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Sniffing Out New Data and Hypotheses on the Form, Function, and Evolution of the Echinopluteus Post-Oral Vibratile Lobe

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Abstract. The performance requirements of ciliary band feeding explain the convoluted forms of many marine invertebrate larvae. Convolutions increase surface area and therefore feeding rates per unit body volume. We review recent advances in morphology, neural development, and behavior at settlement of the echinoid *Lytechinus pictus* and provide new ultrastructural and expression data on larvae of its congener, *L. variegatus*. Larvae of the echinometrid *Colobocentrotus atratus* contain neurons identified by their expression of nitric oxide synthase (NOS), indicating that this character is not unique to *Lytechinus*. We hypothesize that in some echinoids the convoluted shape of the post-oral vibratile lobe (POVL) covaries with the distribution of identified sensory neurons to enable olfaction during settlement. An analysis of variation in structural elaboration of the post-oral transverse ciliary band (PTB) within Echinoida and in feeding larvae of other echinoderm classes indicates that only echinoids, but not all echinoids, possess this novel character; larvae that do are distributed heterogeneously within the class. In recognition of this specialized function for the POVL and surrounding ectoderm, and because it is lobate and grows toward the mouth, we propose naming this structure the adoral lobe.

Introduction

All phytoplanktivorous larvae have evolved mechanisms to swim and concentrate particulate food from seawater. As suspension feeding is the main function of such larvae, the evolution of many larval forms is intimately connected with and constrained by this primary requirement (Strathmann, 1978, 1987; Emlet, 1991). The swimming and feeding mechanism employed by larvae of several marine phyla employs cilia arranged into bands. Ciliary bands consist of multiple rows of cilia used to direct either seawater containing particulate food past the mouth or individual particles of food into the mouth. This is accomplished by a single-band upstream particle-capture system in echinoderms and hemichordates, but by an opposed-band feeding system in several lophotrochozoan phyla (Strathmann, 1971; Strathmann *et al.,* 1972; Hart, 1991). In both cases larvae can simultaneously swim and clear a volume of surrounding seawater of food using the same organ system. For earlier accounts of echinoderm larval feeding, see Gemmill (1914, 1916), Runnström (1918), and Tattersal and Sheppard (1934).

Ciliary bands are frequently present on ridges of the larval body, a feature that optimizes their functionality by increasing the volume of water moved with each swimming stroke (Emlet, 1991). Other than placing ciliary bands on body ridges, there are three mutually inclusive solutions to increasing the amount of food that larvae of a given volume can collect per unit time. Larvae can grow longer cilia, grow more cilia, or increase the total length of the ciliary band (Emlet and Strathmann, 1994). Among echinoderm, hemichordate, and lophophorate larvae, substantial increases in clearance rates are effected exclusively by increasing the length of the ciliary band per unit body volume (Strathmann, 1971; Hart, 1991). The main evolutionary outcome is larvae with convoluted shapes. Conversely, larvae that do not need to feed to reach the juvenile stage have repeatedly evolved into less convoluted forms (Strathmann, 1978). This evolutionary pattern is not unique to echinoderms (for reviews, see Strathmann, 1978; Hart, 2000). Thus, although

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Abbreviations: ADL, adoral lobe; EI, elaboration index; NDN, NOS-defined neurons; NOS, nitric oxide synthase; POVL, post-oral vibratile lobe; PTB, post-oral transverse ciliary band.

not explaining their disparities in body plan, food-capturing performance has provided a functional and morphological perspective from which to understand the varied and peculiar forms that characterize many suspension-feeding larvae of benthic invertebrates.

Among echinoderm planktotrophs, echinoid and ophiuroid pluteus larvae solved the problem of increasing ciliary band length per unit body volume by evolving (sometimes very long) arms supported by a calcite endoskeleton. When pluteus larvae begin feeding they have two or four arms. As the larvae increase in size, more arms develop. The range of arm number among different species of fully grown echinoid larvae is from 2 to 12 (Mortensen, 1921, 1931, 1937, 1938), raising the question of why there is variation in arm number, particularly since larvae of several species can change the length of larval arms in response to food levels (Boidron-Metairon, 1988; Strathmann *et al.,* 1992; Sewell *et al.*, 2004), thereby modifying maximum clearance rates in a fluctuating and food-limited environment (Hart and Strathmann, 1994).

Arms, however, do not constitute the totality of echinoid larval convolutions. In addition to arms as appendages that increase the length of the ciliary band, many echinoid larvae contain vibratile lobes (in Fig. 1, for example, compare larvae 1, 13, 20 to larvae 2, 10, 15). Like arms, vibratile lobes are epidermal outgrowths lined by ciliary band. They can be contrasted with *bona fide* arms as they are not supported by skeletal rods. Because echinoid vibratile lobes are lined by ciliary band and develop later in larval life as larvae increase in size, it may be safely assumed that they function in the same manner as arms: to increase total clearance rates (Emlet and Strathmann, 1994). McEdward (1984) calculated that 32% of the ciliary band of the sand dollar *Dendraster excentricus* was contained on tissues other than its eight arms. For a given arm number, this percentage will increase among larvae bearing vibratile lobes. However, we know of no study that has asked whether echinoid vibratile lobes, like arms, display plasticity in size in response to feeding levels. Wray (1992) cited reports by Strathman (1971) and Hart (1991) as indicating that larvae with vibratile lobes and epaulettes swim faster. Strathmann (1971) interprets lobes as locomotory specializations, but we could find no specific reference (*i.e.,* considering vibratile lobes separately from epaulettes) in either that report or in Hart (1991) to a known relationship between swimming speed and vibratile lobes. Although it is clear from personal observations made by CDB that larvae with epaulettes swim faster, it has not been shown that any or all vibratile lobes, and particularly those that are anatomically independent of epaulettes (such as the POVL, or post-oral vibratile lobe), have this effect on swimming speed.

There is currently no functional explanation for the large degree of variation in the presence (range 0–10; Mortensen, 1921, 1931, 1937, 1938) and size of vibratile lobes across taxa. On the basis of parsimony, vibratile lobes are interpreted as symplesiomorphies of crown group echinoid larvae, with the attendant interpretation that in various lineages, some vibratile lobes have been lost and then regained (Wray, 1992, and others). Covariance between the presence and absence of different lobes was not explicitly tested in Wray (1992), but it is evident that some lobes have been lost and regained independently of others. The significance of variation in the presence, size, degree of elaboration, and number of vibratile lobes among echinoplutei is particularly puzzling in the context of the feeding performance arguments described above (Fig. 1). Why do some epidermal outgrowths not contain skeletal rods (vibratile lobes), while others do (arms)? Whereas all feeding echinoplutei have arms, not all have vibratile lobes. Why? Are vibratile lobes in different taxa equivalent in their function? Are there performance constraints on the placement and shape of vibratile lobes? Are evolutionary reversals between arms and vibratile lobes (as interpreted in Wray, 1992) common? Does the presence or absence of vibratile lobes correlate to other larval characters?

Aside from feeding and avoiding death, the other biological role that larvae have is to settle to the benthos and complete metamorphosis after sufficient juvenile tissues develop. In many larvae this involves the capacity to sense chemical cues from benthic habitats. Cameron and Hinegardner (1974) and Burke (1983) showed that echinoid larvae, juveniles, or both can sense dissolved chemicals in their environment and transduce that information into a decision to complete metamorphosis. If these dissolved chemicals originate at some distance from the sensory tissues, this type of chemosensation is described as olfaction.

No evidence of spatially restricted chemosensation for echinoid larval structures was available until Bishop and Brandhorst (2007) proposed such a function for the post-oral vibratile lobe (POVL) of *Lytechinus pictus* echinoplutei. Here we use "post-oral" instead of "ventral" (used in Wray, 1992) or "antero-ventral" (used in Emlet, 1988) as it is more indicative of its location between the post-oral arms, but these three terms all describe the same structure. The POVL contains neurons that differentiate as late as early juvenile rudiment formation, and project axons to the preoral neuropile. These neurons were initially discovered by Bishop and Brandhorst (2007) because they express nitric oxide synthase (NOS), an enzyme that produces NO, previously shown to regulate the timing of metamorphosis in *L. pictus* (Bishop and Brandhorst, 2001). Subsequently it was determined that NOS-defined neurons (NDN) also express synaptotagmin (Bishop and Brandhorst, 2007), a pan-neural protein in echinoids (Nakajima *et al.,* 2004). Assuming that these NOS-defined neurons are specified locally, these data reveal a novel neurogenic region in the echinopluteus that, temporally, develops somewhat independently from the lar-

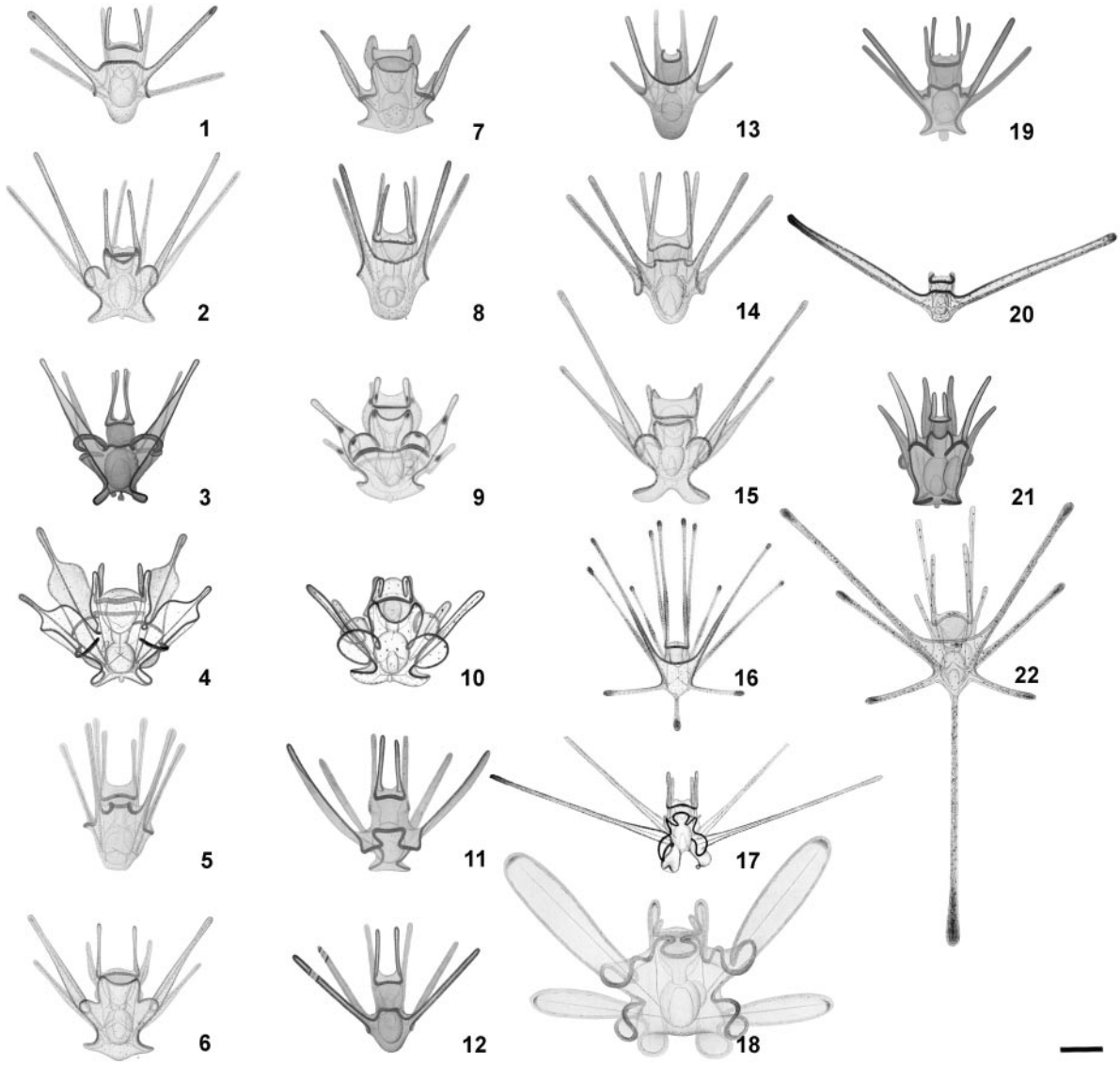

Figure 1. A collage of 22 digitized and scaled drawings of echinoid larvae from Mortensen (1921, 1931, 1937, 1938) that are used for analysis in this work. With the exception of *Temnotrema scillae*, all larvae are viewed from the ventral side. 1. *Echinodiscus auritus*, Leske (Astriclypeidae). 2. *Heterocentrotus mammilatus* Lamarck (Echinometridae). 3. *Mespilia globulus* Linnaeus (Temnopleuridae). 4. *Temnotrema scillae* Mazzetti (Temnopleuridae). 5. *Echinocyamus pusillus* Van Phelum (Fibulariidae). 6. *Echinometra mathei* De Blainville (Echinometridae). 7. *Tripneustes esculentes* Leske (Toxopneustidae). 8. *Fibularia craniolaris* Leske (Fibulariidae). 9. *Nudechinus gravieri* Koehler (Toxopneustidae). 10. *Tripneustes gratilla* Linnaeus (Toxopneustidae). 11. *Evechinus chloroticus* Valen (Echinometridae). 12. *Dendraster excentricus* Eschscholz (Dendrasteridae). 13. *Arachnoides placenta* Linnaeus (Arachnoididae). 14. *Laganum depressum* Lesson (Laganidae). 15. *Prionocidaris baculosa* Lamarck (Cidaridae). 16. *Echinocardium cordatum* Pennar (Loveniidae). 17. *Eucidaris metularia* Lamarck (Cidaridae). 18. *Clypeaster humilis* Leske (Clypeasteridae). 19. *Heliocidaris tuberculata* Lamarck (Echinometridae). 20. *Diadema setosum* Leske (Diadematidae). 21. *Strongylocentrotus franciscanus* Agassiz (Strongylocentrotidae) 22. *Lovenia elongata* Gray (Loveniidae). Scale bar = 250 μm. Additional taxa analyzed but not figured here are *Lytechinus pictus* Lamarck (Toxopneustidae) and *Colobocentrotus atratus* Linnaeus (Echinometridae).

val nervous system. Before and during this period of neurogenesis, the POVL develops from a featureless stretch of ciliary band spanning the post-oral arms in young larvae into a lobate structure with a convoluted shape in fully grown larvae. On the dorsal side of the larva there is no such pattern of neurogenesis, nor does the ciliary band extend across this region of the larva. Collectively, these structural and developmental data led to the conclusion that the POVL is a specialized structure (Bishop and Brandhorst, 2007).

Observation and experimental analyses of larval behavior

during settlement and metamorphosis support this hypothesis. Videomicroscopy indicates that, in a laboratory setting, larvae adopt stereotypical postures during settlement and use epaulettes to generate intermittent bursts of flow that are directed past the ventral surface in an anterior-to-posterior direction (Bishop and Brandhorst, 2007, supplemental data). No locomotion takes place while the epaulettes are beating in this intermittent fashion. Surgical removal of the adoral lobe disrupts these and other settlement behaviors and delays the onset of metamorphosis in response to biofilm, providing experimental evidence that this region of the larva is specialized for functions other than increasing clearance rates or swimming speed. Interestingly, the POVL and the neurons therein in *L. pictus* are developmentally and therefore epigenetically linked: larvae from which the POVL was surgically removed regenerated it, including the NOS-defined neurons.

The available evidence supports the hypothesis that the POVL of *L. pictus* is an olfactory organ that detects chemical cues during settlement. We have not proposed a function for this putative olfactory organ other than during settlement, but it remains possible that dissolved chemicals can also be sensed with this structure while the larva is swimming. In this regard, it is worth mentioning that larvae lacking the POVL can initiate settlement and the completion of metamorphosis, albeit significantly more slowly than those with an intact POVL (Bishop and Brandhorst, 2007).

Genomic data from *Strongylocentrotus purpuratus* strongly supports the possibility that sea urchins are capable of olfaction. Raible *et al.* (2006) identified 979 rhodopsin-type G protein coupled receptors (GPCR) in the *S. purpuratus* genome, constituting more than 3% of all predicted genes. As in vertebrates, this constitutes the single largest family of genes in the *S. purpuratus* genome (Raible *et al.,* 2006). The *L. variegatus* genome is likewise predicted to contain a large family of rhodopsin-type GPCRs, and thus the likelihood that sea urchins in general are genetically equipped for olfaction is high. However, because olfaction *per se* requires specialized morphological and behavioral characters (*i.e.,* noses, antennae; discussed below) in addition to the molecular basis of chemoreception, genomic data are permissive but not diagnostic of an olfactory sense. Here we distinguish between olfaction and the broader category of chemosensation by noting that olfaction is a specialized chemosense that involves detection of odors (chemicals dissolved in air or water) that originate at some distance from the sensory structure. It is in this context that we hypothesize and provide data about the POVL.

We now have a new perspective from which to view the morphology and function of the POVL and vibratile lobes in general. Do all echinoid larvae possess this character or is it restricted to certain lineages? If restricted in its distribution, why? Are other vibratile lobes specialized? In this report, we examine in *L. variegatus* the ultrastructure of the ciliary band that lines the POVL and identify cells that have the structural properties of sensory cells. We show that larvae of *Colobocentrotus atratus* possess NDNs in their POVL. Using images from T.H. Mortensen's major contributions to echinoderm larval development and form, we analyze the degree and distribution of structural elaboration of ectoderm lined by the post-oral transverse ciliary band (PTB) of 24 larvae in the Echinoidea and hypothesize that the structure of the POVL can be intrepreted with respect to fluid dynamic principles associated with olfaction.

Materials and Methods

Animals

Previous studies by CDB have been conducted on *Lytechinus pictus*. Because the present studies were conducted in Nova Scotia, for reasons of availability we switched to *L. variegatus*. Late prism stage or early feeding stage larvae in 100-ml tissue culture bottles or 50-ml conical tubes were mailed to us at ambient temperature by Tom Capo (Rosenstiel School of Marine and Atmospheric Science, University of Miami). Very little mortality was observed from this method of shipment. Specimens of *Colobocentrotus atratus* exposed at low tide in the vicinity of Kewalo Marine Laboratory (Honolulu, HI) were collected in summer 2005. Individuals were spawned by intracoelomic injection of 0.5 mol l^{-1} KCl. Larvae of both species were reared using standard methods (Strathmann, 1987), except that some cultures of *L. variegatus* were not stirred. Cultures of both species were grown at 23–25 °C in unfiltered natural seawater and produced competent larvae in about 3 weeks.

NADPH diaphorase assay and NOS immunohistochemistry

The NADPH diaphorase assay was performed as described in Bishop and Brandhorst (2001) using pre-weighed vials of NADPH (Sigma, St. Louis, Cat# NO411-15VL). Images were collected using a Zeiss Axiophot microscope. NOS immunohistochemistry was performed at Kewalo Marine Laboratory (Honolulu, HI) in summer 2005 on advanced *C. atratus* larvae as in Bishop and Brandhorst (2007). Images were collected on a Zeiss LSM510 confocal microscope.

Scanning electron microscopy

Larvae were deciliated prior to fixation by immersion in 2× Millipore filtered seawater (MFSW) for 10 min and then fixed for 1.25 h in 2.5% glutaraldehyde in MFSW. The deciliation treatment was performed to afford greater visibility of the morphology of the POVL. After several rinses in water, larvae were post-fixed for 1 h in 2% aqueous osmium tetroxide (OsO_4). Larvae were rinsed several times in distilled water, dehydrated in ethanol, critical-point dried,

and platinum/gold sputter-coated (Polaron SC7620). Larvae were mounted on aluminum stubs using conductive graphite adhesive (EM Sciences, Hatfield, PA) and viewed on a Hitachi FE-SEM, model S-4700.

Transmission electron microscopy

For TEM, larvae were fixed in 2.5% glutaraldehyde for 1–2 h, rinsed four times in phosphate buffered saline (PBS) and then post-fixed in 2% OsO_4 (10% solution in 1.25% NaHCO3, diluted five times in PBS) for 1 h. Larvae were then rinsed four times in PBS, dehydrated in an ethanol series, and transferred to propylene oxide for two 5-min incubations. Epon 812 resin was added to larvae at a 1:2 resin/solvent ratio for 1 h, followed by a change in 2:1 resin/solvent ratio for 3 h. A toothpick was used to transfer individual larvae to molds containing 100% resin and manipulated to orient the ventral side of the larva toward the block face. After an incubation in −20 Hg vacuum for 30 min at 70 °C, larvae were incubated at 70 °C overnight under ambient pressure to polymerize the resin.

Larvae stained using the NADPH diaphorase assay were initially fixed in a 2% glutaraldehyde, 1% formaldehyde solution in phosphate buffer pH 7. After the diaphorase assay was completed (as per Bishop and Brandhorst [2001] except that incubations were done at room temperature), larvae were processed in the same manner as larvae prepared for standard TEM. Embedded specimens were first sectioned at 1–2 μm until the POVL could be identified after a brief incubation in 1% toluidine blue. Ultrathin sections for TEM were cut at 60 nm on a Reichert-Jung Ultracut E microtome using a diamond knife. Serial sections were mounted on copper grids and stained with 2% uranyl acetate and Reynold's lead citrate to enhance contrast. Sections derived from larvae subjected to the NADPH diaphorase assay were treated very briefly with lead citrate to create low levels of contrast, thereby preserving signal-to-background ratios to better visualize the diformazan product. Sections were viewed and images collected on an FEI Tecnai-12 TEM at 80 kV.

Reconstruction of serial sections

Sixty-seven serial sections of the POVL of *L. variegatus* were collected and ordered using Reconstruct ver. 1.1.0.0 (Fiala, 2005), a free editor designed to facilitate montaging, alignment, analysis, and visualization of serially sectioned material. Briefly, images were aligned and traces of the perimeter of identified cells of interest in each section were made. A three-dimensional volume-rendered model of these traces was then generated. One model was generated with and one without adjacent cells.

Morphometric analyses

All planktotrophic echinoid larvae from Mortensen (1921, 1931, 1937, 1938) that were fully grown (by his assessment or that contained a juvenile rudiment) and drawn from the ventral perspective (22 in total) were digitized. The resulting images were then scaled according to the absolute size of the original drawings and the magnification given in the figure legends. To ensure that larval sizes derived in this way were accurate, we compared the size of *D. excentricus* calculated from data in Mortensen (1921) to those figured in McEdward (1984). We calculated a larval body length of 423 μm for Mortensen's larva compared to 425 μm for McEdwards's. Using the lasso tool in Photoshop CS2, the PTB was copied from each larval image to create a new image. Confocal images of the PTB of *C. atratus* and *L. pictus* were processed similarly to generate a trace of the PTB. Images of the PTB corresponding to 24 species were printed and then measured. The PTB was scored for the presence of (i) any structural elaboration and, among larvae with some structural elaboration, (ii) the degree of structural elaboration. The number generated from our measurement of structural elaboration represents a scale-free elaboration index (EI). This method assumes that (i) Mortensen's drawings were equivalent in their accuracy, (ii) the error created in measuring a two-dimensional representation of a three-dimensional structure is equivalent across all larvae measured, and (iii) Mortensen's drawings represent the mean phenotype. To test for an allometric association between larval size and elaboration of the PTB, EI values were regressed against larval size. Body length, measured from the apex to the base, was used as a proximate indicator of larval size. *Colobocentrotus atratus* and *Clypeaster humilis* were not included in this analysis as larval length data were not available for either species. Although *C. humilis* was figured by Mortensen, he drew it from a slightly posterior perspective, precluding a measurement of larval length.

We assigned discrete character states to each larva on the basis of a trinary character model. Larvae having zero structural elaboration of the PTB (EI $=1$) were scored with a "0"; those having an EI between 1.01 and 1.3 were scored "1"; and those having an EI above 1.3 were scored "2." We chose to create a 1.31+ character state because *Colobocentrotus atratus* represents the minimum EI value for which we have data about NOS expression in the POVL. Since our hypothesis is that curvature of the POVL is important for function, any larvae having an EI value above 1.3 are included in character state 2. Thus, *C. atratus* is used as a reference point for conservatively estimating taxa that are likely to contain NOS-defined neurons in the PTB. We also expressed the variation in EI values as a binary character: larvae having EI values of 1 were assigned character state 0, while larve with EI values >1 were assigned character state 1. The binary character model allows an assess-

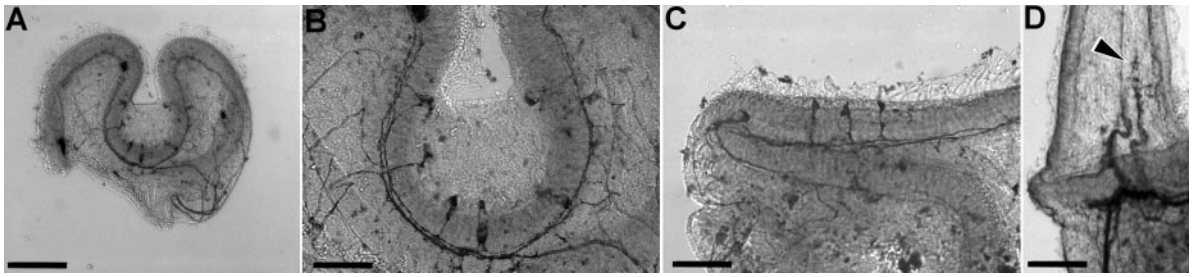

Figure 2. NADPH diaphorase histochemical staining of *Lytechinus variegatus* competent larvae. (A) A post-oral vibratile lobe (POVL) dissected after the staining reaction was complete. (B) Higher magnification view of (A). (C) A folded POVL yielding a lateral view of cells therein. (D) The base of the right anterolateral arm showing the termination of axonal projections from cells in the POVL (arrowhead). Scale bar in A = 50 μm; B–D = 10 μm.

ment of the history of any structural elaboration of ectoderm between the post-oral arms and does not distinguish between degrees of elaboration. Using Mesquite (ver. 2.5) the history of these character states was traced onto a phylogeny adapted from McEdward and Miner (2001) and Littlewood and Smith (1995). Data from Kinjo *et al.* (2008) were used to clarify relationships in the Echinometridae. Maximum parsimony tracing methods (ordered 0 - 1 - 2) were used, as this model reflects the likelihood that the structural elaboration of the PTB is progressive. We polarized the ancestral character state in the Echinoida by placing *Holothuria atra* (Holothuridae) as an outgroup. Auricularia larvae figured in Mortensen's works are invariant in having no structural elaboration of the PTB (*i.e.,* character state 0). Unpublished observations of advanced *H. atra* larvae by CDB support this designation.

Results and Discussion

The first step in our analysis was to ensure that the nitric oxide synthase (NOS)-defined neurons (NDN) previously identified in *Lytechinus pictus* were also present in larvae of their congener *L. variegatus*. Using the NAPDH diaphorase histochemical assay for NOS, it is evident that *L. variegatus* also contains cells in the post-oral vibratile lobe (POVL) that share similarity in structure, distribution, and orientation with those documented in *L. pictus* (Fig. 2)

We then examined the POVL using transmission electron microscopy (Fig. 3). In accord with data collected at the light microscopic level for both species, the ciliary band lining the POVL of *L. variegatus* contains a small number of polarized cells interposed among individual ciliary band cells (Fig. 3C). The apex of these polarized cells breaches the surface of the ciliary band and contains a microvillar array (Fig. 3F–H, J). The base of this cell is broad (Fig. 3C). Confocal images from Bishop and Brandhorst (2007) indicate that two axons project laterally in opposite direction from NDN in this region of the larva. As cell bodies become polarized, each axon becomes closely apposed and can no longer be resolved using light microscopy. We created 67 serial sections through a single polarized cell and computationally reconstructed the cell from the resulting images, showing the basic dimensions and shape of this cell and that its microvillar array extends into the medium beyond the surface of adjacent epithelial cells (Fig. 3H, J). Interestingly, one of the adjacent ciliary band cells projects through a space in the cell, basal to the cell soma (Fig. 3H–J arrowheads). It appears from our reconstruction that these projections may have, at least partially, fused with each other. We don't fully understand this intracellular arrangement.

To ensure that the cells we described at the ultrastructural level are the same as those identified at the light level, we took advantage of the fact that the NADPH diaphorase assay generates an insoluble and electron-dense diformazan product. Production of diformazan has been exploited in other studies to identify cells at the TEM level that express NOS (Darius *et al.,* 1995). We stained larvae using the NADPH diaphorase assay, generated ultrathin sections of the POVL, and identified non-ciliary band cells that contain the diformazan product (Fig. 3E).

The ultrastuctural data indicate that there are non-ciliated cells in the ciliary band lining the POVL that possess the principal characteristic of olfactory neurons: exposure to the exterior environment. However, because the epidermis of pluteus larvae is mostly one cell layer thick, exposure to the environment is less diagnostic of olfactory receptor neurons (ORNs) than it would otherwise be. Whereas cells in the POVL contain a microvillar array, vertebrate ORNs typically possess a dendritic tuft composed of several cilia. It is not widely appreciated that vertebrates contain both ciliated and microvillar type olfactory neurons (Eisthen, 1992). For example, sharks, rays, and ratfish contain only microvillar olfactory neurons, whereas lampreys, snakes, frogs, and turtles contain only ciliated ones. Hagfish, bony fishes, and salamanders contain both in the same olfactory epithelium (Eisthen, 1992). Therefore, the lack of a cilium does not

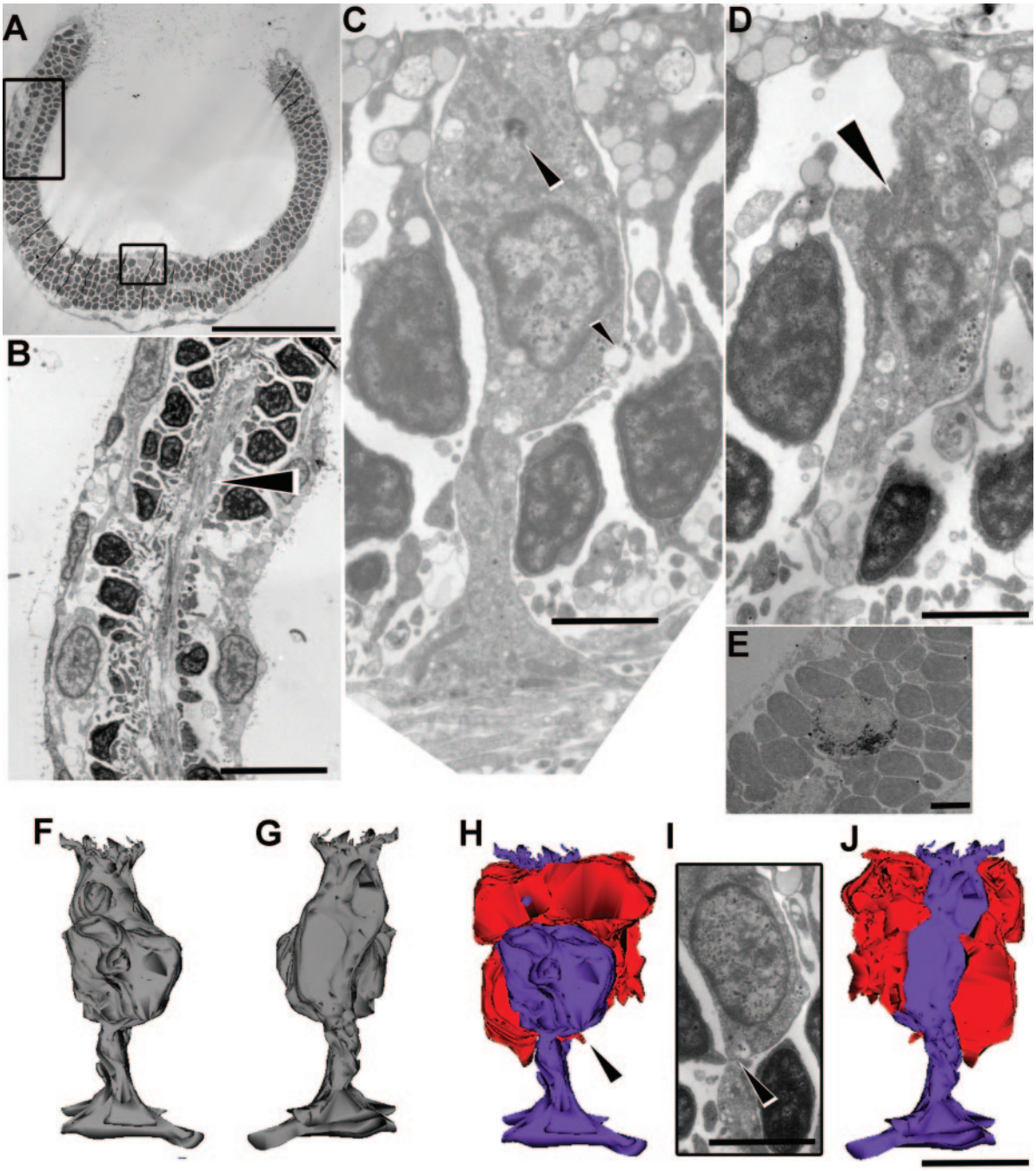

Figure 3. Transmission electron micrographs of putative sensory neurons in the post-oral vibratile lobe (POVL) of *Lytechinus variegatus* and reconstructions of images taken from 67 serially arranged sections of a cell in the POVL. (A) Low-magnification view of the POVL. The rectangular and square boxes indicate the regions from which the images in (B) and (C) respectively, were taken. (B) Tract of axons projecting along the right side of the POVL (arrowhead) through the ciliary band. (C) Section 15 of the series showing polarized cellular morphology, microvillar projections at the apex, a secretory vesicle (small arrowhead), and a basal body (large arrowhead) situated 1.75 μm from the apex. (D) Section 10 of the series showing a dense population of mitochondria apical to the nucleus (arrowhead). (E) The POVL showing the deposition of the diformazan product from the diaphorase reaction. (F, G) Ventral and dorsal view of a reconstructed putative sensory cell in the POVL. (H) Same cell as in (F, G) with adjacent epithelial cells included in red. Arrowhead points to a process originating from a ciliary band cell that projects through the sensory cell. (I) Section 19 of the series corresponding to the reconstructed process in (H). (J) Same reconstruction as in (H), but rotated 180°. Scale bars in (A) = 50 μm, (B) = 5 μm, (C–E, I) = 2 μm, (F–J) = 4 μm.

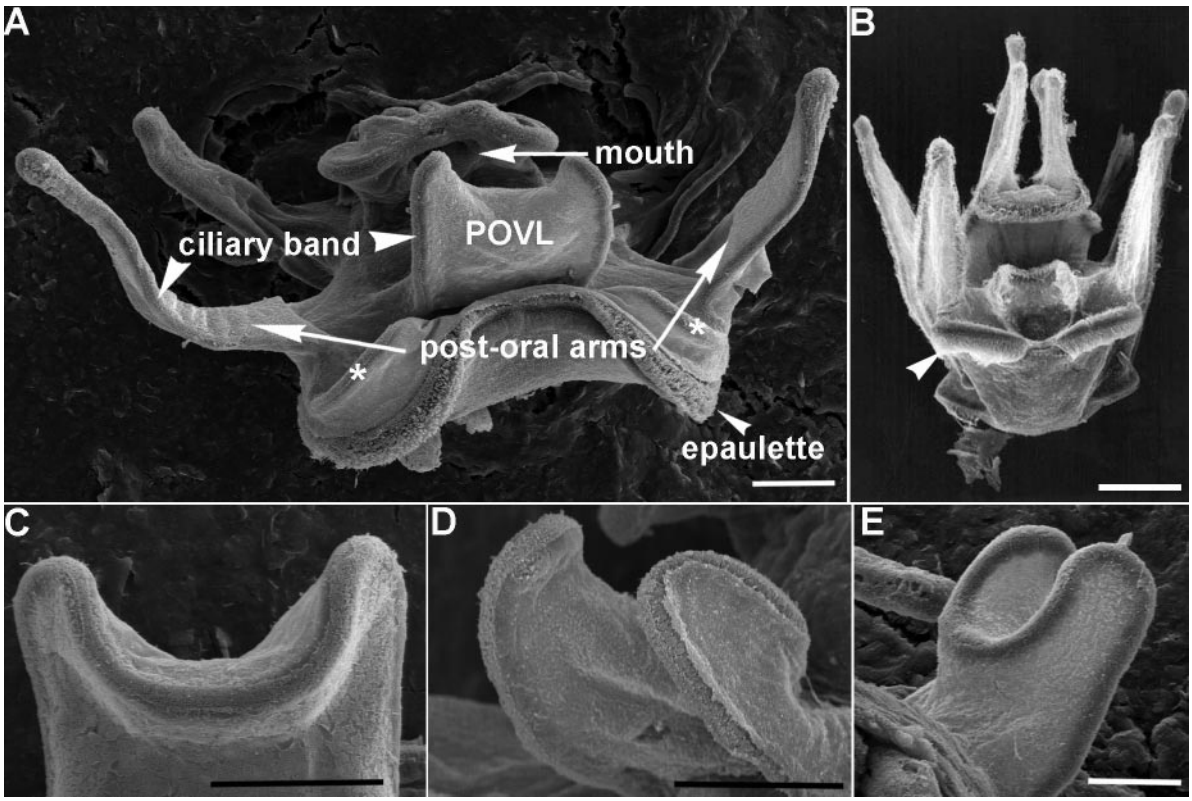

Figure 4. Scanning electron micrographs of advanced deciliated *Lytechinus variegatus* (A, C–E) and *Strongylocentrotus franciscanus* (B) larvae. (A) Ventral view of a *L. variegatus* larva that was deciliated prior to fixation in order to enhance the view of the post-oral vibratile lobe (POVL). The oral hood has also been surgically removed because it frequently collapses over the POVL during sample preparation, obscuring the view. Asterisks indicate two smaller vibratile lobes lateral to the POVL. (B) Ventral view of an advanced *S. franciscanus* larva (reproduced with permission from T. Lacalli, University of Victoria). Arrowhead points to an epaulette. (C–E). Deciliated POVLs from *L. variegatus* larvae viewed from (D) an apical perspective, (E) a ventral perspective, and (F) the left. This latter image was flipped horizontally. Scale bars in (A, B) = 150 μm, (C–E) = 50 μm.

argue against the designation of cells in the POVL as being chemosensory in nature; possession of a microvillar array is not diagnostic either. Notably, birds contain olfactory receptor neurons having both cilia and an extensive array of microvilli (Eisthen, 1992). The apex of the cell in Figure 3C contains a basal body, suggesting the possibility that although we did not observe one, this cell is capable of extending a cilium.

We suggest naming the POVL that we describe herein as the adoral lobe (ADL) to (i) reflect its growth toward the mouth, (ii) distinguish it from adjacent vibratile lobes (present on some larvae), and (iii) recognize a specialized function. In our use of the term ADL, we are referring to the entire ectodermal lobe between the post-oral arms (Fig. 4A,C–E) including the ciliary band and NDNs. In contrast to the condition in young larvae (Bishop and Brandhorst, 2007), the ciliary band associated with the ADL appears discontinuous with the ciliary band on the post-oral arms (Fig. 4A). The comparable region from *Strongylocentrotus franciscanus* larvae (Fig. 4B) appears much smaller and is not as prominently placed relative to the post-oral arms. In the absence of data to the contrary, vibratile lobes other than the ADL (*e.g.,* Fig. 1, posterior lobes on *Prionocidaris baculosa*) may still be assumed to function to increase clearance rates or swimming speed, or both. Because *L. pictus, L. variegatus,* and *Colobocentrotus atratus* are the only taxa for which we have data supporting specialization of the POVL, for the remainder of this work we use the term ADL to refer solely to the POVL of these taxa.

In our opinion, the principal observation that requires explanation is the distribution and orientation of the putative chemosensory neurons relative to the shape of the surrounding ectoderm in which they reside. That is, the possession of a vibratile lobe between the post-oral arms does not predict an olfactory function, but rather its convoluted shape and the distribution of neurons within it does. Next we hypothesize that the shape of the ADL and the distribution of sensory cells therein relates to principles of form associated

with olfaction, and that the ADL, as defined, is a morphological innovation of some echinoid planktotrophs.

A hypothesis of functional relationships

The convoluted shape of the ADL and the distribution of putative sensory cells therein are functionally related, and this relationship corresponds to biophysical constraints associated with olfaction. Because epaulettes generate flow past this region of the larva during settlement, they are also associated with the functionality of the ADL.

In aquatic systems, relationships between structure and function have been analyzed in detail for antennae (arthropod olfactory organs), laying the foundations for our understanding of the biophysical principles that govern the olfactory sense. Some of those principles must be considered in a discussion of the structure of a novel and rather small putative olfactory organ, such as the one proposed here.

While larger scale turbulent flow is important for the arrival of odorants to the vicinity of olfactory organs, smaller scale laminar flow and molecular diffusion are the means by which odorants are brought into contact with receptors (DeSimone, 1981). Because molecular diffusion is not an efficient mechanism for transporting odorants to receptors, animals with an olfactory sense have specialized behaviors and morphologies to increase the efficiency of odor capture and retention. There are two ways that this can be done: animals can either move the medium past sensory tissues (*e.g.,* sniffing in mammals, wing fanning in male silkworm moths) or move sensory tissues through the medium (*e.g.,* antennal flicking in crustaceans, tongue flicking in snakes). Much like the manner in which analyses of flow and feeding performance have informed our understanding of larval shapes (Strathmann, 1971; Emlet, 1991), the manipulation of either sensory organs or flow invokes questions about how fluid moves around olfactory structures and what the consequences are for their form.

The nature of fluid flow around a structure depends on the relative magnitude of inertial and viscous forces, often expressed as the Reynolds (Re) number. Because of the no-slip condition (Vogel, 1981), water in direct contact with a structure does not move relative to it. As a result, flow past a structure generates a velocity gradient between it and free-stream flow. If flow past a small structure is slow, Re values will be low and the boundary layer formed by this velocity gradient will be large relative to that structure. Conversely, if flow past the same structure is fast, the velocity gradient will be relatively steep and the boundary layer correspondingly small. This is important for olfaction because the size of the boundary layer delimits the efficiency of encounter rates between odorants and receptors (Koehl, 2006).

For example, arthropods use antennae to detect chemicals dissolved in fluid by flicking antennae back and forth through the medium. The antennae of *Panulirus argus* (spiny lobster), and those of other decapod crustaceans, are composed of repeated hair-like elements called aesthetascs, which are the functional units of olfaction (Atema, 1977; Grünert and Ache, 1988; Stuellet *et al.,* 2000). Olfactory receptor neurons detect odorants in the fluid that occupies the space between aesthetascs. Importantly, the array of aesthetascs acts as a paddle at low antennal velocity, in which fluid is excluded from the inter-aesthetasc space, or as a sieve at high antennal velocity, in which fluid penetrates the inter-aesthetasc space (Koehl, 2001; Goldman and Koehl, 2001). The reason for this difference in leakiness is that at low speeds the boundary layer is larger than the space between adjacent aesthetascs, whereas the converse is true at higher velocities (Koehl *et al.,* 2001). As a result, contact of odorants with receptors is dependent on the velocity with which fluid passes over the aesthetasc field, as well as the size of the space between individual aesthetascs. When antennae are flicked, the down stroke is much faster than the upstroke, so it is during the down stroke that fluid penetrates the space between individual aesthetascs and odorants arrive close enough to receptors to be efficiently sensed (Koehl *et al.,* 2001). Odorant-rich fluid is then retained in the inter-aesthetasc space for the entire duration of the upstroke and resting period, creating a sampling interval (Reidenbach *et al.,* 2008). This temporal variation in the penetration of odorant-rich fluid into the vicinity of receptors in crustaceans is the functional equivalent of sniffing in mammals (Schmidt and Ache, 1979) and arises through a combination of structure and behavior. Thus, in general terms, efficient interaction of odorants with receptors drives the biophysical demands of olfactory performance.

We hypothesize that at the low Reynolds (Re) numbers that operate on echinoid larvae during swimming, little fluid penetrates into the center of the ADL and flows past the sensory neurons therein. However, when epaulettes drive current at higher velocities past the ADL, Re increases and fluid penetrates to the base of the ADL, transporting odorants to the vicinity of sensory neurons. This hypothesis, schematized in Figure 5, is testable by a careful and quantitative analysis of fluid flow around the ADL during normal swimming and settlement, and by observing flow regimes around dynamically scaled physical models of the ADL. In complement, the comparative method, as detailed below, can be used to test the notion that the presence, distribution, and orientation of sensory neurons in the ADL relate to the overall morphology and position of the ADL.

H_0: The presence of NDNs (or a chemosensory cell detected by any other means) is unrelated to structural elaboration in the post-oral transverse ciliary band (PTB). Or, presence of NDNs $\neq$ presence of structural elaboration of post-oral ectoderm and ciliary band.

H_A: The shape of the ADL and the presence and distribution of NDNs therein are functionally linked and there-

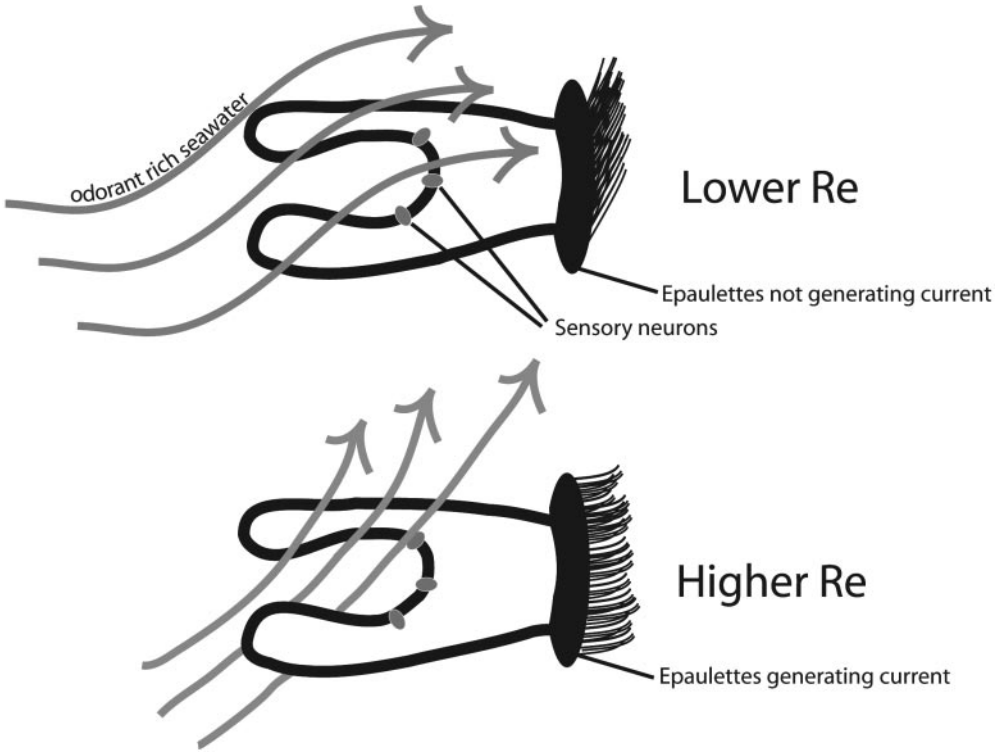

Figure 5. A schematic model hypothesizing the degree to which flow penetrates the adoral lobe (ADL) at low and high Reynolds numbers (Re). The difference between low and high Re values is thought to arise from the difference in flow between normal swimming speeds compared to beating of the epaulettes during settlement, a behavior we have previously observed (Bishop and Brandhorst, 2007). Calculation of flow through the ADL will have to consider the fact that it is lined by ciliary band. Digital video of currents generated by epaulette ciliary beating during settlement is available from CDB upon request.

fore co-vary. Or, presence of NDNs = presence of structural elaboration of the post-oral ectoderm and ciliary band.

Testing these hypotheses requires a comparative investigation with appropriate taxonomic sampling. Support for the null hypothesis requires evidence that NDNs occur in the region of ciliary band between the post-oral arms among larvae with no structural elaboration of that region (*i.e.,* no *bona fide* ADL). Failure to demonstrate this would provide support for the alternate hypothesis. In the absence of access to the broad assemblage of taxa required to conduct this test, we now analyze variation in the structural elaboration of the PTB.

Distribution and origins

NOS expression data on *C. atratus* (Echinometridae) (Fig. 6) indicates that the ADL as we have defined it is not unique to lytechinids (Toxopneustidae). Because we do not have access to a diverse set of planktotrophs from additional taxa, we cannot yet determine whether the ADL is present and functions similarly to lytechinids in enough taxa to distinguish between an echinoid apomorphy and that of smaller clades such as Toxopneustidae + Echinometridae. However, we can examine structural variation in the PTB and predict which taxa are likely to have an ADL. Wray (1992) concluded that ventral vibratile lobes are symplesiomorphies of crown group echinoid taxa. This conclusion, based upon maximum parsimony methods, requires an unambiguous assessment of the presence or absence of vibratile lobes. Our arguments above suggest that the presence of a lobe *per se* may not indicate specialized function, whereas its shape may. Therefore, while we accept the conclusion that vibratile lobes are ancestral to crown group taxa, we do not assume here that the presence of a POVL is equivalent to the presence of an ADL.

A casual inspection of images from Mortensen (1921, 1931, 1937, 1938) indicates that many larvae clearly do not possess a POVL, others have varying degrees of structural elaboration of the PTB and surrounding ectoderm, and still others have structural elaboration comparable to, or greater than, lytechinids. For comparison, and as an outgroup analysis, no planktotrophic larvae from any of the other three echinoderm classes (Holothuroida, Asteroida, Ophiuroida) drawn by Mortensen (1921, 1931, 1937, 1938) display any significant structural elaboration of the PTB. Assuming that

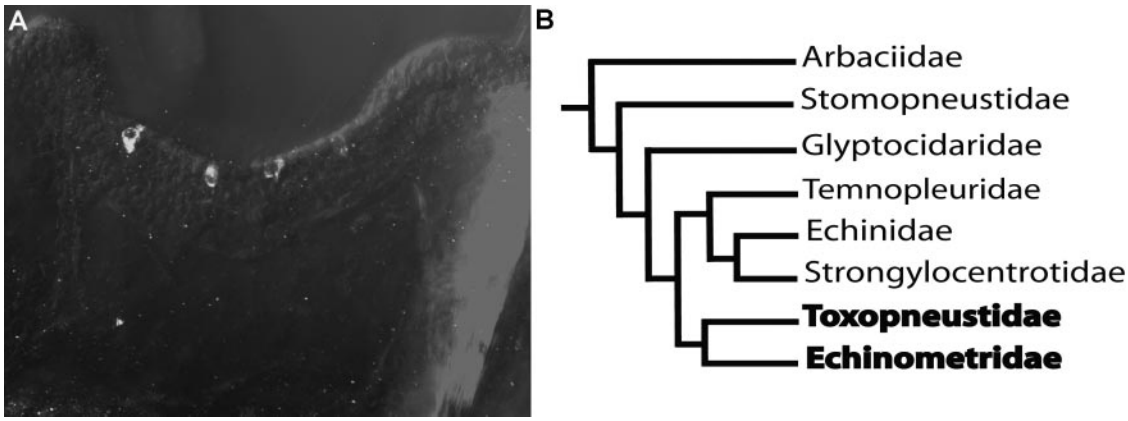

Figure 6. (A) Immunohistochemical detection of NOS in the adoral lobe of *Colobocentrotus atratus.* This image is a composite of a DIC and fluorescence image. (B) The phylogenetic relationship of Echinometridae to Toxopneustidae, indicating that the presence of NOS in the cells of the post-oral vibratile lobe likely predated the divergence of these taxa. The phylogeny of regular urchins was adapted from Littlewood and Smith (1995) and McEdward and Miner (2001).

this section of ciliary band is homologous among these taxa, its structural elaboration in echinoids becomes an interesting question in character origin. Is the ADL a morphological novelty and if so, how is this character distributed phylogenetically in the echinoids? Is there a difference in morphology between a POVL and an ADL?

We have analyzed the shape of the PTB in 22 larvae figured in Mortensen (1921, 1931, 1937, 1938) as well as in larvae of *L. pictus* and *C. atratus*. In his compendia of echinoderm larval development and morphology, the Danish zoologist Ole Theodore Jensen Mortensen (1868–1952) reared and produced detailed drawings of echinoderm embryos and larvae corresponding to 117 taxa from localities around the world. Aside from an obvious enchantment with echinoderm larvae, his desire to study their development was also motivated by his conviction (common in that era, and later supported by Wray, 1992) that larval forms had "classificatory value," particularly for establishing phylogenetic relationships at the family level. Amidst the unity of type within classes of phytoplanktivorous echinoid larvae, morphological variations, particularly in the endoskeleton, contained phylogenetic signal. In the absence of live material, Mortensen's high-quality drawings of echinoderm larvae remain a key resource for analyzing certain aspects of variation in echinoderm larval form. With that in mind, we present the following analysis as a first approximation toward predicting the presence of an ADL in other echinoid taxa.

We examined variation in the structural elaboration of the PTB among 24 species representative of 14 families. Because, as in other parts of the larval body, the PTB is positioned on the ridge of the POVL, an analysis of the structural elaboration of the PTB is used as a proximate measure of overall structural elaboration of this region of the larva. It is important to note that we make three assumptions about the use of Mortensen's drawings (see Methods) for our morphometric analyses. These assumptions temper the strength of our conclusions.

The most obvious result of this analysis is that interspecific variation in elaboration of this region of the larva is approximately continuous. This result challenges our ability, based on shape alone, to unambiguously categorize the presence or absence of an ADL from either live material or images thereof (as done here and in Wray, 1992) and to make the attendant predictions about the presence of sensory neurons (Fig. 7) . To address this problem, we created an elaboration index (EI) that represents a quantitative assessment of structural elaboration. Because larval size also varies, we chose a scale-free method (see Fig. 7A and Methods). If feeding peformance is the primary explanation for larval convolutions, then larval size should correlate positively with the EI values that we generated. As an estimator of larval size, we measured the length of the larval body (excluding arms) along the antero-posterior axis (Fig. 7A). Larval length was then regressed against EI values (Fig 7B).

We found that EI scales positively but weakly with larval size, suggesting that larval size is not a good explanation for structural elaboration of this region of the larval body. This is an interesting result because it provisionally supports alternate explanations for convolutions of larval form in addition to those described above. We use "provisionally" here because larval length is only one metric of larval size and more comprehensive morphometric analyses might generate a significant positive allometric relationship between the elaboration of the PTB and larval body size.

We performed a character-tracing excercise to (i) examine the ancestry of structural elaboration of post-oral ectoderm in echinoids as determined by the shape of the PTB, (ii) assess the homology of such elaborations, and (iii)

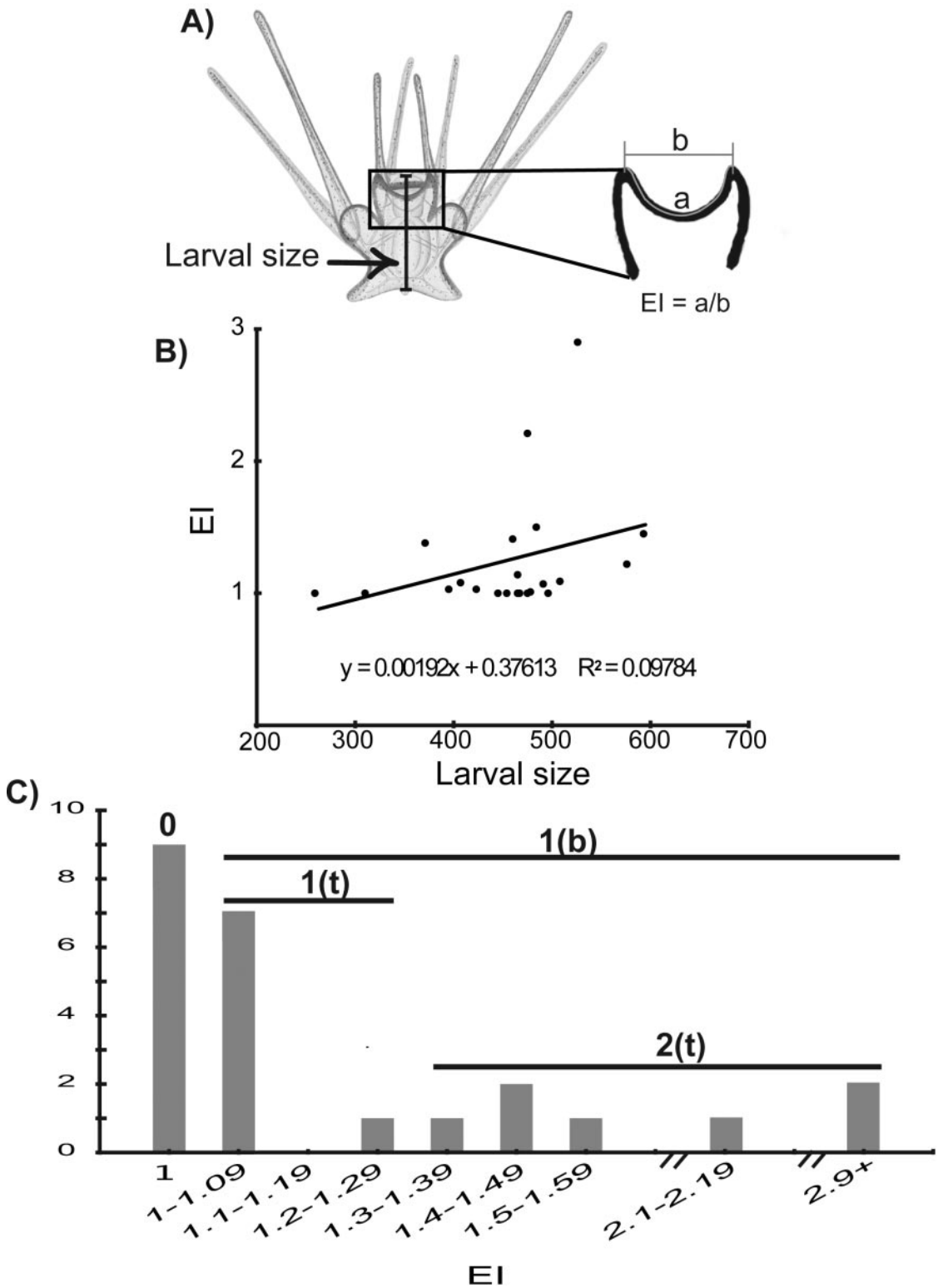

Figure 7. (A) Schematic diagram of the methods used to estimate the elaboration index (EI) and larval length. Mean larval length is 461.87 ± 79.92 μm, and the mean EI value is 1.41 ± 0.89 (see Methods for details). (B) A regression of EI values against larval lengths for 22 taxa. *Colobocentrotus atratus* and *Clypeaster humilis* were not included in this analysis as larval length data were not available. (C) Distribution of EIs among the taxa listed in Fig. 1 plus *C. atratus* and *Lytechinus pictus*. Horizontal bars indicate the coding scheme for each character model: (t) = trinary model; (b) = binary model.

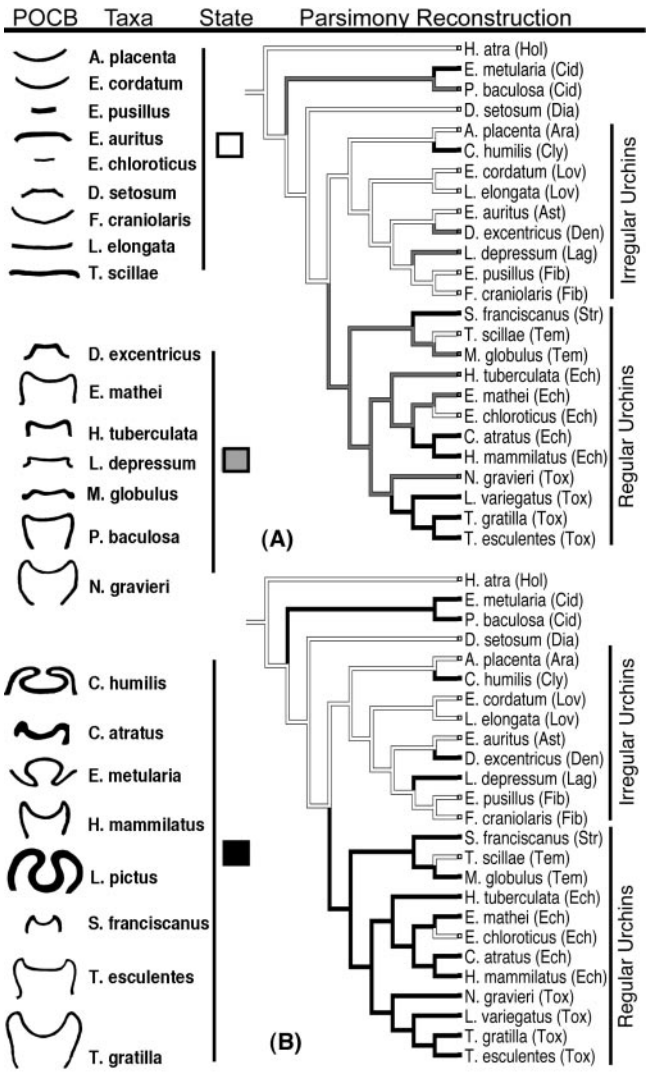

Figure 8. Cropped and arrayed post-oral transverse ciliary bands (PTBs) from digitized larvae in Fig. 1 as well as those corresponding to *Lytechinus pictus* and *Colobocentrotus atratus.* PTBs are grouped according to their character state. Character states were then traced on a phylogeny using maximum parsimony, with ordered character states (see Methods). The ancestral character state was polarized by including *Holothuria atra* as an outgroup. (A) Character-state ancestry using a 3-character state, ordered (0 - 1 - 2), maximum parsimony model (12 steps). Character state 0 is white, character state 1 is grey, and character state 2 is black. Taxa with larvae having at least as much structural elaboration along the PTB as *C. atratus* have evolved five times. (B) Character-state ancestry using a 2-character state, ordered (0 - 1 - 2), maximum parsimony model (7 steps). There are no grey traces in (B) because character states 1 and 2 have been collapsed. Taxa with larvae having any structural elaboration along the PTB have evolved five times. Phylogenetic relationships inferred from Littlewood and Smith (1995), McEdward and Miner (2001), and Kinjo *et al.* (2008).

identify taxa suitable for future comparative analyses (Fig. 8). We performed these traces using either a binary or trinary character-state model (see Methods for details), with the trinary model being a more conservative estimate of which taxa are likely to possess NOS-defined neurons along the PTB. Both models suggest that structural elaboration of post-oral ectoderm is not ancestral to the Echinoidea; both models indicate that structural elaboration, and hence the POVL, has been derived independently multiple times (Fig. 8).

Taxa having larvae with elaborate PTBs (*i.e.,* EI $>$ 1.3) are concentrated in, but not exclusive to, the regular urchins; larvae from representatives of Cidaridae (earliest diverging extant echinoid clade) and Clypeasteridae (earliest diverging extant Irregularia clade) both have PTBs with a high EI. These and their closely related taxa with low EI values will be of particular interest in future studies.

This distribution suggests differences in the manner in which natural selection is acting on the form of echinoid larvae exclusive of arm length and number. Alternatively, this pattern could have resulted from the retention of structural elaboration in some lineages due to cannalization and/or compromise in structure because a specialized role for the ADL may not preclude the contribution of the PTB to clearance rates or swimming speed. According to our hypothesis of functional relationships, we predict that NDNs are present in larvae having character state 2 (Fig. 8A, lineages with black traces). However, taxa having character state 1 are of particular interest because they have an intermediate degree of structural elaboration in PTB. Wray (1992) concluded that the ventral vibratile lobe (*i.e.,* the POVL/ADL here) has been lost in several lineages. Are these taxa with character state 1 in the process of evolving or devolving an ADL proper? Only a careful examination of larvae belonging to this group, with attention to the presence or absence of sensory cells that we characterize here, can answer this question. Anecdotally, *C. atratus* larvae contain fewer NDNs than lytechinids. Because *C. atratus* also has a lower EI (1.31) than *L. pictus* (2.9), there may also be a relationship between EI values and numbers of NOS-defined cells.

Conclusions

Olfaction has been described only for chordates and arthropods. We provide ultrastructural data from the sea urchin *Lytechinus variegatus* that supports the hypothesis of the post-oral vibratile lobe (POVL) as a simple olfactory structure. Further support for this hypothesis would include identification of the expression of G protein coupled receptors (GPCR) in neurons in the POVL, or a demonstration that cells in the POVL bind identified molecules derived from natural settlement cues. To emphasize the specialization of the POVL and to distinguish unequivocally between it and other lobes in the vicinity, we propose renaming the POVL the adoral lobe (ADL). The shape of the aboral and oral ectoderm and the ciliary band that comprise the ADL, particularly in relation to the distribution and orientation of

putative sensory cells therein, evokes a functional morphological explanation. We hypothesize that the shape of the ADL is a consequence of fluid dynamic principles that similarly govern the shape of olfactory structures in other animals and that the presence of sensory neurons and structural elaboration of the post-oral transverse ciliary band (PTB) (and surrounding oral and aboral ectoderm) will covary. In analyzing the shape of the PTB among 22 taxa drawn by T. H. Mortensen, it is apparent that there is much variation, the significance of which remains to be seen. A first approximation reveals a weak positive allometric relationship between the degree of structural elaboration of the PTB and larval size. These analyses provide a foundation upon which to collect and interpret in a statistically sound manner the appropriate comparative data required to test hypotheses about functional relationships between ADL shape and the distribution of sensory neurons therein.

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