Lipoproteins and Heat Shock Proteins as Measures of Reproductive Physiology in the Soft Shell Clam *Mya arenaria*

by

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B.S. Marine Science
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Submitted in partial fulfillment of the
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Abstract

Reproduction is an important physiological process in marine bivalve molluscs. Experiments were designed to examine the role of lipoproteins and heat shock proteins in normal physiological processes of the soft shell clam, *Mya arenaria*, including reproduction.

The distribution of ^{14}C triolein, an injected lipid tracer, occurred rapidly and was unaffected by the early stages of the disease hematopoietic neoplasia. These results, in combination with other evidence, provide support for the hypothesis that plasma is the primary conduit for lipid transport in the soft shell clam. A very high density lipoprotein (VHDL) was isolated from clam plasma. The association of only small amounts of the lipid tracer with the VHDL suggest that it may not play a large role in lipid transport. The lack of information on the turnover rate of the lipoprotein, however, makes this result uncertain.

Additionally, the expression of the heat shock protein 70 family was measured in the gill and gonad of clams from severely (New Bedford Harbor), moderately (Cotuit Bay), and negligibly (Little Buttermilk Bay) contaminated natural populations. Site differences in hsp 70 expression were observed only in the gill of clams from the New Bedford Harbor population, suggesting that a threshold exists for the induction of the stress response. Effects of the reproductive cycle, however, were observed in both gill and gonad tissues. In New Bedford Harbor, the stress associated with the later stages of the reproductive cycle (ripe, spawning, and spent) appears to interact synergistically with the contaminant effect. The interpretation of a stress response associated with exposure to environmental contaminants, therefore, must include a careful consideration of the effect of natural physiological processes, including reproduction, on the expression of hsp 70.

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Chapter 1. Introduction

Sublethal effects of contaminants and disease have been examined at all levels of biological organization, from subcellular biochemistry to communities and ecosystems. Population level effects are generally considered to be among the most interesting, especially for purposes of resource management. Effects at higher levels, however, are more difficult to measure and are less sensitive to low concentrations of contaminants. Biochemical effects are easier to measure and more sensitive, thereby providing early warning signals for sublethal contaminant exposure and effects. One way to correlate biochemical effects to disturbances at the population level may be to choose subcellular events that are involved in the reproductive process.

Reproduction is one of the most energetically expensive physiological processes in the life of a bivalve mollusc. Gametes contain lipid in structural components (eg. membranes) and as compounds for energy storage. Lipid stores deposited in oocytes sustain lecithotrophic larvae until they are able to begin feeding (Gallagher et al., 1986). The concentration of lipid in gametes is an important factor in determining larval viability (Helm et al., 1973; Gallagher and Mann, 1986). Protein synthesis is also required for the production of gametes.

Alterations in the reproductive process have been observed in populations of bivalve molluscs from contaminated habitats and in bivalves exposed to lipophilic organic contaminants under laboratory conditions. In these studies, reproductive processes are affected in a number of ways, including effects on the quantity and quality of gametes (Lowe and Pipe, 1986; Lowe and Pipe, 1987; Lowe, 1988; Sunila, 1988) and changes in the balance of energy allocation between basal metabolic and reproductive demands (Lowe and Pipe, 1985; Lowe and Pipe, 1986; Lowe and Pipe, 1987; McDowell Capuzzo et al., 1989).

Two potential biomarkers with important roles in both basal metabolism and the reproductive cycle in a wide variety of organisms are lipoproteins and heat shock proteins. Both lipoproteins and heat shock proteins may be affected by diseases or exposure to contaminants (Barclay and Barclay, 1980; Lewis, 1980; Opplt, 1980; Wieland and Seidel, 1980; Borlakoglu et al., 1989; Borlakoglu et al., 1990a; Borlakoglu et al., 1990b; Kaufmann, 1990; Sanders, 1990; Winrow et al., 1990; Sanders et al., 1991; Veldhuizen-Tsoerkan et al., 1991; Köhler et al., 1992; DeNagel and Pierce, 1993; Monaghan and Bradley, 1993; Sanders and Martin, 1993; Young et al., 1993; Ryan and Hightower, 1994; Brown et al., 1995). The roles of lipoproteins and heat shock proteins were examined in the soft shell clam, *Mya arenaria*.

The soft shell clam, *Mya arenaria*

The soft shell clam, *Mya arenaria*, is an economically and ecologically important bivalve mollusc that is indigenous to the northeastern coast of the United States. Introduced populations of *M. arenaria* are also found along the west coast of the United States. In New England, *M. arenaria* is found on intertidally-exposed flats and in the subtidal zone. Natural populations of the soft shell clam support a large commercial fishing industry in Massachusetts, Maine, and Chesapeake Bay, as well as a recreational fishery throughout New England.

The internal anatomy of the soft shell clam (Figure 1.1) is similar in many aspects to that of typical bivalve molluscs. The foot of the soft shell clam is used to burrow into a range of sediment types, from mud to sand. The siphon is extended into the water column for filter feeding, in which water is drawn in through the incurrent siphon, passed over the gills, and pumped out through the excurrent siphon. In *M. arenaria*, the gonad and digestive gland form a complex in the visceral mass, unlike some other bivalves (eg. mussels, oysters) in which the gonad is part of the mantle, which also covers the tissues and secretes the shell. The soft shell clam has an open circulatory system; the hemolymph is distributed throughout the tissues and pools in sinuses near the adductor muscles.

Soft shell clams have separate sexes which can only be

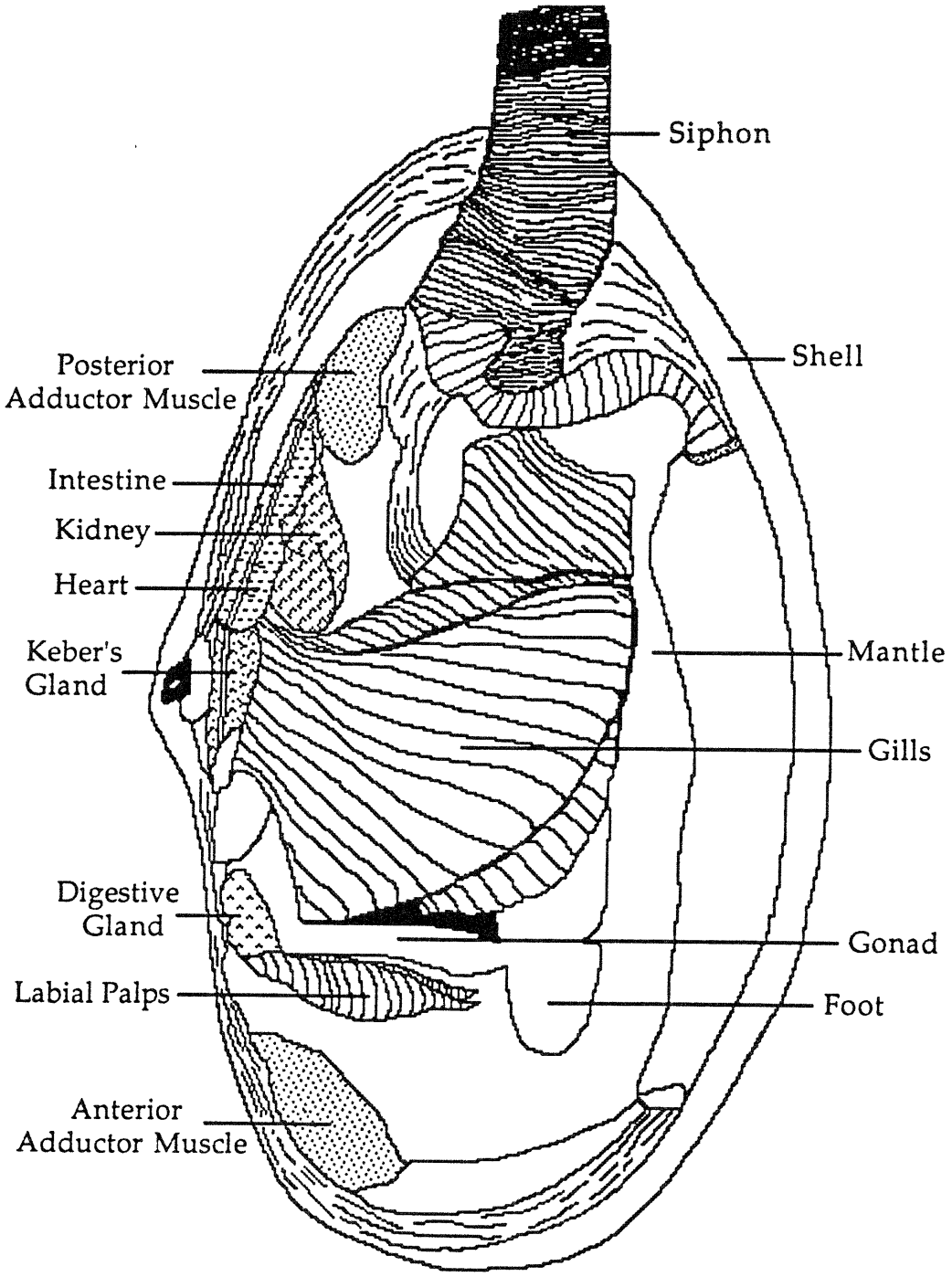

Figure 1.1. Internal anatomy of the soft shell clam, *Mya arenaria* (Potts, 1993).

distinguished histologically. The sex ratio of natural populations is approximately 1:1. The seasonal reproductive cycle is regulated by water temperature and food availability, although spawning is not completely synchronous within a population. It is generally considered that *M. arenaria* from south of Cape Cod spawn twice a year, while populations north of the Cape spawn only once (Ropes and Stickney, 1965).

The reproductive cycle of the soft shell clam is a continuous process that has been divided into five stages by light microscopic observations: (1) indifferent, (2) developing, (3) ripe, (4) spawning, and (5) spent (Ropes and Stickney, 1965). In the indifferent stage, cellular differentiation is not observed within the gonadal follicles. During the developing stage, gametes form on the basal membrane of the follicle, eventually extending into the follicle lumen. Ripe gametes fill the follicle lumen, awaiting the spawning stage, when some ripe gametes remain in the follicle lumen, while others have been spawned. In the spent stage, follicle cells form a thin layer covering the basement membrane of the follicle; unspawned gametes may be observed in the process of being resorbed.

Lipoproteins

Stored energy reserves may be mobilized during periods of reproductive activity to fuel the development of the gonad and

the production of gametes (Giese, 1959; Sastry and Blake, 1971; Vassallo, 1973; Gabbott, 1975; Pollero et al., 1979; Taylor and Venn, 1979; Joseph, 1982; Barber and Blake, 1985; Mathieu and Lubet, 1993; Napolitano and Ackman, 1993). Nutrient storage in the soft shell clam is primarily in the form of triglycerides in the digestive gland-gonad complex. Stored nutrient reserves may be mobilized from the digestive gland to provide the energy required for gamete production.

The circulatory system connects the organs and may, therefore, function as the conduit for the distribution of lipids to the appropriate tissues and cells. A potential problem is that lipids are not soluble in the aqueous environment of the hemolymph. Any potential lipid transport system must, therefore, sequester the hydrophobic lipids during transport and release them at the target tissue or cell.

Lipoproteins fulfill the requirements for a lipid transport system. One or more apolipoproteins combine with lipids containing polar groups to form a capsule which surrounds the hydrophobic lipid core (Figure 1.2). One note is that invertebrate lipoproteins lack cholesteryl esters, which are an important structural component of the depicted mammalian low density lipoprotein (LDL). The capsule structure is composed of a monolayer of phospholipid and sterol (Davis, 1991). The lipids in the core can then be transported through the hemolymph. Uptake of lipids into the

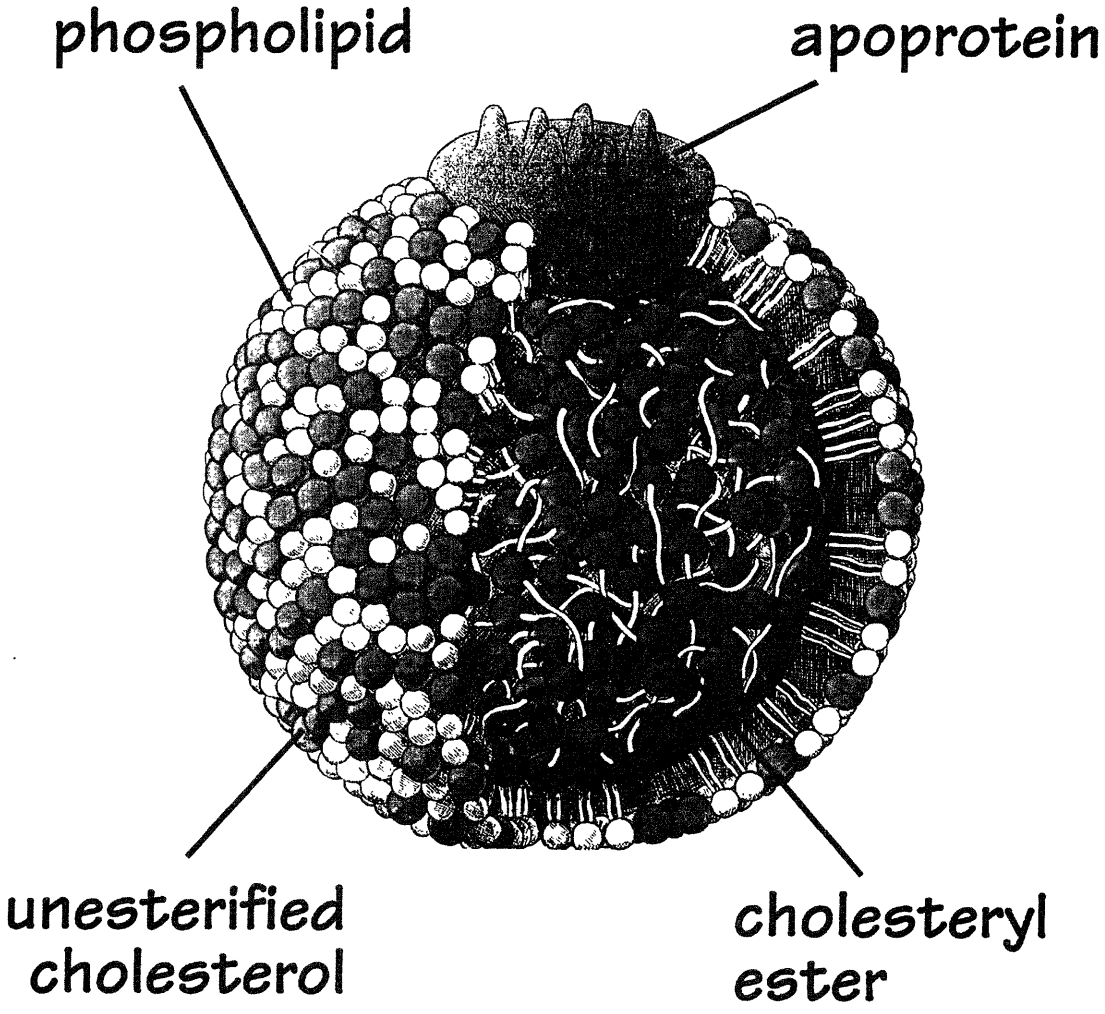

Figure 1.2. A schematic diagram (Brown and Goldstein, 1984), illustrating the structure of human low density lipoprotein (LDL). The core cholesteryl esters are sequestered from the aqueous plasma by a capsule of phospholipid, free cholesterol, and apoprotein. LDL is the main transporter of cholesterol in human blood.

cell may be mediated by a cell surface receptor that specifically recognizes the apolipoprotein, or may occur by non-specific mechanisms; either the core lipid alone or the entire lipoprotein may enter the cell (Brown and Goldstein, 1984; Schneider, 1991).

Heat shock proteins

The classic mechanism for protein synthesis involves the hypothesis of self-assembly, which proceeds from the discovery of *in vitro* renaturation of the protein ribonuclease (Anfinsen, 1973). As a result of this observation, it became clear that the linear amino acid sequence is both necessary and sufficient to produce the functional conformation of a protein. The self-assembly hypothesis suggests that the polypeptide chain interacts with itself after emerging from the ribosome to assume a folded conformation of lower free energy.

An alternative to the self-assembly hypothesis, referred to as the molecular chaperone hypothesis, has been proposed. Molecular chaperones are defined as "a family of cellular proteins which mediate the correct folding of other polypeptides, and in some cases their assembly into oligomeric structures, but which are not components of the final functional structures" (Ellis, 1990). The molecular chaperones do not provide steric information, but promote

attainment of the proper conformation by preventing improper interactions between potentially complementary surfaces (Ellis, 1987). It remains unclear whether molecular chaperones are required for the synthesis of all proteins, or only under certain circumstances.

In 1962, Ritossa observed puffs in the salivary gland chromosomes of *Drosophila* in response to high temperatures. The appearance of these puffs coincided with the synthesis of new proteins (Tissieres and Martin, 1973); these proteins have been named heat shock proteins (hsps). Heat shock proteins are a group of highly conserved proteins that are generally classified into subfamilies by molecular weight; within a subfamily, hsps of similar molecular weight appear to have similar functions. The major subfamilies of heat shock proteins are hsp 70 (known as DnaK in prokaryotes), hsp 60 (also known as chaperonin 60 and, in prokaryotes, groEL), hsp 90, low molecular weight hsps (whose molecular weight and number are highly species specific), and ubiquitin.

Some of the hsp genes are activated by the accumulation of denatured proteins in the cell (Ananthan et al., 1986). Pelham (1986) proposed a model for the function of hsps in which heat shock causes protein denaturation; hsps bind to the exposed hydrophobic surfaces, preventing aggregations. ATP hydrolysis causes a conformational change in the hsp leading to substrate release. This model describes the general function of the hsp 70 family. Hsp 60 stabilizes proteins in

the intermediate stages of folding (Martin et al., 1991) and appears to provide a surface where ATP dependent protein folding takes place. Hsp 90 binds steroid receptors and some protein kineases (Burel et al., 1992), apparently keeping them in an inactive state. The low molecular weight hsps appear to be hormonally and/or developmentally regulated (de Jong et al., 1993); their function is unclear. Ubiquitin is involved in the recognition and degradation of misfolded and abnormal proteins (Burel et al., 1992).

In addition to being required for normal cellular processes, heat shock proteins can be induced, or expressed at higher concentrations, under stressful conditions. The heat shock or stress response is universal, and inducible by heat and other stressors (Lindquist, 1986). The common mechanism of the stressors inducing this response is that they cause protein damage and denaturation; hsps bind to the damaged proteins, reducing the pool of free hsps in the cell, and leading to induced and/or increased hsp synthesis (Hightower, 1991).

The synthesis of hsps appears to be autoregulated. Regulation of hsp transcription occurs through a positively acting transcription factor, heat shock factor (hsf), which binds to the promoter heat shock element (hse). The active form of hsf is oligomeric; each monomer appears to interact with one of the variable number of nGAAn boxes which comprise the hse (Craig and Gross, 1991). Hsf is always present in the

cell, but its capability for DNA binding and high transcriptional activity are increased by heat and other inducers of the heat shock response (Sorger, 1991).

A model for the regulation of the heat shock response suggests that heat shock damages proteins, increasing the number of substrates competing for free hsps, and thereby reducing the pool of free hsps present in the cell. In the simplest model, hsf is normally bound to hsp 70, which may maintain the hsf in an inactive monomeric state (Craig and Gross, 1991; Sorger, 1991; Welch, 1992). Heat shock causes hsp 70 to dissociate from hsf in order to bind to the damaged proteins. Alternate models include the possibility that the inactive hsf is complexed to another protein cofactor (not hsp 70); the cofactor is denatured under heat shock conditions, releasing the hsf (Welch, 1992). A third possibility is that the heat shock directly causes a conformational change in the hsf (Sorger, 1991). Regardless of the nature of the protein involved in maintaining the hsf in an inactive state, the active hsf binds to the hse and causes the transcriptional activation of the hsp genes, in a process that may involve phosphorylation (Sorger, 1991). Ultimately, all of these processes result in the synthesis of heat shock proteins, leading to increased hsp concentration in the cell.

Contaminant and disease effects

Contaminant effects on physiological processes, including reproduction, are commonly observed in marine bivalve molluscs. The most commonly seen effects are the atresia and resorption of gametes, reallocation of energy reserves between reproduction and metabolic demands, and histological abnormalities, especially of the gill tissue. These effects may involve both lipoproteins and heat shock proteins. Gamete atresia and deformed gill tissue may both involve the denaturation and/or degradation of cellular proteins; as a result, heat shock protein expression could be altered in these tissues in response to sublethal contaminant exposure. In addition, gamete resorption and differential allocation of energy stores suggest that lipoproteins, as lipid transport vehicles, are also affected by contaminant exposure. The synthesis and composition of lipoproteins may also be affected by contaminant exposure (Borlakoglu et al., 1990b; Lee, 1993).

The relationship between lipoproteins and contaminants may also involve a role for lipoproteins in the transport of hydrophobic xenobiotic compounds. Lipophilic organic contaminants, including polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are associated with lipoproteins in birds (Borlakoglu et al., 1989; Borlakoglu et al., 1990a; Borlakoglu et al., 1990b), fish (Denison et al., 1981; Denison and Yarbrough, 1985), and mammals (Souès et al., 1989). While the lipophilicity of these contaminants would

suggest that they are associated with the lipid portion of the lipoprotein, in some studies the contaminants appear to be primarily associated with the apoprotein (Denison and Yarbrough, 1985; Borlakoglu et al., 1989; Borlakoglu et al., 1990a). As a result of non-specific, low affinity binding to protein, the xenobiotics are primarily associated with the high density lipoproteins (Borlakoglu et al., 1989; Maillou and Nimmo, 1993), which have higher protein concentrations than LDL or VLDL. In contrast, however, contaminants have also been found in the lipid fraction of lipoproteins (Lee, 1993) and in association with less dense lipoproteins (Borlakoglu et al., 1989; Souès et al., 1989)

In addition to contaminant effects, natural populations of bivalves are also subject to diseases. One disease that impacts the soft shell clam is hematopoietic neoplasia (Hn). Hn is a proliferative disease of the hemocytes of the soft shell clam that was first identified in clams from oil spill sites in Maine (Yevich and Barszcz, 1977). The disease appears to be caused by a retrovirus (Oprandy et al., 1981) and is not strictly correlated with contaminant exposure. Clams with Hn may undergo remission, retain a stable chronic infection level, or progress to greater severity levels and ultimately die (Cooper et al., 1982; Brousseau and Baglivo, 1991).

Diseased clams have an altered hemocyte cytoskeletal structure (Moore et al., 1992) and are unable to phagocytize

foreign particles (Beckman et al., 1992). Reinisch et al. (1984) suggested that Hn hemocytes have higher lipid concentrations than normal hemocytes. Hn clams are in poorer physiological condition than normal clams (Leavitt et al., 1990). Poorer physiological condition and altered hemocyte lipid levels suggest that lipid transport processes may be affected in Hn clams. Additionally, heat shock proteins may associate with components of the cytoskeleton (Lin, 1987; Welch and Mizzen, 1988); expression levels and intracellular location of members of the heat shock protein family, therefore, may be altered in Hn clams.

In order to fully investigate the role of lipoproteins and heat shock proteins in a response to disease or sublethal contaminant exposure, however, the function of these proteins in normal organisms must be examined. Lipoproteins and heat shock proteins were studied in the soft shell clam *Mya arenaria* to determine the role of these proteins in physiological processes, with some attention to alterations in their function in clams exposed to lipophilic organic contaminants and the disease hematopoietic neoplasia. Lipid transport and lipoproteins are discussed in chapters 2 and 3, while heat shock protein expression is described in chapters 4, 5, and 6.

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Chapter 2. Lipid transport in the soft shell clam

Background

Marine bivalve molluscs utilize lipids as structural membrane components and as energy storage compounds in both larval and adult life stages. Lipids may be derived from endogenous (De Moreno et al., 1980; Zandee et al., 1980; Kluytmans et al., 1985) or exogenous (Langdon and Waldock, 1981; Napolitano and Ackman, 1992; Robinson, 1992) sources. Exogenously derived fatty acids may be modified by esterification (Pollero et al., 1985), elongation (Langdon and Waldock, 1981; Pollero et al., 1983), and oxidation (Pollero et al., 1983). Once lipids are synthesized or ingested and absorbed, they are available to the various tissues for utilization or storage. The process by which lipids are distributed from the source tissues via the hemolymph to other tissues for metabolism and/or storage is termed lipid transport.

Absorbed nutrients in excess of energy required for maintenance metabolism can be stored. Energy may be stored for use during periods of reduced nutrient uptake or increased energetic requirements (Lawrence, 1986). During starvation and periods of low food availability, reserve lipids are rapidly metabolized (Riley 1976; Abad et al., 1995). Many published studies suggest that molluscan energy stores may

also be mobilized during periods of reproductive activity to fuel the development of the gonad and the production of gametes (Giese, 1959; Sastry and Blake, 1971; Vassallo, 1973; Gabbott, 1975; Pollero et al., 1979; Taylor and Venn, 1979; Joseph, 1982; Barber and Blake, 1985; Mathieu and Lubet, 1993; Napolitano and Ackman, 1993). In some species of scallops, however, stored reserves do not seem to be important during gonad development (Ansell, 1974; Thompson, 1977). In other scallop species, the initiation of gametogenesis may be dependent on stored reserves, while the final maturation of gametes relies on the availability of food resources at some time during gonad development (Robinson et al., 1981). It has also been suggested that at least some of the nutrients used for gamete production are recycled, coming from the resorption of the products of oocyte atresia (Coe and Turner, 1938; Le Pennec et al., 1991).

The primary sites (and biochemical forms) of nutrient storage in bivalves are the digestive gland (lipid) and, primarily in scallops, the adductor muscle (glycogen). Several lines of evidence suggest that nutrient reserves are mobilized from the digestive gland to the gonad during gonadal maturation and gamete production. While lipid is mobilized from the digestive gland throughout the year, the rate of loss increases during oogenesis (Barber and Blake, 1985). Specific fatty acids (16:1 n-7 and 18:1 n-9) may be tracers of lipid transport between the digestive gland and the developing gonad

(Napolitano and Ackman, 1993). Additionally, if gonadal growth is suppressed, nutrients accumulate in the digestive gland (Sastry and Blake, 1971). As depicted in Figure 2.1, neuroendocrine control of gametogenesis and glycogen metabolism, by gonial mitosis-stimulating factor and glycogen-mobilization hormone respectively (Mathieu et al., 1991), may be correlated by the action of hypothetical feedback loops (Mathieu and Lubet, 1993).

Lipid transport in marine and freshwater molluscs has also been traced from the digestive gland to other tissues, including gonad, by the transfer of label from administered ^{14}C lipids (Vassallo, 1973; Allen, 1974; Gabbott, 1975; Allen, 1977; De Moreno et al., 1977; Allen and Conley, 1982; Huca et al., 1984; Barber and Blake, 1985; Pollero et al., 1985; Pollero, 1987; Pollero and Heras, 1989; Heras and Pollero, 1990; Pollero et al., 1992; Garin and Pollero, 1995). While these studies showed that radiolabelled lipid was transferred from the digestive gland to the gonad, the process by which the lipid was transported in the hemolymph was not investigated. The experiment described in this chapter was designed to investigate lipid transport processes in the soft shell clam *Mya arenaria*, including a consideration of alterations in lipid transport function associated with diseased hemocytes.

Materials and Methods

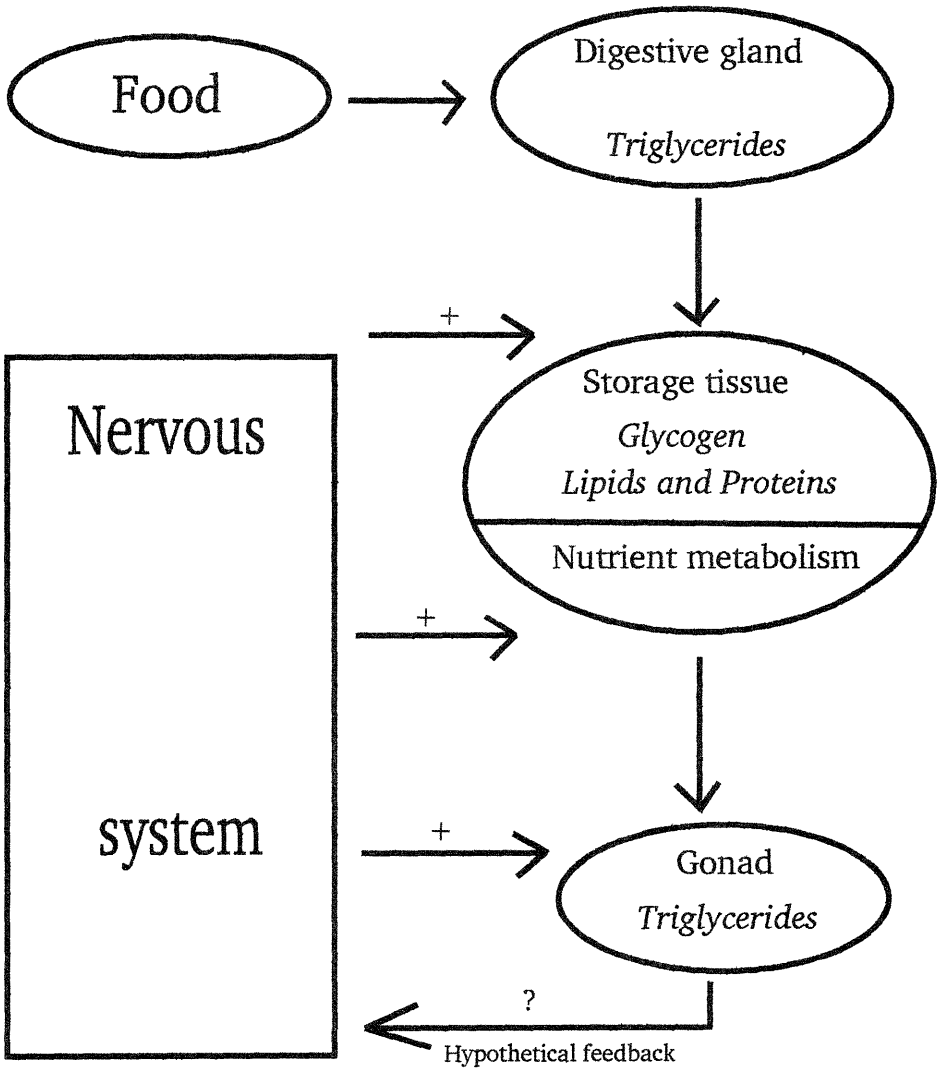


Figure 2.1. A model, adapted from Mathieu and Lubet (1993), depicting the potential sites of neuroendocrine regulation of energy and resource mobilization in organs of bivalve molluscs.

Clams

Soft shell clams were collected by hand digging from the intertidal zone in Little Buttermilk Bay, Bourne, MA. The clams were brought back to the lab and held for up to 6 months in a flow-through seawater system (30 -31 $^{0}/_{00}$) at ambient temperature until they were used in the experiment. The clams were not fed while they were being held (other than phytoplankton which might have been present in the unfiltered seawater), or at any time during the experiment. A total of 49 clams were injected with the radiolabel. The mean length of clams used was 56.43 ± 3.24 mm (range 50.88 - 63.23 mm) and the mean live weight was 26.39 ± 4.49 g (range 17.86 - 36.82 g); live weight is the total weight of a live clam, including shell, tissue, and extracellular fluid. Neither the sex nor the reproductive status of the individual clams was determined, as this information can only be obtained by destructive sampling. Most clams in LBB at this time of year (December - January), however, are indifferent (48 %) or in the early stages of gamete development (47 %) (Potts, 1993; results of the experiments presented in chapters 5 and 6).

Radiotracer injection experiment

Each clam was injected subcutaneously into the siphon with 5 µCi (50 µL) of [^{14}C-carboxyl] triolein (Du Pont NEN NEC-674) with a specific activity of 112 mCi/mmol. The triolein was dissolved in a solvent carrier of 1:1 toluene:ethanol, and

stored at -20 °C under a nitrogen atmosphere. Under these storage conditions, NEN determined that the rate of decomposition of the labeled triolein is less than 1 % for four months from the date of purification. All of the lot of label was used within 2 months of receipt of the shipment. One sample of the injected dose of the compound was retained as the injected dose and treated in the same way as the tissue samples. This sample was used to determine the disintegrations per minute (dpm) of the injected dose; this information was used to calculate the percent recovery of the injected dose and to convert the dpm data to pmol triolein.

Injected clams were held in an aerated static seawater system for 0.25, 0.5, 1, 2, 3, 4, 4.5, 6, 8, 12, 16, 20, 24, and 48 hours post injection. The seawater temperature was 2-6°C, which approximated the ambient water temperature during the experimental period and is the temperature at which rapid gametogenesis occurs in local populations. At the time of sacrifice, hemolymph was collected and the following tissues were dissected out: gonad, digestive gland (including the crystalline style), foot, gill, adductor muscles, siphon, and "mantle" (all remaining soft tissues). The tissues were homogenized and either the entire tissue or a subsample was placed into a 20 mL polyethylene scintillation vial. The hemolymph was centrifuged at 4,800 x g for 10 min and plasma was collected. The cell pellet was washed three times with 1 mL of 0.45 µm filtered seawater. The homogenized tissues were

dissolved in 2 mL ScintiGest Tissue Solubilizer (Fisher SX10-100) in a 60°C drying oven. The tissue solutions were bleached with the addition of 250 µL 30% H_2O_2 to each vial in order to correct for differences in quenching between the tissues; 100 µL acetic acid was added to each vial to correct for the chemiluminescence which can be associated with the presence of hydrogen peroxide or tissue solubilizers. Prior to counting, 10 mL of ScintiVerse BOA (Fisher BP454-4) liquid scintillation cocktail was added to each vial.

Radioactivity of the samples was measured on a Beckman LS1801 Liquid Scintillation Counter. A ^{14}C quench curve was used to determine counting efficiency, which was used to convert the measured counts per minute (cpm) to dpm (dpm = cpm/counting efficiency). An assumption was made that the quench curve was equally appropriate for all of the tissues. The sample counts were repeated if the random coincidence monitor (a measure of chemiluminescence) was above ≈20 % or if the H number (a method of measuring quench) was outside of the limits between which the quench curve is valid; counts were repeated until the chemiluminescence or quenching parameters fell within the appropriate limits. Although the label may not have remained in the form of the injected triolein over the course of the experiment, dpm values were converted to pmol triolein equivalents to provide a biologically meaningful unit for comparisons.

The amount (pmol triolein) and concentration (pmol

triolein/ g soft tissue wet weight) of label were determined for each sample. All values were corrected for recovery of the injected dose; the values reported have been adjusted to 100 % recovery by dividing the label measured in the sample by the percent recovery of the injected dose. Percent recovery of the injected dose varied among clams, but was not correlated to elapsed time (mean = 17.8 %, range = 0.4 - 55.5 %). A separate analysis of the subset of clams with ≥ 20 % recovery of the injected dose did not show any substantial differences from the analysis of the entire data set; for the analyses reported in this chapter, data from all of the injected clams were used. Data from replicate clams with the same elapsed time were averaged, producing a mean and standard error for the time point. Additionally, the ratio of the plasma to hemocyte triolein concentration was determined.

Hemolymph volume determination

Hemolymph (plasma + hemocytes) volume data for *Mya arenaria* were not available, so total hemolymph volume was estimated from literature values for other bivalve species: *Ostrea edulis* (George et al., 1978), *Crassostrea gigas* (George et al., 1978), *Anodonta cygnea* (Potts, 1954), *Mytilus californianus* (Martin et al., 1958; Thompson et al., 1978), and *Margaritana margaritifera* (Martin et al., 1958). In each of the published studies, hemolymph volume, which is generally considered to be synonymous with extracellular fluid volume,

was calculated from the dilution of an injected inulin tracer; the mean hemolymph volume (in mL) in these studies was calculated to be 51.1 % of the soft tissue wet weight (range 46 - 55 %). Hemolymph volume (in mL) is generally reported as a percent of the wet soft tissue weight of the animal, although Robinson and Ryan (1988) reported that a regression of hemolymph volume to shell length was more consistent, since the hemolymph percent of wet weight varied over the reproductive cycle (presumably with the change in wet weight of the gonad, since hemolymph volume was constant). The calculated regression for *Mercenaria mercenaria* was not used in this study because *M. arenaria* and *M. mercenaria* have such different body shapes.

Hemolymph volume collected for radioactivity quantification ranged from 0.8 - 1.1 mL per clam, a subsample of the total hemolymph volume. Because it was impractical to drain all of the hemolymph from each clam, counts on the subsample of hemolymph collected were adjusted to reflect total hemolymph volume. Hemolymph volume (in mL) was calculated as 51.1 % of the total wet weight of the soft tissues; hemolymph mass (in g) was determined from hemolymph volume by using the hemolymph density, which was determined to be 1.05 ± 0.003 g/mL. All of the plasma and hemocyte data reported in this chapter have been corrected to reflect the total hemolymph volume of the individual clams.

Hematopoietic neoplasia assay

Hematopoietic neoplasia (Hn) stage was determined for 45 of the 49 injected clams. At the time of sacrifice, 0.5 mL of hemolymph was collected from the adductor muscle sinus of each clam. Hn cells were stained by the immunoperoxidase method (Smolowitz and Reinisch, 1986) using an anti-Hn monoclonal antibody (Miosky et al., 1989). The slides were examined under a light microscope and the percent of Hn cells was determined.

Results

Figures 2.2 and 2.3 depict the time course of label distribution throughout the clams. The total measured label in the various tissues reflects both the concentration of label in the tissue and the mass of the tissue (ie. content = concentration x mass). Most of the label is found in siphon and mantle, the largest tissues. To correct for the difference in mass among the tissues, the data were replotted in Figures 2.4 and 2.5 using concentration units (pmol triolein per g wet weight). One important result is that within the first hour post injection much of the label has already left the siphon. Over the duration of the experiment, the trend is for decreasing triolein in the siphon and mantle while triolein increases in the gill and the adductor muscle, although the error bars around each point are large. Variability among individual clams masks any other trends.

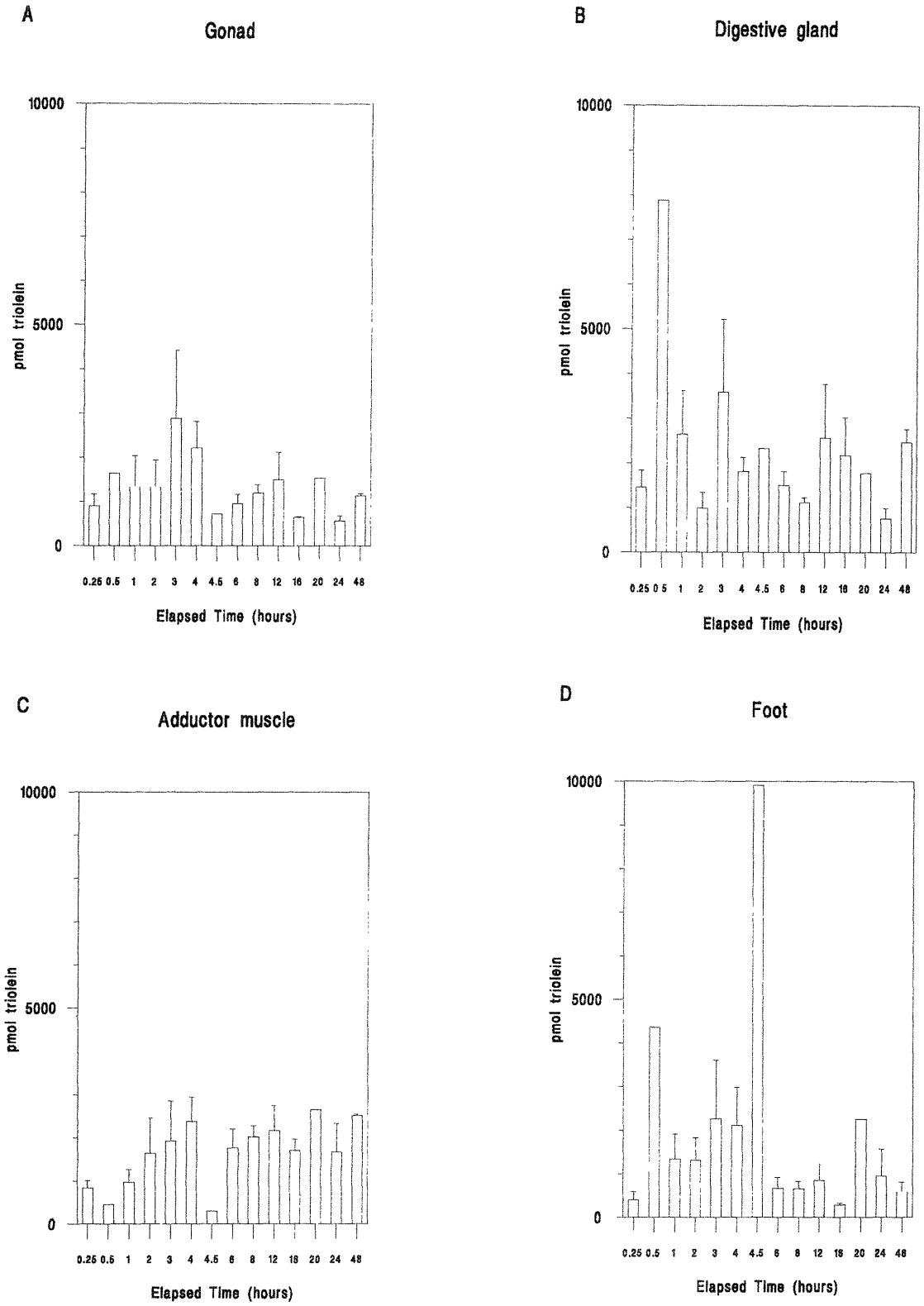
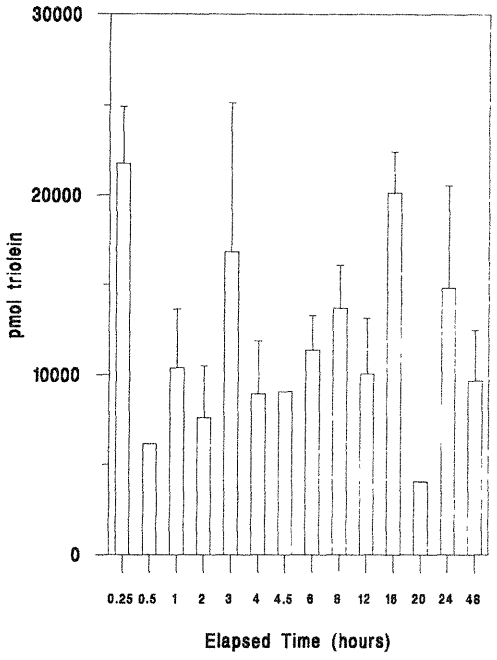


Figure 2.2. Total label (pmol triolein) in individual tissues of the soft shell clam. Tissues shown are: A-gonad, B-digestive gland, C-adductor muscle, D-foot, E-siphon, F-mantle, G-gill, H-plasma, I-hemocytes, and J-total hemolymph. Values are mean ± standard error at each time point.

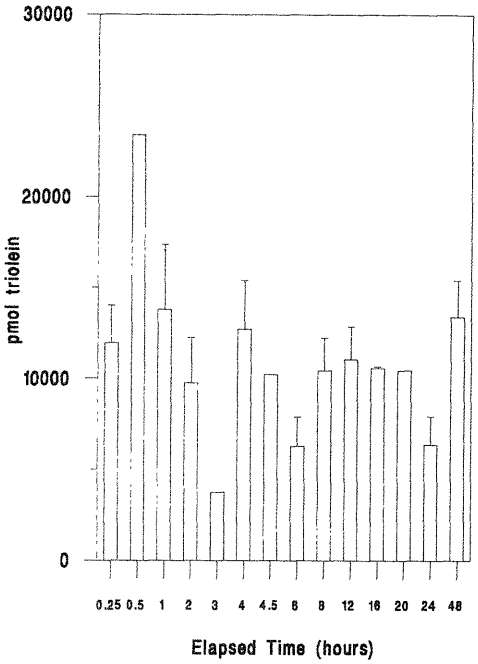
E

Siphon



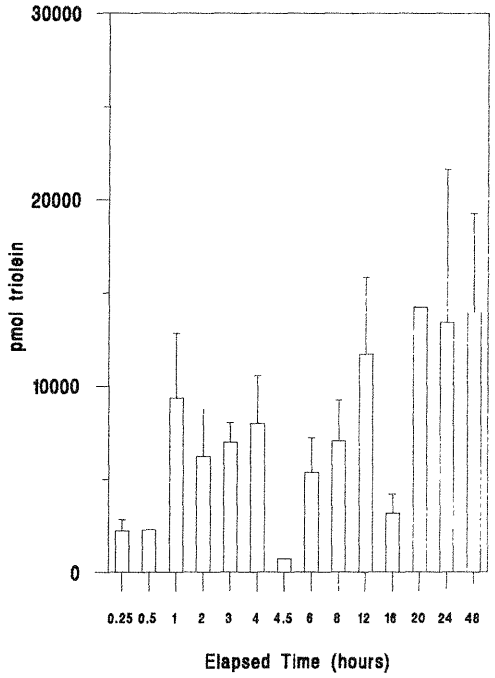
F

Mantle

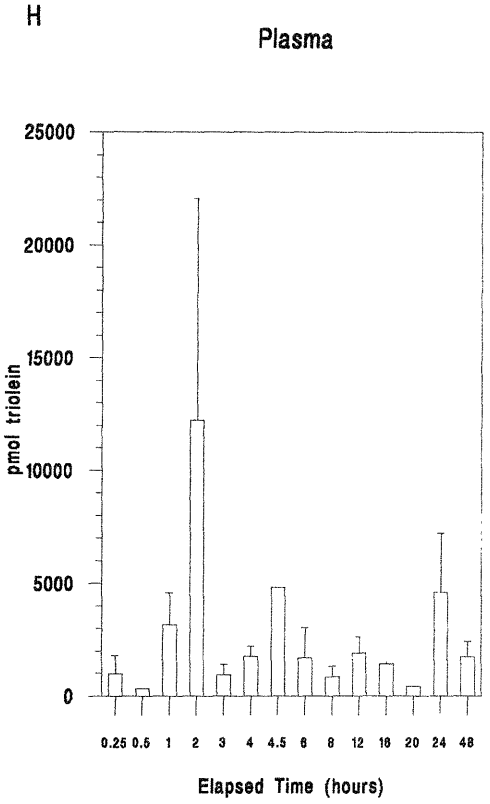


G

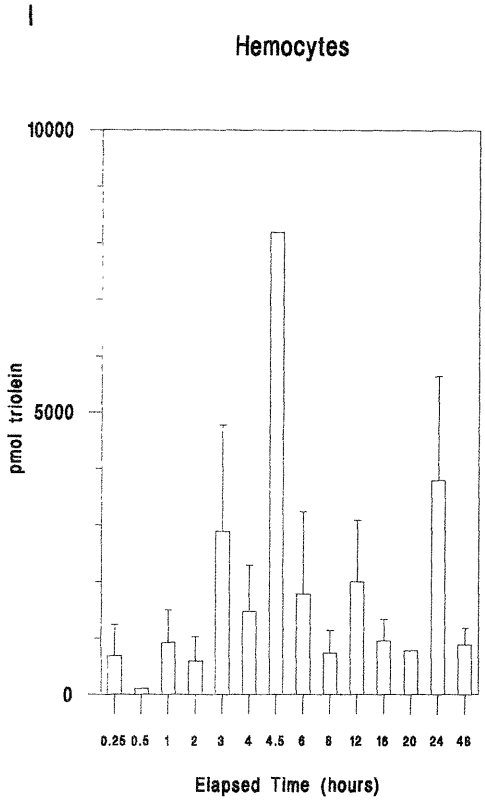
Gill



H


Plasma
25000
20000
15000
10000
5000
0
pmol triolein
0.25 0.5 1 2 3 4 4.5 6 8 12 16 20 24 48
Elapsed Time (hours)

I


Hemocytes
10000
5000
0
pmol triolein
0.25 0.5 1 2 3 4 4.5 6 8 12 16 20 24 48
Elapsed Time (hours)

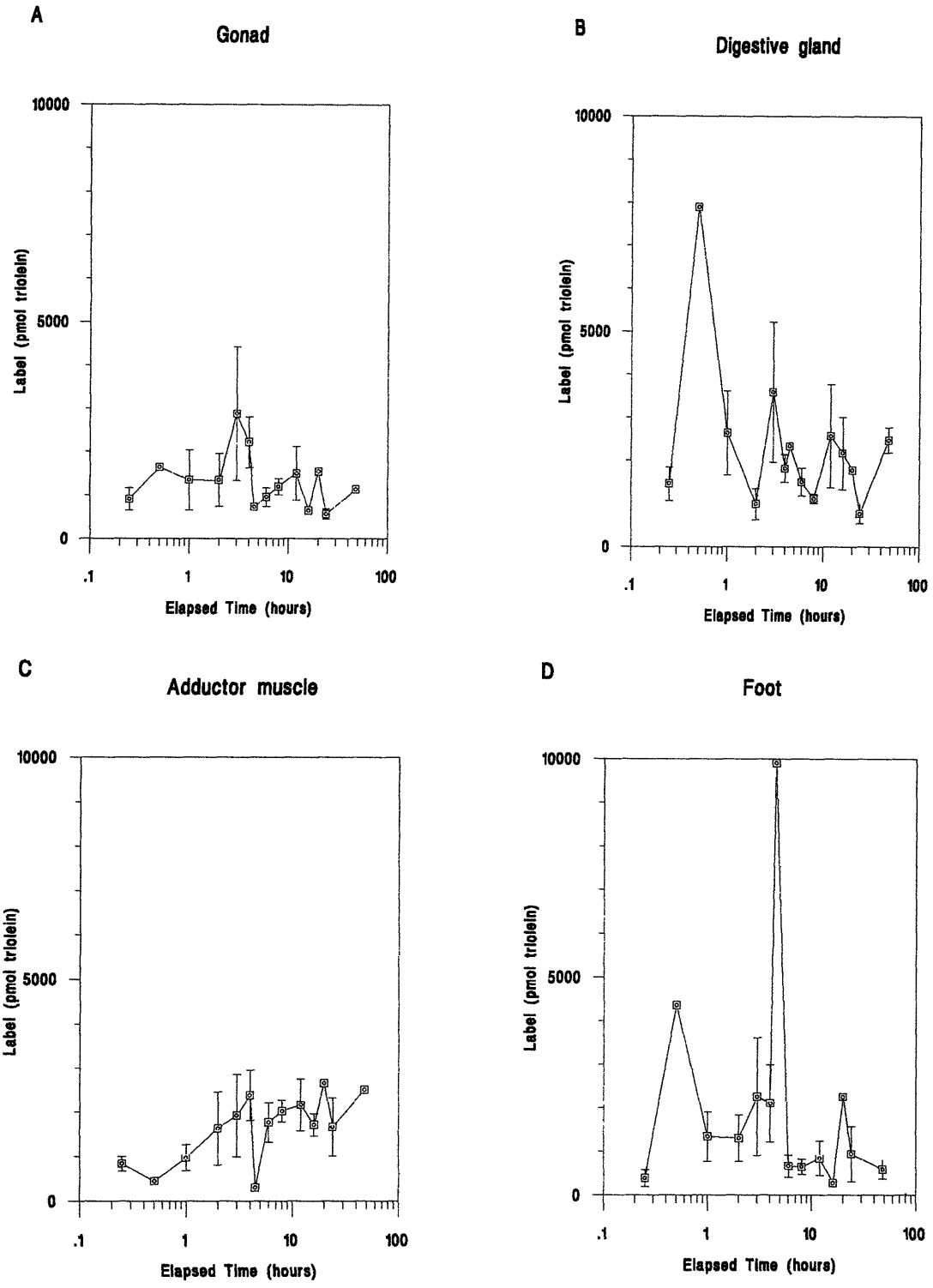
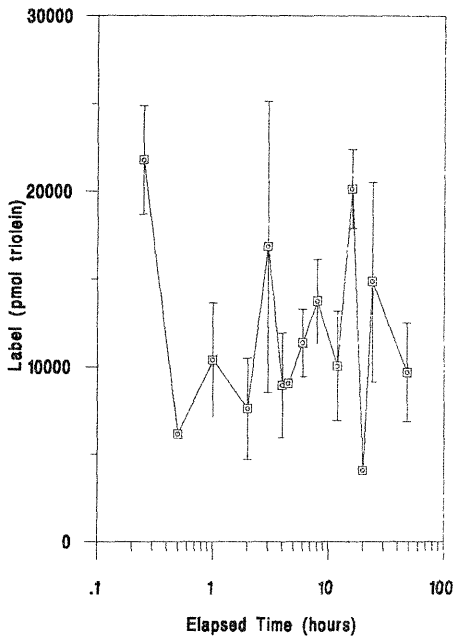
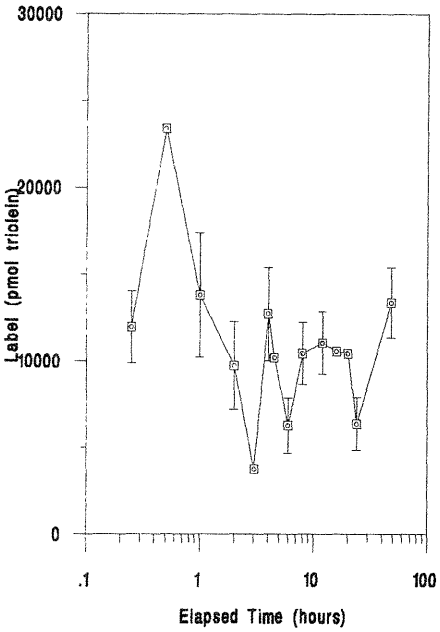


Figure 2.3. Time course of total label (pmol triolein) distribution in individual tissues of the soft shell clam. Tissues shown are: A-gonad, B-digestive gland, C-adductor muscle, D-foot, E-siphon, F-mantle, G-gill, H-plasma, I-hemocytes, and J-total hemolymph. Values are mean ± standard error at each time point.

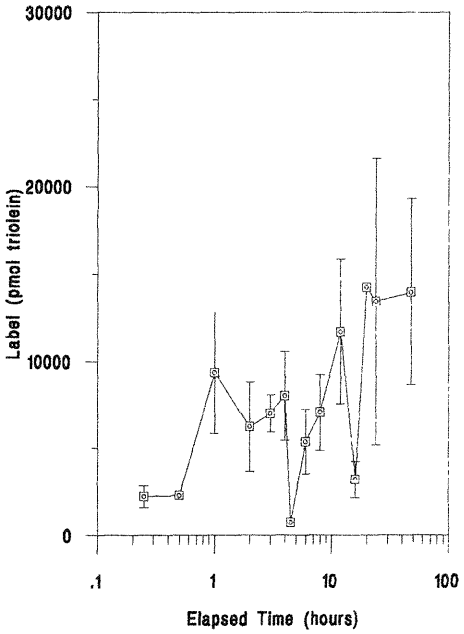
E


Siphon
30000
20000
10000
0
Label (pmol triolein)
.1
1
10
100
Elapsed Time (hours)

F

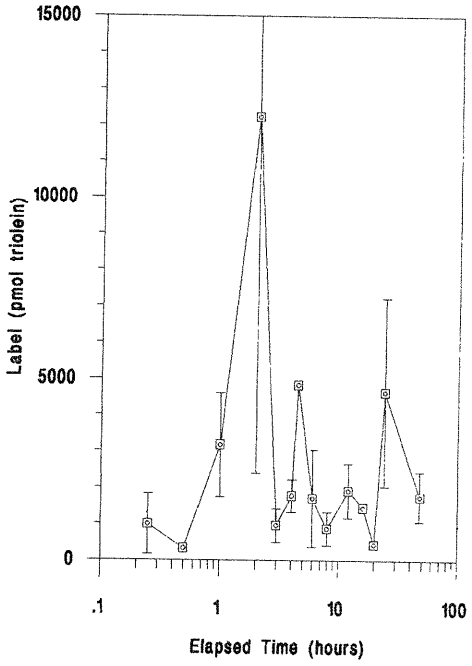

Mantle
30000
20000
10000
0
Label (pmol triolein)
.1
1
10
100
Elapsed Time (hours)

G


Gill
30000
20000
10000
0
Label (pmol triolein)
.1
1
10
100
Elapsed Time (hours)

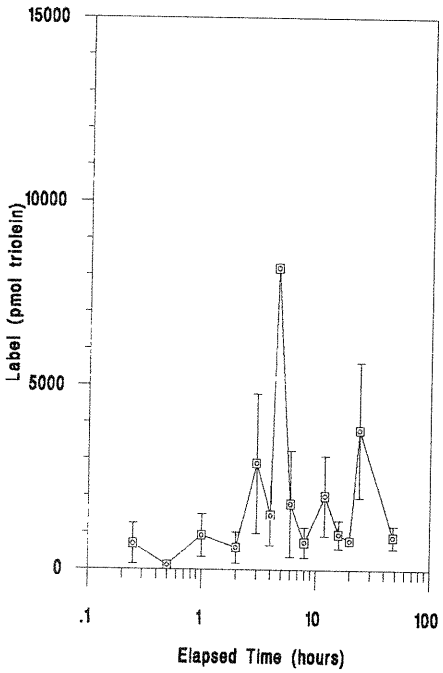
H

Plasma



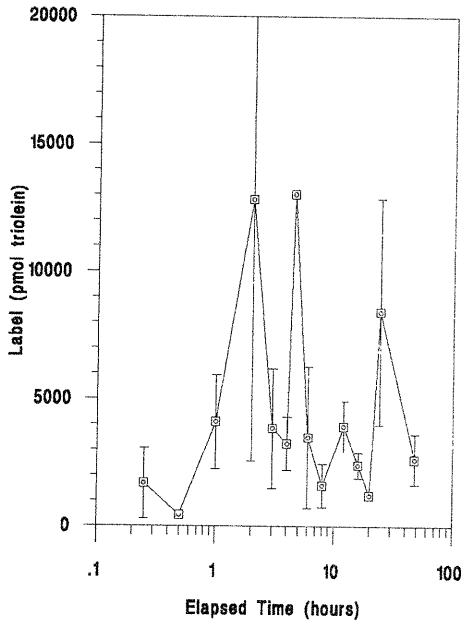
I

Hemocytes



J

Hemolymph



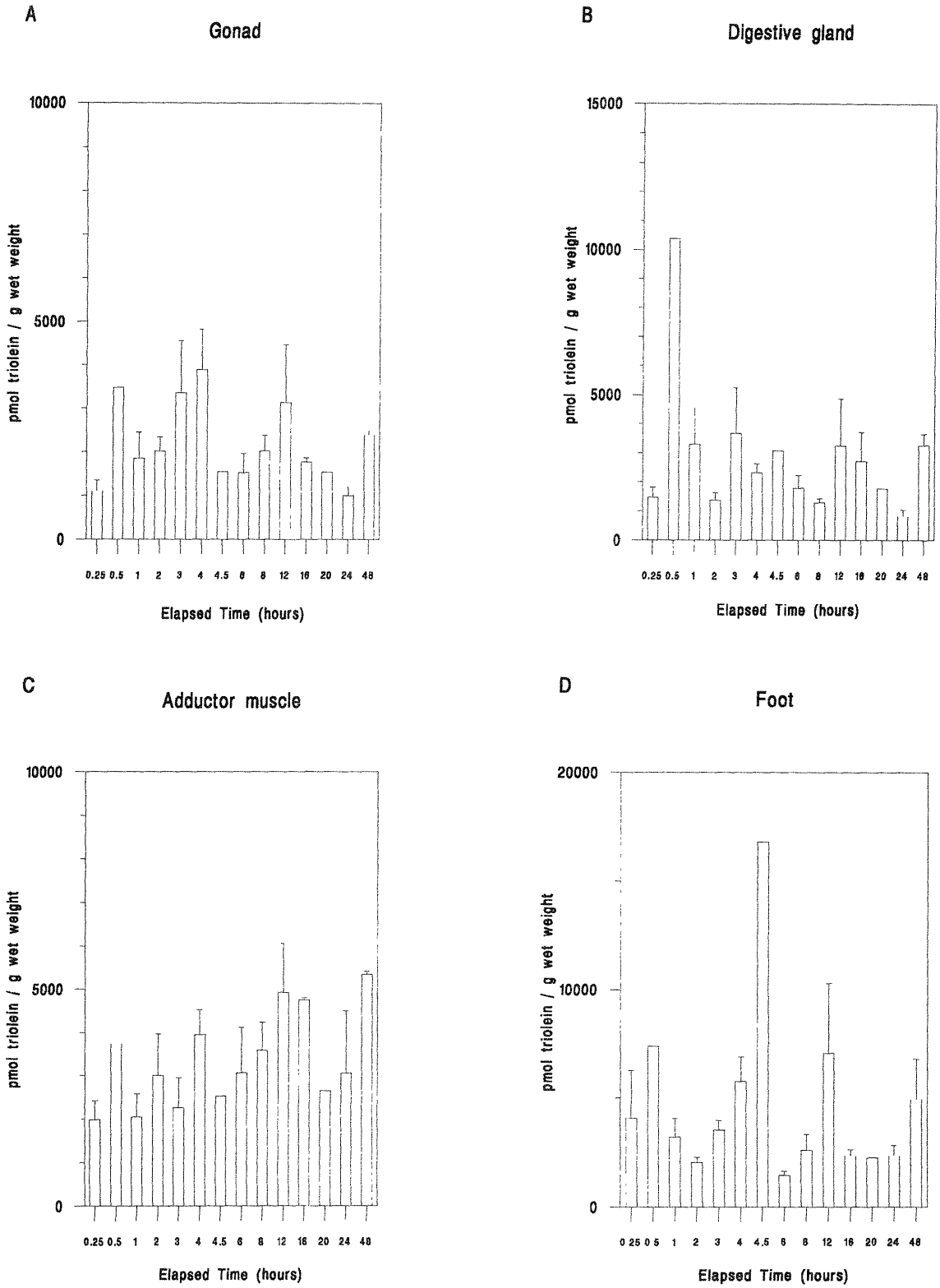
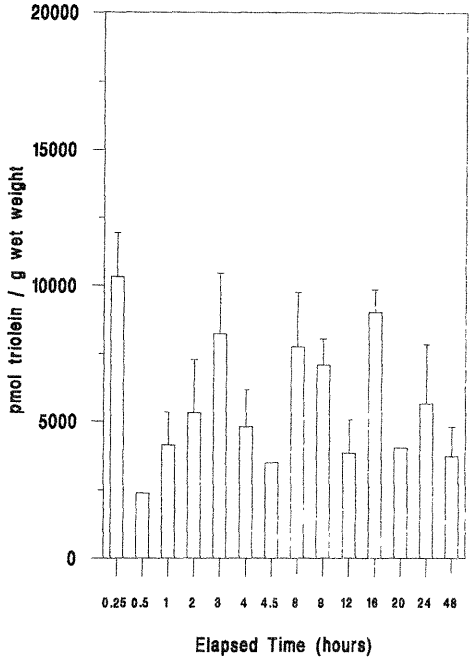


Figure 2.4. Label concentration (pmol triolein per g wet weight) in individual tissues of the soft shell clam. Concentrations for plasma, hemocytes, and hemolymph are based on the wet weight of the total hemolymph. Tissues shown are: A-gonad, B-digestive gland, C-adductor muscle, D-foot, E-siphon, F-mantle, G-gill, H-plasma, I-hemocytes, and J-total hemolymph. Values are mean ± standard error at each time point.

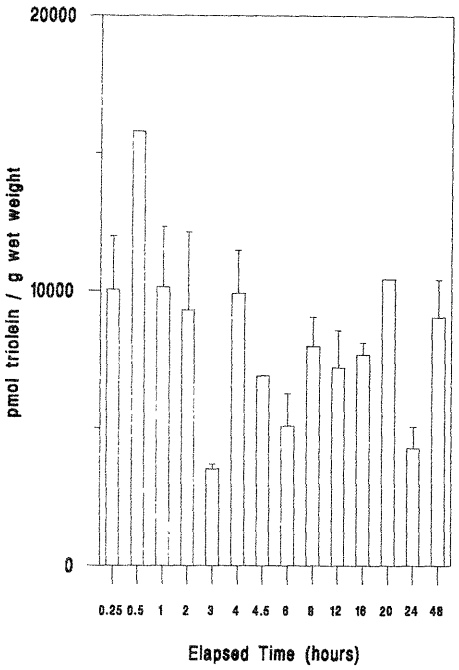
E

Siphon



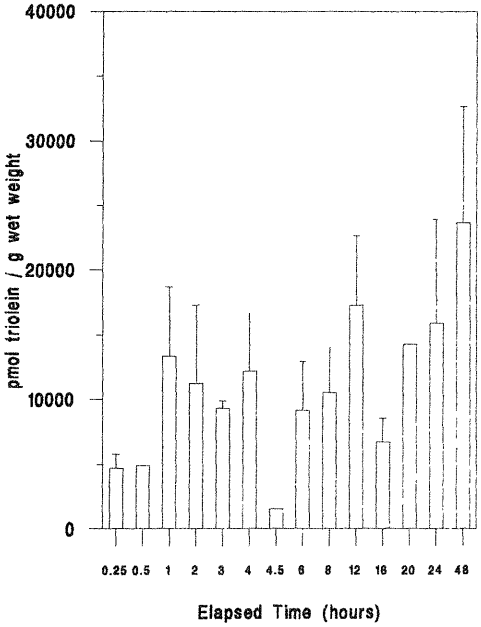
F

Mantle

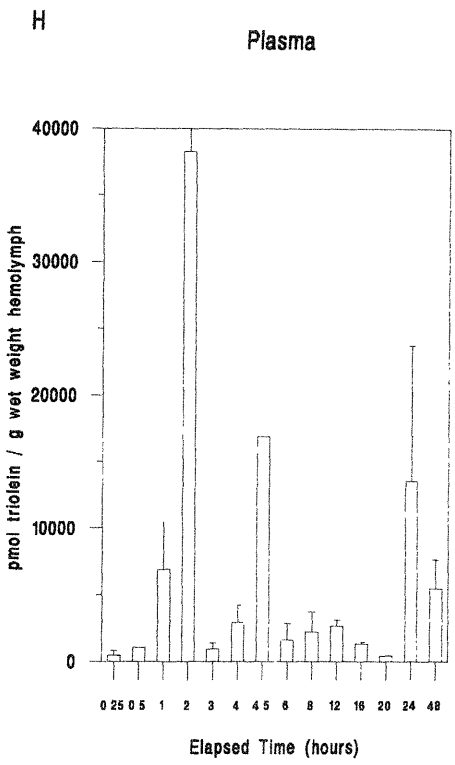


G

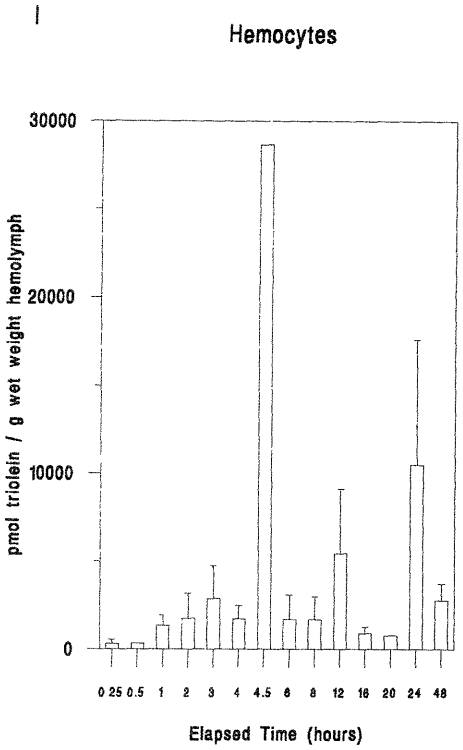
Gill



H


Plasma
40000
30000
20000
10000
0
pmol triolein / g wet weight hemolymph
0 25 0 5 1 2 3 4 4 5 6 8 12 16 20 24 48
Elapsed Time (hours)

I


Hemocytes
30000
20000
10000
0
pmol triolein / g wet weight hemolymph
0 25 0.5 1 2 3 4 4.5 6 8 12 16 20 24 48
Elapsed Time (hours)

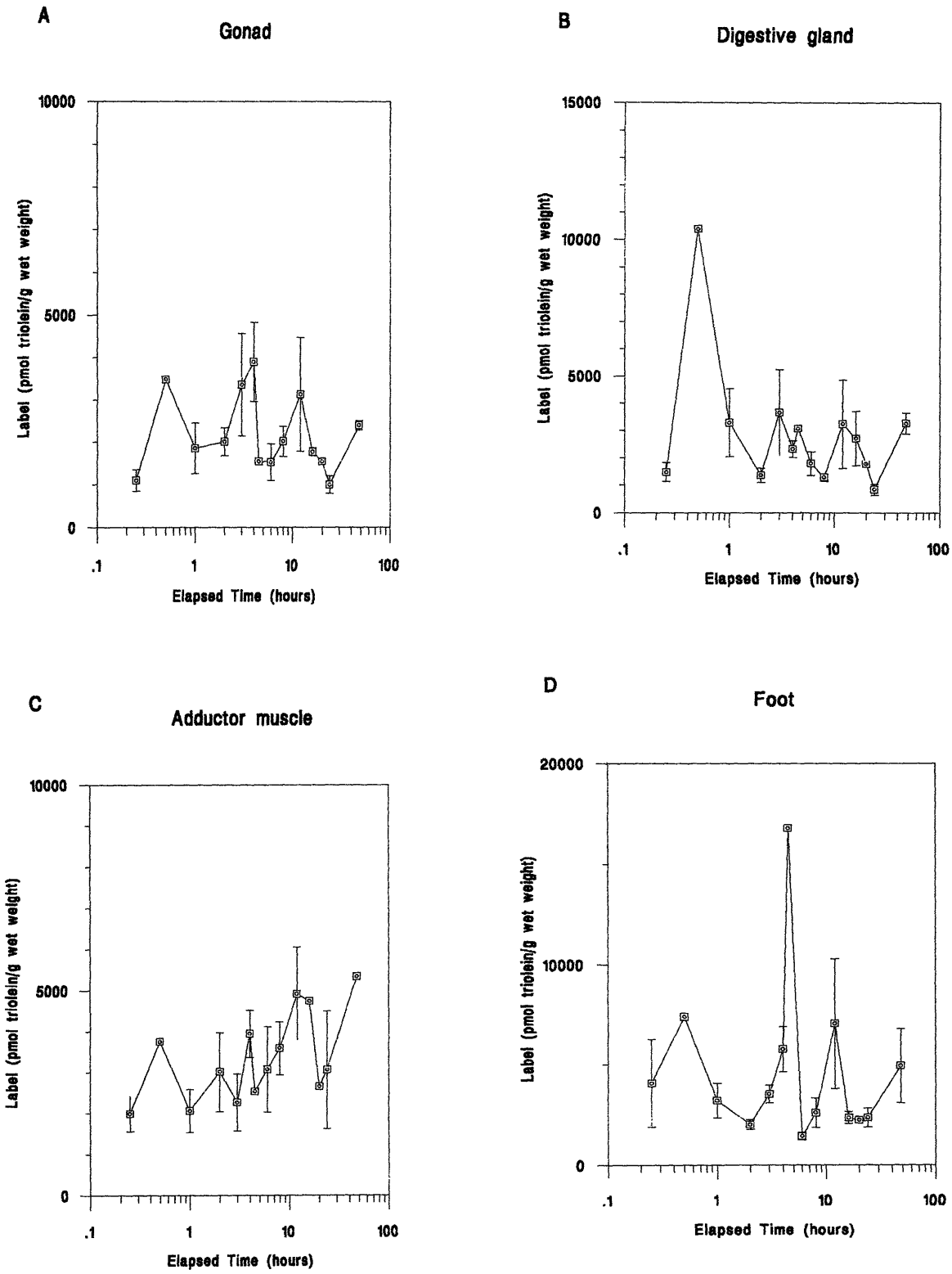
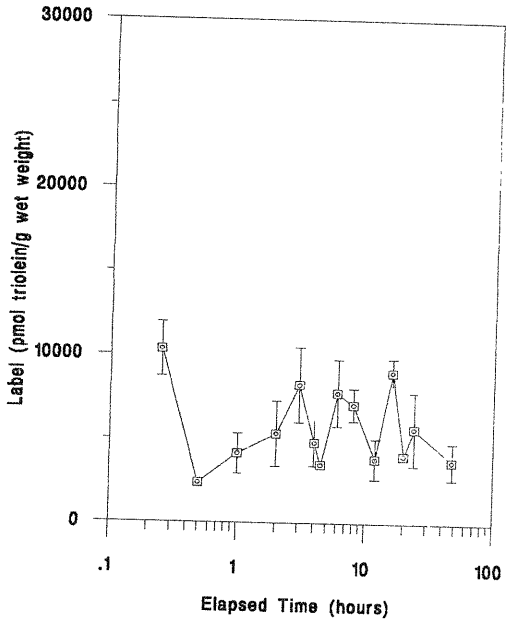
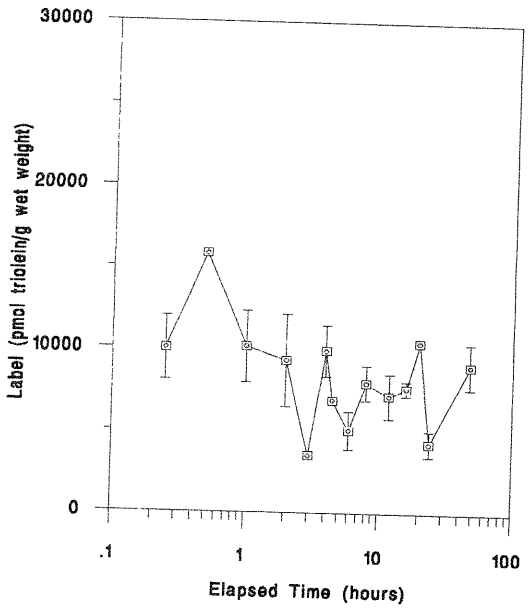


Figure 2.5. Time course of label concentration (pmol triolein per g wet weight) in individual tissues of the soft shell clam. Concentrations for plasma, hemocytes, and hemolymph are based on the wet weight of the total hemolymph. Tissues shown are: A-gonad, B-digestive gland, C-adductor muscle, D-foot, E-siphon, F-mantle, G-gill, H-plasma, I-hemocytes, and J-total hemolymph. Values are mean ± standard error at each time point.

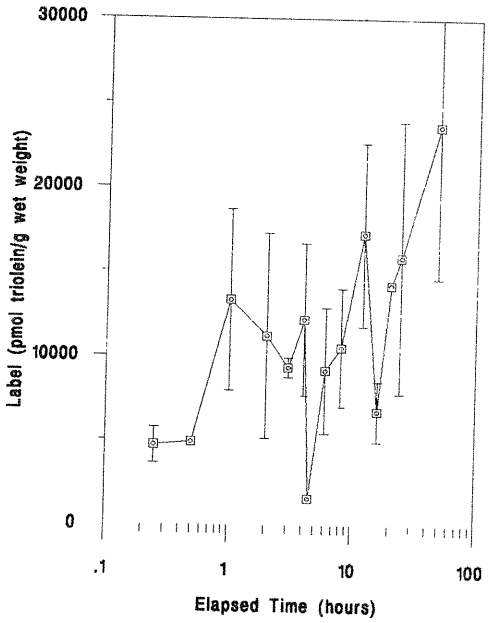
E


Siphon
30000
20000
10000
0
Label (pmol triolein/g wet weight)
.1
1
10
100
Elapsed Time (hours)

F

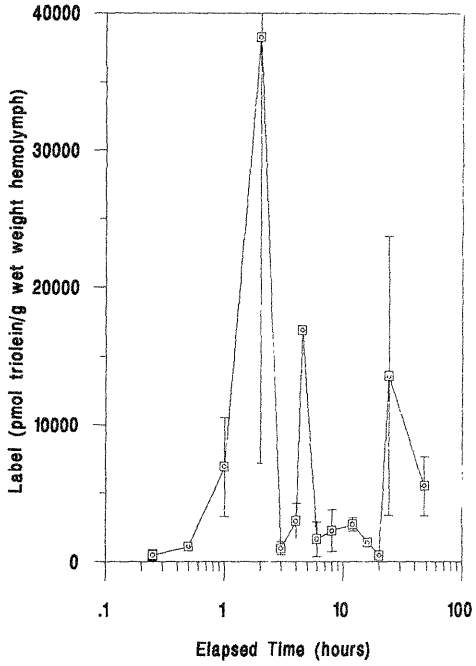

Mantle
30000
20000
10000
0
Label (pmol triolein/g wet weight)
.1
1
10
100
Elapsed Time (hours)

G


Gill
30000
20000
10000
0
Label (pmol triolein/g wet weight)
.1
1
10
100
Elapsed Time (hours)

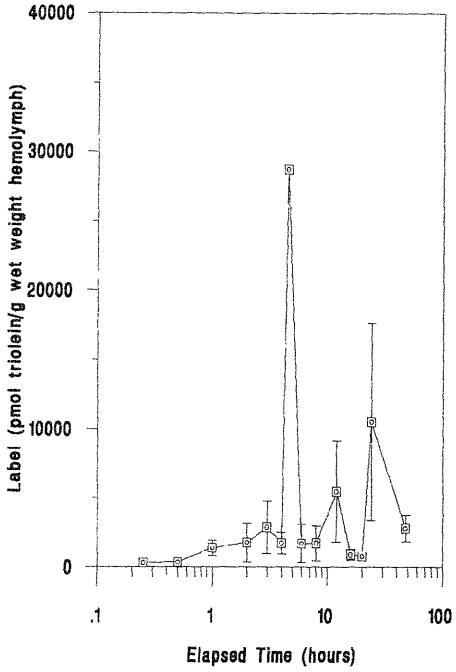
H

Plasma



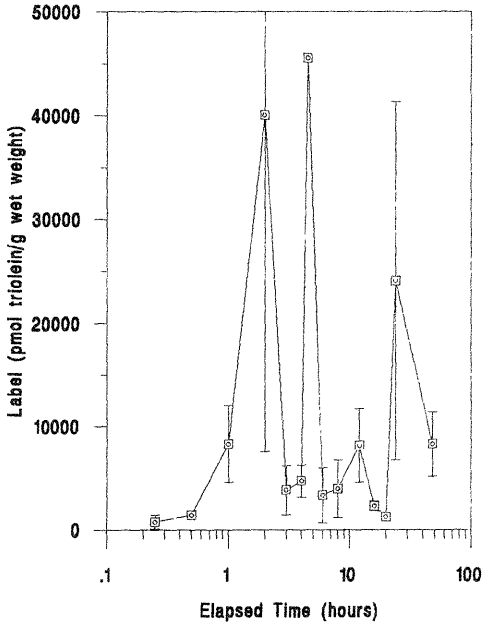
I

Hemocytes



J

Hemolymph



In order to estimate the relative importance of the two hemolymph components, plasma and hemocytes, in the transport of injected triolein, the ratio of the label concentration in plasma to cells was calculated (Figure 2.6). The plasma to cell ratio is significantly higher ($p = 0.04$) within the first 4 hours (mean = 4.5) than after 4.5 hours (mean = 1.6). Early in the time course of the experiment, within 4 hours of label administration, significantly more of the lipid is found in the plasma than in the hemocytes. The plasma to cell ratio is not correlated with the percent of Hn hemocytes ($R^2 \approx 0$).

The concentration of label in the tissues is given in Table 2.1, along with hematopoietic neoplasia status. While many of the clams used in this study had Hn, only 2 showed neoplasia in more than 10% of the circulating hemocytes, and most had disease levels of less than 1%. If all of the time periods and disease stages are pooled, there is no significant difference ($p = 0.85$) between the Hn ($n = 26$) and normal clams ($n = 17$) in the plasma to cell ratio. Small sample size ($n \leq 4$) prevents the comparison of Hn and normal clams within the same time point.

Discussion

Within the constraints of the experimental design and the individual variability of the clams, it is possible to determine a few points about lipid transport in *Mya arenaria*. First, distribution of the label to tissues occurred rapidly,

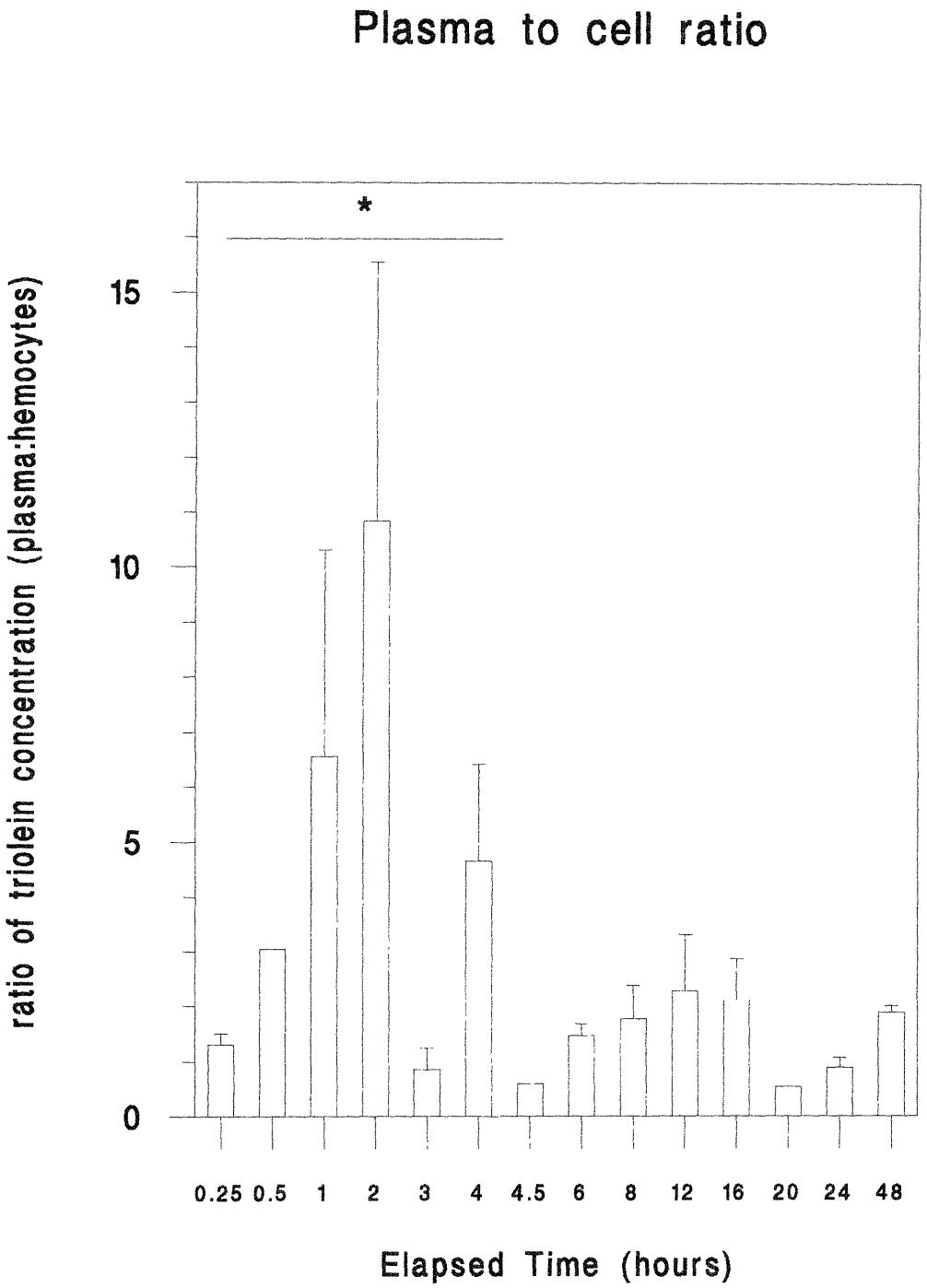


Figure 2.6. Ratio of label concentration (pmol triolein/g wet weight hemolymph) in plasma and hemocytes. Each bar represents the mean ± standard error of the ratios of the clams measured at that time point. The average of the ratios in the first four hours (0.25 - 4 hours) is significantly higher than the average after 4.5 hours (4.5 - 48 hours) ($p = 0.04$).

Table 2.1. Label concentration (pmol triolein/g wet weight) of individual tissues, plasma to cell ratio, percent of the injected dose which was recovered, and hematopoietic neoplasia diagnosis (% Hn cells) of the 49 clams injected in this experiment.

Elapsed	Label concentration (pmol triolein per g wet weight)											plasma/	% dose
Time(hrs)	Gonad	DG	Foot	Gill	Muscle	Mantle	Siphon	Plasma	Cells	Hemolym	Hn %	cells	recovered
0.25	2315.09	2602.43	2514.53	3559.25	1234.93	10923.81	6093.94	32.51	27.56	60.07	0	1.180	0.75
	596.09	524.43	1281.65	2260.78	2130.20	11514.39	12777.58	0.82	0.46	1.28	0.1	1.783	21.99
	1230.17	1663.80	1755.80	10179.43	1480.99	3639.66	8177.38	31.43	62.69	94.12	0	0.501	3.35
	476.31	1059.41	1114.64	3291.78	1524.39	9899.20	10353.06	35.79	32.79	68.58	4.1	1.091	17.50
	1518.42	1494.47	3257.28	4998.11	4608.39	17631.19	9432.25	22.14	21.85	43.99	0	1.013	21.17
	1325.34	2793.34	18045.32	7078.43	2045.05	14640.10	6164.84	2991.51	2008.46	4999.97	0	1.489	1.34
	263.57	175.43	549.78	1431.79	908.84	1977.44	19313.42	40.38	18.80	59.18	0	2.148	36.82
mean	1103.57	1473.33	4074.14	4685.65	1990.40	10032.26	10330.35	450.65	310.37	761.03		1.315	
std dev	661.35	911.07	5764.99	2820.43	1139.77	5178.67	4266.57	1037.37	693.46	1730.74		0.502	
std err	249.97	344.35	2178.96	1066.02	430.79	1957.35	1612.61	392.09	262.10	654.16		0.190	
0.5	3483.61	10376.98	7389.95	4880.51	3750.76	15808.07	2382.37	1068.24	351.70	1419.94	nd	3.037	0.38
1	4435.41	3514.38	3277.36	16289.58	1483.58	9368.16	1096.10	0.89	0.93	1.82	1.7	0.957	25.81
	927.28	1569.69	579.57	9377.74	1493.57	3791.13	6983.42	6688.56	3319.78	10008.34	0.3	2.015	18.12
	776.18	999.19	4025.96	1001.32	2248.69	13638.34	7671.23	3892.08	2300.84	6192.92	nd	1.692	27.65
	1697.03	1735.42	6237.50	4851.02	4206.36	17431.66	2091.53	22335.19	965.62	23300.81	2.2	23.130	12.64
	1435.49	8622.84	1909.34	35119.31	852.98	6512.93	2898.32	1564.08	314.80	1878.86	0	4.968	18.48
mean	1854.28	3288.30	3205.95	13327.79	2057.04	10148.44	4148.12	6896.16	1380.39	8276.55		6.552	
std dev	1332.96	2796.91	1920.77	12025.33	1162.10	4888.06	2666.74	8042.48	1250.51	8276.12		8.401	
std err	596.12	1250.82	859.00	5377.89	519.71	2186.01	1192.60	3596.71	559.25	3701.19		3.757	
2	2807.31	1866.57	2421.27	6897.49	3535.88	12316.84	7620.16	3.42	0.44	3.86	0	7.773	35.93
	1510.69	1432.74	1442.41	25711.13	4734.02	13194.94	7802.16	347.34	126.80	474.14	0.2	2.739	19.58
	1722.30	774.35	2223.80	984.20	748.56	2420.19	570.14	114421.9	5198.57	119620.5	nd	22.010	41.96
mean	2013.43	1357.89	2029.16	11197.61	3006.15	9310.66	5330.82	38257.55	1775.27	40032.82		10.841	
std dev	567.96	449.03	422.65	10542.72	1669.62	4885.47	3367.13	53856.51	2421.19	56277.29		8.161	
std err	327.91	259.25	244.02	6086.84	963.95	2820.62	1944.01	31094.07	1397.87	32491.71		4.712	
3	5058.82	5894.09	4168.70	8493.60	3235.54	3765.63	5080.68	1608.48	5558.98	7167.46	0.6	0.289	1.79
	1651.92	1418.75	2897.03	10160.97	1285.91	3258.99	11387.96	284.15	201.57	485.72	18.8	1.410	20.28
mean	3355.37	3656.42	3532.87	9327.29	2260.73	3512.31	8234.32	946.32	2880.28	3826.59		0.850	
std dev	1703.45	2237.67	635.83	833.68	974.82	253.32	3153.64	662.17	2678.71	3340.87		0.560	
std err	1204.52	1582.27	449.60	589.50	689.30	179.12	2229.96	468.22	1894.13	2362.35		0.396	
4	4910.40	3063.02	6429.39	6995.81	5249.18	9552.21	3363.88	319.23	44.73	363.96	0	7.137	0.96
	1137.00	1732.92	3217.86	6736.59	1737.27	7318.03	3652.06	2296.14	5652.77	7948.91	0	0.406	3.27
	947.46	1042.68	1554.36	2337.71	2320.53	12624.26	10586.86	2514.40	205.67	2720.07	0.5	12.225	45.14
	6988.76	2348.52	10245.43	6153.90	4463.62	6161.65	8021.22	1857.07	2058.34	3915.41	0	0.902	4.75
	3415.17	2707.82	6817.37	15696.70	5556.46	16993.51	1645.17	898.48	883.18	1781.66	0.7	1.017	7.63
	5942.24	3007.60	6298.83	35133.97	4316.97	6771.17	1580.18	9817.47	1573.94	11391.41	0	6.238	24.88
mean	3890.17	2317.09	5760.54	12175.78	3940.67	9903.47	4808.23	2950.47	1736.44	4686.90		4.654	
std dev	2284.67	724.83	2772.65	11022.27	1426.99	3836.05	3355.69	3165.01	1888.60	3811.89		4.308	
std err	932.71	295.91	1131.93	4499.82	582.57	1566.06	1369.96	1292.11	771.02	1556.20		1.759	

Elapsed		Label concentration (pmol triolein per g wet weight)										plasma/	% dose
Time(hrs)	Gonad	DG	Foot	Gill	Musole	Mantle	Siphon	Plasma	Cells	Hemolym	Hn %	cells	recovered
4.5	1543.46	3070.03	16792.44	1551.03	2528.58	6894.08	3492.63	16888.70	28688.75	45577.45	nd	0.589	4.54
6	1247.09	1776.66	1401.01	2779.37	1759.88	6762.74	10202.52	50.17	25.05	75.22	1.1	2.003	46 77
	310.68	401.89	856.36	1041.87	320.98	1456.42	12541.13	49.84	28.81	78.65	0	1.730	45.42
	2653.12	2584.27	1828.68	13699.19	4929.66	7399.18	6252.12	347.36	281.87	629.23	0	1.232	8.60
	1892.05	2407.27	1714.77	19085.82	5253.28	4677.27	2094.87	6020.66	6472.77	12493.43	0	0.930	18.85
mean	1525.74	1792.52	1450.21	9151.56	3065.95	5073.90	7772.66	1617.01	1702.13	3319.13		1.474	
std dev	860.11	857.16	376.93	7512.36	2091.56	2318.54	3974.67	2545.35	2756.30	5301.58		0.418	
std err	430.06	428.58	188.47	3756.18	1045.78	1159.27	1987.33	1272.67	1378.15	2650.79		0.209	
8	1818.02	656.97	989.19	1508.63	2128.60	10264.77	9424.30	128.32	157.10	285.42	0	0.817	55.54
	1034.71	1432.61	1375.05	3834.16	2195.04	5845.09	8849.96	18.40	4.32	22.72	0	4.259	47.78
	1226.44	1766.84	1557.85	26968.41	2119.58	3544.07	5139.81	537.63	254.35	791.98	0.4	2.114	21.28
	2080.69	1238.34	1484.41	13585.33	4450.84	9352.80	4803.69	560.76	1180.95	1741.71	0.3	0.475	8.70
	2221.22	1314.24	4839.39	4487.19	6413.10	7766.68	9976.67	1635.15	32.48	1667.63	0.7	50.343	22.84
	3734.07	1325.63	5393.45	12693.39	4192.64	11055.95	4239.94	10554.72	8661.69	19216.41	0.4	1.219	5.16
mean	2019.19	1289.11	2606.56	10512.85	3583.30	7971.56	7072.40	2239.16	1715.15	3954.31		1.777	
std dev	877.83	329.89	1790.89	8631.05	1597.79	2609.17	2381.55	3755.29	3132.08	6855.35		1.357	
std err	358.37	134.68	731.13	3523.61	652.29	1065.19	972.26	1533.09	1278.67	2798.68		0.607	
12	1539.36	1134.09	1402.08	33962.13	3939.72	3498.89	1428.54	1591.84	753.06	2344.9	57.4	2.114	29.20
	1568.94	1514.18	1841.84	18877.24	3020.06	9358.96	2243.16	4186.78	743.71	4930.49	0.2	5.630	21.79
	1626.88	1445.53	7494.11	4707.07	8797.20	10224.66	7707.90	2759.03	2156.64	4915.67	1.3	1.279	13.64
	7783.84	8862.22	17476.44	11388.83	3912.60	5636.90	4041.14	2205.88	18138.87	20344.75	0.3	0.122	0.91
mean	3129.76	3239.01	7053.62	17233.82	4917 40	7179.85	3855.19	2685.88	5448.07	8133.95		2 286	
std dev	2687.22	3249.72	6479.42	10881.41	2270.36	2736.31	2416.88	959.87	7349.56	7128.05		2.056	
std err	1343.61	1624.86	3239.71	5440.71	1135.18	1368.15	1208.44	479.93	3674.78	3564.02		1.028	
16	1645.54	4116.79	1946.67	9276.36	4661.94	8284.47	7859.01	1517.39	1426.95	2944.34	3.6	1.063	8.78
	1902.67	1293.08	2768.00	4183.13	4826.60	7007.70	10199.15	1231.18	387.79	1618.97	0.2	3.175	9.20
mean	1774.11	2704.94	2357.34	6729.75	4744.27	7646.09	9029.08	1374.29	907.37	2281.66		2.119	
std dev	128.57	1411.86	410.67	2546.62	82.33	638.38	1170.07	143.11	519.58	662.69		1.056	
std err	90.91	998.33	290.38	1800.73	58.22	451.41	827.36	101.19	367.40	468.59		0.747	
20	1538.97	1774.06	2251.09	14239.87	2648.88	10436.95	4055.39	425.19	784.54	1209.73	3.7	0.542	4.94
24	742.05	587.43	3745.35	49388.29	1748.32	3037.82	620.03	21.87	0.13	22	0.6	168.231	26.71
	1330.95	1657.90	2047.07	2821.83	1933.46	5403.49	3692.58	6873.17	6075.73	12948.9	0	1.131	4.20
	551.13	566.91	1058.77	2402.69	1052.31	2763.30	12937.86	502.91	1048.04	1550.95	0.3	0.480	16.65
	637.79	402.34	1500.07	20157.39	1089.29	2959.20	1225.84	58792.18	42155.13	100947.3	1.3	1.395	5.86
	1752.82	990.99	3438.59	4560.80	9456.49	7164.69	9797.23	1631.57	3154.70	4786.27	6.1	0.517	31.45
mean	1002.95	841.11	2357.97	15866.20	3055.97	4265.70	5654.71	13564.34	10486.75	24051.09		0.881	
std dev	463.78	452.03	1059.54	18007.66	3219.29	1741.87	4878.88	22745.20	15969.73	38706.78		0.394	
std err	207.41	202.16	473.84	8053.27	1439.71	778.99	2181.90	10171.96	7141.88	17310.20		0.197	
48	2246.02	3799.91	7554.11	36410.31	5431.08	7102.25	2207.36	2445.86	1424.28	3870.14	0.4	1.717	1.82
	2549.31	2713.69	2317.95	10911.08	5240.17	10969 30	5266.90	8545.52	4144 84	12690.36	0.3	2 062	0.93
mean	2397.67	3256.80	4936.03	23660.70	5335.63	9035.78	3737.13	5495.69	2784.56	8280.25		1.889	
std dev	151.65	543.11	2618.08	12749.62	95.45	1933.52	1529.77	3049.83	1360.28	4410.11		0.172	
std err	107.23	384.04	1851.26	9015.34	67.50	1367.21	1081.71	2156.56	961.86	3118.42		0.122	

with a large portion of the label accumulating in tissues other than the siphon within 15 minutes. Most of these tissues were only connected to the siphon via the hemolymph; in order to get from the siphon to the foot, for instance, the label must have been carried in the hemolymph. In many tissues, label content is greater than total hemolymph label content, implying that the tissue distribution of the label does not simply reflect differences in hemolymph volume among the tissues.

As a first consideration, the rapid distribution of the label suggests that the elapsed timepoints chosen in this study failed to capture all of the dynamics of lipid transport. Many other published studies have relied on a snapshot of label distribution at one time point after label administration, generally at 4 or 8 hours. Published studies are contradictory on the time course of label leaving the site of administration. Activity in the "source" organ was highest in some experiments (Allen and Conley, 1982; Pollero, 1987), while in others (Barber and Blake, 1985; Pollero and Heras, 1989; Garin and Pollero, 1995), tracer concentrations were higher in other "recipient" organs. Huca et al. (1984) found that *Diplodon delodontus* fed tripalmitate retained more than half of the activity in the stomach within the first hour, but that concentrations in other soft tissues exceeded that in the stomach after six hours.

The distribution of lipophilic compounds in *Mya arenaria*

provide support for the observed rapid transport of lipid in the soft shell clam. Disofenin, a small lipophilic molecule, was distributed throughout *Mya arenaria* tissues within 2 - 30 minutes after injection into the adductor muscle; preferential accumulation in the kidney was seen within 1 hour (Burn et al., 1993). Potts (1993), however, suggested that this process is slower at 6 ^{0}C than at 18 ^{0}C (the temperature used in the Burn et al. study). Additionally, Moreno et al. (1992) have studied the uptake and accumulation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in *Mya arenaria* from an *in vivo* seawater exposure. The initial distribution (within 1-2 hours) of TCDD correlated with perfusion rates, implying that the TCDD was transported to some tissues in the hemolymph. Steady state conditions, where TCDD distribution was correlated with tissue lipid content, were reached at about 9 hours.

Second, plasma seems to be the primary route of distribution of the label, at least within the first four hours. Additional support for the role of plasma is that the relative importance of plasma and hemocytes was not altered in clams with diseased hemocytes, at least at the early stages of disease. Since hemocytes have been suggested to play a role in lipid transport (Hoskin and Hoskin, 1977; Allen and Conley, 1982; Pollero, 1987; Pollero and Heras, 1989), one hypothesis of this experiment was that clams with hematopoietic neoplasia would demonstrate alterations in the role of hemocytes in

lipid transport. Although most diseased clams were only in the initial stages of hematopoietic neoplasia, the one clam in which over half of the circulating hemocytes were abnormal showed no differences in label distribution between hemocytes and plasma.

The greater change in plasma label concentration versus hemocyte label concentration seen in this experiment, while not supporting the hypothesis that hemocytes are involved in lipid transport, is supported by published studies of lipid transport in the freshwater bivalve *Diplodon delodontus*. In feeding experiments with labelled palmitate, oleate, linoleate, and triolein, Pollero et al. (1985) found more label in plasma than hemocytes after 45 minutes; the plasma to cell ratios ranged from 2.2 to 8.1. After 4.5 hours of incubation with labelled cholesterol, the plasma to cell ratios were close to one, ranging from 1.3 - 1.5 (Pollero, 1987). Contradictory evidence for ^{14}C palmitate injected into the adductor muscle, however, suggest that plasma label concentration was higher than the hemocyte concentration (ratio = 2.3) after 4.5 hours of incubation (Pollero and Heras, 1989). Triglycerides and cholesterol, however, may be transported by different mechanisms. All of the evidence still appears to support the conclusion of Allen and Conley (1982) that the relative importance of plasma and hemocytes in lipid transport is unclear due to variability among individuals. It remains to be determined, however, how lipid

transported by hemocytes would be taken up by the target tissues.

Caution must be taken in the interpretation of the results of this experiment, however, because the label was introduced to the clams by injection into the siphon. In published studies, lipid tracers were often administered orally or through the stomach; this would more closely match the natural uptake of lipids in food. Passage through the digestive system may affect tracer distribution if specialized lipid absorptive tissues are part of the initial stages of lipid transport. Lipid would not ordinarily enter a clam via the tissues of the siphon; the transport process as observed here may not be representative as a result. Siphon was chosen as the injection site in this study as it was the only tissue into which a known quantity of label could be reproducibly administered; feeding studies generally lack data on the quantity of tracer ingested by the organism.

The recovery of the administered label was relatively low in this experiment. One possible reason for the low recovery is that the clams may have eliminated some of the label into the seawater immediately after injection. A possible result of the introduction of the label into the seawater medium is that label accumulation, particularly in the gill, could have occurred directly from the medium, without transport from the injection site via the hemolymph. If this is true, one expectation is that clams used in later stages of the

experiment, in which the concentration of the label in the medium would have been highest, would have demonstrated higher uptake in the gill. This effect was not observed. The lipophilic nature of the tracer may have caused the label to adhere to the sides of the container or to be adsorbed onto particulates in the seawater, rather than being available for uptake across clam membranes.

From this experiment, it appears that plasma may be the primary means of lipid transport between organs in the soft shell clam. In the aqueous environment of the hemolymph, hydrophobic lipids must be sequestered during transport and released at the target tissue or cell. Macromolecular lipoprotein complexes have been suggested as the mechanism by which ingested lipids are transported to tissues for storage and stored lipid is mobilized to the gonad for the production of gametes. Evidence for the presence of lipoproteins in the hemolymph of *Mya arenaria* will be discussed in Chapter 3.

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Chapter 3. Identification and characterization of a lipoprotein in the soft shell clam *Mya arenaria*

Background

Once lipids are synthesized or ingested and absorbed, they must be transported throughout the body to sites of utilization or storage. Macromolecular lipoprotein complexes facilitate the transport of lipids in the circulatory system by sequestering the hydrophobic lipids from the aqueous hemolymph during transport. Mammalian lipoproteins have been classified on the basis of their flotation density (Table 3.1) as determined by density gradient centrifugation. The centrifugation process, however, can modify lipoprotein composition (Chapman, 1980; Fielding and Fielding, 1991).

The lipid transport cycle in mammals consists of four major lipoprotein classes (chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL)) and two loops. An exogenous loop transports ingested dietary lipids from the site of absorption in the gut to the liver for metabolism and/or storage. The endogenous loop transports lipids from storage pools to target organs for metabolism; this loop includes a homeostatic feedback cycle (reverse cholesterol transport). The roles of the mammalian lipoproteins in these lipid transport pathways are depicted in Figure 3.1. An additional lipoprotein class,

Table 3.1. The hydrated densities and functions of the mammalian lipoprotein classes (adapted from Scanu et al., 1982).

Classification of mammalian lipoproteins

Lipoprotein	Density	Function
chylomicron	< 0.940 g/mL	formed in intestine after fatty meals; transport triglycerides, phospholipids, and sterol esters between liver and intestine
VLDL	0.940 - 1.006	formed in liver for transport of endogenous triglycerides (major); formed in intestine for transport of dietary lipids (minor)
LDL	1.006 - 1.063	transport of cholesterol (esterified)
HDL	1.063 - 1.210	reverse cholesterol transport

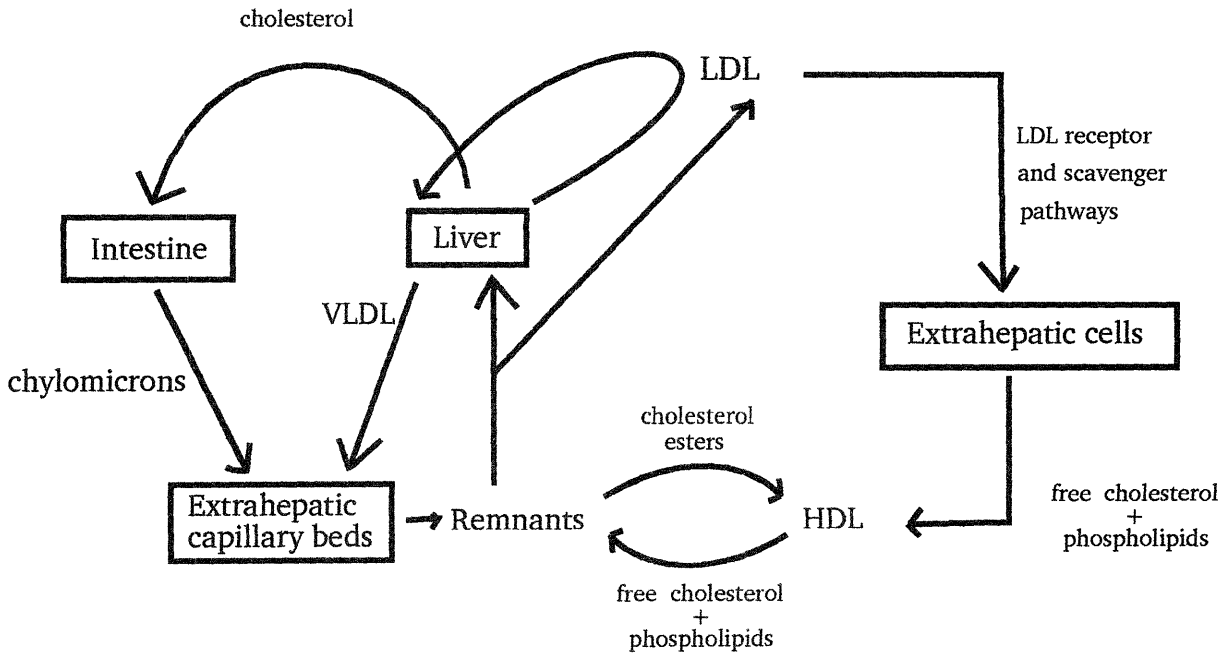

Figure 3.1. A general model of lipid transport in mammals, adapted from Havel (1980).

very high density lipoprotein (VHDL, density > 1.21 g/mL), is not found in mammals but is common in invertebrates.

The nature of lipid transport in bivalve molluscs is not necessarily similar to the process in mammals and other vertebrates. Bivalves and many other invertebrates have an open circulatory system instead of the closed system of vertebrates. Since lipoproteins transport lipids in the blood, the open nature of the circulatory system should be a significant factor in determining the properties of a lipid transport system.

Lipoproteins in invertebrates

Lipid transport has been studied primarily in two groups of invertebrates with open circulatory systems: insects and crustaceans. In insects, the fat body is the analog of the vertebrate liver and adipose tissue. Neutral lipids are stored as triglycerides, but are released into the hemolymph as diglycerides in a high density lipoprotein complex. The HDL is 44% lipid, of which 56% is in the form of diglycerides and 26% is phospholipid. The triglycerides of the fat body and the diglycerides of the hemolymph HDL have a different fatty acid composition, indicating that the fatty acids of the fat body are compartmentalized in some way (see review by Gilbert and Chino, 1974).

In crustaceans, lipid is stored in the hepatopancreas as triglyceride. Lipoproteins are formed, possibly in the F-

cells of the hepatopancreas (Lee, 1991), to transport lipid to other tissues. Different numbers of lipoproteins have been found in different species of decapods. Lee and Puppione (1978) found a HDL_3 ($1.12 < d < 1.21$ g/mL) in the spiny lobster *Panulirus interruptus*; the predominant lipid component was phospholipid (88%). The blue crab *Callinectes sapidus* also has a HDL_3 ($1.12 < d < 1.21$ g/mL) composed of a 112 kDa peptide with phosphatidyl choline as the primary lipid (Lee and Puppione, 1988). The dungeness crab, *Cancer magister*, was found by electrophoresis to have two lipoprotein peptide bands (Allen, 1972); the two peptides may be components of one lipoprotein or indicate the presence of two different lipoproteins. Phospholipids (phosphatidyl ethanolamine and phosphatidyl choline) were the predominant lipids in dungeness crab hemolymph, accounting for 65-82% of the total hemolymph lipids (Allen, 1972).

Teshima and Kanazawa (1980a,b) traced the uptake of ^{14}C tripalmitin in the prawn *Penaeus japonicus*. Labelled lipids in the serum were associated with three lipoproteins: HDL_2 ($1.063 < d < 1.125$ g/mL), HDL_3 ($1.125 < d < 1.21$ g/mL) and VHDL. The amount of radiolabel in the VHDL fraction continued to increase after the HDL activities decreased, suggesting that VHDL has a different functional role (Teshima and Kanazawa, 1980a). The VHDL role might be similar to that of the endogenous lipid transport loop in mammals. The lipid in the HDL_3 and VHDL fractions was almost entirely in the form of

phospholipid (69-87%), whereas the HDL_2 fraction contained significant amounts of free sterols, diglycerides, and free fatty acids in addition to the 40-47% phospholipid (Teshima and Kanazawa, 1980b).

In general, lipid transport in decapod crustaceans appears to be facilitated by one or more high density lipoproteins in which the predominant lipid is a phospholipid. Lipid transport processes in insects and crustaceans seem to be similar, with the primary difference being the form in which the lipid is transported: diglycerides in insects and phospholipid in crustaceans. In either case, the main (and possibly only) lipoprotein appears to be a HDL in the upper portion of the density range for this class. In contrast, mammals and other vertebrates have a greater variety of lipoproteins. Furthermore, vertebrate HDL carries cholesterol, which is found in only small amounts in arthropod lipoproteins.

Lipoproteins have also been found in marine (Allen, 1977; Allen and Conley, 1982) and freshwater (Pollero et al., 1985; Pollero, 1987; Pollero and Heras, 1989) molluscs. In all cases, free fatty acids have been suggested as the form of lipid which is associated with lipoproteins. After an injection of ^{14}C palmitate into the freshwater bivalve *Diplodon delodontus*, Pollero and Heras (1989) found that the label was incorporated into LDL (50%), HDL (13%) and VHDL (6.5%) fractions. The label was found primarily in the free fatty

acids of the LDL (Pollero and Heras, 1989), and in free sterols and triglycerides of HDL and phospholipids of VHDL (Pollero, 1987). A conceptualized pathway of lipid transport in molluscs is depicted in Figure 3.2.

Female specific lipoproteins

Another general feature of lipid transport in invertebrates is the presence of a female specific hemolymph lipoprotein that is either similar or identical to an ovarian lipoprotein. Wallace et al. (1967) suggested that the major HDL of invertebrate eggs should be called lipovitellin in order to standardize the terminology. The term lipovitellin was initially used to describe the major HDL in the yolk of hen eggs. Ovarian lipoproteins are now called lipovitellins; the corresponding female specific hemolymph lipoproteins are often called vitellogenins. Vertebrate lipovitellins have a lipid content of 16 - 22 % and a molecular weight of 4.2 - 4.5 x 10^{5} Da (Wallace et al., 1967).

In insects, a VHDL is found in both hemolymph and yolk; the features of this lipoprotein were reviewed by Gilbert and Chino (1974). The VHDL is 10% lipid of which 50% is phospholipid and 34% is diglyceride. In addition, the VHDL also has a carbohydrate component, making it a glycolipoprotein. An interesting feature of the female specific transport of lipid into insect ovaries is that the hemolymph VHDL is only able to incorporate lipid from the HDL

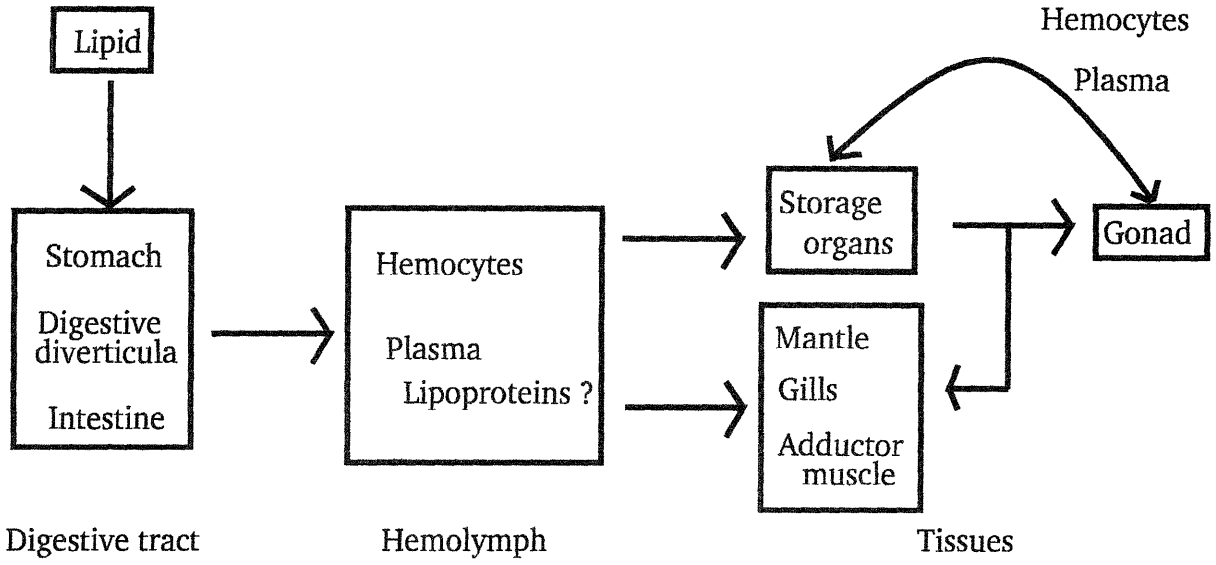

Figure 3.2. A model of lipid transport in molluscs, adapted from Allen and Conley (1982). The relative role of hemocytes and plasma in lipid transport is unclear. The storage organs may include digestive gland, mantle, or adductor muscle, depending on the species.

that serves as the lipoprotein for general lipid transport in both males and females.

Female specific high density lipoproteins have been found in a number of crustaceans (Wallace et al., 1967; Kerr, 1969; Fielder et al., 1971; Lui et al., 1974; Lee and Puppione, 1978; Eastman-Reks and Fingerman, 1985; Lee and Puppione, 1988; Quinitio et al., 1989; Quinitio et al., 1990; Tirumalai and Subramoniam, 1992). The number and molecular weight of peptides appear to vary among species. The apolipoprotein may be composed of two to seven peptides; the HDL of most species contains both 80 and 100 kDa peptides. In general, the HDL lipovitellins of crustaceans contain about 30% lipid and trace amounts of carbohydrate.

Female specific lipoproteins have also been identified in some species of marine bivalves. Zagalsky et al. (1967) found a carotenoid containing glycolipoprotein in the ovary of the scallop *Pecten maximus*; the glycolipoprotein is approximately 10% lipid. The lipid is in the form of phospholipid (61%) and cholesterol (25%). Suzuki et al. (1992) found a female specific hemolymph lipoprotein and a related ovarian protein in *Crassostrea gigas*. The ovarian glycolipoprotein has a molecular weight of approximately 500 kDa and is composed of seven peptides. The origin of the hemolymph protein is unknown; the authors presumed that the ovarian protein was produced inside the ovary because antibody staining was localized to this organ. Lee and Heffernan (1991) found that

a 56 kDa peptide is the major water soluble peptide in the eggs of *Crassostrea virginica*, *Mercenaria mercenaria*, and *Argopecten irradius concentricus*. In *C. virginica* and *M. mercenaria*, this peptide is part of a VHDL that also contains phospholipids (≈80%), with smaller amounts of free sterols and sterol esters; the VHDL is 80% protein, 17% lipid and 2% carbohydrate.

In the radiotracer experiment described in the previous chapter, a lipid label was found in organs with only hemolymph connection to the site of injection within 30 minutes, suggesting a role for hemolymph in the transport of lipid in the soft shell clam. In the first 4 hours after injection, more of the label was found in the plasma than in hemocytes. The purpose of the experiments described in this chapter was to describe the relative roles of hemocytes and plasma in lipid transport, and to characterize plasma lipoproteins in clams.

Materials and Methods

Clams

All of the clams used in these experiments were collected by hand digging from the intertidal zone in Little Buttermilk Bay, Bourne, MA. They were held for up to 6 months in a flow-through unfiltered seawater system (30 - 31 $^{0}/_{00}$) at ambient temperature until needed. The clams were not fed. In one

trial experiment, plasma proteins of identified ripe male and female clams were separated by SDS-PAGE.

The sex, reproductive stage, and hematopoietic neoplasia status of clams used in the remaining experiments presented in this chapter were not determined because the lipoprotein separation occurred within hours of plasma collection to avoid the problem of alteration of the composition of lipoproteins during storage; determination of these parameters was not done after the centrifugation steps because plasma from several clams was pooled in order to obtain the required volume. Although reproductive stage was not determined in individuals, most of the clams in the Little Buttermilk Bay population at the same time of year (December/January) were in indifferent (48 %) or early developmental (47 %) stages (Potts, 1993; results of experiments in chapters 5 and 6). The Little Buttermilk Bay soft shell clam population has been found to have a relatively low (17 %) incidence of Hn (Leavitt, et al., 1990; Potts, 1993).

Hemolymph composition

Hemolymph was collected by syringe from the adductor muscle sinus of soft shell clams. Pooled hemolymph samples were centrifuged at 4,800 x g for 45 minutes at 15 °C to pellet the hemocytes. Hemocyte and plasma lipids were extracted sequentially in 1:2 and 2:1 chloroform:methanol and quantified gravimetrically on a Cahn 29 electrobalance (Sasaki

and Capuzzo, 1984). Hemocyte and plasma proteins were quantified by the method of Lowry et al. (1951) as modified by Hartree (1972), using bovine serum albumin as a standard.

Floating lipoprotein fraction composition

Hemolymph was collected from the adductor muscle sinus and centrifuged as above to pellet the hemocytes. The plasma fraction was subjected to a modification of the standard lipoprotein density gradient centrifugation protocol (Redgrave et al., 1975; Chapman et al., 1979). Plasma density was adjusted to 1.25 g/mL by the addition of solid KBr. Ethylenediaminetetra-acetic acid (EDTA, 1 mM), sodium azide (2 mM), aprotinin (0.1 %), and phenylmethylsulfonyl fluoride (PMSF, 2 mM) were added. The plasma was laid over a 1.27 g/mL KBr solution and centrifuged at 170,000 x g for 13 hours at 15 °C in a Beckman L8-M ultracentrifuge with a 70.1 Ti fixed angle rotor. The floating lipoprotein fraction (top 1 - 1.5 mL) was collected.

Plasma and the lipoprotein fraction were dialyzed against dialysis buffer (0.1 M NaCl, 1 mM EDTA, 2 mM NaN_3, pH 8.0) for 24 - 48 hours. After dialysis, the samples were concentrated by lyophilization before lipid extraction. Lipid and protein assays were performed as described above. Lipid and protein concentrations were corrected for the recovery of an internal standard, human HDL (Sigma L-2014).

Lipoprotein isolation and composition

Plasma was separated as described above. Prior to ultracentrifugation, the plasma was concentrated by ultrafiltration using Millipore 10,000 NMWL filter units (UFC 3 LGC NB). EDTA (1 mM), sodium azide (2 mM), aprotinin (0.1 %), and 0.1 M PMSF (2 mM) were added to the concentrated plasma. Plasma was added to a density gradient formed by 1.019, 1.063, 1.21, and 1.27 g/mL KBr solutions and centrifuged at 170,000 x g for 17 hours at 17 °C. Fractions (1 mL each) were collected by puncturing the bottom of the tubes. Density was determined by refractometry. Lipid concentration of the fractions was determined as described for the hemocyte and plasma samples. Protein concentration was determined by microplate BCA assay (Redinbaugh and Turley, 1986) with bovine serum albumin as a standard.

Radiotracer experiment

The radiotracer ^{14}C triolein (5 µCi, specific activity 112 mCi/mmol) was injected into the siphon of 7 clams, as described in the previous chapter. Hemolymph was collected by syringe from the adductor muscle sinus 15 minutes after injection. Plasma was separated from hemocytes by centrifugation at 4,800 x g for 45 minutes at 4 °C. The plasma was subjected to density gradient ultracentrifugation as described above, except the plasma was not preconcentrated. Fractions were collected in 1 mL increments from the bottom of

the tubes. Fractions from replicate tubes were pooled. Density was determined by refractometry. Lipid and protein concentration of the fractions were determined as described for the floating lipid fraction. Radioactivity was determined by adding 1 mL of each pooled fraction to 10 mL of scintillation cocktail and measuring the mean dpm of triplicate counts as described in the previous chapter; dpm was then converted to pmol triolein, after correcting for background and quench.

Results

The majority of hemolymph lipid (72 %) and protein (78 %) in *Mya arenaria* is carried in the plasma (Table 3.2). Within the plasma, the lipid (72 %) is primarily associated with the floating lipoprotein fraction with a density of less than 1.21 g/mL (Table 3.3). Only 23 % of the total plasma protein is found in the same lipoprotein fraction (Table 3.4).

A VHDL (1.20 - 1.22 g/mL) was isolated from the plasma by density gradient ultracentrifugation. Although this fraction could be considered to be either a HDL or a VHDL because the density range is on the border of the two lipoprotein classes, it falls into the very upper end of the broad range of HDL densities, and so will be considered to fall more naturally into the VHDL class. The VHDL is 80 % protein and 20 % lipid by weight (Table 3.5).

Table 3.2. Lipid and protein concentrations in the hemolymph of various molluscan species.

Lipid

Plasma	Hemocytes
Mya arenaria (this study)	
48.0 ± 2 **µg/mL hemolymph** (72.3 %)	18.4 ± 5 **µg/mL hemolymph** (27.7 %)
Placopecten magellanicus (Thompson, 1977)	
137 ± 24 (59.3 %)	94 ± 14 (40.7 %)
Crassostrea gigas (Allen and Conley, 1982)	
125 ± 55 (65.1 %)	67 ± 18 (34.9 %)
Diplodon delodontus (Pollero et al., 1985)	
80 - 260 (77.7 - 88.4 %)	23 - 34 (11.6 - 22.3 %)
Ampullaria caniculata (Pollero et al., 1992)	
127 - 210 (70.2 - 75.5 %)	54 - 68 (24.5 - 29.8 %)
Mya arenaria (D.F. Leavitt, unpublished data)	
	108 ± 96

Protein

Plasma	Hemocytes
Mya arenaria (this study)	
596 ± 10 **µg/mL hemolymph** (78.1 %)	167 ± 4 **µg/mL hemolymph** (21.9 %)
Placopecten magellanicus (Thompson, 1977)	
1530 ± 280 (75.7 %)	492 ± 131 (24.3 %)
Diplodon delodontus (Pollero et al., 1985)	
600 - 1800 (80.7 - 82.2 %)	130 - 430 (17.8 - 19.3 %)

Table 3.3. Lipid concentrations in the plasma and the floating lipoprotein fraction (density < 1.21 g/mL) of *Mya arenaria* compared to lipid in the plasma of various molluscan species.

Lipid concentration			
Plasma		**Lipoprotein fraction**	
Mya arenaria (this study)			
79.1 ± 28	**µg lipid/mL plasma**	57.2 ± 7 (72.3 %)	**µg lipid/mL plasma**
40 - 300			
Lee, 1991			
molluscan hemolymph			
200 - 840			
Bayne, 1973			
(*Mytilus edulis*)			
137 ± 24			
124 ± 28			
Thompson, 1977			
(*Placopecten magellanicus*)			
40			
Allen, 1977			
(*Cryptochiton stelleri*)			
42 ± 7	Fed, gonads developing		
100 ± 22	Starved, gonads developing		
125 ± 55	Fed, sexually mature (=ripe?)		
Allen and Conley, 1982			
(*Crassostrea gigas*)			
80 - 260			
Pollero et al., 1985			
(*Diplodon delodontus*)			
127 - 210			
Pollero et al., 1992			
(*Ampullaria caniculata*)			

Table 3.4. Protein concentrations in the plasma and the floating lipoprotein fraction (density < 1.21 g/mL) of *Mya arenaria* compared to protein in the plasma of various molluscan species.

Protein concentration	
Plasma	**Lipoprotein fraction**
Mya arenaria (this study)	
596 ± 10 **µg protein/mL plasma**	139 ± 40 (23.3 %) **µg protein/mL plasma**
1530 ± 280 1450 ± 360 Thompson, 1977 (*Placopecten magellanicus*)	
30 Allen, 1977 (*Cryptochiton stelleri*)	
1140 ± 310 Thompson et al., 1978 (*Mytilus californianus*)	
890 ± 60 Fed, gonads developing 1300 ± 230 Starved, gonads developing 1410 ± 380 Fed, sexually mature (=ripe?) Allen and Conley, 1982 (*Crassostrea gigas*)	
600 - 1800 Pollero et al., 1985 (*Diplodon delodontus*)	

Table 3.5. Lipid and protein compositions of lipoproteins in various molluscan species.

Lipoprotein lipid and protein composition	
Lipid	**Protein**
Mya arenaria (this study)	
Floating lipoprotein fraction (d < 1.21 g/mL)	
57.2 ± 7 µg/mL plasma (29 % lipid by weight)	139 ± 40 µg/mL plasma (71 % protein by weight)
VHDL (1.20 - 1.22 g/mL)	
10.3 µg/mL plasma (19.5 % lipid by weight	42.5 µg/mL plasma (80.5 % protein by weight)
Diplodon delodontus (Pollero, 1987; Pollero and Heras, 1989)	
HDL (1.074 - 1.117 g/mL)	
22.6 ± 2.4 %	77.4 ± 2.4 %
VHDL (1.244 - 1.256 g/mL)	
5.6 ± 1.6 %	94.4 ± 1.6 %
LDL (1.04 - 1.054 g/mL)	
50.3 %	49.7 %
Crassostrea virginica and *Mercenaria mercenaria* (Lee and Heffernan, 1991)	
VHDL (1.21 - 1.31 g/mL)	
17 %	80 %
Ampullaria caniculata (Pollero et al., 1992; Garin and Pollero, 1995)	
HDL (1.10 - 1.15 g/mL)	
34.4 %	65.6 %
LDL (1.029 - 1.048 g/mL)	
21.1 (44.5 %)	26.3 (55.5 %)

Most of the remaining plasma lipid is found in the floating fraction, whereas the remaining plasma protein is at the bottom of the density gradient (Figure 3.3). The distribution of the injected radiolabelled triolein in the ultracentrifugation fractions is shown in Figure 3.4. The VHDL fraction contains 9 % of the total recovered label, which is similar to the background concentration of label. Most of the label is found in the highest (1.25 - 1.27 g/mL) and lowest (1.16 - 1.17 g/mL) density fractions (36 and 32 %, respectively).

Discussion

Lipid concentrations in molluscan plasma are at the low end of the range of plasma lipid concentrations in marine invertebrates (Lee, 1991). The low concentration has made the study of lipid transport in molluscan plasma difficult (Vassallo, 1973). A few conclusions about the importance of the plasma lipoprotein in the soft shell clam can, however, be reached from the results of the experiments described in this chapter.

The majority of the hemolymph lipid and protein is found in the plasma fraction. Lipid and protein concentrations in plasma and hemocytes in *Mya arenaria* are low compared to literature values from other mollusc species. The disparity in lipid and protein concentration may be true species

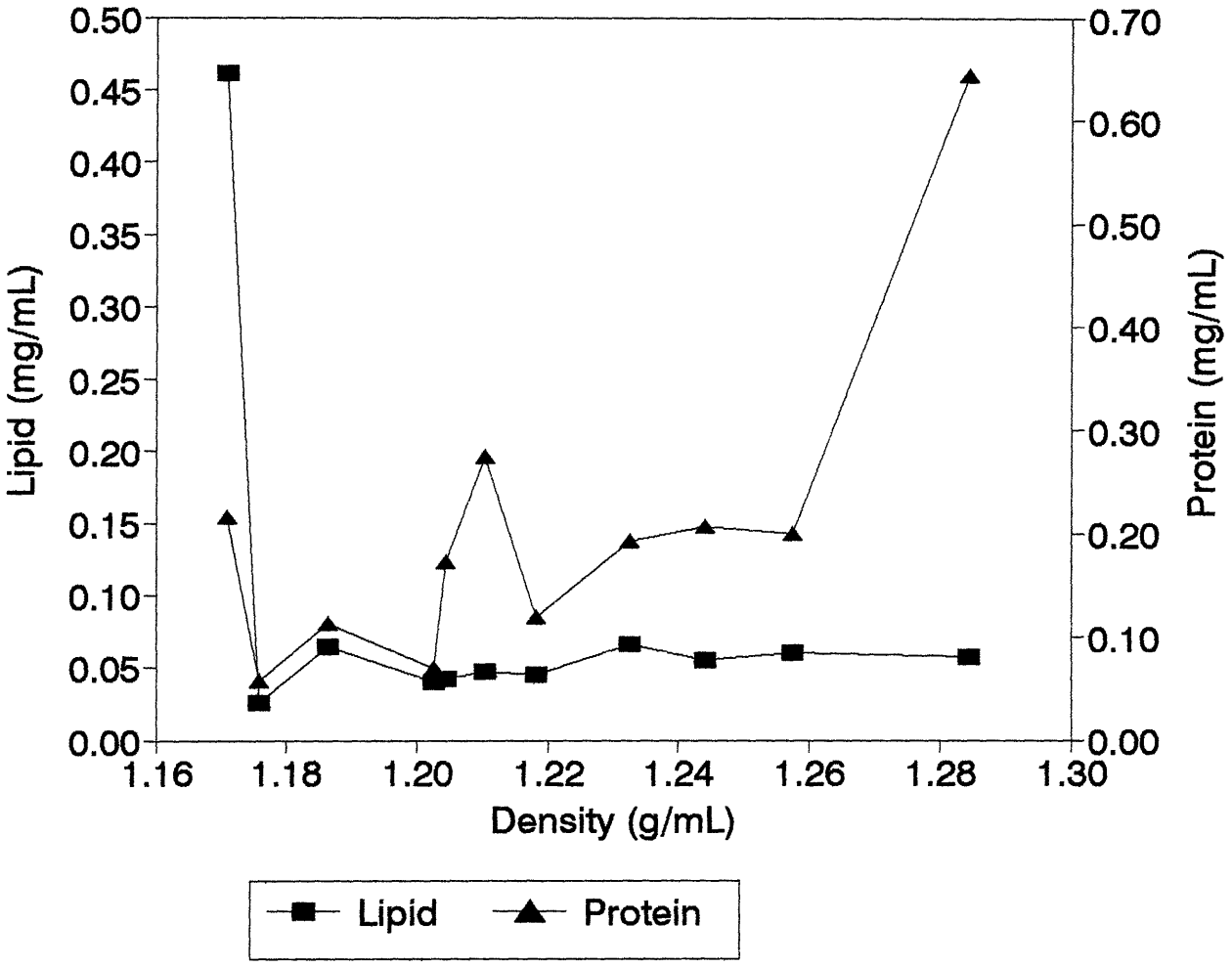

Figure 3.3. Lipid and protein concentration in the density gradient centrifugation fractions of *Mya arenaria* plasma. The protein peak in the density range 1.20 - 1.22 g/mL corresponds to a very high density lipoprotein (VHDL).

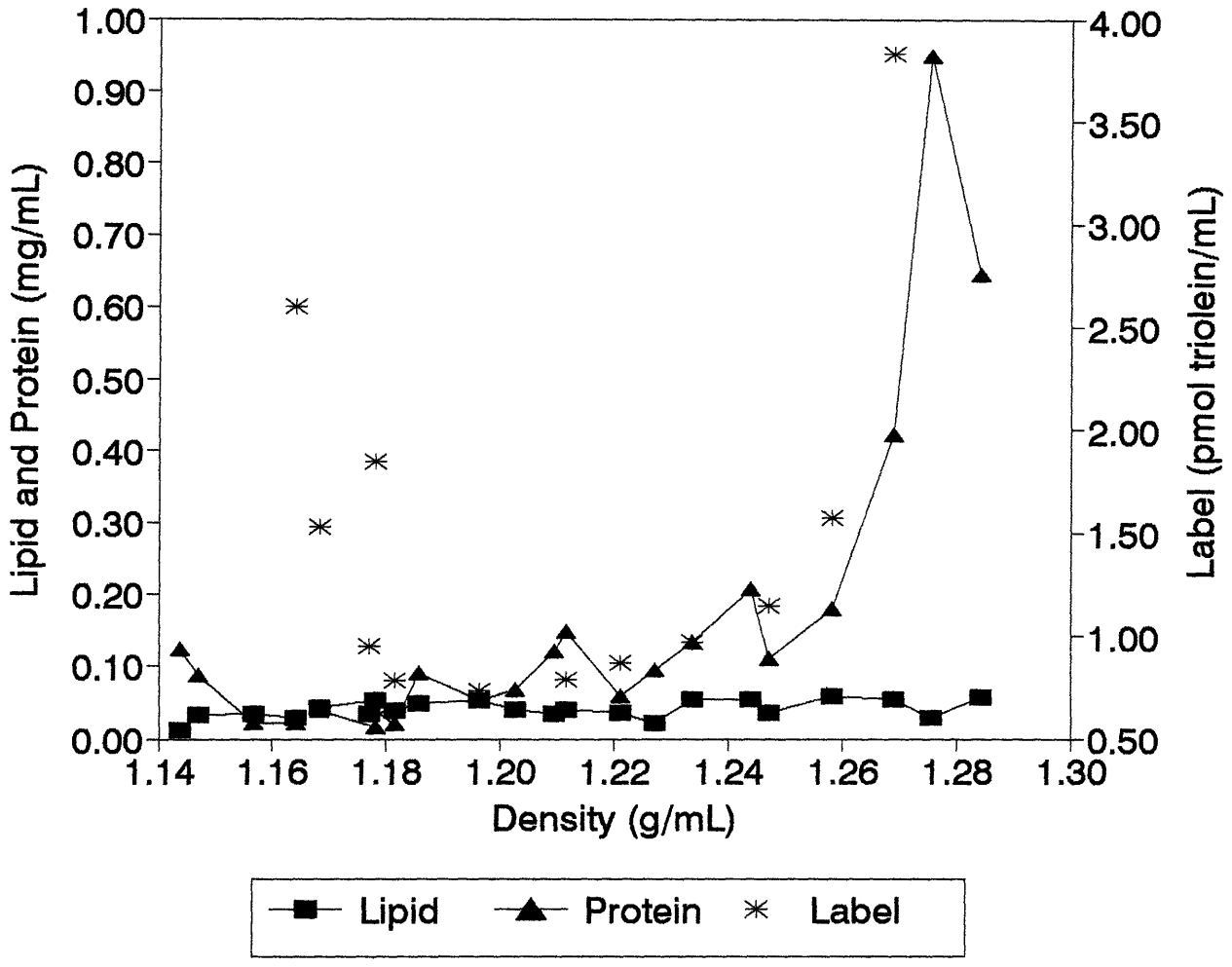

Figure 3.4. Distribution of lipid, protein, and the injected lipid tracer ^{14}C triolein in the density gradient centrifugation fractions of *Mya arenaria* plasma. The protein peak in the density range 1.20 - 1.22 g/mL corresponds to a very high density lipoprotein (VHDL).

differences, or may be attributable to differences in the physiological condition of the specimens or to differences in methodology. Lipid and protein relative concentrations in plasma and hemocytes, however, are similar in all of the species.

The plasma of *Mya arenaria* contains at least one lipoprotein. The very high density (1.20 - 1.22 g/mL) lipoprotein is 80 % protein and 20 % lipid. The remaining plasma lipid may be free fatty acids or triacylglycerols associated with denatured proteins (R.F. Lee, personal communication). The majority of the plasma proteins are found at the bottom of the density gradient. The presence of additional lipoproteins of lower densities is not ruled out. The difficulty in isolating lipoproteins from soft shell clam plasma, however, necessitated the identification of lipoproteins on the basis of the protein peak, which is most clear in the VHDL fraction because it has the highest protein concentration.

The results obtained in these experiments with *Mya arenaria* are generally in good agreement with published studies in other molluscan species. Hemolymph lipid and protein are concentrated in the plasma. Pollero and colleagues, however, have found more than one lipoprotein in the bivalve *Diplodon delodontus* (Pollero, 1987; Pollero and Heras, 1989) and the gastropod *Ampullaria caniculata* (Pollero et al., 1992; Garin and Pollero, 1995). Attempts to

successfully separate lower density lipoprotein classes in the soft shell clam were unsuccessful, probably because of the low lipid concentration in the plasma. The results presented here do not rule out the presence of an LDL or HDL in *Mya arenaria*; the failure to observe an LDL or HDL may be a function of methodology or the physiology of the specimens used rather than a true species difference. The density gradient used for the separation of lipoproteins, for example, may need to be further optimized to resolve the lower density lipoprotein classes.

The isolated VHDL, however, contains only a relatively small amount (9 %) of an injected lipid tracer. This is consistent with the nature of a VHDL, which has more protein and less lipid than lipoproteins of lower densities. Pollero and Heras (1989) also isolated a VHDL, from a freshwater clam injected with ^{14}C palmitate, and reported that 6.5 % of the total hemolymph label was associated with the VHDL fraction. After the samples were recentrifuged on a different density gradient, a LDL and HDL were also identified. These fractions contained 50.1 and 12.9 % of the total hemolymph label, respectively. While the low lipid concentration of the VHDL suggests that the role of VHDL in lipid transport in bivalve molluscs may be relatively minor, no determination of the relative importance of various lipid transport pathways can be made without information on lipid flux; this study only produced a snapshot of the distribution of lipid among the

various plasma fractions.

The density classes containing the greatest percentage of label (36 %) in this experiment also had the highest protein concentration and highest density. While it is expected that most of the protein will be found at the bottom of the density gradient, the high label levels were not anticipated. One possibility is that *Mya arenaria* plasma contains another, higher density, VHDL which was not resolved in this experiment. Another possibility is that the ultracentrifugation methods employed to separate the lipoprotein disrupted the integrity of the lipoprotein (Chapman, 1980; Fielding and Fielding, 1991). The most logical explanation, however, is that the labelled lipid is non-specifically associated with an albumin-like protein and that the amount of associated lipid was not sufficient to raise the density of the albumin-like protein out of the total protein fraction at the bottom of the gradient. In other animals, lipid (especially free fatty acids) are often transported in association with albumin (Pollero et al., 1985). Although invertebrates do not have albumins (Doolittle, 1984), they may have albumin-like proteins that serve a similar function.

The other large pool of labelled lipid (32 %) was found at the top of the density gradient, in the lowest density fractions. At first glance, this would indicate that the labelled lipid was found free in the plasma. The hydrophobic

nature of lipids, however, makes it unlikely that they would be found free in the aqueous plasma. Another possibility is that the ultracentrifugation procedure altered the structural integrity of the lipoprotein, allowing the lipid to float to the top during the centrifugation process. The conditions used in density gradient centrifugation (high centrifugal force and high salt concentrations) are known to modify lipoprotein composition (Chapman, 1980; Fielding and Fielding, 1991). The results of this experiment, however, support the potential that another, lower density, lipoprotein (possibly a HDL) is present in the plasma of the soft shell clam. All of the lowest density fractions in this experiment fall well within the range of densities of the mammalian HDL class. Under similar density gradient conditions, Pollero and Heras (1989) found only a VHDL in the plasma of *Diplodon delodontus*. Upon recentrifugation of the samples in a gradient designed to resolve the lower portion of the density range, however, they were able to identify a LDL and HDL from the same sample. The majority of the injected ^{14}C palmitate label was recovered from these LDL and HDL fractions.

Comparison of SDS-PAGE gels (not shown) of hemolymph from identified ripe male and female clams showed no discernable band differences to indicate the presence of a female specific peptide. While this suggests that there is no female specific lipoprotein in hemolymph of ripe soft shell clams, the potential existence of a female specific lipoprotein with non-

female specific peptides is not ruled out. Additionally, female specific lipoproteins may be found in the soft shell clam only during certain stages of the reproductive cycle which were not examined.

The female specific lipoproteins of bivalves have been identified primarily in extracts of mature gonads or oocytes. Osada et al. (1992) and Suzuki et al. (1992) suggested that the lipovitellin is synthesized within the oocytes. Recently, Lee et al. (1996) have proposed that, in *Mercenaria mercenaria* vitellin is synthesized in the digestive diverticula, assembled and/or modified in the follicle cells, and accumulated in oocytes. The role of hemolymph in lipid transport for egg production now seems to be relegated to a post spawning cleanup of atretic oocytes (Suzuki et al., 1992; Lee et al., 1996); vitellin has been immunologically identified in hemocytes only after spawning (Lee et al., 1996). While this process may be effective in species anatomically similar to *M. mercenaria* (including *M. arenaria*), the question remains whether the same model can be valid in bivalve species in which the digestive gland and gonad are not in such close physical proximity. If the digestive gland is involved in vitellogenesis in these species, the hemolymph may play an important transport function during oocyte development.

The low concentration of lipid and lipid tracer in the VHDL fraction of *Mya arenaria* suggest that this fraction may

not play an important role in lipid transport processes in the plasma of the soft shell clam. The role of the VHDL in lipid transport, however, cannot be determined without information on the turnover rate of this fraction. Additionally, the VHDL may serve an important function in the physiology of the clam by serving as a carrier for a lipid enzyme or a lipid soluble hormone. An investigation of this potential role of lipoproteins was outside the scope of the experiments presented in this chapter.

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Chapter 4. Preliminary assessment of hsp 70 expression in the soft shell clam *Mya arenaria*

Background

The hsp 70 family of heat shock proteins is composed of proteins with molecular weights in the range 68 - 78 kDa. Some of these proteins are inducible by heat shock and other stress events; other members of the family are constitutively expressed, although their expression may be increased as part of the stress response (Burel et al., 1992). The constitutive and inducible forms of hsp 70 may be functionally similar (Hightower, 1991). While one isoform of hsp 70 is found in the mitochondria, most members of the family are located in the cytoplasm and may translocate to the nucleus and nucleolus as part of the stress response. Organisms may have multiple isoforms of hsp 70 that differ in molecular weight and/or isoelectric point. In the literature, unless the molecular weight of the protein is specified, the term hsp 70 is used broadly to refer to the members of the hsp 70 family in general, without specifying the number or molecular weight of the isoforms.

Bradley and Ward (1989) found one hsp 70, or possibly a doublet, in *Mya arenaria* by SDS-PAGE; the molecular weight of the hsp 70 was not given. In contrast, Smerdon et al. (1995) found 4 hsp 70 isoforms in *Mytilus edulis* by two dimensional

SDS-PAGE (molecular weights 70, 72, 72, 78 kDa). The 72 and 78 kDa proteins are constitutively expressed; heat shock induced the 70 kDa protein and increased the level of expression of the 72 kDa protein.

The constitutive and inducible forms of hsp 70 appear to be functionally similar, with the induction of the heat shock response regulating the total pool of hsp 70 in the cell (Mizzen and Welch, 1988; Welch and Mizzen, 1988). The inducible forms of hsp 70 may differ from the constitutive forms only because the inducible forms lack introns, allowing them to be rapidly synthesized in the stressed cell (Lindquist, 1986).

The role of the hsp 70 family is to stabilize unfolded proteins. In normal cells, hsp 70 binds to nascent polypeptides (Beckmann et al., 1990; Ellis, 1990; Gething and Sambrook, 1992) and also facilitates membrane translocation of proteins into the mitochondria (Ungerman et al., 1994) and the endoplasmic reticulum (Chirico et al., 1988) by maintaining the proteins in an unfolded, translocation-competent state. In the heat shock or stress response, hsp 70 associates with denatured proteins and ribosomes (Ananthan et al., 1986; Pelham, 1986; Parsell and Sauer, 1989; Welch, 1991; Burel et al., 1992; Welch, 1992). Hsp 70 is also involved in targeting proteins for degradation in lysosomes (Chiang et al., 1989). The bacterial homolog of hsp 70, DnaK, appears be essential for the refolding of denatured proteins (Gaitanaris et al.,

1990; Skowyra et al., 1990). ATP hydrolysis is required for the dissociation of the hsp 70-polypeptide complex; the energy of hydrolysis causes a conformational change in hsp 70. The requirement for ATP hydrolysis may provide a timed release mechanism, allowing the peptide to follow the correct folding process in a regulated fashion (Gething and Sambrook, 1992).

The stress response is induced by the accumulation of denatured proteins in the cell (Ananthan et al., 1986; Parsell and Sauer, 1989). The common mechanism of heat and chemical stressors is the denaturation of proteins or proteotoxicity (Hightower, 1993). Many chemicals have been shown to induce the stress or heat shock response, including ethanol, certain heavy metal ions (As, Cd, Zn, Cu, Hg, Pb, Ag), sulfhydral reagents, steroid hormones, chelating agents, pyridoxine, methylene blue, glucosamine, deoxyglucose, sodium dodecylsulfate, tributyltin, ß-naphthoflavone, and benzo(a)pyrene (Lindquist, 1986; Lindquist and Craig, 1988; Köhler et al., 1992; Bradley, 1993; Monaghan and Bradley, 1993; Steinert and Pickwell, 1993). The response may also be induced by anoxia, RNA and DNA viruses, and reactive oxygen intermediates (Lindquist, 1986; Kaufmann, 1990; Winrow et al., 1990).

Since the expression of hsp 70 increases in response to generic stress events, as long as these events damage proteins, hsp 70 has been suggested as an excellent biomarker of general stress for environmental toxicology (Sanders, 1990;

Bradley, 1993; Steinert and Pickwell, 1993; Ryan and Hightower, 1994). Whereas numerous studies have examined the heat shock or stress response in lab situations, very few field tests have been done. There is some concern that the heat shock response is transient (Sanders, 1990; Welch, 1992), bringing into question the utility of the stress response for environmental monitoring of populations chronically exposed to environmental contaminants under natural conditions. When *Mytilus edulis* was exposed to elevated temperatures for a period of two months, however, the stress response remained for the duration of the experiment (Sanders et al. 1992), suggesting that the stress response is not transient in organisms experiencing chronic severe heat stress.

Hsp expression in *Mytilus edulis* has been increased in the lab by exposure to many inducers, including heat shock (Sanders, 1988; Veldhuizen-Tsoerkan et al., 1990; Sanders et al., 1992; Smerdon et al., 1995), cadmium (Sanders, 1988; Veldhuizen-Tsoerkan et al., 1990; Brown et al., 1995), tributyltin (Steinert and Pickwell, 1993), and copper (Sanders et al., 1991; Sanders et al., 1994a). Hsps have also been induced by the organic compounds benzo(a)pyrene and ß-naphthoflavone in medaka and channel catfish, respectively (Monaghan and Bradley, 1993). A stress response was also seen in *Escherichia coli* exposed to benzene, chlorpyrivos, 2,4-dichloroaniline, dioctylphtalate, hexachlorobenzene, pentachlorophenol, trichloroethylene, and

tetrapropylbenzosulfonate (Blom et al., 1992); some of the induced stress proteins were hsps, while others were specific to the inducing chemical. All of these hsp-inducing stressors are potential environmental contaminants.

A few investigations of hsp concentrations in organisms collected from natural populations have been published. *Mytilus trossulus* hsp 70 expression is positively correlated with *in situ* body temperatures in the intertidal zone (Hofmann and Somero, 1995). The stream fish *Pimephales promelas*, *Salmo trutta*, *Ictalurus natalis*, and *Ambloplites rupestris* showed significant seasonal variation in hsp 70 concentration, with the highest levels in the spring, at the season of greatest temperature increase, but not highest absolute temperature (Fader et al., 1994).

Veldhuizen-Tsoerkan (1991) and colleagues transplanted mussels from a relatively unpolluted area to sites in the Western Scheldt of the North Sea contaminated with cadmium, copper, zinc, nickel, chromium, lead, PCBs, and pesticides. After 5 months, the transplanted animals showed no elevations in hsp synthesis, as measured by incorporation of ^{35}S-methionine. Sanders and Martin (1993) measured elevated hsp 70 concentrations in some specimens of *Mytilus edulis* and various fish species collected from contaminated sites and archived as part of the NOAA National Status and Trends (NS&T) Program. All of the mussels used in this study were collected from NS&T sites ranked among the top 20 sites in the country

for at least one of the measured contaminants. Although no samples from relatively uncontaminated natural populations were used in the study, the levels of hsp 70 in the tissue samples were compared to laboratory-held controls. Most of the sites show elevated hsp 70 concentrations relative to the laboratory controls, reaching values for laboratory heat shocked samples in some instances.

The intent of the group of experiments described in the next three chapters was to determine whether hsp 70 expression could be seen in natural populations of the soft shell clam *Mya arenaria*. The observed levels of hsp 70 in gonad and gill (chapters 5 and 6, respectively) are discussed in relation to possible stress response inducers, including contaminant exposure, sex and reproductive stage. The experiments described in this chapter were designed as a preliminary survey of hsp 70 expression in natural populations of the soft shell clam *Mya arenaria.*

Materials and Methods

Sample collection and processing

Soft shell clams were collected from the intertidal zone in New Bedford Harbor and Little Buttermilk Bay (Figure 4.1) by hand digging. The clams from the Cotuit site were collected subtidally. Ten clams from each site were collected in August 1994. The water temperature was recorded at each

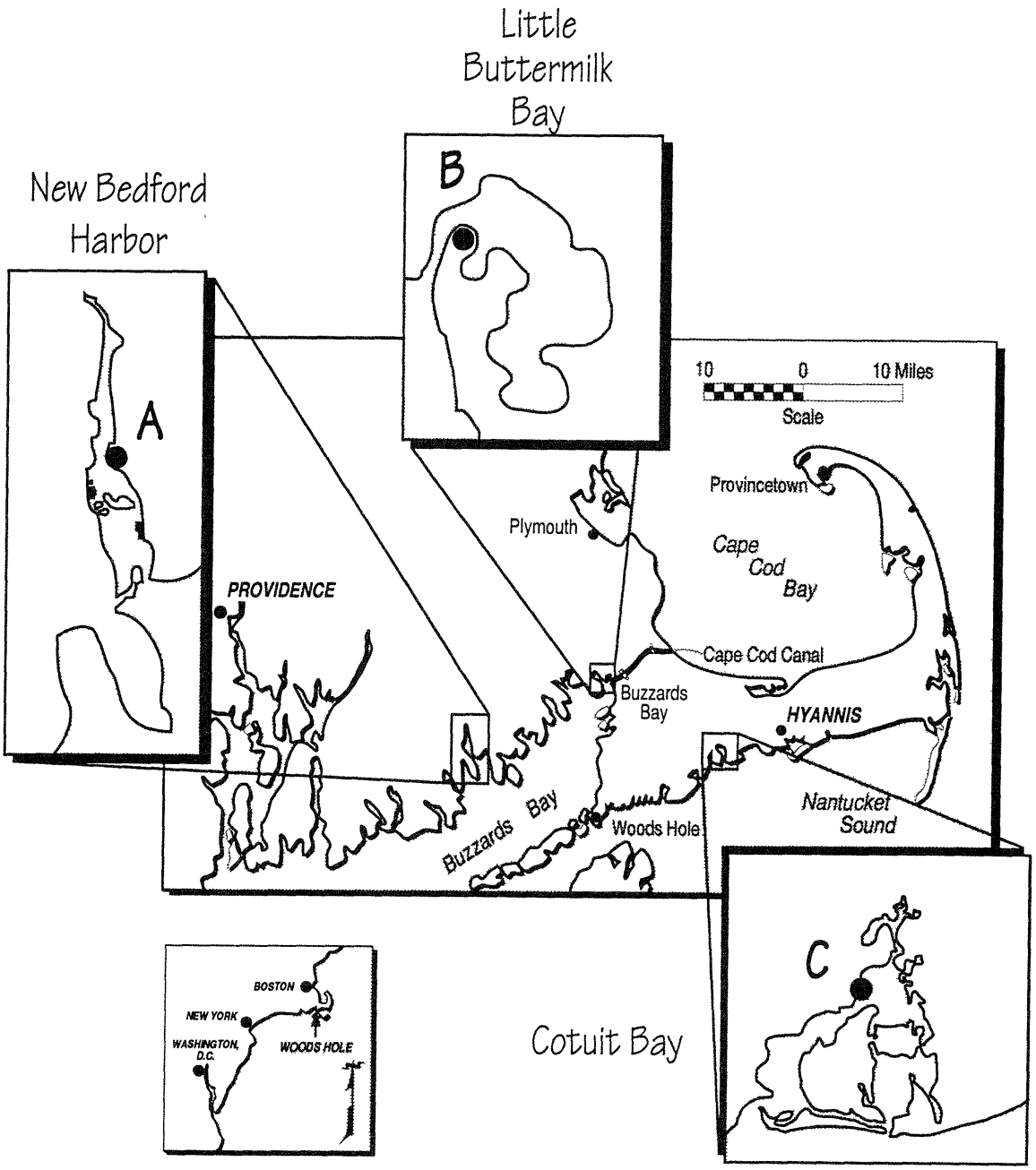

Figure 4.1. A map depicting the location of the collection sites for *Mya arenaria*.

site.

The clams were transported back to the lab on ice and processed immediately. Length and total wet weight of each clam was recorded before sacrifice. The tissues were dissected, rinsed in Tris buffer (0.5 M, pH 8), and roughly chopped. Tissues collected included gill, gonad, digestive gland, foot, adductor muscles, and siphon. The tissues were stored at -70 °C for no more than a day, until they could be homogenized in homogenization buffer (66 mM Tris, pH 7.5, 0.1 % Nonidet, 0.1 mM PMSF, and 0.1 % aprotinin); the hypotonic buffer lyses cells and mitochondria (Sanders et al., 1992). After homogenization, the samples were sonicated for 30 minutes and then stored at -70 °C. The homogenized samples were centrifuged for 90 minutes at 4 °C and 150,000 x g in a Beckman L8-M ultracentrifuge with a 70.1 Ti fixed angle rotor. The supernatant was retained and stored at -70 °C. Total soluble protein was measured by microplate BCA assay using bovine serum albumin as a standard (Redinbaugh and Turley, 1986).

Western blotting

Proteins (100 µg total protein per sample) were denatured in Laemmli (1970) sample buffer, boiled for 5 minutes, separated on 4-20 % gradient SDS-PAGE gels (BIO-RAD 161-0903) and blotted onto 0.45 µm nitrocellulose membranes (Towbin et al., 1979). Hsp 70 purified from bovine brain (Sigma H-1523)

was used as a positive control. The nitrocellulose membranes were blocked for 1 hour at 42 °C in Tris buffered saline (TBS)/milk (5 % non-fat dry milk in 20 mM Tris, 0.5 M NaCl, pH 7.5). The blots were incubated for 1 hour in monoclonal antibody (clone BRM-22) raised against bovine brain hsp 70 (Sigma H-5147) diluted 1:1000 in TBS/milk. Goat anti-mouse horseradish peroxidase-conjugated secondary antibody (BIO-RAD 170-6516) was used with 3,3'-diaminobenzidine (DAB) substrate (Sigma D-4418) to visualize the probed antigen.

Enzyme linked immunosorbent assay (ELISA)

Dynatech Immulon 4 96 well plates (Fisher 14-245-153) were loaded with 100 µg total protein per well; the samples were diluted to equal volume (100 µL) with Tris buffered saline (20 mM Tris, 0.5 M NaCl, pH 7.5). Replicate wells were loaded for each sample. Samples were incubated overnight at 4 °C before rinsing 3 times with 200 µL wash buffer (0.5 M NaCl, 20 mM Na_2HPO_4, and 0.05 % Tween, pH 7.2). The wells were blocked for 1 hour at room temperature with 100 µL PBS-Blotto (4 % non-fat dry milk in 0.15 M NaCl, 10 mM Na_2HPO_4, pH 7.2). Optimal dilutions of the primary and secondary antibodies were initially determined by a standard checkerboard assay. The samples were incubated with 100 µL of primary antibody (clone BRM-22 monoclonal anti-hsp 70) at a dilution of 1:1000 in PBS-Blotto for 1 hour at room temperature. The wells were rinsed in wash buffer before incubation with secondary antibody (goat

anti-mouse conjugate) diluted 1:1000 in PBS-Blotto for 1 hour at room temperature. After a final rinse cycle, 100 µL of enzyme substrate (20 mL 0.1 M sodium citrate, 1 mL 40 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma A-1888), and 20 µL H_2O_2) was added and the reaction was allowed to proceed for 30 minutes in the dark at room temperature. Absorbance of the samples at 405 nm was measured on an Emax microplate reader (Molecular Devices).

Mean optical density at 405 nm was determined for each of the samples after discarding replicate wells with absorbances more than one standard deviation from the mean as spurious outliers. Optical density values were converted to ng hsp 70 per mg total protein by using purified bovine brain hsp 70 to derive a standard curve. Samples with mean hsp 70 concentrations ≤ 0 were regarded as being below the detection limit of the assay and were assigned a hsp 70 concentration of 0 in all further calculations.

Heat shock experiments

Hsp 70 expression after heat shock was examined in two experiments. In one study, clams and mussels (*Mytilus edulis*) from Little Buttermilk Bay were divided into two treatment groups: control and heat shocked. Heat shocked clams (n = 3) and mussels (n = 3) were exposed to 32 °C flow-through seawater for 24 hours; the control clams (n = 3) and mussels (n = 3) were maintained in flow-through seawater at the

ambient temperature of 21 °C. In another season, when the ambient water temperature was lower, clams (n = 3 per temperature) were maintained at ambient temperature (14 °C) or subjected to a heat shock of 25 °C for 24 hours. In both of these experiments, gill and gonad tissues were removed immediately after the heat shock and processed as described in a previous section. Hsp 70 expression was measured by ELISA.

Comparisons of commercially available anti-hsp 70 antibodies

Three commercially available anti-hsp 70 monoclonal antibodies were compared by ELISA (clone BRM-22, Sigma; clone 3a3, Affinity BioReagents; and clone N27F3-4, StressGen). The Sigma antibody (clone BRM-22) was produced with purified bovine brain hsp 70 as an immunogen. The Affinity BioReagents antibody (clone 3a3) was produced with recombinant human hsp 70 expressed in *E. coli* as an immunogen; the human hsp 70 epitope recognized by this antibody appears to be in a region of the protein involved in stress-induced nucleolar localization (S.P. Murphy and R.I. Morimoto, unpublished data). The StressGen antibody (clone N27F3-4) was produced with hsp 70 isolated from human HeLa cells as an immunogen. Each antibody was tested on the same five samples: hsp70 purified from bovine brain and four clam samples (2 gill and 2 gonad). Each antibody was tested at the same dilution (1:1000). The significance of hsp 70 concentration differences, as measured by the different antibodies, was

evaluated by paired t-tests.

The cross reactivity of the three antibodies was also examined by western blotting. Clams were subjected to a heat shock of 25 °C (n = 3) or maintained at the ambient temperature of 14 °C (n = 3) for 24 hours. The gill was removed from the clams immediately after the exposure period; the samples were processed in a previous section. The samples (250 µg total protein) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted as described in the western blotting section. Replicate blots were probed separately with each of the three tested antibodies, at a dilution of 1:1000.

Results

Hsp 70 in *Mya arenaria* reacts with the commercially available monoclonal anti-hsp 70 antibody raised against bovine brain hsp 70 (Sigma, clone BRM-22) and is present in clams collected from natural populations as determined by western blotting (Figures 4.2 and 4.3). The antibody recognizes only one band of approximately 70 kDa in each sample.

Hsp 70 concentrations in gill and gonad of soft shell clams and blue mussels, as measured by ELISA, were not significantly increased in animals which were heat shocked by a temperature increase from 21 to 32 °C (Figure 4.4). In

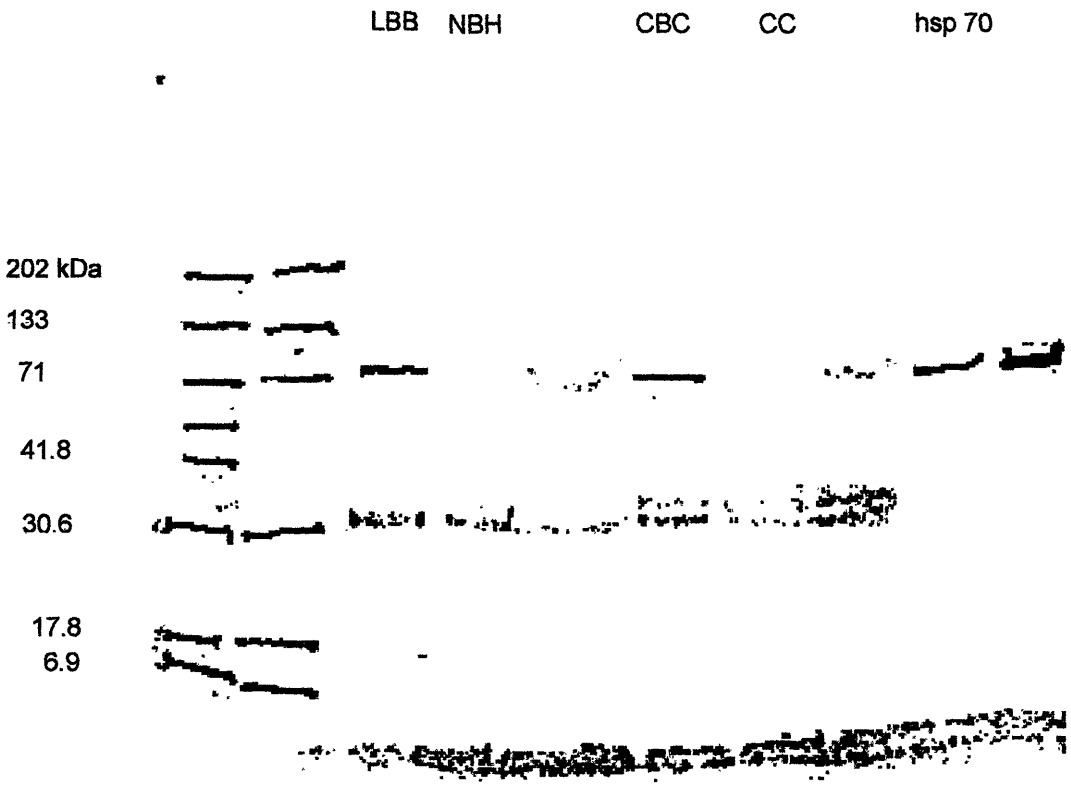

Figure 4.2. A western blot demonstrating the recognition of an approximately 70 kilodalton band in clam peptides probed with the anti-hsp 70 antibody clone BRM-22 (Sigma). Lanes 1 and 2 were loaded with molecular weight standards, and lanes 9 and 10 contain bovine brain hsp 70 as a positive control. Gill tissue samples (250 µg per lane) collected in September 1994 from Little Buttermilk Bay, New Bedford Harbor, the Cotuit Beach Club, and the Cotuit Control site were loaded in lanes 3, 4, 6, and 7, respectively. The staining seen in lanes 5 and 8 is the result of carryover from adjacent lanes. The bands seen between 30 and 40 kDa are breakdown products of hsp 70 during lengthy storage (more than one year at -70 °C); this crossreactivity was not seen in western blots run at the same time as the ELISAs.

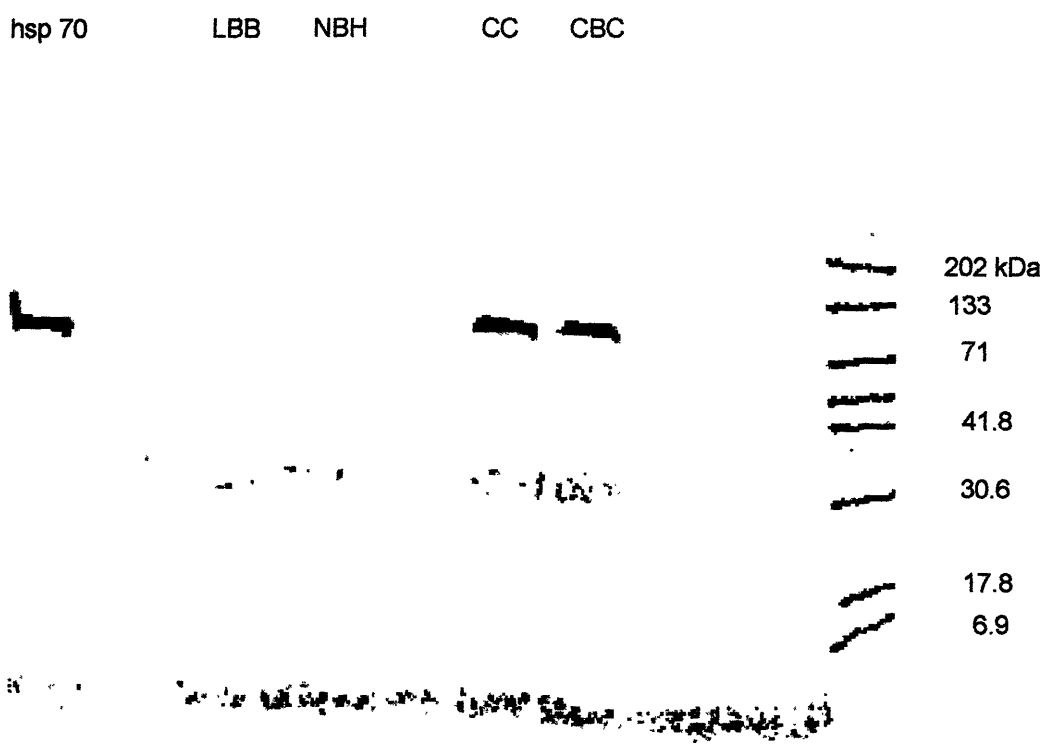

Figure 4.3. A western blot demonstrating the recognition of an approximately 70 kilodalton band in clam peptides probed with the anti-hsp 70 antibody clone BRM-22 (Sigma). Lane 10 was loaded with molecular weight standards, and lane 1 contains bovine brain hsp 70 as a positive control. Gill tissue samples (250 µg per lane) collected in January 1995 from Little Buttermilk Bay, New Bedford Harbor, the Cotuit Beach Club, and the Cotuit Control site were loaded in lanes 3, 4, 6, and 7, respectively. The bands seen between 30 and 40 kDa are breakdown products of hsp 70 during lengthy storage (more than one year at -70 °C); this crossreactivity was not seen in western blots run at the same time as the ELISAs.

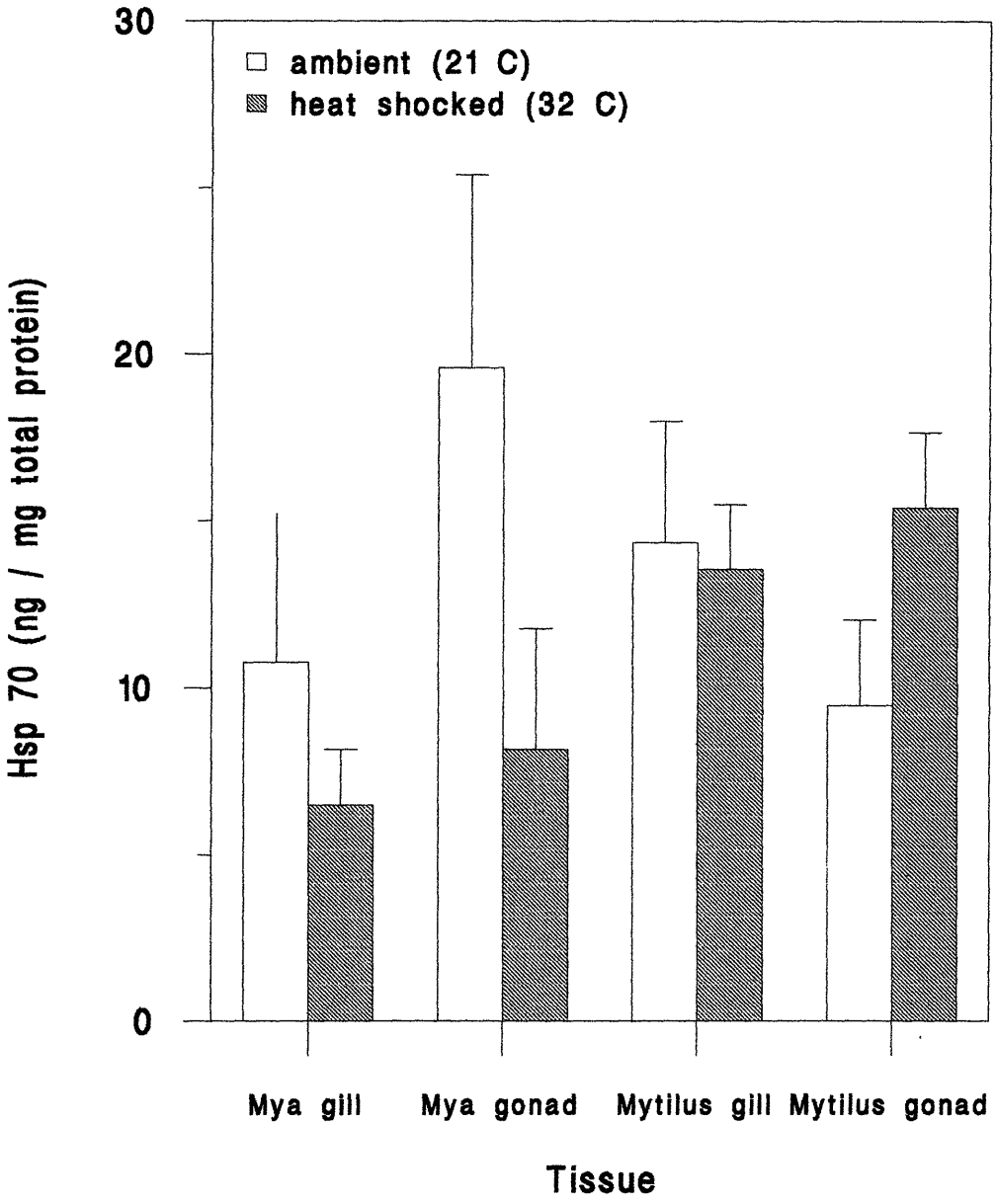

Figure 4.4. The concentration of hsp 70 in heat shocked and control clams (*Mya arenaria*) and mussels (*Mytilus edulis*). The animals were heat shocked for 24 hours and were sacrificed immediately, without a recovery period at ambient temperature. Each bar represents the mean ± standard error (n = 3).

contrast, clams subjected to an 11 °C heat shock from 14 to 25 °C showed elevated hsp 70 expression in the gonad but not in the gill (Figure 4.5).

Differences in hsp 70 concentration among various tissues of the soft shell clam (gill, gonad, foot, siphon, adductor muscle, and siphon) are depicted in Figure 4.6. In general, gill had the highest hsp 70 concentration, followed by foot, siphon and gonad. Hsp 70 concentration in the adductor muscle was low. Concentrations in the digestive gland were the most variable.

The primary between site difference observed was the high hsp 70 concentration in the digestive gland of clams from the Cotuit site. Site differences can also be observed in the gill and gonad, with lower hsp 70 expression in clams collected from Little Buttermilk Bay. The lack of other between site differences may be due to the low sample size (n = 10).

A comparison of the commercially available monoclonal antibodies revealed few differences in cross reactivity of the antibodies with hsp 70 from soft shell clam gill and gonad or bovine brain (Table 4.1). The only significant difference was that the concentration of hsp 70 in the samples as measured with the BRM-22 clone (Sigma) was significantly ($p < 0.05$) higher than the concentration as measured with the StressGen antibody (clone N27F3-4). In contrast, comparisons of the antibodies by western blotting showed similar cross

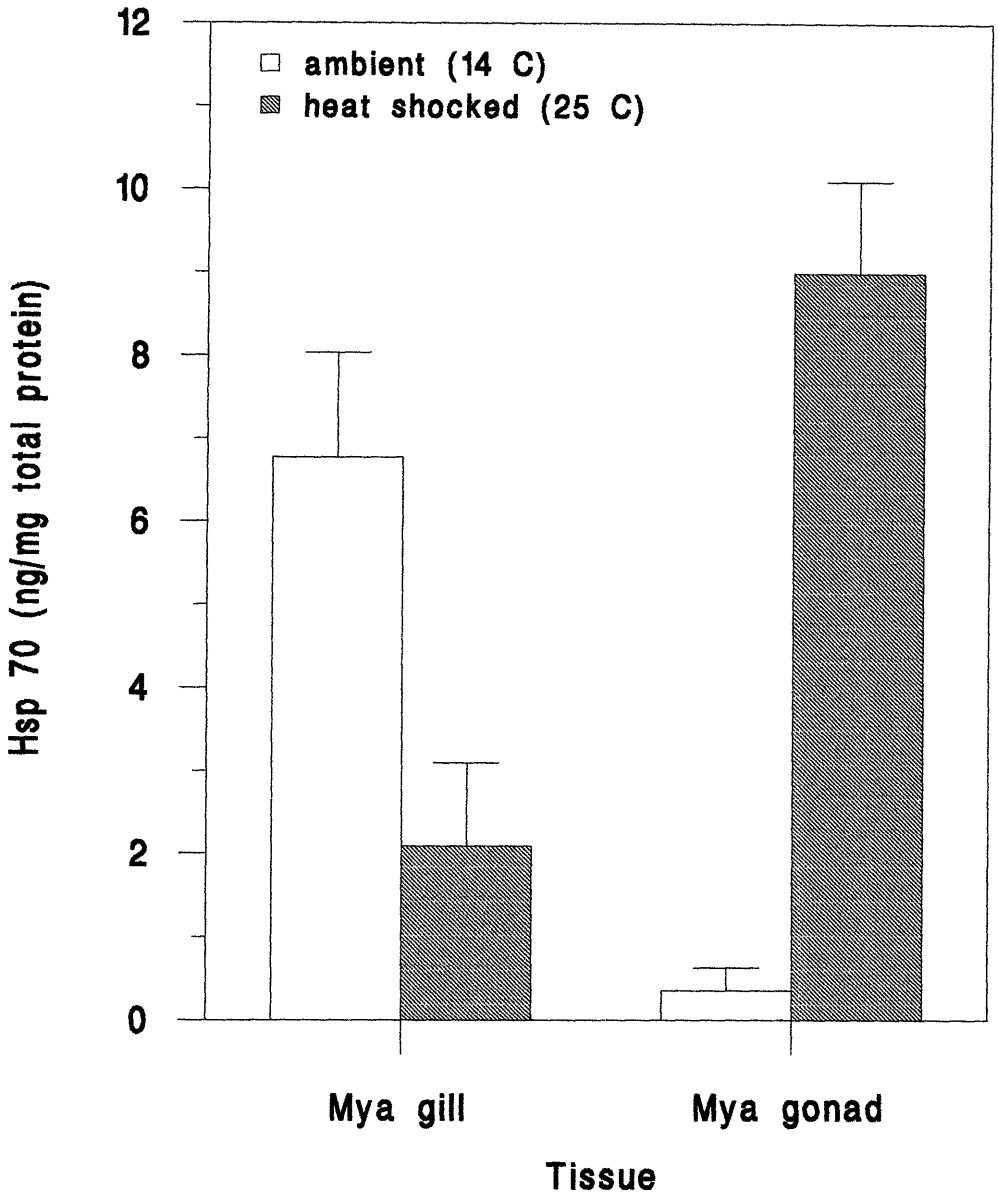

Figure 4.5. The concentration of hsp 70 in heat shocked and control clams (*Mya arenaria*). The animals were heat shocked for 24 hours and were sacrificed immediately, without a recovery period at ambient temperature. Each bar represents the mean ± standard error (n = 3).

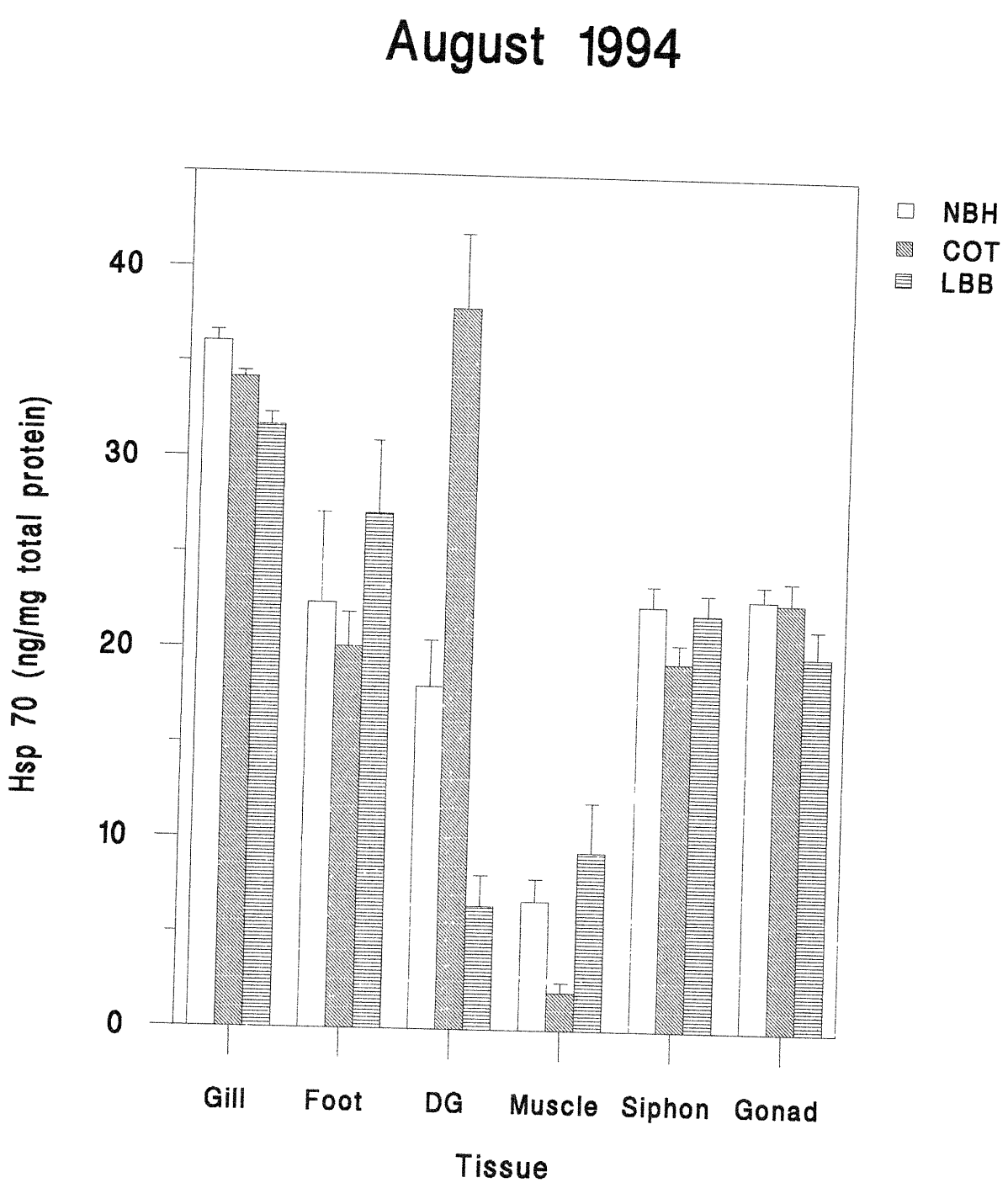

Figure 4.6. Tissue differences in hsp 70 concentration (ng/mg total protein) in *Mya arenaria*. All of the clams used in this experiment were collected in August 1994. Each bar represents the mean ± standard error (n = 10 clams per site).

Table 4.1. A summary of the hsp 70 concentrations of four clam samples and a positive control (bovine brain hsp 70) as measured by ELISA using three commercially available anti-hsp 70 monoclonal antibodies.

	Hsp 70 (ng/mg total protein)				Hsp 70 (ug)
Antibody	Clam 1	Clam 2	Clam 3	Clam 4	Hsp 70
BRM-22 (Sigma)	33.66	45.94	41.38	48.75	0.12
3a3 (Affinity BioReagents)	29.80	57.52	36.12	32.96	0.14
N27 F3-4 (StressGen)	23.84	32.96	28.75	28.75	0.14

reactivities for the BRM-22 clone (Figure 4.7) and the N27F3-4 clone (Figure 4.8) while the 3a3 clone did not cross react with any of the clam samples (Figure 4.9).

Discussion

Mya arenaria contains one protein of approximately 70 kDa which cross reacts with a monoclonal antibody to hsp 70. This protein is present in tissues of clams collected from natural populations, as observed by both western blotting and ELISA. The initial observation that the BRM-22 antibody recognized a hsp 70 band in *Mya arenaria* was made by western blotting. Subsequent measurements of hsp 70 concentration were all made by ELISA. The ELISA assay was developed to increase the number of samples that could be processed. High individual variability is seen in the expression of hsp 70 in whole animals in laboratory (Fargnoli et al., 1990) and field experiments (Yu et al., 1994); the variability problem makes the interpretation of western blot results difficult, and requires large sample sizes for statistical analysis which can only be obtained by using ELISA. In the samples which were analyzed by both western blot and ELISA, the expression patterns of hsp 70 as measured by the two methods were similar.

Induction of hsp 70 in the gill by heat shock was not seen in these experiments. Two possible explanations for this

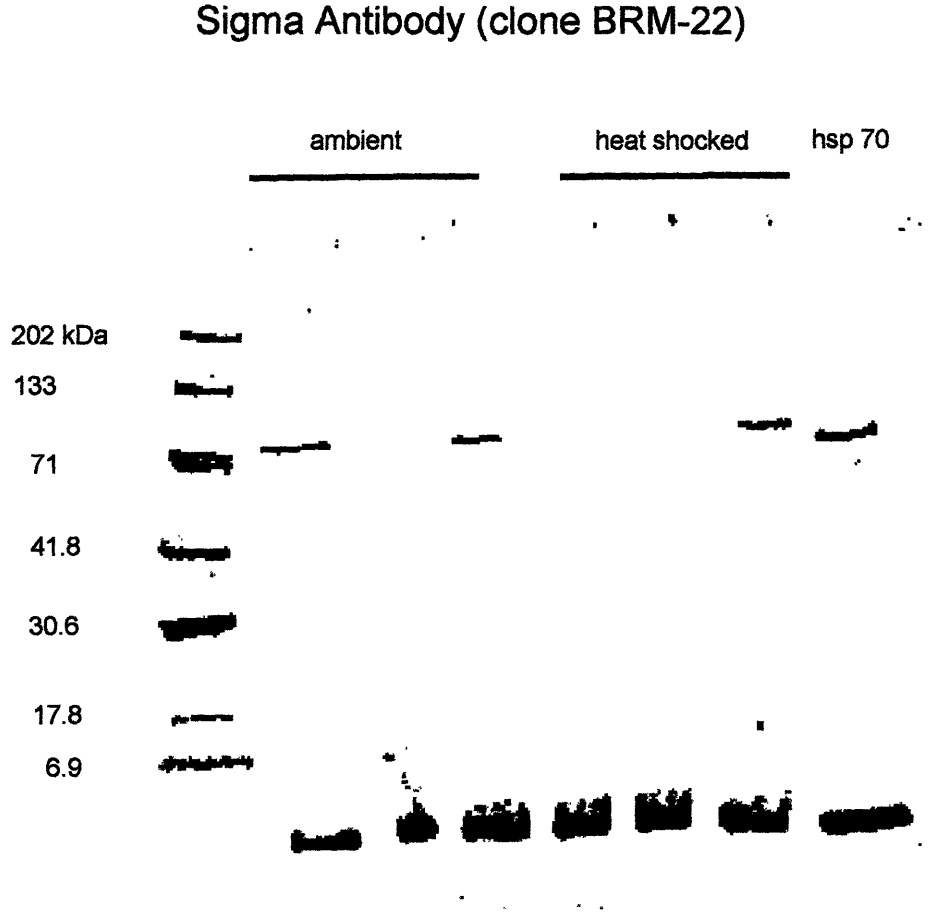

Figure 4.7. A western blot demonstrating the recognition of an approximately 70 kilodalton band in clam peptides probed with the anti-hsp 70 antibody clone BRM-22 (Sigma). Lane 2 was loaded with molecular weight standards, and lane 9 contains bovine brain hsp 70 as a positive control. Gill tissue samples (250 µg per lane) from clams maintained at ambient temperature (14 °C) and at a heat shock temperature (25 °C) were loaded in lanes 3-5 and 6-8, respectively.

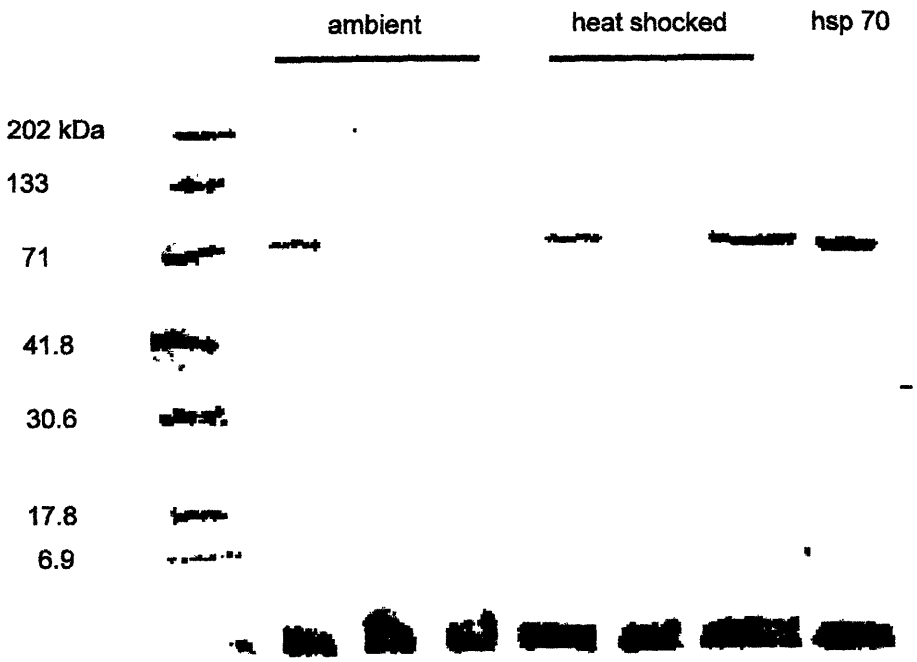

Figure 4.8. A western blot demonstrating the recognition of an approximately 70 kilodalton band in clam peptides probed with the anti-hsp 70 antibody clone N27F3-4 (StressGen). Lane 2 was loaded with molecular weight standards, and lane 9 contains bovine brain hsp 70 as a positive control. Gill tissue samples (250 µg per lane) from clams maintained at ambient temperature (14 °C) and at a heat shock temperature (25 °C) were loaded in lanes 3-5 and 6-8, respectively.

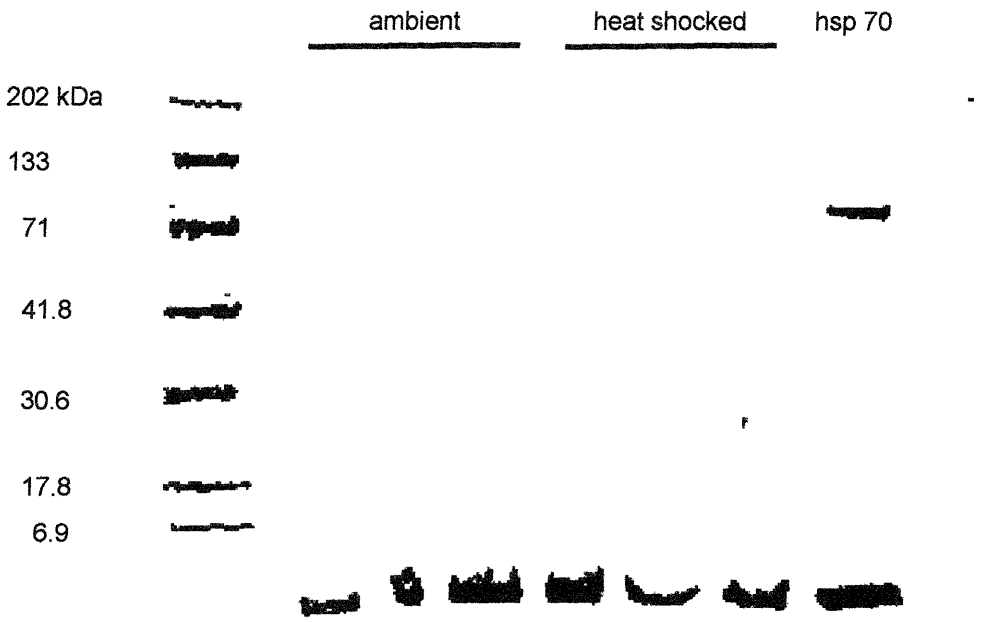

Figure 4.9. A western blot demonstrating the nonrecognition of hsp 70 band in clam peptides probed with the anti-hsp 70 antibody clone 3a3 (Affinity BioReagents). Lane 2 was loaded with molecular weight standards, and lane 9 contains bovine brain hsp 70 as a positive control. Gill tissue samples (250 µg per lane) from clams maintained at ambient temperature (14 °C) and at a heat shock temperature (25 °C) were loaded in lanes 3-5 and 6-8, respectively.

result are that (1) hsp 70 was already maximally expressed at the lower temperature (14 or 21 °C) or (2) the BRM-22 antibody is specific for constitutively expressed hsp 70 in *Mya arenaria*. The heat shock temperatures used in these experiments (25 and 32 °C) are at the extreme end of the temperature range tolerated by *M. arenaria*. As a result, hsp 70 induction by heat shock may already have been at a maximum in the animals held at ambient temperature. The small sample size may also have been a factor in the observed pattern, particularly because of the observed tissue differences at the lower temperatures (Figure 4.5).

Additional support for the hypothesis that hsp 70 expression is already at a maximum at the ambient temperatures is that hsp 70 concentrations declined at the higher temperature, suggesting that protein synthesis was shut down. While induction of hsp 70 at 27 °C in *Mytilus edulis* has been described previously (Sanders et al., 1992), higher temperatures were not investigated. Continuous induction of hsp 70 at 27 °C (Sanders et al., 1992) suggests that the animals were under severe stress. A decrease in protein synthesis, as measured by ^{35}S-methionine incorporation, was observed in mussels held at 30 °C for 4 to 16 hours (Veldhuizen-Tsoerkan et al., 1990). These results suggest that clams and mussels heat shocked at 25 and 32 °C for 24 hours are under severe stress in which protein synthesis is reduced and, consequently, hsp 70 induction is not observed.

The heat shock experiments are preliminary, however, and the interpretation of the results is constrained by the small sample size and by the ambient water temperatures at the time of year in which the experiements were conducted.

The other possible conclusion is that the antibody used does not recognize inducible forms of hsp 70 in bivalves, although it recognizes both constitutive and inducible forms of hsp 70 in mammals (Sanders et al., 1994b). The recognition patterns of the most commonly used anti-hsp 70 antibodies are summarized in Table 4.2. The only definitive statement that can be made about antibody recognition is that it is quite variable. Sanders et al. (1994b) tested the commercially available anti-hsp 70 monoclonal antibodies in a variety of species. In *Mytilus edulis* and *M. californianus*, the BRM-22 antibody reacted with a 78 kDa peptide, whereas the other tested antibodies (3a3, Affinity BioReagents and N27, StressGen) reacted to a 72 kDa peptide in the same species. A polyclonal anti-hsp 70 antibody recognizes a doublet at 70 and 72 kDa in *M. edulis* and a single band (molecular weight not given) in *M. californianus*. Smerdon et al. (1995) detected 4 hsp 70 isoforms in *Mytilus edulis* using the monoclonal antibody clone 5a5 (Affinity BioReagents), in contrast to the results of Sanders et al. (1994b), who found no cross reactivity of this clone in any of the tested fish and invertebrate species, including *M. edulis*.

A small test of the response of three antibodies with

Table 4.2. A summary of the recognition patterns of the most commonly used anti-hsp 70 antibodies.

Antibody	Species	hsp 70 isoform(s)	Reference
polyclonal (anti-human hsp 70)	mammals	70,72 kDa	Sanders et al., 1994b
	Mytilus edulis	70,72 kDa	"
	Mytilus californianus	approx. 70 kDa	"
	Macoma nastuta	approx. 70 kDa	"
	Crassostrea virginica	approx. 70 kDa	"
	M. edulis	not given	Sanders and Martin, 1993
	M. edulis	74 kDa	Sanders et al., 1994a
	M. edulis	70,72 kDa mantle 70 kDa gill	Sanders et al., 1992
	Mya arenaria	not given (doublet ?)	Bradley and Ward, 1989
C92 F3A-5 (StressGen)	mammals	70 kDa	Sanders et al., 1994b
	M. edulis	none	
N27 F3-4 (StressGen)	mammals	70,72 kDa	Sanders et al., 1994b
	M. edulis	72 kDa	"
	M. californianus	72 kDa	"
	M. edulis	approx. 70 kDa	Steinert and Pickwell, 1993
3a3 (Affinity BioReagents)	mammals	70,72 kDa	Sanders et al., 1994b
	M. edulis	72 kDa	"
	M. californianus	72 kDa	"
5a5 (Affinity BioReagents)	humans (HeLa cells)	70,70,72,75 kDa	Murphy et al., unpublished
	fish and invertebrates	none	Sanders et al., 1994b
	M. edulis	70,72,72,78 kDa	Smerdon et al., 1995
BRM-22 (Sigma)	mammals	72,73 kDa	
	mammals	70 kDa	Sanders et al., 1994b
	M. edulis	78 kDa	"
	M. californianus	78 kDa	"
	M. arenaria		this study

clams from natural populations in the ELISA protocol revealed few differences. Hsp 70 concentrations as measured with the BRM-22 clone were the same or higher than the concentrations measured with the other tested antibodies, suggesting that the cross reactivity of the clam hsp 70 was similar with all of the antibodies. When compared with the results of Sanders et al. (1994b), these data suggest that either the samples contained no inducible isoforms of hsp 70, or that the BRM-22 antibody recognizes both constitutive and inducible forms of hsp 70 in *M. arenaria*. Further studies will be required to completely characterize the cross reactivities of the various commercially available antibodies and to determine the validity of comparing results obtained by using different antibodies. For the purposes of the remaining experiments described in chapters 4-6, only the Sigma antibody (clone BRM-22) was used; the protein recognized by the BRM-22 antibody will be referred to as hsp 70.

Tissue specific differences were seen in hsp 70 concentration in the soft shell clam. Generally, the highest concentrations were found in tissues in direct contact with the environment (gill, foot, and siphon). Similar levels were also seen in gonad while adductor muscle, another internal organ, had low concentrations. Digestive gland showed the greatest variability in hsp 70 concentration; the clams were not allowed to depurate before dissection, so the digestive gland variability may reflect differences in feeding among the

clams.

Further experiments to examine the potential causes of hsp 70 expression in gonad and gill are discussed in following chapters (chapters 5 and 6, respectively). Gonad and gill were chosen for further investigation because both tissues are known to be impacted by contaminants and because the expression of hsp 70 in both tissues is potentially affected by normal physiological processes, including reproduction.

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Chapter 5. Hsp 70 expression in gonadal tissue of *Mya arenaria*: Differences related to reproductive stage and collection site

Background

Reproduction is one of the major stress events in the life of soft shell clams and other organisms; clearly, however, the reproductive process is considered to be a normal part of the life cycle. Since heat shock proteins are involved in the synthesis of new proteins (Beckmann et al., 1990; Ellis, 1990; Gething and Sambrook, 1992) and in the stabilization and degradation pathways for abnormal or damaged proteins (Ananthan et al., 1986; Pelham, 1986; Chiang, 1989; Parsell and Sauer, 1989; Welch, 1991; Burel et al., 1992; Welch, 1992), one of the normal functions of heat shock proteins may be differential expression in the gonad during the reproductive cycle.

One family of heat shock proteins, the low molecular weight hsps, are known to be developmentally regulated, at least in part through the action of hormones (Kurtz et al., 1986; Vazquez, 1991; Billoud et al., 1993; de Jong et al., 1993). The induction of hsps is uncoupled during development, in contrast to the coordinate induction during heat shock (Kurtz et al., 1986). The hsp 70 family also exhibits some differential expression that appears to be developmentally

regulated. Mouse embryos at the blastocyst stage have high levels of active hsp transcription factor and high synthesis of constitutive hsps (Burel et al., 1992). The first proteins synthesized from transcribed zygotic genes in mouse embryos are members of the hsp 70 family (Bensaude et al., 1983).

Different patterns of hsp expression are also seen in gonadal tissue. In *Drosophila*, constitutive hsp 70 expression is elevated in the ovary (Kurtz et al., 1986). In amphibians, hsp 70 that is strictly inducible in somatic tissues is constitutively active in oocytes and follicle cells during oogenesis (Billoud et al., 1993). The inhibition of hsp induction seen in *Drosophila* oocytes (Lindquist, 1986) and early mouse embryos could be the result of the high levels of hsps that are already present in these cells (Morange et al., 1984). In male mice, two members of the hsp 70 family are expressed in the testes during spermatogenesis (Zakeri and Wolgemuth, 1987; Zakeri et al., 1988).

The functions of hsp 70, including stabilization of nascent polypeptides (Beckmann et al., 1990; Ellis, 1990; Gething and Sambrook, 1992), solubilization of denatured proteins (Ananthan et al., 1986; Pelham, 1986; Parsell and Sauer, 1989; Welch, 1991; Burel et al., 1992; Welch, 1992), and involvement in protein degradation pathways (Chiang et al., 1989), suggest that hsp 70 may be expressed in the gonad during and after gametogenesis. Expression of hsp 70 linked to the reproductive cycle may interfere with the

interpretation of hsp 70 expression as a biomarker of contaminant effects. The experiment described in this chapter, therefore, attempts to describe the nature of hsp 70 expression during the reproductive cycle of clams collected from contaminated and reference populations.

Materials and Methods

Sample collection and processing

Soft shell clams were collected from four sites in Massachusetts: the intertidal zone in Little Buttermilk Bay (LBB) and New Bedford Harbor (NBH) and the subtidal zone at two adjacent sites in Cotuit (Figure 4.1). Ten clams were collected from each site in August 1994; the sample size was increased to 20 clams per site at the remaining sampling times in September 1994, and January, March, and May 1995. Ambient water temperature was recorded at each site. The clams were transported to the lab on ice. A subsample of the gonad was removed from each clam and processed as described in chapter 4.

New Bedford Harbor has been designated as a Superfund site because of the high concentrations of PCBs found in the sediments. As an urban harbor, NBH also has high levels of petroleum-derived hydrocarbons and some heavy metals. The samples for this study were collected at Tin Can Island, a location within the zone that is closed to commercial

shellfishing. Little Buttermilk Bay is a relatively pristine control site that is generally open to commercial and recreational diggers; LBB is occasionally closed to shellfishing for management reasons or because of high fecal coliform counts. The two Cotuit sites are designated Cotuit Beach Club (CBC) and Cotuit control (CC). The Cotuit Bay Shores Beach Club is a private beach. During World War II, the site was used by the military to practice amphibious landings and was known as Camp Candoit; in 1990, residents began to notice pools of what appeared to be oil on the surface of the sand at some spots. The site appears to have been contaminated with petroleum hydrocarbons of unknown origin, probably as the result of leakage from an old underground fuel storage tank. Clams were collected subtidally at the Cotuit sites because the scheduled remediation of the former Camp Candoit by the U.S. Army Corps of Engineers included the disturbance of the intertidal zone at this site. Although this site is closed for shellfish harvesting, adjacent sites, including the Cotuit control site, are commercially harvested.

Reproductive stage

A subsample of the gonad was collected at the time of processing and preserved in 10 % formalin in filtered seawater (0.45 µm). The tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin at the Marine

Biological Laboratory, Woods Hole. The sections were examined by light microscopy and staged according to the criteria proposed by Ropes and Stickney (1965). The five reproductive stages are indifferent, developing, ripe, spawning, and spent. The sex of each clam was also determined.

Chemical analysis

Five clams were collected from each site in September 1995, and from the two Cotuit sites in May 1995 and stored at -70 °C; the samples were shipped on dry ice to the laboratory of Dr. Damian Shea at North Carolina State University for chemical analysis, according to the procedures outlined by Shea and Seavey (1994). Soft tissue wet weight, dry weight, and lipid content were recorded for the pooled samples. The samples were extracted in 1:1 dichloromethane:acetone and passed through clean up steps, including processing through silica/alumina columns and by gel permeation chromatography. Sample extracts in dichloromethane were analyzed for polycyclic aromatic hydrocarbons (PAHs) and linear alkyl benzenes (LABs) by gas chromatography/mass spectrometry (GC/MS). Extracts were solvent-exchanged with isooctane before the analysis of polychlorinated biphenyls (PCBs) and chlorinated pesticides by gas chromatography/electron capture detection (GC/ECD). Sample recoveries were corrected for the recovery of blanks and internal standards (PAH and LAB: phenanthrene-d_{10}, benzo[*a*]pyrene-d_{12}, naphthalene-d_8,

acenaphthene-d_{10}, 1-phenyldodecane, chrysene-d_{12}, and perylene-d_{12}; PCBs and pesticides: tetrachloromethylxylene, dibromooctafluorobiphenyl, PCB-112 (Cl_5), and PCB-197 (Cl_8)).

ELISA

The concentration of hsp 70 in each gonad sample was determined by ELISA, as described in the chapter 4.

Statistical analysis

The hsp 70 data (ng/mg total protein) were transformed by taking the square root of the hsp 70 concentration; the transformed data more closely fit the assumptions of analysis of variance (ANOVA), including normality and homogeneity of variance, and therefore the transformed data were used in the ANOVA test. Significant differences between hsp 70 concentrations in the gonad were examined by three-way analysis of variance with the fixed factors site, reproductive stage, and sex. The analysis was conducted with the statistical software SPSS for Windows (SPSS, Inc., Chicago). Post hoc multiple comparisons were conducted with the Student-Newman-Keuls test ($\alpha = 0.05$).

Results

From the chemical analysis of tissues from clams collected at the four sample sites (Tables 5.1, 5.2 and 5.3),

Table 5.1. Concentration of polycyclic aromatic hydrocarbons (PAHs) in the soft tissues of *Mya arenaria* collected from New Bedford Harbor, Cotuit Beach Club, the Cotuit Control site, and Little Buttermilk Bay. Contaminant concentrations are given in ng per g dry weight.

	NBH 9/95	CBC 5/95	CBC 9/95	CC 5/95	CC 9/95	LBB 9/95
wet weight (g)	41.28	21.37	16.49	29.71	22.11	41.09
dry weight (g)	4.73	1.95	2.21	3.62	1.80	5.26
total lipid (g)	0.24	0.12	0.14	0.15	0.12	0.17
% lipid	5.14	6.08	6.37	4.02	6.65	3.24
PAH (ng/g dry weight)						
naphthalene	47.68	59.57	83.45	30.97	128.64	28.68
2-methylnaphthalene	30.96	17.80	36.55	9.84	52.93	8.28
1-methylnaphthalene	14.92	8.02	19.65	4.28	30.66	4.07
2,6-dimethylnaphthalene	27.68	49.08	30.24	31.85	94.00	3.60
fluorene	2.20	4.94	8.05	2.83	12.56	1.65
1-methylfluorene	21.16	2.17	4.41	1.18	4.17	3.98
biphenyl	14.21	7.23	10.98	4.22	12.72	2.12
acenaphthylene	24.64	4.94	15.44	3.62	12.01	1.21
acenaphthene	2.57	1.44	4.02	0.44	5.14	0.52
dibenzofuran	4.56	6.45	13.36	4.73	15.07	3.21
dibenzothiophene	1.48	2.29	4.54	1.38	5.43	0.77
phenanthrene	11.08	29.94	62.30	14.71	77.15	7.97
retene	15.45	6.47	10.60	6.68	16.42	2.62
perylene	9.90	5.00	9.55	20.62	8.63	2.34
dibenz[a,h]anthracene	12.14	0.00	3.55	0.00	1.60	0.00
anthracene	28.60	6.97	12.28	3.63	10.74	1.10
1-methylphenanthrene	14.86	7.79	15.86	4.61	17.25	1.47
fluoranthene	124.30	60.65	97.81	39.30	121.87	15.28
pyrene	146.57	54.04	85.52	38.20	101.76	12.04
benz[a]anthracene	67.22	11.37	24.87	9.07	26.72	2.96
chrysene	149.56	20.80	43.01	18.22	48.47	6.37
benzo[b]fluoranthene	108.10	10.42	23.69	11.23	27.00	3.70
benzo[k]fluoranthene	100.64	12.01	25.68	12.21	26.31	3.75
benzo[e]pyrene	167.04	19.11	29.55	15.36	30.47	5.40
benzo[a]pyrene	33.13	10.60	26.79	8.99	30.78	2.38
indeno[1,2,3-c,d]pyrene	39.65	3.22	18.37	4.81	9.21	1.26
benzo[g,h,i]perylene	157.73	16.78	27.30	15.40	25.93	5.47
coronene	13.15	2.95	6.66	4.22	4.89	0.95
Total PAH	1391.16	442.05	754.09	322.57	958.54	133.14

Table 5.2. Concentration of polychlorinated biphenyls (PCBs) in the soft tissues of *Mya arenaria* collected from New Bedford Harbor, Cotuit Beach Club, the Cotuit Control site, and Little Buttermilk Bay. Contaminant concentrations are given in ng per g dry weight.

		NBH 9/95	CBC 5/95	CBC 9/95	CC 5/95	CC 9/95	LBB 9/95
wet weight (g)		41.28	21.37	16.49	29.71	22.11	41.09
dry weight (g)		4.73	1.95	2.21	3.62	1.80	5.26
total lipid (g)		0.24	0.14	0.12	0.15	0.12	0.17
% lipid		5.14	6.37	6.08	4.02	6.65	3.24
PCBs (ng/g dry weight)	IUPAC number						
2,4	8	42.74	0.00	0.00	0.00	0.00	0.00
2,2'5	18	309.78	0.00	0.00	0.00	0.00	0.00
2,4,4'	28	453.66	0.00	0.00	0.18	0.00	0.00
2,2'3,5'	44	357.33	0.00	0.00	2.27	0.00	0.00
2,2'5,5'	52	825.00	36.23	23.74	3.20	0.00	0.55
2,3'4,4'	66	859.96	0.00	0.00	1.83	1.24	1.52
3,3'4,4'	77	635.12	0.00	0.00	0.06	0.10	0.17
2,2'4,5,5'	101	888.91	0.85	1.62	2.00	3.32	0.90
2,3,3'4,4'	105	231.33	0.00	2.08	0.00	0.00	0.00
2,3'4,4',5	118	849.93	6.43	5.93	1.26	4.80	1.90
3,3'4,4'5	126	50.99	0.65	0.92	0.74	1.66	0.00
2,2'3,3',4,4'	128	85.18	0.00	0.00	0.00	0.00	0.19
2,2'3,4,4'5	138	432.98	0.00	0.00	0.00	0.00	1.78
2,2'4,4'5,5'	153	541.54	13.79	11.02	6.65	19.65	3.60
2,2'3,3'4,4'5	170	16.77	0.00	11.30	0.00	0.00	0.00
2,2'3,4,4'5,5'	180	38.29	0.00	0.00	0.00	0.00	0.00
2,2'3,4,5,5'6	187	56.13	0.00	0.00	0.00	0.00	0.75
2,2'3,3'4,4'5,6	195	2.45	0.00	0.00	0.00	0.00	0.00
2,2'3,3'4,4'5,5'6	206	1.38	0.00	0.00	0.00	0.00	0.00
2,2'3,3'4,4'5,5'6,6'	209	0.54	0.00	0.00	0.00	0.00	0.00
Total PCB		6680.00	57.95	56.60	18.19	30.77	11.36

Table 5.3. Concentration of organochlorine pesticides and linear alkyl benzenes (LABs) in the soft tissues of *Mya arenaria* collected from New Bedford Harbor, Cotuit Beach Club, the Cotuit Control site, and Little Buttermilk Bay. Contaminant concentrations are given in ng per g dry weight.

	NBH 9/95	CBC 5/95	CBC 9/95	CC 5/95	CC 9/95	LBB 9/95
wet weight (g)	41.28	21.37	16.49	29.71	22.11	41.09
dry weight (g)	4.73	1.95	2.21	3.62	1.80	5.26
total lipid (g)	0.24	0.14	0.12	0.15	0.12	0.17
% lipid	5.14	6.37	6.08	4.02	6.65	3.24
LABs (ng/g dry weight)						
phenyldecanes	0.00	37.70	37.40	14.13	44.17	4.34
phenylundecanes	74.58	34.93	48.17	15.26	62.76	17.74
phenyldodecanes	18.88	21.94	39.50	9.69	37.55	22.29
phenyltridecanes	71.38	70.15	78.74	67.70	110.88	50.33
phenyltetradecanes	210.34	94.64	171.14	52.32	50.03	64.03
Total LABs	375.18	259.36	374.95	159.10	305.40	158.73
Pesticides (ng/g dry weight)						
Hexachlorobenzene	0.14	0.00	0.00	0.00	0.00	0.00
Lindane (gamma-HCH)	2.05	0.00	0.00	0.94	0.00	0.00
Heptachlor	0.00	0.20	0.00	0.00	0.61	0.15
Aldrin	0.00	0.00	0.00	0.00	0.00	0.00
Heptachlor epoxide	27.47 *	2.38	2.92	1.11	2.66	0.15
cis-Chlordane	0.00	5.89	0.00	0.00	0.00	0.00
trans-Nonachlor	1.23	6.66	7.60	0.00	0.00	0.67
Dieldrin	0.00	1.94	1.45	1.44	1.94	0.94
Endrin	0.00	1.52	1.62	1.55	3.46	1.95
Mirex	0.00	0.00	0.00	0.00	0.00	0.00
Total Pesticides	30.89	18.60	13.58	5.04	8.67	3.87
DDT and Metabolites (ng/g dry weight)						
2,4'-DDE	59.26 *	0.00	0.00	0.36	0.00	0.75
4,4'-DDE	63.07	5.97	5.94	3.30	6.44	4.49
2,4'-DDD	0.00	0.00	0.56	0.75	1.49	0.00
4,4'-DDD	82.32	6.83	5.73	0.00	0.00	2.63
2,4'-DDT	0.00	0.00	0.00	0.00	0.00	0.00
4,4'-DDT	0.00	0.00	0.00	0.00	0.00	0.00
Total DDT	204.65	12.80	12.24	4.41	7.93	7.87

* indicates the possibility of a coeluting PCB

New Bedford Harbor is clearly the most contaminated site, with the highest concentrations of total PCBs, chlorinated pesticides, and total PAHs. The majority of the PCBs in NBH have 3 to 6 chlorine substituted positions. In addition, NBH clams contain high levels of the higher molecular weight PAH congeners. Total LABs, tracers of domestic sewage sludge, are similar at all sites. Since the contaminant profiles at the two Cotuit sites were not significantly different, the two sites were combined and will collectively be referred to as Cotuit (COT).

The mean hsp 70 concentration, standard error, and sample size for all of factor combinations are presented in Table 5.4. The sample size of each group varies because sex and reproductive stage could not be determined at the beginning of the experiment. The ANOVA table (Table 5.5) indicates that three factors were significant: reproductive stage, sex, and the interaction of site and reproductive stage.

Males had significantly higher hsp 70 concentrations than females (Figure 5.1). The highest hsp 70 levels are found after spawning (Figure 5.2). Hsp 70 concentrations in spent clams are significantly higher than those in clams in the developing and spawning stages; the other reproductive stages are not significantly different from each other. No significant difference was observed among the sites (Figure 5.3). The only significant interaction effects are site by reproductive stage. Spawning clams from Cotuit have

Table 5.4. Hsp 70 concentration in the gonad of the soft shell clam, *Mya arenaria*, broken down by sex, reproductive stage, and site.

		NBH		COT		LBB	
Reproductive stage		Female	Male	Female	Male	Female	Male
Indifferent							
	mean	5.99	9.36	2.45	5.06	0.33	9.44
	std error	3.12	4.20	2.45	1.62	0.33	2.83
	n	9	7	2	9	6	13
Developing							
	mean	3.72	7.08	4.73	4.37	2.45	5.54
	std error	0.94	1.72	2.35	0.65	1.00	1.80
	n	18	12	41	33	19	14
Ripe							
	mean	0.00	19.00	6.91	12.33	3.50	0.00
	std error	0.00	19.00	2.03	2.65	2.50	
	n	5	2	11	6	6	1
Spawning							
	mean	4.11	5.36	9.09	9.59	3.71	3.14
	std error	3.99	2.44	2.39	1.50	1.16	0.97
	n	9	20	20	35	8	12
Spent							
	mean	11.90	24.30	11.15	8.10	5.76	14.85
	std error	5.42		2.97	1.77	2.55	6.85
	n	6	1	10	3	9	2

Table 5.5. The results of the three way ANOVA performed on the square root-transformed hsp 70 concentrations in the gonad of the soft shell clam, *Mya arenaria*.

Source of Variation	Sum Squares	DF	Mean Squares	F	p
Within + Residual	916.57	319	2.87		
Site	11.37	2	5.68	1.98	0.140
Reproductive stage	34.91	4	8.73	3.04	0.018
Sex	32.79	1	32.79	11.41	0.001
Site by Reprod.	46.41	8	5.8	2.02	0.044
Site by Sex	5.59	2	2.8	0.97	0.379
Reprod. by Sex	12.18	4	3.05	1.06	0.376
Site by Reprod. by Sex	21.37	8	2.67	0.93	0.492
Model	178.64	29	6.16	2.14	0.001
Total	1095.21	348	3.15		
R-squared =	0.163				
Adjusted R-squared =	0.087				

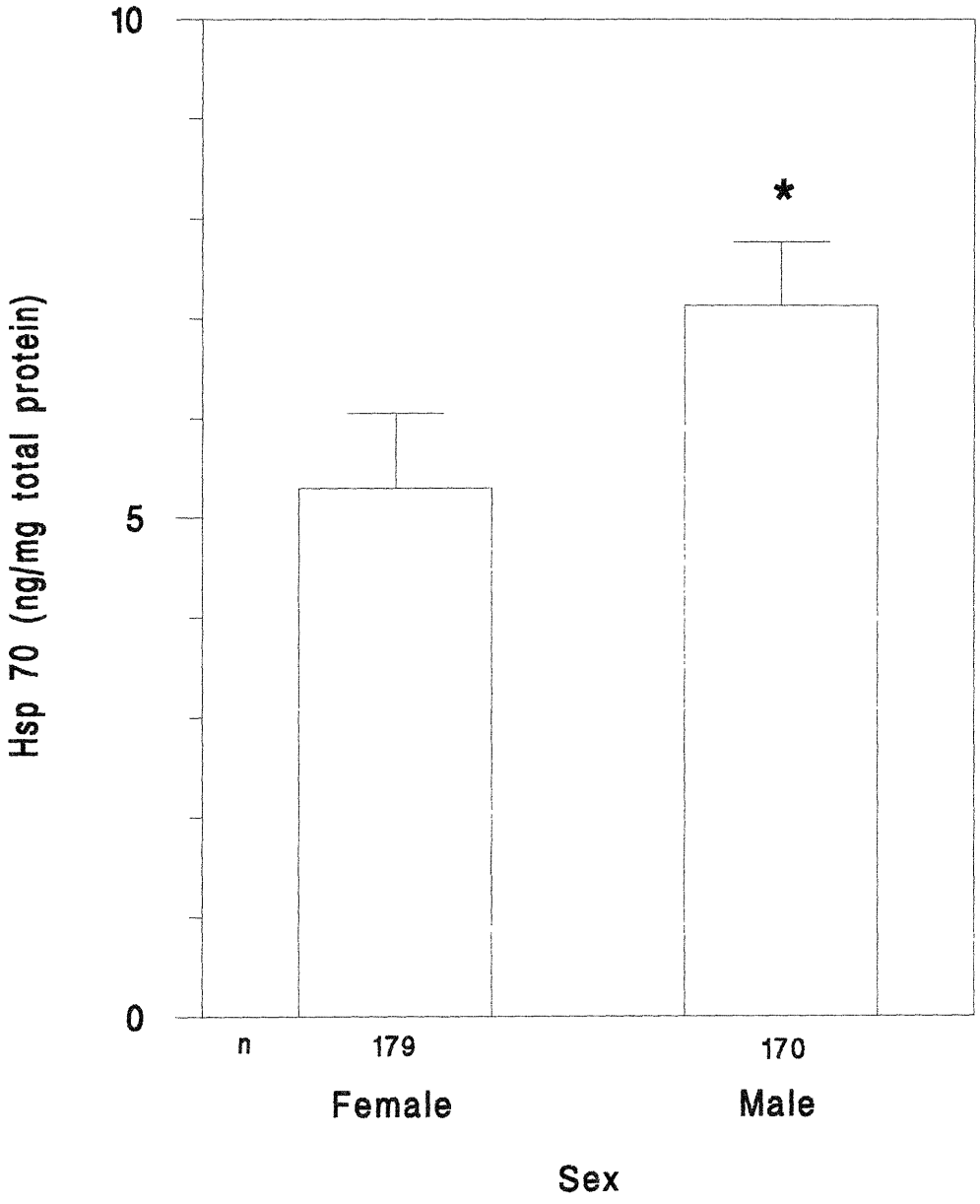

Figure 5.1. Hsp 70 concentrations in the gonad of male and female *Mya arenaria*. All of the sites and reproductive stages have been combined. Each bar represents the mean ± standard error for the listed sample size. The * represents a statistically significant difference ($p < 0.05$).

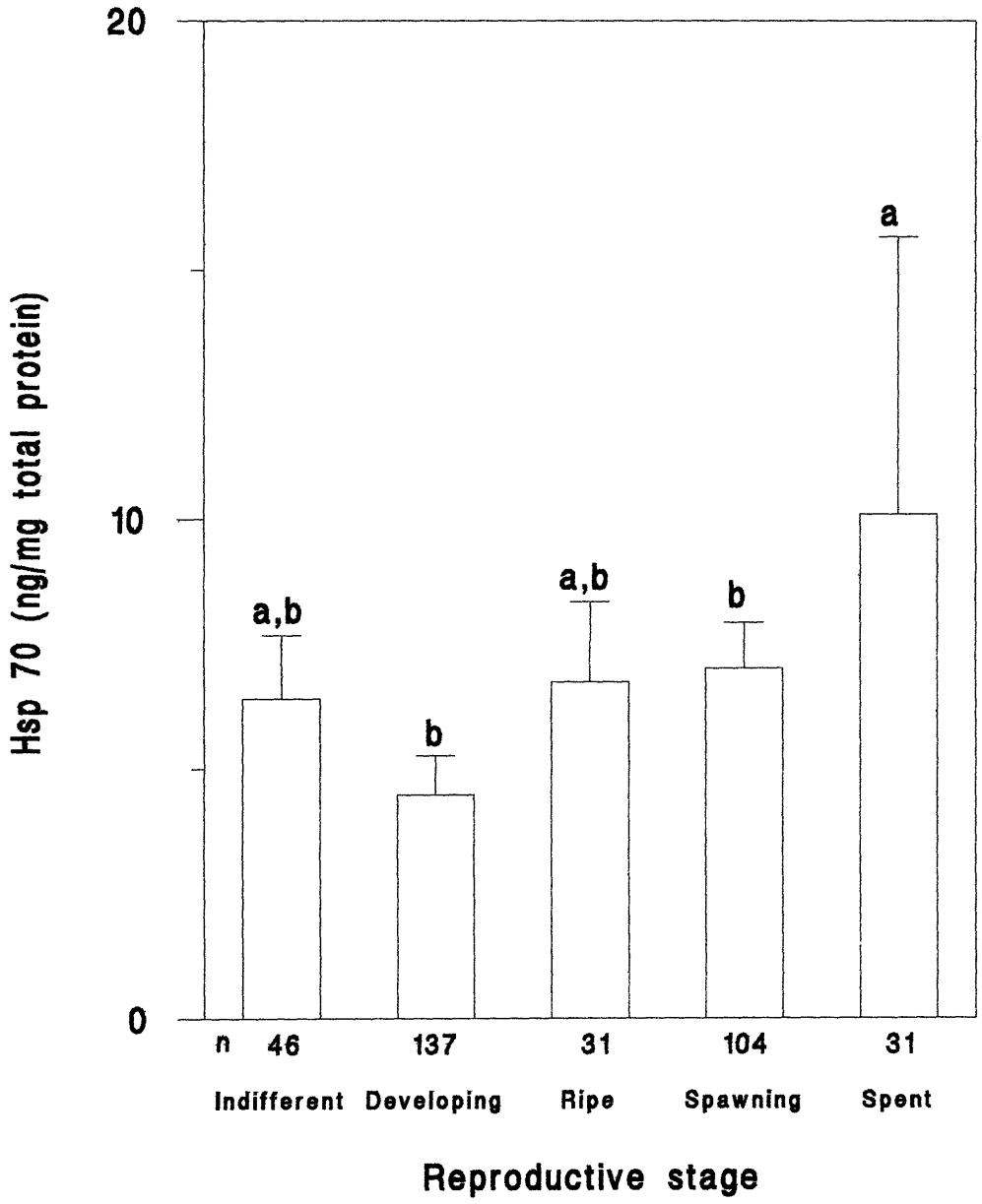

Figure 5.2. Hsp 70 concentrations in the gonad of *Mya arenaria* throughout the reproductive cycle. All of the sites and sexes have been combined. Each bar represents the mean ± standard error for the listed sample size. The stages marked a and b are significantly different from each other ($p < 0.05$). The stages marked a,b are not significantly different from any of the other stages.

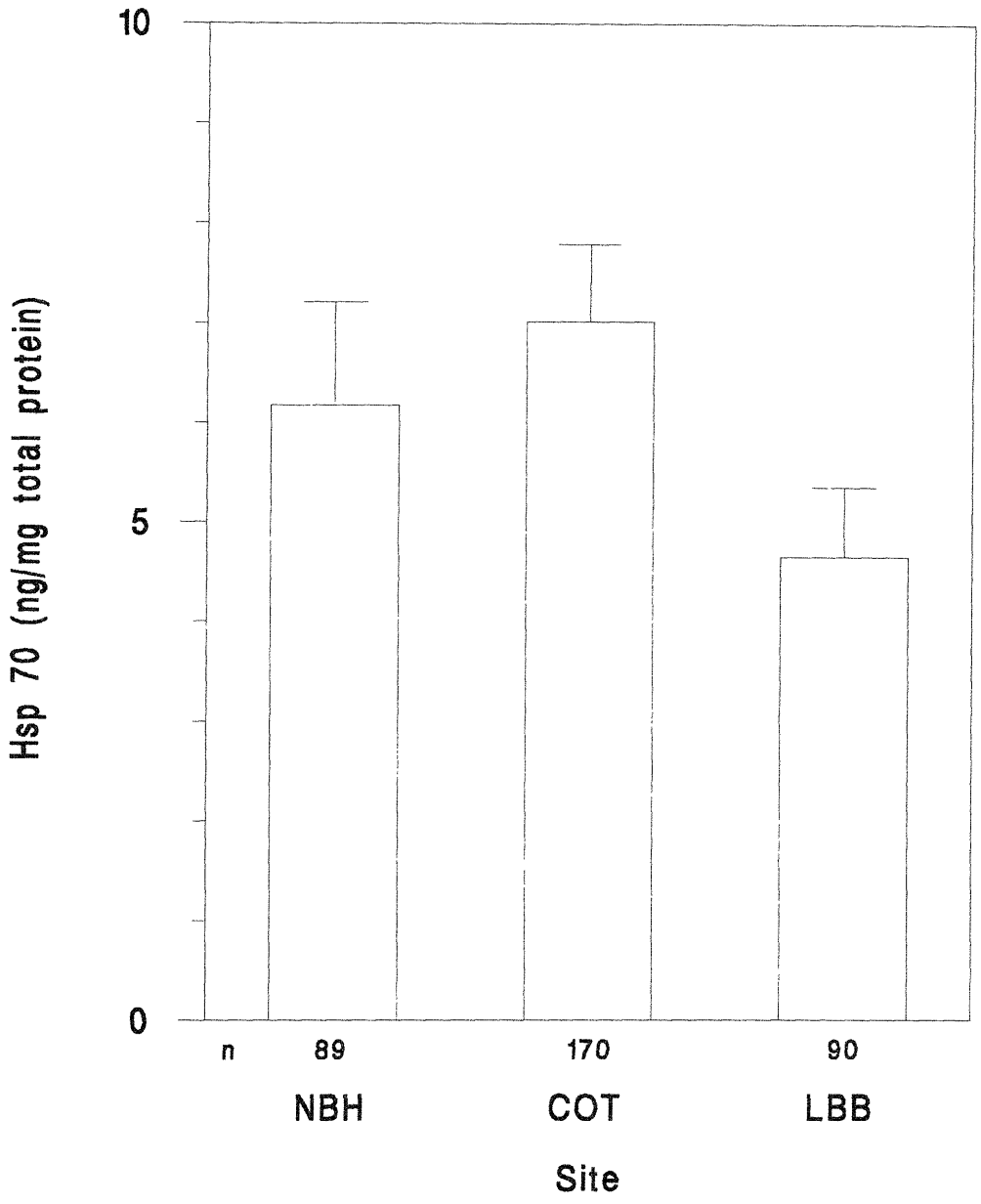

Figure 5.3. Site differences in hsp 70 concentrations in the gonad of *Mya arenaria*. All of the reproductive stages and sexes have been combined. Each bar represents the mean ± standard error for the listed sample size. There are no significant differences between the sites ($p > 0.05$).

significantly higher concentrations of hsp 70 than spawning clams in NBH and developing clams in Cotuit (Figure 5.4).

Discussion

The differences in hsp 70 concentration observed in the gonad of *Mya arenaria* appear to be associated only with the reproductive cycle, and not with exposure to contaminants. First, gonad hsp 70 concentrations are higher in males than in females. This result is not surprising for two reasons: (1) sperm have a higher protein concentration than eggs, which have high lipid levels, and (2) abnormal spermatogonia are not uncommon in male gonads, particularly during the indifferent stage (Ropes and Stickney, 1965; Sunila, 1988). An additional factor, which is true in many organisms but has not been demonstrated in bivalves, is that sperm development is extremely sensitive to high temperatures; high hsp concentrations in male reproductive organs may be required to protect sperm during development and to dispose of the products of abnormal sperm development (Lindquist, 1986). Hsp 70 expression, therefore, may be higher in males because of increased protein synthesis and degradation of abnormal proteins, compared to females.

Hsp 70 concentration also varied over the reproductive cycle, reaching a maximum after spawning when the gonads were spent. During this stage, unspawned gametes may be resorbed

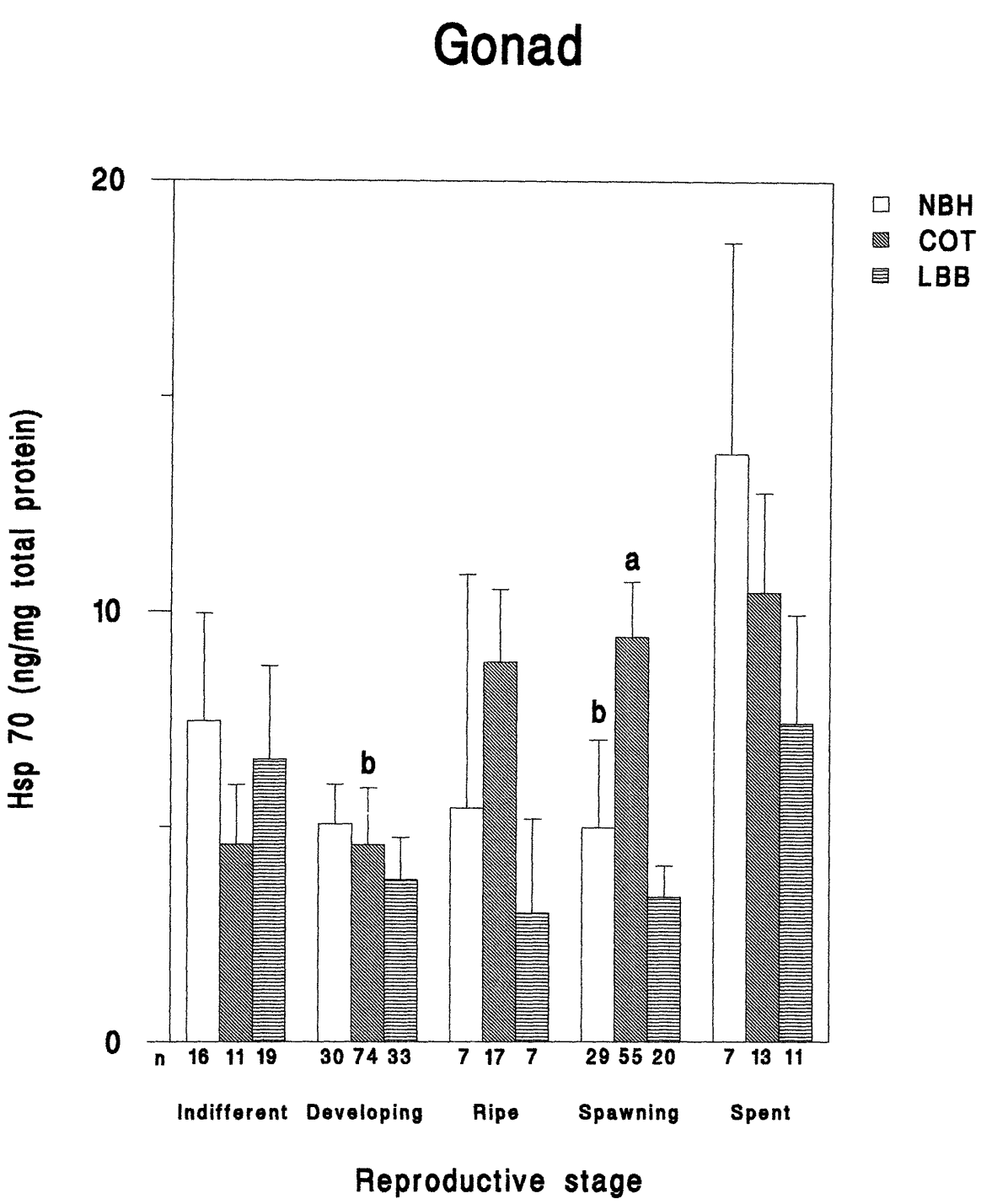

Figure 5.4. Site differences in hsp 70 concentrations in the gonad of *Mya arenaria* throughout the reproductive cycle. The sexes have been combined. Each bar represents the mean ± standard error for the listed sample size. Site-reproductive stage combinations marked a and b are significantly different from each other ($p < 0.05$). The other observed differences are not statistically significant.

(Sunila, 1988) in a process that is likely to involve protein degradation. Sanders (1993) hypothesized that hsp expression would be elevated by normal physiological processes that involve the rapid breakdown and/or reorganization of tissues, including the resorption of gametes. The significant interaction term, site by reproductive stage, suggests that gamete resorption may begin earlier in the reproductive cycle in the Cotuit population. The reason for this difference in the Cotuit clams is unknown.

One test of the hypothesis that hsp 70 expression during the reproductive cycle is a function of both protein synthesis and protein degradation may be to determine the differential expression of hsps 60 and 70 during the reproductive cycle. If the hypothesis is correct, hsps 60 and 70 would both be expressed during periods of protein synthesis, while hsp 70 alone would be expressed during periods of protein degradation. The assumption is that hsps 60 and 70 function in concert during protein synthesis, while hsp 60 expression would not be required to correctly refold proteins that are destined to be degraded. In addition, ubiquitin expression should be correlated with hsp 70 expression only when proteins are being degraded, provided that gamete resorption occurs via the standard pathways for protein degradation.

Hsp 70 concentration in the gonad was not different between contaminated and control sites. Two possible interpretations of this results are: (1) hsp 70 expression is

not increased by contaminant exposure in natural populations of *Mya arenaria*, or (2) the gonad is protected from contaminant effects. Evidence in support of the second interpretation is provided by detailed comparisons of the reproductive effort of mussels and soft shell clams from New Bedford Harbor. Although both species had similar body burdens of both PCBs and PAHs, reproductive effort was unaffected in populations of *Mya arenaria* whereas *Mytilus edulis* showed highly significant reductions in ovarian output and development (McDowell, 1995). This conclusion will be further explored in the next chapter, which discusses hsp 70 concentrations in the gill tissue of the same clams used in this study.

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Chapter 6. An evaluation of the potential use of heat shock protein 70 as a biomarker of environmental contamination in the gill of *Mya arenaria* collected from natural populations

Background

The use of hsp concentrations as a biomarker of general stress for environmental monitoring has been widely proposed (Sanders, 1990; Bradley, 1993; Steinert and Pickwell, 1993; Ryan and Hightower, 1994), but most research efforts to date remain focused on determining dose-dependent induction by various chemicals under controlled laboratory conditions. The general stress response of increased hsp concentration in response to a wide variety of proteotoxic stressors suggests that the interpretation of an observation of elevated hsp levels in a natural population may be confounded by the number of potential causes of that increase, including both contaminant and "natural" effects. The "natural" effects may include age (Fargnoli et al., 1990; Blake et al., 1991), temperature (Lindquist, 1986; Lindquist and Craig, 1988; Sanders, 1988; Sanders et al., 1991; Bols et al., 1992; Dietz and Somero, 1992; Hofmann and Somero, 1995), season (Fader et al., 1994), food availability (Werner-Washburne et al., 1989; Sanders, 1990), anoxia (Lindquist and Craig, 1988; Nover, 1991), and effects linked to the reproductive cycle (Morange et al., 1984; Kurtz et al., 1986; Lindquist, 1986; Zakeri and

Wolgemuth, 1987; Zakeri et al., 1988; Billoud et al., 1993). Many of these effects are not independent.

The experiment described in this chapter was designed to examine the potential for the use of hsp 70 concentration in the gill of the soft shell clam as a biomarker of exposure to environmental contaminants in natural populations. The potentially confounding effects of sex and reproductive stage were also measured. Water temperature was not used as a variable for several reasons, including the fact that reproductive stage and water temperature are not independent, and the fact that soft shell clams live buried in the sediment, so the relationship between water temperature and organismal body temperature is unclear. Gill tissue was chosen because it is in close contact with the external environment.

Materials and Methods

Subsamples of gill tissue were collected from the same clams used for gonad tissue analysis in chapter 5. All chemical and statistical analyses of the gill samples were conducted as described for the gonad samples. The two Cotuit sites were combined, as described in the interpretation of the contaminant chemistry data in chapter 5.

Results

The mean hsp 70 concentration, standard error, and sample size for all of the levels of the factors are presented in Table 6.1. The ANOVA table (Table 6.2) indicates that three effects were significant: site, and the interactions of site by sex and site by reproductive stage.

Hsp 70 concentrations in the gills of clams collected in New Bedford Harbor were significantly higher than the other sites (Figure 6.1). No significant differences were seen between the sexes (Figure 6.2) or among the reproductive stages (Figure 6.3).

In Little Buttermilk Bay only, males have significantly higher hsp 70 concentrations than females (Figure 6.4). Little Buttermilk Bay males are intermediate between the New Bedford Harbor clams and the Cotuit clams and Little Buttermilk Bay females. The site by reproductive stage interaction is more complicated (Figure 6.5). New Bedford Harbor is consistently higher than Cotuit and Little Buttermilk Bay; this difference is most pronounced in the later stages of the reproductive cycle (ripe, spawning, and spent clams), although the site difference is significant only in the ripe and spawning stages. In New Bedford Harbor, spent clams have significantly higher hsp 70 concentrations in the gill than developing clams; the other stages are not significantly different. For Cotuit, the only significant difference is that hsp 70 concentrations in the gill of developing and spawning clams are higher than in ripe clams.

Table 6.1. Hsp 70 concentration in the gill of the soft shell clam, *Mya arenaria*, broken down by sex, reproductive stage, and site.

		NBH		COT		LBB	
Reproductive stage		Female	Male	Female	Male	Female	Male
Indifferent							
	mean	19.13	23.79	24.10	8.44	5.83	16.25
	std error	3.29	3.14	6.10	3.15	2.48	4.37
	n	9	7	2	9	6	13
Developing							
	mean	14.49	13.88	11.61	12.46	13.26	21.35
	std error	4.09	4.01	1.90	1.93	2.24	3.73
	n	18	12	41	33	19	14
Ripe							
	mean	31.80	34.50	3.82	0.50	3.00	9.00
	std error	1.39	2.50	1.19	0.50	1.69	
	n	5	2	11	6	6	1
Spawning							
	mean	25.03	25.01	18.66	10.65	6.06	12.03
	std error	3.49	2.65	2.76	2.15	3.02	2.79
	n	9	20	20	35	8	12
Spent							
	mean	28.10	43.80	16.59	4.20	15.18	27.90
	std error	4.18		5.48	4.20	4.73	3.90
	n	6	1	10	3	9	2

Table 6.2. The results of the three way ANOVA performed on the square root-transformed hsp 70 concentrations in the gill of the soft shell clam, *Mya arenaria*.

Source of Variation	Sum Squares	DF	Mean Squares	F	p
Within + Residual	1302.43	319	4.08		
Site	140.53	2	70.26	17.21	< 0.001
Reproductive stage	16.03	4	4.01	0.98	0.418
Sex	1.58	1	1.58	0.39	0.534
Site by Reprod.	163.66	8	20.46	5.01	< 0.001
Site by Sex	61.98	2	30.99	7.59	0.001
Reprod. by Sex	6.7	4	1.67	0.41	0.801
Site by Reprod. by Sex	23.92	8	2.99	0.73	0.663
Model	421.49	29	14.53	3.56	< 0.001
Total	1723.92	348	4.95		
R-squared =	0.244				
Adjusted R-squared =	0.176				

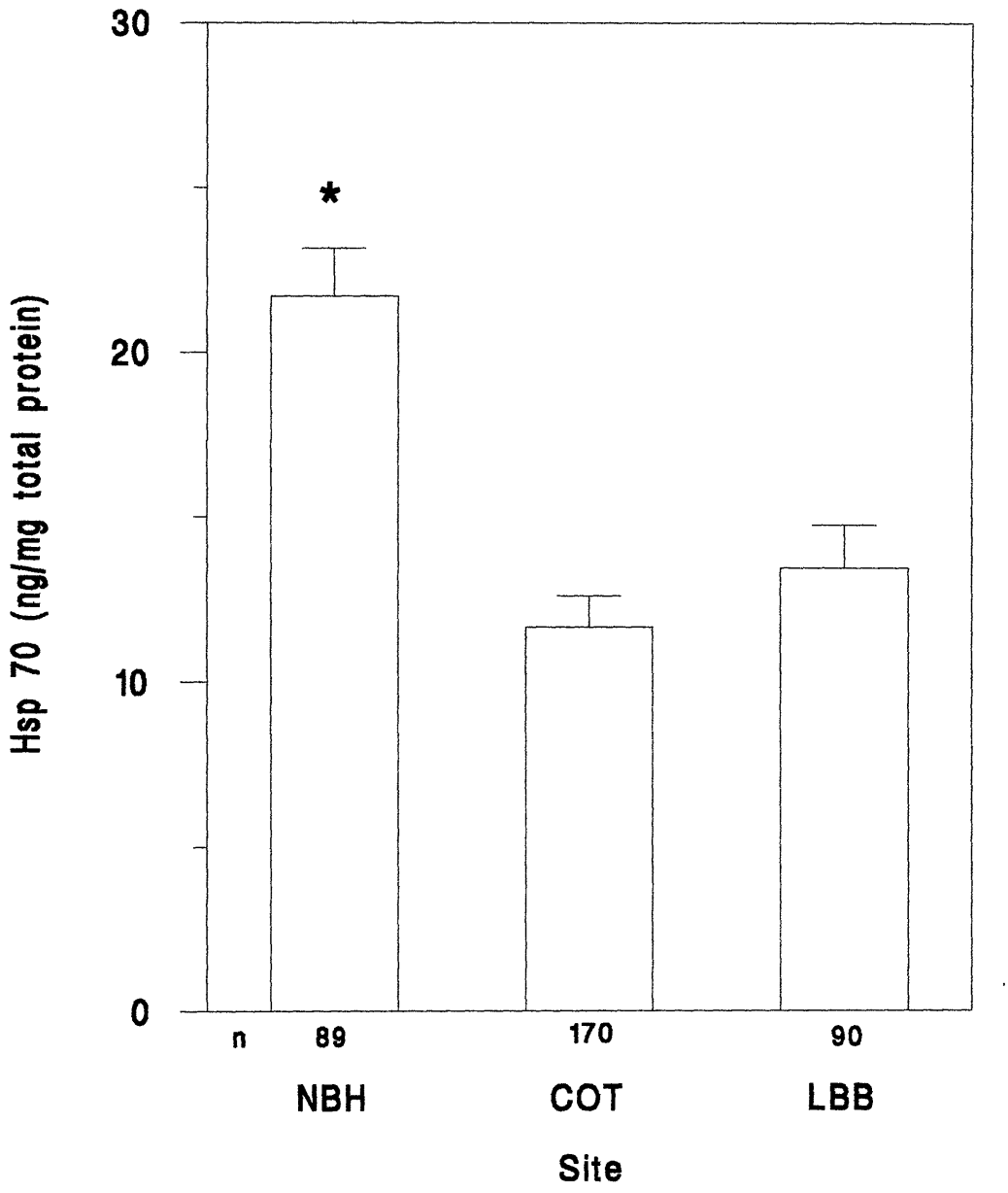

Figure 6.1. Site differences in hsp 70 concentration in the gill of *Mya arenaria*. The sexes and reproductive stages have been combined. Each bar represents the mean ± standard error for the listed sample size. The * denotes sites at which the concentration of hsp 70 in the gill is significantly higher than the concentration at the other sites ($p < 0.05$).

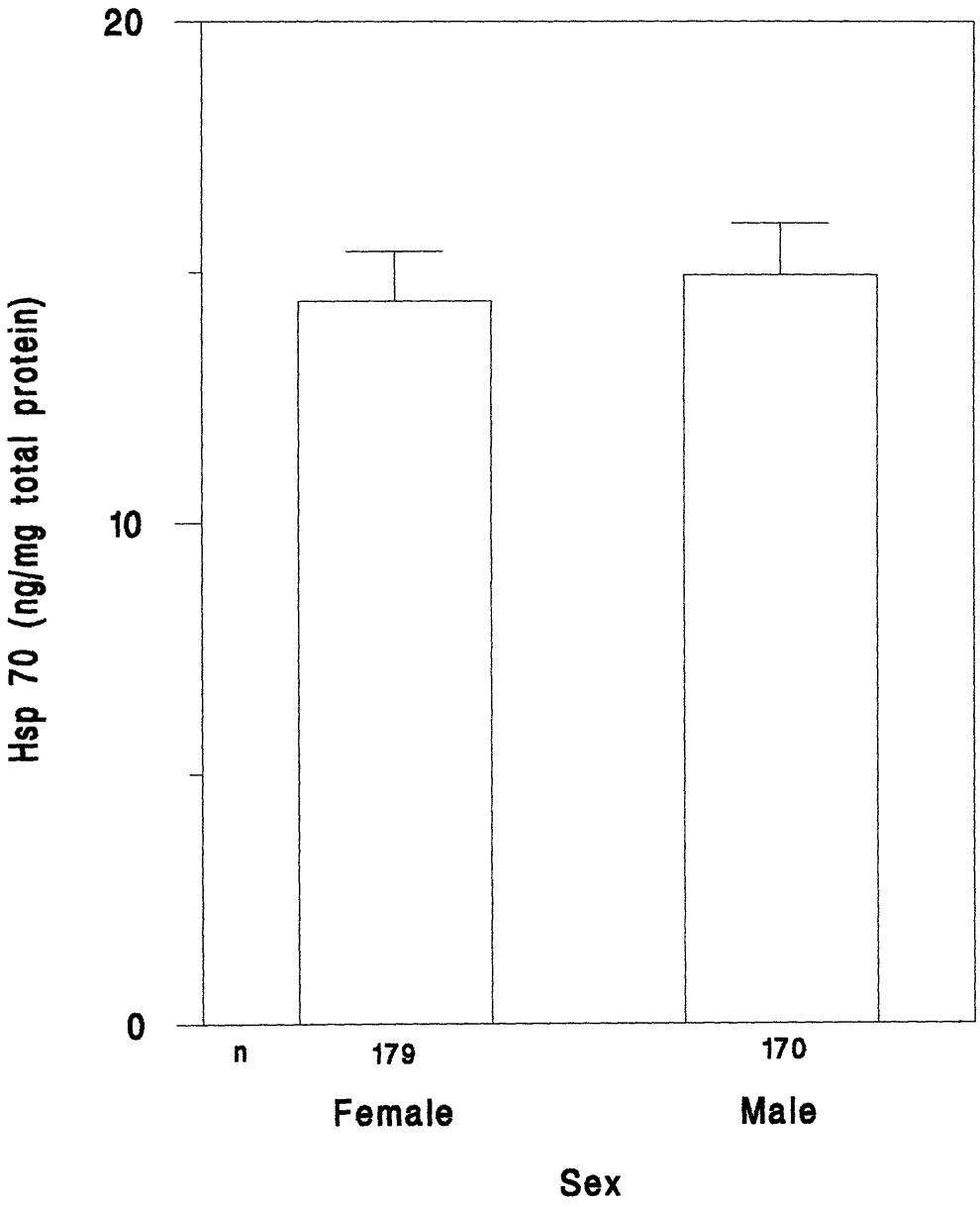

Figure 6.2. Hsp 70 concentrations in the gill of male and female *Mya arenaria*. The sites and reproductive stages have been combined. Each bar represents the mean ± standard error for the listed sample size. The sexes are not significantly different ($p > 0.05$).

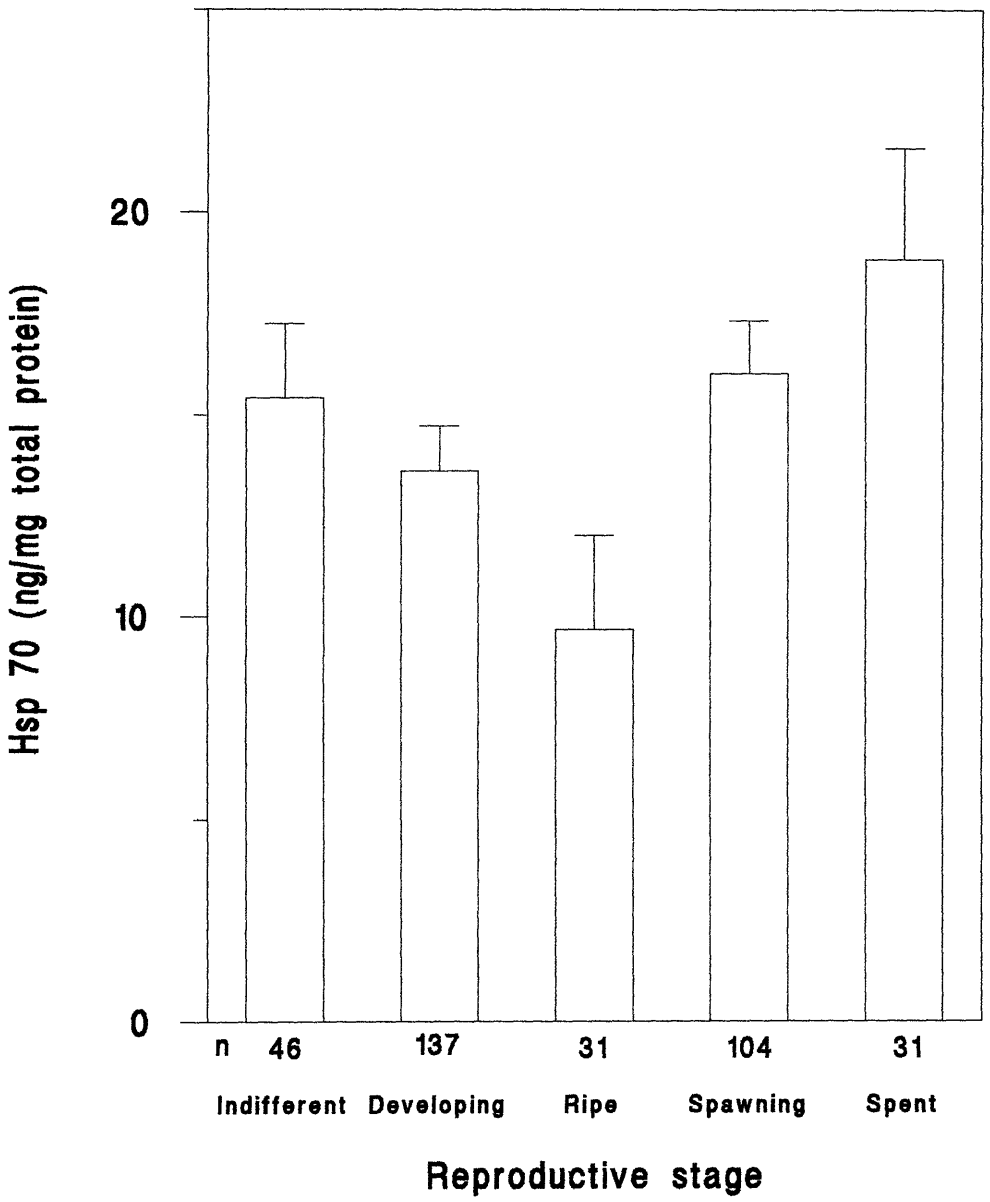

Figure 6.3. Hsp 70 concentrations in the gill of *Mya arenaria* throughout the reproductive cycle. The sites and sexes have been combined. Each bar represents the mean ± standard error for the listed sample size. There are no significant differences between the reproductive stages ($p > 0.05$).

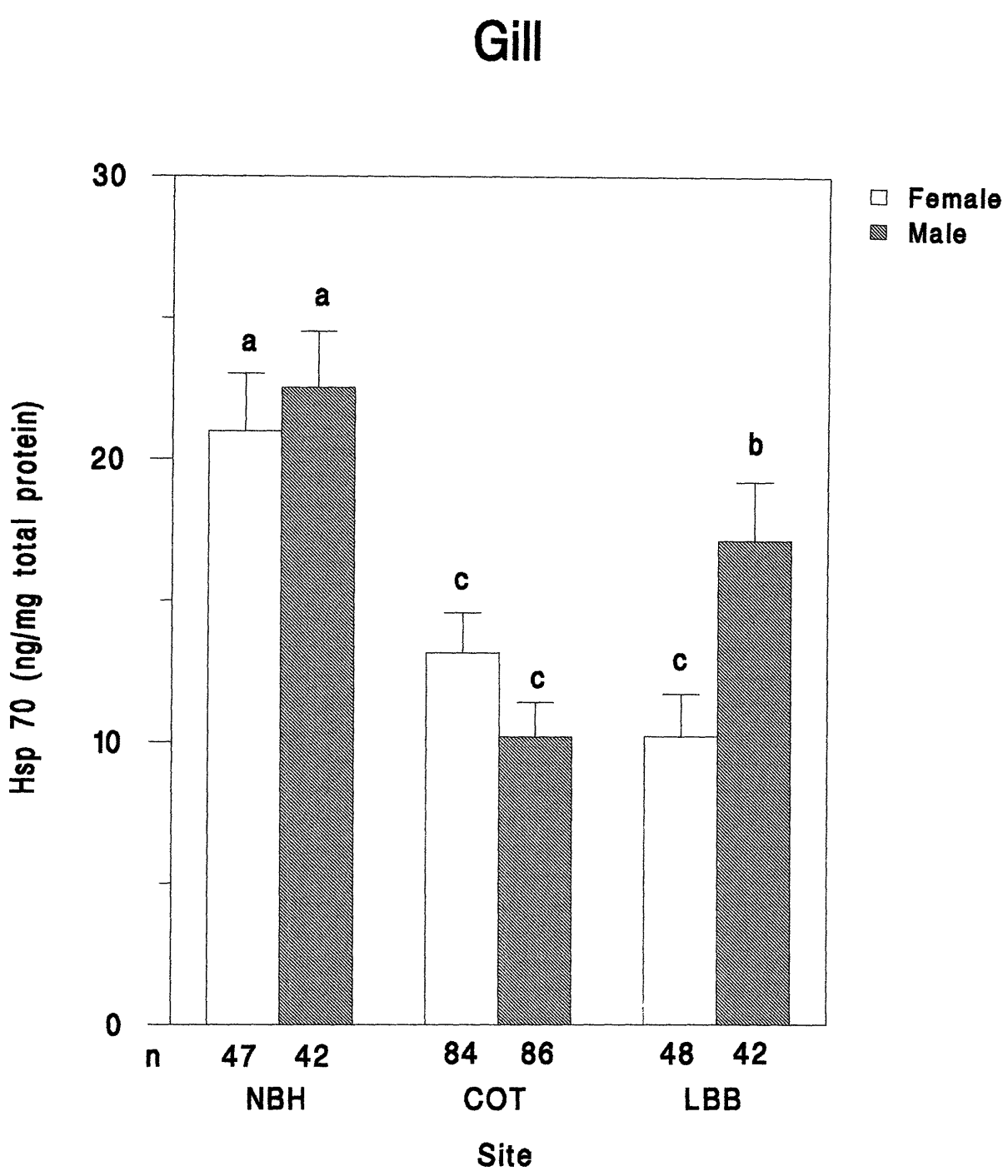

Figure 6.4. Site differences in hsp 70 concentrations in the gill of male and female *Mya arenaria*. The reproductive stages have been combined. Each bar represents the mean ± standard error for the listed sample size. The site-sex combinations marked a, b, and c are significantly different from each other.

Gill

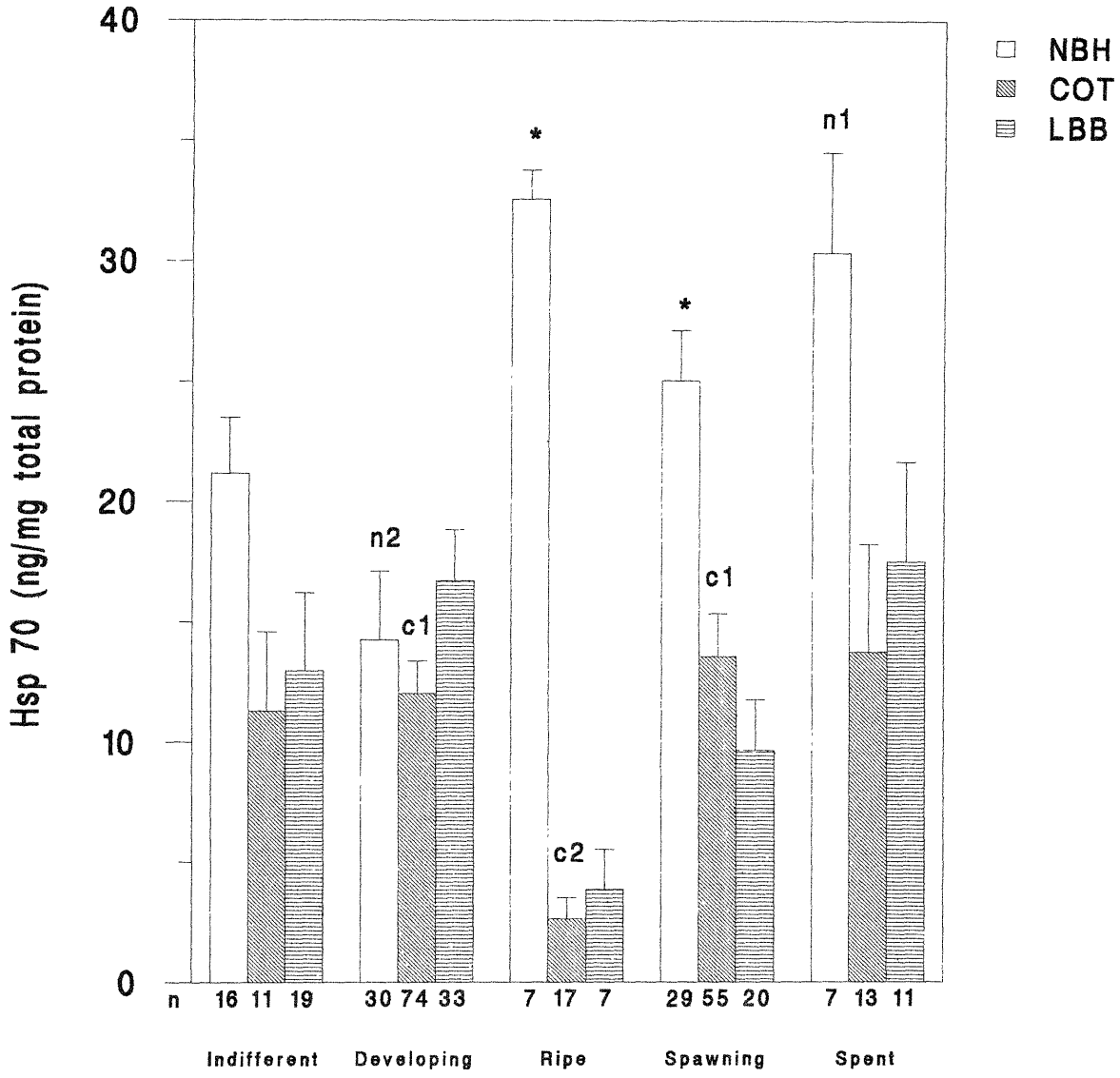

Figure 6.5. Site differences in hsp 70 concentrations in the gill of *Mya arenaria* throughout the reproductive cycle. The sexes have been combined. Each bar represents the mean ± standard error for the listed sample size. The * represents sites which are significantly higher ($p < 0.05$) than the other sites at the same reproductive stage. The NBH reproductive stage marked n1 is significantly higher ($p < 0.05$) than the stage marked n2. The Cotuit stages marked c1 are significantly higher ($p < 0.05$) than the stage marked c2. The other observed differences are not statistically significant ($p > 0.05$).

In Little Buttermilk Bay, the differences in hsp 70 expression among the reproductive stages are not significant.

Discussion

Site differences were observed in the concentration of hsp 70 in the gill, in contrast to the results of the gonad analyses of the same samples. Gill tissue from clams collected in New Bedford Harbor, which is much more highly contaminated than the Cotuit or Little Buttermilk Bay sites, had significantly elevated expression of hsp 70. Other effects on the gills of bivalves in New Bedford Harbor include DNA strand breaks (Nacci et al., 1992) and alterations in gill function as measured by clearance rate (Nelson and Gutjahr-Gobell, 1990). Hightower (1993) suggested that the induction of hsps in response to proteotoxicity shares conceptual similarities to DNA repair mechanisms induced by genotoxicity; the observed strand breaks and hsp 70 elevation may be related in this manner.

An inflammatory response, which may be associated with changes in cell morphology and the cytoskeletal structure, is also common in gills from bivalves exposed to contaminants (Sunila, 1988). Under stressful conditions, heat shock proteins are often associated with the intermediate filaments of the cytoskeleton (Lin, 1987; Welch and Mizzen, 1988) and hsp expression has been correlated with other inflammatory

diseases, especially arthritis (Winrow et al., 1990). These observed effects support the hypothesis that contaminant exposure in the New Bedford Harbor population of *Mya arenaria* results in damage to the gill tissue and the induction of the stress response.

The lack of elevated expression of hsp 70 in the samples from the Cotuit site suggests two possible interpretations: (1) hsp 70 in *Mya arenaria* is not elevated in response to petroleum hydrocarbon contaminants, or (2) there is a threshold level of chronic pollutant stress below which the stress response is not induced. Although few studies have examined hsp 70 elevation in response to lipophilic organic contaminants, Monaghan and Bradley (1993) saw increased levels of hsp 70 in fish exposed to ß-naphthoflavone and benzo(a)pyrene and Werner (1994) observed elevations in hsp 70 levels in amphipods exposed to dieldrin and fluoranthene, suggesting that at least some petroleum hydrocarbons (and other lipophilic contaminants) can induce the stress response in aquatic animals.

The results, therefore, suggest that a threshold exists for both individual and population level stress responses. The threshold nature of the stress response has been demonstrated under laboratory conditions (Mizzen and Welch, 1988; Welch and Mizzen, 1988; Köhler et al., 1992; Zanger et al., 1996) and suggested as a potential problem in the application of hsp concentrations as environmental biomarkers

(Sanders, 1990; Yu et al., 1994). Studies of populations of soil invertebrates exposed to cadmium and lead, for example, have shown that there is a threshold below which the stress response is not induced (Köhler, et al., 1992; Zanger et al., 1996).

Expression of hsp 70 was different between male and female clams only in Little Buttermilk Bay, where males had significantly higher hsp 70 concentrations; the difference was consistent across the reproductive stages. The reason for this observed difference is unknown. There is also a significant interactive effect of site and reproductive stage. The observed elevation in hsp 70 levels in New Bedford Harbor clams is more pronounced at the later stages of the reproductive cycle, especially at the ripe and spawning stages. One interpretation of this effect is that the interaction of reproductive and contaminant stresses is additive or synergistic, with the combination of the effects producing a greater stress and a higher hsp 70 concentration than either one alone. In *Hydra attenuata*, for example, the stress response to a combination of sodium azide and heat shock was greater than the response to either individual stressor (Bosch et al., 1988).

In conclusion, the results presented in this chapter suggest that hsp 70 concentrations in the gill of soft shell clams have some potential to serve as a general biomarker of sublethal effects of contaminants, provided that any

interfering effects are considered and controlled. The limitations on the interpretation of hsp 70 concentration defined in chapters 4-6 include tissue differences, interactive effects of the reproductive cycle, and the threshold required for the induction of the heat shock response. Large sample sizes and a combination of several biomarkers will probably be required for a complete interpretation of the stress response.

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Chapter 7. Conclusions and suggestions for future research

The process of lipid distribution and the potential role of lipoproteins were discussed in chapters 2 and 3. The general conclusions are that the injected lipid label was rapidly distributed throughout the clam. Additionally, no alterations in lipid distribution patterns were observed in clams in the early stages of hematopoietic neoplasia, although a confirmation of this effect awaits an experiment specifically designed to investigate the effects of Hn, including the advanced stages of the disease where hemocyte function is impaired and the neoplastic cells invade the tissues. The evidence suggests that the plasma may have been responsible for the observed distribution of the injected triolein.

The plasma of *Mya arenaria* contains at least one lipoprotein, in the very high density range (1.20 - 1.22 g/mL). The isolated VHDL, however, contains only a small amount of the lipid tracer at the timepoint 15 minutes after the injection of the label. The majority of the tracer is associated with the lower density fractions, or with the total protein pool. Refinement of the density gradient centrifugation protocol to optimize the separation of lower density lipoprotein classes should allow further resolution of the distribution of the injected triolein.

While these results suggest that the VHDL plays only a

relatively minor role in lipid transport, the question cannot be answered without an investigation of the turnover rates of the various lipid pools. For example, a lipid pool with a quick turnover but a small lipid concentration may be more important in lipid transport than a high concentration pool which is not distributed rapidly. The VHDL may play an important role in lipid transport if the flux of lipid through this pathway is high.

Additionally, comparisons of the results of the experiments described in chapters 2 and 3 suggest that the route of administration of the label may be a significant factor in determining the transport pathways and the ultimate distribution of the label. Unfortunately, an appropriate means of feeding a known amount of a radiolabelled lipid to bivalve molluscs remains to be determined. If the techniques become available, a comparison of the lipid transport process in fed and starved animals may be used to define storage pools and metabolically active organs. In conjunction with these experiments, lipid class analysis to determine the fate of the injected triglyceride may be useful in defining the transport system and in interpreting the observed distribution pattern of the label because the measurement of total radioactivity does not differentiate between the injected triglyceride and the potential fatty acid hydrolysis products and their derivatives.

Additional factors of potential interest that were

outside the scope of this study are the effects of sex and reproductive stage on lipid transport. In order to fully investigate these effects, a model species in which the animals can easily be sexed and staged would be more appropriate than using the soft shell clam. Alternatively, a reliable non-destructive assay for sexing and staging clams would be required; no such assay currently exists.

A second group of experiments, described in chapters 4 - 6, were designed to investigate the heat shock or stress response in natural populations of *Mya arenaria*. The soft shell clam has a heat shock protein of approximately 70 kilodaltons that is recognized by a commercially available anti-hsp 70 monoclonal antibody. The expression of hsp 70 was measured in the gill and gonad of male and female clams in various stages of the reproductive cycle from contaminated and reference populations. Hsp 70 concentrations in the gonad were influenced by sex and reproductive stage, but not by site specific effects, including the presence of contaminants. Hsp 70 expression was higher in males and in spent clams. These effects implicate hsp 70 in protein synthesis and degradation. Both of these roles are supported by the numerous literature concerning the functional role of hsp 70 in the stabilization of unfolded or denatured polypeptide chains (see reviews by Ellis, 1990; Burel et al., 1992; Gething and Sambrook, 1992; Welch, 1992).

The expression of hsp 70 in the gill of the same clams

demonstrated both the advantages and drawbacks of the use of hsps as biomarkers of sublethal effects of contaminants. On the positive side, hsp 70 concentration in the NBH population was significantly higher than the concentration in the control site, LBB. On the other hand, hsp 70 expression at the moderately contaminated Cotuit site was not significantly elevated above the control. These results suggest that a threshold for induction exists in natural populations, potentially limiting the use of hsp 70 as a biomarker to only the most severely impacted sites. The stress response was also influenced by reproductive stage, suggesting a synergistic effect of contaminant exposure and stress from reproduction.

Confirmation of the conclusions from the hsp experiments awaits laboratory studies of the hsp inducing ability of environmentally relevant lipophilic organic contaminants, both singly and in mixtures. Laboratory studies of potential inducers may also help to interpret the hsp 70 expression patterns from sites like NBH, which are characterized by high levels of a broad suite of contaminant classes. Further constraints on the interpretation of the stress response are imposed by the lack of knowledge of the recognition patterns of the commonly used anti-hsp 70 antibodies. To date, different investigators have obtained different results with the same antibody in the same or similar species.

Laboratory experiments will also be required to separate

the effects of the reproductive cycle and temperature on hsp 70 expression. As the reproductive cycle in *Mya arenaria* is driven, in part, by water temperature, this separation is somewhat artificial. Nevertheless, the contributions of these two factors could potentially be separated in a laboratory experiment in which animals in each of the reproductive stages are acclimated to different water temperatures.

Finally, differential expression of other hsps, notably hsp 60 and ubiquitin, would provide further evidence for the interpretation of the various roles of hsp 70 in the cell. Hsps 60 and 70 should function in concert during protein folding, while hsp 70 and ubiquitin may have related functions in the degradation of abnormal proteins. The broad functions and nature of inducers of the hsps suggest that they will have, at best, limited utility as biomarkers of environmental contamination. The interpretation of stress responses will be constrained by the effects of physiological processes, including reproduction, as "natural" stressors.

The large sample sizes used in the investigation of hsp 70 expression in the gill and gonad tissues provided sufficient power in the ANOVA F test to distinguish the statistically significant differences between the levels of the different factors. The sample size required to ensure that a test using hsp 70 as a biomarker of contaminant exposure and effect has sufficient power depends on the magnitude of the difference in hsp 70 expression between the

study sites. The sample size can be reduced if the expected difference is increased. One way to achieve this is to hold constant any other potentially confounding variables. For example, a careful selection of sampling times at which all of the animals are in the same reproductive stage and the temperature difference between the sites can be minimized or eliminated, will maximize the contribution of the site variable to the observed differences and decrease the sample size required to achieve the same power.

In summary, the experiments described in the preceding chapters have investigated the response of lipoproteins and heat shock proteins of the soft shell clam, *Mya arenaria*, to various physiological processes, including those involved in reproduction and in sublethal responses to disease and lipophilic organic contaminants. The application of lipoproteins and heat shock proteins as biochemical markers that link the cellular and population levels of effects via the physiological process of reproduction is complicated by the number of factors which potentially influence the expression of these markers. The interpretation of any observed pattern must therefore include a careful consideration of the effect of natural physiological processes, as well as alterations of these processes as a result of diseases or contaminant exposure.

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Appendix. Raw heat shock protein data

August 1994

Site	Water Temp.	Clam	Length (mm)	Wet Weight (g)	Sex	Reproductive Stage	hsp 70 (ng/mg total protein) Gill	Gonad
NBH	29 C	1	62.00	42.96	F	spent	38.16	25.16
		2	70.05	52.29	F	spent	37.6	26.94
		3	54.10	33.32	M	spawning	36.05	23.98
		4	69.53	57.68	M	spent	43.77	24.30
		5	66.76	43.53	M	indifferent	36.62	20.69
		6	66.91	50.57	F	spent	33.81	19.30
		7	67.83	47.02	F	indifferent	35.63	22.35
		8	63.94	38.90	F	indifferent	32.83	21.47
		9	67.20	52.79	M	indifferent	34.51	24.54
		10	64.77	34.64	M	spawning	33.25	19.25
CBC	23 C	1	81.64	100.07	F	spawning	37.74	24.78
		2	84.48	82.20	F	spent	34.09	20.94
		3	79.93	77.37	M	spawning	32.97	24.08
		4	89.30	94.60	M	spawning	35.49	25.23
		5	78.10	65.21	F	spent	36.48	23.26
		6	71.67	54.24	F	spawning	32.13	24.61
		7	63.55	35.42	F	spent	35.07	14.32
		8	67.11	33.09	F	spawning	32.41	17.91
		9	62.16	30.99	F	spent	33.11	24.97
		10	59.11	29.85	F	spawning	33.88	26.28
LBB	25 C	1	70.19	49.96	F	spent	37.04	22.44
		2	67.64	40.54	M	indifferent	41.39	23.18
		3	68.83	43.72	M	indifferent	30.86	17.46
		4	60.70	24.93	F	spent	32.41	13.28
		5	60.19	22.02	M	spent	31.56	21.75
		6	61.18	28.03	M	indifferent	32.41	21.32
		7	68.19	38.12	M	developing	30.16	20.14
		8	64.99	33.29	M	indifferent	32.83	27.32
		9	61.08	28.41	M	indifferent	29.88	10.98
		10	62.19	27.78	M	developing	30.58	20.64

August 1994

		hsp 70 (ng/mg total protein)			
Site	Clam	DG	Muscle	Foot	Siphon
NBH	1	8.76	7.71	28.75	21.22
	2	13.67	4.55	45.59	20.67
	3	19.28	9.46	6.65	18.70
	4	9.81	9.11	11.56	18.25
	5	15.07	16.48	13.32	18.68
	6	22.79	12.62	7.36	21.33
	7	17.18	8.06	7.36	25.38
	8	12.97	0.00	45.94	24.27
	9	25.60	0.00	37.17	28.73
	10	35.42	0.00	20.33	26.68
CBC	1	21.04	3.50	21.39	17.32
	2	23.10	4.90	12.27	20.83
	3	31.20	4.55	11.56	20.27
	4	27.27	1.04	30.86	18.49
	5	40.85	1.39	22.09	17.25
	6	60.84	0.69	17.53	16.90
	7	43.13	1.74	18.93	27.08
	8	36.47	0.00	21.39	22.09
	9	40.33	2.09	17.18	16.62
	10	55.41	0.00	27.70	17.25
LBB	1	0.00	3.50	48.04	17.46
	2	6.65	9.11	29.80	19.39
	3	4.20	32.26	20.68	19.69
	4	6.65	13.67	7.71	23.55
	5	0.00	5.95	10.51	24.09
	6	1.74	5.60	24.19	23.07
	7	17.53	1.74	28.75	25.18
	8	10.51	10.86	24.54	28.69
	9	7.36	4.55	41.73	18.36
	10	10.51	6.65	35.42	20.55

September 1994

Site	Water Temp.	Clam	Length (mm)	Wet Weight (g)	Sex	Reproductive Stage	hsp 70 (ng/mg total protein) Gill	Gonad
NBH	22 C	1	92.41	111.92	M	indifferent	20.37	17.88
		2	91.57	110.57	M	spawning	25.08	14.37
		3	89.66	100.81	M	spawning	18.81	9.46
		4	82.80	94.66	M	developing	23.12	3.85
		5	78.25	74.18	F	spawning	28.21	0.00
		6	79.52	82.12	M	spawning	52.52	0.00
		7	78.13	70.42	M	spawning	19.59	0.00
		8	78.72	73.93	M	developing	32.13	2.44
		9	75.97	73.76	M	spawning	21.94	0.00
		10	74.82	69.79	M	spawning	12.92	0.00
		11	72.92	72.46	F	spawning	21.94	1.04
		12	74.66	64.90	F	spent	19.98	0.00
		13	69.96	57.74	M	spawning	22.73	0.00
		14	69.49	51.14	F	spawning	19.20	0.00
		15	67.79	48.88	M	spawning	21.16	0.00
		16	63.38	41.42	M	spawning	1.16	0.00
		17	64.45	37.88	F	spawning	0.00	0.00
		18	62.89	35.14	F	developing	0.00	0.00
		19	57.89	27.25	M	spawning	1.95	0.00
		20	55.01	11.33	M	spawning	16.06	0.00
COT (CBC)	19 C	1	68.78	42.92	F	spawning	29.00	4.90
		2	67.55	39.01	M	spawning	28.61	0.00
		3	64.66	40.29	M	spawning	25.08	8.76
		4	64.27	39.26	M	spawning	34.88	8.76
		5	61.07	32.01	F	spawning	28.21	9.46
		6	62.94	30.90	M	spawning	12.92	11.92
		7	60.45	28.70	M	spawning	24.29	3.50
		8	62.90	35.18	M	spawning	23.51	18.93
		9	59.16	24.92	M	spawning	24.69	7.36
		10	56.05	20.61	F	spent	25.08	0.00
		11	48.98	20.02	F	spawning	20.37	0.00
		12	54.50	23.53	F	spawning	24.69	0.00
		13	47.96	13.40	M	spawning	9.00	0.00
		14	54.41	20.93	M	spawning	27.43	0.00
		15	52.05	15.75	M	spawning	19.20	0.00
		16	56.66	23.48	M	spawning	21.94	0.00
		17	62.58	31.29	F	spawning	18.81	0.00
		18	51.33	17.79	F	spawning	18.41	0.00
		19	51.75	20.11	M	spawning	25.08	0.00
		20	61.25	26.26	F	spawning	17.24	0.00

September 1994

Site	Water Temp.	Clam	Length (mm)	Wet Weight (g)	Sex	Reproductive Stage	hsp 70 (ng/mg total protein) Gill	Gonad
COT	19 C	1	92.07	106.15	M	spawning	10.96	0.00
(CC)		2	69.77	54.96	F	spawning	9.00	0.00
		3	66.40	45.10	M	spawning	0.00	0.00
		4	63.04	32.71	F	spawning	16.06	0.00
		5	64.31	30.37	M	spawning	1.56	1.39
		6	61.82	29.06	M	spawning	0.00	0.00
		7	61.06	27.71	M	spawning	9.79	19.98
		8	60.71	28.05	M	indifferent	0.00	9.46
		9	62.29	26.51	M	spawning	0.00	1.04
		10	60.19	27.72	F	spawning	2.73	5.25
		11	54.62	21.23	M	spent	0.00	8.41
		12	50.74	17.98	F	spawning	3.52	30.86
		13	54.33	21.25	F	spawning	0.00	13.67
		14	49.35	16.05	M	spawning	0.00	11.21
		15	55.54	19.63	M	spawning	0.00	10.86
		16	51.56	15.08	M	spawning	0.00	8.41
		17	56.81	24.00	F	spawning	0.00	0.00
		18	58.47	25.94	M	spawning	1.16	5.25
		19	51.53	17.67	F	spawning	23.12	7.00
		20	52.60	18.01	F	spawning	25.86	11.92
LBB	19 C	1	80.98	62.22	F	spawning	12.14	9.11
		2	80.49	52.05	M	spawning	23.90	8.06
		3	76.70	57.53	M	spawning	16.06	9.27
		4	74.37	57.42	F	spawning	13.32	5.95
		5	73.07	47.33	M	indifferent	0.00	15.42
		6	72.55	50.80	F	spent	0.00	2.44
		7	72.09	42.37	F	spent	0.00	1.39
		8	71.90	44.52	F	spawning	0.00	1.39
		9	70.10	37.22	F	spawning	1.16	3.50
		10	69.03	38.72	F	spawning	0.00	0.00
		11	68.59	41.89	F	spawning	0.00	1.04
		12	69.93	38.45	F	spawning	0.00	7.00
		13	69.06	42.14	F	spent	26.25	4.90
		14	67.23	39.86	F	spawning	21.94	1.74
		15	66.56	45.15	M	spawning	19.98	7.36
		16	66.40	32.19	M	spawning	25.86	3.85
		17	66.57	36.87	M	spawning	16.06	0.69
		18	65.57	39.66	F	spent	10.96	7.36
		19	61.77	29.57	F	spent	12 92	0.00
		20	63.55	34.77	M	spawning	18.41	2.44

January 1995

Site	Water Temp.	Clam	Length (mm)	Wet Weight (g)	Sex	Reproductive Stage	hsp 70 (ng/mg total protein) Gill	Gonad
NBH	0 C	1	75	54.78	M	indifferent	16.56	0.00
		2	73	50.80	F	indifferent	17.88	3.15
		3	73	51.54			9.46	0.00
		4	72	57.08	F	indifferent	17.28	0.00
		5	70	54.22	F	indifferent	9.30	0.00
		6	70	50.60	F	developing	18.13	0.00
		7	73	46.86	M	developing	19.46	0.00
		8	71	42.45	F	spent	13.46	0.00
		9	66	41.49	F	developing	15.29	0.00
		10	68	39.41	M	indifferent	17.04	0.00
		11	62	31.33	F	indifferent	21.40	0.00
		12	63	29.60	F	indifferent	17.69	0.00
		13	63	28.89	F	indifferent	14.57	6.65
		14	66	39.05	F	developing	23.62	2.09
		15	61	29.56	M	developing	27.71	1.04
		16	56	20.21	F	developing	22.84	0.00
		17	55	20.44	F	developing	40.73	0.00
		18	55	18.81	M	developing	17.24	0.00
		19	48	13.88	F	developing	23.05	0.00
		20	47	13.49	F	developing	64.84	0.00
COT (CBC)	0 C	1	84	103.63	M	indifferent	2.22	2.80
		2	82	71.55	F	developing	1.07	0.00
		3	75	62.75	F	developing	0.55	0.00
		4	72	57.08	F	developing	9.20	0.00
		5	74	64.47	F	developing	1.82	0.00
		6	69	41.97	M	developing	6.66	0.00
		7	68	40.48	F	developing	18.84	0.00
		8	61	31.96	M	developing	19.94	0.00
		9	64	39.93	F	developing	24.16	0.00
		10	64	43.53	F	developing	9.95	0.00
		11	68	38.55	M	indifferent	22.97	0.00
		12	61	36.78	F	developing	12.72	0.00
		13	64	30.60	M	indifferent	21.83	0.00
		14	58	30.43	M	indifferent	15.03	0.00
		15	58	29.82	M	developing	15.00	0.00
		16	55	26.27	F	indifferent	17.69	0.00
		17	54	21.86	F	developing	18.42	0.00
		18	56	23.87	M	developing	48.91	0.00
		19	54	22.45	M	developing	7.31	9.11
		20	53	22.43	M	developing	16.70	7.36

January 1995

Site	Water Temp.	Clam	Length (mm)	Wet Weight (g)	Sex	Reproductive Stage	hsp 70 (ng/mg total protein) Gill	Gonad
COT	0 C	1	83	67.43	F	developing	57.08	6.30
(CC)		2	80	83.60	M	developing	9.47	2.09
		3	74	53.87	F	developing	18.76	4.55
		4	78	56.04	F	developing	11.02	2.09
		5	73	45.19	M	developing	20.06	0.00
		6	70	41.14	M	developing	16.69	2.44
		7	70	37.18	M	developing	16.72	1.74
		8	65	35.94	F	developing	19.54	0.69
		9	63	32.53	M	developing	19.61	0.69
		10	62	31.03	M	indifferent	9.70	5.25
		11	62	30.44	M	indifferent	3.79	5.25
		12	61	29.87	F	developing	18.48	3.85
		13	65	30.69	F	developing	0.45	0.00
		14	61	29.88	F	developing	0.00	2.80
		15	58	22.65	M	developing	0.00	3.85
		16	57	26.93	F	developing	4.85	10.51
		17	56	24.50	F	developing	0.29	0.69
		18	56	18.16	F	developing	3.23	0.00
		19	56	20.82	F	developing	0.90	0.00
		20	55	17.80	M	developing	0.00	0.00
LBB	0 C	1	74	49.31	M	developing	60.13	0.00
		2	72	43.56	M	indifferent	17.99	0.00
		3	70	48.09	F	spent	17.09	0.00
		4	74	50.84	F	developing	4.46	0.00
		5	70	35.34	F	developing	3.44	12.27
		6	74	39.18	F	indifferent	0.00	2.44
		7	70	46.23	F	indifferent	1.18	0.00
		8	69	45.08	M	indifferent	0.22	0.69
		9	71	35.84	F	indifferent	0.00	0.00
		10	63	34.91	M	indifferent	0.00	2.44
		11	63	31.40	M	indifferent	0.00	0.00
		12	62	30.15	F	indifferent	12.25	0.00
		13	64	26.78	M	indifferent	0.48	2.44
		14	59	24.71	M	indifferent	6.79	0.00
		15	59	24.68	F	indifferent	10.11	0.00
		16	56	33.97	F	developing	5.94	0.00
		17	55	25.90	F	developing	0.00	0.00
		18	58	20.68	F	developing	0.00	0.00
		19	59	23.96	F	developing	0.00	0.00
		20	64	29.06	F	developing	0.00	0.00

March 1995

Site	Water Temp.	Clam	Length (mm)	Wet Weight (g)	Sex	Reproductive Stage	hsp 70 (ng/mg total protein) Gill	Gonad
NBH	7 C	1	51.49	27.27	F	developing	19.63	7.71
		2	58.19	24.81	M	indifferent	18.23	0.00
		3	52.09	19.34	F	developing	17.53	0.00
		4	56.48	23.90	F	developing	7.71	4.90
		5	52.65	25.35	M	developing	0.00	14.02
		6	55.21	26.85	F	developing	0.00	5.25
		7	54.86	22.97	M	developing	2.80	18.58
		8	60.27	33.78	F	developing	0.00	12.62
		9	54.43	24.53	M	developing	1.74	8.76
		10	43.95	13.07	F	developing	7.00	6.65
		11	50.83	14.63	M	indifferent	22.79	2.44
		12	52.87	17.79	M	developing	8.06	9.81
		13	60.62	31.41	M	developing	0.00	5.60
		14	52.08	16.52	F	indifferent	5.60	0.00
		15	45.36	10.82	M	developing	34.72	9.11
		16	52.85	18.81	F	developing	0.00	8.06
		17	48.97	16.10	F	developing	0.00	7.36
		18	47.35	15.66	F	developing	0.00	4.55
		19	46.50	14.37	F	developing	0.00	7.71
		20	51.40	16.01	M	developing	0.00	11.92
COT (CBC)	9 C	1	90.20	99.51	M	developing	21.39	9.81
		2	84.68	103.77	F	developing	9.46	11.21
		3	62.66	27.66	M	developing	7.71	3.15
		4	64.52	32.19	F	developing	34.36	0.00
		5	65.28	35.68	M	developing	14.37	4.55
		6	62.85	32.82	M	developing	28.05	5.60
		7	65.41	30.86	F	developing	18.58	0.00
		8	64.53	31.19	M	developing	0.00	4.90
		9	68.94	50.30	F	developing	12.97	3.50
		10	67.31	39.93	F	developing	16.48	0.00
		11	66.14	44.29	F	developing	10.51	0.00
		12	52.44	15.57	M	developing	0.00	4.55
		13	48.31	13.61	F	developing	36.47	5.95
		14	58.93	24.05	F	developing	0.00	3.15
		15	54.69	26.10	M	developing	0.00	4.55
		16	53.92	18.11	F	developing	17.53	0.00
		17	56.48	22.70	M	developing	0.00	6.30
		18	37.05	6.97	F	developing	0.00	0.00
		19	64.69	33.93	M	developing	4.20	3.50
		20	69.09	49.85	F	developing	0.00	0.00

March 1995

Site	Water Temp.	Clam	Length (mm)	Wet Weight (g)	Sex	Reproductive Stage	hsp 70 (ng/mg total protein) Gill	Gonad
COT	9 C	1	91.44	113.21	M	developing	1.74	15.77
(CC)		2	84.81	101.79	M	developing	12.97	9.11
		3	66.83	37.40	M	developing	0.00	5.95
		4	63.20	38.52	M	developing	0.00	9.46
		5	60.58	25.32	F	developing	3.85	0.00
		6	59.49	25.27	F	developing	0.00	15.07
		7	56.06	25.33	F	developing	18.23	0.34
		8	58.55	26.23	M	spawning	12.62	4.90
		9	53.47	17.85	F	developing	15.07	7.36
		10	52.25	18.65	M	developing	15.42	9.46
		11	49.10	13.14	M	developing	11.92	4.55
		12	49.36	16.18	F	developing	14.37	95.40
		13	48.39	14.72	F	developing	23.49	0.00
		14	46.44	14.30	F	developing	13.67	0.00
		15	50.10	14.81	M	developing	14.37	0.00
		16	47.40	12.80	M	developing	19.98	5.25
		17	45.49	12.20	F	indifferent	30.16	4.90
		18	44.48	10.39	M	developing	32.96	3.85
		19	50.79	13.93	M	developing	13.67	4.90
		20	45.91	12.49	M	developing	14.37	2.09
LBB	9 C	1	43.35	9.73	M	indifferent	18.93	2.09
		2	50.85	15.86	M	developing	25.24	2.80
		3	52.51	16.25	F	developing	19.63	0.00
		4	46.48	12.86	F	developing	21.74	0.00
		5	51.62	17.21	F	developing	22.09	0.00
		6	43.94	10.35	M	developing	16.83	0.34
		7	48.90	14.73	F	developing	21.04	6.65
		8	46.49	12.74	M	developing	14.72	3.15
		9	47.52	13.41	M	developing	14.37	2.09
		10	51.02	15.45	F	developing	23.49	0.00
		11	62.38	28.30	M	developing	19.98	4.90
		12	43.17	10.75	M	developing	20.68	8.41
		13	55.35	18.22	F	developing	22.09	12.97
		14	48.55	14.26	M	developing	18.93	1.04
		15	53.15	18.61	F	developing	25.60	0.00
		16	55.50	24.32	F	developing	19.63	7.36
		17	57.02	22.86	F	developing	15.77	6.65
		18	56.39	31.03	M	developing	23.84	4.55
		19	46.05	15.19	F	developing	21.04	0.69
		20	47.65	13.64	M	developing	18.58	3.85

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Site	Water Temp.	Clam	Length (mm)	Wet Weight (g)	Sex	Reproductive Stage	hsp 70 (ng/mg total protein) Gill	Gonad
NBH	14 C	1	73.45	61.64	F	spawning	31.56	36.47
		2	65.89	42.62	M	ripe	36.82	37.52
		3	61.66	37.85	M	spawning	34.36	39.98
		4	62.03	34.14	M	ripe	32.26	0.00
		5	54.28	24.73	M	spawning	34.01	0.00
		6	56.54	30.54	F	spawning	29.10	0.00
		7	49.75	15.00	M	spawning	31.21	0.00
		8	47.09	13.43	M	spawning	26.30	0.00
		9	48.23	14.10	M	spawning	28.05	0.00
		10	49.01	11.98	F	spent	25.95	0.00
		11	59.60	22.00	F	spawning	31.28	0.00
		12	53.11	20.36	F	ripe	33.31	0.00
		13	52.06	17.28	M	spawning	32.26	0.00
		14	43.12	12.46	F	ripe	35.07	0.00
		15	42.77	9.41	F	ripe	28.40	0.00
		16	45.45	10.80	F	ripe	29.10	0.00
		17	44.08	11.19	M	spawning	31.56	0.00
		18	42.96	10.17	F	ripe	34.36	0.00
		19	43.79	9.46	F	spawning	31.56	0.00
		20	42.53	9.19	F	spawning	32.26	0.00
COT (CBC)	14 C	1	81.81	84.25	F	ripe	11.21	15.77
		2	78.88	72.54	F	ripe	3.85	0.00
		3	72.06	52.14	F	ripe	5.95	11.56
		4	64.86	38.59	M	spawning	0.00	18.23
		5	72.07	52.13	F	ripe	0.00	6.30
		6	63.45	34.89	M	spawning	0.00	0.69
		7	69.91	42.25	F	ripe	0.00	6.30
		8	69.19	41.76	F	developing	0.00	0.00
		9	66.69	43.70	F	ripe	0.00	18.58
		10	63.59	32.45	M	ripe	0.00	13.32
		11	62.53	33.70	M	ripe	0.00	17.18
		12	61.53	33.64	M	ripe	0.00	20.68
		13	56.96	27.77	F	ripe	9.11	9.46
		14	62.50	38.50	F	ripe	0.00	0.00
		15	60.34	29.65	F	ripe	0.69	7.71
		16	60.38	32.03	M	ripe	2.80	10.51
		17	55.70	23.29	M	spawning	0.00	6.65
		18	55.78	24.96	F	ripe	4.90	0.00
		19	56.26	21.61	F/M	ripe	5.60	0.00
		20	56.39	18.96	F	spent	2.09	0.00

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Site	Water Temp.	Clam	Length (mm)	Wet Weight (g)	Sex	Reproductive Stage	hsp 70 (ng/mg total protein) Gill	Gonad
COT	14 C	1	79.01	71.15	F	developing	0.00	6.65
(CC)		2	78.79	71.14	M	indifferent	0.00	10.16
		3	69.89	46.54	F	developing	0.00	12.27
		4	63.49	36.25	M	spawning	0.00	30.86
		5	70.33	44 33	F	spent	0.00	3.15
		6	65.02	37.59	F	spent	0.00	9.46
		7	69.77	45.54	M	spent	0.00	10.51
		8	69.58	43.19	M	spawning	0.00	15.77
		9	65.42	45.51	M	spawning	0.00	21.04
		10	63.69	32.33	M	ripe	0.00	2.44
		11	64.73	38.26	M	spawning	0.00	18.58
		12	61.06	25.39	M	spawning	0.00	11.21
		13	63.25	28.84	F	spent	0.00	10.16
		14	63.20	30.72	F	spent	0.00	5.60
		15	60.13	23.46	M	spawning	0.00	19.98
		16	65.15	27.61	M	indifferent	0.00	12.62
		17	53.62	22.58	M	ripe	0.00	9.81
		18	51.44	15.23	F	spawning	0.00	3.50
		19	53.70	20.48	M	spawning	0.00	12.97
		20	53.04	18.23	M	spawning	3.85	12.27
LBB	14 C	1	70.15	49.68	F	spent	0.00	0.00
		2	54.06	16.53	M	spawning	0.00	2.80
		3	52.77	21.48	F	ripe	0.00	15.07
		4	54.24	18.01	M	spawning	0.00	2.44
		5	50.97	17.34	F	developing	7.00	0.00
		6	55.98	19.89	M	developing	0.00	5.60
		7	52.90	19.37	F	developing	20.33	0.00
		8	47.92	18.33	M	spent	24.19	7.71
		9	50.43	18.73	M	developing	4.55	0.00
		10	49.02	16.97	F	ripe	1.74	5.95
		11	51.78	17.97	M	spawning	7.71	1.04
		12	53.96	18.95	M	spawning	14.02	0.00
		13	56.43	20.73	F	ripe	6.30	0.00
		14	50.85	17.10	F	ripe	0.00	0.00
		15	52.81	18.67	F	ripe	0.34	0.00
		16	50.05	17.06	M	spawning	1.74	0.00
		17	49.64	16.94	M	spawning	0.00	0.00
		18	47.85	14.28	F	ripe	10.16	0.00
		19	49.28	15.56	F	indifferent	11.92	0.00
		20	53.06	19.54	M/F	ripe	9.11	0.34

Biographical Note

The author was born in Annapolis, Maryland on January 6, 1969. As a young child, she learned about fishing and the water from her grandfather and later learned about science from numerous teachers, including Miss Sarah Bell and Dr. John E. Reynolds, III. She received a B.S. with high honors in Marine Science from Eckerd College in May of 1990, with an honors thesis supervised by Dr. Carm Tomas at the Florida Marine Research Institute and entitled The Relationship Between Phosphate Availablity and Toxin Content in the Florida Red Tide Dinoflagellate *Gymnodinium breve*. She eagerly anticipates beginning a postdoc with Dr. Karl Fent of the Swiss Federal Institute of Environmental Science and Technology in Duebendorf, Switzerland this fall.