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SHORT COMMUNICATION

Laser ablation of the apical sensory organ of *Hydroides elegans* (Polychaeta) does not inhibit detection of metamorphic cues

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ABSTRACT

Larvae of many marine invertebrates bear an anteriorly positioned apical sensory organ (ASO) presumed to be the receptor for settlement- and metamorphosis-inducing environmental cues, based on its structure, position and observed larval behavior. Larvae of the polychaete *Hydroides elegans* are induced to settle by bacterial biofilms, which they explore with their ASO and surrounding anteroventral surfaces. A micro-laser was utilized to destroy the ASO and other anterior ciliary structures in competent larvae of *H. elegans*. After ablation, larvae were challenged with bacterial biofilmed or clean surfaces and percentage metamorphosis was determined. Ablated larvae were also assessed for cellular damage by applying fluorescently tagged FMRF-amide antibodies and observing the larvae by laser-scanning confocal microscopy. While the laser pulses caused extensive damage to the ASO and surrounding cells, they did not inhibit metamorphosis. We conclude that the ASO is not a required receptor site for cues that induce metamorphosis.

KEY WORDS: Apical sensory organ, *Hydroides elegans*, Larval development, Metamorphosis

INTRODUCTION

Communities of animals that inhabit the benthos in the seas depend on successful recruitment of very small larvae for their establishment and maintenance. Most of these animals produce larvae that spend hours to months swimming and growing in the plankton before they are developmentally competent to settle and metamorphose (Crisp, 1974; Chia and Rice, 1978; Hadfield, 1998). Then, they must locate an appropriate site for settlement; one that will provide the right habitat requirements and access to appropriate food and other conspecific individuals with which to mate and produce new generations. Each larva, equipped with few simple sensory organs, must detect unique cues from habitats and settle selectively onto the 'right' one (Hadfield and Paul, 2001). Critical questions regarding site-specific larval recruitment concern how and where on their bodies, larvae detect habitat-specific cues.

Major focus has been on the apical sensory organ (ASO), a principal cellular structure of many different types of larvae across many phyla (Nielsen, 2004; Wanninger, 2009; Hadfield, 2011; Marlow et al., 2014). Its development is typically complete at the onset of larval swimming, either as the embryos develop in the plankton or are released from parental broods or benthic egg masses, and it degenerates at the onset or quickly after metamorphosis (Marlow et al., 2014; Nelson et al., 2017). ASOs are considered homologous across lophotrochozoan phyla. Representatives of unrelated taxa within the cnidarians and bilaterians have ASOs of similar structure (Chia and Koss, 1979; Nielsen, 2004; Byrne et al., 2007; Nielsen, 2008; Miyamoto et al., 2010; Rawlinson, 2010; Hadfield, 2011; Hindinger et al., 2013; Marlow et al., 2014). The ASO, located at the anterior pole of the larva that is directed forward during swimming, consists of an apical tuft (AT) of sensory cilia arising from a cluster of sensory neurons that lie just anterior to major ganglia (Conklin, 1897; Beklemishev, 1964; Page, 2002; Yuan et al., 2008; Steinmetz et al., 2010; Marlow et al., 2014). In most taxa, the cells of the ASO are serotonergic or FMRF-amidergic (reviewed by Lacalli, 1994 and Marlow et al., 2014). The unique evolutionary constancy of the ASO suggests that its function is conserved across many larval types.

The AT and its associated cells have long been suspected to be the site of detection of cues for settlement and metamorphosis (Hadfield, 2011), although experimental evidence for this exists for only a few species (e.g. Hadfield et al., 2000). As they prepare to settle, larvae of many phyla behave in a manner that suggests they are testing the substratum (Segrove, 1941; Marsden and Anderson, 1981; Gabilondo et al., 2013; Nelson et al., 2017); they swim close to the surface at slower speeds than when swimming in the plankton and they orient themselves with their apical end toward the substratum, so the AT brushes the surface (Barnes and Gonor, 1973; Nott, 1973; Marsden and Anderson, 1981; Zimmer, 1991; Gabilondo et al., 2013; Nelson et al., 2017).

The globally distributed serpulid polychaete *Hydroides elegans* (Haswell 1883) has emerged as a useful model for studying larval settlement, especially induction by specific biofilm bacterial species (Hadfield et al., 1994; Unabia and Hadfield, 1999; Lau et al., 2002; Huang and Hadfield, 2003; Nedved and Hadfield, 2009; Huang et al., 2012; Freckelton et al., 2017; Vijayan and Hadfield, 2020). Evidence for cue detection is strong: larvae of *H. elegans* typically will not settle in the absence of a biofilm, and induction of metamorphosis by bacteria requires contact with a biofilm (Hadfield et al., 2014). Furthermore, while numerous biofilm bacterial species will induce metamorphosis, most do not (Unabia and Hadfield, 1999; Lau and Qian, 2001; Vijayan and Hadfield, 2020).

There are at least three potential sites for chemoreception of bacterial cues on the episphere of competent larvae of *H. elegans*, the first being the ASO, which is prominent at the apex of the episphere. Cilia of the AT project from the ASO into the environment (Fig. 1A). Lacalli (1984) reconstructed the ASO in metatrochophore larvae of another serpulid polychaete *Spirobranchus polycerus* and found it to consist of 12 cells. Five multi-polar, apical cells project processes that constitute most of the apical plexus. The AT arises from two of these apical cells. The remaining seven cells encapsulate the multi-polar apical cells (A1–A5) (Lacalli, 1984).

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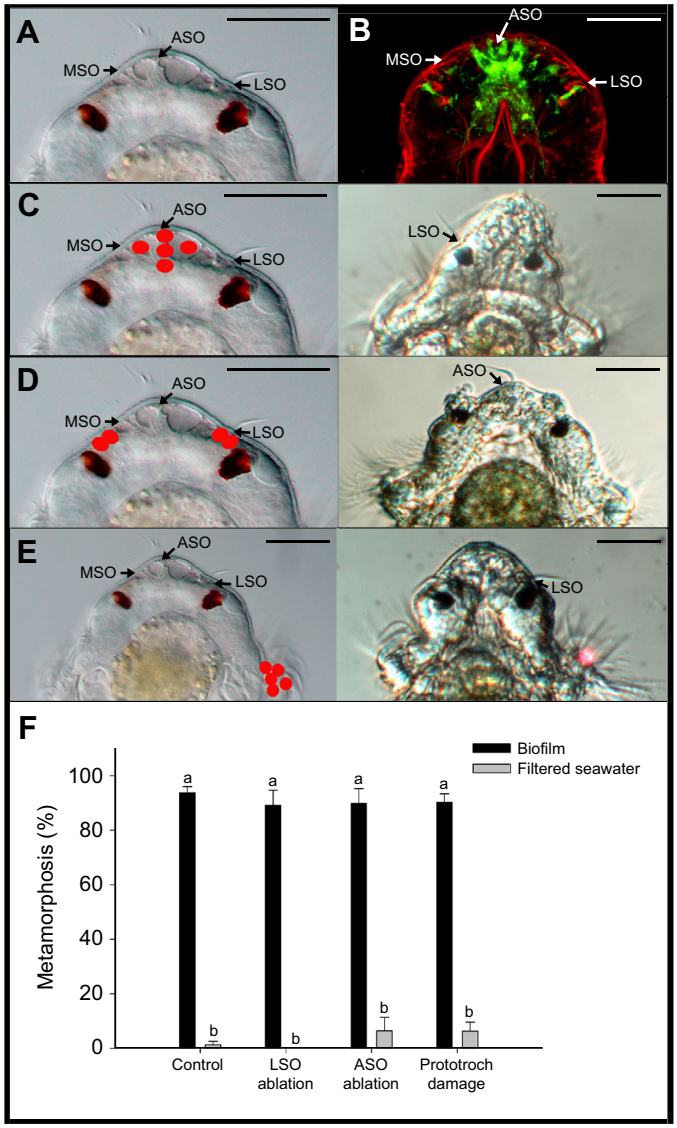

Fig. 1. Location of targeted cells and damage caused by laser ablations of the anterior sensory organ (ASO) and surrounding sensory cells in larvae of *Hydroides elegans*. (A) Morphology of the episphere of a competent larva. (B) Location and morphology of FMRFamide-immunoreactive cells (green) in the episphere of a competent larva. (C) Left, locations of five laser pulses administered to ASO; right, morphological damage caused by 5 pulses. (D) Left, location of 4 laser pulses administered to LSOs; right, damage caused by 4 pulses to LSOs. (E) Left, location of 5 laser pulses administered to the right side of the prototroch; right, damage caused by five pulses to prototroch. (F) Percentage of larvae that metamorphosed 14–16 h after ablation of the cells within the ASO (5 pulses), LSOs (2 pulses on each) and cells in the prototroch (5 pulses). *N*=4 replicate dishes. Dishes contained 12–24 larvae. The experiment was performed once. Kruskal–Wallace test, $P<0.0006$. A Dunnett's test compared treatments against the control treatment (Biofilm, 0 Pulse). Data show means±s.e.m. Bars with the same letter are not significantly different from the control. ASO, apical sensory organ; MSO, medial sensory organ; LSO, lateral sensory organ. Scale bars: 25 µm.

In competent larvae of *H. elegans,* there are four sensory cells in the ASO and an additional 12–4 cells in the cerebral ganglia that are strongly immunoreactive to FRMF-amide antibodies. Anti-serotonin antibodies label only cells within the cerebral ganglia and do not label cells within the ASO (Nedved, 2010) (Fig. S1). While there are currently no serial reconstructions of the ASO of *H. elegans*, the number and morphology of immunoreactive cells indicate it is morphologically similar to the ASO of *S. polycerus.*

On each side of the ASO are two additional sensory patches. Most distal are the lateral sensory organs (LSOs, Fig. 1A), each including two cilia-bearing, FMRF-amide positive cells (Fig. 1B). The medial sensory organs (MSOs, Fig. 1B) lie between the LSOs and the ASO. One sensory cell within the MSO is FMRF-amide positive and projects a fiber into the cerebral ganglia.

The goal of the research presented here was to test the hypothesis that the ASO is the receptor for bacterial settlement cues in *H. elegans*. We employed micro-laser destruction of the targeted ciliary organs (ASO, MSOs, and LSOs) followed by microscopic observations of larvae exposed to FMRF-amide antibodies to determine the extent of the damage and bioassays to determine if larvae with destroyed sensory organs could still detect bacterial cues and respond to them.

MATERIALS AND METHODS

Culture of larvae

Adults of *Hydroides elegans* were collected from Pearl Harbor, HI (21°21′25.5″ N, 157°57′35.9″ W) and maintained in flowing seawater at the Kewalo Marine Laboratory of the University of Hawai'i at Mānoa. Diecious adult worms were induced to spawn by removal from their tubes. Fertilization occurred in 0.22 µm filtered seawater (FSW), and development progressed to the feeding trochophore stage in ∼12 h. Larvae were cultured using the methods of Nedved and Hadfield (2009). Briefly, larvae were maintained in FSW at a density of 10 larvae ml^{-1} at 25–26°C. Larvae were fed the single-celled alga *Isochrysis galbana* (Tahitian strain) at a concentration of 6×10^4 cells ml^{-1}. The culture vessels and FSW were changed daily to minimize buildup of bacterial films. Larvae became metamorphically competent 5 days post-fertilization; competent larvae were used in all experiments and assays.

Laser ablation of sensory cells

Larvae were relaxed in 3.7% $MgCl_2$ in FSW. Five larvae were pipetted in a small volume of seawater onto a Rain-X®-coated microscope slide. Larvae were covered with a #1 coverslip that had its edges supported by a double thickness of tape, which served to immobilize the larvae. By moving the coverslip, larvae were rolled until their dorsal sides were facing up. Cell ablations were performed using a XYclone infrared laser (Hamilton Thorne, Beverly, MA) mounted to a 20× objective on a Zeiss Axiophot upright microscope at 100% power. The laser was aimed at the selected location on the larva and 500 µs pulses were fired the desired number of times (Fig. 1). This procedure was then repeated for each larva on the slide. Two different types of controls were utilized in all ablation experiments. A laser control was implemented by focusing on the right side of the prototroch and administering five pulses. A set of handling controls was also implemented. For these controls, a subset of larvae was pipetted, immobilized and rolled on a slide. No laser pulses were applied in this treatment. After ablation, the coverslips were carefully removed, and the water droplet containing the ablated larvae was removed from the slide by pipetting. Larvae were deposited into dishes of FSW and allowed to recover for 1 h before use.

Induction of metamorphosis in treated and untreated larvae

After experimentation, both ablated and unablated larvae were split into two treatments: (1) exposed to a complex natural bacterial biofilm at least 2 weeks old or, (2) kept in FSW, both in 35 mm Petri dishes. Unablated, handling control larvae were exposed to a

bacterial biofilm and used as a positive control (0 pulses+Biofilm). Handling control larvae were kept in FSW and used as negative control treatments (0 pulses+Filtered Seawater) in metamorphic assays. Four replicate dishes were used for each treatment, and 7–28 larvae were added to each dish. The proportion of larvae that had metamorphosed was determined after 14–16 h. Proportional data were transformed by the arc-sine of the square root of the variate (Sokal and Rohlf, 1981). Transformed data failed the Shapiro–Wilks test for normality, and the transformed means were compared using the non-parametric Dunnet's test using the Analyse-it (v. 5.65) statistical software for Microsoft Excel (Analyse-it Software, Ltd; https://analyse-it.com). Means and standard errors were back-transformed to percentages and plotted in SigmaPlot (v. 12.3, Jandel Scientific Software Inc.).

Visualizing damage to sensory cells

The immunocytological methods described below were modified from Croll and Chiasson (1989) to determine the expression of the neuropeptide FMRF-amide in the developing larvae of *H. elegans*. FMRF-amide antibodies were chosen because they label cells of the ASO, as well as sensory cells in the LSOs and MSOs, plus cells within the cerebral ganglia, and axons in the cerebral commissure.

The laser-ablation methods described above were administered to additional larvae. After recovering, each larva was pipetted into a separate well of a 24-well plate that also contained a chip of biofilmed microscope slide. Larvae were monitored once an hour, and if a larva had begun to metamorphose and had secreted a primary tube, the chip was transferred to a dish containing ice-cold 3.7% $MgCl_2$ in FSW for 5 min to anesthetize the larva. Although these larvae had irreversibly begun the metamorphic cascade, they still retained the ASO, MSO and LSO sensory complexes. After larvae were relaxed, an eyelash brush was used to gently coax each metamorphosing larva from its primary tube. Larvae were then fixed for 1 h in 4% paraformaldehyde in FSW (4°C). After fixation, larvae were washed (3×5 min) in phosphate buffered saline (PBS; 50 mmol l^{-1} Na_2PO_4, 140 mmol l^{-1} NaCl, pH 7.4). Larvae were then washed (3×30 min) in PBT (PBS+0.01% Triton X-100) and further incubated for 20–30 min in blocking solution (PBT+5% v/v heat-inactivated normal goat serum). Larvae were incubated for 2 days at 4°C in the polyclonal antibody anti-FMRF-amide (raised in rabbit, ImmunoStar, Hudson,WI, cat. no. 20091, lot no. 1807001), at a dilution of 1:500 (v/v in blocking solution). After incubation in primary antibodies, larvae were subjected to three 2–3 min washes with PBT, followed by three washes (30 min) in PBT, and then incubated in blocking solution (30 min). Larvae were subsequently incubated for 12–24 h at 4°C in Alexa Fluor®488 goat anti-rabbit (1:1000 dilution, Molecular Probes, cat.no. A-11008). After incubation in the secondary antibodies, larvae were washed several times in PBS, immersed overnight in 3:1 glycerol:PBS, and mounted on glass microscope slides in the PBS:glycerol solution.

In order to visualize the position of labeled nerves within each larva, fluorescently tagged phalloidin was used as a counterstain to provide morphological landmarks within the larvae. A 1:500 dilution of Alexa Fluor® 594 phalloidin (Molecular Probes, cat. no. A-12381) was co-incubated with the secondary antibody solutions. Labeled larvae were examined using a Zeiss LSM 710 scanning confocal microscope (Zeiss, Germany) equipped with the appropriate laser and filter combinations. Digital images of optical sections of the preparations were produced using the Zen imaging software package (v. 4.3, Zeiss) and single plane projections of these images, which have a greater depth of focus than single images, were produced using the same software package. Composite plates of these images were constructed using Adobe Photoshop CC2019 (Adobe Systems, San Jose, CA), and the brightness and contrast of each figure was adjusted to provide consistency within the plate.

Immunocytochemistry of untreated larvae

The previously described immunocytological methods were used to document the position of cells expressing the neurotransmitter serotonin. Identical procedures were employed with the following exception, competent larvae of *H. elegans* were incubated in a polyclonal primary serotonin antibody (raised in rabbit, ImmunoStar, Hudson,WI, cat. no. 20080, lot no. 1807001), used at a dilution of 1:500 (v/v in blocking solution).

RESULTS AND DISCUSSION

Ablation of the ASO and MSOs

Five laser pulses administered to the ASO region caused extensive damage to the ASO itself, the apical tuft and the MSO; 198 of the 200 larvae survived the treatment. These pulses also damaged epithelial cells adjacent to the ASO, changing the shape of the episphere in some larvae (Fig. 2C). Rarely, one FMRF-amide-positive cell remained in metamorphosing worms (Fig. 1C and Fig. 2C,F, and Table S1). Despite loss of the cells underlying the apical tuft and MSO cilia, 91% of ablated larvae completed metamorphosis when exposed to a biofilm (Fig. 1F).

Ablation of the LSO

Four laser pulses administered to the LSOs ablated all lateral sensory cells in 73% of larvae examined (Fig. 1D and Fig. 2B). In eight larvae, at least one (of two on each side) FMRF-amide-positive cell associated with either the left or right LSO remained. These lateral pulses left the ASO, MSO and the cerebral ganglion undamaged but destroyed the sensory cilia of the LSOs. (Fig. 2B,E). Of larvae that had been exposed to the lateral laser pulses, 90% completed metamorphosis when exposed to a biofilm (Fig. 1F).

Ablation of prototroch cells

As control experiments, laser ablations targeted cells in the prototroch, which are neither sensory nor FRMF-amide immunoreactive. Most (99%) larvae survived either 5 or 9 laser pulses to the right side of their prototrochs. The episphere was not damaged (Fig. 1E), and 93% of these larvae metamorphosed when exposed to a biofilm, a result that is consistent with the response of larvae that have not been subjected to a laser pulse (Fig. 1F).

Ablation of all known sensory cells in the larval episphere

Because ablation of the LSOs, MSOs or ASO did not inhibit sensing of metamorphic cues and induction of metamorphosis, we used nine laser pulses to ablate all five of these sensory organs in 82 larvae (Fig. 3A,B). Survival of these larvae was high (95%), and 88% metamorphosed when exposed to a biofilm (Fig. 3C). That is, destruction of the ciliary organs hypothesized to be the sites of bacterial cue detection was not effective in reducing the larval response to the cue. Importantly, the loss of neurons within the ASO did not, alone, induce settlement and metamorphosis.

Because laser ablations of the ASO and nearby sensory cells did not prevent larvae of *H. elegans* from sensing metamorphic cues, we conclude: (1) if the ASO is a detector for bacterial settlement cues, it is not alone in this respect; other receptors must be present in other anterior regions of the larva; and (2) the function of the ASO may be

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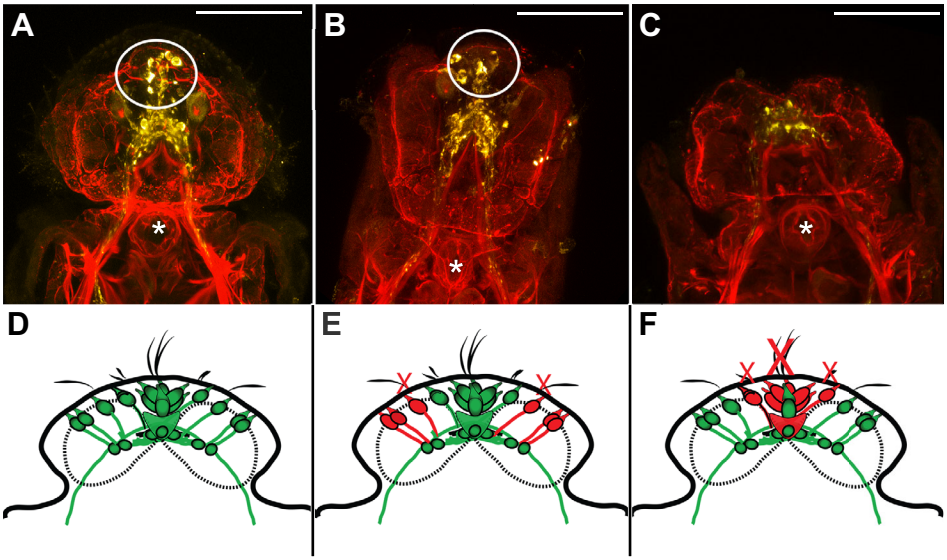

Fig. 2. Cellular damage by laser ablation in the apical sensory organ, medial sensory ciliated organs and lateral sensory ciliated organs in *H. elegans*. (A–C) Anti-FMRFamide immunoreactive cells (yellow) in a metamorphosing larva after (A) 5 laser pulses to prototroch, (B) 4 lateral pulses damaged sensory cells underlying lateral sensory cilia and medial sensory cilia (LSOs) and (C) 5 laser pulses caused extensive damage to the ASO. Preparations were co-labeled with phalloidin (red). White circles indicate the position of ASO. Asterisks show the position of the larval mouth. (D) Schematic diagram of FMRFa-positive cells in an intact larva (*N*=30). (E) Schematic diagram of damage caused by 4 lateral laser pulses (*N*=30). (F) Schematic diagram of damage caused by 5 laser pulses to the ASO and MSOs. *N*=36 Green cell bodies and fibers are FMRFa positive and undamaged. Red color indicates cells destroyed by laser pulses. Red Xs overlay cilia destroyed by laser. Scale bars: 50 µm.

for a different sensory modality, discussed below. Given that pre-settling larvae of *H. elegans* search for cues by swimming across substrata with their anteroventral regions pressed against the surface, it may be that bacterial-cue receptors are located on cilia of the ventral portions of the prototroch, metatroch or food groove, especially around the mouth. We have observed that bacteria are actively swept into the mouth by the actions of prototroch, metatroch and food-groove cilia (Vijayan and Hadfield, 2020), and it is possible that settlement receptors lie inside the mouth. This location has previously been suggested by Biggers et al. (2012) for the polychaete *Capitella teleta*. However, ablation of these oral regions would be difficult without inflicting other major damage. It remains possible that the anterior sensory structures deleted in our experiments do detect bacterial cues, but not exclusively.

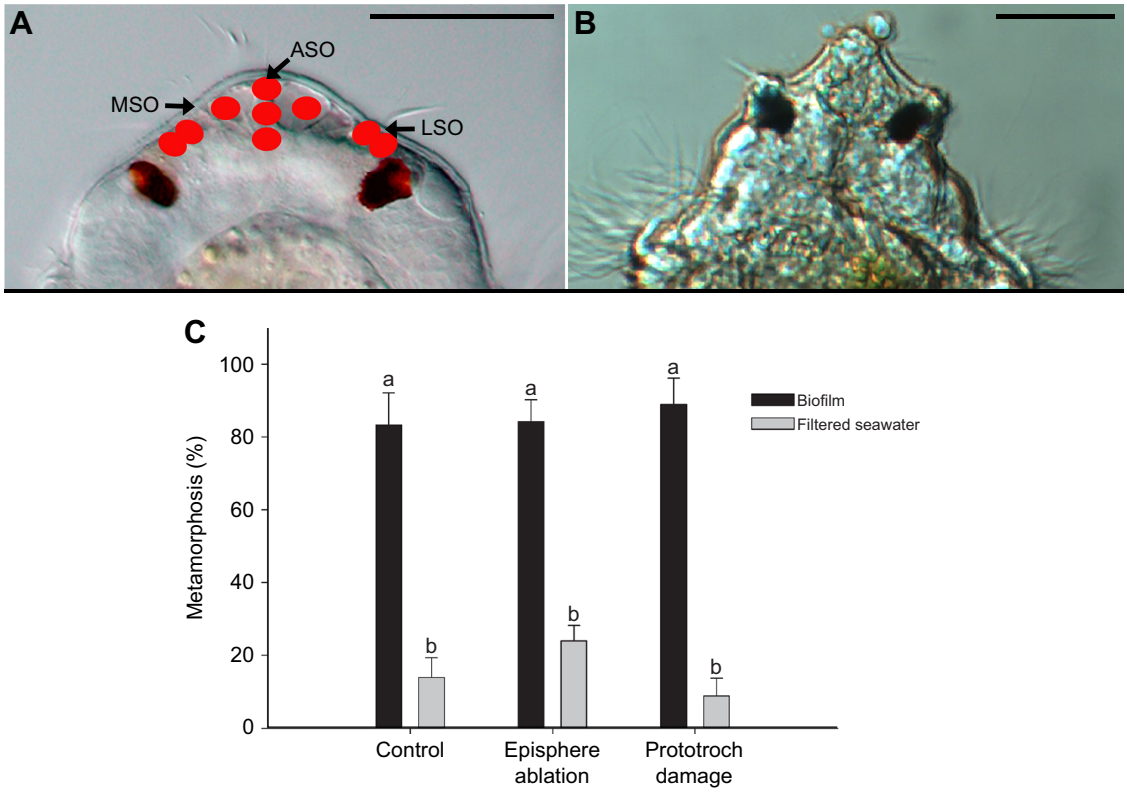

Fig. 3. Destruction of the ASO and surrounding tissues does not prevent larvae of *H. elegans* from metamorphosing. (A) Location of 9 laser pulses administered to the episphere of larvae. (B) Damage caused by nine laser pulses. (C) Percentage of larvae that metamorphosed after laser ablation. 0 pulses=handling controls; Episphere ablation results are from larvae receiving 9 laser pulses to episphere as indicated in A. Prototroch damage results are larvae receiving 9 laser pulses to right side of prototroch (as shown in Fig. 1E). *N*=4 replicate dishes; dishes contained between 5 and 20 larvae. The experiment was performed once. Kruskal–Wallace test, *P*=0.002. A Dunnett's test compared treatments against the control treatment (Biofilm, 0 Pulse). Data show means ±s.e.m. Bars with the same letter are not significantly different from the control. Scale bars: 25 µm.

Based on both developmental and molecular evidence, the ASOs of annelid and molluscan larvae are considered homologous structures (Nielsen, 2004; Marlow et al., 2014). The ASO of veliger larvae of the nudibranch *Phestilla sibogae* is the sensor for metamorphic cue, and its ablation prevents larvae from sensing the cue and undergoing metamorphosis (Hadfield et al., 2000). However, this function for the ASO may not be completely conserved even within the gastropods. Observations of pharmacologically manipulated, encapsulated, larval-stage embryos of freshwater snails, led Voronezhskaya and Khabarova (2003) to propose that the main function of the ASO was to inhibit metamorphosis until an appropriate moment. A similar function for the ASO was suggested for larvae of *Tritia (Ilyanassa) obsoleta*, i.e. a subset of cells within the ASO acts as an inhibitor of programmed cell death of the ASO, and stimulation of these cells by a mixture of cues from bacteria and diatoms triggers both metamorphosis and the destruction of the ASO (Froggett and Leise, 1999; Leise et al., 2004; Hens et al., 2006). If the ASO of *H. elegans* serves a similar function, its removal should result in metamorphosis, which did not occur in our experiments (Fig. 3C).

If the ASO is not a receptor for settlement cues, what is its role in the biology of the larvae of *H. elegans*? Marlow et al. (2014) proposed that ASOs evolved as multimodal structures that may have been co-opted for different functions in different lineages. We have observed that apical cilia of swimming larvae of *H. elegans* are stiff, but bend when a larva makes a turn (our unpublished results). It is thus possible that it functions in positional sensing in a turbulent world (see Koehl and Cooper, 2015), rather than, or in addition to, chemo-sensing. Marsden and Anderson (1981), observing that contact of the apical tuft caused larvae of the serpulid polychaete *Galeolaria caespitosa* to stop swimming and change direction, suggested the ASO may act as a mechanoreceptor. Several lines of evidence suggest that the sensory cells within the ASO of larvae of *Platynereis dumerilii* directly influence the speed of ciliary beat in its prototroch and may directly influence the position of the larva in the water column (Conzelmann et al., 2011; Verasztó et al., 2017; Bezares-Calderón et al., 2018).

Finally, cells within the ASO of *P. dumerilii* produce an orthologue of the myoinhibitory peptide (MIP), which induces settlement of both competent and pre-competent larvae but does not induce metamorphosis on its own (Conzelmann et al., 2013). It is currently unknown if cells within the ASO of *H. elegans* produce MIP, but laser ablation of the ASO and surrounding cells did not prevent competent larvae from settling and metamorphosing. Indeed, it is possible that the ASO in *H. elegans* is important in mechano- and chemo-sensing of settlement sites, but impulses from the ASO alone do not provide the ultimate signal to attach and metamorphose, implying a role for additional, as yet undiscovered, anteroventral receptors.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: B.T.N., M.G.H.; Methodology: B.T.N., M.G.H.; Formal analysis: B.T.N., M.L.F.; Investigation: B.T.N.; Resources: M.G.H.; Data curation: M.L.F.; Writing - original draft: B.T.N., M.L.F., M.G.H.; Writing - review & editing: B.T.N., M.L.F., M.G.H.; Supervision: M.G.H.; Funding acquisition: B.T.N., M.G.H.

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Data availability
Data are available from figshare under accession numbers: 13585052, 13585172 and 14782296.

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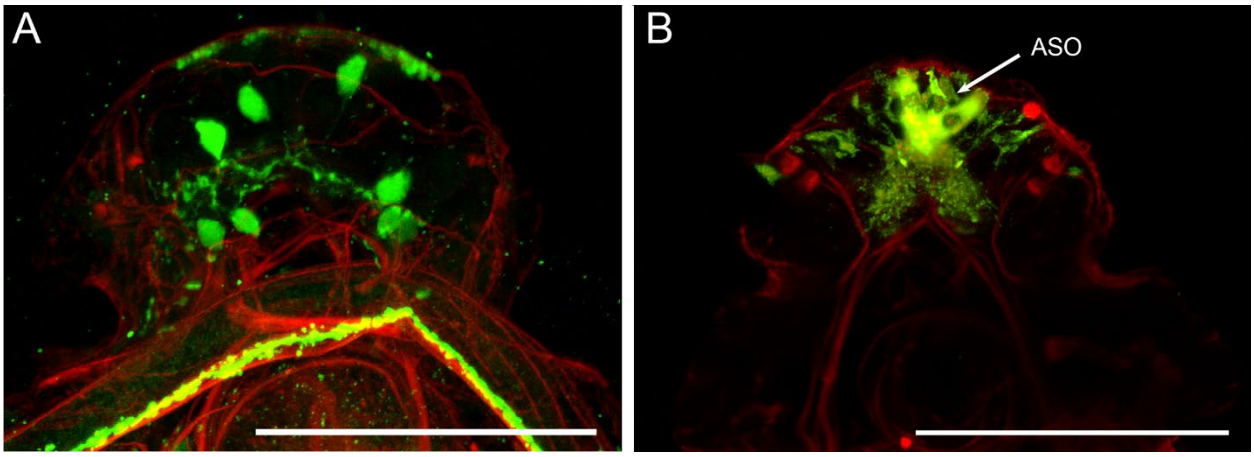

Fig. S1. Neuronal development in episphere *of competent larvae of H. elegans.* A. Serotonin immunoreactivity in episphere of larva. Eight cell bodies were labeled by serotonin antibodies in the cerebral ganglia of larvae. No sensory cells in the episphere of competent larvae express serotonin. C. FMRF-a immunoreactivity in episphere of larva. Four sensory cells were immunoreactive for FMRF-a in the ASO or larvae. Scale bars = 25 μm.

Table S1. Percentage of larvae having FMRF-a positive cells destroyed by laser ablation.

Treatment	Larvae with all cells destroyed in LSO (%)	Larvae with all cells destroyed in MSO (%)	Larvae with all cells in ASO destroyed (%)
4 Lat Pulses	87	0	0
5 Pulses	0	100	92
Prototroch	0	0	0