

Musculature Associated with the Water Canals in Freshwater Mussels and Response to Monoamines *In Vitro*

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Abstract. The gills of freshwater mussels perform many functions that depend on water flow through the water canals and channels. Regulation of water flow depends in part on ciliary activity and in part on the contraction of musculature underlying the gill filament and water channel epithelium. Obliquely striated muscles control water canal openings (ostia) at the base of the filaments and also at the entry into the water channel (internal ostia, IO). The muscles adjacent to the ostia are oriented in an anterior-posterior direction (perpendicular to gill filaments), and those controlling the internal ostia are oriented in a dorso-ventral direction (parallel to gill filaments). Small bundles of fibers radiate from the major dorso-ventral IO muscle bands and appear to insert at the base of the water canal epithelial cells at the canal-channel junction. Both muscular bands are closely associated with the branchial nerves in the gill. When gills are exposed to 10^{-5} M serotonin *in vitro*, both ostial openings dilate and gill ciliary activity increases. The net result of serotonin treatment is an increase in ciliary activity, a maximal opening of the water canal ostia, and, presumably, an increase in water flow through the gill.

Introduction

The gill in freshwater mussels is responsible for many of the functions associated with water flow through the animal. For example, ion transport, feeding, reproduction, and respiration are all dependent on the pattern of water

flow through the gill. The gill ciliary activity generates the force for water flow (Riisgard and Mohlenberg, 1979; Jorgensen, 1982, 1989; Paparo, 1988; Silvester, 1988; Sleigh, 1989), and water flow has been calculated from data characterizing ciliary activity (Jorgensen 1989; Sleigh, 1989). The pattern of flow through the gill begins with water moving across the gill filaments and through the ostial (O) openings that lead into the water canals. From the canals, water flow is directed through the internal ostia (IO), into the central water channels that conduct water into the suprabranchial chamber, and then out through the excurrent siphon (see Fig. 1).

The specializations found in the various gill epithelia indicate that ion transport and perhaps respiration take place across the internal epithelial lining the water canals and channels (Kays *et al.*, 1990). The epithelial cells of the gill showing the most enzymatic activity for carbonic anhydrase are located on the internal epithelial surfaces (Kays *et al.*, 1990). In addition, the cells showing the most oxidative activity form the epithelia lining the canals. The ciliated epithelia lining the filaments do not appear to contain any of the specializations associated with ion transporting cells and are larger (apical to basal surface) than one would expect for gas exchange. They appear to be providing protection, as well as the driving force for water flow.

While ciliary activity may be the principal driving force for water flow, the pattern of flow may be regulated by the muscles present in the gill tissue. In oysters, the gill musculature and vascular changes control the diameter of the ostia (Galtsoff, 1964; Nelson, 1941; Nelson and Allison, 1940) and influence the rate of water flow through the gill (Nelson, 1941; Nelson and Allison, 1940). Similar regulation of ostial diameter by muscles in unionid gills

Received 12 December 1990; accepted 8 March 1991.

Abbreviations: Acetylcholine (ACh); Epinephrine (Epi); Gamma Aminobutyric Acid (GABA); Internal Ostia (IO); Norepinephrine (Nor-epi); Ostia (O); Scanning Electron Microscopy (SEM); Transmission Electron Microscopy (TEM)

would control water flow in response to the osmoregulatory (Dietz and Graves, 1981), respiratory, and even the reproductive needs of the animal (Silverman, 1989; Richard *et al.*, 1991). Muscular control also offers the possibility of blocking flow into the water channels. Previously we showed that the water channels of the Lampsilinae are functionally occluded during reproduction (Silverman *et al.*, 1987).

In the research reported here, we have used morphological techniques to describe the musculature associated with the water canals of the freshwater unionid gills. We have also demonstrated that serotonin, a well-known inhibitor of muscle contraction in a number of molluscan systems (Twarog 1954; Cambridge, 1959; Twarog and Cole, 1972; Jorgensen, 1976; Satchell and Twarog, 1978; Kobayashi and Hasimoto, 1982), causes the canal musculature to relax, allowing the water canals to expand. Serotonin increases ciliary activity in a variety of marine mussels (Gosselin *et al.*, 1962; Aiello and Guideri, 1966; Aiello, 1970; Paparo and Murphy, 1975; Jorgenson, 1976; Capatane *et al.*, 1978; Paparo, 1980; Sanderson and Satir, 1982; Sanderson *et al.*, 1985), and dopamine depresses ciliary activity in marine bivalves (Catapane *et al.*, 1978; Paparo, 1980). However, we report here that both serotonin and dopamine increase gill ciliary activity in the freshwater unionids.

Materials and Methods

Animal maintenance

The unionid mussels *Anodonta grandis* and *Ligumia strostrata* were collected from ponds near Baton Rouge, Louisiana. The animals were maintained in aerated artificial pondwater (0.5 mM NaCl, 0.4 mM CaCl₂, 0.2 mM NaHCO₃, and 0.05 mM KCl) at 25°C and were allowed to acclimate to laboratory conditions for a week before being used. The mussels were only studied during the non-reproductive season so the gills were not being employed to brood larvae.

Preparation of gills for light and transmission electron microscopy

We opened the clams by cutting the adductor muscles, thereby exposing the lateral and medial demibranch (gill) pairs. The gills were excised and placed in a Ringer's solution designed for freshwater mussels (5.0 mM CaCl₂, 0.5 mM KCl, 5.0 mM NaCl, 5.0 mM NaHCO₃ and 5.0 mM Na₂SO₄) or a 30 mM tris(hydroxymethyl)amino-methane (tris-HCl) buffer solution, pH 7.8. After several minutes, the gills were removed and flattened on a polystyrene petri dish or pinned to a wax base.

Gills were fixed in 2% glutaraldehyde (EM grade) in 30 mM tris-HCl containing 1 mM ethylenediaminetetraace-

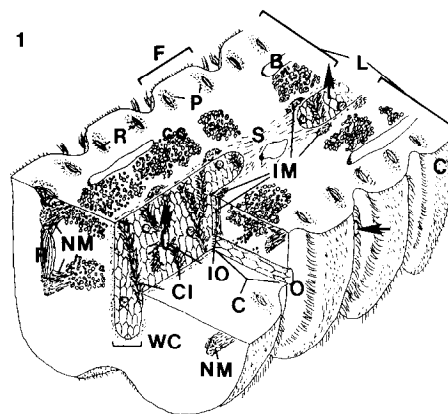


Figure 1. A schematic representation of the gill of *Ligumia strostrata* modified from Kays *et al.* (1990). The gill consists of an ascending and descending lamella (L) organized as filaments (F) surrounding central water canals (WC). The lamellae are joined by connective tissue septa (S). The filaments are supported by discontinuous calcified chitinous rods (R) and an associated mucopolysaccharide matrix (P). Extensive extracellular calcium concretions (cc) are located in the connective tissue underlying the filaments. Blood sinuses (B) also occur in this region. Water enters the gill through ostia (O) located at the base of the filaments. The ostia open into water canals (C) which lead into the WC. The opening of the water canal into the WC is designated as the internal ostia (IO). Water moves into the WC, and is directed dorsally to the suprabranchial chamber. The general direction of water flow through the gill is indicated by the arrows. Associated with the rods are anterior-posterior oriented bands of muscle (NM); these bands are associated with nerve fibers which are oriented in the same direction. This musculature flares and inserts onto adjacent chitinous rods at discontinuities in the rods. The muscle bands alternate with ostial openings at the base of the filaments. Associated with the internal ostia are bands of muscle (IM) oriented in the dorsal-ventral direction; these bundles of muscle also are associated with nerve fibers. Water canal epithelial cells are non-ciliated microvillar cells. Ciliated cells (CI) are oriented in rows in the water channel; the epithelial cells forming the border of the IO are also ciliated (not to scale); the top and bottom of the figure are dorsal and ventral, respectively; anterior is to the left and posterior to the right).

tic acid (EDTA), pH 7.8 (Silverman *et al.*, 1983, 1987). Alternatively, glutaraldehyde was added directly to the gills in freshwater mussel Ringer's solution with or without EDTA. Gills were exposed to the fixative for 2 h. During fixation, the gills were cut dorso-ventrally (parallel to gill filaments) into strips of 5–8 mm. Following fixation, gill strips were washed three times for 5 min each in either 30 mM tris-HCl or phosphate buffer, pH 7.8, and post-fixed in 1% aqueous osmium tetroxide for 1 h. After osmication, the gill strips were washed three times for 10 min each in deionized water and then dehydrated in a graded ethanol series (10 min in 50%, 70%, 80%, 90%, 95%, and 3 × 10 min in 100%). Two resins, Spurr's low viscosity (Polysciences, Inc.) and LR White hard grade (EMS, Inc.), were used. Gill strips to be embedded in LR White were placed in a 1:1 resin/ethanol mixture for 20 min and then into 100% LR White resin for 24 h. Fol-

lowing the overnight incubation, fresh LR White resin was added, and the gill strips were embedded flat in aluminum pans at 60°C for 48 h. Gill strips embedded in Spurr's resin were initially placed in 100% propylene oxide, 3 × 20 min, followed by graded propylene oxide/resin series (20 min at 1:1, 1:2, 1:3, 1:4, 3 × 1 h at 100% resin, and 100% for 24 h) and final embedding in fresh resin at 60°C for 48 h.

Gills were sectioned for light microscopy with a Reichert-Jung Ultracut E ultramicrotome at 0.5–2.0 μm thickness with glass knives and for transmission electron microscopy (TEM) at 60–90 nm thickness with a diamond knife. Sections for light microscopy were stained according to a tribasic staining procedure developed by Grimley (1964). The gill was sectioned in two planes: (1) anterior-posterior cross-sections, and (2) frontal sections (*en face*) across the surface of the filaments. Sections for light microscopy were examined and photographed with a Nikon Microphot-FXA microscope. Sections for TEM were stained with 3% uranyl acetate for 2 min followed by Reynolds' (1963) lead citrate for 2–5 min. The sections were examined with a JOEL 100 CX transmission electron microscope operating at 80 kV.

The light micrographs of isolated chitinous rods were prepared by cutting gills into 5–6 mm longitudinal strips along the filaments and incubating the strips in calcium-free Ringer's solution containing 1000 U/ml collagenase IV (Sigma, St. Louis). After 12 h, the rods were collected by repeated centrifugations of the collagenase treated gill at 50 × *g* for 5 min and viewed with an Nikon Diaphot inverted microscope.

Neurotransmitter application

Gills were excised and placed in individual polystyrene petri dishes containing freshwater mussel Ringer's, pH 7.8. Gills were cut in half dorso-ventrally, and incubated in the Ringer's solution for 30 min with changes every 10 min. The diameter of the ostia and the internal ostia, and gill movement, were monitored with an inverted Nikon Diaphot microscope for 5 min before fixing. One of the gill halves was fixed without being exposed to putative transmitter substances by the addition of an equal volume of fixative (4% formalin and 4% glutaraldehyde in mussel Ringer's, pH 7.8) directly onto the tissue. Before fixing the control, we placed the other half of the bisected gill in a mussel Ringer's solution containing 10⁻⁵ M neurotransmitter [acetylcholine, dopamine, gamma aminobutyric acid (GABA), epinephrine, norepinephrine, or serotonin], pH 7.8.

Preparation of gills for scanning electron microscopy

After fixation, the gills were washed in a 30 mM tris-HCl or phosphate buffer, pH 7.8, for 15 min with changes

every 5 min, and then osmicated in 1% aqueous osmium tetroxide for 1 h. After osmication, gills were washed in deionized water for 15 min with changes every 5 min. Fine micro-dissection tools were used to separate the opposing gill lamellae by severing the interlamellar septa and exposing the central water channel epithelium (see Fig. 1). Gills were cut dorsal-ventrally (parallel to filaments) every 6–10 mm and dehydrated in the graded ethanol series. The tissues were then stacked perpendicular to one another and wrapped in lens paper to ensure that they would remain flat during critical point drying. Gills were critical point dried (Denton Vacuum, Inc.) and mounted on stubs. The water channel epithelium was oriented as the facing surface. Specimens were sputter coated with gold/palladium (20 nm) and viewed with a Hitachi S-500 scanning electron microscope (SEM) with a working distance of 30 mm, operated at 25 kV.

Neurotransmitter effect on the canal ostial diameter

The dimensions of the internal ostia of gills exposed to transmitter substance were obtained from scanning electron micrographs and assessed quantitatively. Samples for scanning electron microscopy were selected at random from control and treated tissues that had remained flat after critical point drying. Three or more tissues were selected from each treatment group, and three low magnification micrographs of each sample were taken from the first three separate fields of the water channel region brought into view. Image-analysis was performed on the resulting SEM negatives. Ostial surface area and other average ostial dimensions (*i.e.*, perimeter and diameter) were calculated from digitized images using densitometry and stereology software (Image-1/AT IM5000). Statistics are based on paired Student's *t*-tests with significance set at *P* < 0.05.

Assay of gill ciliary activity

While the gross muscular responses in the gill to various neurotransmitters was being monitored, ciliary activity was observed to increase when the gills were exposed to serotonin or dopamine. The changes in lateral ciliary activity in *Ligumia* gills were assayed by the procedures of Paparo (1980). Ciliary beats per second was determined by synchronizing the activity with the rate of flashing of a calibrated strobe light. Gills were placed in dishes designed to allow pondwater to flow through at a rate of 0.5 ml/min. Initial measurements of the rate of ciliary beating in pondwater over 60 min were used as control values. The effect of serotonin or dopamine on the ciliary activity was analyzed after the petri dish contents were replaced with fresh pondwater containing either serotonin or dopamine and then continuing the flow at 0.5 ml/min with

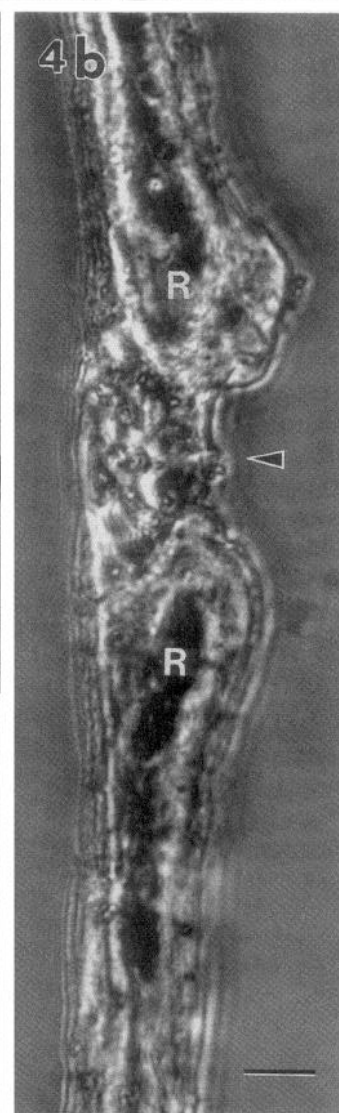
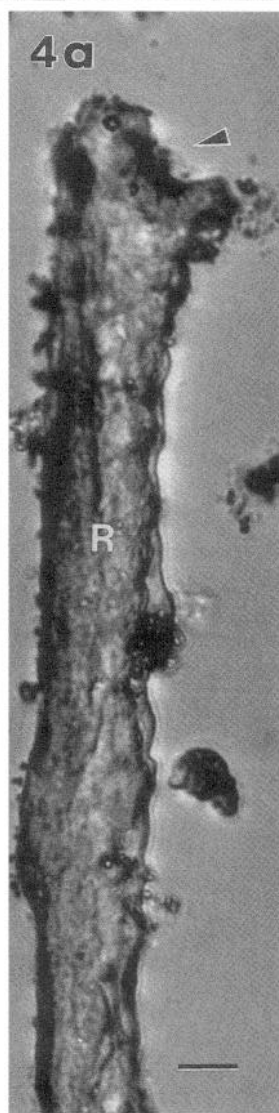
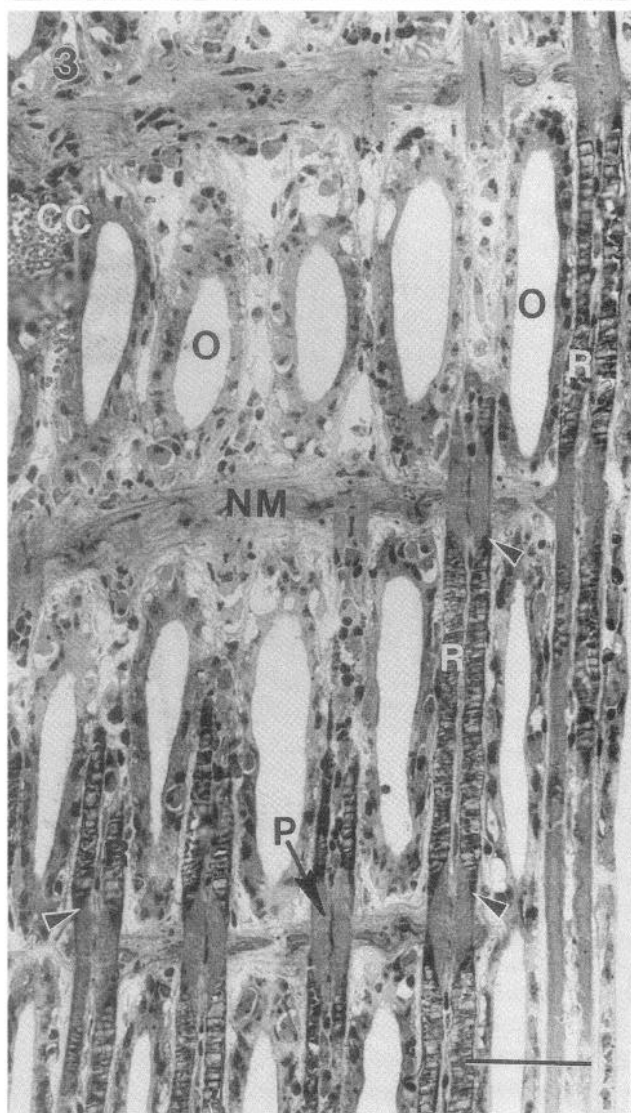
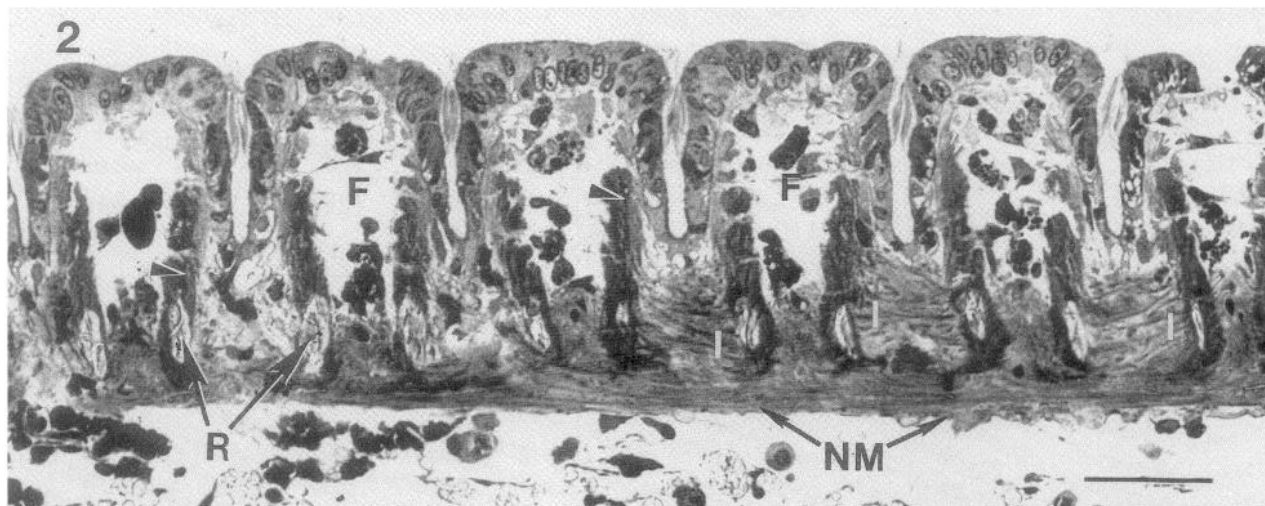


Figure 2. Light micrograph of a cross-section of the gill of *Anodonta* (anterior/posterior to the left/right). Underlying the filaments (F) is the muscular band (NM) that traverses in an anterior-posterior direction. The muscles insert (I) onto the calcified chitinous rods (R) that support the filaments. The insertion occurs as fibers of muscle extend from the main band and contacts each rod. The rods are surrounded by a mu-

fresh pondwater containing the appropriate neurotransmitter. This application of the neurotransmitter solution was maintained for 1 h; then the pondwater was removed and replaced by fresh pondwater lacking neurotransmitter, and the gill was monitored for another hour. The concentrations of serotonin and dopamine tested ranged from 10^{-6} to 10^{-4} M.

Rhodamine-123 treatment of gill explants

Mitochondrial activity in epithelial cells of the water canal was demonstrated with a mitochondrial fluorescence stain, rhodamine-123 (Johnson *et al.*, 1980), a positively charged lipophilic molecule that interacts specifically with mitochondrial membranes. Isolated gills were examined with a confocal imaging system supplied by Bio-Rad Laboratories (Richmond, California). Gills were excised from animals, and the interlamellar septa were cut to expose the central water channel. The gills were incubated for 20 min in pondwater containing 10 μ g/ml rhodamine-123. Following incubation, the gills were placed on glass slides and covered with coverslips with petroleum jelly at the corners. The samples were viewed under a Nikon Microphot-FXA microscope equipped for confocal imaging. Serial images along the length of the water canals were captured by digitizing on the image enhancement computer.

Results

Canal-associated musculature

Two bands of musculature are directly associated with the water canals of the gill. Located at the base of the filaments are relatively thick bands of musculature, 70–72 μ m and 20–23 μ m in diameter for *Anodonta* and *Ligumia*, respectively. These muscle bands underlie the fil-

aments and are oriented in the anterior-posterior direction along the entire length of the gill (Fig. 2). The muscle bands occur periodically, alternating with rows of ostia located at the base of the filaments (Fig. 3). Muscle bands occur approximately every 335 μ m and every 180 μ m in *Anodonta* and *Ligumia*, respectively. The rows of ostial openings connect the mantle cavity to the water canal at the base of the filaments (Figs. 1–3). The muscle bands lie perpendicular to, and run between, septations in the parallel calcified chitinous rods that support the filaments (Figs. 3, 4). The muscle appears to be attached to the end of the rods (Fig. 4). Indentations are observed at the separation points along the individual discontinuous chitinous rods. Contraction of the muscle bands pulls the rods of adjacent filaments together, reducing the water canal opening to a slit oriented in the dorsal-ventral direction. There is little musculature located in the underlying connective tissue in the vicinity of the water canal (Fig. 2) except where the water canal approaches the basal surface of the central water channel epithelium.

Another distinct band of musculature is located at the base of the canal near the opening of the water canal into the water channel (Fig. 5). These muscular bands are approximately 21 μ m in thickness in *Ligumia* and are oriented dorso-ventrally (Fig. 6). The muscle bands are on either side of rows of canals and send muscle fibers into the base of the canal epithelium, that forms the IO (Figs. 6–9). This musculature appears to have the ability to control the diameter of the IO (Fig. 9).

Neurotransmitter effects on canal-associated musculature

The two muscular systems described above responded similarly to the exogenous putative neurotransmitters to which the gills were exposed. The addition of serotonin to the gills *in vitro* resulted in an immediate relaxation of

copolysaccharide substance that appears darkly stained in this micrograph (arrowheads). Ostia are not visible in this micrograph as they alternate with muscular bands along the base of the filaments (see Fig. 3). Bar = 50 μ m.

Figure 3. Light micrograph of a gill from *Anodonta* cut across the face of the filaments in the dorso-ventral plane. This section is below the base of the filaments and demonstrates the alternation of water canal ostia (O) with nerve-muscle bands (NM). The periodicity of this alternation of structures is readily apparent. Note that the section is cut through the calcified rods (R). The rods taper (arrowheads) and become discontinuous every 335 μ m, and it is at this tapered site that the muscle bands interact with the rods. Mucopolysaccharide (Silverman *et al.*, 1983) material (P) associated with the rods is evident at the tapered sites. A few calcium concretions (CC) are seen in the connective tissue of this micrograph. Bar = 100 μ m.

Figure 4. Whole mounts of calcified chitinous rods from gills digested with collagenase. (a) Is a portion of a rod in which soft tissue has been completely digested away. Rods are discontinuous allowing the nerve muscle tract to pass in the anterior-posterior direction between adjacent filaments. The ends of the rods where muscles attach are flared and indented (arrowhead). (b) Is partially digested with collagenase and the remains of soft tissue/mucopolysaccharide (arrowhead) can be seen inserting on two rods (R) oriented end to end. The internal darker portion of the rod is calcified. The less dense perimeter contains layers of less calcified mucopolysaccharide. Bars in a and b = 2 μ m.

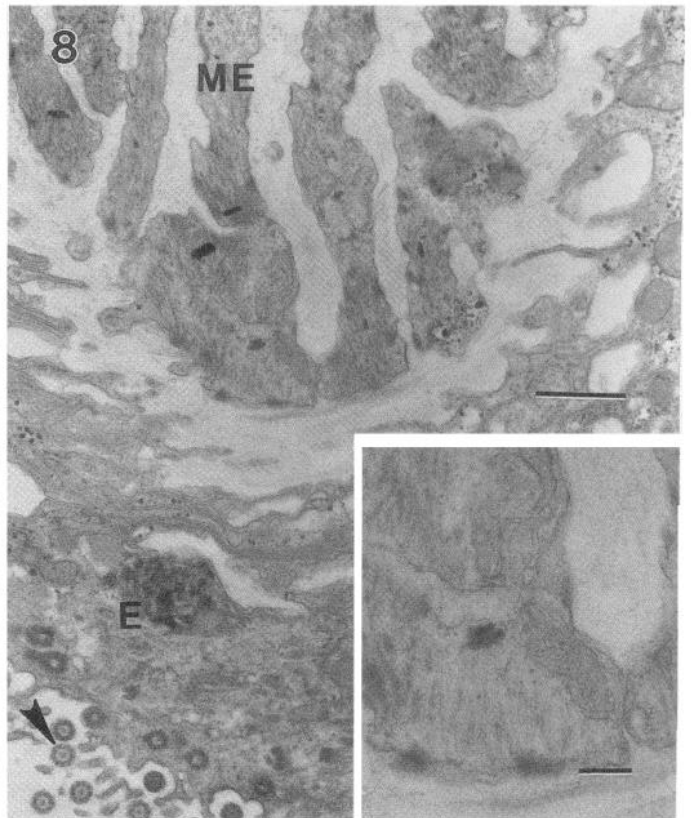
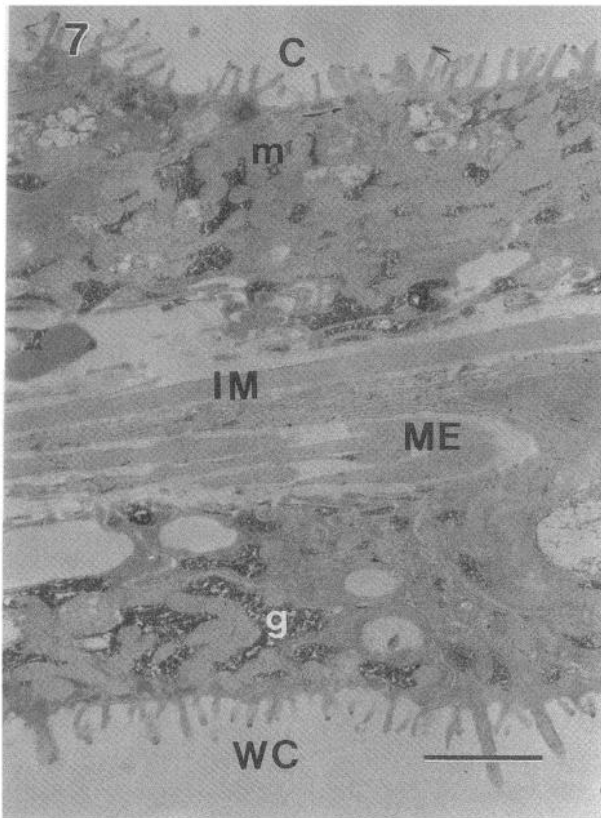
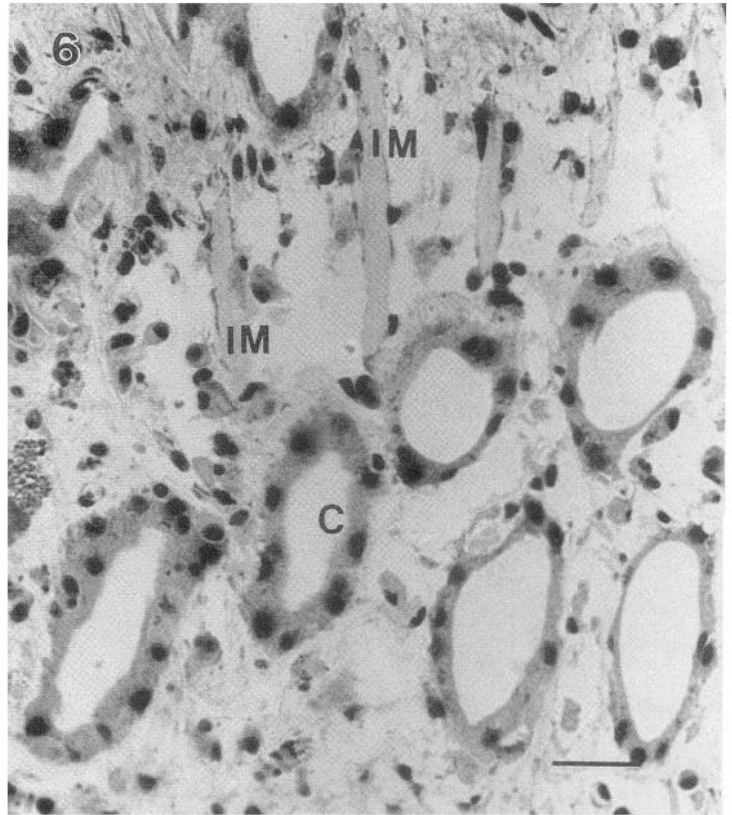
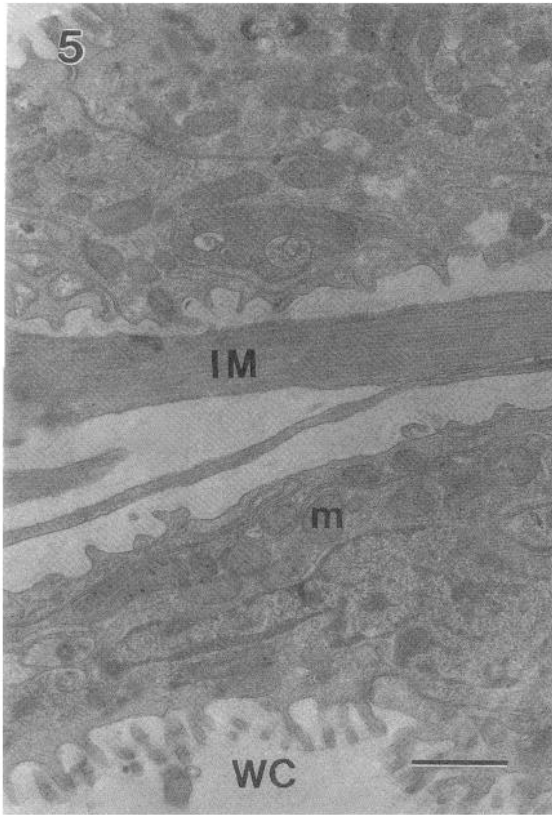


Figure 5. A TEM of the base of the water channel of a *Ligumia* gill. The water channel (WC) is located at the bottom of the micrograph. The epithelium of a water canal near its junction with the water channel is located at the top of the micrograph. Note the major obliquely striated muscle band (IM) located in the connective tissue at the base of the water channel epithelium. Mitochondria (m) are indicated in the mi-

the external and internal musculature, thereby increasing the diameter of the ostia (Fig. 10) and the internal ostia (Fig. 11). These results were visible by gross observation of the preparations with an inverted light microscope (Fig. 10). The relaxation was maintained throughout the 5-min observation period. In contrast, none of the other transmitters tested appeared to have any visible effect.

The internal ostia were examined by SEM and the average dimensions of the internal ostia were measured in control and experimental treatment groups (Table I). The average dimensions of the internal ostia in a serotonin-treated gill are 2–3 fold larger than controls (Table I, Fig. 11). The internal ostia of the controls have a distinct long axis (height) showing indented edges (Figs. 11, 12). The fully relaxed serotonin-treated ostia have a smooth, uniform oval to circular shape (Figs. 11, 13). The addition of acetylcholine, dopamine, GABA, epinephrine, or norepinephrine caused no observable changes in the size of the internal ostia, implying that the contractile state of the gill musculature was not affected by these agents (Table I).

Rhodamine-123 treatment

The muscular control of canal ostia, and the regulation of water flow through the canal, are consistent with our hypothesis that the water canal epithelial cells are a major site of ion transport in the gill. When gills were incubated in rhodamine-123, fluorescence was specifically localized with the mitochondria-rich cells of the canal epithelium. When the intact living gill was visualized with confocal optics, the cells lining the water canals displayed the greatest fluorescence because of their high mitochondrial content (Fig. 14). Optical sectioning shows that this high activity is in every water canal epithelial cell and extends along the entire length of the water canal.

Gill ciliary activity

In vitro, gills incubated in pondwater showed a consistent rate of ciliary activity (15 beats/s) during the 1-h control observation (Fig. 15). Addition of 10^{-4} M serotonin caused an immediate increase in ciliary activity that peaked within 20 min at 24 beats/s. The high ciliary rate was maintained until serotonin was removed 40 min later, and the ciliary beat returned to base line about 40 min thereafter.

Dopamine had an effect on ciliary activity, but it differed from that of serotonin (Fig. 15). The increased ciliary activity peaked at 20 beats/s upon the addition of 10^{-4} M dopamine, but 40 min were needed to reach the peak ciliary rate. After dopamine was removed, the ciliary activity immediately dropped to baseline. The response to both dopamine and serotonin was dose-dependent as shown in Figure 16.

Discussion

Most studies of water flow through eulamellibranch gills stress the role of ciliary activity as the driving force for water flow (Jorgenson, 1982; Silvester 1988; Sleight, 1989). Indeed, many flow measurements and coupled mathematical analyses indicate that ciliary activity is sufficient to account for the water flow (Jorgenson, 1989; Sleight, 1989). These models treat water canals as hollow tubes of fixed dimensions for the calculations. While such models are useful for studying water flow through gill systems, they are constrained by the underlying assumptions. The measured 2–3 fold difference in the internal ostia dimensions between control and serotonin-treated gills indicates that effective water canal size and its regulation are potentially important factors to be considered for water flow through the gill of unionids. The substantial increase in ostial size coupled to the increase in ciliary activity known

tochondria-rich water canal epithelial cells. Bar = 1 μ m.

Figure 6. A light micrograph of a face section through the gill of *Ligumia*. The section is cut through the gill just above the base of the water channel epithelium. Located between and associated with the canals (C) are bands of muscle (IM) traversing in a dorsal/ventral direction. These muscle bands alternate with rows of canals and send fibers to the base of the canal epithelial cells. Muscle bands are not seen in every location between water canals as the section is at a slightly oblique plane. Bar = 60 μ m.

Figure 7. Low magnification TEM of *Ligumia* gill indicating that the major internal muscle band (IM) lying at the base of the water channel (WC) epithelium branches and has numerous muscular extensions (ME). These extensions eventually end in the region of the internal ostia with several muscle fibers inserting at the base of the water canal (C) epithelium (see Fig. 8). Most of the cytoplasm of the two epithelia observed is occupied by glycogen (g) and mitochondria (m). Bar = 2 μ m.

Figure 8. Higher magnification TEM of *Anodonta* gill showing that the muscle extensions end in thin, finger-like processes consisting of only a few muscle fibers. These fibers are obliquely striated fibers, and the inset indicates the presence of thick and thin filaments. The muscle is inserting in the basal region of the water canal near the water channel epithelium (E) and has hemidesmosome-like electron-dense material at the muscle-connective tissue interface (inset). Note that in this region of the water channel epithelial cells are ciliated (arrowhead). Bar = 1 μ m; inset bar = 0.25 μ m.

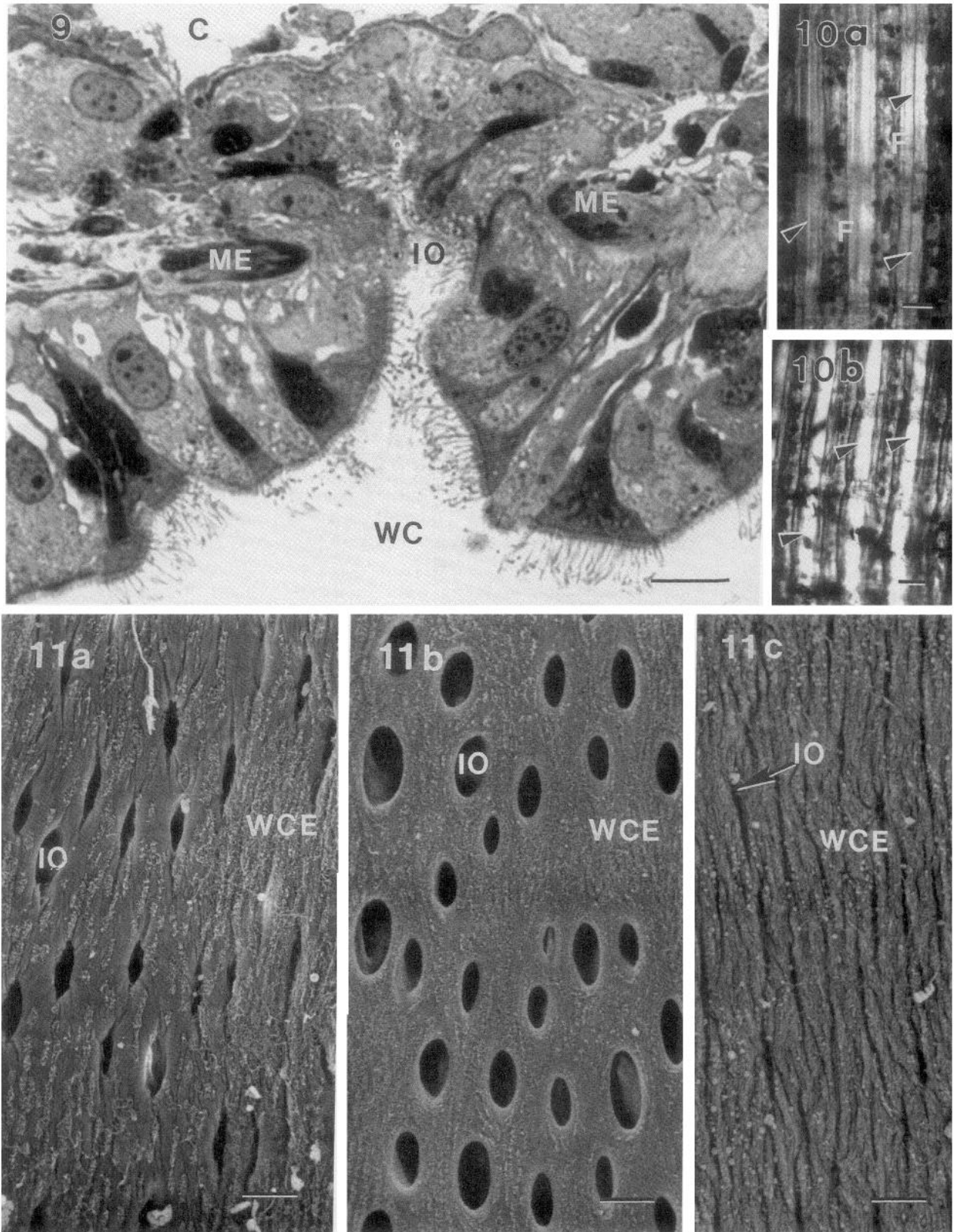


Figure 9. A light micrograph from *Anodonta* gill showing the finger-like muscle extensions (ME) on either side of a water canal (C) at the base of the epithelial cells where the canal enters the water channel (WC) at the internal ostia (IO). Bar = 60 μ m.

to occur with serotonin in some bivalves (Gosselin *et al.*, 1962; Aiello and Guideri, 1966; Aiello, 1970; Paparo and Murphy, 1975; Jorgenson, 1976; Capatane *et al.*, 1978; Sanderson and Satir, 1982; Sanderson *et al.*, 1985; this study) makes canal size regulation an important, underestimated contributor to water flow regulation. The three-fold difference in ostia dimensions with serotonin treatment may be an over-estimate of the normal conditions based on the potential for some partial contraction of the muscle. We developed our methods to minimize fixation artifact, but the microscopic preparations would likely lead to reduced ostial dimensions (shrinkage) rather than enlargement. Our data demonstrate the potential range within which the mussels can regulate canal openings and water flow with the ostial musculature.

The general orientation of the musculature associated with the ostia and internal ostia was the same in the two unionid genera we examined and is thus likely to be the generalized pattern for the unionids. These muscles are obliquely striated and have not been well-characterized (Ridewood, 1903; Ortmann, 1911; Kays *et al.*, 1990; Richard *et al.*, 1991). Their organization and their association with the ostia suggest that the axes for movement and for regulation of the two openings are different. The ostia are regulated by the muscles. When they contract, adjacent chitinous rods of adjacent filaments are pulled toward one another closing the ostia. During relaxation, the tension on the rods is released, allowing the filaments to separate and the ostia located at the base of the filaments to open. Such a mechanism suggests that the gill as a whole would have a "postural tone" under normal conditions. This can be confirmed by watching the accordion-like movements of the gill due to spontaneous contractions, and the expansion of the gill when relaxed following the addition of serotonin. The muscle bands at the IO are perpendicular to the muscle bands at the ostia. They are oriented dorso-ventrally along the gill axis and in close

Table 1

Average internal ostia size in Ligumia subrostrata gills following exogenous treatment with biogenic amines

	Height	Width	Perimeter
ACh	39.7 ± 8.0	12.1 ± 3.2	93.9 ± 21.2
ACh-control	33.1 ± 9.1	12.3 ± 2.9	82.2 ± 22.1
Dopamine	24.3 ± 0.9	12.3 ± 1.5	65.9 ± 3.3
Dopamine-control	30.6 ± 0.9	10.3 ± 0.5	75.6 ± 1.9
Epi	34.5 ± 5.3	11.3 ± 1.4	81.9 ± 11.9
Epi-control	27.4 ± 5.3	10.2 ± 1.4	67.2 ± 11.5
Norepi	29.9 ± 4.6	9.4 ± 1.3	71.4 ± 11.2
Norepi-control	31.9 ± 1.9	11.3 ± 2.9	74.9 ± 7.5
GABA	22.5 ± 1.8	15.9 ± 1.9	64.6 ± 6.3
GABA-control	23.8 ± 4.5	13.2 ± 1.2	60.4 ± 9.0
Serotonin	57.9 ± 2.7*	24.4 ± 1.1*	147.3 ± 6.9*
Serotonin-control	33.1 ± 3.8	10.0 ± 0.9	78.3 ± 8.2

All measurements are in microns. Height and width refer to the longest and shortest axes of the oval shaped ostia, respectively. Data are means ± standard error (n ≥ 3), ACh = Acetylcholine, Epi = Epinephrine, GABA = Gamma Aminobutyric Acid, Norepi = Norepinephrine.

* Significantly different from controls, $P < 0.01$.

proximity to the IO; they exert control by sending a few muscle fibers to the base of the epithelial cells surrounding the IO. When these muscle bands contract, the inserting fibers pull on the IO, creating an elongated shape, and causing an indented appearance on the edges of the IO. Increased muscular contraction elongates and closes the opening. The SEM, TEM, and bright field images, comparing control to serotonin-treated gills, are all consistent with this proposed mechanism of action.

The results reported here indirectly suggest that serotonin is a relaxing agent for the muscle bands we have described. While the results have not been confirmed electrically, they are consistent with such experiments in other molluscan systems (Cambridge, 1959; Twarog and Cole, 1972; Satchell and Twarog, 1978; Kobayashi and

Figure 10. Comparison of whole mount light micrographs of control (a) and serotonin-treated (b) *Anodonta* gills. The micrographs show the surface of the filaments, allowing observation of the ostia (arrowheads). In (a), the control gill ostia are barely discernable as the lighter areas because the adjacent filaments (F) are pulled toward one another closing the space between filaments. Gills in this condition have few ostial openings. In contrast, a gill treated with 10^{-5} M serotonin (b) shows filaments that are farther from one another allowing ostial openings to enlarge. Bars in a and b = 50 μ m.

Figure 11. Scanning electron micrographs of the water channel epithelium (WCE) of *Ligumia* showing the internal ostia (IO) as they enter the water channel. (a) Is an untreated control gill. Note both the size and shape of the IO openings. The long axis (height) has a dorsal/ventral orientation. Their edges, particularly those on the dorsal and ventral ends, tend to have an indented appearance. (b) Is a gill that has been treated with 10^{-5} M serotonin. Relaxation of musculature allows the IO to fully expand. The ostia still have a dorsal-ventral orientation although not nearly as pronounced. The ostia have an oval to round shape and the indentations seen in (a) are absent. The oval orientation is likely due to the orientation of the underlying musculature. (c) Is a gill that has been treated with pH 5 buffer to stimulate full contraction. Note the exaggerated dorsal-ventral orientation and deep indentations of the ostial edges oriented in the same direction. The "pull" by the underlying musculature has occluded the IO opening. Bars in a, b and c = 10 μ m.

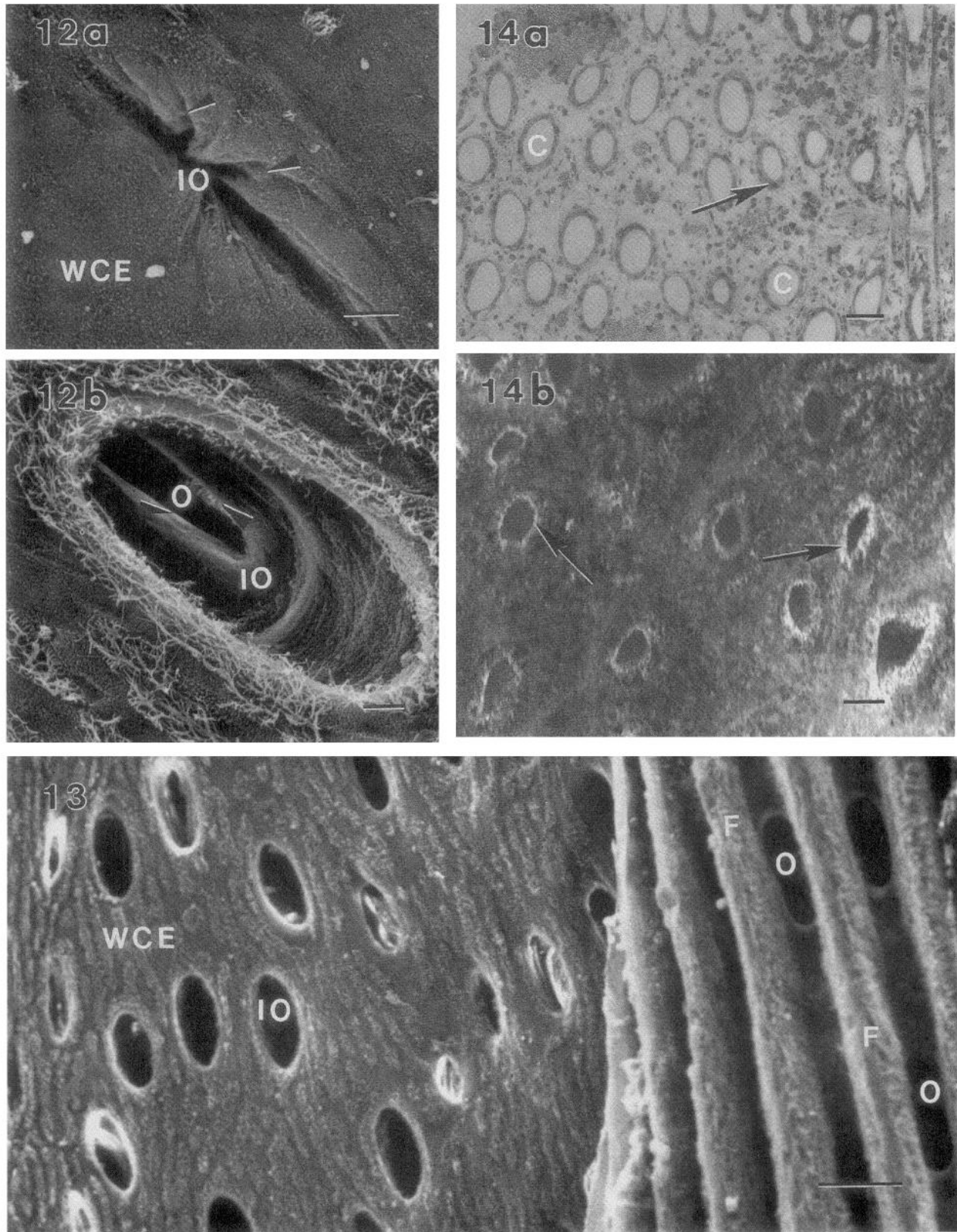


Figure 12. High magnification SEM micrographs of *Ligumia* gills viewing the water channel epithelium (WCE). (a) Is from a control gill showing the elongation in the dorsal-ventral direction. This internal ostium is almost completely occluded. The indentations of the IO border are evident (arrows) as well. (b) Is an internal ostium from a serotonin-treated ($10^{-5} M$) gill. The dorsal-ventral longitudinal orientation is evident.

Hasimoto, 1982). Acetylcholine, dopamine, norepinephrine, epinephrine, and GABA were neither excitatory nor inhibitory in our bioassay. These results clearly do not exclude any of these substances as putative excitatory transmitters because bath application may not allow these agents to reach their targets. We were able to demonstrate a dose-response relationship of ciliary beat for both serotonin and dopamine, but our bioassay was not sufficiently sensitive, and so no dose-response relationship for serotonin-induced muscular relaxation was demonstrated only an all-or-none relaxation response occurred.

Although these muscles have previously been ignored, their importance to the functions of the unionid gill should not be overlooked. Evidence demonstrating that the gill is the predominant site of ion regulation in unionid mussels is convincing (Dietz and Findley, 1980; Dietz and Graves, 1981; Dietz and Hagar, 1990). Further, more evidence is accumulating (Kays *et al.*, 1990; this study) that the epithelial cells of the water canals are important osmoregulatory cells. The high mitochondrial content and activity (as demonstrated here by rhodamine-123 experiments), surface area calculations, considerable basal and lateral membrane infolding, and the high levels of cytochrome oxidase activity (Kays *et al.*, 1990) shown by these cells all suggest osmoregulatory function. Coordinated muscle and ciliary activity may allow finer control and a wider range of regulation, including a shut-down of water flow. The coordinated control of ciliary and muscular activity is apparent, at least in response to serotonin.

No-flow conditions do occur in some unionid species during reproduction. In the Lampsilinae, the central water chambers housing embryos are physiologically isolated from the water flow through the mantle cavity (Silverman

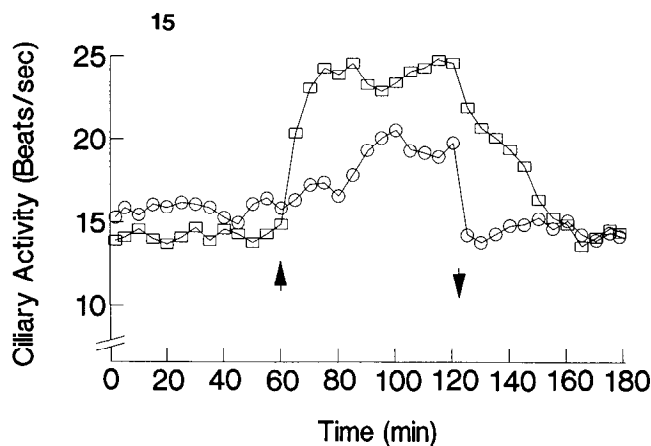


Figure 15. Lateral ciliary activity of a representative (of 5) *Ligumia* gill in response to application of exogenous serotonin and dopamine. Gills were exposed either to 10^{-4} M serotonin (open squares) or 10^{-4} M dopamine (open circles) for 1 h. Initiation of the treatment is indicated by the upward pointing arrow and termination by the downward pointing arrow.

et al., 1987; Richard *et al.*, 1991). We speculate that the mechanism for reduced water flow into the brood chamber is, in part, regulation by canal ostial musculature.

Mathematical treatments of the hydrodynamics of water flow through mussel gills do not completely fit the available data. Silvester (1988) has recently concluded, after an elegant treatment of *Mytilus* gill ciliary mechanics, that faster flow than can be accounted for by known ciliary activity actually occurs. Indeed, his final statement, "one should perhaps be alert to the possibility that other systems in the mussel may be contributing to the pumping performance" (Silvester, 1988), could allude to the possibility

The IO opening is not indented as seen in (a). This field allows a clear view through the IO and into the water canal. At the filament side of the water canal is an ostium that is delimited by the filaments on either side of the ostium. This is evident by the dorsal-ventral, straight edges (arrowheads) of the ostium. The muscle bands underlying the two ostial openings lie perpendicular to one another (not shown), but both bands work to close their respective opening in the dorsal-ventral direction. Bars in a and b = 10 μ m.

Figure 13. An SEM micrograph of *Ligumia* gill exposed to 10^{-5} M serotonin. The gill has been prepared so that the left hand side of the micrograph has one lamella removed to expose the water channel epithelium (WC) while the right side of the micrograph contains an intact lamella and filaments (F). Between the filaments, the ostia (O) are visible and fully open, displaying the dorsal-ventral long-axis orientation. They clearly demonstrate the limitations on their size being set by the inter-filament distance. The space between filaments is controlled by muscle inserting on the chitinous rods supporting the filaments. The internal ostia (IO) show the same orientation. Bar = 50 μ m.

Figure 14. Face view of *Ligumia* gills showing the mid-region of the water canals (C) passing from the gill filaments into the water channel. (a) Is a control light micrograph of fixed tissue similar to that seen in Figure 6. Note the size of the water canal epithelial cells (arrow). These cells have previously been shown to be high in cytochrome oxidase activity (Kays *et al.*, 1990). (b) Is an optical section through a living gill accomplished using confocal imaging techniques. The gill has been treated with rhodamine-123 to highlight mitochondrial location. The major fluorescence corresponds to the cytoplasm of the water canal epithelial cells (arrows). The canal cells are the major site of active mitochondria. There is some auto-fluorescence associated with other epithelia of the gill, but it is minor compared with that seen in the water canal epithelial cells. Bars in a and b = 50 μ m.

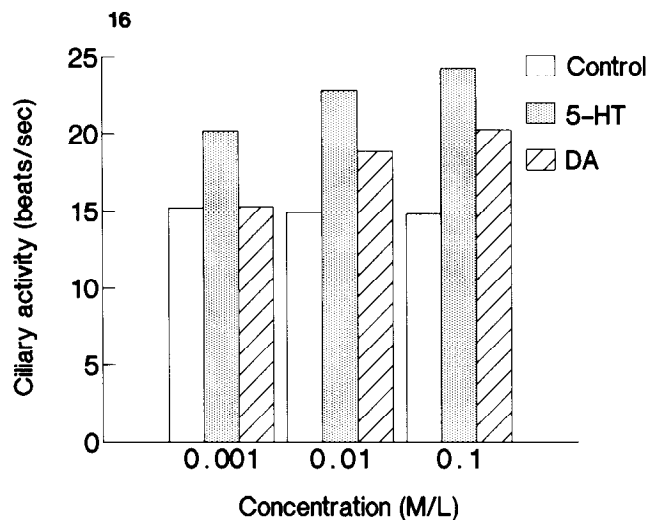


Figure 16. Dose-response relationship of lateral ciliary beat in *Ligumia* gills following the application of serotonin or dopamine. The last three ciliary rate measurements of the initial control and treatment periods were averaged for each gill, and the average of five gills are presented (standard error < 0.5 beats/s).

of muscular activity aiding ciliary function. Indeed, the anterior-posterior and dorsal-ventral contractions of the muscle bands in the gill may provide additional driving force through an accordion-like motion. The current study indicates that these muscles are likely to be important contributors to water flow dynamics across the molluscan gill, at least for the unionids.

Acknowledgments

We thank Dr. A. Paparo for determination of ciliary activity, Ms. Beckey Demler of the LSU Basic Sciences Microscopy Center for technical help, and Ron Bouchard for photographic assistance. All image analysis was done in the Microscopy Facility. This work comprises a portion of an MS thesis (Louisiana State University) by DBG. This work was supported by a NSF grant DCB88-02320.

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