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Potential roles of smell and taste in the orientation behavior of coral-reef fish larvae: insights from morphology

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An ontogenetic analysis of the olfactory organ and the number and distribution of internal taste buds was carried out in two neon gobies (*Elacatinus lori* and *Elacatinus colini*) with a goal of revealing morphological trends that might inform an understanding of the roles of olfaction and taste in larval orientation and settlement behavior. The pattern of development of the olfactory organ is unremarkable and enclosure of the olfactory epithelium occurs concurrently with metamorphosis and settlement in both species. Like other gobies, juvenile and adult *E. lori* and *E. colini* have one olfactory lamella, but they lack the accessory nasal sacs (present in some gobies)

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that could facilitate active olfactory ventilation (*i.e.*, sniffing). A small number of taste buds are present at hatch with most found in the caudal region of the buccal cavity (on gill arches, roof of mouth). As taste bud number increases they demonstrate an anterior spread to the lips, buccal valves and tongue (*i.e.*, basihyal). In the absence of an obvious ventilatory mechanism for the olfactory sacs, the water flows through the buccal cavity that occur with cyclic gill ventilation may allow the internal taste buds to play a role in chemical-mediated orientation and reef-seeking behavior in larval fishes.

KEYWORDS

Caribbean, Gobiidae, gustation, olfaction, sensory ecology, taste bud

Significance Statement: Most marine fishes have pelagic larvae that orient in the water column and actively swim towards settlement sites, but the sensory basis for this behavior is still a subject of debate. The morphological analysis of the developing olfactory and gustatory systems in two goby species reported here suggests that if chemical cues help larvae orient towards settlement sites on reefs, it is probably the numerous taste buds of the gustatory system and not the flat olfactory sensory epithelium, that mediates this critical behavior.

1 | INTRODUCTION

Most marine fishes have a pelagic larval phase, during which larvae spend days, weeks, or even months in the water column before they locate and reach a suitable settlement site and recruit into the juvenile population. Until the late 1990s, it was generally assumed that patterns of larval

distributions, dispersal and settlement were the result of physical (not behavioral) processes (Williams *et al.*, 1984; Roberts, 1997). However, this view has been challenged by recent field and laboratory studies that found that pelagic marine fish larvae can swim at considerable speeds and demonstrate directional behaviors in open water (Fisher, 2005; Leis, 2006; Jones *et al*., 2009; Fisher & Leis, 2009; reviewed in Leis *et al*., 2011; M. Foretich, J. E. Majoris, R. Chaput, C. L. Di Persia, E. Schlatter, P. M. Buston, C. B., Paris, unpubl. Data; J. E. Majoris, K. Catalano, D. Scolaro, J. Atema, P. M. Buston, unpubl. data). Furthermore, patterns of larval dispersal described using genetic techniques seem more restricted than would be predicted by purely physical oceanographic models (D'Aloia *et al*., 2015; Williamson *et al*., 2016; Almany *et al*., 2017). These findings suggest that larval swimming and orientation behaviors have the potential to directly influence patterns of dispersal and ultimately the structure of adult populations.

Leis (1991) first suggested that the reef and its resident organisms are a source of sensory cues that could allow fish larvae to orient toward and locate settlement sites and subsequent studies addressed the role of auditory, visual, chemical, or magnetic cues in this critical behavioral task (Lecchini *et al*., 2005; Dixson *et al*., 2008; Leis *et al*., 2011; O'Connor and Muheim, 2017; Foretich *et al*., 2017; Morais, *et al*., 2017; Majoris, *et al*., 2018). The chemical composition of the ocean, a potential source for chemosensory cues, is variable at different spatial scales as the result of both biotic and abiotic processes (Kingsford *et al*., 2002). Also, chemicals of biotic origin (*e.g.*, amino acids, fatty acids) in particular, form gradients that could be used by fish to locate their source on a reef (Gerlach *et al*., 2007).

The role of chemoreception in larval orientation behavior has been examined under both experimental laboratory and field studies using a wide range of coral-reef taxa, in particular. Larvae respond to chemical cues derived from reefs by changing swimming speed and direction

(*e.g.*, Apogonidae and Pomacentridsae Paris *et al*., 2013), actively choosing habitats (*e.g.*, pomacentrids, Lutjanidae; Lecchini & Nakamura, 2013) and discriminating between lagoon water and ocean water (*e.g.*, an apogonid; Atema *et al*., 2002). Larvae are attracted to chemical cues originating from island vegetation (*e.g.*, anemonefish *Amphiprion* Bloch & Schneider 1801; Dixson et al., 2008) and from the seagrass habitats (a lutjanid; Radford *et al*., 2012) where they typically settle. In addition, chemical cues from adult conspecifics presumably allow larvae to identify high quality settlement sites, thus adding a social component to settlement behaviour (a pomacentrid; Sweatman, 1988; Lecchini *et al*., 2005). The chemical identity of the cues used in these studies had not been determined, but a recent study showed that dimethyl sulphide (produced in large quantities by coral-reef organisms) affects behaviour of late-stage coral-reef larvae (primarily pomacentrids), but not the larvae of an open water species (mahi mahi *Coryphaena hippurus* L. 1758; Foretich *et al*., 2017). These studies provide compelling evidence for the role of chemical cues in orientation and settlement behaviour in the settlement stage larvae. However, many of them have assumed that these behaviours are mediated by olfaction, often using the terms olfactory and chemosensory interchangeably, with chemical cues referred to as odours. However, few of these studies (Sweatman, 1988; Wright *et al.*, 2005; Lecchini *et al.*, 2005b) have provided the necessary anatomical and experimental evidence to demonstrate the role of olfaction, in particular, in mediating larval behaviour.

It has been stated repeatedly that larval fishes have well-developed sensory systems (Lara, 2008, Arvedlund, 2007) and it has been asserted that their sensory acuity increases during ontogeny (Teodósio, *et al.*, 2017), but these terms are rarely defined or quantified in a morphological or functional context. It is known that the major sensory organs are present (in some form) at hatch and that they continue to develop through the larval period with suggestions

of ontogenetic trends in function (*e.g.*, vision: Shand, 1997; Shand, *et al.*, 1999, 2002; lateral line: Bird & Webb, 2014; Becker *et al.*, 2016). In coral-reef fishes, data on the developmental morphology of the olfactory organ are available for a small number of species (Lara, 1999, 2008; Arvedund *et al.*, 2006, 2007) and taste bud morphology and distribution are only known in the adults of a few species (Fishelson, *et al.*, 2004; Fishelson & DeLarea, 2006).

The olfactory system of bony fishes undergoes a simple morphological transition during the larval stage with important functional implications (reviewed in Kasumyan, 2004). A bilateral pair of olfactory epithelia appears in embryos just prior to hatch (Kawamura *et al*., 1990; Diaz *et al*., 2002; Arvedlund & Kavanagh, 2009) or in larvae soon after hatch (Iwai, 1980; Pankhurst & Butler, 1996; Kawamura *et al*., 2003). Each olfactory epithelium increases in size (Kawamura & Munekiyo, 1989; Kawamura *et al*., 1989), sinks into a shallow pit and is then enclosed in a blind sac (the olfactory organ) leaving one or typically two (incurrent and excurrent) nares open, which link the lumen of the water-filled olfactory sac and the external environment. Among families of coral-reef fishes, enclosure of the olfactory organ is complete prior to or at settlement and regardless of larval period duration (Atema *et al*., 2002; Lara, 2008; Leis *et al*., 2011; J. M. Leis, unpubl. data). After enclosure, the development of lamellae (epithelial folds comprising the olfactory rosette) provides more surface area for additional olfactory neurons, the density of which may also increase with fish size (Lara, 2008). The number and complexity of lamellae varies (Yamamoto *et al*., 1979; Lara, 2008), but the olfactory epithelium in adult gobies, for instance, has been described as unilamellar (Yamamoto *et al*., 1979; Belanger *et al*., 2003; Sarkar, *et al*., 2014). The olfactory nerve (I), composed of the axons of the ciliated olfactory sensory neurons, projects to the olfactory bulb where they synapse with mitral cells that form the olfactory tract and project to the telencephalon and then the diencephalon (Hara, 2000).

To be effective as olfactory stimuli, water-soluble molecules at suprathreshold concentrations must come in contact with and bind to specific membrane receptors on the sensory neurons in the olfactory epithelium. Furthermore, water in contact with the sensory epithelium must be renewed periodically (*i.e.*, sniffing) to avoid sensory fatigue. Motile cilia of non-sensory cells located in the olfactory epithelium of larvae may move water across the epithelium allowing molecules to bind with sensory membrane receptors (Iwai, 1980). During the larval period, ontogenetic increases in larval swimming speed (Blaxter, 1986; Leis & Carson-Ewart, 1997; Fisher *et al*., 2005; Leis *et al*., 2006, 2009; J. E. Majoris, K. Catalano, D. Scolaro, J. Atema, P. M. Buston, unpubl. data) and the increasing frequency of intermittent bursts of swimming will increase the Reynolds number (*Re*, the ratio of inertial to viscous forces) experienced by a larva (McHenry & Lauder, 2005). This would also serve to decrease the thickness of the boundary layer around the larva increasing the probability of contact between molecules serving as a potential chemical cue and the olfactory epithelium (Cox, 2008). It would also explain why younger larvae with weak swimming ability, which operate in a low *Re* regime with a relatively thick boundary layer, may not be able to detect or respond to olfactory cues (Morais, *et al*., 2017; O'Connor, *et al*., 2017).

After enclosure of the olfactory epithelium (typically at metamorphosis), cilia may still contribute to water movement in the olfactory organ, but water could also move into and out of the nares if the prevailing current or the fish's swimming speeds is high enough to move water into the small nares (*e.g.*, as a result of secondary flow and the Bernoulli effect; Verraes, 1976; Cox, 2008). Enclosure of the olfactory organ earlier during the larval period, suggests that olfactory-mediated behaviour is important during the larval period (*e.g.*, anemonefish: Arvedlund & Kavanagh, 2009; salmonids: Hara & Zielinski, 1989; Jahn, 1972; Verraes, 1976).

In post-settlement individuals, after the olfactory organ is enclosed, one or more accessory nasal sacs (non-sensory diverticulae of the olfactory organ) develop. They are thought to facilitate consistent ventilation of the olfactory organ when compressed by the movements of motile skeletal elements involved in cyclic respiratory gill ventilation (Ismail, 1986; Nevitt, 1991; Webb, 1993; Hansen & Zielinski, 2005; Belanger, *et al*., 2006). A simple application of a soluble dye at the incurrent naris in post-settlement apogonids showed pulsed movement of water out of the excurrent naris (Atema *et al*., 2002) indicating a cyclic renewal of water over the olfactory epitheium.

The taste buds of the gustatory system are most often implicated in feeding behaviour in larval, juvenile and adult fishes (Hara, 1994; Leguen, 2017), but roles in orientation and social behaviours have also been suggested (de la Hoz *et al*., 2014). In contrast to the bilateral pair of relatively large olfactory organs found on the head, small taste buds (pear-shaped clusters of elongate sensory and non-sensory cells) may be found on small papillae or level with the epithelial surface (taste bud types I, II, III; Reutter, 1974). Internal taste buds are present at hatch or appear within 2 weeks post-hatch and continue to increase in number through ontogeny (Iwai, 1980; Noakes & Godin, 1988; Komada, 1994; Kawamura *et al*., 2003; Kawamura & Munekiyo, 1989; Kawamura *et al*., 1989). They are distributed on the lips and within the buccal cavity (internal taste buds) and on the skin of the head, body and tail (external taste buds; *e.g*., catfishes, order Siluriformes: Atema, 1971; Cyprinidae: Gomahr, *et al*., 1992; zebrafish *Danio rerio* (Hamilton 1822): Hansen *et al*., 2002, Ohkubo, *et al*., 2005; Atherinopsidae: de la Hoz, 2014; Mullidae, McCormick, 1993; sand goby *Pomatoschistus minutus* (Pallas 1770): Whitear, 1971; Cichlidae, J. F. Webb, unpubl. data). The sensory cells in taste buds are innervated by the facial (VII), glossopharyngeal (IX) or vagus (X) nerve, depending on their location (internal,

external: Atema, 1971; Hara, 1994; Fishelson & Delarea, 2004) that project to primary gustatory centers in the brain (Hara, 2000).

The goal of this paper is to describe the ontogeny of the olfactory organ and ontogenetic trends in the number and distribution of internal taste buds in order to understand the potential contribution of olfaction and gustation to larval orientation and reef-seeking behaviour in two species of gobies and compare them with other gobies and other coral-reef taxa. The two focal species in this study are the line snout neon goby *Elacatinus lori* Colin 2002 and its congener *Elacatinus colini* Randall & Lobel 2009. Both lay demersal eggs and newly hatched larvae are 3.7 mm in length. Larvae emerge from host tube-sponges occupied by their parents and move into the water column where they are dispersed up to 10 km (D'Aloia *et al*., 2011; Majoris *et al*., 2018a). In the laboratory, their yolk reserves are depleted within 24 h; larvae undergo flexion at *c.* 10 9days post hatch (dph) and settle after 28–58 dph when they are 9.0–9.5 mm; (Figure 1). The fact that these fish can be reared through settlement (Majoris *et al*., 2018a) has enabled this first detailed morphological analysis of sensory ontogeny in gobioids. This study forms part of a larger effort to understand the physical and biological processes that influence population connectivity in these species (D'Aloia et al., 2015; Lindo *et al*., 2016; Majoris et al., 2018a,b; M. Foretich, J. E. Majoris, R. Chaput, C. L. Di Persia, E. Schlatter, P. M. Buston, C. B., Paris, unpubl. data; J. E. Majoris, K. Catalano, D. Scolaro, J. Atema, P. M. Buston, unpubl. data).

2 | METHODS

2.1 Fish capture

Mated pairs of *E. lori* and *E. colini* were collected from reef habitats near Carrie Bow Caye, Belize (16° 48′ 09′′ N, 88° 04′ 55′′ W) and housed in 75 l aquaria in a flow-through seawater laboratory at the International Zoological Expeditions (IZE) field station on South Water Caye, Belize (16° 49′ N, 88° 05′ W), or in a recirculating seawater system at Boston University, USA. Ontogenetic series of *E. lori* and *E. colini* larvae were reared in 76 l cylindrical black bins and fed a variety of cultured and wild-caught zooplankton (Figure 1). A detailed description of broodstock maintenance and larval rearing methods can be found in Majoris *et al*. (2018). Additional post-settlement *E. lori* and *E. colini* (settlers) were collected from reef habitats within the South Water Caye Marine Reserve. Field research in Belize and the export of samples from Belize was carried out with the approval of the Belize Fisheries Department. Fish were immersed in cold seawater (2–4° C) for 2 min and then fixed in cold (2–4° C) 10% formalin in seawater (or in phosphate-buffered saline; PBS) for at least 2 min for anatomical study, which is consistent with American Veterinary Medical Association guidelines on euthanasia of small warm-water fish. Care was taken to ensure that fish did not contact ice directly. Chemical anaesthetic was not used for several reasons, in particular because fixation must occur prior to death to avoid post-mortem changes at the cellular level and ensure quality of the histological data and specimens prepared for SEM.

2.2 | Scanning electron microscopy

Elacatinus lori hatched and reared in the Buston Laboratory at Boston University in 2015 (n = 10, 0–45 dph, 2.5 mm notochordal length (L_N), 11 mm standard (L_S) were fixed in cold (2–4° C) 4% formalin in seawater. Additional *E. lori* hatched and reared in Belize in 2016 (n = 24, 0–44 dph, 3 mm L_N, 8 mm L_S), were fixed in cold 4% formalin in PBS and transferred through an

ascending sucrose series (5, 10, 20% sucrose in PBS) prior to dehydration (see below). *Elacatinus lori* settlers, wild-caught in Belize (n = 5, 9–18 mm L_S; 2011) were fixed in cold 4% formalin in seawater. *Elacatinus colini* hatched and reared at Boston University in 2014 (n = 11, 10–70 dph, 4.5 mm L_N, 10.5 mm L_S) were fixed in cold 4% formalin in seawater. Subsequently all fish were dehydrated in an ascending ethanol series [50%, 70% (overnight), 85%, 95%, 100% x 3], critical-point dried out with liquid CO_2 (Tousimis Samdri 780A), mounted on aluminum stubs with adhesive carbon discs, sputter coated with platinum (15 nm; Leica MED 020; www.leica.com) and viewed with a Zeiss NTS Supra 40VP SEM (www.zeiss.com) at 3 kV and a working distance of 10 mm.

2.3 | Histology

Elacatinus lori raised in Belize in 2015 and 2016 (n = 33, 0–44 dph, 3 mm L_N, 11 mm L_S), wild-caught *E. lori* settlers collected at Belize in 2015 and 2016 (n = 5, 9.5–14 mm L_S) and *E. colini* raised at Boston University in 2015 (n = 25, 0–50 dph, 3.5-15 mm L_N or L_S) were prepared for plastic-resin histology. Fish > 6 mm L_S were decalcified in Cal-Ex (Thermo Fisher Scientific; www.thermofisher.com) for 2 h (6–7.5 mm L_S), 3.5 h (8–8.5 mm L_S), or 7–8 h (> 8.5 mm L_S) and rinsed in PBS for 2 h. Fish were dehydrated in an ascending ethanol series (to 95% ethanol), infiltrated overnight in glycol methacrylate resin (Technovit 7100, Electron Microscopy Sciences; www.emsdiasum.com) and embedded in fresh resin to which polymerization agent had been added. Most of the fish were double embedded, individual fish were first embedded in small resin blocks and then 6–8 small resin blocks were re-embedded in a single larger block of resin to allow sectioning in the transverse plane (cross-sections). Sections (5 µm thickness, using a

Leica 4M2265 motorized microtome with tungsten carbide knife) were individually mounted out of distilled H_2O onto clean slides, dried overnight, stained with 0.5% aqueous cresyl violet for 5 min, rinsed in running tap water, air-dried overnight and coverslipped with Entellan (Electron Microscopy Sciences).

Elacatinus lori settlers wild-caught in 2011 (n = 8, 9–17 mm L_S) and larvae reared at Boston University in 2015 in addition to 3 wild-caught settlers (total of 7 fish, 0–30 dph, 3 mm L_N, 15 mm L_S) were decalcified in Cal-Ex for 2 h (6.0–7.5 mm L_S) or 7–8 hours (> 8.5 mm L_S) and rinsed in PBS for 2 h. Then they were dehydrated in ascending series of ethanol and t-butyl alcohol, infiltrated in Paraplast (Thermo Fisher Scientific) for 4 h under vacuum and individually embedded. Blocks were sectioned at a thickness of 8 µm, serial sections were mounted on slides subbed with 10% albumin in 0.9% NaCl, stained with a modified Hall-Brunt quadruple (HBQ) stain (Hall, 1986) and coverslipped with Entellan.

A subset of the histological material prepared (as above) was analysed quantitatively (*E. lori*, n = 28; *E. colini*, n = 9). The length of the olfactory epithelium (rostro-caudal axis) was calculated as the number of transverse sections containing sensory tissue multiplied by section thickness (5µm for plastic-resin sections). In each individual, images were captured at positions 25%, 50% and 75% along the rostro-caudal length of the left sensory epithelium (or the right epithelium, if the left one was damaged), with a Zeiss AxioCam MRc camera mounted on a Zeiss AxioImager1 compound microscope and analyzed using ImageJ 1.45s (NIH; www.imagej.nih.gov). Sensory epithelium width was determined in images of each of the three sections by measuring the epithelium half way between the apical cell surface and basement membrane; epithelium thickness (from apical surface to basement membrane) was also measured

in the middle of the epithelium in the same three sections. The mean of three measurements for each variable was used in subsequent analyses.

Individual taste buds *c.* 15–20 μm in diameter) were present in multiple sections, so other landmarks (*e.g.*, eye, skeletal features) were used to distinguish between individual taste buds to ensure accurate counts. Taste buds were counted in four regions: oral jaws, lips and buccal valves; roof of the buccal cavity; floor of the buccal cavity, including the basihyal and basibranchial (the tongue, *i.e.*, basihyal); gill arches, including taste buds located between pharyngeal teeth. In order to assess rostro-caudal distribution of taste buds within the buccal cavity, the head of each individual was divided into ten equal bins along the rostro-caudal axis, extending from the lips to the anterior end of the oesophagus (beyond which taste buds were not found) and the total number of taste buds was calculated for each bin.

3 | RESULTS

On the day of hatch, a pair of round olfactory sensory epithelia is found rostro-medial to the eyes in both *E. lori* and *E. colini* (Figure 2a). Each sensory epithelium (Figure 2b) appears to include two cell types: ciliated sensory cells with long, dense cilia and cells with shorter single cilia or microvilli. As fish grow, the olfactory epithelium elongates rostro-caudally and sinks into a shallow pit (Figures 3b–d, 4a–c). At 7–9 mm L_S (*c.* 30–50 dph) the lateral edges of the skin start to grow towards each other at a point mid-way along the length of the olfactory epithelium (Figures 3d, 4d) and fuse forming a bridge, leaving anterior and posterior openings, the nares (Figures 3d, 4e). Histology and SEM reveal that the enclosure of the bilateral pair of olfactory organs is complete in post-settlement individuals (9.5–10 mm L_S). Histological examination of a

pre-settlement and a post-settlement *E. colini* of the same size and age (36 dph, 9.5 mm L_S; reared in Belize) revealed that the olfactory epithelium is enclosed only in the post-settlement individual (Figure 3d,e) suggesting that the process of enclosure occurs relatively quickly following settlement. After enclosure, the anterior naris elongates into a tube with a terminal opening (Figure 5) and within the olfactory organ the length and width of the sensory epithelium continues to increase in proportion to body size (Figure 6 and Table 1). They increase at similar rates in the two species, showing more than a three-fold increase between day of hatch and the time of settlement. In contrast, the thickness of the sensory epithelium increases much more slowly (Figure 6). Neither lamellae nor accessory nasal sacs are present in either larvae or in post-settlement individuals of up to 14 mm L_S (*E. lori*) and 15 mm L_S (*E. colini*).

Taste buds are apparent in the epithelium lining the buccal cavity (Figure 2c,d). A small number (5–6) of taste buds are present in *E. lori* and in *E. colini* on the day of hatch and total taste bud number increases such that > 500 taste buds are present in the buccal cavity of post-settlement individuals (Figure 7). Taste buds are found on the gill arches (including those between the pharyngeal teeth), with the rest distributed on the tongue (*i.e.*, basihyal), roof of the mouth and on the oral jaws (including lips and buccal valves; Figure 8). Ontogenetic trends in taste bud number vary by location within the buccal cavity (Figure 9). Taste buds on the gill arches and on the tongue increase in number gradually (Figure 9a,b). In contrast, on the oral jaws and on the roof of the mouth, there are few taste buds until just prior to settlement when the number of taste buds increases rapidly (Figure 9c,d). The differences in the timing and rate of increase in taste bud number among different regions in the buccal cavity result in an overall shift in the relative distribution of taste buds over time (Figure 10). In young larvae, the vast majority of taste buds are located in the posterior half of the buccal cavity, but as settlement

approaches, proportionally more taste buds are found in the anterior half of the buccal cavity (on the oral jaws, including the lips).

4 | DISCUSSION

The analysis of the ontogeny of the olfactory organ and taste buds in larval and juvenile *E. lori* and *E. colini* reveals simple trends in the size and enclosure of the olfactory organ and in the number and distribution of taste buds; these are similar to the few descriptions available for other species. The results of this analysis suggest that larvae may use two different mechanisms for acquiring chemical cues. Larvae may need to swim at speeds sufficient to reduce the thickness of the boundary layer allowing chemical cues to contact the ciliated olfactory epithelium located on the surface of the head. Alternatively, normal cyclical gill ventilation moving water past the internal taste buds in the buccal cavity and between the gill arches facilitating contact of chemical cues with taste buds in the lining of the buccal cavity and on the gill arches. This latter mechanism suggests that the gustatory system may play an unappreciated role in chemosensory-based orientation behaviour.

4.1 | Ontogeny of the olfactory organ

Among gobiids, the olfactory organ had only been described in juveniles and adults of a few species (Belanger *et al*., 2003; Arvedlund *et al*., 2007; Kuciel 2013; Kim *et al*., 2016). Here, we have shown that the timing and pattern of development of the olfactory organ in *E. lori* and *E. colini* is typical of that found in other species of gobies and in teleost fishes more generally. The

olfactory epithelia are present on the skin at hatch, increase in size through the larval period and the enclosure of the olfactory organ in a blind sac coincident with metamorphosis and settlement. The presence of non-sensory motile cilia in association with the sensory epithelium could not be confirmed with histology or SEM, although it appears that a second class of either microvillous or ciliated cells is present in larval or juvenile *E. lori* or *E. colini*. In larvae, prior to enclosure, motile cilia could generate flows that bring chemical cues into contact with the olfactory epithelium. However, in the absence of motile cilia, larvae would have to swim (continuously or episodically) at speeds sufficient to increase *Re* and reduce the boundary layer so that chemical stimuli can come into contact with the olfactory epithelium. In larval *E. lori* and *E. colini*, swimming speed increases with larval size though not as much as in some other species (McHenry and Lauder, 2005; J. E. Majoris, K. Catalano, D. Scolaro, J. Atema, P. M. Buston, unpubl. data).

Post-settlement *E. lori* and *E. colini* have a flat olfactory epithelium without lamellae, which is similar to what is described as a unilamellar epithelium in the adults of other gobies (Belanger *et al*., 2003; Sarkar, *et al*., 2014). This is in contrast to the higher numbers of well-developed lamellae described in other coral-reef species at comparable stages (wrasses: Lara, 2008; an apogonid, *Ostorhinchus doederleini* (Jordan & Snyder 1901): Atema *et al*., 2002; damselfishes, *Amblyglyphidodon leucogaster* (Bleeker 1847), a clownfish *Amphiprion polymnus* (L. 1758) a cardinalfish *Cheilodipterus quinquelineatus* Cuvier 1828: M. R. Lara, unpubl. data). Unfortunately, functional correlates are not known for interspecific variation in the timing of the onset of lamellar development (Iwai, 1980; Noakes & Godin, 1988; Kawamura *et al*., 1990; Kawamura *et al*., 2003; Arvedlund *et al*., 2007; Lara, 2008; Arvedlund & Kavanagh, 2009) or in the ultimate number of lamellae present in adult fishes (Hansen & Zielinski, 2006; Arvedlund &

Kavanagh, 2009; Yamamoto *et al*., 1979). Nevertheless, if juvenile and adult *E. lori* or *E. colini* depend on olfaction to mediate critical behaviours, then presence of a simple lamellar morphology, as found in other gobies, must be sufficient to detect meaningful olfactory cues. It should be noted, however, that a recent study (Majoris et al., 2018) showed that the choice of settlement habitat (sponge type) in *E. lori* settlers seems to be dependent on visual cues rather than chemosensory cues.

The enclosure of the olfactory organ, which occurs at settlement in *E. lori* and *E. colini* changes the hydrodynamics of water flow over the sensory epithelium (Cox, 2008). Thus, after settlement, a different mechanism probably brings water into contact with the sensory epithelium.. Accessory nasal sacs facilitate olfactory sampling of the chemical environment in a range of fish species (Webb, 1993; Kasumyan, 2004). Among coral-reef fishes, accessory nasal sacs (which are easily seen in histological material) are known in cardinalfishes (*O. doederleini*, Atema *et al*., 2002; *C. quinquelineatus*: Y. Hu, J. E., Majoris, P. M. Buston & J. F. Webb, unpubl. data) and damselfishes (*A. leucogaster*: Y. Hu, J. E., Majoris, P. M. Buston & J. F. Webb, unpubl. data). They are also found in gobies from freshwater and estuarine habitats (male round goby, *Neogobius melanostomus* (Pallas 1814): Belanger *et al*., 2003; Korean sand goby, *Favonigobius gymnauchen* (Bleeker 1860), Kim *et al*., 2016), but are absent in post-settlement *E. lori* and *E. colini* (this study). After settlement, *E. lori* and *E. colini* use their pelvic discs (a post-metamorphic feature of gobioids and gobiids, in particular: Thacker, 2011; VanTassell et al., 2011) to attach themselves to the surface of the large tube sponges that they inhabit (D'Aloia et al., 2011; Majoris et al., 2018). One might speculate that, in the absence of accessory nasal sacs (and thus an obvious mechanism for active olfactory ventilation), the combination of water flows generated by their filter feeding sponge host and difference in the length of their anterior and

posterior nares (Figure 5) could generate secondary flow through the olfactory system of an *E. lori* or *E. colini* residing within a sponge tube (as per Vogel, 1994, Cox, 2008). An ecomorphological study of olfactory morphology among taxa inhabiting habitats that experience different hydrodynamic regimes (*e.g.*, sponge *v.* non-sponge dwelling species) might provide insights into the importance of olfaction in the juveniles and adults of gobies.

4.2 | Ontogeny of taste bud distributions

Among coral-reef fishes, taste bud morphology and distribution are known for the adults of only a few species (Fishelson, *et al*., 2004; Fishelson & DeLarea, 2006) including gobies (Fishelson & DeLarea, 2006). Here we have described ontogenetic trends in the number and distribution of taste buds for the first time in two coral-reef fishes. We have shown that internal taste buds are present at hatch in the buccal cavity of both *E. lori* and *E. colini*. They resemble the taste buds of adults in other gobies (Fishelson & DeLarea, 2004). The number of internal taste buds is higher in both *E. lori* and *E. colini* than in the larvae of several other coral-reef taxa at comparable sizes (*A. leucogaster, A. polymnus, C. quinquelineatus*: Y. Hu, J. E., Majoris, P. M. Buston & J. F. Webb, unpubl. data), suggesting that gustation may be more important for *Elacatinus* spp. when compared with these other species.

Taste bud number increases through the larval stage in *E. lori* and *E. colini*, so that there are hundreds of taste buds in the buccal cavity at the time of settlement. The anterior spread of taste buds in the buccal cavity (Figure 10) has been noted in other fishes (bluefin tuna *Thunnus orientalis* (Temminck & Schlegel 1844): Kawamura *et al*., 2003; a flatfish, *Rhombosolea tapirine* Günther 1862: Pankhurst & Butler, 1996; *A. leucogaster, A. polymnus*, cardinalfish *C.*

quinquelineatus: Y. Hu, J. E., Majoris, P. M. Buston & J. F. Webb, unpubl. data). This ontogenetic pattern may be a simple consequence of an increase in the availability of surface area that can be occupied by taste buds as a fish grows, or may indicate an adaptive ontogenetic shift that reflects the way in which chemical cues are assessed. In adult fishes, for instance, external taste buds (*e.g.*, on barbels) are used to locate food, often at a distance, but internal taste buds are used to screen food items before deciding to swallow or expel them (Atema, 1971; Hara, 1994; Finger *et al*., 2000; Clemens & Rubenheim, 2006; Yashpal *et al*., 2009). In early larvae of *E. lori* and *E. colini* the taste buds, found predominantly in the caudal half of the buccal cavity, probably function in the acceptance of small prey items that are large relative to buccal volume in these small larvae. In older larvae and post-settlement juveniles, the taste buds present on the lips and on the oral jaws (Figure 1c) may facilitate preliminary chemosensory assessment of prey items prior to final acceptance by taste buds found more caudally in the buccal cavity, including those between the pharygeal teeth and on the gill arches.

4.3 | Chemoreception in larval orientation behaviour : what about taste?

In most studies of the role of chemoreception in larval orientation behaviour, it had been assumed that the olfactory system mediates orientation behaviour (Gerlach *et al*., 2007; O'Connor *et al*., 2017; Arvedlund & Kavanagh, 2009). However, the gustatory and olfactory systems of adult fishes detect some of the same classes of organic molecules (*i.e.*, amino acids, bile salts) at comparable concentrations (10^{-7}–10^{-9} mol^{-1} and 10^{-11}–10^{-12} mol^{-1}, respectively: Hara, 1994), which must bind to specific membrane receptors located on the sensory cells of the olfactory epithelium or taste bud (Hara, 1994). In addition, the pattern and timing of the

development of the olfactory organ revealed in this study suggest that there are functional limitations on the ability of the olfactory system of larvae to sample the chemical environment to guide orientation behaviour. The ability of a larva to respond to chemical cues is only possible if larval swimming speed is high enough to increase *Re* (McHenry & Lauder, 2005), which will reduce the thickness of the boundary layer, thus allowing water containing potential chemical cues to come into direct contact with the sensory epithelium.

In contrast, the presence of taste buds on all surfaces of the buccal cavity in *E. lori* and *E. colini* larvae, especially on the gill arches, provides another possibility for chemosensory detection of potential orientation cues. Internal taste buds could continuously sample the chemical environment as a simple by-product of normal gill ventilation. When cyclic gill ventilation replaces cutaneous respiration for gas exchange in small larvae, water is consistently moved through the buccal cavity, past the taste buds located on the gill arches and through the opercular cavity. In the laboratory, *E. lori* larvae have been observed actively ventilating their gills at 20 dph (J. E. Majoris, pers. obs.), when 125 taste buds are already present in the buccal cavity (of a small larva, 6.5 mm L_S). It has been shown that external taste buds, including those on barbels, allow fishes to detect chemical cues in the water, presumably leading them to a food source (Hara, 1994) and that internal taste buds assess the presence and quality of food and its ultimate acceptance or rejection (Hara, 1994). However, it has also been suggested that internal taste buds on the gill arches of adult fish can detect suspended or dissolved materials as water moves between them (Iwai, 1963). Thus, if one or more elements of the chemical signature of a reef (representing a source of food, habitat or social cues) are detected by taste buds, then chemical sampling of the environment by the gustatory system may indeed play a role in the ability of larval fishes to detect and orient towards a reef.

In conclusion, this morphological study contributes to our understanding of sensory ontogeny in the Gobiidae, the most speciose family of fishes on coral reefs and the most speciose family of marine fishes (Nelson *et al*., 2016). It has shown that the olfactory and gustatory systems of the pelagic larvae of congeners (*E. lori*, *E. colini*) lack notable morphological specializations. It also suggests that in the absence of a mechanism for active olfactory ventilation (sniffing) in larvae, normal cyclic gill ventilation would bring a consistent flow of water into contact with the numerous taste buds in the buccal cavity, especially those on the gill arches. This could provide a reliable chemical sampling mechanism that allows larvae to use chemical cues to orient towards a reef. Integrated anatomical, behaviour al and physiological analyses will be necessary to test this hypothesis explicitly and the task of experimentally teasing apart the relative contributions of olfaction and taste will present a considerable challenge. Ultimately, the ontogeny of the different sensory modalities in larval fishes (olfaction, taste, audition, vision, magnetoreception, and mechanoreception) and their relative roles in larval orientation behaviour (*e.g.*, sensory hierarchy: Teodósio, *et al*., 2016) will need to be addressed.

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Figure Captions

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1 Change A, B etc to (a), (b) etc. throughout.

FIGURE 1. Ontogenetic series of lab-reared *Elacatinus lori*. (a) 3.4 mm notochord length (L_N), (b) 4.0 mm L_N, (c) 4.4 mm L_N, (d) 5.2 mm standard length (L_S), (e) 7.7 mm L_S, (f) 8.3 mm L_S, (g) 9.0 mm L_S, (h) 9.2 mm L_S. Scale bars = 1 mm.

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FIGURE 2. Olfactory epithelium and taste buds in *Elacatinus lori*. (a) Circular, ciliated olfactory epithelia on the surface of the head of a 18 days post hatch (dph; *c.* 4.5-5 mm standard length; L_S) larva. Scale bar = 100 µm. (b) Enlargement of the sensory epithelium in (a) showing dense cilia on the apical surface of the sensory epithelium. Scale bar = 10 µm. (c) Lower jaw (7 mm L_S, 30 dph) with a line of taste buds behind the teeth of the oral jaw. Scale bar = 10 µm. (d) Higher power image of the 2nd taste bud from the right in (c), revealing the short, dense microvilli that emerge from the centre of the taste bud, on the apical surface of the sensory cells (not visible) that lie beneath the epithelial cells surrounding the taste bud. Scale bar = 2 µm.

FIGURE 3. Development of the olfactory organ in *Elacatinus colini*. Scanning electron micrographs of the olfactory organ showing the change in the sensory epithelium and its enclosure in the olfactory sac. (a) The ciliated sensory epithelium is open and in a shallow depression (10 days post hatch (dph), 4.5 mm notochord length; L_N); (b) the depression elongates (20 dph, 5.5 mm L_N) and (c) the sensory epithelium is in an elongated pit (30 dph, 8.0 mm standard length; L_S), and (d) the olfactory sac starts enclosing over the sensory epithelium (50 dpf, 9.0 mm L_S), which will leave two nares in post-settlement individuals (also see Figure 5).

FIGURE 4. Transverse sections at the rostro-caudal midpoint of the sensory epithelium (►) in *Elacatinus colini*. (a) Sensory epithelium is on the surface of the head in a young larva (13 days post hatch (dph), 5 mm standard length; L_S). (b) Sensory epithelium sinks into a shallow pit at 20 dph (6 mm L_S). (c) At 30 dph, 8.5 mm L_S, the epithelium is still in a shallow pit (►). (d) Skin has partially extended over the pit just before settlement (36 dph, 9.5 mm L_S); ↔, Thickness of epithelium. (e) Post-settlement individual of the same size and age as in (d), in which the olfactory epithelium is enclosed, indicating that the process of enclosure occurs quickly at the approximate time of settlement. (f) Enclosed olfactory organ in an older post-settlement individual (50 dph, 12.5 mm L_S). All scale bars = 100 μm.

FIGURE 5. Post-settlement (juvenile) *Elacatinus lori*. (a) Dorsal view of post-settlement individual. (b) Transverse section at level of anterior (Ant.) nares, showing tube that extends the naris, (c) Transverse section at level of posterior (Pos.) nares. The longer naris, which is raised further from the body–boundary layer will probably experience higher flow and lower

pressure facilitating water movement through the olfactory organ as flow generated by their filter feeding sponge host passes over the body.

FIGURE 6. Ontogeny of the size of the olfactory epithelium in *Elacatinus lori* (O, □, △) and *Elacatinus colini* (●, ■, ▲). O, ●, rostro-caudal length of olfactory epithelium; □, ■, width of olfactory epithelium, including additional surface area provided by lamellae; △, ▲, thickness of olfactory epithelium (from apical surface to basement membrane). See Table 1 for statistics. L_N, Notochord length; L_S, standard length.

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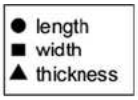
2 Delete 

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FIGURE 7. Total number of internal taste buds in the buccal cavity through ontogeny in *Elacatinus colini* (O) and *Elacatinus lori* (●). L_N, Notochord length; L_S, standard length.

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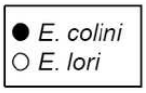
2 Delete 

FIGURE 8. Internal taste buds (▼) in different locations in the buccal cavity (bc) of post-settlement *Elacatinus lori* (12 and 14 mm Standard length; L_S): (a) on the ventral lip; (b) on buccal valve (v) and oral jaw; (c) on tongue (*i.e.* basihyal); (d) gill arches (c – cartilage); (e)

on roof of mouth; (f) between pharyngeal teeth; (g) enlarged view of a taste bud on the oral jaw (as in (b); v – buccal valve); (h) taste buds on the gill arch (also see (d). Scale bars = (a)–(f) 100 μm, (g)–(h) 50 μm.

FIGURE 9. Number of taste buds in different regions of the buccal cavity *v.* fish notochord (L_N) and standard length (L_S), derived from histological material in *Elacatinus colini* (O) and *Elacatinus lori* (●) on (a) gill arches (including taste buds between pharyngeal teeth), (b) roof of the mouth, (c) tongue (*i.e.* basihyal) and (d) the oral jaws (including lips and buccal valves).

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1 Replace gill arches with (a), roof of mouth with (b), tongue with (c) and oral jaw with (d).

2 Delete all x-axis numeral from (a) and (b).

3 Change 2x x-axis label to single centred to L_N or L_S (mm).

4 Delete All y-axis numerals from (d).

5 Replace 2x y-axis label with single centred Taste buds (*n*)

FIGURE 10. Distribution of taste buds along the rostro-caudal axis (anterior→posterior) of the buccal cavity in (a) *Elacatinus lori* and (b) *Elacatinus colini* from day of hatch (0 days post hatch; dph), through settlement, and in post-settlement individuals. LN, notochord length; LS, standard length; ——, *c.* time of settlement. * = wild-caught, post-settlement *Elacatinus lori* (unknown ages).

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1 Replace NL with L_N and SL with L_S throughout.

2 Delete all the hash symbols and insert (*n*) after each -axis label.

TABLE 1. Regression analysis of olfactory organ development in *Elacatinus lori* and *Elacatinus colini.* Length of the olfactory epithelium (in rostro-caudal axis), width of the olfactory epithelium (in medio-lateral axis; at rostro-caudal midpoint) and thickness of the olfactory epithelium (apical surface to basement membrane; at rostro-caudal midpoint) are regressed against fish standard length (L_S). See FIG 6. and text for additional details.

Dimension	***Elacatinus lori***	***Elacatinus colini***
Length	$y = 24.262x + 2.801$, $r^2 = 0.88$, $P < 0.001$	$y = 24.83x + 20.289$, $r^2 = 0.83$, $P < 0.001$
Width	$y = 33.085x - 62.481$, $r^2 = 0.89$, $P < 0.001$	$y = 29.129x - 49.549$, $r^2 = 0.95$, $P < 0.001$
Thickness	$y = 2.0973x + 25.811$, $r^2 = 0.43$, $P < 0.001$	$y = 1.715x + 28.112$, $r^2 = 0.50$, $P < 0.02$

Figure 1

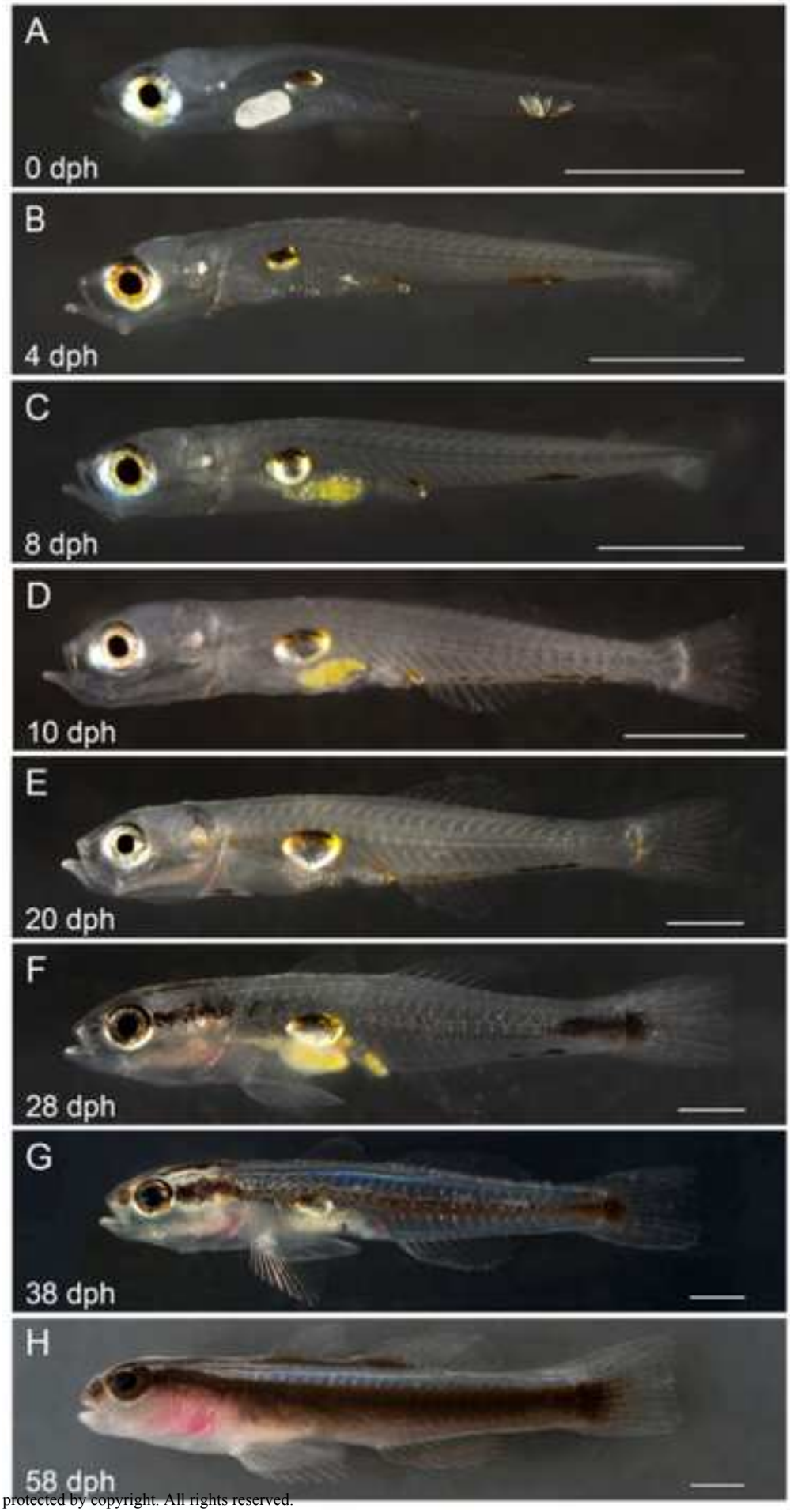
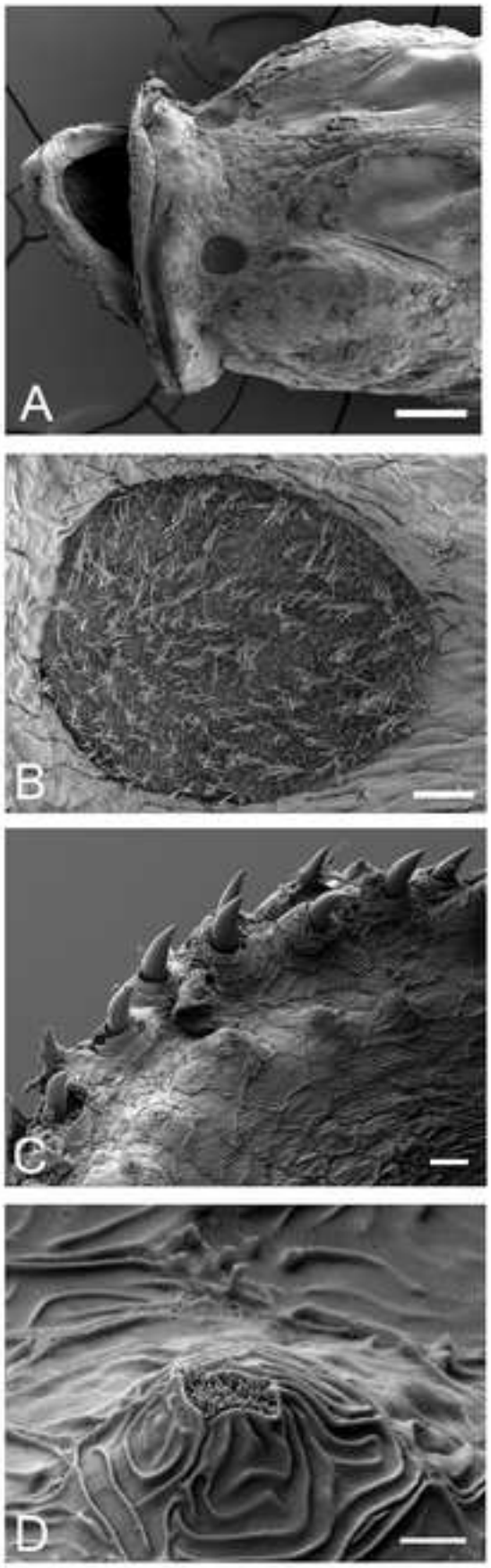


Figure 2



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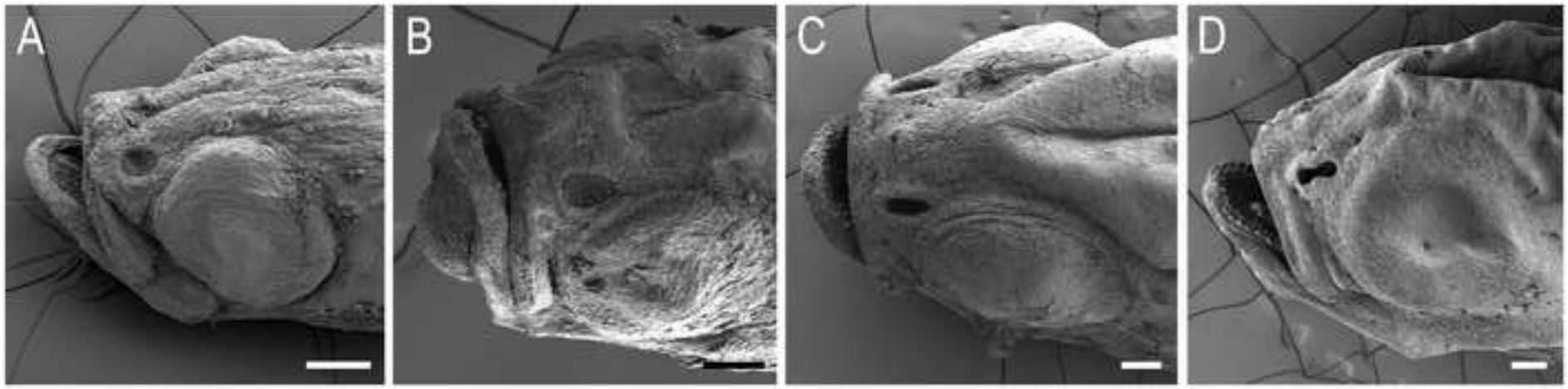


Figure 4

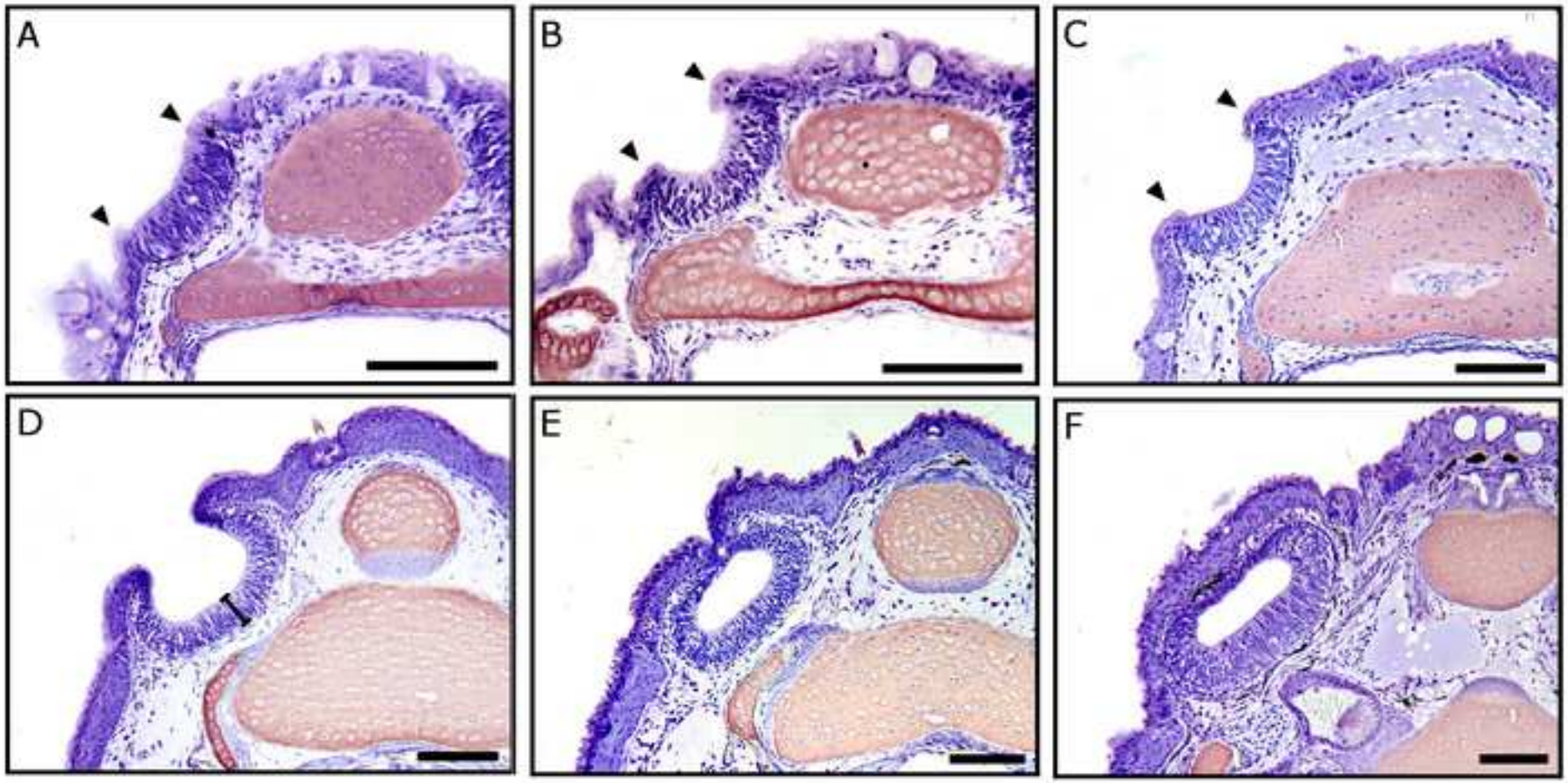


Figure 5

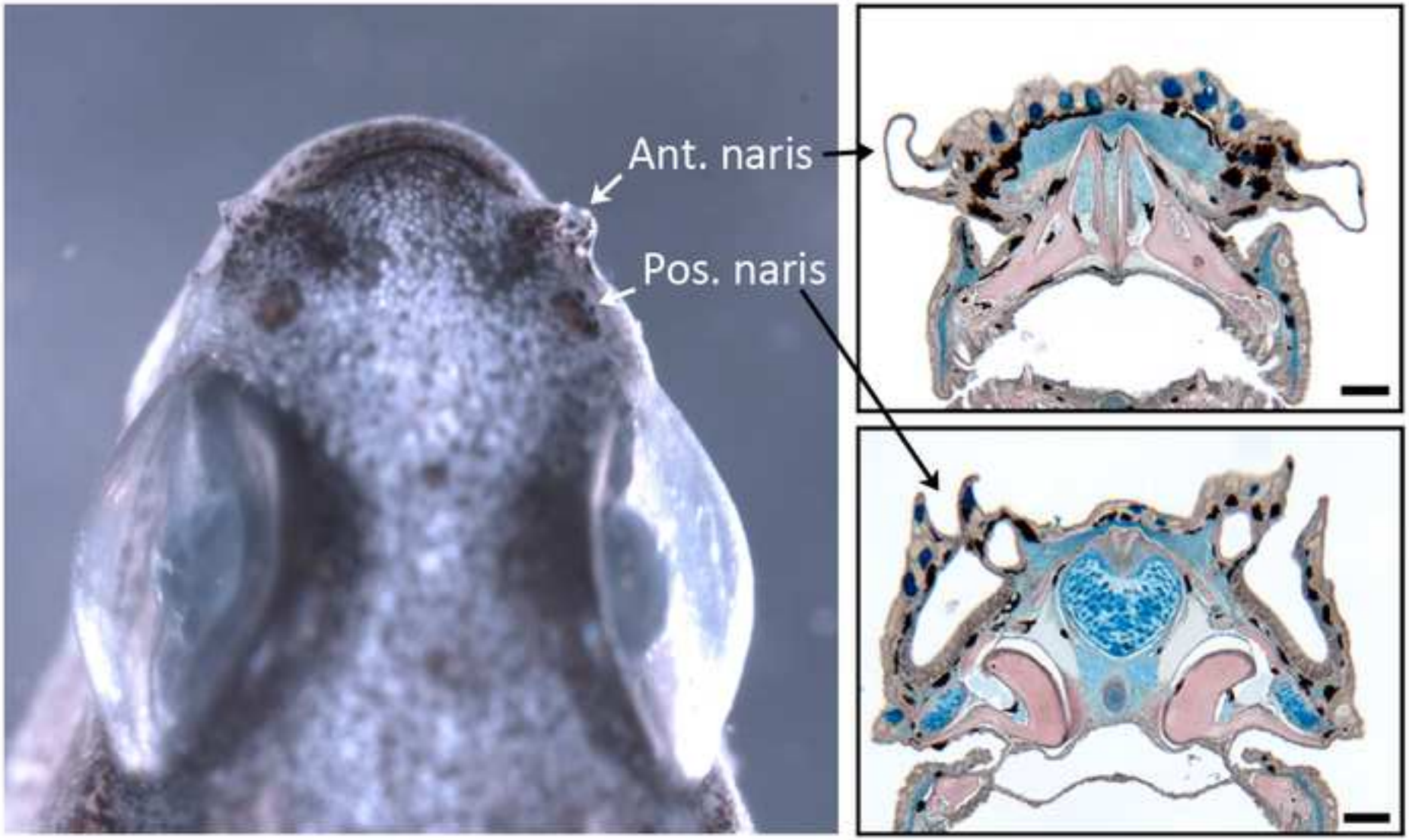
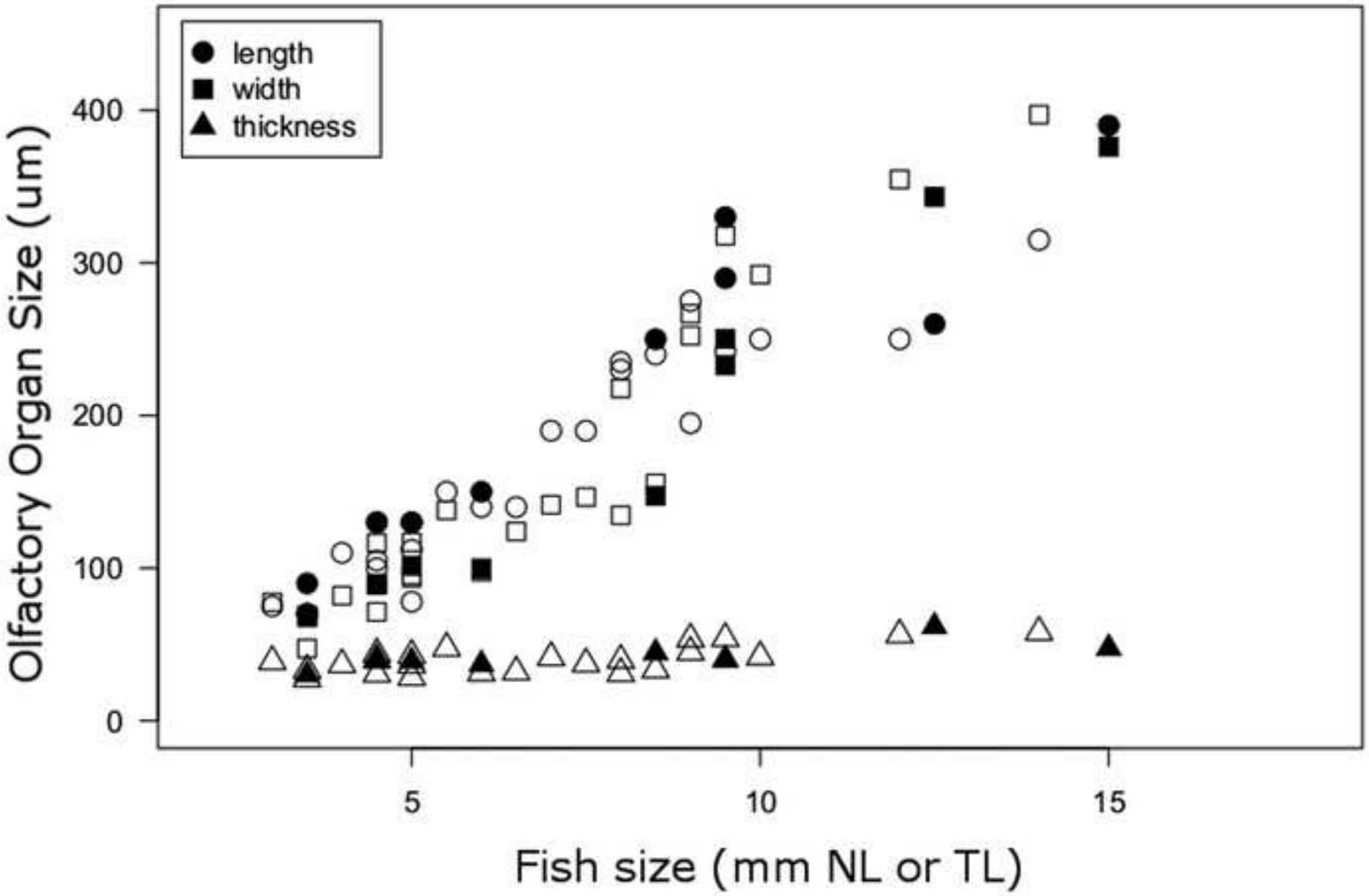


Figure 6



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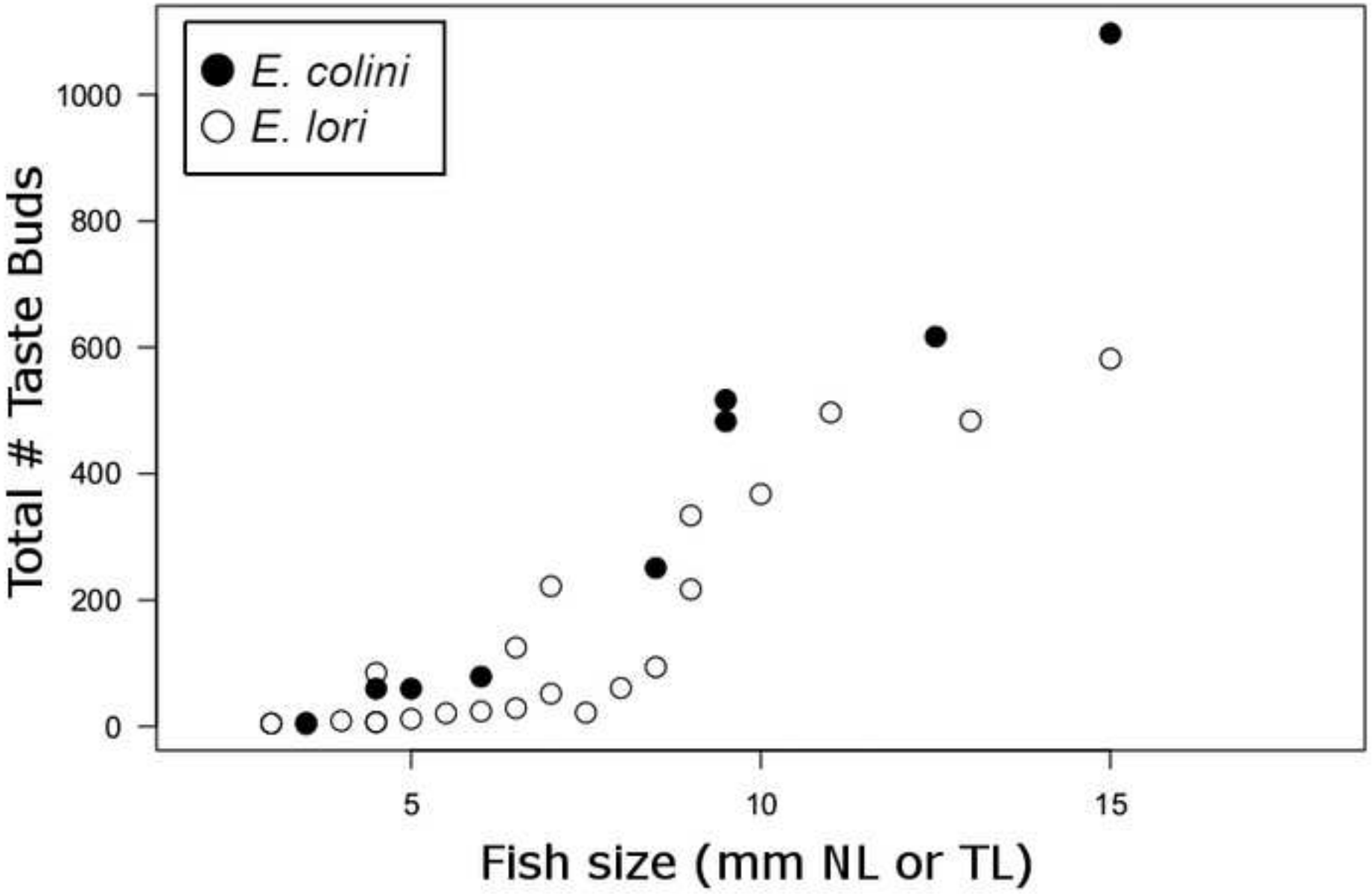

Figure 8

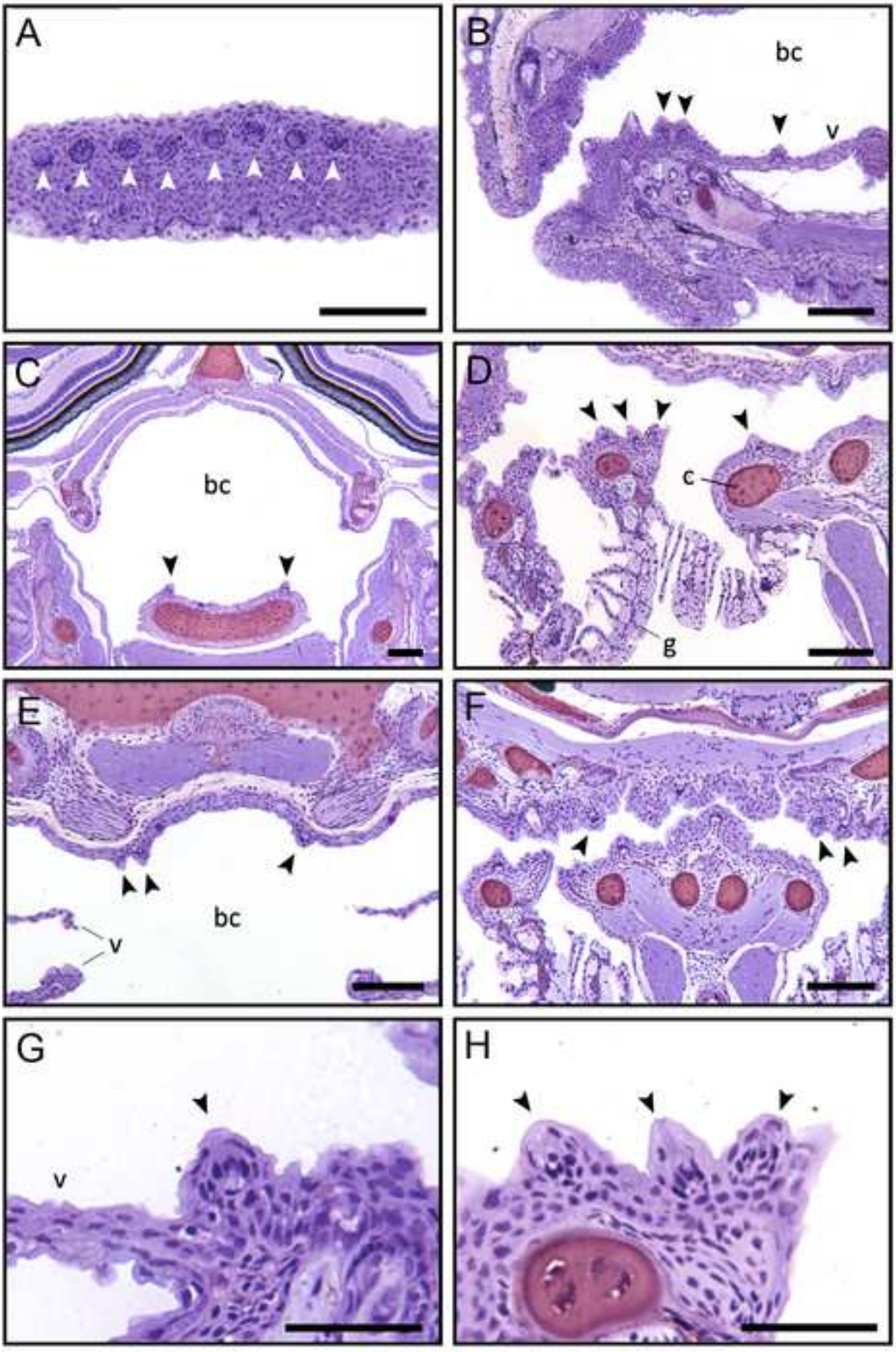

Figure 9

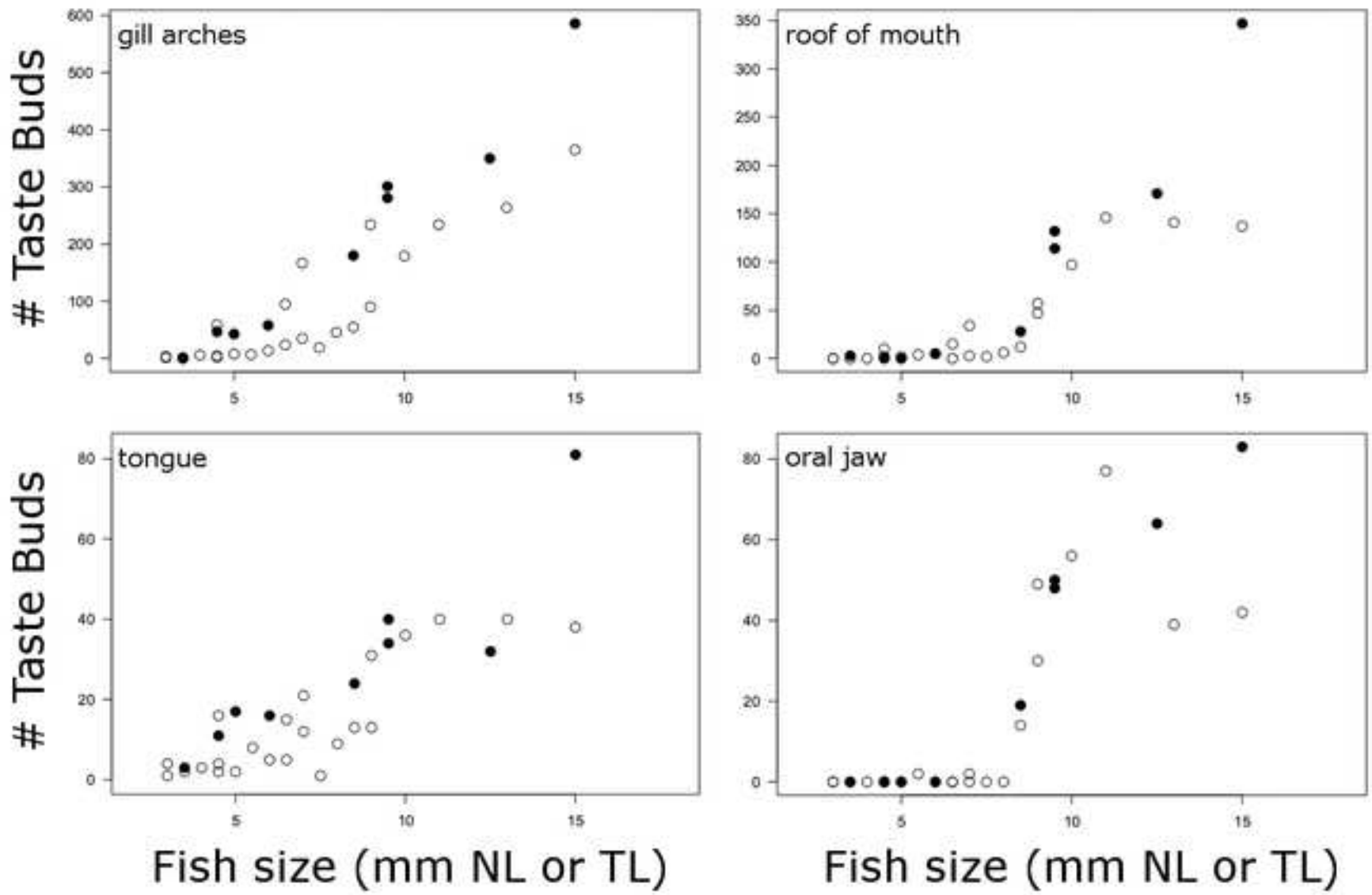

Figure 10