Shrinking to fit: fluid jettison from a haemocoelic hydrostatic skeleton during defensive withdrawals of a gastropod larva

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Although most of the basic animal body plans are supported by hydrostatic skeletons consisting of fluid maintained at constant volume, studies on how animals have solved biomechanical scaling dilemmas during evolution of large body size have emphasized cases where skeletons are formed by rigid solids. Larvae of gastropod molluscs swim using ciliated velar lobes supported by a constant volume hydrostatic skeleton. Defensive behaviour involves rapid withdrawal of the velar lobes and foot into a protective biomineralized shell. Some gastropod larvae grow to giant size and the velar lobes enlarge allometrically, but the lobes and foot of many can still withdraw completely into the mineral-stiffened shell. I dyed internal fluid of a large gastropod larva with fluorescein to show that fluid supporting the extended velar lobes is expelled from discrete release sites during defensive withdrawals. Scanning electron microscopy suggested that release sites are distinctive papillae on the upper velar epidermis. Ultrathin sections revealed that branched tracks of microvilli-free membrane on the surface of these papillae were formed by very thin epithelial cells, which may rupture and re-anneal during and after defensive withdrawals. Behaviours facilitated by fluid discharge from a haemocoelic (non-coelomic) body compartment have been rarely reported among aquatic invertebrates, but may be more widespread than currently recognized.

Keywords: allometry; hydrostat; ontogenetic scaling; defence; veliger

1. INTRODUCTION

Change in body size has been pervasive during animal evolution (Alroy 1998; Kingsolver & Pfennig 2004; Hunt & Roy 2006) and body size affects almost every aspect of how organisms function and interact with their environment (Schmidt-Nielsen 1984; Brown & West 2000). For example, as animal bodies get larger they are subject to a number of scaling constraints inherent to musculoskeletal systems. Ways in which evolution has circumvented these constraints have been extensively explored for organisms in which rigid solids provide the major skeletal elements (e.g. Alexander et al. 1979; Alexander 1981; Carrier 1996; Biewener 2005). However, most of the more than 30 major body plans that have emerged during animal evolution are largely supported by hydrostatic skeletons, including all of the many ‘worm’ phyla, the Cnidaria, the water vascular system of echinoderms, and many anatomical components of molluscs. Relatively recent studies have unveiled some surprising mechanical properties and functional roles for hydrostatic skeletons (Kier & Smith 1985; Taylor & Kier 2003) and have analysed the nature of constraints during ontogenetic scaling (Quillin 2000; Thompson & Kier 2002; McHenry & Jed 2003).

Many marine gastropods, like most other marine invertebrates, have a complex life cycle that incorporates a planktonic larval stage (Thorson 1950; Jägersten 1972). Swimming by gastropod veliger larvae is powered by a tract of long cilia that runs along the periphery of two velar lobes extending from the larval head (Fretter 1967). Gastropod larvae also have a biomineralized shell, partially occupied by visceral organs, which provides a protective retreat for the velar lobes and foot during defensive withdrawals.

Basal clades of gastropods have a pelagic, non-feeding larval stage that rarely exceeds 350 μm in shell size (e.g. Bandel 1982; Hadfield & Strathmann 1990; Kay & Emlet 2002), but feeding larvae have arisen in three derived clades (Haszprunar et al. 1995; Ponder & Lindberg 1997). Among feeding larvae, cilia of the velar lobes facilitate both swimming and food capture (Strathmann & Leise 1979), and both the shell and soft tissues of feeding larvae grow between hatching and metamorphic competence. One of these derived clades, the Caenogastropoda, includes many species with larvae that attain sizes of 1–5 mm in maximum shell dimension (Scheltema 1971; Thiriot-Quiecux 1971; Perron 1981; DiSalvo 1988; Romero et al. 2004). The increased body mass of these giant gastropod larvae is correlated with allometric lengthening of the ciliary band that runs around the periphery of the velar lobes. The lobes consequently increase disproportionately in size relative to the shell and often subdivide to form multiple lobes.

The outstretched posture of the extended velar lobes is supported by a hydrostatic skeleton that must maintain constant fluid volume. However, allometric scaling of the velar lobes during enlarging larval body size presents a potential dilemma for the defensive withdrawals of the velar lobes and foot if space within the larval shell does not increase at the same rate as size of the foot and velum. Although there are no empirical data on ontogenetic
scaling of shell volume and soft tissue volume for gastropod larvae, there often appear not to be insufficient vacant space within the rigid shell of caenogastropod larvae to accommodate the visceral organs, foot and oversizer velar lobes if constant volume of haemocoelic fluid is maintained. Nevertheless, many large gastropod larvae can fully withdraw all the body components into the shell. How is this possible?

I dyed haemocoelic fluid of veliger larvae of the caenogastropod, *Euspira lewisii* (Gould 1847), with fluorescein to test the possibility that the large velar lobes and foot are able to withdraw into the biomineralized shell by releasing fluid from the internal haemal compartment. Results indicating that fluid jetisson does occur during withdrawals of the foot and velum were extended by using scanning and transmission electron microscopy (SEM and TEM) to characterize the functional morphology of the velar release sites.

2. MATERIAL AND METHODS

Methods for culturing larvae of *E. lewisii* have been described previously by Pedersen & Page (2000) and protocols for preparing specimens for SEM and TEM followed those of Page (2002). Larvae at four stages of development up to 28 days post-hatching (dph) were examined by SEM. At 28 dph, larvae of this species had completed approximately two-thirds of the growth and development that is typically achieved at the onset of crawling behaviour and metamorphic competence (Pedersen & Page 2000). Velar release papillae (see below) from three larvae at 28 dph were thin sectioned for study by TEM. Contrast and brightness of light and electron micrographs were adjusted using Adobe Photoshop software.

To dye haemocoelic fluid of larvae with fluorescein, larvae at 19, 20, 24, 28 and 33 dph were initially anaesthetized with artificial seawater having augmented [Mg$^{2+}$] and reduced [Ca$^{2+}$] (Audesirk & Audesirk 1980). A salinity refractometer was used to ensure that the artificial seawater was isoosmotic to local seawater used for larval culture. The larvae readily recovered from this gentle form of anaesthesia. Anaesthesia was required because extensive preliminary trials indicated that a dilute solution of fluorescein, which has low toxicity (Miller et al. 2004) and is routinely used as an ophthalmic diagnostic tool (Gonzales 1985; Berkow et al. 2003), nevertheless induced larvae of *E. lewisii* to withdraw into their shells. Withdrawal prevented movement of dye into the haemocoel because the operculum of the foot sealed soft tissues inside the shell. The anaesthetizing artificial seawater was gradually replaced over 2–3 hours with a solution of 0.015% fluorescein sodium salt (Sigma) dissolved in the anaesthetizing artificial seawater. After this incubation, larvae were rinsed in several changes of anaesthetizing artificial seawater, was gradually replaced over 2–3 hours with a solution of 0.015% fluorescein sodium salt (Sigma) dissolved in the anaesthetizing artificial seawater. After this incubation, larvae were rinsed in several changes of anaesthetizing artificial seawater, without dissolved fluorescein, and then pipetted into normal seawater within a plastic Petri dish. The Petri dish was placed under a stereomicroscope fitted with a video camera. Larvae began recovering from anaesthesia almost immediately after exposure to normal seawater and bright light from a fibre optic illuminator stimulated withdrawal of the foot and velar lobes into the shell. A few areas of larval soft tissue typically adhered to the Petri dish, which facilitated mounting of the shells for electron microscopy. In these experiments, the speed of withdrawal was slower than normal withdrawals because larvae were still partially anaesthetized. I did not see evidence of haemal fluid discharging from epithelium of the foot.

To promote visualization of fluorescein, light entering the light guide of the fibre optic illuminator was passed through a 460 nm/50 nm bandpass filter and reflected light from the specimen was passed through a 514 nm long pass filter (Chroma Technologies) that was placed in the head of the microscope immediately below the video camera. However, in many cases, fluorescein could be seen without the use of filters.

I used a Sony DXC-950P colour video camera (750 line resolution; 25 frames s$^{-1}$) to record withdrawal behaviour of more than 80 larvae that had been previously incubated in fluorescein solution. Output from the video camera was recorded on videotape using a Sony DSR-20P digital videocassette recorder. Video sequences were then imported into a computer running Adobe Premiere Elements v. 2.0. This software also allowed frame-by-frame analysis of videotaped withdrawal behaviours.

3. RESULTS

Larvae of *E. lewisii* hatched with relatively small, circular velar lobes extending from the left and right sides of the head (figure 1a). Between 8 and 10 days after hatching, the lobes began to bifurcate so as to generate a total of four lobes (figure 1b), each of which continued to elongate during later development (figure 1c). Duplication and elongation of the velar lobes resulted in allometric elongation of the tract of ciliated cells running along the periphery of each lobe. The scaling exponent for the relationship between ciliary tract length and width of the shell aperture for larvae of *E. lewisii* as they grew between hatching and onset of crawling behaviour (a proxy for metamorphic competence) was 2.02 (M. Lesoway & L. Page 2007, unpublished observation).

At the onset of velar lobe bifurcation, a patch of red pigment appeared at the apex of each of the four incipient lobes. Each pigment patch surrounded a small central area of non-pigmented epithelium, which appeared as a translucent ‘button’ against a red background (figure 1b). When larvae were incubated in fluorescein to dye internal haemal fluid and then briefly rinsed and viewed microscopically with appropriate optical filters for excitation and emission wavelengths of fluorescein, each velar button appeared as an isolated spot of bright fluorescence due to lack of obscuring pigment in the overlying velar epidermal epithelium (figure 1d; fluorescence shining through the button at the apex of one of the velar lobes is indicated by an arrow). When larvae with fluorescein loaded into fluid within the internal haemocoel pulled the foot and velar lobes into the shell, fluorescein-dyed haemal fluid abruptly escaped from the transparent button at the apex of one or more of the velar lobes (figure 1d–g). Dye was often emitted as four streams, each stream originating from the apex of one of the four velar lobes (see electronic supplementary material). In these experiments, the speed of withdrawal was slower than normal withdrawals because larvae were still partially anaesthetized. I did not see evidence of haemal fluid discharging from epithelium of the foot.
Figure 1. Growth of velar lobes and evidence of haemal release sites within the velar lobes of *E. lewisii* larvae. (a) Apical view of a recently hatched larva, showing foot (f) and hemispherical velar lobes (vl). Scale bar, 50 μm. (b) Larva at 10 days post-hatching (dph), showing onset of velar lobe bifurcation; note the transparent release ‘buttons’ (arrows) within the pigment patch at the apex of each velar lobe. Scale bar, 100 μm. (c) Larva at 59 dph, showing four elongate velar lobes. Scale bar, 500 μm. (d–g) Selected frames from a video recording showing the release of fluorescein-dyed haemal fluid (arrowheads) from the release site of cuboidal epithelial cells of the velar papilla (figure 2c). The release site is indicated by an arrow in (d). Scale bar, 200 μm. (h) SEM of the apex of a velar lobe, showing the haemal release papilla (arrow). Scale bar, 20 μm. (i) Higher magnification of release papilla. Scale bar, 5 μm.

Views of the upper surface of the velar lobes with scanning electron microscopy showed that velar release sites for haemal fluid (‘buttons’) corresponded to the position of a distinctive papilla at the apex of each velar lobe (figure 1h). Each papilla had a branched tract of microvilli-free membrane running over its surface (figures 1i and 2a). Transmission electron microscopy showed that these microvilli-free tracts on the surface of papillae were areas where a very thin squamous epithelial cell was the only partition between the internal haemocoel and external seawater (figure 2b,c). This highly squamous epithelial cell extended from the base of surrounding cuboidal epithelial cells of the velar papilla (figure 2c). Two out of 12 larvae fixed at 28 dph and examined by SEM had velar papillae that were ruptured open (figure 2d,e). The rupture appeared to occur along the tract of microvilli-free membrane, suggesting that these were zones of weakness within the velar epithelium. Although larvae younger than 28 dph had only one release papilla at the apex of each velar lobe when examined by SEM, evidence of additional papillae beginning to form was seen in larvae examined at 28 dph (figure 2d, arrowhead).

Although experiments using fluorescein-dyed haemal fluid were done only on larvae of *E. lewisii*, I have also seen papillae on the upper velar surface of two other caenogastropod larvae, the lamellariid *Marrenina stearnsii* (Dall 1871) and the neogastropod *Anphissa columbiana* (Dall 1916), when metamorphically competent larvae of these species were examined with SEM. The velar papillae of these species were morphologically similar to those of *E. lewisii*, in having a tract of microvilli-free membrane running across the surface (figure 2f). Papillae were occasionally ruptured open along this naked tract of membrane (figure 2g).

4. DISCUSSION

Large gastropod larvae function with a body design inherited from a much smaller, non-growing ancestral larva (Haszprunar et al. 1995; Ponder & Lindberg 1997). Allometric scaling of the velar lobes during evolutionary and ontogenetic size increase has allowed continued ability to swim and to capture sufficient food. However, disproportionate enlargement of velar lobes relative to the bimineralized shell may have, in some cases, posed problems for the inherited defensive behaviour of these larvae, which involves full withdrawal of the velar lobes and foot into the protective interior of the shell. My results provide evidence that at least one species of large gastropod larva and, possibly, larvae from two other gastropod families have resolved this dilemma by discharging fluid from specialized velar release sites during withdrawals of the foot and velum into the shell. This result helps explain how the outstretched posture of large velar lobes can be supported by internal fluid contained at constant volume (i.e. a hydrostatic skeleton), even when this volume of fluid appears too great to allow the velar lobes and foot to fit within the rigid shell during protective withdrawals.

Electron microscopy showed that specialized fluid release sites within the apical epidermis of the velar lobes consist of areas where highly flattened epithelial cells provide the only partition between the internal haemocoel and the external medium. These sites may literally rip open when contracting muscles elevate internal fluid
pressure. This hypothesis requires that ruptured membrane of release sites subsequently re-anneal, perhaps using a membrane fusion mechanism similar to that employed during phagocytosis.

Release of fluid from an internal, non-coelomic body compartment has also been reported for the adult stage of *E. lewisii* and other moon snails (Bernard 1968). The large foot of these snails is inflated with external seawater taken in through pores in pedal epidermal epithelium and distributed throughout the foot in a specialized haemal compartment. When the foot is withdrawn into the shell, the seawater escapes via the pores. However, unlike the release sites in the velar lobes of the larval stage, the pores in the adult foot are apparently not transient. Inflation of the foot with external seawater and release of this seawater during protective withdrawal is unique to adults of moon snails, and may suggest that velar release pores in the larval stage are also unique to this family of gastropods. Nevertheless, observations by SEM also revealed papillae on the velar lobes of at least two other species of caenogastropods, *M. stearnsii* and *A. columbiana*, which are very similar to the release pores in the velar lobes of *E. lewisii*.

Loss of internal fluid during defensive withdrawals of *E. lewisii* larvae implies that reinflation of the velar haemal compartment with fluid must occur. Although the most probable source of fluid is surrounding seawater, further research is necessary to reveal how this uptake occurs. Previous studies have demonstrated that dehydrated terrestrial slugs can rapidly transport water into their haemocoel by opening a paracellular route through pedal epithelium (Uglem et al. 1985). I found that re-extension of velar lobes by *E. lewisii* after their full withdrawal into the shell requires much more time (more than 3 min) than the very rapid withdrawal phase, which might be consistent with intake of fluid across the velar epithelium via a paracellular route. Import of fluid via a paracellular route is an appealing possibility because the basal lamina of velar epithelium could act as an ultrafilter to prevent bacteria from entering the haemocoel.
To date, well-documented cases of fluid release from a haemocoelic compartment as part of a normally occurring behaviour appear to be limited to the retracting foot of adult moon snails, as described above, and a behaviour known as ‘easy bleeding’ or ‘reflex bleeding’ among certain groups of insects (Happ & Eisner 1961; Muller & Brakefield 2003; Burrett et al. 2005) and a genus of mites (Yoder et al. 2006). Reflex bleeding appears to have evolved multiple times independently among insects and involves the release of a droplet of haemal fluid, often from distinct sites on the body, in response to trauma. In some cases, the released haemal fluid is known to incorporate defensive allomones or it can incapacitate appendages of small predators when cloting of released haemal fluid occurs. Although known cases of release and import of fluid into haemocoelic compartments are rare, these exchanges would easily escape notice when organisms are aquatic, as fluids are colourless and openings in epithelia are subtle. The phenomenon may be more widespread among invertebrates than is currently recognized.

Whereas fluid exchange between a haemocoel and the external environment has been rarely reported, cases of fluid exchange between a coelomic compartment and the exterior are widespread and well known. Indeed, many coelomate invertebrates have peritoneum-lined ducts between the coelom and external environment to facilitate exchange of fluids (Shinn et al. 1990). In these cases, movement of fluids and materials between internal and external compartments can be controlled by peritoneal myoepithelial cells acting as sphincters. Examples of ducts of this type include metanephridial ducts (Ruppert & Smith 1988) and gonoducts among animals like polychaete worms that store gametes within a coelom (Clark 1964). Shinn et al. (1990) have argued that the perivisceral coelom of holothurians functions primarily as a hydrostatic skeleton, and the primitive function of transrectal coelomoducts in these echinoderms is to regulate fluid volume within this coelom. A similar function is postulated here for the release papillae of the velar lobes of E. lewisi, although the internal compartment of E. lewisi larva is a haemocoel rather than a coelom.

Gigantism among gastropod larvae has been facilitated by evolution of larval feeding and the capacity for growth during the larval stage, but evolution of large size has required adjustments to larval design. Allometric enlargement of the velar lobes and the tract of cilia that permit swimming and feeding is a major innovation but disproportionate enlargement of the velum relative to the shell could potentially prevent full withdrawal of the velar lobes into the shell. Results reported here suggest that allometric enlargement of velar lobes relative to shell size has, in some cases, been accompanied by novel release sites for haemal fluid so that the internal hydrostastic skeleton of the velar lobes can shrink during defensive withdrawals.

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REFERENCES


Schmidt-Nielsen, K. 1984 *Scaling: why is animal size so important?* Cambridge, UK: Cambridge University Press.


