Loss of Sensory Elements in the Apical Sensory Organ During Metamorphosis in the Nudibranch \textit{Phestilla sibogae}

GUADALUPE J. RUIZ-JONES* AND MICHAEL G. HADFIELD†
Kewalo Marine Laboratory, University of Hawai‘i at Manoa, 41 Ahui St., Honolulu, HI 96813

Abstract. Larvae of the nudibranch \textit{Phestilla sibogae} are induced to metamorphose by a water-borne chemical cue released by the adult nudibranch’s prey, the coral \textit{Porites compressa}. In competent larvae, the apical sensory organ (ASO) includes five serotonergic parampullary neurons; five ampullary neurons, the ampullae of which are filled with sensory cilia; and a basal neuropil. After sensing the coral cue, the ASO undergoes radical morphological changes: a deterioration of sensory elements in the ASO and serotonergic axons originating from them to innervate the velum. Three hours after metamorphic induction, the velar lobes are lost, the serotonergic axons begin to break apart, the five parampullary neurons begin to degenerate, and the five ampullary neurons retract away from the epidermal surface. The extent of deterioration evident by this time suggests that the parampullary and ampullary components of the ASO are no longer functional. By 10 h after metamorphic induction, labeling of the ciliary bundles in the ampullary neurons has disappeared, and it is likely that these cells have degenerated. The results presented here provide evidence that the sensory neurons of the ASO and probably the entire organ are solely larval structures that do not persist into the adult sensory-nervous system in \textit{P. sibogae}.

Introduction

Most marine invertebrates produce free-living larvae that undergo metamorphosis, a morphogenetic event that transforms the larva into the juvenile form of the species. For many species, metamorphosis is triggered by the presence of a specific chemical cue that may be associated with their prey or ideal post-metamorphic habitat (Crisp, 1974; Hadfield and Paul, 2001). The tropical Indo-Pacific nudibranch \textit{Phestilla sibogae} Bergh 1905 (Gastropoda, Opisthobranchia, Nudibranchia) has served as a model organism for studying metamorphosis in gastropod species (e.g., Bonar and Hadfield, 1974; Hadfield and Pennington, 1990; Miller and Hadfield, 1990; Pires et al., 1997; Hadfield et al., 2000; Bishop et al., 2008). Adult \textit{P. sibogae} prey on the coral \textit{Porites compressa} Dana 1846, and competent larvae of \textit{P. sibogae} are induced to metamorphose by a water-borne chemical cue released by \textit{P. compressa} (Hadfield and Pennington, 1990).

Successive stages during the metamorphosis of larvae of \textit{P. sibogae} were classified by Bonar and Hadfield (1974). In laboratory conditions, a competent larva (stage B) placed in metamorphic inducer begins the process of metamorphosis by attaching to the substratum and losing its velar lobes (stage C). The larva next loses attachment to its shell (stage D), and then it evacuates the shell (stage E). Stage E is characterized by a distinct hump-backed body morphology. Next, the visceral hump is integrated into the body (stage F), and the juvenile progressively flattens. For the next 2 days the body continues to flatten and elongate (stage G), and the rhinophores and cerata develop until the form of a small adult is achieved (Bonar and Hadfield, 1974).

In molluscan larvae, as well as those of some other lophotrochozoan phyla, detection of external metamorphic cues is thought to be the function of the apical sensory organ.
(ASO) (summarized in Hadfield, 2011). In the Gastropoda, ASOs have been identified in larvae of the Heterobranchia, Caenogastropoda, Patellogastropoda, and Neritimorpha. In all of these groups the ASO consists of three types of cells: ampullary neurons, or “sensory cups,” and serotonergic types I and II parampullary neurons, terms first coined by Chia and Koss (1984) and modified by Kempf et al. (1997). A ciliary tuft is present on the external surface of the larva near the openings of the ampullary neurons (Bonar, 1978; Kempf et al., 1997; Hadfield et al., 2000; Page and Parries, 2000; Page, 2002; Page and Kempf, 2009). Kempf et al. (1997) reported that the elongate cilia on the ciliary tuft cells are motile and that the cells lack emergent axons, leading to the probability that, contrary to the supposition by Chia and Koss (1984), the ciliary tuft cells are not sensory. There is variation among gastropods in the number and placement of each of the ASO cell types.

Bonar (1978) first identified the three cell types in the ASO of larvae of *P. sibogae* by using transmission electron microscopy; he noted (1) five or six flask-shaped cells with sensory cilia filling a lumen that opens to the anterior surface; (2) supporting cells with numerous microvilli; and (3) vacuolated cells. For several reasons, Bonar (1978) concluded that the flask-shaped cells (later termed ampullary neurons by Chia and Koss [1984]) have a sensory function. First, their cilia differ from typical motile cilia in that their basal rootlets are very short or missing, the axonal microtubules lack dynein arms, and the few cilia that slightly protrude through the apical neck at the epidermal surface lie parallel to the apical end of the cell. Secondly, axons project from the base of the flask-shaped cells into the nervous mass at the base of these cells, which Bonar (1978) interpreted to be the cerebral commissure (later determined by Kempf et al. [1997] to be a neuropil at the base of the ASO sensory elements; see below). Moreover, the openings of the flask-shaped cells are located between the velar lobes at the entrance to the mantle cavity in an ideal location to sample water moved by the ciliary activity of the velum (Bonar, 1978).

Kempf and Page (2005) confirmed that there are five ampullary (Amp) neurons (the flask-shaped cells of Bonar [1978]) with internal cilia in the ASO of near-competent larvae of *P. sibogae* by labeling these neurons with an anti-acetylated tubulin antibody. Each Amp neuron has an external opening (ampullary pore) through which the cilia extend to the surface of the larva very near the openings of the Amp cells and the Para II cells located lower in the ASO and lacking sensory dendrites (Kempf et al., 1997). From the ASO neuropil, these axons appear to extend into the corresponding cerebral ganglion neuropil. On each side, the axons then branch, with one process extending into the ipsilateral velar lobe and the other ultimately innervating the contralateral velar lobe (Kempf et al., 1997). Because axons from the Para II neurons innervate the velar lobes, it was suggested that the ASO is also involved in regulating velar function (Kempf et al., 1997; Page and Parries, 2000; Page, 2002; Croll, 2006; Kempf, 2008; Page and Kempf, 2009). The precise role of the Para I neurons is unknown, but their structure and the presence of dendrites projecting to the epidermal surface imply a sensory role (Kempf et al., 1997; Croll, 2006).

Marois and Carew (1990) identified the ASO in veliger larvae of *Aplysia californica* (an opisthobranch gastropod), but termed it an “apical ganglion” in their 1997 publications. The sensory cells in the ASO of larvae of *A. californica* include Amp neurons, parampullary (Paramp) neurons, and ciliary tuft cells (Marois and Carew, 1997a). The velar lobes are innervated by axons originating from serotonergic Paramp neurons in the ASO (Marois and Carew, 1997b). In
A. californica, the loss of the velar lobes occurs within 24 h of metamorphic induction, and the ASO disappears by 48 h post-induction (Marois and Carew, 1997c). Marois and Carew (1997c) attributed the loss of the Paramp neurons in the ASO of A. californica during metamorphosis to cell death. Marois and Carew (1997c) used transmission electron microscopy to study the metamorphosing larvae of A. californica, and reported that the “apical ganglion, together with its serotonergic cells, is resorbed at metamorphosis.”

Available evidence suggests that receptor cells in the ASO of larvae of P. sibogae are responsible for detecting the metamorphic chemical cue and initiating metamorphosis (Bonar, 1978; Kempf et al., 1997; Hadfield et al., 2000). Hadfield et al. (2000) demonstrated that the cells in the ASO of P. sibogae must be functional for the metamorphic cue to be sensed. When DASPEI (2-(4-dimethylaminostyryl)-N-ethylpyridinium iodide)-stained cells in the ASO were irradiated with UV light and destroyed, the larvae were incapable of detecting the metamorphic cue from Porites compressa. Because exactly five cells in the ASO—the number of Amp neurons—were stained by DASPEI and the dye is known to stain mitochondria, it was very likely that the mitochondria-rich Amp neurons in the ASO were destroyed in the treatment, and thus were credited with the role of detecting the metamorphic inducer (Hadfield et al., 2000). Hadfield and Koehl (2004) reported the rapid cessation of ciliary beat on the velum and the velum’s partial retraction when tethered larvae of P. sibogae were engulfed in narrow filaments of water containing the dissolved metamorphic cue, responses that would cause rapid sinking in freely swimming larvae. While including no evidence as to the receptor for the cue, Hadfield and Koehl’s (2004) observations suggest a close tie between sensing the inducer and velar activity consistent with the innervations reported by Kempf et al. (1997) and others.

The study reported here examined the structure of the ASO in larvae of P. sibogae during metamorphosis. We asked, does the ASO remain intact throughout metamorphosis and become incorporated into the juvenile nervous system, or does it disappear once metamorphosis has been initiated? Specifically, what is the appearance of the Para II neurons in stage C larvae and later, and do the Amp neurons, presumed to be receptors for the metamorphic inducer, deteriorate after metamorphosis has commenced, because they have no known role in juveniles and adults of P. sibogae? This supposition was strongly supported by Marois and Carew (1997c) in their study of the fate of the ASO during metamorphosis of A. californica.

**Materials and Methods**

**Rearing** Phestilla sibogae

Adults of Phestilla sibogae were maintained in flow-through seawater tables at the Kewalo Marine Laboratory, University of Hawaii. Larvae of P. sibogae were reared according to the protocol reported in Hadfield et al. (2000). For the experiments described here, day 0 is the day on which the eggs were laid, and day 6 is when the egg masses were mechanically disrupted to free the swimming larvae. Our experiments were performed with larvae ages days 8 through 11—that is, 2 to 5 days after hatching.

**Preparation of larvae for immunolabeling**

Larvae of P. sibogae at the following developmental stages were collected and prepared for immunolabeling: metamorphically competent larvae at ages 8–11 days and metamorphic stages were examined in larvae from days 10 and 11 that were fixed every hour between 1 and 10 h, 24 h, and 26 h after initial exposure to the metamorphic inducer. Larvae were examined at metamorphic stages B, C, D, F, and G (Bonar and Hadfield, 1974). To prepare seawater containing the metamorphic inducer, pieces of living Porites compressa were placed in a beaker with seawater and aerated for 24 h (coral water). Competent larvae were placed into the filtered coral water for the designated period of time. Larvae at the various developmental stages (competent non-induced and metamorphosing) were relaxed in isotonic MgCl$_2$ for 15–20 min prior to fixation. The larvae were then fixed in 3.7% formaldehyde in filtered seawater for 24–48 h at 4 °C. After fixation, the larval shells were decalcified with 100 mmol l$^{-1}$ ethylene diamine tetraacetic acid in deionized water for 20–30 min. The larvae were then rinsed three times with phosphate-buffered saline (PBS) (pH 7.4) for 5 min per rinse and stored in PBS at 4 °C until they were needed for immunolabeling.

**Immunocytochemistry**

The protocol used for immunocytochemical labeling was derived from methods described by Kempf and Page (2005) and Croll (2006). After being stored in PBS, the larvae were rinsed twice in 0.1% Triton X-100 in PBS (PBT) for 20 min per rinse. The larvae were then incubated in blocking solution (1.0% bovine serum albumin [Sigma, Saint Louis, MO; catalog # B-4287] in PBT) for 25 min at 4 °C. The larvae were next labeled with polyclonal anti-serotonin (5-HT) (ImmunoStar, Hudson, WI; catalog # 20080) and monoclonal anti-acetylated tubulin (Sigma, Saint Louis, MO; catalog # T-6793) primary antibodies. Polyclonal anti-5-HT (raised in rabbit) was applied at a 1:400 dilution. Monoclonal anti-acetylated tubulin (raised in mouse) was applied at a 1:20 dilution. The larvae were incubated in the primary antibodies diluted together in blocking solution for ≈48 h at 4 °C. Next, the larvae were rinsed three times in PBS for 5 min per rinse, followed by two rinses in PBT for 20 min each. The larvae were then incubated in blocking solution for 25 min at 4 °C. The larvae were then treated with polyclonal goat anti-rabbit (Alexa Fluor 488) (Invitrogen,
Carlsbad, CA; catalog # A-11008) and monoclonal goat anti-mouse (Alexa Fluor 594) (Invitrogen, Carlsbad, CA; catalog # A-11032) secondary antibodies diluted together at a 1:400 dilution in blocking solution for 48 h at 4 °C. After this, the larvae were rinsed three times in PBS for 5 min per rinse and stored in 75.0% glycerol in PBS (v/v) until they were mounted on glass slides for examination. Controls for this experiment included (1) incubating larvae in primary antibodies only, to check for autofluorescence, and (2) incubating larvae in secondary antibodies only. In neither control was any fluorescence detected, nor were any cells labeled.

Confocal microscopy

Larvae of P. sibogae were examined for serotonergic and acetylated tubulin immunoreactivity with a Zeiss LSM 510 laser scanning confocal microscope. Stacks of scanned optical sections were compiled to make 3D projections of the larval body with the ASO in a visible position. We examined 11 competent larvae and 6–16 larvae at each stage of metamorphosis observed. Image brightness and contrast were adjusted with Adobe Photoshop CS3 10.0.1 (Adobe Systems Inc.).

Results

In larvae of Phesitlla sibogae, the Paramp neurons were serotonergic immunoreactive and the Amp neurons were acetylated tubulin immunoreactive, as previously reported by Kempf et al. (1997) and Kempf and Page (2005), respectively. Additionally, the cilia on the velar lobes and on the larval foot were acetylated tubulin immunoreactive. The structure of the apical sensory organ (ASO) was very similar in all competent larvae of P. sibogae examined. As reported by others, the ASO of these larvae had five Paramp neurons, five Amp neurons, a neuropil, and cells bearing a ciliary tuft (Figs. 1A, B, C). The ASO was bilaterally symmetrical, with the two round Para II neurons being the most lateral cells in the ASO and bilaterally positioned to each side. The three elongate Para I neurons were located between and anterior to the Para II neurons, with the middle Para I neuron positioned on the midline of the ASO. The five Amp neurons were positioned dorsal to the Para I neurons and directly posterior to the ciliary tuft, which was located on the preoral surface (Fig. 1C). It was evident that serotonergic axons originating from Paramp neurons innervate the velar lobes (Fig. 1B, C). The ASO neuropil, which includes serotonergic axons from the Paramp cells, is positioned posterior to the Para I and Amp neurons, between the Para II neurons, and dorsal to the cerebral commissure (Fig. 1B). In larvae that had been in metamorphic inducer for 1 h (image not included), the structure of the ASO remained identical to that seen in competent larvae. Larvae at this early stage of metamorphosis retained velar lobes.

About 25% of larvae that had been in metamorphic inducer for 2 h (image not included) had lost their velar lobes. In about 75% of larvae that retained velar lobes, the ASO was intact. In larvae without velar lobes (stage C), the five Paramp neurons had begun to break apart into smaller pieces, while the five Amp neurons appeared unchanged. Additionally, the serotonergic axons that had innervated the velum were beginning to fragment, although larger segments of axons were still present (image not included).

More than 50% of larvae that had been in metamorphic inducer for 3 h had lost their velar lobes. In larvae that retained velar lobes, the ASO appeared to be unchanged. In those without velar lobes (stage C), the five Amp neurons appeared to be contracted into a curl but still located in the same general location—central in the ASO—as they were in competent larvae (Fig. 1D). The Paramp neurons were no longer visible. The presence of serotonergic immunoreactive cell fragments and blebs in the region previously occupied by the Paramp neurons suggests that the Paramp neurons had undergone cell death (Fig. 1D). In stage C larvae, what were earlier continuous serotonergic axons with varicosities innervating the velar lobes now appeared as fragmented vesicular remnants of immunoreactive material, as opposed to the serotonergic axons innervating the foot, which were intact (Fig. 1D).

The majority of larvae that had been in metamorphic inducer for 4 h had lost their velar lobes (stage C). In these larvae, the five Amp neurons were morphologically distorted and had clearly lost their organization relative to the epidermal surface and ciliary tuft, which was no longer visible (Fig. 2A). These neurons had moved to new positions below the epidermis, perhaps by differential growth or some unknown mechanism in this region. At this stage of metamorphosis, the ASO appeared to be deteriorating, as indicated by the disruption of the organization of the Amp neurons and the loss of the five Paramp neurons (Fig. 2A).

Larvae that had been in metamorphic inducer for 5, 6, 7, and 8 h (images not included) appeared very similar to one another. Larvae at this stage had lost their velar lobes (stage C or stage D), and the five morphologically distorted Amp neurons were still present and scattered throughout the region previously occupied by the ASO. Small remnants of the fragmented serotonergic components (neurons and axons) in the ASO remained visible.

In larvae that had been in metamorphic inducer for 9 h (stage C or stage D) (image not included), there was a noticeable reduction in the amount of serotonergic immunoreactive cell and nerve fiber debris in the region where the ASO had been located. Between one and four distorted Amp
neurons were visible near the cerebral ganglia, bilateral to the cerebral commissure.

Larvae that had been in metamorphic inducer for 10 h (stage C or stage D) lacked all remains of the ASO and its associated neurites (Fig. 2B). The ciliary bundles of the five Amp neurons were no longer visible, and there was no remaining cell debris (blebs) from their degeneration. The antibody used to identify the Amp neurons labeled the

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**Figure 1.** Serotonergic (yellow-green) and acetylated tubulin (red) immunoreactivity in competent and metamorphosing larvae of *Phestilla sibogae*. Scale bar in all images is 20 μm. In panels A, B, and D, dorsal is up; in C, posterior is up. (A) Day 10 competent larva under DIC: the apical sensory organ (ASO) is medial to the eyes and velar lobes (the cilia of the velar lobes are acetylated tubulin immunoreactive). (B) Day 10 (same larva as in A) and (C) day 9 competent larvae: the five ampullary neurons (red) are positioned dorsal to the five serotonergic parampullary neurons (green; three type I and two type II, with the ASO neuropil located between the type II parampullary neurons); the ciliary tuft is located on the epidermal surface medial to the velar lobes; serotonergic axons, originating from the parampullary neurons and the ASO neuropil, innervate each velar lobe; the serotonergic neurons of each cerebral ganglia are positioned bilaterally to the ASO. (D) 3 h into metamorphosis: the velum has been shed; the five parampullary neurons are no longer visible; the five ampullary neurons (small arrowheads) appear as a clump within the ASO region and their shape has changed; the deterioration of the serotonergic axons (large arrowheads) is evident in the area where the velar lobes were previously attached; the foot is visible (the cilia on the foot are acetylated tubulin immunoreactive). A, ampullary neurons; ASO, apical sensory organ; C, cilia on velum; CG, cerebral ganglia; CT, ciliary tuft; E, eye; F, foot; N, ASO neuropil; NF, nerve fibers (axons); P1, type I parampullary neurons; P2, type II parampullary neurons; V, velar lobe.
ciliary bundles and probably did not label the perikarya of the Amp neurons, and thus the ultimate fate of these cells was not determined.

Larvae that had been in metamorphic inducer for 24 h and 26 h (stage F or stage G) appeared identical to each other. Evidence of the ASO was completely missing in these early-stage juveniles, and the cerebral ganglia were in their definitive positions (Fig. 2C).

It is important to note that not all larvae lost their vela when the majority of larvae at the same metamorphic stage did. A small fraction of larvae placed in inducer for up to 8 h had not initiated metamorphosis. In these larvae the ASO remained intact, as indicated by the structure of the Paramp and Amp neurons (image not included).

**Discussion**

The apical sensory organ (ASO) in gastropod larvae has been recognized as a larval sensory structure (Bonar, 1978; Kempf et al., 1997; Marois and Carew, 1997c; Hadfield et al., 2000; Page and Parries, 2000; Page, 2002; Page and Kempf, 2009). The confocal laser microscopic images collected and examined in the present study reveal that the ampullary and parampullary components of the ASO in
larvae of *Phestilla sibogae* undergo morphological changes and eventually disappear during metamorphosis (Figs. 1D; 2A, B, C). Because these cells are the major sensory components of the ASO, their disappearance strongly suggests the complete loss of the organ during metamorphosis. However, reports on a number of gastropod species differ in their descriptions of the timing and fates of cells during metamorphosis. In veligers of *P. sibogae*, breakdown of the sensory elements of the ASO begins simultaneously with the loss of the velar lobes. Similarly, in *Aplysia californica*, the ASO disappears after the velar lobes detach (Marois and Carew, 1997c). Marois and Carew (1997c) suggested that the serotonergic neurons in the ASO of *A. californica* undergo cell death during metamorphosis. In the prosobranch gastropod *Ilyanassa obsoleta*, the cells in the ASO demonstrated evidence of apoptosis as early as 12 h after induction by serotonin or 7-nitroindazole (Gifondorwa and Leise, 2006). Gifondorwa and Leise (2006) found that cells in the ASO of *I. obsoleta*, including five serotonergic neurons (presumably homologous to the five serotonergic parampullary neurons in the ASO of *P. sibogae*), underwent some form of programmed cell death during metamorphosis. In *I. obsoleta*, the ASO was no longer present 72 h after metamorphic induction, although evidence of its degeneration (cell debris and blebs) persisted (Gifondorwa and Leise, 2006). Gifondorwa and Leise (2006) found that in the nudibranch *Aeolidiella stephanieae*, five serotonergic neurons (also homologous to the five parampullary neurons in the ASO of *P. sibogae*) lie within the apical organ (i.e., the ASO); two cells in the ASO were labeled by serotonin or 7-nitroindazole (Gifondorwa and Leise, 2006). Gifondorwa and Leise (2006) noted that these cells lost their sensory function early in the developmental transition. Although it would require structural investigation, eventually, their internal cilia can no longer sense stimuli from the external environment. Whether the ampullary neurons undergo apoptosis has not been determined, but because they became detached and misshapen as early as 3 h after the onset of metamorphosis (Fig. 1D), it seems certain that these cells lost their sensory function early in this important developmental transition. Although it would require structural investigation, ultimately at the level of electron microscopy, to provide definitive evidence for the complete loss of the ASO during metamorphosis of *P. sibogae*, the data presented here strongly imply that its major sensory elements, the five serotonergic parampullary and five ampullary neurons, begin to degenerate soon after the onset of metamorphosis and are no longer apparent by 10 h after metamorphic induction (Fig. 2B). It is noteworthy that the ASOs of gastropod larvae all appear to contain two distinctive clusters of sensory cells, but only a single sensory role has thus far been demonstrated for the organ. The question thus remains, what does the other set of sensory cells detect? Definitive proof of the specific roles of each of these two or three cell types awaits experimental investigation, but such studies will be very difficult with animals as small as gastropod larvae.

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**Literature Cited**


