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Comparative Structure and Function of Intrinsic Gill Muscles in Freshwater Bivalve Molluscs.

Scott Medler
Louisiana State University and Agricultural & Mechanical College

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COMPARATIVE STRUCTURE AND FUNCTION OF INTRINSIC GILL MUSCLES IN FRESHWATER BIVALVE MOLLUSCS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
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in partial fulfillment of the
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in

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by
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DEDICATION

To my Dad, Byron W. Medler, who always encouraged.

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ABSTRACT

The lamellibranch gill is used for various vital functions ranging from food capture to ion regulation. The foundation for many of these functions is the transport of water through the gill. This dissertation examines the organization of intrinsic gill muscles and the associated connective tissue in the freshwater bivalve *Dreissena polymorpha* with comparisons made to *Corbicula fluminea* and *Toxolasma texasensis*. Gill muscles can be divided into two groups: those associated with the connective tissue sheets that underlie external and internal gill epithelia; and those encased in connective tissue bands oriented perpendicular to the bases of gill filaments. The sets of muscles are oriented to serve complementary functions of drawing filaments together and of reducing ostial openings, thus affecting water flow through the gill. The supportive tissue in the gills of each of the three species is composed of a similar extracellular matrix with interspersed muscle fibers. These matrices consist of a periodic acid Schiff-positive tissue supported by small collagen fibrils as determined by morphological and biochemical examination. The ultrastructure of the muscle fibers associated with the connective tissue corresponds with a known type of smooth muscle. The gill muscles of *D. polymorpha* contract in response to acetylcholine and FMRFamide but relax with serotonin application. External calcium is required for muscle contraction and a proper balance between NaCl and KCl is critical for the maintenance of maximal responsiveness. Acclimation to hyperosmotic conditions is dependent in part on the activity of a ouabain-sensitive Na^+/K^+ ATPase. The gills of all three species show a common relationship between live gill area and dry body mass. Each of the animals have comparable ostial dimensions and possess the ability to control these dimensions through muscular tone. The similarity in structure and apparent function of the intrinsic gill muscles in each of the species examined suggests that the muscles are an important, conserved feature of the bivalve gill.

CHAPTER 1

INTRODUCTION

Filter-feeding bivalves can play a central role in the ecology of aquatic ecosystems when they occur in high densities. Dame (1993) compared the functional role of filter-feeding marine bivalves to that of bison on the great plains of North America, where a single group of organisms plays a dominant role in grazing. Filtration of suspended particles by bivalves can influence ecosystem function largely through material uptake and subsequent biodeposition (Jørgensen 1990; Dame 1993). These processes are important because they may ultimately affect parameters other than material fluxes, such as nutrient processing and retention (Dame 1993). The importance of bivalve filter-feeding to an ecosystem is largely determined by the size of the bivalve population relative to the resident water volume. The entire resident water volume in certain sites may be filtered every 0.7 to 13 days depending on the filtration capacity and water volume and may represent eutrophication control (Cloern 1982; Officer et al. 1982; Smaal and Prins 1993). In fact, in the Netherlands, *Dreissena polymorpha* is being studied as a tool in eutrophication control (Smit et al. 1993). In the Great Lakes region of the U.S., zebra mussels (*D. polymorpha*) can exhibit significant filtration capacities. Bunt et al. (1993) estimated that small zebra mussels were capable of pumping between 39 and 96% of the water column per day at a western Lake Erie site and Fanslow et al. (1995) estimated that a zebra mussel population at a Lake Huron site could filter between 0.2 and 1.3 times the resident volume per day. Further, using a bioenergetics model approach, Madenjian (1995) estimated that about 25% of the primary production in western Lake Erie was consumed by the zebra mussel population. These tremendous filtration capacities may lead to dramatic ecosystem effects such as enhanced water clarity, increased macrophyte and cyanobacterial densities, shifts in energy from pelagic to benthic foodwebs, and enhanced biomagnification of toxins to higher trophic levels (MacIssac 1996). Heavy zebra mussel infestations serve as an extreme example of the potential influence of filter-feeding bivalves on the ecosystem.

The ability of bivalves to exploit a filter-feeding lifestyle is intimately related to gill structure. During the Ordovician, small bivalve deposit feeders are thought to have developed an enlarged gill that not only served a respiratory function, but also allowed small particles to be filtered from the water column (Cope 1993). The small gills that primarily served a respiratory function are still represented in a few extant bivalve groups as the protobranch type of gill. The originally expanded gill allowing filter-feeding was presumably similar to the filibranch gill found in many extant bivalves (Cope 1993). While these filibranch gills are believed to have arisen in a single group of animals early in the evolution of bivalves, the eulamellibranch gill type has arisen independently several times from the filibranch gill in several bivalve groups (Cope 1993). In all filter-feeding bivalves, water is drawn between filaments by lateral ciliated cells and into the central portion of the gill. The difference between the filibranch gill and the eulamellibranch gill is that in the latter gill type the filaments are connected by sheets of subfilamentar tissue, while the filibranch gill is composed of less united filaments (Fig. 1.1). All of the animals in this study possess the eulamellibranch type of gill.

Theoretical models of the bivalve gill (Foster-Smith 1976; Jørgensen et al. 1986) have been developed in order to understand the basic processes involved in filtration. These models are simplified versions of the gill that compartmentalize the bivalve into a series of canals that can be represented by pressure heads as water moves through the animal. Simplifying assumptions include estimates of the dimensions of the water passageways through the gill. For example, Foster-Smith (1976) assumed the water canals through the gill to be circular tubes 60 μm in diameter and 100 μm long, while Jørgensen et al. (1986) modelled these as rectangular slots 40 μm in diameter and 200 μm long. Both of these models predict that the largest changes in pressure through the system is from the water leaving the animal via the excurrent siphon and secondarily from the water moving through the interfilament spaces of the gill. Based on these findings,

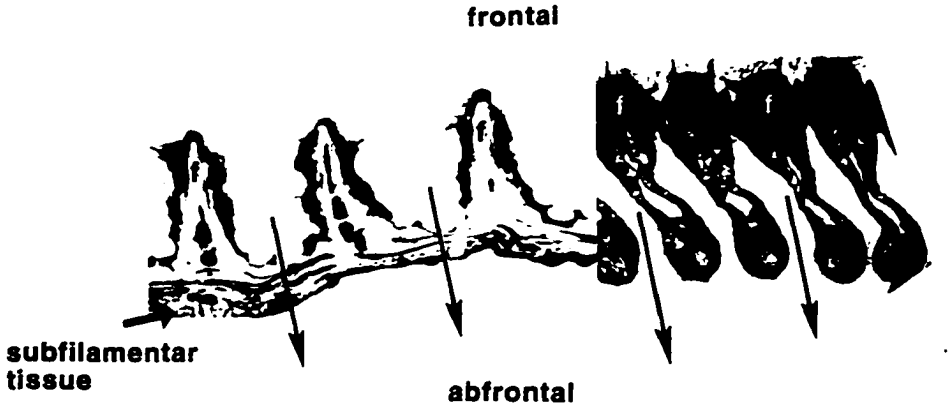

Fig. 1.1. A comparison between the eulamellibranch gill (left side; exemplified by *D. polymorpha)* and the filibranch gill (right side; exemplified by *M. edulis).* In both gill types, water flows between filaments (f) from the frontal side of the gill to the abfrontal side. The filaments are joined by sheets of subfilamentar tissue in eulamellibranchs, but are much more independent in the filibranch gill. Water flows through water canals in the subfilamentar tissue in the eulamellibranch gill (not shown). (The total thickness of the lamellae shown is 55 μm for *M. edulis* and 25 μm for *D. polymorhpha*). Light microscopy.

Foster-Smith (1976) argued that the excurrent siphon was the most important adjustable resistance factor for the bivalve pump, believing that the ostial dimensions could not change fast enough to be the primary adjustable factor. Jørgensen and coworkers (Jørgensen et al. 1986; Jørgensen et al.1988; Jørgensen 1989; Jørgensen 1990) have indicated that the interfilament distance is the most important adjustable factor in the bivalve pump because this distance affects the interference between opposing lateral ciliated tracts. Overall, the capacity of the bivalve pump to transport water is determined by the pump characteristics (ciliary beat) and the system resistances (dimensions of the water passageways) (Foster-Smith 1976). Whether the dimensions of the gill affect the ciliary activity directly or affect the resistance to water flow, significant changes in these dimensions should affect water flow through the animal. Interestingly, Silvester (1988) compared measured pump capacities with theoretical ciliary capacities based on widely accepted parameters. He found that the ciliary activity alone was insufficient to account for measured pump capacities and suggested that there might be some factor unaccounted for in the current theoretical models.

The contribution of the intrinsic musculature to basic gill function is almost completely unknown. Jørgensen (Jørgensen et al. 1988; Jørgensen 1990) has indicated that the interfilament distance is controlled by muscles of the gill axis, the filaments being drawn together as the gill axis is retracted. This description completely ignores the fact that an extensive musculature within the gill itself is a common feature of the bivalve gill. With the exception of the studies of Setna (1930), Elsey (1935), Atkins (1943) and later by Gardiner et al. (1991), the descriptions of intrinsic gill muscles have been largely anecdotal. Elsey (1935) indicated that variation in ostial dimension, locally or through the gill as a whole, was accomplished by the interaction of blood pressure, elastic skeletal material, and intrinsic gill muscles and this variation could affect water flow. Atkins (1943) believed that slight contractures of the muscles worked in a coordinated fashion

with the gill cilia, leading to fine regulation of feeding mechanisms. She further thought that the violent contractions of the gills, for example those preceding rapid closure of the valves and the subsequent expulsion of water from the animal, were for the protection of the gills. Further study of the general anatomy, regulatory control, coordination, and basic function of these muscles has been almost non-existent. In his comprehensive study of oyster feeding mechanisms, Nelson (1960) wrote, "Too little study, anatomical and physiological, has been made of musculature of oyster's gills, and of relation of gill movements to operation of ciliary mechanisms." He concluded that the muscular movements were important in feeding and that "further research in this field should yield rich returns."

The initial and major focus of this dissertation is the basic structure and function of the intrinsic gill musculature of *Dreissena polymorpha* (Pallas 1771; Superfamily Dreissenacea) and their possible contribution to the regulation of water flow through the bivalve gill. This species is a relatively recent inhabitant of North America, apparently released from ballast water near Detroit, MI in 1985 (Hebert et al. 1989) and may have originated from the Black Sea/Caspian Sea region of Europe (Smirnova et al. 1993; Ludyanskiy 1993) . The animals have quickly spread throughout the Great Lakes region and into the Mississippi River system (Ram and McMahon 1996). In addition to *D. Polymorpha*, comparative analysis of *Corbicula fluminea* (Müller 1774; Superfamily Corbiculacea) and *Toxolasma texasensis* (I. Lea 1857; Superfamily Unionacea) is reported. The dreissenids and corbiculids share a common lineage (Nuttall 1990; Morton 1993) and underwent rapid radiation into freshwater habitats during the Miocene. The unionids have inhabited freshwater since the Triassic (Haas 1969) and are the only one of the three groups native to North America. The groups exhibit differences in physiology, morphology, and life history traits important to the general biology of the animals. For example, one important character, unique among the three species to *D. polymorpha*, is

the possession of a heteromyarian condition (Morton 1993) which has allowed the exploitation of a hard substratum. This heteromyarian form is characterized by a reduction in the anterior adductor and byssal retractor muscles as well as a flattening of the ventral margin and neotenic retention of the byssus. This form has played an important role in the success of *D. polymorpha* (Morton 1993). Other differences include variability in the ion transport characteristics of these species (Dietz et al. 1994; Dietz et al. 1996; Dietz et al. 1997; Horohov et al. 1992). It seems reasonable that interspecific differences may also exist between the general structure and function of the integral gill muscles. The similarities and differences observed between the species may give insight into the general function of these gill muscles.

In this dissertation, Chapter 2 focuses on the functional organization of the muscles and associated connective tissue elements in the gill of *D. polymorpha* and their apparent ability to regulate water flow. This section not only shows the general scheme of how the muscles and connective tissue function together, but also demonstrates that the active musculature responds to known transmitter substances. Included are comparisons to *C. fluminea* and *T. texasensis* which show that these species have the same basic ability to alter their gill dimensions as *D. polymorpha*.

Chapter 3 provides analysis of muscle cell ultrastructure and partial characterization of the connective tissue in all three species. All of the gills are made of a similar collagenous connective tissue matrix with interspersed smooth muscle fibers. Although the presence of collagen has been generally accepted for a number of years, this is the first explicit identification of collagen as the supportive tissue in the bivalve gill. The smooth muscles are similar structurally and correspond to a specific smooth muscle ultrastructure found in other molluscs and in echinoderms.

Chapter 4 begins study on the effects of ionic composition on gill muscle function in *D. polymorpha*. Muscle contraction was monitored indirectly by measuring changes in

gill dimension over time. Extracellular Ca^{2+} is an absolute requirement for contraction and the balance between Na^+ and K^+ is also important. The muscles can acclimate to a hyperosmotic environment as long as the appropriate concentration of K^+ is present. This acclimation process can be partially blocked by ouabain, demonstrating the importance of a Na^+/K^+ ATPase for the response. Overall, these muscles respond to their ionic environment as one might expect from active smooth muscles.

Finally, Chapter 5 examines the overall dimensions among the three species' gills and the ability of their intrinsic muscles to alter these dimensions. Differences in the ability of freshwater bivalves to filter bacterial sized particles is largely dependent on the structure of the ciliated cells of the gills. Although there are differences in the absolute size and dimensions of the gills, the relative size and dimensions in all three species are uniform.

The comparative studies indicate that active smooth muscles and associated collagenous connective tissue are a common element of the eulamellibranch gill. The muscle fibers respond to neural transmitters and work with the connective tissue skeleton to affect the dimensions of the gill. Despite significant differences in certain anatomical parameters of the gills, the overall size and dimensions seem to be fixed. Together, these results suggest that the intrinsic muscles are a conserved feature that are important for basic gill function.

CHAPTER 2

FUNCTIONAL ORGANIZATION OF INTRINSIC GILL MUSCLES IN *DREISSENA POLYMORPHA* AND RESPONSE TO TRANSMITTERS *IN VITRO*[*]

[*] Reprinted with permission of Invertebrate Biology. Originally published as:
Functional organization of intrinsic gill muscles in zebra mussels, *Dreissena polymorpha* (Mollusca: Bivalvia), and response to transmitters *in vitro*
Scott Medler and Harold Silverman
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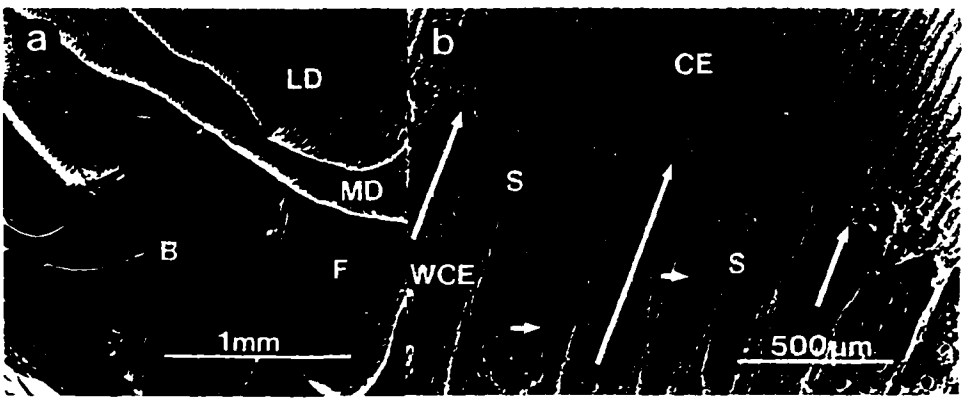
Lamellibranch gills are often the major site of gas exchange, ion transport, and nutrient capture depending on the species of bivalve (Barrington 1979; Dietz 1985; Pearse et al. 1987; Kays et al. 1990; McMahon 1991). In some bivalves, the gill also serves as a reproductive brood chamber (Ortmann 1911; Silverman et al. 1985; Silverman 1989; Tankersley and Dimock 1992; Tankersley 1996) or the organ housing symbiotic organisms that produce nutrients for the bivalve (Southward 1986; Cavanaugh et al. 1987; Dando and Spiro 1993). All of these vital functions can be influenced or regulated by the amount of water flowing through the gill. While there are clearly variations among bivalve families (Ridewood 1903; Atkins 1937), the major force generating water flow in a eulamellibranch is provided by the lateral ciliated cells on adjacent filaments (Jørgensen 1975; Silvester 1988; McMahon 1991). The movement of these cilia propels water into the mantle cavity through an incurrent siphon. Inside the mantle cavity, water is drawn into the gill through external ostia that lead into water canals (Pearse et al. 1987; Gardiner et al. 1991; McMahon 1991). Water moves through these canals into a central water channel via internal ostia, and then travels dorsally through the water channel to the suprabranchial chamber (Barrington 1979; Pearse et al. 1987; Gardiner et al. 1991; McMahon 1991) (see Fig. 2.1). Water exits the animal through the excurrent siphon.

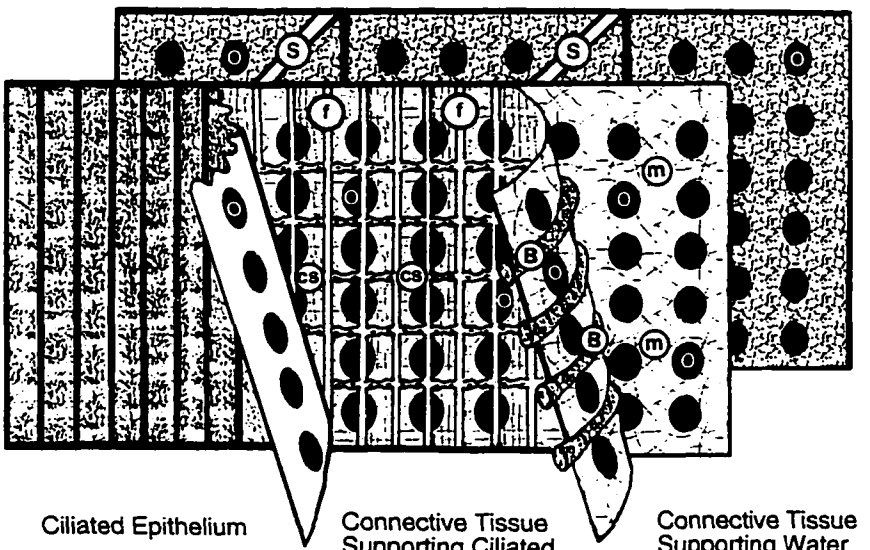
While the driving force for water movement is ciliary activity, there are several control points in this pathway that are under muscular control. For example, the incurrent and excurrent siphons vary greatly in their diameter in response to the tone of integral muscles. Foster-Smith (1976) characterized the excurrent siphon as the most important adjustable resistance factor influencing water flow through several bivalve species. In their model of the bivalve pump, Jørgensen et al. (1986) supported this view with the estimation that the excurrent siphon accounts for the largest pressure head in the water pump. Lei et al. (1996) observed changes in excurrent siphon diameter of *Dreissena polymorpha* in response to changes in particle concentration. Water flow may also be

Fig. 2.1. General organization of the gills in *D. polymorpha* with respect to the gross morphology of the animal.
(a) A lateral demibranch (LD) and a medial demibranch (MD) compose a gill on each side of the animal. The byssus (Bs) and foot (F) of the animal are also seen. SEM.
(b) A higher magnification micrograph of a single demibranch showing the outer ciliated epithelium (CE) and the internal structure of the demibranch where the outermost lamella has been removed. Water moves past the ciliated epithelium and into the central water channel via water canals (not shown). These water canals empty into the central water channel through internal ostia (small arrows) in the water channel epithelium (WCE). The central water channel is partitioned into water tubes by the septa (S) which connect opposing gill lamellae. Water moves dorsally (large arrows) through the water tubes into the suprabranchial chamber (not shown) before exiting through the excurrent siphon (not shown). SEM.
(c) A diagrammatic composite of the elements of a demibranch (not to scale). The outermost layer is the ciliated epithelium. Supporting the ciliated epithelium is a loose connective tissue sheet, filament supports (f), and cross-struts (cs) between filaments. Bands of connective tissue and muscle fibers (B) lie deep to the base of the filament supports and are continuous with the deep portion of the cross-struts. A second connective tissue sheet supports the water channel epithelium. The hemocoel of the gill lies between the connective tissue sheet supporting the ciliated epithelium and the connective tissue sheet supporting the water channel epithelium. Associated with both loose connective tissue sheets are arrays of muscle fibers (m) that radiate in many directions. External and internal ostia (O) are associated with the external and internal portions of the gill, respectively. Septa (S) connecting the lamellae are continuous with the water channel epithelium and the associated connective tissue sheets.



C

Water Channel Epithelium



controlled by integral gill muscles that control interfilament and ostial dimensions. In the Jørgensen et al. (1986) model of the bivalve pump, the interfilament canals collectively are considered to be the second largest pressure head in the pump, accounting for over 30% of the total pressure in the system. Increases in pressure are inversely related to the square of the interfilament distance. Jørgensen (1989) also indicated that the distance between opposing bands of lateral cilia, controlled by the width of interfilament canals, is "the main factor in determining pump pressure and flow rate in bivalves". Other studies have demonstrated a relationship between ostial dimension and water flow, particle filtration, and filtration efficiency (Nelson and Allison 1940; Dral 1968; Foster-Smith 1975, 1976)

Many studies have documented neural control of the bivalve gill (reviewed by Paparo 1988). Application of exogenous transmitters has been known for some time to affect ciliary activity (Aiello 1960; Aiello 1962; Aiello and Guideri 1964). The effects of transmitters on intrinsic musculature (Gardiner et al. 1991) and overall pumping rate (Jones and Richards 1993) have been examined more recently. Previous accounts reported that the gill muscles of *D. polymorpha* respond to exogenous transmitters (Duncan et al. 1994; Medler and Silverman 1994). Acetylcholine, FMRFamide (Phe-Met-Arg-Phe-NH_2), and serotonin are all physiologically important in a variety of molluscan species. Although there is considerable variation in response between and even within a species, FMRFamide and acetylcholine are often excitatory neurotransmitters, while serotonin is often inhibitory or neuromodulatory (Muneoka and Twarog 1983).

This study examines the role of connective tissue elements, intrinsic gill muscles, and their ability to alter the dimension of water passageways in a homorhabdic eulamellibranch gill. *Dreissena polymorpha* is a non-native freshwater species recently introduced into North America from the Caspian Sea/Black Sea region of Europe (Smirnova et al. 1993; Ludyanskiy 1993) and represents a phylogenic group distinct from

the previously studied bivalves. I report here that the musculature in the gill of *D. polymorpha* responds to exogenous acetylcholine, FMRFamide, and serotonin. Stimulation by FMRFamide and acetylcholine caused muscle contraction in the gill while serotonin caused muscle relaxation. Observation of live gill tissue revealed complex and dynamic changes in interfilament distance and internal ostial area consistent with regulation of water flow. These changes are related to the contraction of the intrinsic musculature integrated with the associated connective tissue of the gill. Incidental observations of *Corbicula fluminea* and *Toxolasma texasensis* were consistent with those made in *D. polymorpha.*

METHODS

Animals and maintenance

Dreissena polymorpha (Pallas 1771) were collected from Lake Erie at the mouth of the Raisin River in Monroe, Michigan and from the Mississippi River near Plaquemine, Louisiana. *Corbicula fluminea* (Müller 1774) and *Toxolasma texasensis* (I. Lea 1857) were collected from ponds near Baton Rouge, Louisiana. The animals were maintained in artificial pondwater (0.5 mM NaCl, 0.4 mM $CaCl_2$, 0.2 mM $NaHCO_3$, 0.05 mM KCl, and 0.2 mM $MgCl_2$) in aerated aquaria under laboratory conditions of approximately 22-25° C and 12h light / 12h dark cycles.

General gill preparation

Gills were excised by freeing the gill from its dorsal attachment with forceps and placed in a Ringer's solution designed to approximate blood composition of the animals (Dietz et al. 1994) (5 mM NaCl, 5 mM $CaCl_2$, 5 mM $NaHCO_3$, 0.5 mM KCl, 5 mM $NaSO_4$, 0.5 mM $MgCl_2$; 48 mOsm). Calcium-free Ringer's used in various experiments had the same composition as the above solution except that the $CaCl_2$ was omitted and 4 mM EDTA was added. The osmolality and pH were the same in both solutions. Lateral and medial demibranchs were separated by cutting along their dorsal connection.

Muscle labelling with phallacidin

The f-actin of muscle bands was labelled with phallacidin conjugated to a fluorescent marker, NBD [N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin]. Phallacidin is a bicyclic peptide that specifically binds f-actin at nanomolar concentrations in muscle and non-muscle cells from many different plants and animals (Molecular Probes Inc., Eugene, OR). Excised demibranchs were split into single lamellae and stripped of epithelium by placing them in a Ca^{2+}-free Ringer's solution containing 4 mM EDTA, and passing them through the end of a small transfer pipette. Phallacidin application procedures were those recommended by Molecular Probes. Briefly, single lamellae were extracted with a solution of -20° C acetone for 3 to 5 min and then air dried. The lamellae were incubated with phallacidin in Ringer's solution for 20 min at 25° C . Finally, the lamellae were washed twice with Ringer's solution. Samples were observed on a Nikon Microphot FXA using an excitation filter of 450-490 nm and a barrier filter of 520 nm. Gills were also observed with Nomarski illumination to compare structural correlates of labelled areas.

Preparation for transmission electron microscopy (TEM)

Prior to gill excision, hemolymph samples were taken from animals by inserting a 26 gauge needle between the valves and into the pericardial space (Fyhn and Costlow 1975; Dietz et al. 1994). Hemolymph osmolality was measured on a Precision Systems freezing point osmometer. A 2% glutaraldehyde solution was adjusted with phosphate buffer to match the hemolymph osmolality. Excised gills were cut along the filaments into strips about 3 mm wide and quickly fixed by immersion in the glutaraldehyde solution for 1 h. Gills were rinsed twice in phosphate buffer and post-fixed in 1% OsO_4 for 1 h, rinsed twice in phosphate buffer, and dehydrated in a graded ethanol series. Gill strips were embedded in LR White (London Resin Co.) medium grade resin by placing

them in a 1:1 mixture of ethanol and resin for 24 h. They were transferred to 100% resin for 12 h, and embedded flat in fresh resin at 60° C for 24 to 48 h.

Gills were sectioned with a Reichert-Jung ultracut E ultramicrotome at 60-90 nm thickness with glass knives. The gills were sectioned in two planes: (1) in a dorso-ventral plane between adjacent filaments, and (2) in frontal sections (*en face*) across gill filaments and through the muscles surrounding internal ostia. Sections 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 100CX transmission electron microscope operating at 80 kV.

Preparation of gills for scanning electron microscopy (SEM)

Dissected gills were placed in Ringer's solution as described above. In some instances, gills were exposed to acetylcholine, FMRFamide, or serotonin for 10-20 min prior to fixation. The solution was carefully drained and quickly replaced with liquid nitrogen in order to prevent muscular contraction of the gills during fixation. After a few s, the nitrogen was removed and the frozen gills were immersed in the osmotically balanced 2% glutaraldehyde solution for 1 to 4 h. Gills were rinsed in phosphate buffer and post-fixed in 1% OsO_4 for one h. After osmication, gills were rinsed in phosphate buffer and dehydrated in a graded ethanol series. Gills were wrapped in lens paper, critical-point dried, and mounted on stubs. Specimens were sputter coated with a mixture of gold and palladium (20 nm) and viewed with a Cambridge S-260 SEM. Measurements of ostial areas were made by cutting out and weighing printed images. These masses were converted into areas by calibrating with a known area.

Confocal microscopy

Optical sections (2 μm) of the connective tissue structures were observed by confocal imaging techniques with a Noran Instruments Odyssey XL Laser Confocal Microscope. Prior to observation, gills were incubated with a horseradish peroxidase-

FITC (fluorescein-5-isothiocyanate) conjugate at 1 mg/mL. Initially this methodology was used in an attempt to label neural elements of the gill. However, the marker was absorbed into the highly porous connective tissue elements, effectively labelling the connective tissue but not the nerves.

Measurement of demibranch contraction

Excised demibranchs were placed in Ringer's solution on a glass microscope slide such that the demibranch was allowed to float freely in the solution. The solution was then aspirated to leave the demibranch flattened on the surface of the slide. The gill was rapidly immersed in a Ringer's solution containing acetylcholine or FMRFamide at concentrations from 10^{-3} to 10^{-6} M. The transmitter-containing solution was aspirated immediately following its application, leaving the demibranch flat on the slide. During this procedure, gills were observed at a magnification of about 10X with a dissecting microscope and recorded on VHS videotape. Video images were digitized and measured with Image-1 computer software (Universal Imaging Corp.). Gill area was measured prior to transmitter exposure and at timed intervals after transmitter application. Changes in gill area over time were either expressed as the relative reduction from the initial area (% of initial area), or as surface area (mm^2).

Observation and measurement of perfused gill tissue

Internal ostial dimensions were observed using a Nikon Diaphot inverted microscope with Hoffman Modulation optics or Nomarski (DIC) optics at magnifications of 400X or 600X and recorded on VHS videotape. Excised demibranchs were split along the interlamellar septae into ascending and descending lamellae. Individual lamellae were placed in a 200 µl perfusion chamber with the water channel epithelium visible and held in place by nylon mesh. 60 or 70% Ringer's solution perfused the tissue at a flow rate of between 1.5 and 5 mL/min. The solutions perfusing the lamellae were introduced at the bottom of the chamber and aspirated at the top. Thus, water flow was theoretically

unidirectional from bottom to the top of the chamber. Solutions were changed with a valve that switched to a second reservoir.

In addition to control observations with only Ringer's solution bathing the organs, the effects of serotonin, acetylcholine, and FMRFamide were observed at concentrations from 10^{-3} to 10^{-6} M. In some cases ostial dimensions were measured with Image-1 computer software (Universal Imaging Corp.) as described above. Measured values were calibrated with a stage micrometer in the experimental set-up. Recordings used for measurements were from open ostia flat enough to be within a single plane of focus throughout an experiment and reasonably stable in dimension prior to experimental manipulation. Images for figures were processed with Adobe Photoshop (Adobe Systems, Inc.) to optimize image brightness and contrast.

In addition to general observations, pre-treatment ostial areas were statistically compared with post-treatment areas for each of the three transmitters at a concentration of 10^{-5} M. In each case, ostial area was measured immediately preceding transmitter application and again after 5 min of perfusion with a transmitter solution. Pre-treatment and post-treatment areas were compared with paired t-tests (n = 10 for each transmitter). Statistics were performed with SAS version 6.10 (SAS Institute, Cary, NC).

RESULTS

The gill of *Dreissena polymorpha* is of eulamellibranch form whose gross structure has been described previously (Ridewood 1903; Morton 1969). Fig. 2.1 relates the gross organization of the gill and the direction of water movement through it to the more complex organization of the gill. Fig. 2.1c is a diagrammatic composite of the gill showing the position of the connective tissue elements and intrinsic musculature described below.

Connective tissue skeleton

Stripping the epithelial tissue from the underlying supportive tissue in living gills leaves a "skeleton" that has filaments at a maximum distance from one another (see Fig. 2.1c). This "skeleton" has a compressible yet resilient texture. The gill filaments are supported by a fibrous connective tissue matrix (Fig. 2.2a). This matrix is composed of a network of thin fibers that are several nanometers in diameter. Aside from this rather dense homogeneous matrix there are no further supportive structures in the filaments. The filaments do not contain calcified rods as are found in the gills of unionids. The cellular elements associated with the filament connective tissue and vascular sinus are located in the central midline of the filament.

The connective tissue matrix supporting individual filaments is continuous with connective tissue struts that cross the filaments at right angles approximately every 30-80 μm (Fig. 2.2b). This distance can vary with animal size, contractile state, and fixation state. These cross-struts have an elongate figure eight morphology and have the same fibrous appearance as the filament supports (Fig. 2.2). During muscular contraction, the cross-struts bend at their attachment to the filaments and become more parallel to the filaments; they also bend inwardly (Fig. 2.3). The connective tissue supporting the epithelial cells is a thin sheet of loose, but fibrous connective tissue (Fig. 2.1c, 2.2d, 2.4a, 2.4b, 2.5a). One sheet underlies the external ciliated epithelium while a second sheet underlies the internal water channel epithelium. The hemocoel is enclosed between these two sheets of connective tissue and their associated epithelium (see Fig. 2.1c). In areas where the cross-struts occur, the thin sheet of connective tissue dips under the strut so that the strut lies between the sheet and the outer epithelium (Fig. 2.2d). The deep regions of the cross-struts form bands of connective tissue perpendicular to the filaments. These bands are interspersed with muscle fibers (see below).

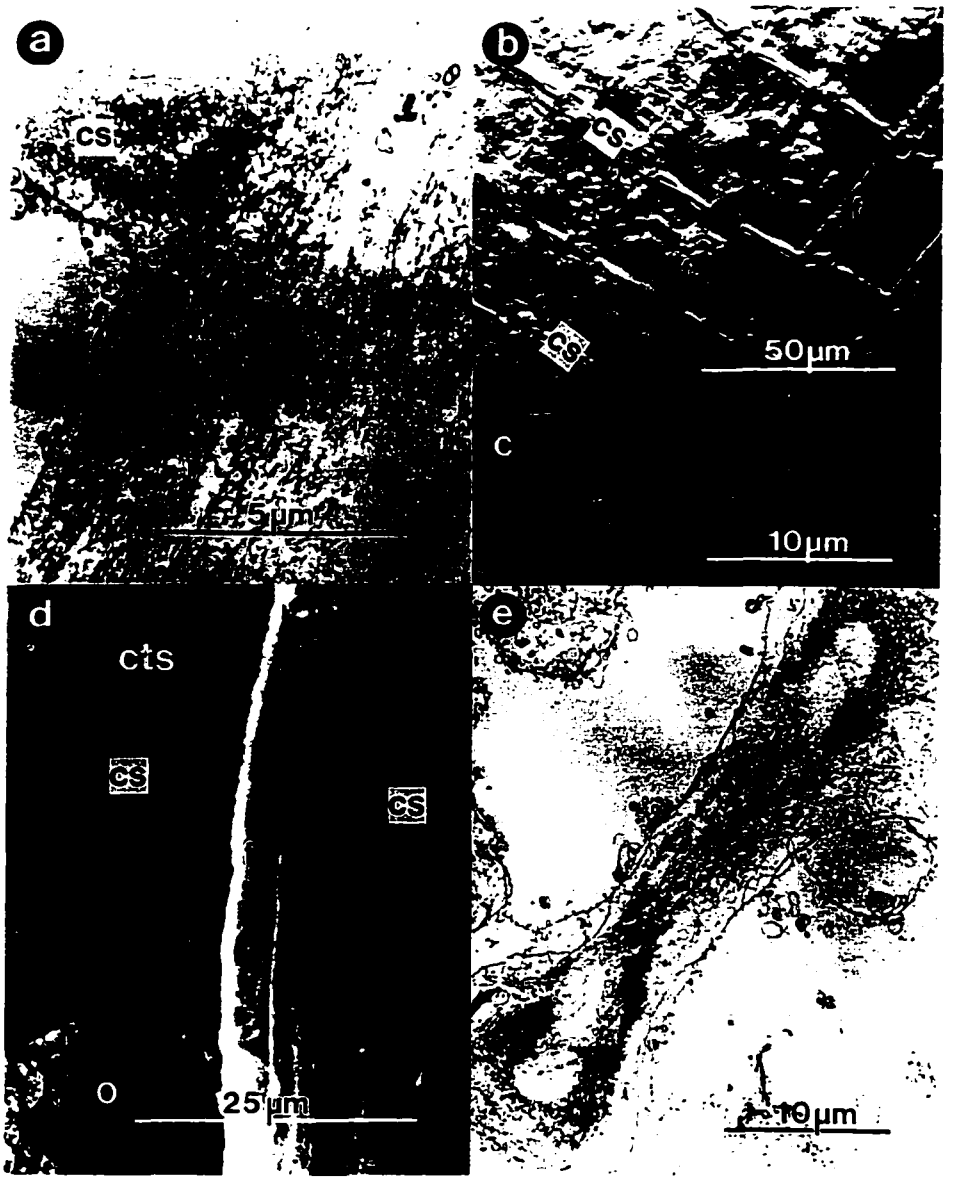


Fig. 2.2. Connective tissue support of the gill of *Dreissena polymorpha*.
(a) Filament support demonstrating the fibrous connective tissue of the structure. Tissue forming a cross-strut (cs) is continuous with the filament support. TEM.
(b) Gill stripped of epithelium to expose underlying connective tissue support. Parallel filaments (f) are separated by perpendicular cross-struts (cs). Nomarski illumination [Differential interference contrast (DIC)]
(c) A 2-µm-thick laser confocal optical section of a cross-strut labelled with horseradish peroxidase-FITC.
(d) A gill stripped of its external ciliated epithelium. The filament supports (f) and cross-struts (cs) overlay the connective tissue sheet (cts) which encloses the hemolymph space. An external ostium (O) is visible. SEM.
(e) Thin section of a cross-strut showing the filamentous nature of the connective tissue. TEM.

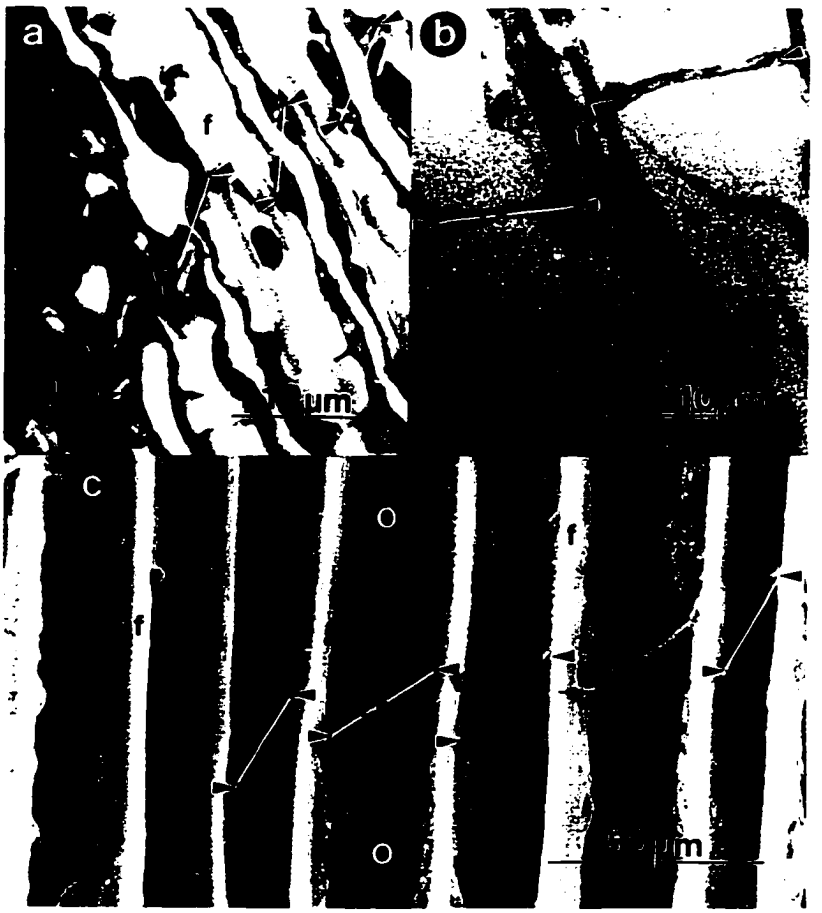


Fig. 2.3. Cross-struts during contraction and at rest.
(a) Frontal section of a contracted gill. The positions of attachment of the cross-struts to the filaments (arrowheads) are shifted with respect to one another, causing the cross-struts (position marked by white lines) to be bent toward a position parallel to the filaments (f). In many instances the middle portion of the cross-struts are bent inwardly, out of the plane of the section. Light microscopy.
(b) Frontal section of a relaxed gill. The cross-struts (cs) are within a single plane and are approximately perpendicular to the filaments. Light microscopy.
(c) Gill stripped of its epithelium and in a partially contracted state. Where filaments are drawn more closely together, the cross-struts are bent toward the parallel position. Those filaments closer to the relaxed state are closer to the perpendicular position. Ostia (O) are visible. SEM.

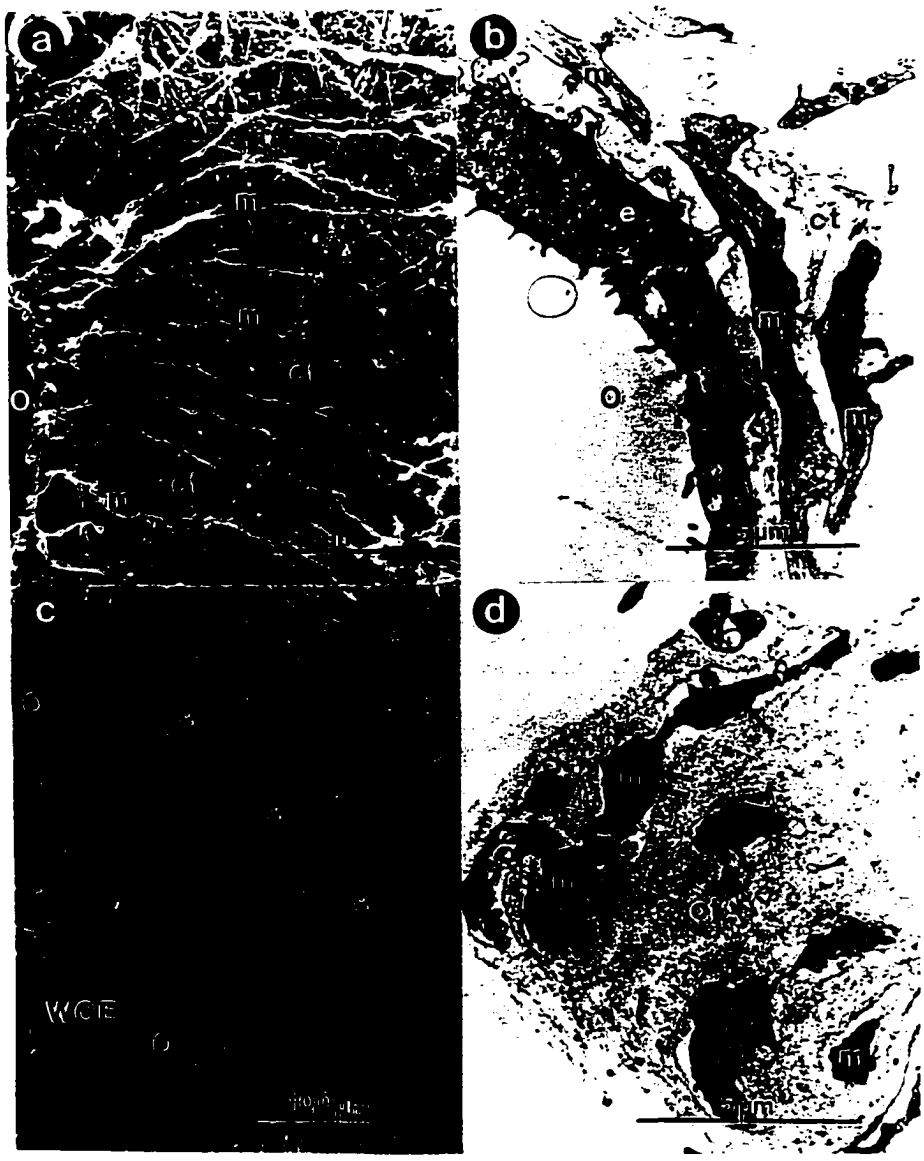


Fig. 2.4. Intrinsic gill muscles.
(a) Hemocoel side of the connective tissue sheet underlying the internal water channel epithelium. Presumptive muscle fibers (m) are closely associated with the connective tissue (ct). The margin of an internal ostium (O) is just visible. SEM.
(b) Frontal section of a gill at the level of an internal ostium (O). The epithelial tissue (e), muscle fibers (m), and connective tissue (ct) near the ostium are clearly visible. TEM.
(c) Bands (B) of connective tissue and muscle, which lie in the hemocoel at the base of filaments. Water channel epithelium (WCE) has largely been stripped away to reveal the bands, but some remains with intact internal ostia (O). SEM.
(d) Cross section of the connective tissue (ct) and muscle fiber (m) bands shown in 4c. The connective tissue filaments are arranged in parallel with the muscle fibers and dense bodies (arrows) connect muscle fibers with the surrounding connective tissue. TEM.

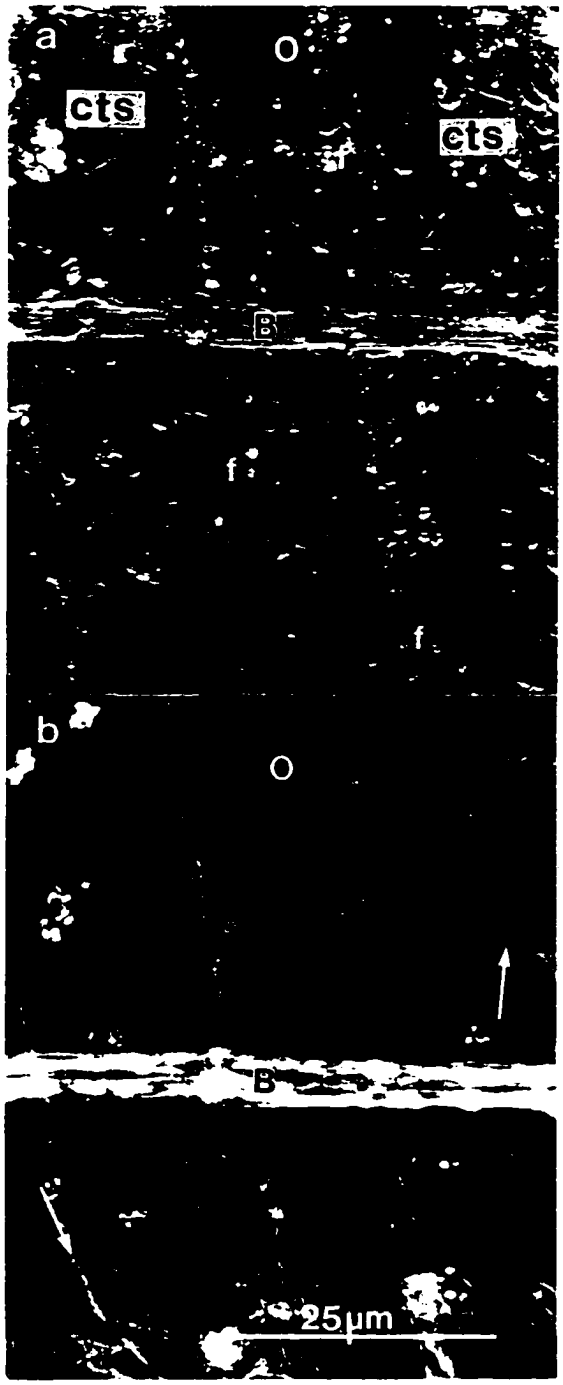


Fig. 2.5. Gill stripped of its epithelium.
(a) Filament supports (f), the connective tissue sheet (cts), ostia (O), and bands (B) crossing the filaments at right angles are visible with Nomarski illumination.
(b) Same gill labelled with phallacidin to reveal f-actin fibers in presumptive muscle bands. Large bands of muscle fibers lie at right angles to the filaments, while fibers associated with the connective tissue sheet form a complex web-like pattern around the ostia (arrows).

Muscle organization

Muscle fibers were identified structurally using TEM and by their positive labelling with NBD-phallacidin. Groups of smooth muscle fibers lie in bands at right angles to gill filaments, and muscle fibers are also found in a web-like orientation in association with the loose connective tissue sheet that underlies the external and internal epithelium (Figs. 2.1c, 2.4 and 2.5a). The muscle fibers crossing the gill filaments are encased in well-organized bands of connective tissue with fiber orientation paralleling the longitudinal axis of the muscle fibers (Fig. 2.4d). These bands lie at the base of the gill filaments in the hemocoel space between the outer ciliated epithelium and the inner water channel epithelium. The individual muscle fibers are attached to the connective tissue fibers by peripheral dense bodies (Fig. 2.4d).

A second major set of muscle fibers is found in the sheets of connective tissue underlying the epithelial layers of the gill (Figs. 2.1c, 2.4a,b and 2.5). Contraction of these muscle fibers causes a reduction in the size of the water canal and the ostia leading to the water channel. These individual muscle fibers are small, generally on the order of 1 - 2 μm in diameter (Fig. 2.4) and of unknown length. Nervous tissue lies in close association with these fibers, but the overall organization of these nerves has not been formally assessed (not shown).

Demibranch contraction

To assess the effects of general intrinsic muscle contraction demibranchs were isolated and exposed to the neurotransmitters acetylcholine and FMRFamide. Gross area of the demibranch was measured over time, with contraction of the musculature resulting in reduced demibranch area. Gill area was reduced following application of a stimulating transmitter, with the greatest reduction occurring in the first 10 to 20 s and then became asymptotic to a level of maximum reduction (Figs. 2.6 and 2.7). Gills returned to Ringer's solution lacking transmitters were observed to relax after several min.

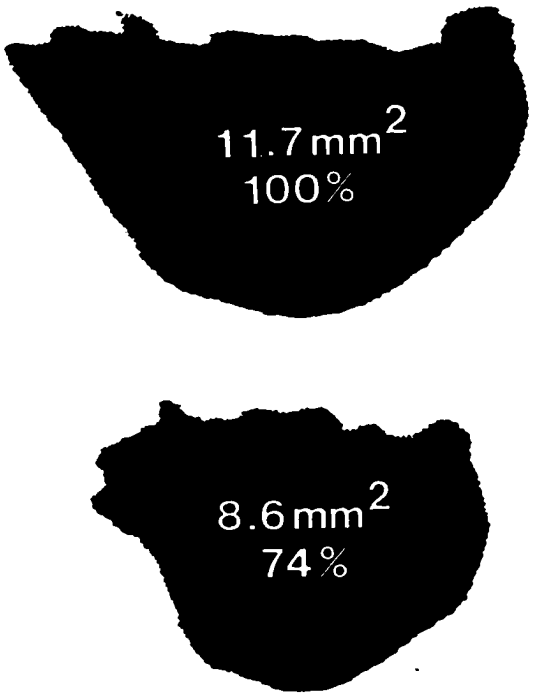


Fig. 2.6. Digitized image of a single demibranch that has contracted after application of acetylcholine. The figure shows the gill just prior to acetylcholine application (top) and at 60 s after the addition of acetylcholine. At both time points, the absolute area (mm^2) is shown above and the relative reduction in gill area (% of initial area) is shown below.

Reduction in demibranch area was concentration-dependent following exposure to either acetylcholine or FMRFamide (Fig. 2.7). Responses to acetylcholine and FMRFamide were similar in magnitude and time course (Fig. 2.7).

Observation of perfused gill tissue

Over 20 h of video-taped observations were made on the internal water channel epithelium and ostia in these preparations. Generally, ostial area decreased in response to either acetylcholine or FMRFamide and ostial area increased following serotonin application. The paired comparisons of pre-treatment and post-treatment areas generally support these observations. Ostial area was reduced to an average of 55% of the pre-treatment value following acetylcholine treatment ($p < 0.01$) and to an average of 67% of the pre-treatment value following FMRFamide treatment ($p < 0.02$) (Fig. 2.8). The ostial area of serotonin-treated preparations increased to an average of 113% of the control value, but this difference was not statistically significant ($p < 0.1$) (Fig. 2.8). In the serotonin treatment group, 7 of the 10 ostia increased in size to an average of 120% of the control area.

Overall, conspicuous changes in ostial size and shape were observed over time, including movements in the gills of *C. fluminea* and *T. texasensis* (Figs. 2.9d-i; 2.10; 2.11). While most ostia were fairly stable in dimension during our observations (Fig. 2.11a), a few ostia were found to be active in their movements even without transmitter application (Figs. 2.10d-f and 2.11b). Transmitter-treated preparations showed a wide range of ostial size and shape over short time periods (Fig. 2.9 and Fig. 2.11c-e). Interfilament distance followed the same pattern of change as ostial area, decreasing when the ostial area decreased and *vice versa*.

DISCUSSION

The major muscles in the gill of *Dreissena polymorpha* cross the base of the gill filaments at right angles and radiate within the thin sheets of connective tissue supporting

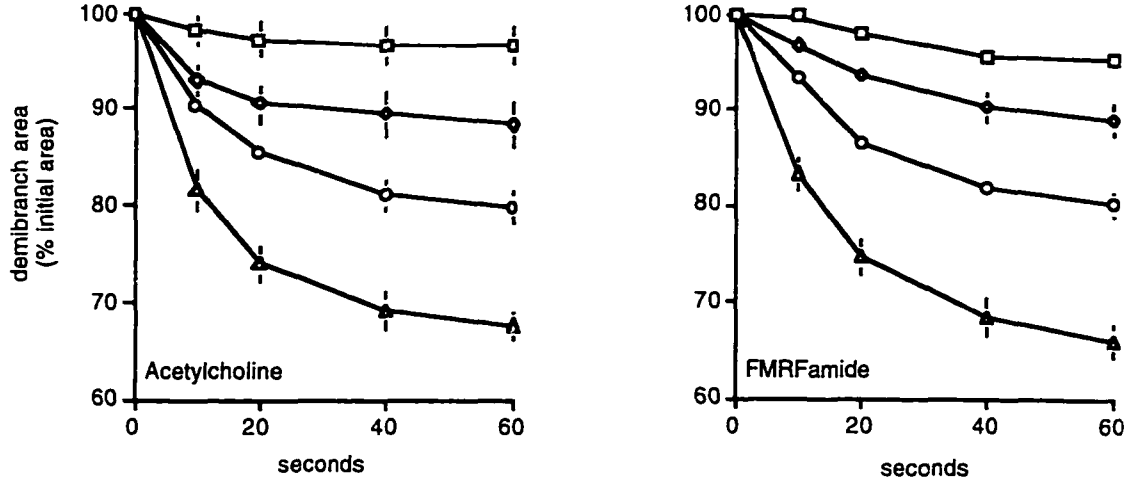


Fig. 2.7. Reduction in gill area (% of initial area) as a function of time in response to exogenous acetylcholine and FMRFamide. Dose-dependent responses to 10^{-6} M (squares), 10^{-5} M (diamonds), 10^{-4} M (circles), and 10^{-3} M (triangles) acetylcholine or FMRFamide are shown. Both experiments used 10 animals, with each of the four demibranchs per animal randomly distributed to a transmitter concentration (Each point is the mean ± se).

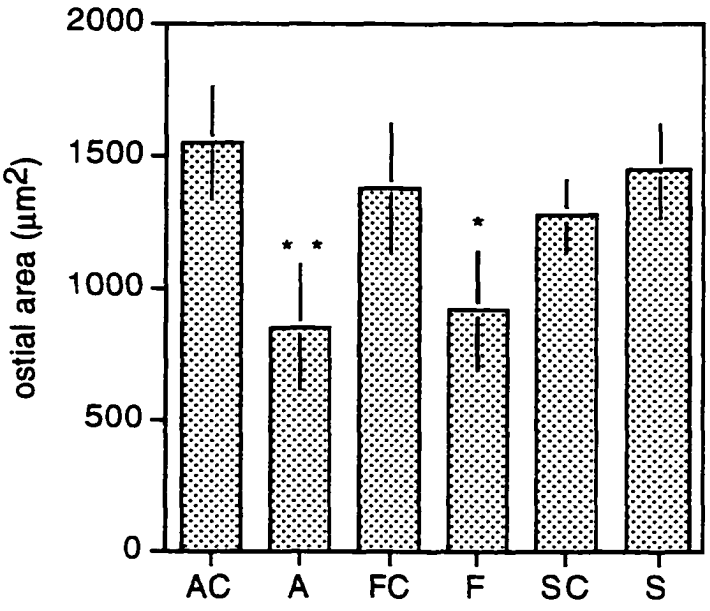
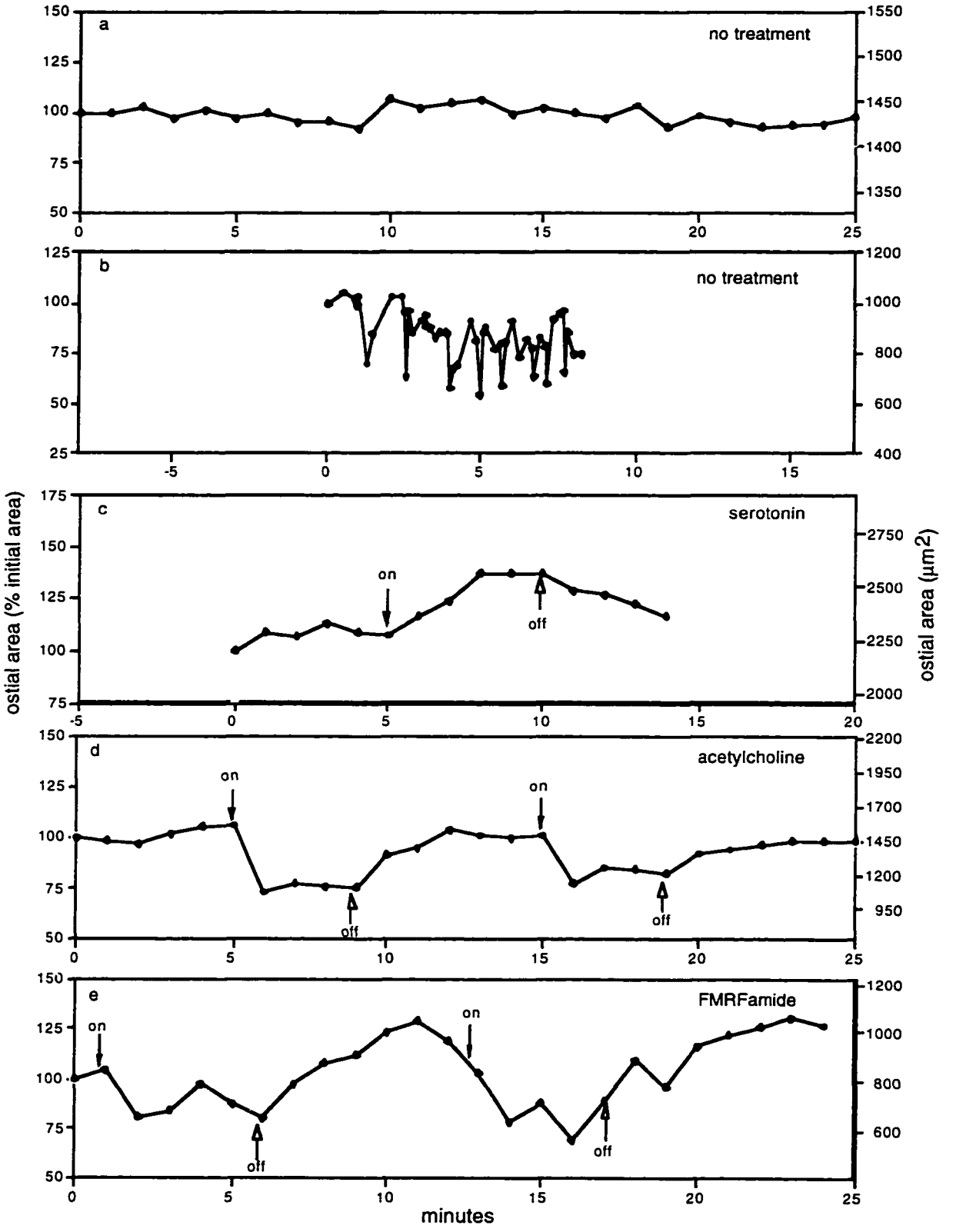


Fig. 2.8. Pretreatment ostial areas (μm^2) for acetylcholine (AC), FMRFamide (FC), and serotonin (SC) and the corresponding ostial areas (A, F, and S) following 5 min of exposure to the respective transmitter. Transmitter concentration in each case was 10^{-5} M. (Each value is mean ± se, n = 10 preparations. * $p < 0.02$; ** $p < 0.01$; p value for the serotonin group was < 0.1.)

Fig. 2.9. *D. polymorpha* ostial areas between gills and for individual ostia over time. (a-c) Examples of internal ostia and water channel epithelium. SEM. (a) Serotonin treated gill (10^{-5} M): average ostial area of 2760 μm^2. (b) Untreated gill: average ostial area of 1444 μm^2. (c) FMRFamide treated gill (10^{-5} M): ostia completely closed (arrows denote dorsal and ventral margins).
(d-f) Digitized video images of a single ostium from a perfused live gill preparation (DIC optics). The asterisks in this series mark two reference cells that are visible throughout the series. The preparation was briefly treated with serotonin (approximately 10^{-5} M) a few min prior to setup in the chamber. (d) Ostium as it is perfused with a control Ringer's solution 1.25 min prior to treatment with FMRFamide (10^{-5} M for 1.5 min). The ostial area is 2525 μm^2. (e) Same ostium 1.16 min after the start of FMRFamide treatment. The ostial area has been reduced to 2013 μm^2 (80% of the area in d). (f) Same ostium 2.5 min following the initial exposure to FMRFamide. The ostium in this figure is completely occluded (arrows denote dorsal and ventral margins).
(g-i) Digitized video images of a single ostium from a perfused preparation (Hoffman modulation optics). (g) Single ostium 2 min prior to treatment with serotonin (10^{-4} M for 1.33 min). The ostial area is 912 μm^2. (h) Same ostium after 1.33 min of serotonin treatment. The ostial area is 2017 μm^2 (221% of the area in g). (i) Same ostium 3.67 min after a return to control Ringer's. The ostial area is 1450 μm^2 (159% of the area in g).

Fig. 2.10. Individual ostia over time in *Corbicula fluminea* (a-c) and *Toxolasma texasensis*(d-f) live gills.
(a) Digitized video images of a *C. fluminea* ostium 1 min prior to treatment with 10^{-3} acetylcholine. The ostial area is 11015 μm^2. (b) Same ostium following 1 min of the treatment and the ostial area has been reduced to 8233 μm^2 (75 % of the area in a). (c) Same ostium following 2.2 min of acetylcholine treatment. Ostial area is 3265 μm^2 (30 % of the area in a).
(d) Digitized video images of a *T. texasensis* ostium just prior to a spontaneous closure. Ostial area is 3200 μm^2. (e) Same ostium 10 s later just following the closure (arrows denote ostial margin). The area has been reduced to 444 μm^2 (14 % of the area in d). (f) Same ostium 30 s following d. Ostium has reopened to 2766 μm^2 (86 % of that in d).

Fig. 2.11. Changes in internal ostial areas from perfused gill tissues.
(a) Ostium receiving continuous Ringer's without transmitter. Overall, ostial area is stable but does vary over time. The movements demonstrated by this ostium are typical of what we observe in control ostia.
(b) Ostium receiving continuous Ringer's without transmitter that was observed to pulsate with a sphincter-like contraction. This ostium's movement is atypical but not unique.
(c-e) Responses in ostial area following the application of exogenous transmitters at a concentration of 10^{-6} M. In each case, "on" arrows indicate initiation of perfusion with the transmitter-containing Ringer's solution and "off" arrows indicate a return to control Ringer's solution. (c) Average changes in two adjacent ostia in response to serotonin application. (d) Average changes in three adjacent ostia in response to acetylcholine application. (e) Average changes in two adjacent ostia in response to FMRFamide application. In each case, the net changes appear to be a result of the response to the applied transmitter and to the endogenous movements of the gill.



the epithelial cells of the gill. While neither of these groups is paired with antagonistic muscles, the anatomical relationship between the main supportive connective tissue elements of the gill and the musculature suggests a functional antagonism. In the absence of contraction, the dense interfilament cross-struts serve a mechanical function in keeping the gill filaments apart. During contraction, the interfilament muscle bands pull the filaments together in the concertina-like motion described for many bivalve gills (Setna 1930; Elsey 1935; Atkins 1943; Jørgensen 1976). Additionally, the contraction of the fibers surrounding the internal ostia act to close the ostia in a sphincter-like manner. Elsey (1935) first suggested an antagonistic relationship between the muscles and connective tissue skeleton in the bivalve gill. Elsey reported that the cross-struts of *Ostrea lurida* and *O. gigas* are straight when muscles are relaxed but become bent as the muscles contract and draw the filaments together. My own observations are consistent with this assessment (Fig. 2.3). Intrinsic gill muscles in unionid bivalves have recently been described for *Ligumia subrostrata* and *Anodonta grandis* (Kays et al. 1990; Gardiner et al. 1991). In these species, muscle bands run perpendicular to gill filaments and insert onto calcified (chitinous) rods. Other muscles are associated with the water canals connecting the outside of the gill with the central water channel. Gardiner et al. (1991) concluded that the muscular elements in these unionids were antagonized by the connective tissue skeleton.

Ridewood (1903) and Elsey (1935) both described the chitinous skeleton of bivalves as elastic and resistant in nature. Our experience with the connective tissue skeleton of *D. polymorpha* is consistent with these descriptions. While the connective tissue support of gill filaments has often been termed chitinous, preliminary data indicate that this connective tissue is similar to other connective tissues in that they contain collagen fibers embedded in a ground substance (see Chapter 3). The individual fibrils from which larger vertebrate collagen fibers are composed are on the order of 20 to 200

nm in diameter, but those composing the reticular fibers of vertebrate connective tissue can be similar in size to those seen here (Kelley et al. 1984).

Experiments with whole demibranchs show that as muscles contract the demibranch shortens mainly in an anterio-posterior direction, thereby decreasing the demibranch area (Fig. 2.6). This reduction proceeds in a dose-dependent manner with either acetylcholine or FMRFamide application (Fig. 2.7). Since the degree of muscle activation is a function of intracellular calcium concentration (Ruegg 1986), it is likely that the differing levels of contraction are correlated with the amount of calcium entering the muscle. Preliminary experiments with calcium-free Ringer's solutions suggest that external calcium is required for muscle contraction to proceed. This dependence on external calcium is not surprising given the small size of these fibers and the apparent lack of a developed intracellular membrane system (Fig. 2.4 b, d). These changes in whole demibranch area are consistent with contraction of the muscle fibers in connective tissue-associated bands that run across the base of the filaments at right angles.

Observation of the water channel epithelium and internal ostia also reveals consistent responses to exogenous transmitter application. Changes in ostial dimension in response to acetylcholine, FMRFamide, and serotonin are rapid and reversible (Fig. 2.11). Acetylcholine and FMRFamide both cause ostial area to diminish. Serotonin generally causes the ostia to open more widely. Apparently, acetylcholine and FMRFamide cause the muscle fibers to contract while serotonin causes the muscle fibers to relax. While the experimental examination of the serotonin effect was not statistically significant, the response to serotonin in many cases is dramatic and compelling (Figs. 2.9g-i and 2.11c). Elsey (1935) observed dramatic changes in the ostial dimensions of the actively functioning gills of *O. lurida* and Gardiner et al. (1991) demonstrated significant increases in ostial dimensions of *Ligumia subrostrata* following serotonin

(10^{-5} M) application. Further work is needed to understand which transmitters are physiologically important for direct muscular control.

Recent *in vivo* observations have documented the apparent muscular control of gill function. Tankersley (1996) made endoscopic observations from the suprabranchial chamber of the unionid *Pyganodon cataracta* and found that the internal ostia were more visible and less constricted during active water pumping. These results were interpreted to be consistent with the muscular control of ostia reported by Gardiner et al. (1991). The rhythmic expansion and contraction of the gills of *P. cataracta* have also been observed and were believed to be a result of cardiac rhythm and subsequent blood movement or to the muscular activity of water canals and ostia (Tankersley and Dimock 1993). Endoscopic observations made on *Placopecten magellanicus* found that concertina-like movements of the gill are important in regulating ingestion volume (Beninger et al. 1992). Ward et al. (1994) observed movements in *Crassostrea virginica* including expansion and contraction of the plicae caused by movements of the ordinary filaments. Observations through the transparent shell of small *Dreissena polymorpha* by Sprung and Rose (1988) found gill expansion following shell opening.

The size of the passageways for water flow are important parameters for bivalve gill function. Models of the bivalve gill have used fixed estimates of interfilament distance or ostial dimension to determine other values like pump capacity and interfilament flow velocity (Foster-Smith 1976; Silvester and Sleigh 1984; Jørgensen et al. 1986; Jones et al. 1993). Our observations on *D. polymorpha* and those of other studies on oysters (Elsey 1935) and freshwater unionids (Gardiner et al. 1991) show that the interfilament distance and ostial dimensions are variable in nature and under muscular control. This study demonstrates that muscular alteration of the water passageways through the gill can be both rapid and dramatic in an excised gill

preparation. The importance of muscle structure and function among bivalve phylogenies and gill types, as well as the extent of gill movements *in vivo* remain to be determined.

CHAPTER 3

CONNECTIVE TISSUE AND MUSCLE FIBERS IN THE GILLS OF FRESHWATER BIVALVES

The bivalve gill is a highly complex organ composed of regionally specialized epithelial tissue attached to a supportive structure of connective tissue, blood spaces, nerves, and muscles (LePennec et al. 1988; Kays et al. 1990; Gardiner et al. 1991; Eble and Scro 1996; Gros et al. 1996; Medler and Silverman 1997). The gill is an important organ involved in nutrition, ion transport, and gas exchange (Dietz 1985; Pearse et al. 1987; LePennec et al. 1988; Kays et al. 1990; Eble and Scro 1996). LePennec et al. (1988) emphasized the need for detailed descriptions of the anatomy of littoral bivalve gills. Indeed, there are gaps in our knowledge of the non-epithelial components of the gill. This comparative study focuses on the composition of the connective tissue and muscle fibers in the freshwater bivalve gill.

The connective tissue of the bivalve gill has historically been termed chitinous (Ridewood 1903; Yonge 1926; Elsey 1935; Atkins 1943), although more contemporary work recognizes the tissues as being composed primarily of collagen and associated muscle fibers (LePennec et al. 1988; Kays et al. 1990; Eble and Scro 1996; Gros et al. 1996; Medler and Silverman 1997). Nevertheless, there are no studies that have explicitly examined the nature of the collagen; the evidence that collagen is the fibrous protein supporting the gill has largely been anecdotal or preliminary (Brown 1952; Ruddall 1955; LePennec 1988; Kays et al. 1990; Gros et al. 1996; Medler and Silverman 1997). The intrinsic muscles of the bivalve gill were described many years ago (Setna 1930; Elsey 1935; Atkins 1943) but have received little attention until recently (Gardiner et al. 1991; Medler and Silverman 1997). The ultrastructure of these muscle fibers is poorly known and has been described for only a single species (Medler and Silverman 1997) . In the present study, the fibrous structural material of the gill is identified and the associated muscle fibers are classified according to their comparative ultrastructure.

Gills of the freshwater bivalves *Corbicula fluminea*, *Dreissena polymorpha*, and *Toxolasma texasensis* were studied. *C. fluminea* and *D. polymorpha* are members of the

subclass Heterodonta (Newell 1965) and share a common evolutionary lineage (Nuttall 1990). *Toxolasma texasensis* is of the subclass Paleoheterodonta (Newell 1965) and is a member of the family Unionidae. As a group, the animals represent the majority of extant freshwater bivalves.

METHODS

Animals and maintenance

Dreissena polymorpha (Pallas 1771) were collected from western Lake Erie and from the Raisin and Huron Rivers in Michigan; and from the Mississippi River near Baton Rouge, Louisiana. *Corbicula fluminea* (Müller 1774) and *Toxolasma texasensis* (I. Lea 1857) were collected from ponds near Baton Rouge, Louisiana. The animals were maintained in artificial pondwater (0.5 mM NaCl, 0.4 mM $CaCl_2$, 0.2 mM $NaHCO_3$, 0.05 mM KCl, and 0.2 mM $MgCl_2$) in aerated aquaria under laboratory conditions of approximately 22 - 25° C.

Preparation of supportive tissues

The supportive elements of the gills were stripped of their epithelia using the method described by Medler and Silverman (1997). Briefly, gills were excised and placed into a calcium-free Ringer's solution to loosen the tissue and mechanically agitated through a transfer pipette to remove the cells. This procedure was used to prepare tissues for periodic acid Schiff (PAS) staining, scanning electron microscopy (SEM), collagen isolation, and gill homogenization (see below).

Periodic acid Schiff (PAS) reactions

Supportive tissues were prepared as described above and split along the interlamellar septae into single lamellae. These lamellae were laid flat on glass slides and air dried. The whole lamellae were exposed to salivary amylase for 10 to 20 min and then stained with a standard PAS reaction, following the methods of Troyer (1980): lamellae were oxidized with 1% periodic acid for 10 min; tissues were rinsed in running tap water

for 5 min and placed in Schiff's reagent (de Tomasi 1936) for 10 min; the stained lamellae were washed in three changes of 0.5% sodium metabisulfite for two min each and washed in running water for 5 min. Finally, the stained lamellae were dehydrated in a graded ethanol series and mounted with Permount. The prepared tissues were examined with a Nikon Microphot FXA light microscope using a green filter.

Preparation of gill sections

Prior to gill excision, hemolymph samples were taken from animals by inserting a 26 gauge needle between the valves and into the pericardial space (Fyhn and Costlow 1975; Dietz et al. 1994). Hemolymph osmolality (approximately 40-60 mOsm) was measured on a Precision Systems freezing-point osmometer. A 2% glutaraldehyde solution was adjusted with phosphate buffer to match the hemolymph osmolality. Excised gills were cut along the filaments into strips about 3 mm wide and quickly fixed by immersion in the glutaraldehyde solution for 1 h. Gills were rinsed twice in phosphate buffer and post-fixed in 1% OsO_4 for 1 h, rinsed twice in phosphate buffer, and dehydrated in a graded ethanol series. Gill strips were embedded in LR White (London Resin Co.) medium grade resin by placing them in a 1:1 mixture of ethanol and resin for 24 h. They were transferred to 100% resin for 12 h, and embedded flat in fresh resin at 60° C for 24 to 48 h.

Gills were sectioned with a Reichert-Jung ultracut E ultramicrotome at 60-90 nm thickness with glass knives, or at 2 µm and stained with toluidine blue. The gills were sectioned in three planes: (1) in cross section transverse to gill filaments (2) in a dorso-ventral plane between adjacent filaments, and (3) in frontal sections (*en face*) across gill filaments. Thin sections were stained with 3% uranyl acetate for 8 min followed by Reynolds' (1963) lead citrate for 2-5 min. Thick sections were examined with a Nikon Microphot FXA and thin sections with a JOEL 100CX transmission electron microscope (TEM) operating at 80 kV.

Preparation of gills for SEM

The supportive structures of the gill were isolated and the whole structure was fixed with 2% glutaraldehyde and 1% OsO_4 as described above. After osmication, gills were rinsed in phosphate buffer and dehydrated in a graded ethanol series. Gills were wrapped in lens paper, critical-point dried, and mounted on stubs. Specimens were sputter coated with a mixture of gold and palladium (20 nm) and viewed with a Cambridge S-260 SEM.

Collagen isolation

Collagen isolation followed a modified version of Murray et al. (1982) designed for the acid extraction of polychaete cuticle collagen. Gills were excised and stripped of their epithelium as described above. The remaining supportive tissues were stirred overnight at 4° C in a neutral salt solution containing protease inhibitors (0.05 M tris-HCl; 1.0 M NaCl; 0.01 M N-ethylmaleimide (NEM); 0.025 M ethylenediaminetetraacetic acid (EDTA). The gills were homogenized with a hand-held glass homogenizer in 0.1 M acetic acid at 4° C and then extracted for at least 24 h in 0.1 M acetic acid at 4° C with stirring. Homogenates were centrifuged at 25,000 g for 30 min. The supernatant was collected and dialyzed against 3 to 4 changes of 20 mM Na_2HPO_4, which caused the collagen to precipitate. Precipitated collagen was collected by low speed centrifugation and freeze-dried. Dried collagen was suspended in deionized water at a concentration of 1 mg/mL and frozen in 50 µL aliquots at -20° C.

Gill homogenization

Whole gill tissues were stripped of their epithelium as described above and homogenized in deionized water at approximately 25° C using a hand-held glass homogenizer. Suspensions (approximately 2 demibranchs/mL) were applied to grids and negatively stained (see below).

Negative staining

Collagen isolates (1 mg/mL) or gill homogenates (approximately 2 demibranchs/mL) were suspended in deionized water. Formvar-coated grids were floated on a drop of the suspension for 10 min. The grids were drained and allowed to air dry. 3% uranyl acetate in 30% ethanol was dripped across the face of the grids and the grids were drained and allowed to air dry. The prepared grids were examined with a JOEL 100CX transmission electron microscope operating at 80 kV.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE)

Collagen proteins were electrophoresed at 20 V for about 12 h on 8% polyacrylamide gels (acrylamide: methylene bisacrylamide = 37:1) following a modified version of Murray et al. (1982). The running buffer consisted of 0.16 M tris-HCl; 1% sodium dodecylsulfate; pH 6.6 with acetic acid. The sample buffer consisted of 0.05 M sodium phosphate buffer, pH 7.4; 20% (w/v) glycerol; 2% mercaptoethanol; 2% SDS; 0.033% phenol red as tracking dye. Samples (50 μg) were loaded on gels with Bovine Type I collagen (Sigma C-9879) and high molecular weight markers (Sigma M-3788) as standards. Gels were stained with 0.1% Coomassie Blue R-250 in a solution of 10% acetic acid and 25% isopropanol for 1 h, and destained with a solution of 10% acetic acid and 10% isopropanol for several h.

Microscopic measurements

All measurements were made from photographic prints to the nearest 0.01 mm with digital calipers. For the estimation of thick filament diameter, 500 thick filaments were measured from several different muscle fibers for each species. To estimate thin:thick filament ratios I counted filaments from several different regions of fibers from each of the three species.

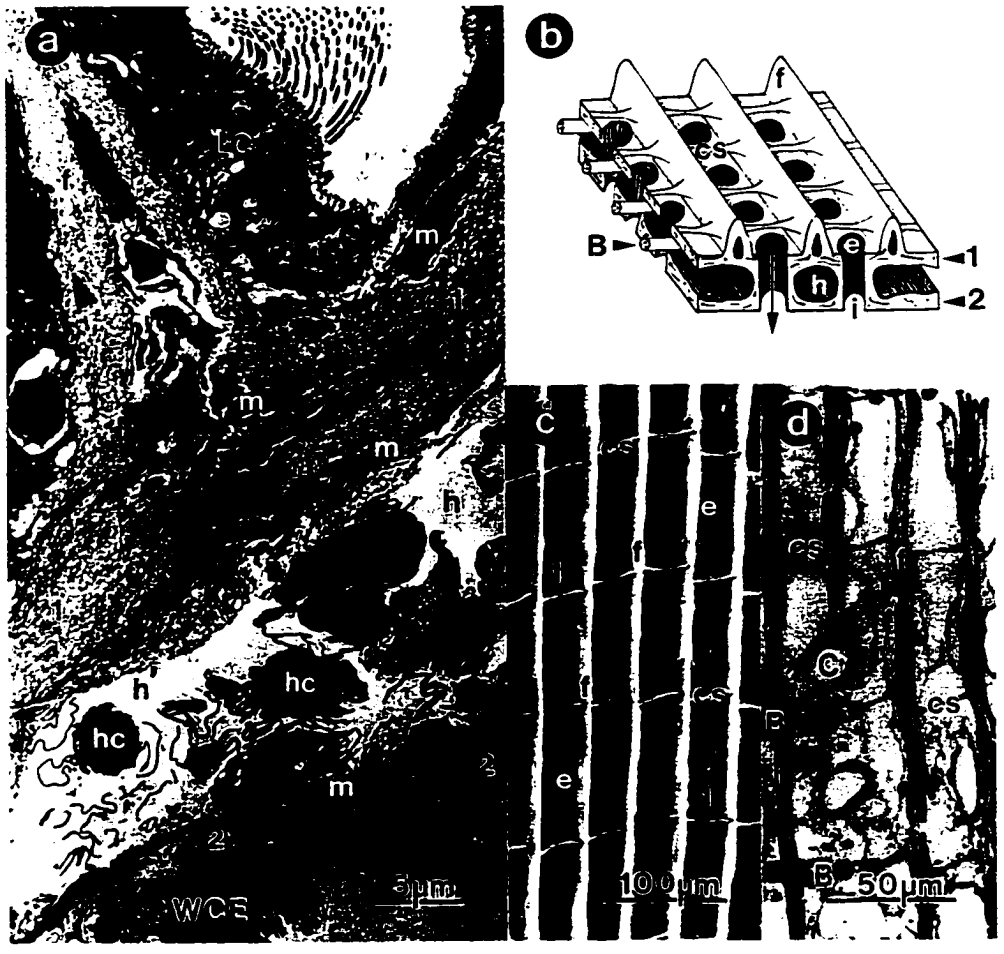
RESULTS

General organization of gill lamellae

The gills of all three species are homorhabdic and the general organization of the gill lamellae forming them is shown in Fig. 3.1. The gill filaments consist of regionally specialized epithelial cells attached to a fibrous connective tissue matrix. The connective tissue in the filaments is continuous with an outer connective tissue sheet at the base of the filaments. A second connective tissue sheet supporting the internal water channel epithelium is separated from the outer sheet by a hemocoel. The muscles of the gills are restricted to the subfilamentar tissue: in the two connective tissue sheets of the lamellae and in bands of connective tissue running perpendicular to the bases of the filaments. The bands of muscle and connective tissue are antagonized by connective tissue cross-struts more superficial to the muscle bands and perpendicular to the filaments.

While the general organization of the gills from the three species is similar, some important differences are noteworthy. The lamella of *T. texasensis*, like those of other unionids, have calcified concretions in the connective tissue sheets and calcified rods at the base of the filaments (Silverman et al. 1983; Gardiner et al. 1991); these structures are absent in *C. fluminea* and *D. polymorpha.* The gills of *T. texasensis* are also generally thicker than in the other species, thus the water canals connecting to the central water channel are longer (on the order of 100 μm in *T. texasensis* versus about 20 μm in *C. fluminea* and *D. polymorpha*). The filaments in *T. texasensis* are alternate with the ostia (as in Fig. 3.1b), while in *C. fluminea* and *D. polymorpha* the external ostia are effectively divided into two by half of the filaments (as in Figs. 3.1c,d). The cross-struts in *C. fluminea* and *D. polymorpha* have an elongate, figure-eight morphology, while those of *T. texasensis* are straight (not shown). The gills of *C. fluminea* are slightly plicate but those of *T. texasensis* and *D. polymorpha* are flat. Grossly, the gills of *C. fluminea* and *D. polymorpha* are more similar in structure.

Fig 3.1. General organization of connective tissue and muscle in the gills of the bivalves studied.
(a) Transverse section through a single lamella of *C. fluminea*. The connective tissue fibers of the filament (f) merge into the connective tissue of the outer connective tissue sheet (1). This connective tissue sheet envelops muscle fibers (m), as does the inner connective tissue sheet (2). Between the two connective tissue sheets is a hemocoel (h) with various hemocytes (hc). Epithelial tissue, including lateral ciliated cells (LC), cover the outside of the gill and water channel epithelium (WCE) lines the central water channel. TEM.
(b) Diagrammatic composite of the common supporting structure of the gills studied (not to scale). Dorso-ventral filaments are supported by a fibrous connective tissue structure (f) that merges into a flat connective tissue sheet (1). These filament supports are held apart by connective tissue cross-struts (cs) that are superficial to bands of connective tissue and muscle fibers (B). A second connective tissue sheet (2) supports the water channel epithelium. Both sheets of connective tissue are interspersed by muscle fibers (wavy black lines). The space between the two connective tissue sheets comprises the hemocoel (h). Water moves through the gill by entering external ostia (e) and into water canals (arrow) which empty into the central water channel via internal ostia (i).
(c) External view of the supportive structure in the gill of *D. polymorpha*. The filament supports (f), cross-struts (cs), outer connective tissue sheet (1), and external ostia (e) are shown. SEM.
(d) 2 µm *en face* section through a gill lamella of *D. polymorpha* showing the internal structure of Fig. 3.1c. Filament supports of varying density (f), cross-struts (cs), muscle/connective tissue bands (B), and water canals (C) are visible. Light micrograph.



Connective tissue

The supportive tissue of the gills (filament supports, cross-struts, and connective tissue sheets) was found to be PAS-positive in each of the three species (Fig. 3.2). Sections of the gills revealed that the connective tissue is composed of small fibrils approximately 15 nm in diameter and having an axial periodicity of about 10 nm (Figs. 3.3a, c, d). In some regions, interfibrillar banding was observed with an axial periodicity of about 55 nm (Figs. 3.3c, d). In some dense regions such as the main support of the filaments, the banding pattern was obscured by a ground substance (not shown).

Collagen isolates were found to contain fibrils approximately 30 nm in diameter with an axial periodicity of about 20-30 nm (Figs. 3.3e-g) . Many of these fibrils consisted of 2 or more individual fibrils, so the dimensions of individual fibrils were difficult to determine. The gill homogenates contained large fibers of up to about 150 nm in diameter with axial periodicities of approximately 55 nm (Figs 3.3h-j). These fibers display the "hole" and "overlap" zones seen in negatively stained vertebrate collagen fibers.

SDS PAGE of collagen samples demonstrated protein bands that are consistent with the banding patterns of vertebrate collagens for all three bivalves (Fig. 3.4). The most prominent bands correspond to collagen alpha chains as inferred by the position of Type I collagen bands. Bands corresponding to gamma and beta chains were also observed but less prominent. The resolution was insufficient to make inferences about the alpha chain composition in these samples.

Muscle fibers

The muscle fibers in the gill are similar in each of the species (Fig. 3.5). They are smooth muscle fibers with a thin:thick contractile filament ratio of approximately 12:1. The electron-dense bodies are generally peripherally placed, and occasionally observed in the internal part of the fiber with no apparent organization (Figs. 3.5a-c). The

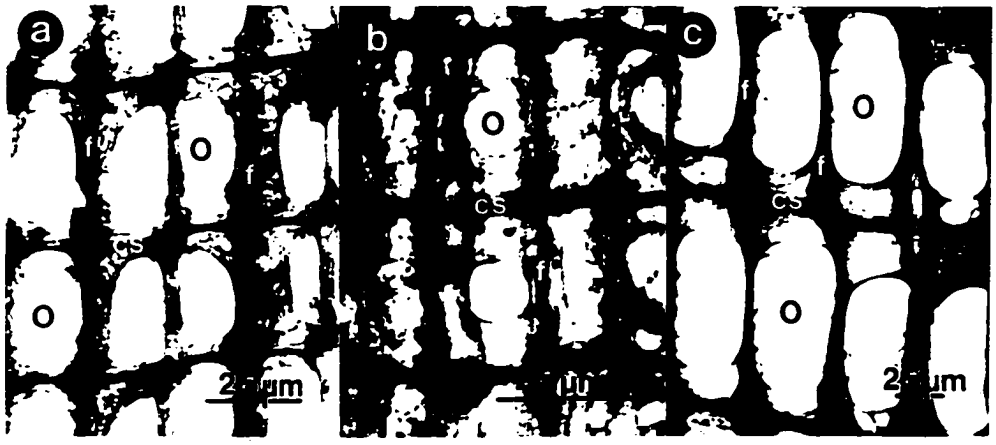


Fig. 3.2. PAS-stained connective tissue elements in the gills of (a) *D. polymorpha*, (b) *C. fluminea*, and (c) *T. texasensis*. All of the major connective tissue regions show a positive PAS reactivity including the filament supports (f), cross-struts (cs), and the connective tissue sheets that delineate ostia (O). Light micrograph.

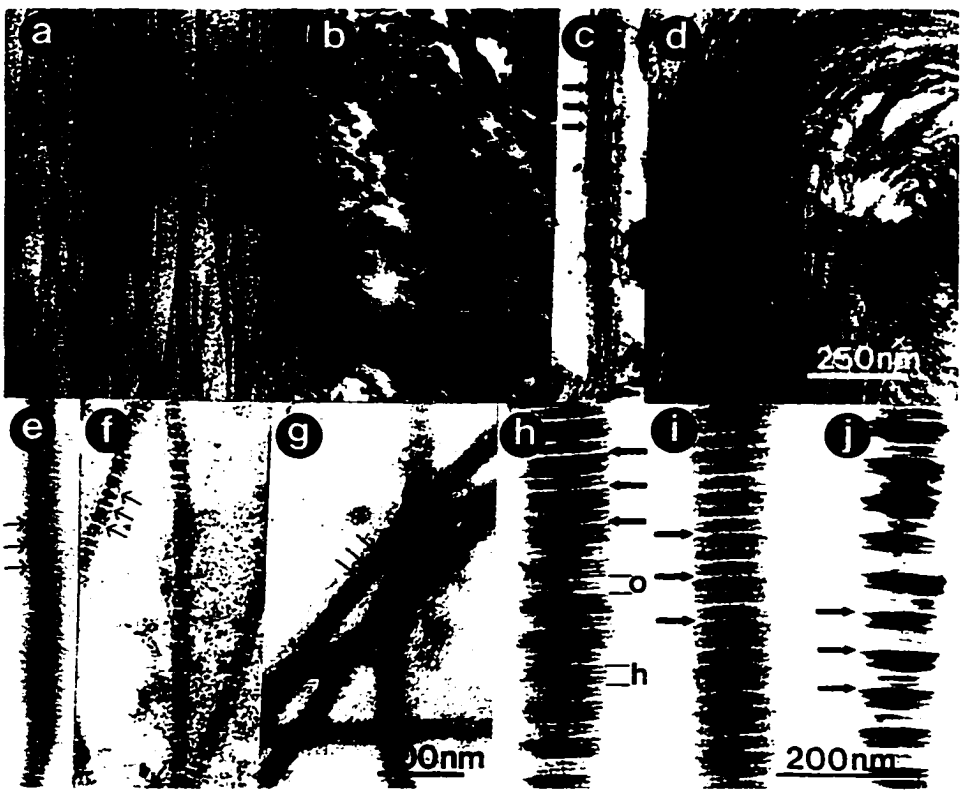


Fig. 3.3. Collagen fibrils (a-g) and fibers (h-j) from the gill tissue. TEM.
(a-d) Fibrils observed in sections of gill tissues of (a) *D. polymorpha*, (b,c) *C. fluminea*, and (d) *T. texasensis*. Axial banding patterns of about 10 nm are visible for each species. A very regular and tightly packed arrangement of fibrils is demonstrated in the section from *C. fluminea* (b). An interfibrillar periodicity of about 55 nm (arrows) is visible in sections from *C. fluminea* (c) and *T. texasensis* (d).
(e-g) Negatively stained collagen fibrils isolated from (e) *D. polymorpha*, (f) *C. fluminea*, and (g) *T. texasensis*. Each of these samples contains fibrils that show a definite axial periodicity of about 20-30 nm (arrows) and are approximately 30 nm in diameter.
(h-j) Collagen fibers from homogenized gill tissues in (h) *D. polymorpha*, (i) *C. fluminea*, and (j) *T. texasensis*. These fibers are large (up to 150 nm diameter) and show an axial periodicity of approximately 55 nm (arrows). Alternating "hole" zones (h in figure) and "overlap" zones (o) characteristic of collagen are visible in each case.

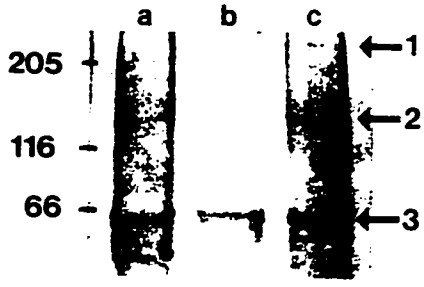


Fig. 3.4. SDS PAGE of collagen isolated from (a) *D. polymorpha*, (b) *C. fluminea* , and (c) *T. texasensis*. The values on the left show the molecular weights of standards in kDa. Those on the right mark the positions of gamma (1), beta (2), and alpha (3) chains inferred from the migration pattern of vertebrate Type 1 collagen. The alpha bands show up most prominently in each case, although other bands are faintly visible.

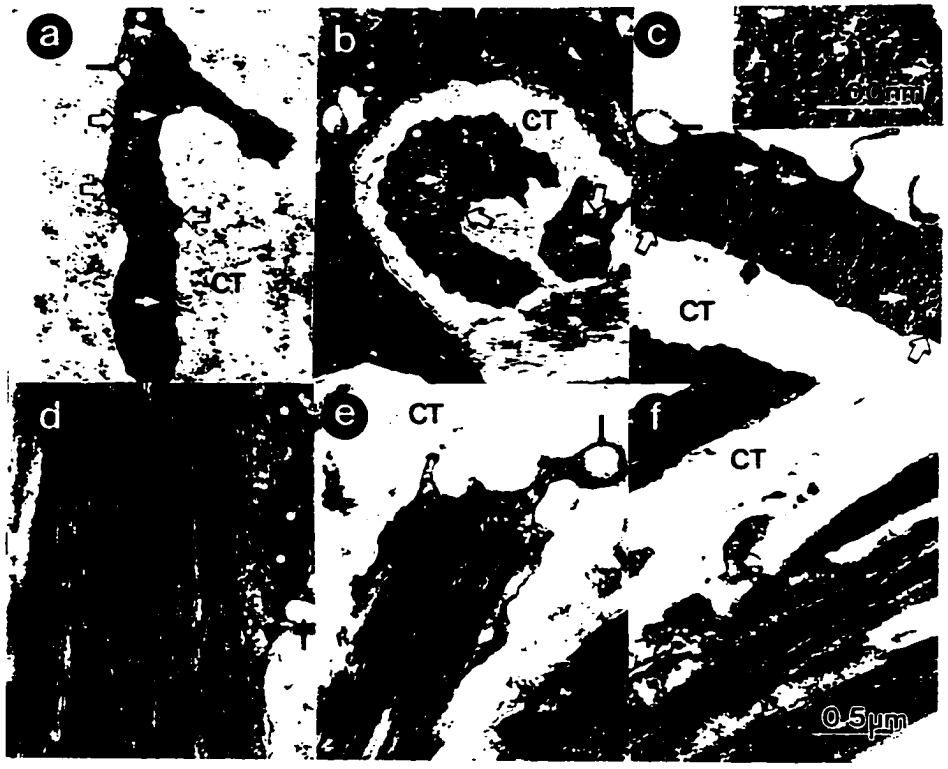


Fig. 3.5. Sections of muscle fibers from (a,d) *D. polymorpha*, (b,e) *C. fluminea*, and (c,f) *T. texasensis*. TEM.
(a-c) Cross sections of muscle fibers from the three species. The muscle fibers show densely packed thick and thin filaments with dense bodies (open arrows), peripheral mitochondria (asterisks), scanty sarcoplasmic reticulum (black arrows), and microtubules (white arrows). The inset in (c) is an enlargement of the fiber to show the structure of microtubules.
(d-f) Longitudinal sections of muscle fibers from the three species. Muscle fibers are always surrounded by a connective tissue matrix (CT).

cytoplasmic organelles and scanty sarcoplasmic reticulum are peripherally placed (Figs. 3.5a, c, d, e), while microtubules are scattered throughout along the long axis of the muscle fibers (Figs. 3.5a-c, inset in c). In cross-section, the muscle fibers vary from ovate to elongate and are only a few microns in diameter. The thick contractile filament diameter in cross section is variable, with the three species having overlapping distributions (Fig. 3.6). The maximum filament diameter is approximately 40 nm in *C. fluminea* and 50 nm in *D. polymorpha* and *T. texasensis* and the minimum diameter is about 11 nm in each of the species.

DISCUSSION

The supportive skeleton of these freshwater bivalve gills is composed mainly of an extracellular matrix of collagen and associated PAS-oxidizable carbohydrates. These reactive carbohydrates probably represent both the glycosylated components of the collagen, a glycoprotein exhibiting varying degrees of glycosylation (Linsenmeyer 1981), and the ground substance in which the collagen is embedded. An abundance of smooth muscle fibers are present throughout this matrix, along with a variety of other cell types not described here. Overall, the composition and organization of these supportive structures are reminiscent of many molluscan organs where muscular systems are encased and embedded by connective tissue layers (Bairati 1985). A few examples of such organs include the bivalve mantle (Morrison 1996), palps (Morse and Zardus 1997), and neural sheath (De Biasi et al. 1985).

The collagen fibrils *in situ* are approximately 15 nm in diameter with an apparent axial periodicity of about 10 nm that may be part of a larger, unresolved pattern. The fibrils are similar in structure to other collagens reported for a number of different molluscan species (Bairati 1985). This similarity is not surprising given the wide distribution and long evolutionary history of collagens in general (Baccetti 1985; Mathews 1985). Negatively stained collagen samples and gill homogenates also

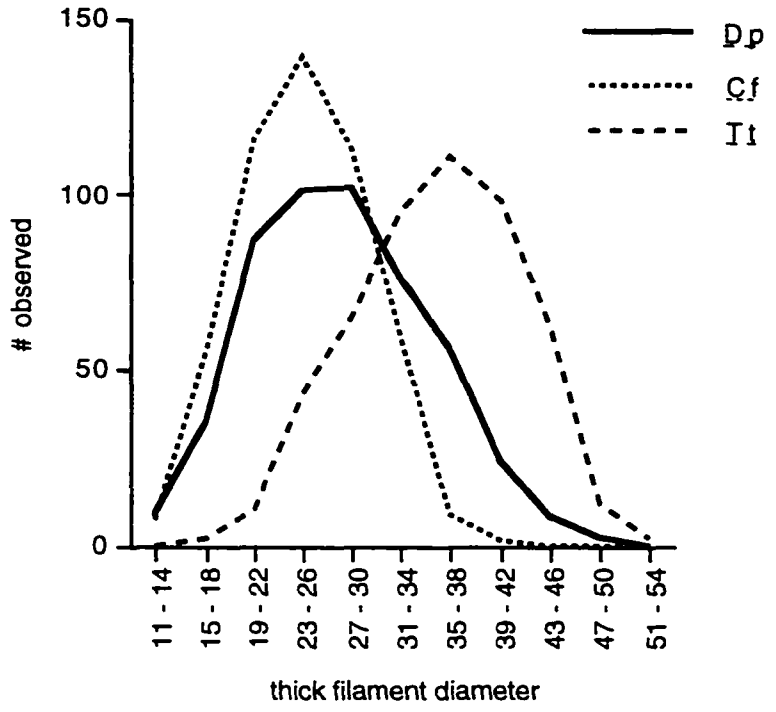


Fig. 3.6. Distribution of thick contractile filament diameter in 4 nm groupings (n = 500). The thickest part of the filament is just over 50 nm in both *D. polymorpha* and *T. texasensis*, but is about 40 nm in *C. fluminea*. The minimum diameter is about 11 nm in each of the species and the mean diameters are 28 nm in *D. polymorpha*, 25 nm in *C. fluminea*, and 35 nm in *T. texasensis*.

demonstrate axial banding patterns similar to one another and to those of collagens in general. While an axial periodicity of 64 nm (Gosline and Shadwick 1983; Kelley et al 1984) or 67 nm (Galloway 1985; Wolfe 1985) is often considered to be characteristic of collagen, the period is variable and dependent on tissue source, preparation, and tensile strength (Trelstad and Silver 1981). The periodicity of approximately 55 nm is reasonably close to that of other collagens, as is the banding pattern. As individual collagen molecules associate into larger fibers they are aligned end to end, with regular gaps left between sequential molecules. The molecules of adjacent rows are staggered and show regions of regular overlap where no gaps exist (Linsenmeyer 1981; Kelley et al 1984 ; Wolfe 1985). The "hole" regions in the large fibers correspond to accumulated stain in the gaps between sequentially packed molecules, while the "overlap" regions are where stain is excluded by the overlapping molecules (Fig. 3.3h-j) (Linsenmeyer 1981; Wolfe 1985).

The appearance of the connective tissue fibers from the isolated samples and from the gill homogenates is consistent with collagen, but is not directly comparable to the fibrils *in situ*. Positive and negative staining patterns are determined by different molecular mechanisms and do not produce the same results (Katayama and Nonomura 1979; Chapman 1985). Further, the large connective tissue fibers observed when the gills are homogenized in deionized water (Fig. 3.3h-j) have never been observed *in situ* and may be formed by reorganization of the smaller fibrils. Nevertheless, it is interesting that the interfibrillar banding patterns *in situ* have a correspondent periodicity of about 55 nm (Fig. 3.3c,d).

The electrophoretic migration pattern of the collagen is consistent with the patterns shown by vertebrate collagens (Miller and Rhodes 1982). Collagens are formed from three intertwined alpha helices (alpha chains), with different collagens made of unique combinations of alpha chains (Miller and Gay 1982; Gosline and Shadwick 1983; Wolfe

1985). Bands representing dimers (beta chains) and trimers (gamma chains) of alpha chains were sometimes resolved in our gels, but not as prominently as the alpha chains. Our resolution of individual bands was too low to make any interpretation about the number or types of alpha chains and further work is required to understand this level of molecular detail for these collagens.

The distribution of contractile thick filament diameter in the muscle fibers is consistent with filaments 40-50 nm in the middle, tapering to about 11 nm at the ends for each of the three species (Fig. 3.6). This type of tapered contractile thick filament is characteristic of other invertebrate smooth muscles (Sobieszek 1973; Castellani et al. 1983; Paniagua et al. 1996). Preliminary observations indicate that the muscle fibers are spindle shaped with a centrally placed nucleus, but the overall length and shape of the muscle fibers are largely hidden within the associated connective tissue and will probably require serial reconstruction to be fully revealed.

Although we have previously described the integral gill muscles in bivalves as obliquely striated (Kays et al. 1990; Gardiner et al. 1991; Medler and Silverman 1997), the differences between obliquely striated and invertebrate smooth muscles often follow a continuum (Morrison and Odense 1974; Paniagua et al. 1996). Based on the arrangement of the electron-dense bodies and on the appearance of the fibers in cross section, the fibers in all three species are more typical of molluscan smooth muscles. Obliquely striated muscles have sarcomeres aligned at a small angle with respect to the fiber long axis, as may be indicated by electron-dense body arrangement (Rosenbluth 1972; Paniagua et al. 1996). Cross sections of the fibers often contain distinct regions representing H bands, I bands, and A bands simultaneously (Rosenbluth 1972; Paniagua et al. 1996). The muscle fibers of the species studied here show no apparent organization between sarcomeres, as the electron-dense bodies are peripheral or occasionally inside the

muscle with no clear organization. Cross sections of the muscles always contain both thick and thin filaments together and do not show organized sarcomeric zones.

These muscle fibers correspond to Matsuno's (1987) invertebrate smooth muscle type B: peripherally-placed cytoplasmic organelles, thick filaments approximately 40 nm in diameter, disordered electron-dense bodies, and scanty sarcoplasmic reticulum. This type of muscle cell is found in other molluscan species as well as in echinoderms, coelenterates, and oligochaetes (Matsuno 1987; Paniagua et al. 1996). According to Matsuno (1987), these are the most prevalent muscles in the motile organs of molluscs and echinoderms where they provide body support and movement. The similarity in muscle type between the three bivalve species studied is interesting, given the significant differences in invertebrate muscle structure within (Morrison and Odense 1974; Matsuno 1987; Matsuno 1988; Matsuno and Kuga 1989; Matsuno et al. 1993; Royuela et al. 1995) and between species (Morrison and Odense 1974; Matsuno 1987; Paniagua et al. 1996).

The gross organization of the gills follows a general pattern with some differences between species. Overall, more similarity exists between the most closely related animals, *C. fluminea* and *D. polymorpha*. Nevertheless, the gills of all three species are constructed from very similar collagenous material and smooth muscle, suggesting a conserved function. Early authors proposed that the integral gill muscles work in a coordinated fashion with the ciliated cells of the bivalve gill (Yonge 1926; Elsey 1935; Atkins 1943). We have recently demonstrated that the active gill musculature in unionid and dreissenid bivalves responds to neurotransmitters and is capable of altering the dimensions of the water passageways in excised gills (Gardiner et al. 1991; Medler and Silverman 1997). Similar movements have also been observed in the gills of *C. fluminea* and *T. texasensis* (Medler unpublished observations). The biological significance of the muscles and associated connective tissue may lay in the regulation of water flow through

the gill (Gardiner et al. 1991; Tankersley 1996; Medler and Silverman 1997), but further study is needed to determine all of their functions.

CHAPTER 4

IONIC EFFECTS ON INTRINSIC GILL MUSCLES IN *DREISSENA POLYMORPHA*

Dreissena polymorpha, the zebra mussel, is a relatively recent inhabitant of fresh water with records dating only to the Miocene (Nuttall 1990), as compared to the Triassic for the unionids (Haas 1969). Ionic tolerances described for *D. polymorpha* differ from the tolerances seen in the unionids. Zebra mussels do not survive in deionized water (Nichols 1993; Ram and Walker 1993; Dietz et al. 1994), while unionids survive for months under such conditions (Dietz et al. 1994). The rapid turnover of ions and the lack of ability to reduce ionic losses in a dilute medium are an indication of the incomplete adaptation to fresh water by *D. polymorpha* (Wilcox and Dietz 1995). Fischer et al. (1991) found that relatively low levels of K^+ were toxic to *D. polymorpha* and the animals do not tolerate exposure to hypertonic NaCl challenges (Horohov et al. 1992). Nevertheless, the presence of some K^+ is essential to the animals and is important for modulating the effects of elevated NaCl (Dietz et al. 1994; Dietz et al. 1996; Dietz et al. 1998; Wilcox and Dietz 1998). Finally, *D. polymorpha* has a Mg^{2+} requirement not found in other freshwater bivalve species (Dietz et al. 1994).

While populations of *D. polymorpha* have been found inhabiting European estuaries with salinities as high as 12 ppt. (reviewed by Strayer and Smith 1993), the species exhibits a limited salinity tolerance (Wilcox and Dietz 1997) which is influenced by ambient temperature (Kilgour et al. 1994). Fong et al. (1995) found that reproductive events are depressed upon acute exposure to elevated salinities, but that this depression is significantly reversed after several days of acclimation. The epithelium in *D. polymorpha* is leaky by comparison with other freshwater bivalve species, showing significant paracellular solute exchange between the bathing medium and the blood (Dietz et al. 1995; Zheng and Dietz 1998). When *D. polymorpha* are exposed to dilute artificial sea water (ASW), they become isosmotic with the bathing medium (Wilcox and Dietz 1997) and experience increases in blood ions from the bath, namely Na^+, Cl^-, K^+, and Mg^{2+}

(Wilcox and Dietz 1997). Alteration of muscle function might be expected when these ions are elevated in the blood. For example, Horohov et al. (1992) and Wilcox and Dietz (1995) hypothesized that imbalances in K^+ lead to a disruption of the electrochemical potentials vital to excitable tissue function.

The lamellibranch gills of bivalve molluscs are important respiratory and feeding structures. The intrinsic gill muscles in some eulamellibranchs are oriented to control the dimensions and posture of the demibranchs, leading to a likely role in the control of water flow through the gill. We have recently studied the anatomy of the muscles and their responses to selected neurotransmitters in both unionid and dreissenid bivalves (Gardiner et al. 1991; Medler and Silverman 1997). Briefly, the muscles in *D. polymorpha* are arranged in two sets. One set is encased in connective tissue bands at the base of the gill filaments and is oriented so that contraction decreases interfilament distance; the second set is closely associated with the loose connective tissue sheets supporting the external and internal epithelial layers and is responsible for controlling the dimensions of the ostia and water canals of the gill (see Chapter 2). The muscle fibers are small (1-2 μm in diameter) and have only a rudimentary internal membrane system (Chapters 2 and 3; Medler and Silverman 1997). These morphological features suggest a reliance on external calcium for muscle activation. The first part of this study examines the dependence of muscle activity on extracellular Ca^{2+}, while the second examines the effects of elevated Na^+, Cl^-, K^+, and Mg^{2+} on muscle contraction in the gills of *D. polymorpha*.

METHODS

Animal Maintenance

Dreissena polymorpha were collected from Lake Erie at the mouth of the Raisin River in Monroe, Michigan and from the Mississippi River near Plaquemine, Louisiana.

The animals were maintained in artificial pondwater (see below) in aerated aquaria under laboratory conditions of approximately 22 - 24° C.

Solutions

Artificial pondwater (APW) in mM consisted of 0.5 NaCl, 0.4 $CaCl_2$, 0.2 $MgSO_4$, 0.2 $NaHCO_3$, 0.05 KCl (Dietz et al. 1994). Artificial seawater (ASW) in mM consisted of 449.1 NaCl, 27.5 $MgSO_4$, 24.4 $MgCl_2$, 9.9 $CaCl_2$, 6.6 KCl, 2.4 $KHCO_3$, 0.8 KBr, 0.4 H_3BO_3; 1076 mOsm•kg^{-1} total solute concentration; 35 ppt salinity (Chambers and De Armendi, 1979; Wilcox and Dietz 1997). Ringer's solution in mM was made of 5 NaCl, 5 $CaCl_2$, 5 $NaHCO_3$, 0.5 KCl, 5 $NaSO_4$, 0.5 $MgCl_2$; 48 mOsm•kg^{-1} (Dietz et al., 1994). 'Elevated' Ringer's consisted of the same components as the standard Ringer's but with 45 NaCl, 2 KCl, and 5 $MgSO_4$; 130 mOsm•kg^{-1}. Various experimental Ringer's solutions were made by adjusting single components of both Ringer's solutions. Three different Ca^{2+}-free Ringer's solutions were made as follows. One solution was made by omitting $CaCl_2$ and including 1mM EGTA. The difference in osmolality was corrected by the addition of NaCl. In two other cases, either $MnCl_2$ or $CoCl_2$ was substituted for $CaCl_2$. In each of the Ca^{2+}-free or ion-substituted solutions, osmolality and pH were the same as in the Ringer's solution.

Gill Preparation

Gills were excised into the appropriate Ringer's solution by freeing the anterior and posterior connections of the gills with forceps and then gently freeing the gills from along the body of the animal. Lateral and medial demibranchs from each side of an animal were separated by cutting the basal attachment with surgical scissors. Thus, each animal provided four demibranchs which were randomly distributed among treatment groups for all experiments. Lateral and medial demibranchs were considered to be the

same physiologically. Randomly placing tissues from a single animal to each treatment group eliminated between animal differences as a factor in statistical analysis.

Demibranch Contraction Assay

We have previously described an *in vitro* assay designed to examine the response of intrinsic demibranch muscles to neurotransmitters (Chapter 2; Medler and Silverman 1997). Demibranchs from animals were excised into a Ringer's solution and after 30-45 min of equilibration, the demibranchs were placed into a drop of Ringer's solution on a glass slide. The gills were left in this position for about one min to ensure that the muscles of the demibranch had relaxed. The Ringer's solution was gently aspirated to leave the demibranch spread flat across the slide. A solution containing 1 mM acetylcholine (ACH) in Ringer's was quickly applied to the gill and immediately aspirated, leaving the demibranch flat on the slide. Acetylcholine is a dose-dependent stimulator of muscle contraction in the intrinsic gill muscles of *D. polymorpha* (Chapter 2; Medler and Silverman 1997), eliciting strong responses at concentrations near 1 mM . Over the next 1 to 2 min, the demibranch decreases in area as the intrinsic muscles contract. During this procedure, gills were video taped on VHS tape at a magnification of about 10X through a dissecting microscope. Digitized-video-images were analyzed with Image-1 computer software (Universal Imaging Corp). Gill area was measured prior to transmitter exposure and at timed intervals after transmitter application. This process represents tonic contraction and the reduction in gill area approaches an asymptote after about 1 min. The reduction in gill area (% of the initial area) after one min of contraction will be referred to as the 'contractile response'.

It is crucial that the demibranchs start in a relaxed state, since a shrunken or contracted gill is limited in its ability to reduce its area any further. Preparations are uniform and demibranchs are readily flattened on the glass slides as described above. Resting demibranch surface areas (prior to addition of transmitter) were compared

between treatment groups to ensure that shrinkage did not confound the contractile response.

Ca^{2+}-free Experiments

Three experiments were conducted to examine the relationship between extracellular Ca^{2+} and muscle contraction. In one experiment, gills were excised into a Ca^{2+}-free solution containing 1mM EGTA before exposure to the 1 mM ACH. After 1 min of ACH exposure without Ca^{2+}, 1mM ACH in the Ringer's solution with Ca^{2+} was administered to the gills. Contractile responses for each solution were measured. In other experiments, the inorganic Ca^{2+} antagonists $MnCl_2$ or $CoCl_2$ were substituted for $CaCl_2$ in the Ringer's solution, while control gills were placed in 'normal' Ringer's solution. Complementary experiments were also performed in which gills exposed to the antagonists were returned to Ringer's with Ca^{2+} to examine whether the response was reversible. In each of the experiments, the contractile responses were compared with a t-test (n = 10 demibranchs per treatment group).

Artificial Sea Water Experiment

A group of about 30 animals were transferred to 15% artificial seawater (ASW) diluted with artificial pond water (APW) in two salinity steps over four days. Between 80 and 90 percent of these animals survived the transfer and remained alive throughout the experiment. A second group of animals remained in APW. The gills from animals in each of these acclimation regimes were dissected as described above and placed in either 'elevated' Ringer's or Ringer's solution. Thus, the experiment had a 2 X 2 factorial treatment arrangement with one factor being the water to which the animals were acclimated (15% ASW or APW), a second being the Ringer's solution to which the

excised gill was exposed ('elevated' Ringer's or Ringer's). The contractile response was measured for each treatment group (n = 20 demibranchs per treatment group).

$MgSO_4$ Experiment

The effect of $MgSO_4$ on demibranch muscle contraction was examined with both Ringer's solutions. The four excised demibranchs from 10 APW-acclimated animals were distributed to the Ringer's solution or to the 'elevated' Ringer's solution with either 0.5 mM $MgSO_4$ or 5 mM $MgSO_4$. Thus, the experiment had a 2 X 2 factorial treatment arrangement, with one factor being $MgSO_4$ concentration (0.5 mM or 5.0 mM) and the second being the Ringer's solution ('normal' or 'elevated'). The contractile response was measured for each of the demibranchs (n = 10 demibranchs per treatment group).

KCl Experiments

The effects of KCl concentration on muscle contraction in the 'elevated' Ringer's solution were examined in a set of four experiments, with a different NaCl concentration in each experiment (2, 10, 15, 45 mM). The experimental design was to vary KCl concentration (0, 1, 2, 4 mM) while holding NaCl concentration constant for each experiment. All other ions were equal in concentration to those in the 'elevated' Ringer's solution. In each experiment, the four demibranchs from 10 APW-acclimated animals were distributed to one of the four KCl concentrations for about 40 min. The demibranchs were exposed to 1mM ACH in the contraction assay and the contractile responses were measured (n = 10 demibranchs per treatment group).

NaCl Experiments

The effect of NaCl concentration in the 'elevated' Ringer's solution was directly examined in two experiments with different KCl concentrations (0 or 2 mM). In both experiments, the four demibranchs from each of 10 APW-acclimated animals were distributed to 'elevated' Ringer's solutions containing one of four NaCl concentrations

(2, 15, 30, or 45 mM) for about 40 min. Therefore, these experiments were the reciprocal of the KCl experiments: NaCl concentration was variable while KCl concentration was held constant. The contractile response from each experiment was plotted and simple linear regression was used to determine whether the response changed as a function of the NaCl concentration (n = 10 demibranchs per treatment group).

Ouabain Experiment

The role of the Na^+/K^+ ATPase on the ability of demibranch muscles to recover from an acute exposure to 'elevated' Ringer's solution with no KCl was examined. The cardiac glycoside, ouabain, was used to block Na^+/K^+ ATPase activity and KCl was provided to some of the demibranchs following the acute exposure. The demibranchs from 10 APW-acclimated animals were excised and placed into 'elevated' Ringer's solution without K^+ for 25 and 30 min. After this incubation, the demibranchs were distributed to an 'elevated' Ringer's solution containing either 0 or 2 mM KCl. Additionally, each Ringer's solution contained either 0 or 1 mM ouabain. Thus, the experiment had a 2 X 2 factorial treatment arrangement with one factor being the presence or absence of KCl, the second being the presence or absence of ouabain. After about 40 min in these solutions, the demibranchs were exposed to 1 mM ACH in the demibranch contraction assay and the contractile responses were recorded (n = 10 demibranchs per treatment group).

Statistics

In most experiments, the contractile response was compared between treatment groups with an ANOVA. A Tukey post-ANOVA test was then used to make pair-wise comparisons between individual treatment means (experiment-wise error rate = 0.05 in all experiments). In the NaCl experiment, a simple linear regression was used to test whether the contractile response changed as a function of NaCl concentration.

Frequency histograms of the data and of the residuals demonstrated normality of the data distribution, therefore no further transformation was required. Variances were homogeneous between treatment groups. All statistics were performed with SAS version 6.10 (SAS Institute, Cary, N.C.).

RESULTS

Ca^{2+}-free Experiments

In each of the three individual experiments, the gills in the Ca^{2+}-free or ion-substituted solutions failed to contract when exposed to 1 mM ACH, while the control groups contracted normally ($p < 0.0001$ for each experiment) (Fig. 4.1). Demibranchs exposed to $MnCl_2$ or $CoCl_2$ solutions rapidly regain their contractile activity when returned to the Ringer's solution with Ca^{2+} (experiments not shown).

Artificial Seawater Experiment

The gills of animals acclimated to 15% ASW showed the same level of muscle contraction when stimulated by 1 mM ACH in both 'elevated' Ringer's and Ringer's solutions (Fig. 4.2). These responses were the same as those of the APW-acclimated gills stimulated with 1 mM ACH in Ringer's. The gills from APW-acclimated animals exposed to 'elevated' Ringer's solution showed a significant ($p < 0.0003$) depression in the ACH-stimulated contractile response when compared with the other treatment groups (Fig. 4.2).

$MgSO_4$ Experiment

The concentration of $MgSO_4$ (0.5 vs. 5 mM) had no effect on the degree of ACH-induced contraction in either Ringer's solution ($p < 0.591$) (Fig. 4.3). The gills in the 'elevated' Ringer's solution did contract less than those in the Ringer's solution ($p < 0.012$). This depression is consistent with the results of the ASW experiment (Fig. 4.2).

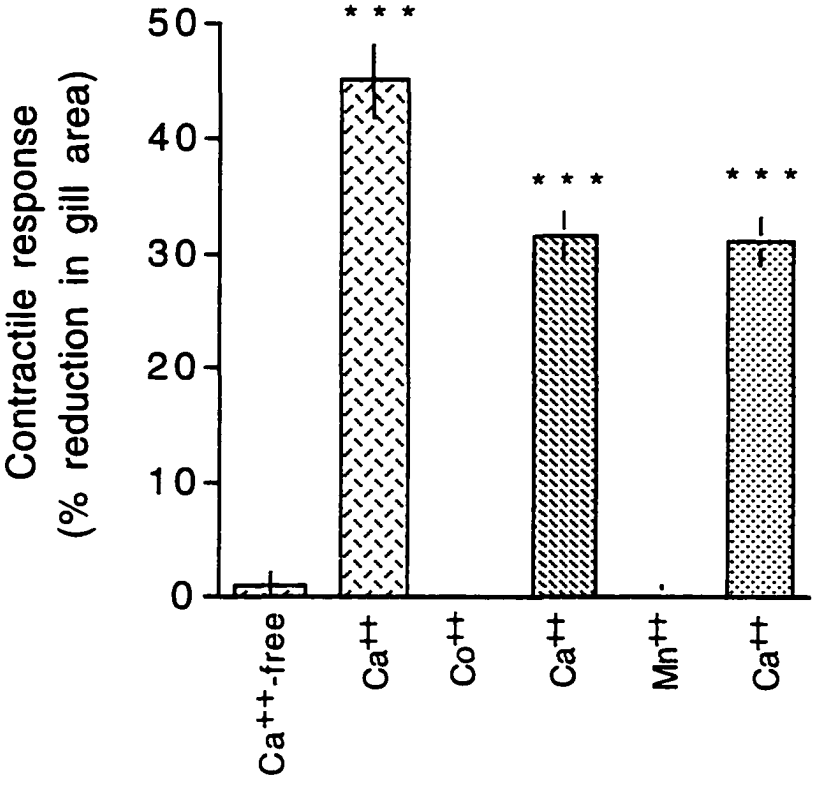


Fig. 4.1. Three independent experiments, each shaded with a different pattern, demonstrating the effect of extracellular Ca^{2+} on muscle contraction. In one experiment, demibranchs were bathed in Ca^{2+}-free Ringer's solution containing 1mM EGTA (Ca^{++}-free). In the second and third experiments, the $CaCl_2$ in the Ringer's solution was replaced by the Ca^{2+} antagonists $CoCl_2$ (Co^{++}) and $MnCl_2$ (Mn^{++}), respectively. In each of the three experiments, the demibranchs with Ca^{2+} available in the Ringer's solution (Ca^{++}) contracted significantly more than those without Ca^{2+} (Ca^{++}-free, Co^{++}, Mn^{++}). (*** $p < 0.0001$; mean ± se, n=10).

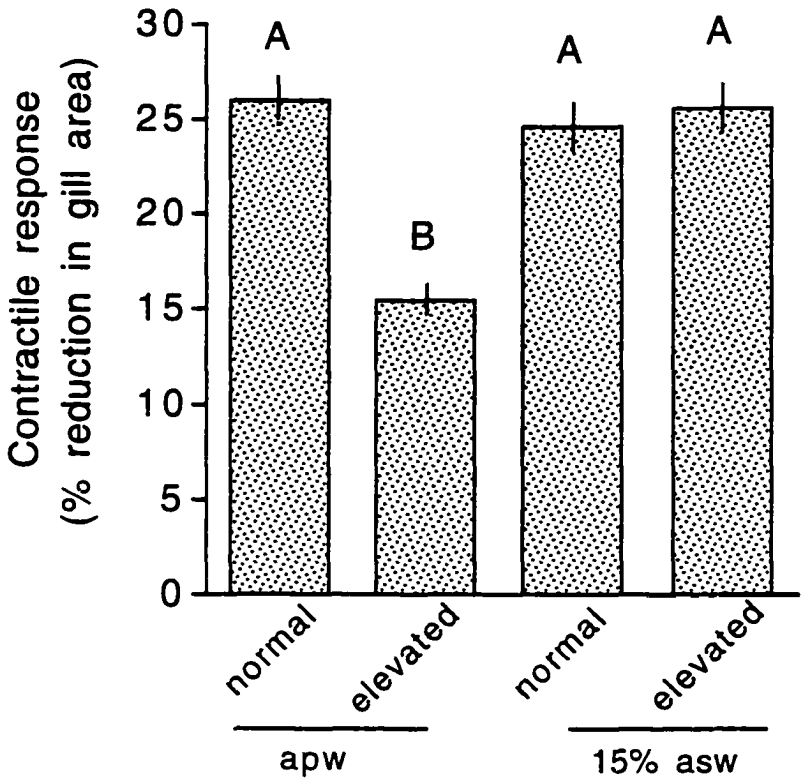


Fig. 4.2. Contractile responses of gills acclimated to 15% ASW (15%) or to APW (apw) and exposed acutely to Ringer's (normal) or to 'elevated' Ringer's (elevated). The APW-acclimated animals acutely exposed to 'elevated' Ringer's solution show depressed contractile activity as compared with the other treatment groups. The 15% ASW acclimated-animals had the same level of demibranch contraction as the APW-acclimated animals in Ringer's. Contractile responses with the same letter are not significantly different from one another when compared by a Tukey post-ANOVA test. (mean ± se, n=20).

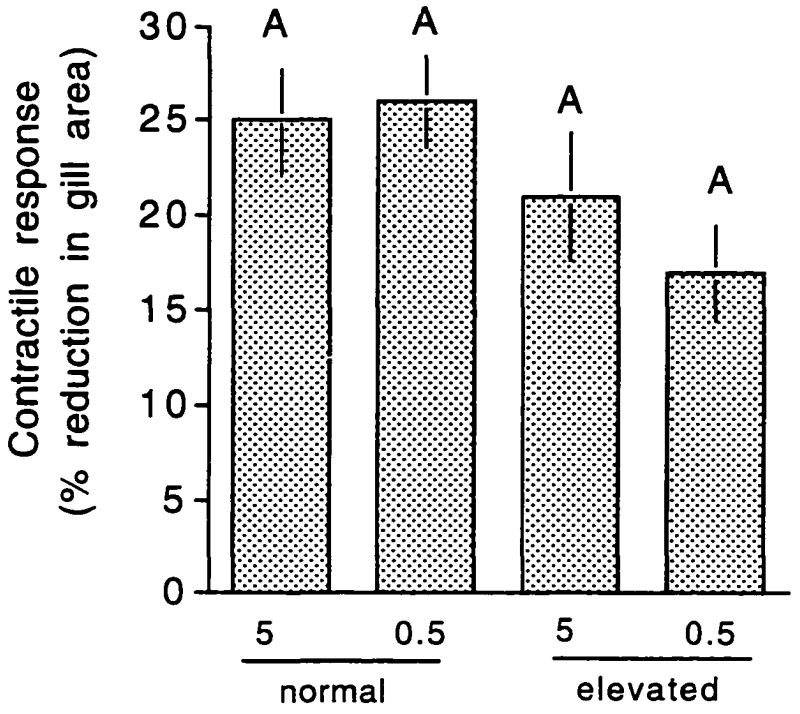


Fig. 4.3. Contractile responses of gills in Ringer's (normal or 'elevated') with different concentrations of $MgSO_4$. The high concentration of $MgSO_4$ was 5.0 mM, while the low concentration was 0.5 mM. $MgSO_4$ concentration did not have a significant effect on muscle contraction. The demibranchs in the 'elevated' Ringer's solution contracted significantly less than those in the Ringer's solution ($p < 0.012$). The contractile responses with the same letter are not significantly different from one another when compared by a Tukey post-ANOVA test. (mean ± se, n=10).

KCl Experiments

The effects of KCl concentration on ACH-induced contraction in APW-acclimated mussel gills were examined in four independent experiments, each with four different NaCl concentrations in the 'elevated' Ringer's solution. Maximum contraction corresponded to either 1 or 2 mM KCl in each of the experiments (Fig. 4.4). Comparison across the four experiments suggests that when KCl is absent from the Ringer's solution, contraction decreases as a function of increasing NaCl concentration.

NaCl Experiments

The effects of NaCl concentration on ACH-induced contraction in APW-acclimated mussel gills were examined in two experiments with either 0 mM or 2 mM KCl in the 'elevated' Ringer's. Contraction of the demibranch muscles significantly decreased as a function of increasing NaCl concentration when the KCl concentration was 0 mM ($p < 0.0001$, $r^2 = 0.36$). However, when 2mM KCl was present the contractile response did not change with increasing NaCl concentration ($p < 0.655$, $r^2 = 0.006$) (Fig. 4.5).

Ouabain Experiment

The effect of ouabain on ACH-induced muscle contraction was examined in both the presence and absence of KCl following an acute 25-30 min incubation in 'elevated' Ringer's without KCl. The interaction effect between ouabain and KCl was significant ($p < 0.0039$), with 2 mM KCl alone showing significantly greater contraction than the other treatments groups (Fig. 4.6). The mean response of the treatment group with KCl alone was over three times larger than the mean response when KCl was present with ouabain and over 1.5 times greater than the response of the group with neither KCl nor ouabain added. The main effect of ouabain was significant ($p < 0.0001$), but this effect is difficult to interpret since the interaction of ouabain and KCl was significant.

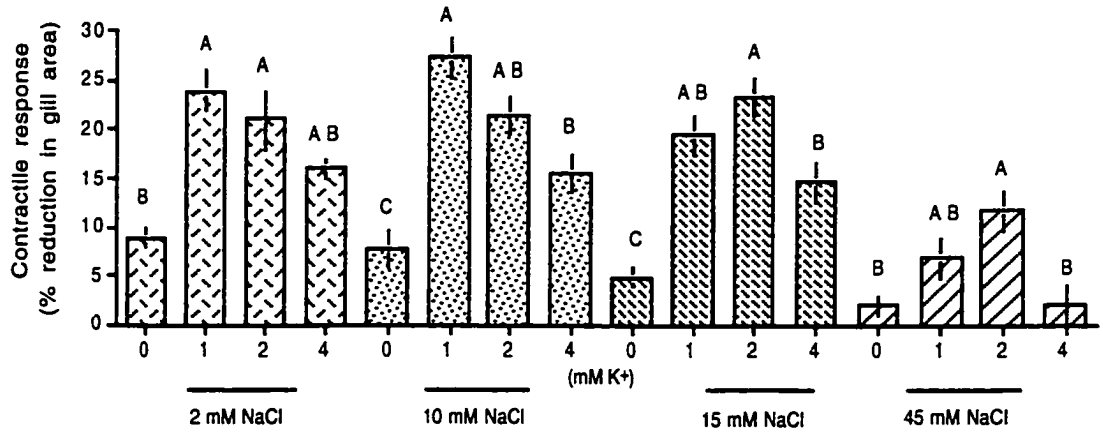


Fig. 4.4. Contractile responses of gills for the four independent KCl experiments, each at a different NaCl concentration indicated by a different shading pattern. The greatest degree of muscle contraction corresponds to either 1 or 2 mM KCl in each experiment. The contractile responses with the same letter within an experiment are not significantly different from one another when compared by a Tukey post-ANOVA test. (mean ± se, n=10).

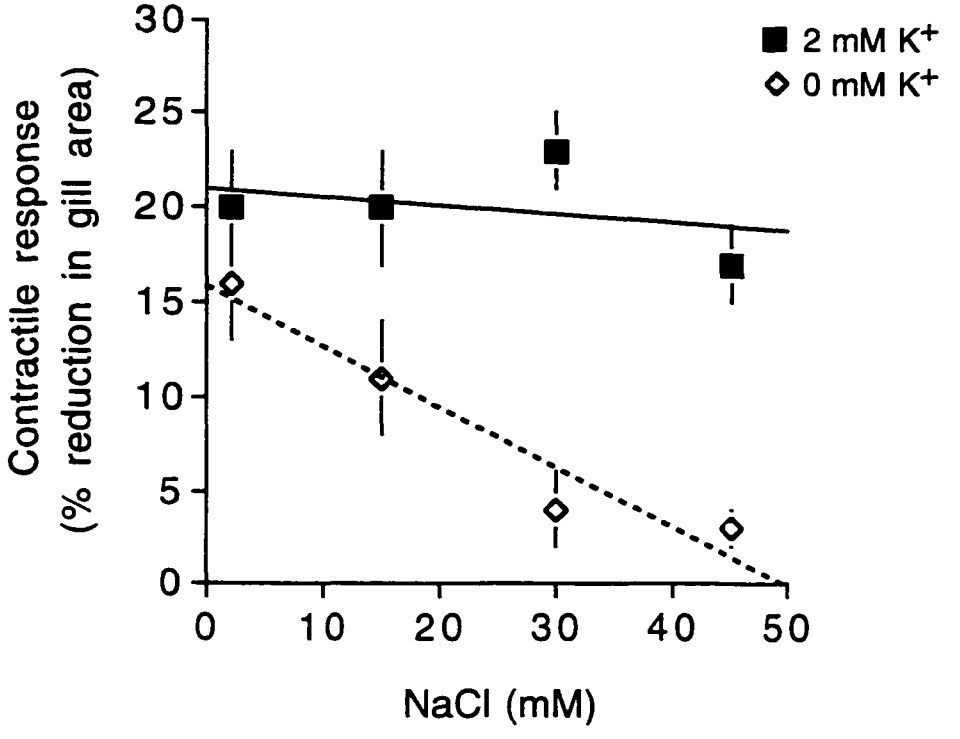


Fig. 4.5. Contractile responses as a function of increasing NaCl concentration in the 'elevated' Ringer's solution with either 0 mM or 2 mM KCl. When no KCl was present in the solution, contraction significantly decreased as a function of increasing NaCl concentration. When 2 mM KCl was provided, no such reduction was found. (mean ± se, n=10).

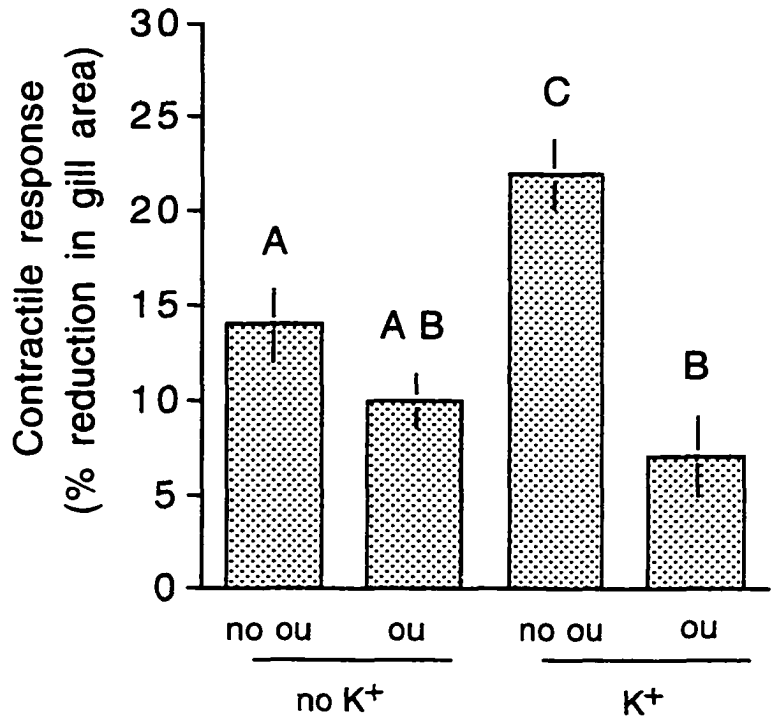


Fig. 4.6. Contractile responses of demibranchs treated with ouabain (0 or 1 mM) and/or KCl (0 or 2 mM). Gills were acutely exposed to 'elevated' Ringer's without KCl. After 25 to 30 min, demibranchs were distributed to treatment groups and allowed to recover for about 40 min. Treatment groups are 'elevated' Ringer's with (K^+) or without (no K^+) KCl and with (ou) or without (no ou) ouabain. When K^+ was present without ouabain, the greatest level of muscle contraction was measured. However, adding ouabain caused a significant reduction of this response. Contractile responses with the same letter are not significantly different from one another when compared by a Tukey post-ANOVA test. (mean ± se, n=10).

DISCUSSION

The gill muscles of *D. polymorpha* are dependent on external calcium for ACH-induced muscle contraction. Removing Ca^{2+} from the Ringer's solution or blocking the effects of Ca^{2+} with inorganic Ca^{2+} antagonists (Co^{2+} or Mn^{2+}) prevents muscle contraction. The small size of the fibers and their lack of a well-developed internal Ca^{2+} storage system (Chapters 2 and 3; Medler and Silverman 1997) are consistent with these findings. Other molluscan muscle fibers also depend on external Ca^{2+} sources for activation, for example the odontophore protractor of *Busycon canaliculatum* (Huddart et al. 1992) and the anterior byssus retractor muscle of *Mytilus edulis* (Miyahara et al. 1993). Mg^{2+} did not affect muscle contraction at the concentrations examined here.

The proper balance between NaCl and K^+ is essential to normal muscle contraction in the gill of *D. polymorpha*. This study shows that K^+ must be present at concentrations of 1 to 2 mM in order to support normal contraction. When exposed to elevated NaCl concentrations, the presence of K^+ ions becomes even more critical for the maintenance of contractility. Consistent with these findings, whole animals cannot survive beyond a few days when exposed to 45 mM NaCl challenge with 0.05 mM K^+ present in the pond water (Horohov et al. 1992; Dietz et al. 1996) and a hyperosmotic stress similar to an acute exposure to 15% ASW results in epithelial cell shrinkage within min (Dietz et al. 1998). The movement of K^+ and other inorganic ions between intracellular and extracellular pools can be an important mechanism in volume regulation in response to osmotic stresses both in invertebrates (Pierce 1982; Gilles 1987; Dietz et al. 1998) and vertebrates (Hoffmann 1987; Hoffmann and Dunham 1995). Furthermore,

K^+ transport in *E. coli* has been particularly well studied, where it also plays a critical role in osmoregulation (Epstein and Laimins 1980; Epstein 1986).

Often during hypoosmotic volume regulation (the case most studied in invertebrates), K^+ efflux from intracellular compartments appears to be a response to reduce swelling during the initial phase of exposure (within min), while changes in the concentration of organic effector molecules such as free amino acids are thought important for volume regulation over a longer readjustment period (within h) (Gilles 1987). While inorganic ion movement is often passive (Gilles 1987), active transport is also important for maintaining ion balance. For example, Na^+/K^+ ATPase activity is involved in the recovery of neural tissues from osmotic or ionic imbalances in polychaetes (Benson and Treherne 1978) and in bivalves (Treherne et al. 1969; Willmer 1978c). In addition, the activity of a ouabain-sensitive sarcolemmal Na^+/K^+ ATPase has been linked to fatigue resistance in vertebrate skeletal muscle, where cellular extrusion of K^+ and accumulation of Na^+ is a contributor to fatigue (Clausen 1996; Nielsen and Overgaard 1996). My results demonstrate that the recovery of muscle contractility following acute osmotic stress in *D. polymorpha* is ouabain-sensitive. The exchange of K^+ and Na^+ in the gill muscles of *D. polymorpha* during hyperosmotic/ionic stress is probably important for cellular osmoregulation and maintenance of an electrical potential by increasing intracellular K^+ while extruding Na^+. Under similar osmotic stress, it has been demonstrated that ouabain-sensitive processes are essential for short-term volume regulation in the gill epithelia of *D. polymorpha* (Dietz et al. 1997).

Osmoconforming animals show continued cellular function over a wide range of ion concentrations. *Mytilus edulis* can acclimate to between 25% and 125% sea water with ionic and osmotic conformity (Willmer 1978a). The nervous tissue from these

animals continues to produce full-sized action potentials after acclimation to 25% sea water (Willmer 1978b). Isolated ventricle strips from three bivalve species continue to function over a wide range of salinities: good contractile activity was found over a range of 40-160% sea water in *Mytilus edulis*, 70-120% sea water in *Ostrea edulis*, and 2-24% sea water in *Anodonta cygnea* (Pilgrim 1953a). Whole animals exhibited an even greater ability to survive salinity changes (Pilgrim 1953a) and showed continued ciliary activity over a wide range of salinities (Pilgrim 1953b).

Despite extreme changes in the ionic composition of the bathing medium, the ionic basis of excitability appears to be the conventional mechanism based on Na^+ and K^+ distribution in invertebrates such as annelids (Carlson and Treherne 1977; Nicholls and Kuffler 1964), snails (Brezden and Gardner 1984; Dorsett and Evans 1989) and bivalves (Treherne et al. 1969; Shigeto 1970; Kidokoro et al. 1974; Willmer 1978b,c). The results of the present study support the necessity for a proper balance between Na^+, K^+, and an absolute requirement for Ca^{2+} to maintain normal gill muscle function in *D. polymorpha*. Whether impaired volume regulation, electro-chemical imbalance, or both are involved in the depression of muscle activity remains to be determined. We cannot separate electrochemical effects on the muscle from osmotic effects since we have not measured membrane potentials. The muscle fibers in the gill of *D. polymorpha* that are responsible for the contractility reported here are small (1-2 μm in diameter) and encased within a connective tissue matrix (Chapter 2; Medler and Silverman 1997), making electrical recordings elusive to date.

D. polymorpha belongs to the bivalve subclass Heterodonta, which contains many brackish water species. A number of these animals are less well adapted to freshwater than, for example, members of the subclass Paleoheterodonta, to which the unionids belong (Deaton and Greenberg 1991). Indeed, *Dreissena polymorpha* is a

freshwater bivalve that shows a marked intolerance for deionized water (Nichols 1993, Ram and Walker 1993, Dietz et al. 1994). Wilcox and Dietz (1995) suggested that *D. polymorpha* is incompletely adapted to fresh water as indicated by the rapid ion turnover and an inability to reduce ion loss in dilute fresh water. The present study demonstrates that APW-acclimated animals have depressed muscle contractility when acutely transferred to 'elevated' Ringer's solution but that animals acclimated to 15% ASW in steps over a few days have normal levels of muscle function.

Wilcox and Dietz (1998) concluded that *D. polymorpha* is a freshwater species in transition from a brackish water ancestry and still has some ability to tolerate oligohaline habitats. However, they also found that the animals could not tolerate large or rapid salinity fluctuations. One factor contributing to the animals' inability to tolerate these fluctuations may be a transient loss of normal muscle function that accompanies such changes. We have demonstrated that ion imbalances can alter normal muscle function in the gills and it is likely that other muscles such as the heart may be adversely affected as well.

CHAPTER 5

COMPARATIVE GILL DIMENSIONS

The filtration rate of many bivalves is directly related to the total gill area (Meyhöfer 1985; Jones et al. 1992; Lei et al. 1996) and some level of plasticity in the relative size of the gill is apparently related to environmental factors (Thiesen 1982; Franz 1993; Payne et al. 1995). Different groups of bivalves exhibit varying abilities to clear bacterial–sized particles from their environment (Møhlenberg and Riisgård 1978; Silverman et al. 1995; Silverman et al. 1997). These differences are probably due to differences in the structure of the latero-frontal cirri of the gill (Moore 1971; Owen and McCrae 1976; Silverman et al. 1996a,b), but some differences may be caused by differences in the relative dimensions of the gills (Silverman et al. 1997).

In addition to ciliary structure and relative gill size, the ostial area available for water flow across the gill may have an important influence on filtration rate (Foster-Smith 1975, 1976; Jones et al. 1993). Ostial dimensions are variable over time (Elsey 1935; Nelson and Allison 1940; Foster-Smith 1976; Jørgensen 1990; Gardiner et al. 1991a; Tankersley 1996; Medler and Silverman 1997) and may be related to water-flow regulation. As glochidia develop in the water channels of unionids, there is a shut down of ostial water flow in the posterior portion of the gills. This represents the extreme case where gill musculature presumably regulates water flow through ostia (Richard et al. 1991).

This chapter examines live gill area as a function of dry body mass and the dimensions of the internal ostia in the freshwater bivalves, *Dreissena polymorpha*, *Corbicula fluminea*, and *Toxolasma texasensis*. Previous studies using these species have shown significant differences in the ability to filter bacterial-sized particles (Silverman et al. 1995; Silverman et al. 1997) with *D. polymorpha* having the highest clearance rates, followed by *C. fluminea* and *T. texasensis*, respectively. While these differences were related to differences in the structure of latero-frontal cirri, differences in

relative gill size or ostial area or both could also contribute to the differences observed in clearance between the three species.

METHODS

Animals and maintenance

Dreissena polymorpha (Pallas 1771) were collected from western Lake Erie and from the Raisin and Huron Rivers in Michigan; and from the Mississippi River near Baton Rouge, Louisiana. *Corbicula fluminea* (Müller 1774) and *Toxolasma texasensis* (I. Lea 1857) were collected from ponds near Baton Rouge, Louisiana. The animals were maintained in artificial pondwater (0.5 mM NaCl, 0.4 mM $CaCl_2$, 0.2 mM $NaHCO_3$, 0.05 mM KCl, and 0.2 mM $MgCl_2$) in aerated aquaria under laboratory conditions of approximately 22 - 25° C.

Measurement of gill dimensions

Excised gills were placed in Ringer's solution diluted to 70% with deionized water. The undiluted solutions contained 5 mM NaCl, 5 mM $CaCl_2$, 5 mM $NaHCO_3$, 0.5 mM KCl, 5 mM $NaSO_4$, 0.5 mM $MgCl_2$; 48 mOsm. Calcium-free Ringer's used in various experiments had the same composition as the above solution except that the $CaCl_2$ was omitted and 4 mM EDTA was added. The osmolality and pH were the same in both solutions. For *C. fluminea* and *D. polymorpha*, gills were placed in the Ca^{2+}-free Ringer's to induce muscle relaxation. Presumably because of extensive calcium concretions and rods in the gills of *T. texasensis* (Silverman et al. 1983; Silverman et al. 1985; Silverman et al. 1987), the same solution did not fully inhibit muscular contraction in this species. Thus, 10^{-4} M serotonin was used to relax the muscles in the gills of these animals (Gardiner et al. 1991a,b). To induce muscle contraction, gills were exposed to 10^{-3} M acetylcholine (ACH) in *C. fluminea* and *D. polymorpha*. The gills of *T. texasensis* appear to be insensitive to ACH. However, they will contract when placed in a low pH environment, or if they are lightly stretched in an anterio-posterior direction.

The gills of *T. Texasensis* were thus placed in a Ringer's solution of pH 4 and mechanically stretched with forceps prior to measurements in order to elicit a contractile response.

The demibranchs from one side of each animal were placed in a solution to induce muscular contraction, while the gills from the other side were placed in a solution to cause relaxation. After 5 minutes in the appropriate solution, gills were laid flat on a microscope slide and photographed. The printed photographs were used to measure gill surface area (mm^2) using Image I software.

After the photographs had been taken, the gills were returned to Ringer's solution without ACH or acidic pH and split into single lamellae along the interlamellar septae. The single lamellae were returned to the appropriate treatment solution and the water channel side of the gill was viewed at a magnification of 200X using either differential interference contrast (DIC) or darkfield optics on a Nikon Microphot FXA in the Socolofsky Microscopy Facility at Louisiana State University. The regions selected for examination were from the central portion of the gill and represented undamaged tissue within a single focal plane. The percentage of the water channel epithelial surface interrupted by ostia was estimated from high contrast photographic prints using point-count stereology (Weibel 1979).

Analysis of gill dimensions

Soft tissue was excised and dried overnight at 90° C for shell-free dry mass determination. Relaxed gill surface areas (mm^2) were plotted as a function of dry mass (mg) and a regression was made through the data with a line having the form: $Y = bX^m$, where b is the elevation of the line and m is the slope. Internal ostial areas from the relaxed and contracted gills of the three species were compared by a 2 X 3 factorial with one factor being the state of contraction (relaxed or contracted) and the second factor being the species. Individual factor means were compared with a Bonferroni/Dunn post-

ANOVA test (experiment-wise error rate = 0.05). The relative contracted gill areas (% of relaxed gill area) were compared among the three species by a one-way ANOVA and individual means were compared with a Tukey post-ANOVA test (experiment-wise error rate = 0.05). Data were normally distributed and the variances were homogeneous. Therefore, the data fit the assumptions of the statistical models and no transformations were performed.

RESULTS

The slopes and intercepts of the regression lines for live gill area plotted as a function of dry body mass were not significantly different among the species. Therefore, a common regression line was used for all three species together: area (mm^2) = 63.396 • dry tissue mass $(mg)^{0.450}$ (Fig. 5.1). The contracted gill ostial areas were always significantly smaller than the corresponding relaxed gills ($p < 0.0001$) (Fig. 5.2). The effect of species was also significant ($p < 0.002$) with *C. fluminea* having significantly more ostial area than the other two species (Fig. 5.2). The interaction effect was not significant ($p < 0.543$). The rank order of contracted gill area (% relaxed area) was: *D. polymorpha* (60%) > *C. fluminea* (75%) > *T. texasensis* (95%) (Fig. 5.3).

DISCUSSION

The relative size of the gill appears to be the same in each of these species, despite differences in phylogeny and gross gill organization. Gill surface area scales as a common function of dry tissue mass in all three species (Fig. 5.1). In fact, this relationship is remarkably similar to the scaling of the filibranch gill in the marine bivalve, *Mytilus edulis* (Fig 1, broken line from Jones et al. 1993). A number of studies have demonstrated that water pumping rate, measured directly or indirectly, is a direct function of total gill surface area (Meyhöfer 1985; Jones et al. 1992; Lei et al. 1996) or total ostial area (Foster-Smith 1975, 1976). Kryger and Riisgård (1988) found that 6 species of

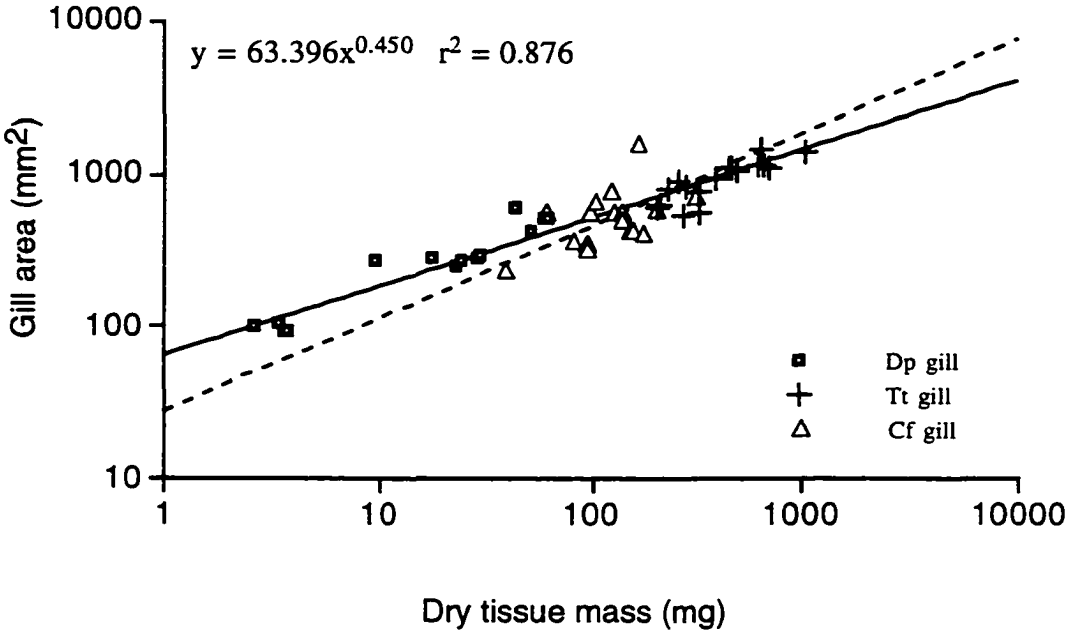


Fig. 5.1. Gill area (mm^2) as a function of dry tissue mass (mg) for the three species of freshwater bivalves. Each of the species gill area falls on the same line: gill area (mm^2) = 63.396 • dry tissue mass $(mg)^{0.450}$. This relationship is similar to that found for *Mytilus edulis* (broken line) by Jones *et al.* (1993) over a similar size range.

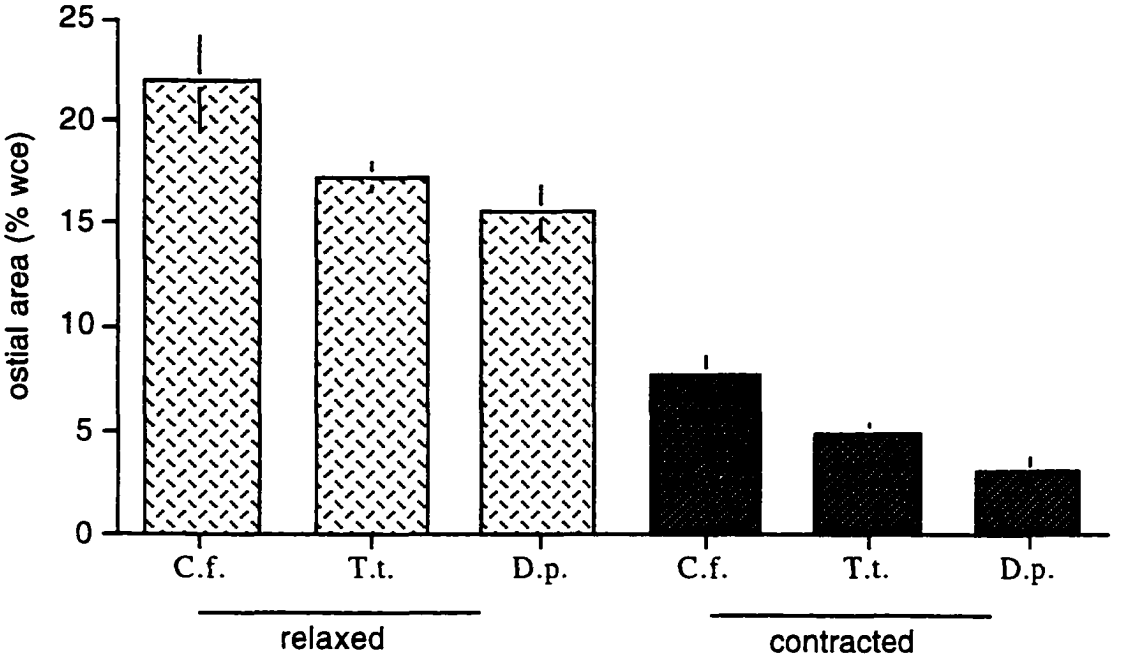


Fig. 5.2. Internal ostial area (% of total water channel epithelium) for the three bivalve species in relaxed and contracted conditions. The mean relaxed area was significantly greater than the mean contracted area ($p < 0.0001$). The species effect was also significant ($p < 0.002$), with *C. fluminea* having more ostial area than the other two species (mean ± se, n=15).

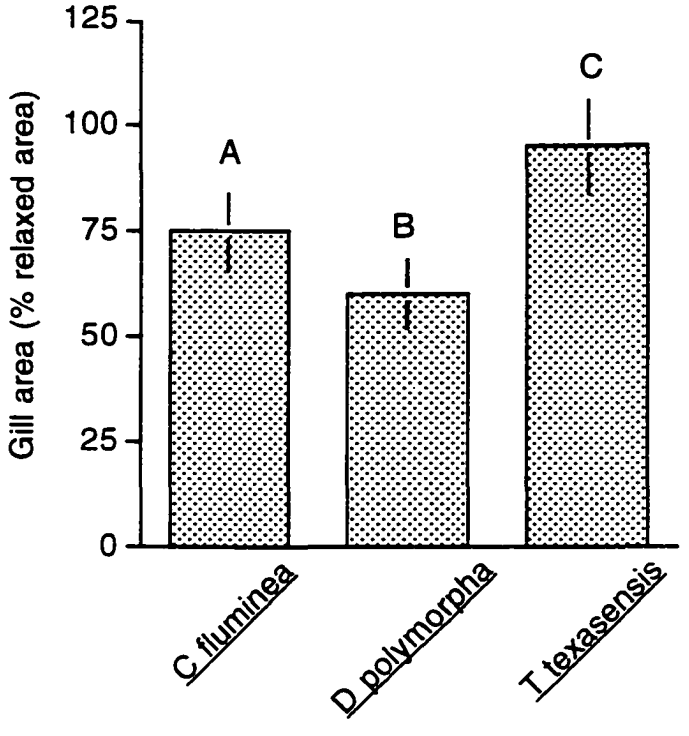


Fig. 5.3. Gill area in the contracted state relative to the relaxed state (% relaxed area). Each species showed significant differences in area (% relaxed area) with *D. polymorpha* showing the greatest change and *T. texasensis* showing the least change in area (mean ± se, n=15 except for *C. fluminea* where n = 11).

freshwater bivalves (including *D. polymorpha*) have the same water pumping rate when corrected for gill area. Similarly, Lei et al. (1996) demonstrated that when corrected for differences in body mass, 4 different species of freshwater bivalves (including *D. polymorpha* and *C. fluminea*) should have the same rate of clearance of particles retained with high efficiency. Significant differences in gill dimension have been found within species from different habitats (Thiesen 1982; Franz 1993; Payne et al. 1995); these differences are apparently related to differences in trophic and other environmental factors. Collectively, the available data suggest that the bivalve gill as a water pump, *per se*, has a fundamental design that tends to keep gill dimension and ostial area within a narrow range in spite of other differences that exist between species. Some fluctuation about this general relationship may exist for animals from different habitats, but overall suspension-feeding bivalves may have been held by a common design constraint.

The scaling of gill size as a function of body weight has important implications. Jones et al. (1993) pointed out that clearance measurements should be carefully corrected for body mass before drawing conclusions from the data. The fact that gill area scales as mass to an exponent of less than 1 means that smaller animals have larger gills on a mass-specific basis. This generalization can have significant biological implications: based on the data presented here, a 20 mg *D. polymorpha* would have a relative gill area of about 12.2 mm^2/mg; while one 100 mg *T. texasensis* would have a relative gill area of only about 5.0 mm^2/mg. Since a large number of zebra mussels often occupy a given area, this large number combined with small sizes will lead to significantly larger gill areas and higher particle clearance rates from the water column, the end result being that exploitative competition may be having negative impacts on the native clam populations. Interference competition occuring when zebra mussels attach to unionids may also be a contributing factor to the mortality of the native clams where zebra mussel infestations have occurred (Schloesser et al. 1996).

Similarity in the capacity of the water pump does not necessarily correspond to similar clearance rates between species. Significant differences in clearance rate exist between freshwater bivalves (including those studied here) when feeding on bacterial sized particles (Silverman et al. 1995; Silverman et al. 1997). These differences are related more to distinct differences in the structure of latero-frontal cirri than to body size. Both *C. fluminea* and *D. polymorpha* have the most complex latero-frontal cirri when compared with unionid species (Silverman et al. 1995; Silverman et al. 1996a,b; Silverman et al. 1997). This complexity apparently allows them to more efficiently capture bacterial sized particles. Møhlenberg and Riisgård (1978) also found differences related to cirral structure among 13 species of marine bivalves in their ability to retain bacterial-sized particles. Thus, the clearance capacities of bivalves appear to be related to the fine structure of the ciliated gill surfaces and total number of cirri but not to differences in the scaling of gill area between species.

Another similarity between the species studied here is the size of the internal ostia of the gill. Ostial area is between 22 and 30% of gill area in *Mytilus* (Foster-Smith 1976; Jones et al. 1992), while the values reported here range from about 16 to 24% of the water channel epithelium in the relaxed condition. *C. fluminea* has a significantly more open gill than *D. polymorpha* (Fig. 5.2), because of the very large ostia found regionally in *C. fluminea*. (Fig. 5.4) These regions in *C. fluminea* appear to correspond to the plicate gill type found in this species; the large ostia correspond to the apex of the plicae, where water canals from several external ostia fuse into a single canal with a common internal ostium. More typical ostia are found near the plical troughs. While the values are in general agreement with those of other studies, the smaller areas of the species studied here reflect the fact that all of these species possess eulamellibranch gills. The base of the filaments in eulamellibranch gills unite within sheets of subfilamentar tissue, through which the ostia and water canals connect the outside of the gill with the inner water

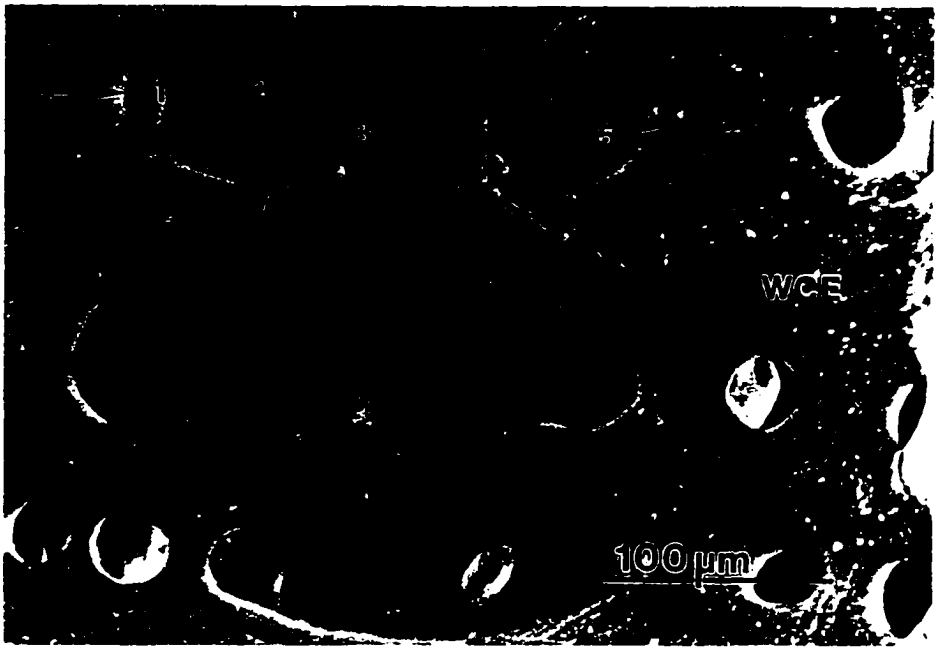


Fig. 5.4. Internal ostia in *C. fluminea* viewed from the inside of the water channel. Regionally, several external ostia may lead into a singe large internal ostia. In one case, 5 external ostia (#'s 1-5) can be seen leading into a single internal ostium (borders denoted by arrows).

channel. This subfilamentar tissue is absent in filibranch gills (refer to Fig. 1.1). Since some the measurements in other studies were from filibranch gills, they are not directly comparable to the values reported here.

All three of the species in this study demonstrate the ability to significantly decrease the internal ostial area of the gill through muscular activity. These muscular adjustments correspond to those reported in Chapter 2 and further demonstrate that each of these species is capable of muscular regulation of gill dimension. The significant differences in the reduction of gross gill area are probably related to the compressibility of the gills. The gills of both *D. polymorpha* and *C. fluminea* were reduced in size significantly more than *T. texasensis*, which has extensive calcification in its gills (Silverman et al. 1983; Silverman et al. 1985; Silverman et al. 1987). Another factor leading to differences may be different levels of responsiveness to the contractile stimuli. For example, the gills of *D. polymorpha* respond dramatically to ACH, while the gills of *C. fluminea* may not be as sensitive to this transmitter. In addition to transmitter specificity, this sensitivity may be related to the higher rates of paracellular exchange that exist across the epithelium in *D. polymorpha*, as compared to those in *C. fluminea* (Dietz et al. 1995; Zheng and Dietz 1998). Thus, the effective ACH concentrations may have been much less in *C. fluminea* .

Overall, the three species show similarity in terms of overall gill dimensions and in the ability to change these dimensions through muscular contraction. This uniformity supports the idea that the general structure and function of the gills in these animals is similar. Studies providing water flow or clearance rates as a function of live gill area are needed to further interpret these relationships.

CHAPTER 6

CONCLUSIONS

The studies presented here indicate that eulamellibranch gills of freshwater bivalves have a well organized muscular system that works in coordination with connective tissue elements to provide the gill with a 'postural tone'. The muscle and connective tissue elements in each of the three species are similar in their structural organization and in their apparent ability to regulate the muscular tone of the gill. The collagenous connective tissue of the gill provides the general support of the gill and is also important for transmitting the forces generated by the active muscle fibers. The response of these muscle fibers to transmitter substances and their sensitivity to the ionic makeup of the bathing solution is evidence that they are physiologically active. Overall, the size and dimensions of the gills are similar in all three species.

From a theoretical standpoint, there are at least three factors that can be addressed in relation to the muscular regulation of the bivalve gill as a water pump. First is the role of the distance between the lateral cilia and its affect on interfilament flow rates. The interfilament flow rate at maximal pump velocities is apparently faster than the ciliary tip speeds and Jørgensen and coworkers have attributed this phenomenon to positive interference between opposing bands of cilia (Jørgensen et al. 1988; Jørgensen 1989; Jørgensen 1990). Conversely, when the distances between the opposing ciliary tracts decrease to a critical level, the interference becomes negative and this interference is possibly the most important factor in the regulation of water flow and pump capacity (Jørgensen et al. 1988; Jørgensen 1989; Jørgensen 1990). Unfortunately, these authors have assigned a passive, indirect mechanism to the regulation of interfilament distance as being a result of muscular activity in the mantle and gill axis, as well as due to valve gape (Jørgensen et al. 1988; Jørgensen 1989; Jørgensen 1990). The work presented here suggests that the regulation of interfilament distance is not indirect, but is in part the result of muscle tone provided by smooth muscle fibers that lie in bands at the base of the filaments.

A second theoretical factor to consider is the role of the resistance to water flow provided by the water canals of the gill. Two prominent models of the bivalve gill (Foster-Smith 1976; Jørgensen et al. 1986) agree that the resistance through the gill is second in importance only to the resistance provided by the excurrent siphon. Foster-Smith (1976) estimated that either the excurrent siphon or the gill ostia would need to reduce their area to 20% of the fully open area to cause a significant reduction in water flow through the gill, assuming that the rest of the system remains unchanged. He further argued that the excurrent siphon was the more reasonable point of regulation because the ostia could not respond quickly. The work presented here clearly demonstrates that the gill dimensions can be rapidly altered (in a matter of seconds) and that ostia held in a relaxed state generally become more than 50% smaller following stimulation and may close completely.

The third factor is the general compliance of the whole gill to water flow. Jørgensen and colleagues (Jørgensen et al. 1988; Jørgensen 1989; Jørgensen 1990) have commented that when the bivalve pump is active, the gill becomes inflated and represents little resistance to water flow. As the pumping activity stops, the gill becomes flaccid and collapses on itself, resulting in high resistances through the gill. In fact, Jørgensen concludes his review of water processing by ciliary feeders by stating, "the rate of water pumping constitutes an emergent property of the spatial geometry of the interfilament canals and the mantle cavity in the fully inflated state of the collapsible pump" (Jørgensen 1989). However, the results presented here show that the degree to which the gill inflates is not only a function of the ciliary activity, but also of the muscular tone of the gill. The relative importance of muscular tone versus ciliary pumping has still not been explicitly examined, but is a critical concept.

In isolation of active water pumping the following observations on the gill can be made. When the muscles of the gill are completely relaxed, as they are following

incubation in a calcium-free medium, the gill becomes maximally expanded and very fluid. As the muscles contract, not only do the dimensions of the gill change, but the gill becomes rigid. Thus, the resistance provided by the gill is not only a passive result of ciliary activity filling the gill, but also from the general muscular tone of the gill. Endoscopic observations by Tankersely (1996) support this interpretation that the overall dimensions of the gill are affected not only by ciliary activity but also gill musculature.

While these studies have focused almost exclusively on the muscle fibers within a single gill lamella, it is important to understand that the muscles in the connective tissue sheets of the lamella are continuous into the interlamellar septa. This is important because it means the dimensions of the water tubes that lead to the suprabranchial chamber are under the same muscular control as the rest of the gill. One can think of multiple levels of muscular regulation of important relative gill dimensions, even though these may or may not function independently in the animal. The interfilament distance can be controlled by the bands of muscle that are antagonized by the connective tissue cross struts; the general ostial dimension can be controlled by the muscles in the connective tissue sheets in the subfilamentar tissue; and, the dimension of the water tubes can be affected by the muscles of the interlamellar septae which are continuous with those in the subfilamentar tissue. Fig. 6.1 gives an interpretation of muscular regulation of the bivalve pump. Inhalant and exhalent siphon diameters affect water flow entering and leaving the animal. These dimensions are also related to valve gape and the position of the mantle edges. Water flow into the gill can be affected by interfilament distance and the dimensions of the ostia and water canals of the gill. Finally, the dimensions of the water tubes and the overall compliance of the gill affect water flow through the gill and into the suprabranchial chamber. Thus, there are several control points through the system that are under direct muscular control. When all of the muscles in the system are relaxed, the pump can

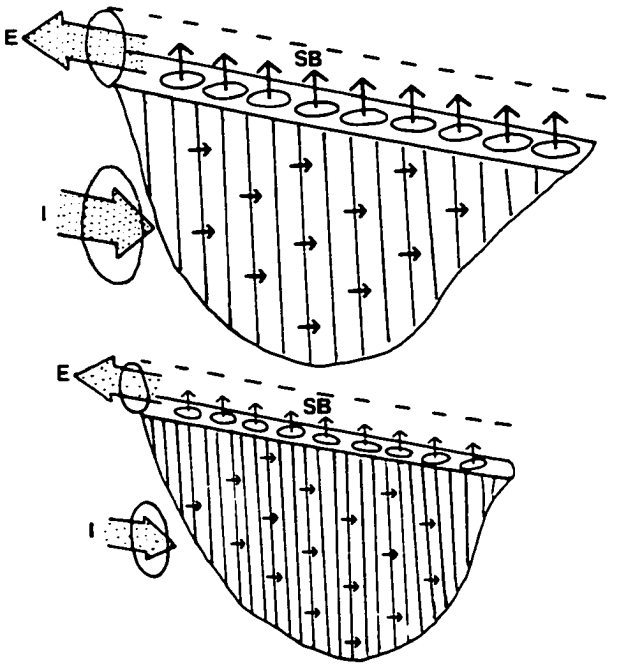


Fig. 6.1. A diagrammatic representation of the changes caused by muscular regulation of the bivalve gill and siphons. The fully relaxed state is shown in the top diagram, while a significantly contracted state is shown below. The sizes of the arrows indicating water movement are intended to suggest the relative magnitude of flow. Excurrent siphon (E); Incurrent siphon (I); Suprabranchial chamber (SB).

operate at maximum capacity (Jørgensen et al. 1988) and this represents the most energetically efficient use of the system.

It is also possible that the muscles in the gills have other functions in addition to regulation of suspension-feeding activities. For example, expulsion of embryos from the gill might be considered an important role for the muscles in the gills of *T. texasensis* and *C. fluminea* . In unionid mussels, the gills and adductors contract rhythmically to expel the brooded larvae (Tankersely 1996). However, the organization of the muscle does not suggest this as a primary or sole function. In the case of *D. polymorpha,* the gill is never used for the development of embryos and yet the muscular development and organization is similar to that seen in the other species. None of my observations were made on animals with developing embryos.

An interesting hypothesis that should be investigated is that the ciliary activity and the muscular activity are coordinated through the branchial nerves. Gardiner et al. (1991) showed that serotonin not only relaxes the gill musculature, but also increases the ciliary activity of the gill. In this situation, the gill would be open to water flow as the driving force for water flow increases. In a complementary fashion, one could envision that conditions leading to reduction in ciliary activity might also lead to an increase in the muscular tone of the gill. Between these two extremes could lie fine tuning of the gill as a water pump. Few experiments or studies in bivalve suspension-feeding are designed to examine the fine-scale regulation suggested here, and indeed most studies are designed to eliminate such regulation as it contributes variability when comparing 'maximal' filtration rates between different species and/or different studies. Improvements in our ability to observe bivalve feeding at various levels of resolution and under various experimental conditions, both *in vivo* and *in vitro* should allow further understanding of the regulation occurring in suspension-feeding bivalves. If pure bulk-flow mechanics driven largely by

ciliary activity and siphonal musculature are truly the only important regulatory components, then regulation is essentially an on-off switching cycle.

Foster-Smith (1975; 1976) demonstrated that pumping rates are a function of total ostial area and that some ostia were contracted under different conditions. Owen and McCrae (1976) suggested that patches of sensory cilia are coordinated with the motor activity of cilia and gill muscles. Unfortunately, very little is known about the organization of the nerves of the gill. Previous studies have demonstrated that branchial nerves lead to the ciliated epithelial cells where they control ciliary activity (see Paparo 1988) and the muscles of the gills are associated with nerves. Whether these sets are branches from the same nerve trunks and whether these systems are coordinated remains to be seen. Overall, the bivalve gill is a remarkably complex structure that seems capable of dynamic integration of sensory cues with motor responses. It is an intriguing possibility that chemical and mechanical cues invoke coordinated ciliary and muscular responses leading to fine regulation of gill function.

Finally, it is possible that the muscles of the gill actively pump water in coordination with the cilia. As an inflated gill contracts, the water will be forced in the direction of normal water flow. The active cilia will then re-inflate the gill and the cycle can continue. A slow but rhythmic contraction cycle could contribute significantly to the water pumping capacity of bivalves. In this light, it is interesting that theoretical ciliary capacities do not add up to measured pump capacities (Silvester 1988) and Silvester concluded that because of this apparent paradox, "one should be alert to the possibility that other systems in the mussel may be contributing to the pumping performance". Using endoscopic techniques, Tankersley and Dimock (1993) observed rhythmic expansion and contraction of the gill in a unionid mussel consistent with such a pumping mechanism. Future endoscopic techniques *in vivo* may begin to answer the question of whether the gill is not only a ciliary pump, but a muscular pump as well.

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APPENDIX

LETTERS REQUESTING AND GRANTING PERMISSION OF USE OF PUBLISHED ARTICLES

Scott Medler
Department of Biological Sciences
Louisiana State University
Baton Rouge, LA 70803

2 December 1997

Dr. Vicki Pearse, Editor of Invertebrate Biology
Marine Sciences, University of California
1156 High St.
Santa Cruz, CA 95064

Dear Dr. Pearse,

I am planning to use our paper on bivalve gill musculature (Medler and Silverman, Invertebr. Biol. 116: 200-212) as a chapter in my dissertation. As such, I need permission to use previously published material. Could you please send me a letter of permission to use this material? Thank you for your time.

Sincerely,
Scott Medler
Scott Medler

Invertebrate Biology

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Division of Invertebrate Zoology, Society for Integrative and Comparative Biology

Dr. Vicki Pearse
Editor, *Invertebrate Biology*
Institute of Marine Sciences
University of California
1156 High St.
Santa Cruz, CA 95064, USA

Internet: vpearse@cats.ucsc.edu
Telephone: 408/459-5065
Fax: 408/459-4882

10 December 1997

Scott Medler
Dept. of Biological Sciences
Louisiana State University
Baton Rouge LA 70803

Dear Scott,

This letter is to indicate full permission for you to use material from your paper in Invertebrate Biology, as a chapter in your dissertation, or in other future publications, provided only that full credit is given to the previously published citation:

Functional organization of intrinsic gill muscles in *Dreissena polymorpha* (Mollusca: Bivalvia) and response to transmitters *in vitro*
Scott Medler and Harold Silverman
Invertebrate Biology 116(3): 200–212.

Best regards,

Vicki Pearse

Vicki Pearse
Editor, Invertebrate Biology

VITA

Scott Medler attended Texas A & M University, where he received a Bachelor of Science in Zoology in 1989. During his time as an undergraduate, he developed interests in comparative physiology. He worked 20 hours a week in a molecular biology laboratory during his last four semesters; an experience that helped to develop basic laboratory skills and introduced him to research. One week prior to graduation, he married another biology student at Texas A & M, Kathryn Fulton. They attended San Diego State University (1989-1992) where Mr. Medler's main area of interest was comparative organismal biology. His Master of Science research involved comparative kidney function in the Anna's hummingbird, *Calypte anna*. The results of this research were reported at the 1991 meeting of the American Society of Zoologists. Teaching responsibilities at S.D.S.U. included introductory biology lab and human anatomy lab. After finishing his course work and research, Mr. Medler left the university without a degree because of irreconcilable differences with his thesis advisor. Two research articles stemming from his work at San Diego State were subsequently accepted for publication. From 1992-1993 he worked in an immunology laboratory at La Jolla Pharmaceutical Company, monitoring antibody titres in a mouse model of systemic lupus. In 1993, he enrolled in the Department of Zoology and Physiology at Louisiana State University to pursue studies in comparative muscle structure and function under the direction of Dr. Harold Silverman. After his first semester of study, he and Kathryn had a son, Peyton A. Medler. Mr. Medler's teaching assistant responsibilities included introductory biology lab, comparative anatomy lab, vertebrate physiology lab, and cellular physiology lab. He is currently teaching an undergraduate human physiology course and coordinating the vertebrate physiology labs. He will receive the degree of Doctor of Philosophy in Zoology from Louisiana State University in May, 1998.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Scott Medler

Major Field: Zoology

Title of Dissertation: Comparative Structure and Function of Intrinsic Gill Muscles in Freshwater Bivalve Molluscs

Approved:

Major Professor and Chairman

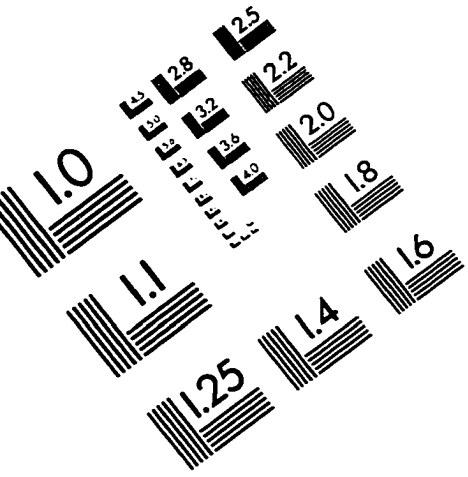
Dean of the Graduate School

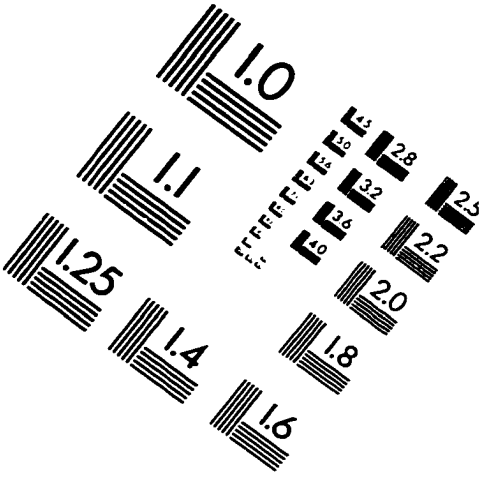
EXAMINING COMMITTEE:

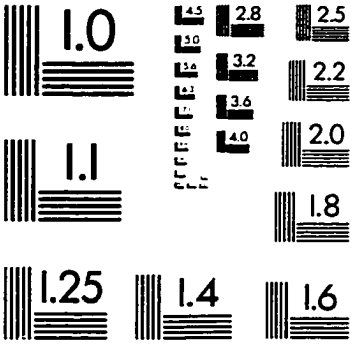
Date of Examination:

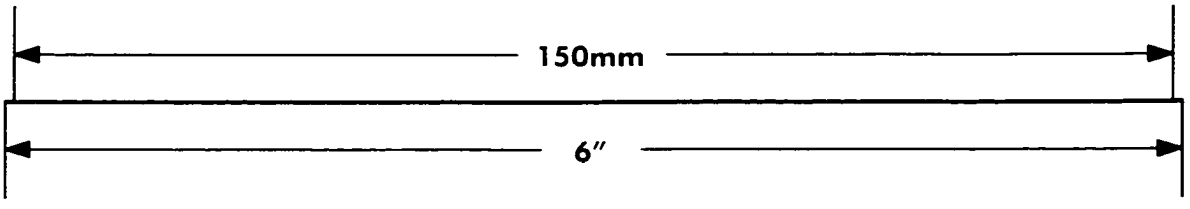
January 15, 1998

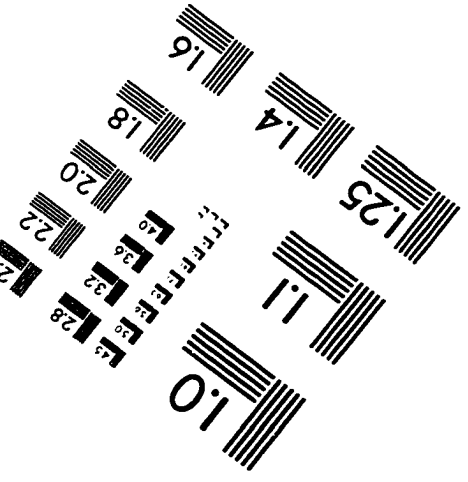
IMAGE EVALUATION
TEST TARGET (QA-3)











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