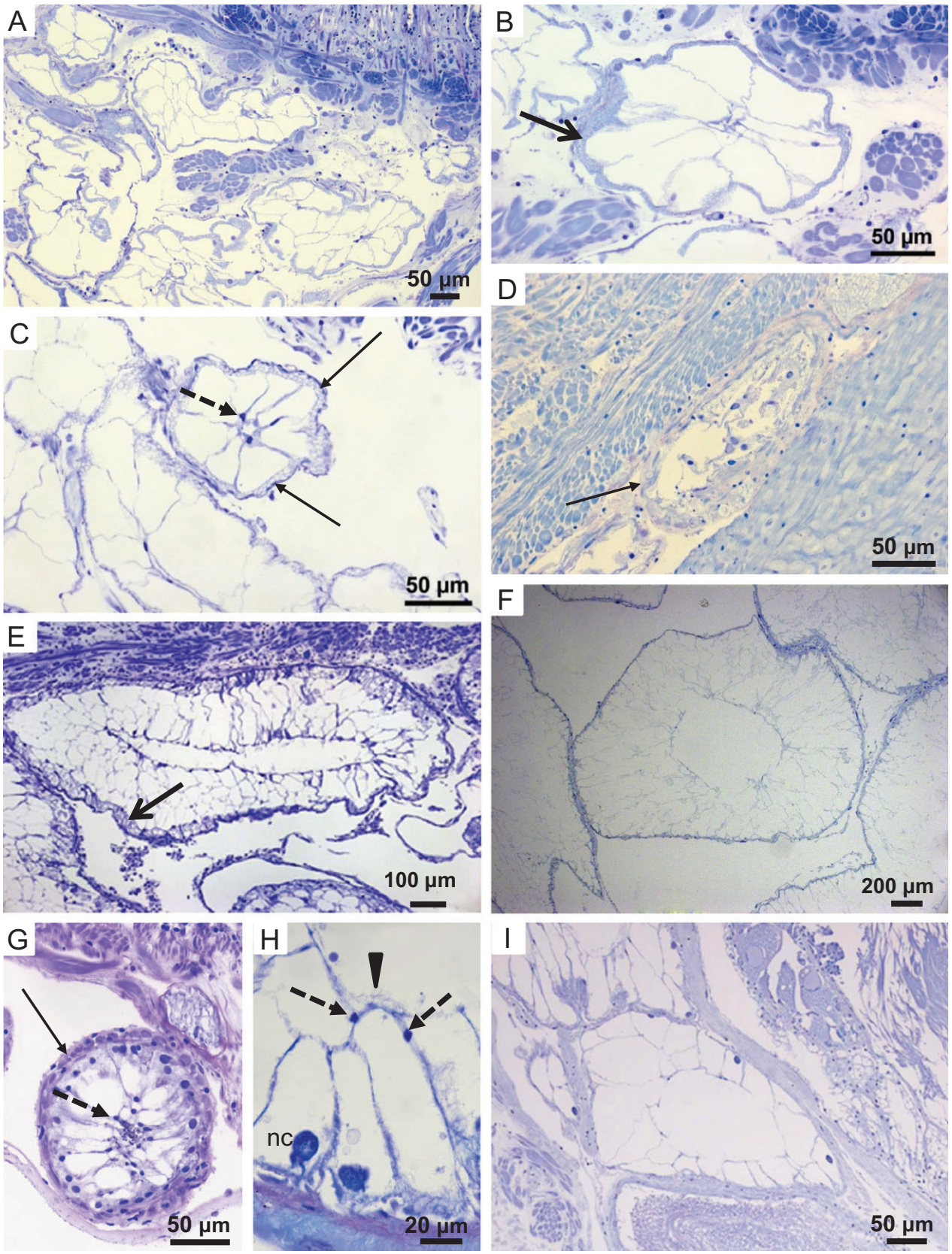

Figure 5. Median buccal glands (MBGs). **A, B.** *Berthellina citrina*. **C.** *Berthellina edwardsi*. **D.** *Pleurobranchus albiguttatus*. **E.** *Bathyberthella antarctica* juvenile. **F.** *B. antarctica* adult. **G.** *Berthella stellata*. **H, I.** *Plocamopherus ceylonicus*. Thick arrow indicates 'spongy' cytoplasm; thick arrowhead indicates cilia originating from thin ciliated cells lying between acid-containing cells; dashed arrows indicate nuclei of ciliated cells; thin arrows indicate muscle fibres/layer. Abbreviation: nc, nucleus of acid-gland cell.

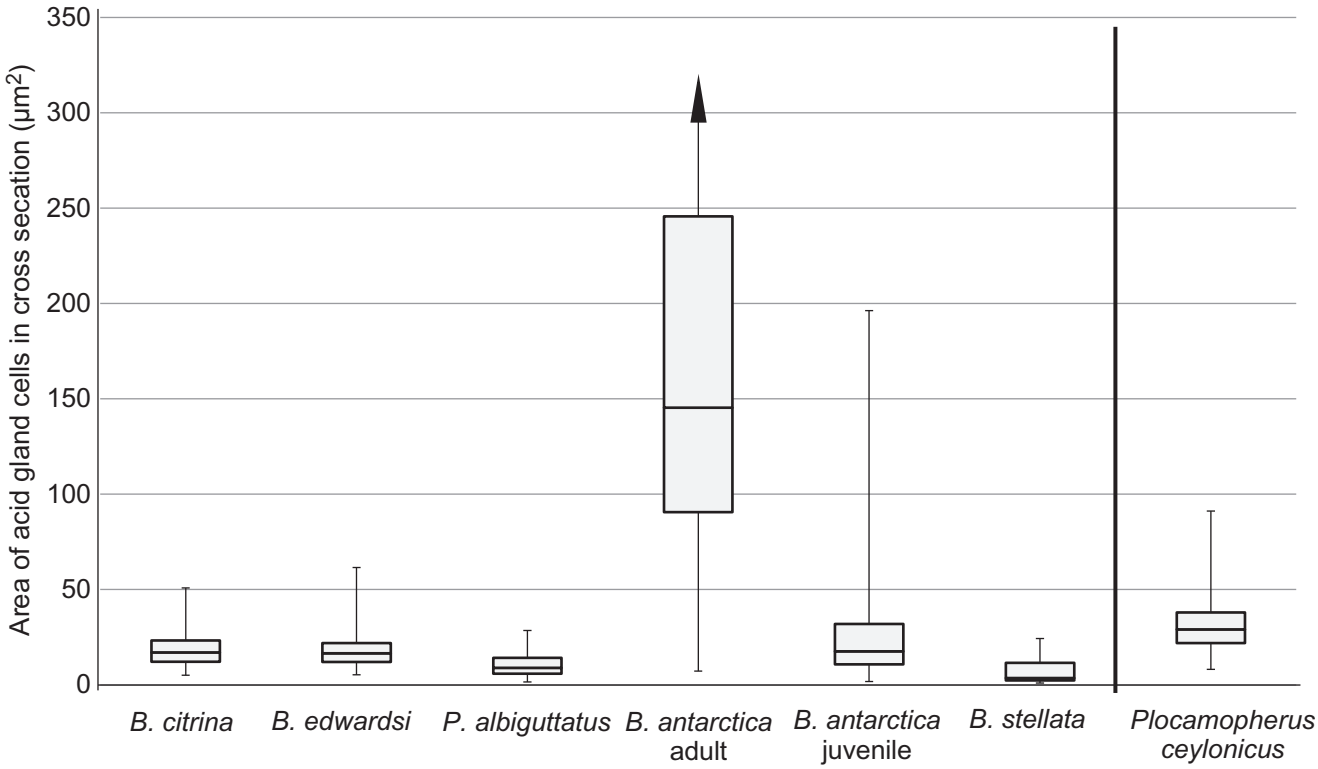

Figure 6. Sizes (areas in cross section) of single cells of median buccal glands (MBGs). For each species, interquartile range (25–75%) is shown by box, extreme values by whiskers and line within box is the median. Maximum value of *Bathyberthella antarctica* was 130 μm^2 (not shown). *Plocamopherus ceylonicus* is separated by a vertical line, indicating that it is the only non-pleurobranchid species.

It has been speculated that settlement of bacteria and protists might be discouraged by mucus (Davies & Hawkins, 1998), which is produced in the epidermal mucus cells and forms a natural biofilm (Wahl *et al.*, 2012), although these cells are rather few in numbers in pleurobranchids compared with many other marine heterobranchs. We speculate that a continuous release of small amounts of acid, without a special release event, might also aid in antifouling, but this requires investigation.

Subepidermal acid glands

Here we confirmed the absence of SAGs for additional *Pleurobranchus* and *Berthella* species, as well as *Bathyberthella* and *Tomthompsonia* (Table 2). These findings mean that the documented occurrence of SAGs within Pleurobranchomorpha is restricted to the genus *Berthellina*. The multicellular glands presumably originated as invaginations of the outer epidermis, which then evolved into larger structures with an enlarged acid-producing area and thus providing a more effective defence. Thompson's (1988) assumption that discharge of acid is actively triggered by muscular contraction seems likely for the two investigated *Berthellina* species, which clearly show a muscular layer around the glands and a surface pore (Fig. 3F). Lack of any ciliated cells in the glands also supports a more active means of secretion than a continuous transport to the surface.

The glandular area in cross sections was observed to be much larger in *B. edwardsi* than in *B. citrina*. Thompson & Slinn (1959) mentioned that repeated stimulation of a small area of the mantle leads to local exhaustion of the capacity to secrete acid. This might result in a decrease of the overall size of the glands and a condensation of the muscle fibres—exactly the conditions observed here in the *B. citrina* specimen, which we therefore suggest might have been stimulated to release acid before fixation.

Our results on the distribution of SAGs throughout the body show a higher presence on the lateral parts of the body compared with the dorsal part. This suggests that a predation is mainly from the benthic community rather than from predators in the water column. Pleurobranchids are nocturnal (Marbach & Tsurnamal, 1973; personal observations) and hide under stones or crevices during daytime. Marbach & Tsurnamal (1973) showed in experiments with predatory fish and crabs the rejection of pleurobranchids as food due their acidic contents. Encounters with fish predators are less likely during the night, but this is not the case for crustaceans, especially larger brachyurans, which can be seen much more often during the night. Furthermore, crabs do not attack from the water column. Thus, we suggest that the lateral sides of the slugs are more prone to attacks and the increase of defensive glands in the lateral notum might be an adaptation to their nocturnal lifestyle and to brachyuran predation. *Pleurobranchaea* species are sometimes voracious hunters on other pleurobranchid species or nudibranchs, thus representing another putative benthic nocturnal predator against which pleurobranchids could be protected (Willan, 1984). Gillette *et al.* (1991) experimented with *Pleurobranchaea californica* and showed its aversion to low pH values.

Median buccal gland

Rudman (1972) described an elongate tubular gland that emerges from the posterior dorsal end of the oral tube in the heterobranch genera *Micromelo*, *Hydatina* and *Bullina* (Acteonoidea). Histological investigations of these three taxa (HW, unpublished data) have shown that the gland is lined by a high columnar epithelium with cells secreting neutral or acidic mucopolysaccharides. A very thick muscle layer surrounds the tube. Therefore this gland, which is often termed an MBG, is histologically and functionally different from the acid gland of the Pleurobranchomorpha and *Plocamopherus*, described here in detail.

Table 2. List of acid-secreting heterobranch species reliably recorded in literature and this study, and their gland types.

Taxonomic classification	Species	EAGs	SAGs	MBG	Reference
Cephalaspidea (Heterobranchia)					
Philinidae	*Philine aperta* (Linnaeus, 1767)	0	+	0	Thompson (1983, 1986, 1988)
	Philine quadripartita (Ascanius, 1772)	0	+	0	Thompson (1960a)
Pleurobranchomorpha (Heterobranchia)					
Pleurobranchaeidae	*Pleurobranchaea californica* (MacFarland, 1966)	+	?	+	Gillette *et al.* (1991)
	Pleurobranchaea maculata (Quoy & Gaimard, 1832)	+	0	+	Thompson (1969, 1988) and Thompson & Colman (1984)
	Pleurobranchaea meckeli (Blainville, 1825)	?	?	+	Schulz (1905), Alvim *et al.* (2014)
	Pleurobranchaea gela Er. & Ev. Marcus, 1966	?	?	+	Alvim *et al.* (2014)
	Pleurobranchaea hedgpethi hamva Er. Marcus, 1961	?	?	+	Alvim *et al.* (2014)
	Pleurobranchaea inconspicua Bergh, 1897	?	?	+	Alvim *et al.* (2014)
	Pleurobranchaea spiroporphyra Alvim *et al.* (2014)	?	?	+	Alvim *et al.* (2014)
Pleurobranchidae	***Bathyberthella antarctica*** Willan & Bertsch, 1987	+	0	+	Wägele & Willan (1994) and this study
	Berthella canariensis Cervera, Gosliner, Gomez & Ortea, 2000	?	?	0	Cervera *et al.* (2000)
	Berthella pellucida (Pease, 1860)	NN	NN	NN	Thompson (1969)
	Berthella plumula (Montagu, 1803)	+	?	0	Thompson (1960a), Gillette *et al.* (1991) and Martynov & Schrödl (2008)
	Berthella stellata (Risso, 1826)	+	0	+	Thompson & Colman (1984), Thompson (1988) and this study (contradicting earlier studies)
	Berthella strongi (MacFarland, 1966)	+	?	?	Gillette *et al.* (1991)
	Berthellina citrina (Rüppell & Leuckart, 1828)	+	+	+	Thompson (1969, 1988), Thompson & Colman (1984), Gillette *et al.* (1991) and this study
	Berthellina edwardsi (Vayssière, 1896)	+	+	+	This study
	Boreoberthella augusta Martynov & Schrödl (2008)			+	Martynov & Schrödl (2008)
	Pleurobranchus albiguttatus (Bergh, 1905)	+	0	+	This study
	Pleurobranchus areolatus (Mörch, 1863)	NN	NN	NN	Edmunds (1968)
	Pleurobranchus forskalii (Rüppell & Leuckart, 1828)	NN	NN	NN	Thompson (1969)
	Pleurobranchus membranaceus (Montagu, 1815)	+	0	+	Thompson & Slinn (1959), Thompson & Colman (1984), Thompson (1988) and Gillette *et al.* (1991)
	Pleurobranchus ovalis (Pearse, 1860)	NN	NN	NN	Thompson (1969)
	Pleurobranchus peronii (Cuvier, 1804)	+	NN	NN	Thompson (1969)
	Tomthompsonia antarctica Thiele, 1912	+	0	0	Wägele & Hain (1991) and this study
Nudibranchia, Euctenidiacea (Heterobranchia)					
Discodoridae	*Discodoris stellifera* (Ihering in Vayssière, 1904)	+	+	0	Edmunds (1968)
	Geitodoris pusae (Er. Marcus, 1955)	+	+	0	Edmunds (1968)
	Geitodoris tema Edmunds (1968)	+	+	0	Edmunds (1968)
	Tayuva lilacina (Gould, 1852)	+	NN	NN	Thompson (1969)
Doridae	*Doris verrucosa* Linnaeus, 1758	-	+	0	Edmunds (1968)
Onchidoridae	*Onchidoris bilamellata* (Linnaeus, 1767)	+	0	0	Edmunds (1968) and Thompson (1988)
Polyceridae	***Plocamopherus ceylonicus*** (Kelaart, 1858)	0	0	+	This study

Taxonomy follows World Register of Marine Species (www.marinespecies.org). Species analysed in this study in bold. Abbreviations: EAGs, epidermal acid glands; SAGs, subepidermal acid glands; MBG, median buccal gland; 0, absent; +, present; NN, no specific location of acid exudation or gland type is mentioned.

MBGs producing sulphuric acid are typical for many genera of Pleurobranchomorpha (Willan, 1987; Wägele & Willan, 1994, 2000). They are missing in the genus *Tomthompsonia* (Wägele & Hain, 1991). Cervera *et al.* (2000) stated that they were absent in *Berthella canariensis* besides several other *Berthella* species, including *B. stellata*. However, our results clearly show a short, unbranched tube opening in the oral tube (Fig. 5G) and exhibiting the same cells as observed in the MBGs of other pleurobranchids. This shows the necessity to re-investigate species that have been described as lacking the MBG. Wägele *et al.* (2006) first mentioned the presence of a MBG in *P. ceylonicus*. This is the only record of this particular glandular organ in another marine heterobranch group outside the Pleurobranchomorpha, but it seems to be absent in all other genera and species of the family Polyceridae (Vallès & Gosliner, 2006).

The MBG can be tubular (*B. antarctica*, *B. stellata* and *P. albiguttatus*) or ramified (*B. citrina*, *B. edwardsi* and *P. ceylonicus*) (Wägele & Willan, 1994; Wägele *et al.*, 2006; this study). The space occupied by the gland varies; we found it smallest in *P. albiguttatus* and *B. stellata*. In general the cellular morphology was similar in all investigated specimens and was also comparable to that of the SAGs in *Berthellina*. Since the MBG originates from the oral tube, it has the same ectodermal origin as the SAGs. However, cells of the MBG are arranged around a lumen with ciliated cells interspersed (Fig. 5H). This contradicts Schulz (1905), who stated that ciliated cells were absent in between the gland cells in *Pleurobranchaea*

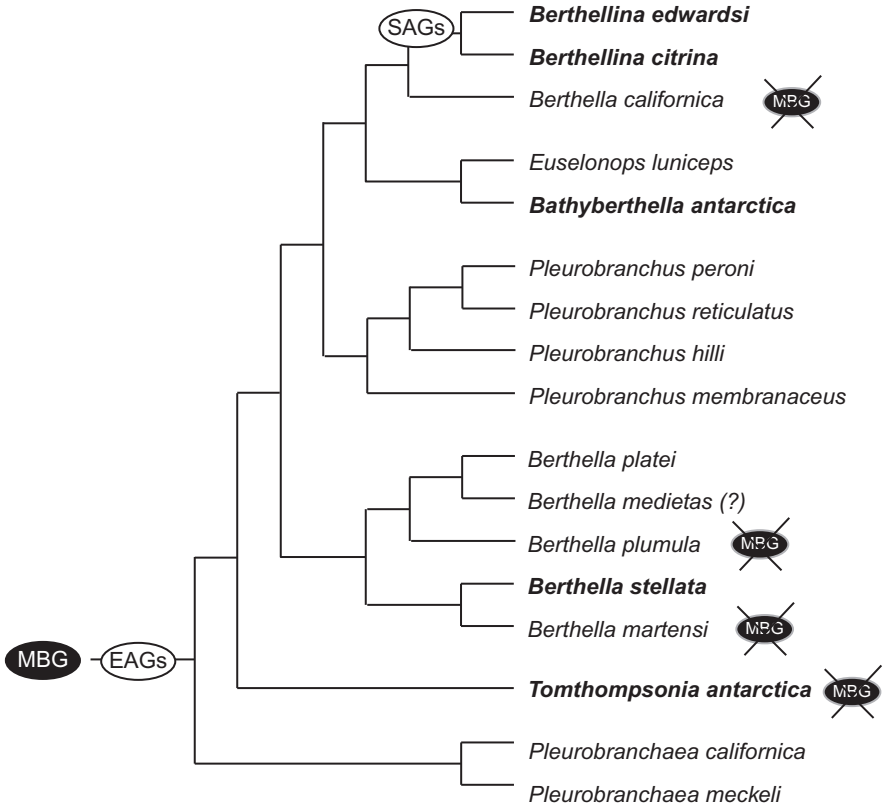

Figure 7. Phylogeny of Pleurobranchomorpha (Baysian-inference phylogram based on four genes, after Göbbeler & Klussmann-Kolb, 2010) with acid glands mapped on the tree. Species investigated in this study in bold. Question mark indicates lack of any information on presence or absence of MBG. Origin of MBG in the stemline of the Pleurobranchomorpha with independent secondary loss in the taxa marked is considered the most likely interpretation of the available data. Abbreviations: EAGs, epidermal acid glands; MBG, median buccal gland (cross indicates assumed loss); SAGs, subepidermal acid glands.

meckeli. Our specimen of *P. albiguttatus* exhibited glandular cells of smaller size and a denser layer of muscles, indicating a likely recent exudation of acid (see Schulz, 1905 for *P. meckeli*). The adult specimen of *B. antarctica* had much larger cells than all other investigated specimens, including the juvenile of this species (Fig. 6). The adult had a body size of about 10 cm, in contrast to the juvenile (and other species studied) of about 3 cm length. This could imply an ontogenetic variation in cell size, but requires further investigation. As described by Schulz (1905) for *P. meckeli*, we noticed areas of 'spongy' appearance in the basal parts of the MBG cells, indicating that acid formation is an ongoing process.

The functions of the MBG are unclear. Potentially, it could be defensive, expelling acidic fluid towards aggressors. However, the anatomy of the gland does not support such a role, since the surrounding muscle layer (which would be required to expel the fluid rapidly) is extremely thin (Fig. 5). Additionally, all species with a MBG have alternative defensive features (EAGs in pleurobranchomorphs; bioluminescence in exuded mucus in *Plocamopherus*, Vallès & Gosliner, 2006). Alternatively, the considerable size of the gland, and the exudation with the help of ciliated cells, suggest an extra-oral digestion of food, or perhaps help to decrease the pH value in the digestive glands. This needs further investigation.

Evolution of acid glands in marine Heterobranchia

Pleurobranchomorpha are usually divided into Pleurobranchidae and Pleurobranchaeidae (Gofas, 2014). Many genera of the Pleurobranchidae, but only *Pleurobranchaea* within Pleurobranchaeidae, have EAGs (see Table 2; Thompson, 1960a, 1969, 1988; Edmunds, 1968; Marbach & Tsurnamal, 1973; Thomson & Colman, 1984; Gillette *et al.*, 1991). According to Göbbeler & Klussmann-Kolb (2010), these two families are sister taxa (although Pleurobranchaeidae were underrepresented in their molecular analysis). We assume, therefore, that EAGs evolved in the stemline of Pleurobranchomorpha (Fig. 7). In phylogenetic analysis of Göbbeler & Klussmann-Kolb (2010), *Tomthompsonia* was not the most basal taxon. This implies either the independent evolution of the MBG several times within the Pleurobranchomorpha or, alternatively, its secondary loss in *Tomthompsonia* and some *Berthella* species. Our findings of a MBG in *B. stellata* using histological methods contradicts previous investigations by dissection and illustrates the necessity to re-investigate other *Berthella* species that have been described without a MBG, e.g. *B. ocellata* and *B. plumula* (Cervera *et al.*, 2000; Martynov & Schrödl, 2008).

Recent findings by Kano *et al.* (2016) indicate a sister relationship of Ringiculidae with Nudipleura, uniting them under the name Ringipleura. Morphological synapomorphies uniting these taxa are mainly characters from the nervous system and the special ontogenetic development of the frontal mantle margin and headshield. In contrast to the highly reduced or lost shell in Pleurobranchomorpha, *Tomthompsonia* has an internal coiled shell similar in shape to that of Ringiculidae (Wägele & Hain, 1991). A more detailed phylogeny of Ringipleura is still awaited. However, the presence of symplesiomorphic morphological characters in *Tomthompsonia* compared with all other Pleurobranchomorpha could imply a rather basal position of this genus and hence that the evolution of a MBG within Pleurobranchomorpha was a single event. Ringiculidae also exhibit glands in the fused mantle/headshield area, which were described as transparent defensive glands by Kano *et al.* (2016). It has yet to be ascertained whether these glands are similar to the SAGs described for *Berthellina*, and to

those described from a few euctenidiacean Discodoridiidae (Table 2; Edmunds, 1968; Thompson, 1988).

The presence of a MBG in the euctenidiacean nudibranch *P. ceylonicus* is exceptional, since there is no indication of its close relationship with Pleurobranchomorpha, so evolutionary convergence is implicated. Further histological investigations of related taxa are necessary to evaluate this situation.

Lifestyle

Pleurobranchomorpha cannot rely on the protection provided by a shell, since it has been internalized, reduced or lost. Acid release is thus a defensive strategy in addition to the commonly displayed camouflage (e.g. the orange *B. citrina* on orange sponges, Marbach & Tsurnamal, 1973) and/or nocturnal activity. The presence of large SAGs only in two *Berthellina* species might suggest a more exposed lifestyle and therefore a need of larger glands, able to produce a greater volume of acid for defence. Whereas other pleurobranchids were collected during the night or underneath coral rubble or stones, all *Berthellina* specimens were sitting on sponges exposed to the open environment during night and day (personal observation), where they were more exposed to predators.

It is not known why Pleurobranchomorpha produce acid for defence (and perhaps antifouling) instead of secondary metabolites, as is common in many heterobranchs. These compounds (often terpenoids) have only been found in a few pleurobranchids (Ciavatta *et al.*, 1995; Wesson & Hamann, 1996; Spinella *et al.*, 1997; Fu *et al.*, 2004; Robert *et al.*, 2006; Tan *et al.*, 2013; Wakimoto, Tan & Abe, 2013; Pereira *et al.*, 2014). A remarkable case reported by Wood *et al.* (2012) is the presence of the highly toxic tetrodotoxin in *Pleurobranchaea maculata*, which is probably sequestered from its food, although the authors doubted a defensive role. Living in a highly diverse environment, it might be advantageous to expel a substance that is repellent at once, without predators learning of adverse effects after feeding. Since toxic compounds have been confirmed in a few pleurobranchid species, it is necessary to investigate others in order to understand the relative importance of acids versus toxic metabolites for defence. Studying *P. maculata*, Gillette *et al.* (1991) indicated that taurine is a feeding deterrent and acts in combination with sulphuric acid. Moustafa *et al.* (2014) also described the presence of high amounts of taurine in the defensive acid secretion of *B. citrina*. Taurine is known as a phagomimic substance and is a component of other repellent substances exuded e.g. by sea hares (Heterobranchia: Anaspidea) and cephalopods (Derby *et al.*, 2007).

Together with other means of defence (cryptic appearance, sequestration of noxious compounds and behaviour), acid secretion is a strategy that has evolved independently several times within Gastropoda. This wide range of occurrence supports the effectiveness of the various acid glands, but the ecological and behavioural aspects of acid secretion, and its evolutionary context, are as yet largely a matter of speculation. Further studies, including less investigated species, are needed.

ACKNOWLEDGEMENTS

We wish to thank Franziska Günter und Alexandra Lischka, who did the first preliminary analyses on pleurobranchids. We thank Annette Klussmann-Kolb who provided the *Plocamopherus ceylonicus* when working in Australia in 1999. Elise Laetz helped in improving the English. David Reid gave valuable comments on the first draft and also helped in amending the English. Additionally we would like to thank two anonymous reviewers for their valuable comments that improved the manuscript. We want to express our sincere thanks to the Alexander Koenig Society who provided financial support.

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