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Physiological Ecology of Stress-Responsive Gene Expression in the American Lobster, Homarus americanus: Molecular Chaperones and Polyubiquitin

By

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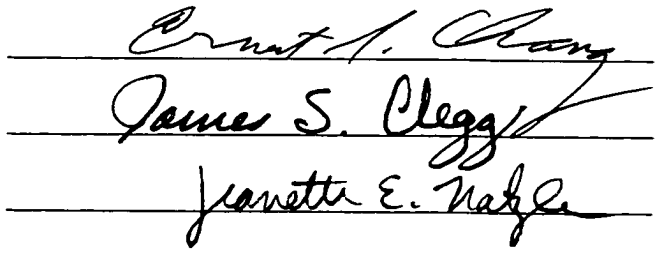

Committee in Charge

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Jeffrey L. Spees
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Physiological ecology of stress-responsive gene expression in the American lobster, Homarus americanus: molecular chaperones and polyubiquitin

Abstract

Homologous molecular probes were used to examine in vivo molecular chaperone and polyubiquitin gene expression patterns in the American lobster, Homarus americanus, an ectothermic marine crustacean. Following long-term laboratory acclimation to temperatures experienced during overwintering in nature, juvenile lobsters failed to elicit the classical "heat-shock response" when subjected to an acute 13 °C thermal stress. Levels of mRNA coding for molecular chaperones (HSC70, HSP70, and HSP90) and polyubiquitin were not induced during thermal stress or recovery in cold-acclimated animals. These results contrasted with those for lobsters acclimated to ambient Pacific Ocean temperatures that experienced an acute stress over an equivalent thermal interval. Ambient-acclimated lobsters displayed significant inductions in the mRNA levels for both molecular chaperones and polyubiquitin. Hyper- and hypo-osmotic stress were found to significantly induce HSP90 and polyubiquitin mRNA levels in ambient-acclimated juvenile lobsters. Additionally, osmotic and thermal stress interactively altered HSP90 and polyubiquitin gene expression in animals that received both types of stress. Physiological changes over the molt cycle significantly affected gene expression in claw muscles undergoing atrophy in preparation for molting. Molecular chaperone and polyubiquitin mRNA levels differed significantly between premolt claw and abdominal muscle, which does not undergo atrophy during the premolt stages.

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Introduction

Structural and enzymatic proteins function optimally under specific conditions of temperature, pH, and osmolarity. For example, some proteins operate well at 0°C (antifreeze glycoproteins from Trematomas sp.) (Lane et al., 2000), while others function efficiently at 100°C or more (Thermus aquaticus DNA polymerase) (Somero, 1995). Under non-optimal conditions, however, proteins may lose their native conformations. Denatured (non-native) proteins are potentially cytotoxic because they can aggregate when regions of exposed hydrophobic amino acids come into contact and sequester. Accordingly, organisms have evolved plastic systems to repair or degrade proteins during thermal or otherwise denaturing stresses. The first two chapters of this dissertation will examine gene expression in two important cellular systems that deal with aberrant proteins, "the heat-shock response" and the ubiquitin-proteasome pathway in the American lobster, Homarus americanus. These chapters will focus on responses to environmental stress. The third chapter explores gene expression for these systems during unique conditions dependent upon the crustacean molt cycle.

Identified by Ritossa (1962), the heat-shock response was originally noted as chromosomal puffing in the salivary glands of Drosophila during thermal stress. Subsequently, this puffing pattern, which is indicative of gene transcription, was found to produce a series of proteins called heat-shock proteins (HSPs) (Tissières et al., 1974). Following an intense and prolific period of study (still on-going after almost 40 years), the production of inducible heat-shock proteins is presently one of the best understood models of transcriptional regulation.

HSPs belong to a class of abundant intracellular molecules called molecular chaperones. Molecular chaperones, so-called because they facilitate the proper folding and conformational status of other proteins, are now known to be ubiquitous among organisms (Feder and Hofmann, 1999). Constitutively expressed chaperones, heat-shock cognate proteins (HSCs), as well as inducible isoforms (HSPs) perform the critical job of folding nascent proteins as they arise from the ribosome. They aid in the synthesis of most cellular proteins. Additionally, they play important roles in intracellular transport, signal transduction, the cell cycle, and stress responses (for reviews see: Morimoto et al., 1990; Hartl, 1996; Nover and Scharf, 1997; Bukau and Horwich, 1998; Morimoto, 1998).

Following cell stress, denatured proteins that can not be salvaged by HSCs/HSPs must be degraded and re-cycled by the cell; this occurs primarily via the ubiquitin-proteasome pathway. Biochemical studies using cell-free lysate from rabbit reticulocytes during the 1970's initiated the study of ubiquitin-mediated proteolysis (Hershko and Ciechanover, 1992). Ubiquitin (Ub) is a highly conserved 76 AA protein degradation signal in eukaryotes (reviewed in: Hochstrasser, 1996; Hershko and Ciechanover, 1998; Pickart, 2000). Eukaryotic genetic coding regions for Ub are repeated in tandem to expedite its production. Following transcription, the "polyubiquitin" transcript (poly-Ub) is translated and post-translationally cleaved into multiple Ub peptides. Through a multi-step enzymatic cascade, branched polymeric chains of Ub are covalently ligated to damaged proteins. These proteins are subsequently destined for proteolysis by a degradative protein complex known as the 26S proteasome. Prior to conjugation with its target, Ub is activated through adenylation at its C terminus by a Ub-activating enzyme,

E1. After attack by the sulfhydryl group of E1, the resulting E1-Ub thiolester is passed to a ubiquitin-conjugating enzyme, E2. E2 enzymes catalyze the ligation of Ub to its substrate (a damaged protein or a regulatory protein subunit) either alone, or with the help of a ubiquitin-protein ligase, E3. It is thought that degradation is accelerated by further addition of multiple Ub peptides. Additionally, the binding pattern of moieties can determine whether an entire protein is degraded, or whether a specific regulatory subunit is removed. A rapidly growing body of research has revealed Ub conjugation to be a key step in a variety of cellular regulatory networks including cell cycle transitions, class I antigen processing, signal transduction, and receptor mediated endocytosis (Hochstrasser, 1996). Protein turnover and regulation via proteolysis are likely to play equally important roles in cellular regulation as does mRNA and protein synthesis.

The lobster is an interesting model for examining gene expression in response to environmental stress because it naturally undergoes long-term seasonal thermal acclimatization and is ectothermic. In addition, lobsters encounter multiple environmental stresses during several life stages and can be used to evaluate interactive stress responses. Finally, the profound physiological changes that occur in lobster in response to ecdysteroids (steroid molting hormones) provide a unique system to study molecular chaperone and ubiquitin expression during steroid hormone regulation, protein synthesis, and protein turnover required for molting. Experimental results examining all of these issues are presented in the chapters that follow.

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Chapter One

Thermal acclimation and stress in the American lobster Homarus americanus: equivalent temperature shifts elicit unique gene expression patterns for molecular chaperones and polyubiquitin

Abstract

Using homologous molecular probes, the influence of equivalent temperature shifts on the in vivo expression of genes coding for a constitutive heat-shock protein (HSC70), heat-shock proteins (HSP70 and HSP90), and polyubiquitin, were examined post-acclimation in the American lobster, Homarus americanus. Sibling, intermolt, juvenile male lobsters were acclimated to thermal regimes experienced during overwintering conditions (0.4±0.3 °C), and to ambient Pacific Ocean temperatures (13.6±1.2 °C), for 4-5 weeks. Both groups were subjected to an acute thermal stress of 13.0 °C, a temperature shift previously found to elicit a robust heat-shock response in ambient-acclimated lobsters. Animals were examined after several durations of acute heat-shock (0.25- 2 h) and after several recovery periods (2- 48 h) at the previous acclimation temperature following a 2 h heat-shock. Significant inductions in HSP70, HSP90, and polyubiquitin mRNA levels were found for the ambient-acclimated group. Alternatively, for the cold-acclimated group, an acute thermal stress over an equivalent interval resulted in no induction in mRNA levels for any of the genes examined. For the ambient-acclimated group, measurements of polyubiquitin mRNA levels showed that hepatopancreas, a digestive tissue, incurred greater "irreversible" protein damage relative to abdominal muscle, a tissue possessing superior stability over the thermal intervals tested.

Introduction

Many ectothermic organisms acclimate and survive over wide temperature ranges. Lacking the heat-producing capacity of endotherms, their metabolism is at the mercy of temperature changes dictated by seasons and disturbance. These thermal fluctuations are not trivial. Critical biological processes including development, growth, and fitness are all temperature-dependent. Responding to thermal variation in the environment, numerous organisms have evolved biochemical adaptations to buffer temperature changes. These adaptations include homeoviscous and homeophasic alteration of cell membranes (Vigh et al., 1998), enhanced protein stability due to specialized amino acid sequence (e.g. Thermus aquaticus DNA polymerase) (Somero, 1995), elevated concentrations of intracellular osmolytes such as glycerol, sorbitol, trehalose, and glucose (Yancey et al., 1982), anti-freeze glycoproteins (Cheng and Chen, 1999), and inducible stress proteins (molecular chaperones, heat-shock proteins, HSPs) (Feder and Hofmann, 1999).

Production of HSPs is a common cellular response to thermal stress. In addition to "chaperoning" nascent polypeptides into their native structures HSPs are known to protect against thermal stress by refolding denatured proteins and preventing protein aggregation (see reviews in Morimoto et al., 1990; Hartl, 1996; Nover and Scharf, 1997; Bukau and Horwich, 1998). Some HSPs such as HSP104 in yeast, Saccharomyces cerevisiae (Sanchez and Lindquist, 1990), are required for induced thermotolerance, a process by which an organism previously exposed to a sublethal temperature can subsequently survive a normally lethal heat stress (Schlesinger et al., 1982; Lindquist and

Craig, 1988; Clegg et al., 1998).

Intense or prolonged thermal stress can permanently damage proteins, promoting ubiquitination and degradation via the 26S proteasome (Varshavsky, 1997; Mykles, 1998). While HSP expression may be viewed as a measure of "reversible" protein damage, ubiquitin production and conjugation are considered signs of "irreversible" protein damage (Parsell and Lindquist, 1993; Hofmann and Somero, 1996). Through a series of enzymatic reactions, the 76 amino acid ubiquitin polypeptide is covalently bound to damaged proteins or regulatory proteins such as transcription factors, cell cycle regulators, kinases, phosphatases, and tumor suppressors (Bonifacino and Weissman, 1998). The presence and patterning of ubiquitin moieties determine the specificity of the 26S proteasome which is signaled to degrade particular peptide subunits or entire proteins (Hochstrasser, 1996; Varshavsky, 1997).

In the American lobster, Homarus americanus, multiple ubiquitin coding regions are transcribed as a single transcript (polyubiquitin) which is post-translationally processed into individual ubiquitin moieties (Shean and Mykles, 1995). Here I use polyubiquitin gene expression as an indirect measure of protein degradation. As the number of proteins permanently damaged by a particular environmental insult increases, greater numbers of polyubiquitin transcripts are required for ubiquitin production to support the degradative pathway. Polyubiquitin expression is correlated with the degradation of myofibrillar proteins during premolt claw atrophy in the land crab, Gecarcinus lateralis (Shean and Mykles, 1995), and in H. americanus (see Chapter Three).

Because of their remarkable ability to maintain homeostasis and metabolism during long-term thermal acclimatization/acclimation, lobsters are interesting organisms in which to study thermal acclimation and stress. Lobsters can acclimate and survive at temperatures ranging from -1.0°C to 30.5°C (Lawton and Lavalli, 1995). While one might expect the lobster to become dormant at temperatures below 5°C (at these temperatures growth is minimal), lobsters are known to be active and even to mate at temperatures in the 0-2°C range. In fact, individuals that reach premolt (stage D_1) before declining autumn temperatures may continue the molting process, eventually shedding the exoskeleton at temperatures as low as 0°C (Waddy et al., 1995).

Throughout the long-term acclimatization to winter thermal regimes lobsters are likely to change metabolically, down-regulating DNA replication, and altering protein synthesis and enzyme reaction rates. Modifications in both subunit expression and isozyme patterns have been studied in response to temperature changes (Hochachka and Somero, 1984; Somero, 1995). To maintain both the function and efficiency of enzymatic reactions during thermal acclimation, the structures of some requisite proteins such as carp (Cyprinus carpio) myosin ATPase are known to display specific temperature-dependent differences. Myosins from 30°C-acclimated carp are about 4 times as thermostable as those from 10°C-acclimated carp (Hwang et al., 1990). Similar types of conformational changes could occur during the long-term thermal acclimatization of overwintering lobsters. Trausch (1976) found that temperature-mediated changes in the configuration of lactate dehydrogenase profoundly influenced its kinetics in abdominal muscle from the lobster Homarus gammarus. Proteins that undergo flexibility changes,

conformational adjustments, or isozyme exchanges over different thermal regimes are likely to differ in stability/lability characteristics (Johnston et al., 1973; Hashimoto et al., 1982; Hwang et al., 1990). In regard to these observations, and those concerning plasticity in the heat-shock response for marine ectotherms (Dietz and Somero, 1992; Dietz, 1994; Roberts et al., 1997; Tomanek and Somero, 1999), I examined thermal acclimation and stress in H. americanus, a eurythermal organism adapted to survive cold over-wintering temperatures as well as thermal shifts during spring and summer reproductive migrations (Crossin et al., 1998). Following acclimation of lobsters to "ambient" and "cold" temperatures I observed whether acute temperature shifts over equivalent intervals (13.0°C) would elicit analogous signs of protein denaturation such as HSP and polyubiquitin expression.

Materials and Methods

Animal Care

Juvenile lobsters (sibling, intermolt males; mean weight 85.3±17.2 g) reared at the Bodega Marine Laboratory (BML) were acclimated (4-5 weeks) to "cold" (0.4±0.3°C, n=44), and "ambient" (13.6±1.2°C, n=44) temperatures. Ambient-acclimated lobsters were maintained in a flow-through aquaculture system, held in individual compartments, and fed shrimp 3 times weekly. Detailed descriptions of the aquaculture system and lobster culture techniques at BML are reviewed elsewhere (Chang and Conklin, 1993; Conklin and Chang, 1993).

Cold-acclimated lobsters were held in 2 L glass jars filled with sea water in a temperature-controlled (±1.0°C) cold room. Each jar was supplied with an airstone, and water was changed once weekly. Water was changed twice during the first week of acclimation to prevent possible build-up of nitrogenous wastes. These animals were fed peeled shrimp, ad libitum, once weekly (before the water change). Generally, animals would not eat more than once a week, likely due to decreased metabolic requirements. Initial transfer to the cold-room occurred in the 2 L glass jars filled with ambient temperature seawater. Over the next 12 h, the water temperature gradually equilibrated to ~0.4°C. Temperatures were monitored and recorded daily for both the ambient- and cold-acclimated groups.

Thermal Stress

Both groups received an acute thermal stress (an elevation of 13.0°C) for 2 h. Ambient-acclimated lobsters were heat-shocked in aerated sea-water in 2 L glass jars previously equilibrated in a temperature-controlled (±0.1°C) water bath. Cold-acclimated lobsters were heat-shocked by returning them directly to compartments in the flow-through seawater system (13.4°C).

Four animals from each group were sacrificed immediately at several time points during the heat stress (0.25, 0.5, 1, and 2 h), and after several recovery periods at the previous acclimation temperature, after the 2 h heat shock (2, 6, 12, 24, 48, and 96 h recovery). Four unstressed control animals were also sacrificed from each acclimation group. For all time points and controls, several samples of abdominal muscle (AM) and hepatopancreas (HP) (=midgut gland) were dissected, frozen in liquid N_2, and stored at

-70°C.

Because of potential jar effects for the cold-acclimated animals relative to the other animals that were acclimated in the flow-through system, I do not make direct statistical comparisons between acclimation groups at particular time points for heat-shock or recovery. There were, however, no statistical differences in abdominal muscle molecular chaperone protein concentrations between controls of either group following acclimation, indicating that the cold-acclimated animals were not pre-stressed prior to the experiment (see Results section).

Molecular Probe Isolation and Northern Analysis

Total RNA was isolated from juvenile H. americanus HP (Totally RNA kit, Ambion) and 5 μg was reverse-transcribed at 37°C using an oligo-dT_{15} primer (Superscript reverse transcriptase, Stratagene protocol). This cDNA was used as template for polymerase chain reaction (PCR). A 500 bp partial clone for lobster HSP70 was amplified using the following degenerate primers (Cochrane et al., 1994):
5' GC[GCT]AAGAA[TC]CA[AG]G[TC][TC]G[ACGT]ATGAAC 3' and
5' GT[AGT]G[AC][CT]TT[AGC]AC[AGC]TC[AG]AAGAT 3'.
Using the same template, a 380 bp partial clone for lobster HSP90 was also isolated using a 5' GGGAAGCACGAGGAGCGGAGG 3' forward primer and an oligo-dT_{15} reverse primer. Strong nucleotide sequence similarity between HSP70 and the constitutive heat-shock protein HSC70 allowed the degenerate HSP70 primers described above to anneal and amplify large segments of an HSC70 cDNA in an "anchored" PCR approach. This involved the dilution, boiling, and centrifugation of an aliquot of a lobster hepatopancreas

cDNA library. One µl of this preparation served as template for separate 50 µl PCR reactions utilizing the HSP70 forward and reverse primers, and SP6 and T7 RNA vector promotor primers. A 2100 bp fragment of HSC70 derived from the HSP70 forward and SP6 reverse reaction was cloned and subsequently digested with EcoRI to yield a 600 bp fragment. This fragment was then subcloned into the pCR2 vector (Invitrogen). Because it consists largely of sequence from the 3' untranslated region of lobster HSC70, this 600bp gene segment was used to measure HSC70 expression specifically as this probe does not hybridize to HSP70 gene sequence (data not shown).

All PCR reactions were performed under the following conditions: 95°C initial denature, 3 min (1 cycle); 95°C, 1 min; 56°C, 1.5 min; 72°C, 2 min (35 cycles); 72°C final extension, 10 min (1 cycle), using Taq DNA polymerase (PCR buffer, 2.5 mM Mg^{2+}, Gibco). PCR products were gel-purified (Qiagen kit) and cloned into the pCR 2.1 or pCR2 vectors (Invitrogen). PCR products were sequenced by the DNA sequencing facility, University of California, Davis or by Davis Sequencing, Davis, CA. NETBLAST searching (NCBI) was used to confirm the identities of PCR products.

Total RNA was isolated from the dissected AM and HP samples (RNAgents kit, Promega), quantified with a spectrophotometer, and equally loaded (15 µg AM total RNA, 25 µg HP total RNA) onto denaturing 1% agarose gels. These gels were washed (15 min, DEPC H_2O) and blotted overnight onto nylon membranes (Magnagraph, MSI). Following UV cross-linking (UV Stratalinker 1800), blots were pre-hybridized (2 h) in 5x SSPE buffer, 50% (w/v) formamide, 5x Denhardt's, 1% SDS, and 100 µg/ml sheared salmon sperm DNA. The partial lobster HSP70 clone was ^{32}P-labeled (Prime-It RmT,

Stratagene), added directly to the prehybridization solution, and allowed to hybridize overnight at 42°C. Following hybridization, the blots were washed twice with 2x SSPE and placed on film for 2 d at -70°C. Following exposure of the film, the blots were stripped with several washes (0.1x SSC, 0.1% SDS, 65°C) until background was minimal and prehybridized, hybridized, and washed as above, except that a partial lobster HSP90, or polyubiquitin (600 bp; gift of Prof. D.L. Mykles) cDNA probe was added. To check for equal loading of RNA, the blots were probed with a 700 bp partial cDNA probe for lobster (H. gammarus) actin (Harrison and El Haj, 1994). Separate blots were run to examine HSC70 mRNA levels with an identical protocol as above using the 600 bp partial clone specific for HSC70 as a probe. Films were scanned on a high resolution scanner, and densitometry was performed with Scion Image 2.1 software.

Western Immunoblotting

Abdominal muscle was homogenized in a hypotonic cell lysis buffer (40 mM HEPES, 5 mM $MgSO_4$, 10 mM KCl, 20 mM NaCl, 1% (w/v) SDS, pH 7.5). Following centrifugation (10 min, 15,000g), aliquots of the resulting supernatants were taken for protein assay (Bio-Rad DC protein assay) and also combined (equal volumes) with 2x SDS sample buffer for SDS-PAGE (Laemmli 1970). SDS-PAGE samples were heated for 5 min at 100°C, and loaded onto 15-well, 13% polyacrylamide gels. Loading was based on equal protein concentration (40 μg). HSP positive controls were loaded onto each of the gels (50 ng of recombinant human HSP70; 50 ng of bovine HSP90; Stressgen). Because the positive controls contained a known amount of pure HSP, their signals were later used to quantify the amount of HSP in each sample. Proteins were

electrophoresed at 150V (Bio-Rad Mini Protean II) until the dye front ran off (~1.3 h), at which time the running buffer was changed, and gels were run for 1 more hour. Previous staining of other gels allowed us to estimate the relative locations of HSP70 and HSP90 following electrophoresis. A razor blade was used to excise the slice of gel containing HSP70 and HSP90 from each 15-well gel (modified from Clegg et al., 1998). These slices were combined and electroblotted (100V, 1 h; Bio-Rad Mini Trans-blot) to a single 0.45 μm nitrocellulose membrane.

After blocking overnight with 5% w/v nonfat dry milk in TBST (150 mM NaCl, 10 mM Tris, 1 ml/L Tween 20, pH 7.4), and washing (TBST), blots were incubated in HSC70/HSP70 antisera for 1 h (1:1000, SPA-822, Stressgen), washed, and incubated in HSP90 antisera (specific to both HSP90α and HSP90β isoforms) for 1 h (1:500, SPA-830, Stressgen). Previous western blots allowed us to distinguish between proteins bound by the HSC70/HSP70 or HSP90 antisera (Spees, unpublished data). Secondary antibody was HRP-conjugated goat anti-mouse IgG (1:1000, 1 h; Sigma).

Proteins were then visualized with a chemiluminescent reaction (CLR). CLR reagents were 25 μl of 90 mM *p*-coumaric acid (Sigma C-9008) and 50 μl of 250 mM 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol; Sigma A-8511) prepared in DMSO. These reagents were mixed in 15 ml of CLR buffer (100 mM Tris, pH 8.5). Immediately before use, they were added to another 15 ml of CLR buffer containing 4 μl of 30% H_2O_2, and poured on the blots. Blots were exposed to film (Kodak Biomax MR single emulsion) for 2 min. Initially, several exposures were made (30 sec, 1 min, 2 min, 5 min) and 2 min was chosen because it appeared to keep signals within the saturation limits of

CLR reagents. Scanning densitometry was done with a high resolution scanner and Scion Image 2.1 software.

To minimize variability, all samples from a single group (n=44) were run on four, 15-well gels (poured from the same batch), and then transferred to a single membrane. Blots for both groups were incubated simultaneously in HSC70/HSP70 antibody, in HSP90 antibody, and in the CLR reagents. They were also exposed to the same films.

Results

HSP70 Gene Expression

There were marked differences in HSP70 gene expression between ambient- and cold-acclimated animals. These differences were apparent in both abdominal muscle (Fig. 1) and hepatopancreas (Fig. 2). All gene expression data were $\log_{10}$-transformed prior to one-way ANOVA to normalize variance. Dunnett's Test was used to compare treatment values to control values following significant ANOVA results.

In ambient-acclimated abdominal muscle, significant differences in HSP70 mRNA levels were detected (ANOVA; p=0.011; Fig. 3A). Further testing of heat-shock and recovery values versus control values indicated significant increases in HSP70 mRNA levels following 1 and 2 h of heat-shock, and 6 and 12 h recovery (post 2 h heat-shock) (Fig. 3A). In contrast, HSP70 mRNA in cold-acclimated abdominal muscle never reached significant levels over those of controls either during the heat-shock or during recovery. Cold-acclimated abdominal muscle showed significant differences among treatments (ANOVA; p=0.001; Fig 3A), but these differences were due to treatment

values being lower than control values. Cold-acclimated abdominal muscle had significant decreases in HSP70 mRNA levels following 1 h of heat-shock, and 48 h recovery (post 2 h heat-shock) (Fig. 3A).

HSP70 mRNA levels in ambient-acclimated hepatopancreas were also significantly different (ANOVA; $p<0.001$; Fig. 4A). Testing of treatments versus control values indicated increases in HSP70 mRNA levels following 1 and 2 h of heat-shock, and 6 h recovery (post 2 h heat-shock) (Fig. 4A). No such induction was observed for cold-acclimated hepatopancreas (ANOVA; $p=0.082$; Fig. 4A).

HSP90 Gene Expression

Differences in HSP90 gene expression were observed between acclimation groups for both abdominal muscle (Fig. 1) and hepatopancreas (Fig. 2). Similar to HSP70 expression, HSP90 mRNA levels in ambient-acclimated abdominal muscle were significantly different (ANOVA; $p=0.003$; Fig. 3B). Animals that received 2 h of heat-shock, as well as those that received a full 2 h heat-shock and 2, 6, 12, or 24 h recovery had significant values over controls (Fig. 3B). In cold-acclimated abdominal muscle HSP90 mRNA levels were significantly different between treatments but not between treatment versus control levels (ANOVA; $p=0.017$; Fig. 3B)

Hepatopancreas HSP90 gene expression patterns were strikingly different between acclimation groups. Ambient-acclimated animals displayed a significant increase in hepatopancreas HSP90 mRNA over control levels at 6 h recovery, but also a significant decrease after 48 h recovery (ANOVA; $p<0.001$; Fig. 4B). Cold-acclimated animals showed a significant inhibition/decrease in expression from basal levels that appear to be

higher than those found in ambient-acclimated controls (compare HSP90 control mRNA levels for ambient- and cold-acclimated hepatopancreas; Fig. 2) (absolute quantitative assessment of these differences, however, is not available without an internal standard). Measurements of HSP90 mRNA levels from cold-acclimated hepatopancreas were significantly less than control values at the 6, 12, 24, and 48 h time points (ANOVA; $p<0.001$; Fig. 4B).

Polyubiquitin Gene Expression

For all tissues examined, under all acclimation regimes, ambient-acclimated hepatopancreas was the only tissue to show significant expression of the polyubiquitin gene (ANOVA; $p<0.001$; Figs. 4C, 5). Expression in ambient-acclimated hepatopancreas was significantly different from control values at 2 and 6 h recovery (post 2 h heat-shock) (Fig. 4C). There were no significant changes in polyubiquitin expression in ambient-acclimated abdominal muscle (ANOVA; $p=0.90$; Fig. 5). Cold-acclimated abdominal muscle polyubiquitin expression was low and I was unable to accurately quantify it (Fig. 1B). Comparison of tissue-specific expression responses for ambient-acclimated abdominal muscle and hepatopancreas indicates that the abdominal muscle protein pool may be more conformationally stable over the thermal interval that I examined.

HSC70 Gene Expression

The pattern of HSC70 gene expression in ambient- versus cold-acclimated animals was consistent with those observed for the other chaperones, HSP70 and HSP90. Inductions of HSC70 mRNA levels were observed in both the abdominal muscle and hepatopancreas of ambient-acclimated animals but not so in the same tissues from cold-

acclimated individuals which received a heat-shock over an equivalent interval (Fig. 6).

Molecular Chaperone Protein Profiles

Representative immunoblot data from ambient- and cold-acclimated abdominal muscle are presented (Fig. 7A, B). Optical density values from samples were normalized against the values derived from HSP standards to quantify the amount of molecular chaperones. These data were $\log_{10}$-transformed and analyzed statistically (ANOVA).

Despite significant increases in molecular chaperone mRNA levels, immunoblotting results from ambient-acclimated abdominal muscle did not reveal an induction in stress proteins (Figs. 7A, 8A). This observation was consistent for the HSC70/HSP70 band (bottom band; ANOVA; $p=0.141$; Fig. 8A), the HSP90α band (top band; ANOVA; $p=0.415$; Fig. 8A), and the HSP90β isoforms (middle bands; scanned together; ANOVA; $p=0.093$; Fig. 8A).

However, some significant differences in HSP protein levels were found among the cold-acclimated animals (Figs. 7B, 8B) for the HSP90α band (top band; ANOVA; $p=0.003$; Fig. 8B) and HSP90β isoforms (middle bands; scanned together; Kruskal-Wallis ANOVA on ranks; $p=0.044$; Fig. 8B). Further testing revealed that the differences detected among the HSP90α bands were significant between treatments but not between treatment values versus control values (Bonferroni t-test). The differences detected among HSP90β isoforms were not significant when examined by multiple comparisons (Dunn's Test). Additionally, there were no significant differences detected among the HSC70/HSP70 bands (bottom band; ANOVA; $p=0.064$; Fig. 8B).

There were no significant differences in abdominal muscle molecular chaperone protein content detected between control samples of ambient- and cold-acclimated animals (HSC70/HSP70, $p=0.328$; HSP90α, $p=0.097$; HSP90β, $p=0.345$; Students t-test).

Discussion

Gene Expression Patterns

Homologous molecular probes were used to quantify H. americanus gene expression in vivo in different tissues and over long-term recovery periods. To my knowledge, the in vivo expression patterns of multiple stress-responsive genes from separate acclimation states has yet to be examined in the detail described here for any invertebrate, including model organisms such as Drosophila.

My results illustrate how ectothermic organisms which naturally undergo long-term seasonal acclimatization to cold temperatures can respond uniquely to equivalent thermal shifts. The lack of molecular chaperone and polyubiquitin gene expression observed in cold-acclimated lobsters contrasts strongly with results from ambient-acclimated animals heat-shocked with an equivalent temperature shift.

Tissue Specific Stress Responses

Not all tissues respond in concert to a given thermal stress. While both abdominal muscle and hepatopancreas of ambient-acclimated lobsters displayed signs of "reversible" protein damage (molecular chaperone expression) in response to an acute thermal stress, only the hepatopancreas showed significant gene expression for polyubiquitin, a sign of increased protein degradation or "irreversible" damage (Mykles, 1998). The protein

pools that make up these tissues may differ considerably in their stability characteristics. Feder and Krebs (1998) reported that the gut of Drosophila larvae stained intensely with trypan blue dye following a severe heat shock, suggesting that it is especially thermosensitive. Interestingly, this tissue also exhibited a prolonged delay in expression of the HSP70 protein relative to other tissues examined. They noted that severe heat shock markedly increased trypan blue staining in many tissues, but in inverse relationship to the times in which these tissues expressed HSP70, an indication of the protective effects of this chaperone. The ability of temperature to alter (and increase) the activity of proteases should also be considered here. Hepatopancreas tissues are rich in digestive enzymes, and this may also account for some of the differences observed between it and abdominal muscle.

The consistent inhibition of HSP90 gene expression in the hepatopancreas of cold-acclimated animals during recovery from acute thermal stress may be due to the interruption of transcriptional processing. Unlike HSP70, which is known to lack introns, HSP90 has several introns and requires splicing to generate a complete message (Yost and Lindquist, 1986). While markedly different from the response of ambient-acclimated animals, this inhibition does indicate that the cold-acclimated animals experienced a "stress" in response to the shift in temperature. Observing that the actin mRNA levels among cold-acclimated animals did not differ significantly, this may relate to the roles of HSP90 in multiple signal transduction pathways which may have reacted to the experimental treatment (Pratt, 1997). For example, HSP90 transcription in H. americanus is influenced by the molt-cycle and in response to ecdysteroids (steroid molting

hormones) (Chang et al., 1999; Spees, unpublished data). The Drosophila ecdysteroid receptor is, in part, activated by HSP90 (Arbeitman and Hogness, 2000) as are most steroid receptors studied to date (Pratt, 1997).

Eurythermal and Stenothermal Characteristics of H. americanus

Lobsters can survive long-term acclimation to 30°C, a temperature close to lethality (~ 32°C) (McLeese, 1956; Reynolds and Casterlin, 1979). Recall that the ambient-acclimated animals in this study received an acute stress up to about 26°C, among the higher temperatures experienced by intertidal juveniles or young adults in nature, but not unreasonably so. Although H. americanus are not native to the Pacific Ocean, they routinely experience the ambient temperatures that they were exposed to in my experiments during on-shore summer reproductive migrations into coves or estuaries of the eastern U.S. (Reynolds and Casterlin, 1979; Lawton and Lavalli, 1995).

While a robust increase in HSP mRNA levels was observed in heat-shocked ambient-acclimated lobsters, I found no induction in the corresponding proteins. Although immunoblotting does not provide direct estimates of protein synthesis, most HSP synthesis inductions are also measurable as increases in HSP protein levels on western blots. It is therefore possible in this case that the translational apparatus was rendered non-functional, potentially due to the acute nature of the thermal stress. McLeese (1956) found that upward acclimation to high temperature (from 14.5°C to 23°C) required a longer time for lobsters, about 22 days, when compared to several fish species. It was noted that full acclimation required one day for the bullhead (Ameiurus nebulosus) (Brett, 1944), the large mouth bass (Huro salmoides) (Hathaway, 1927), and

the marine goby (Gillichthys mirabilis) (Sumner and Doudoroff, 1938). Although lobsters are stereotyped as eurythermal, the temporal scales over which they experience thermal changes in the wild (often over days or seasons rather than minutes or hours) may also confer upon them some stenothermal characteristics over short time scales.

The observed lack of HSP induction in lobsters contrasts with results from other eurythermal ectothermic marine organisms that have been examined. Oysters (Crassostrea gigas; Clegg et al., 1998), snails (Tegula sp.; Tomanek and Somero, 1999), limpets (Collisella sp.; Sanders et al., 1991), and teleost fish (Gillichthys mirabilis; Dietz, 1994), are all able to synthesize and accumulate HSPs following acute thermal stresses of magnitudes equal to or greater than those used in this study. These organisms may be better adapted to acute thermal shifts because of the temporal scales over which they experience temperature changes in nature.

Conclusions and Further Questions

The observed differences in HSP and polyubiquitin responses between ambient- and cold-acclimated lobsters, and between tissues of ambient-acclimated animals may be due to differences in substrates. While the HSP or polyubiquitin systems could themselves differ between acclimation regimes, or between tissues, it is likely that substrate flexibility, conformation, presence/absence, or concentration differences between acclimation groups or tissue types is also important. Denaturation of proteins (substrates) should precede HSP induction and/or activation of ubiquitin/proteasome-mediated degradation.

The results of this study (and those mentioned above) inspire several interesting

questions for biochemists and ecologists alike. For a given organism, what are the first proteins to unfold? Is the identity and order in which particular proteins denature conserved between tissues, acclimatization/acclimation states, or organisms? Do multiple organisms in a given ecological community display similar protein denaturation profiles during a perturbation? Given the current state of scientific and technological progress, I am confident that future investigations in physiological ecology can address many of these exciting questions.

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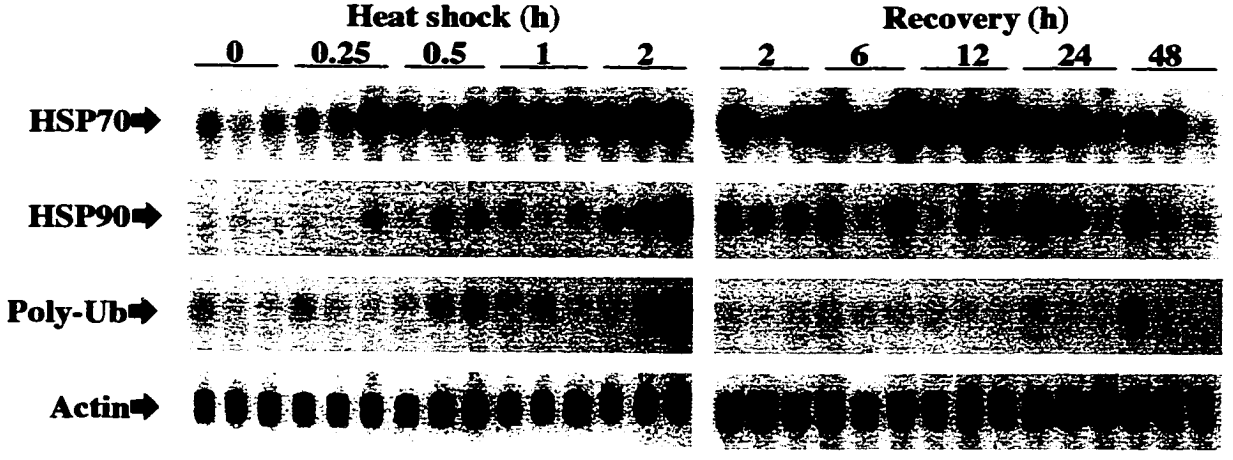
Fig. 1. Abdominal muscle mRNA levels for HSP70, HSP90, polyubiquitin (Poly-Ub), and actin (indicator of equal loading) in A) ambient-acclimated and B) cold-acclimated lobsters during heat-shock and recovery. N=3 for each time point. Data for each acclimation group represent a single gel that was transferred and probed with several cDNAs. Polyubiquitin transcripts were hardly detectable in the cold-acclimated group.

A) Ambient-acclimated abdominal muscle



B) Cold-acclimated abdominal muscle

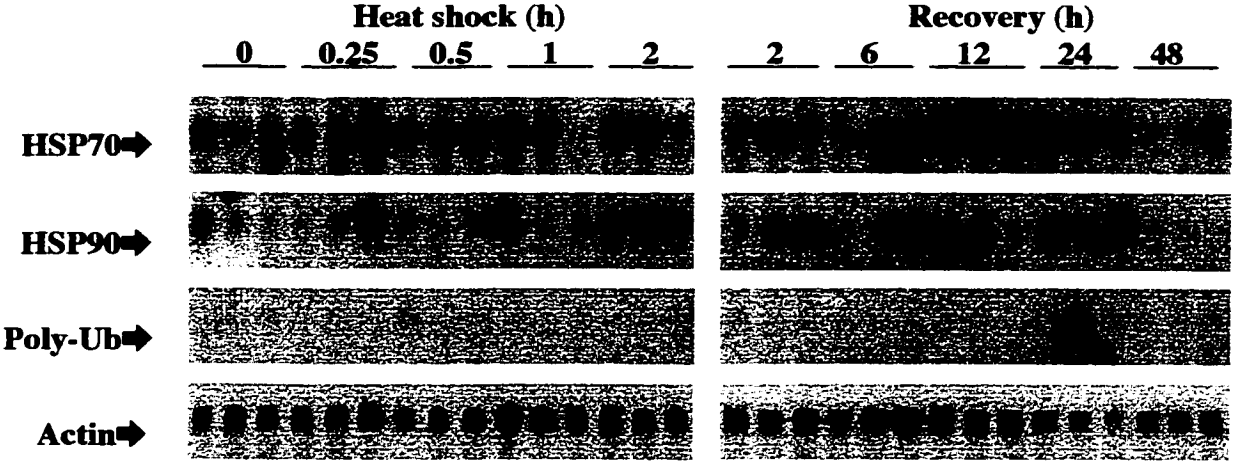
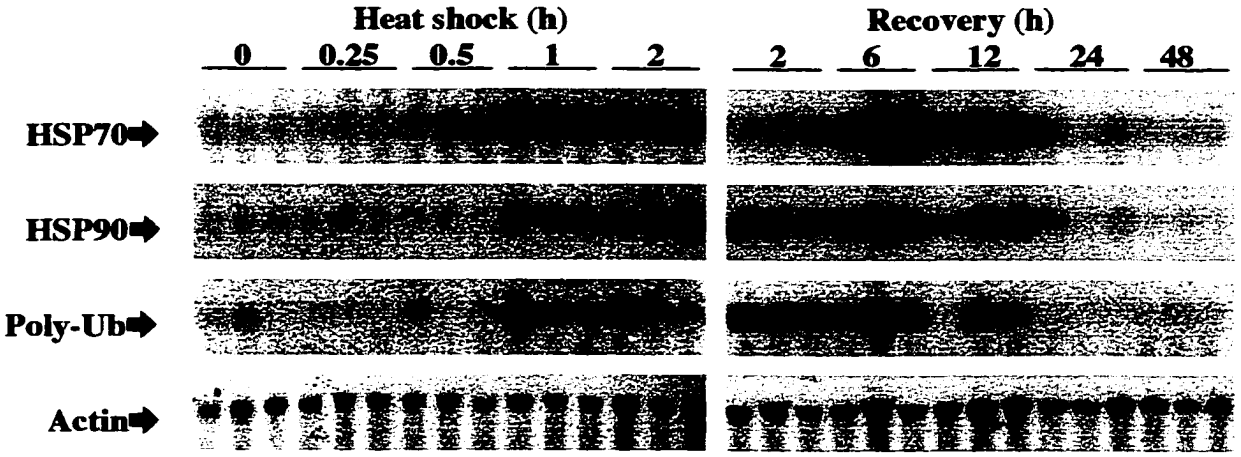


Fig. 2. Hepatopancreas mRNA levels for HSP70, HSP90, polyubiquitin (Poly-Ub), and actin (indicator of equal loading) in A) ambient-acclimated and B) cold-acclimated lobsters during heat-shock and recovery. N=3 for each time point. Data for each acclimation group represent a single gel that was transferred and probed with several cDNAs.

A) Ambient-acclimated hepatopancreas



B) Cold-acclimated hepatopancreas

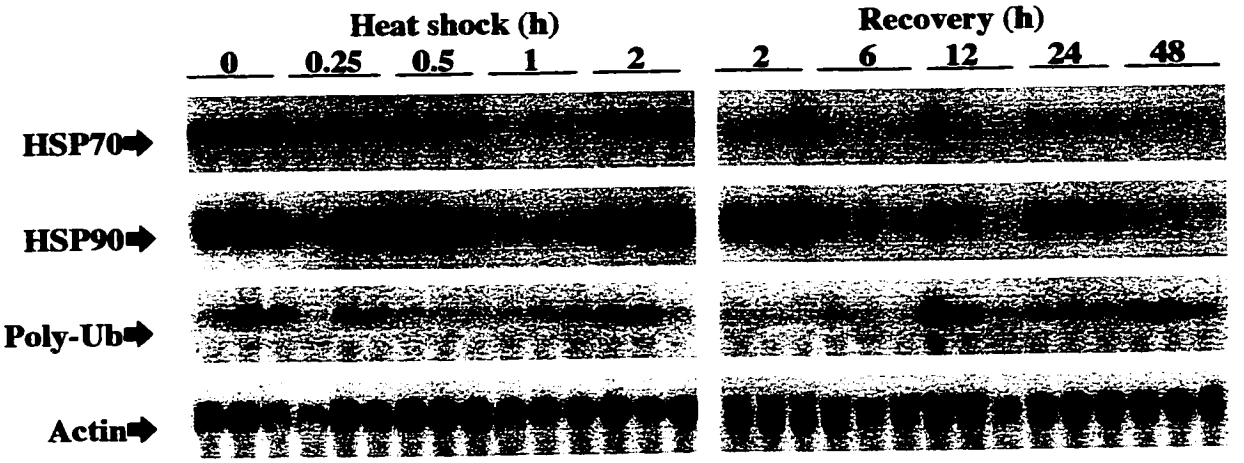
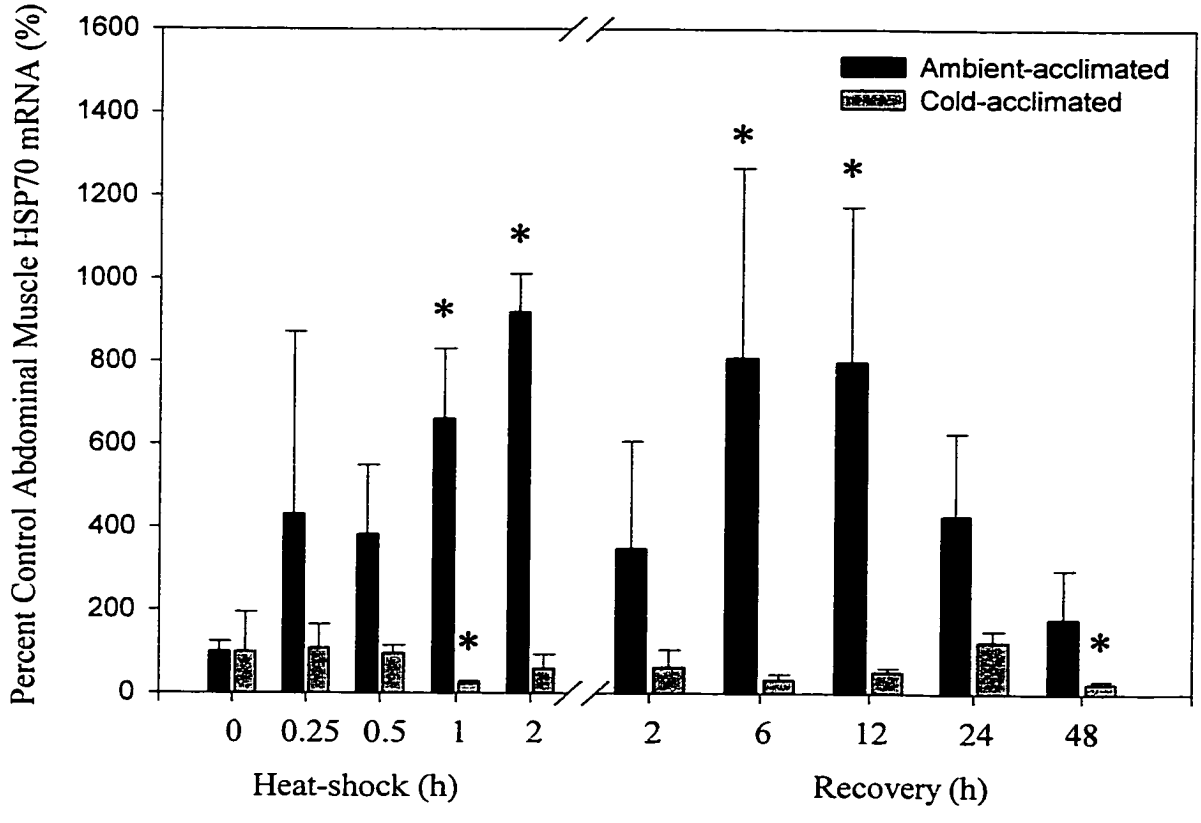


Fig. 3. Comparisons of A) HSP70 and B) HSP90 mRNA levels in abdominal muscle of ambient- and cold-acclimated lobsters during heat-shock and recovery. Data are normalized against actin levels (to control for equal loading) and presented as percent control expression levels. N=3 for all time points. Error bars represent one S.D. of the mean. Asterisks denote statistical significance from control (no heat-shock) mRNA levels within an acclimation group (* $p<0.05$).

A) Abdominal muscle HSP70 mRNA levels



B) Abdominal muscle HSP90 mRNA levels

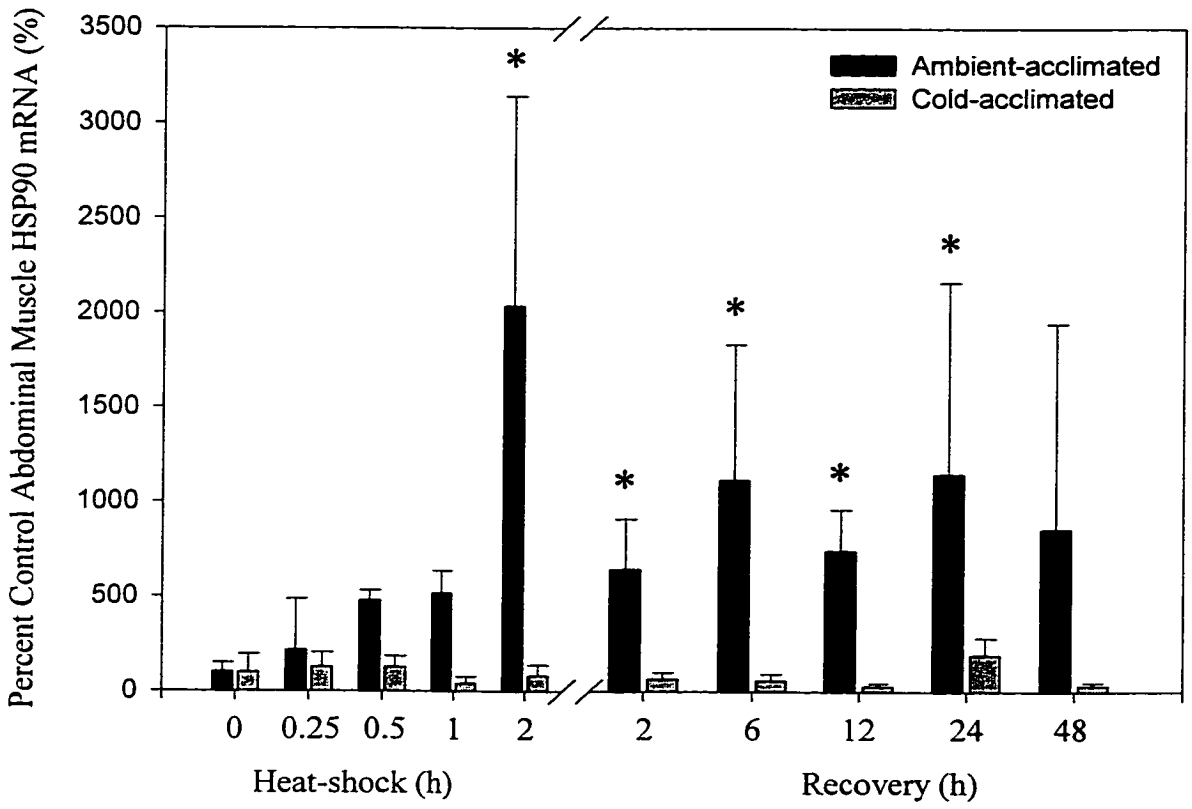
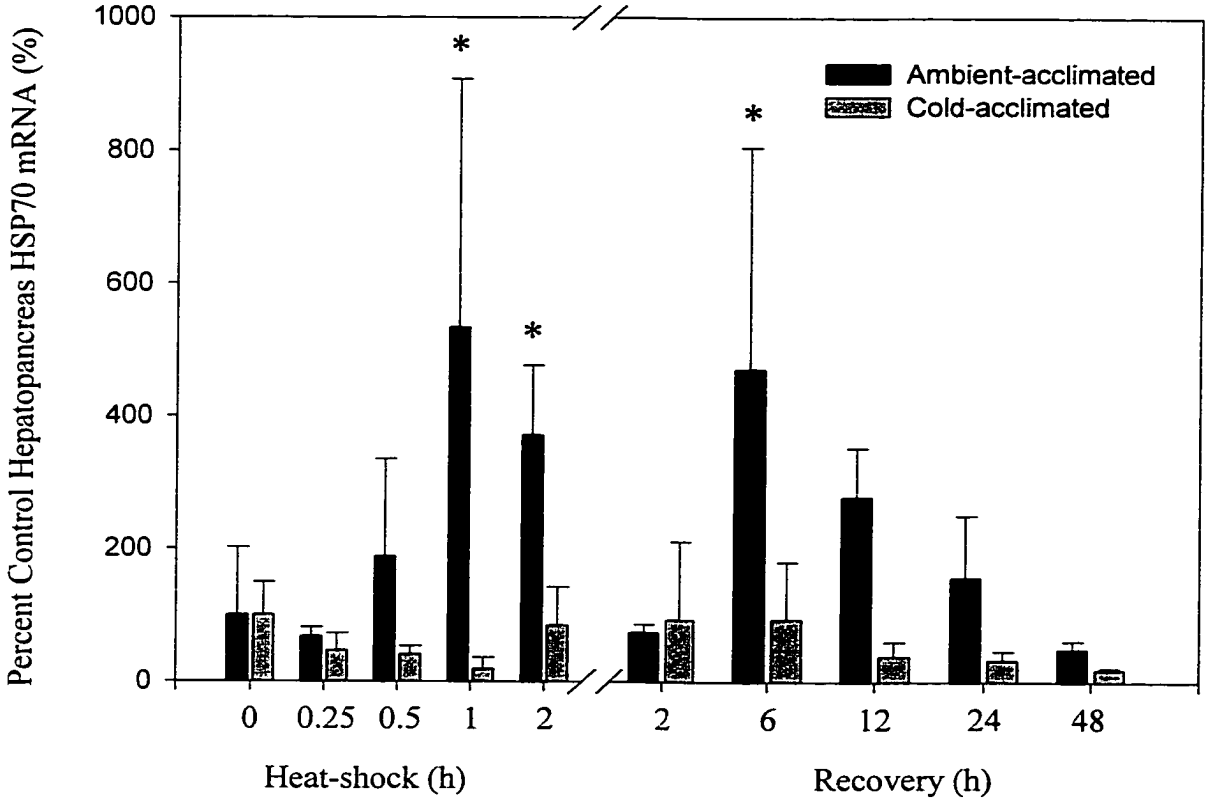
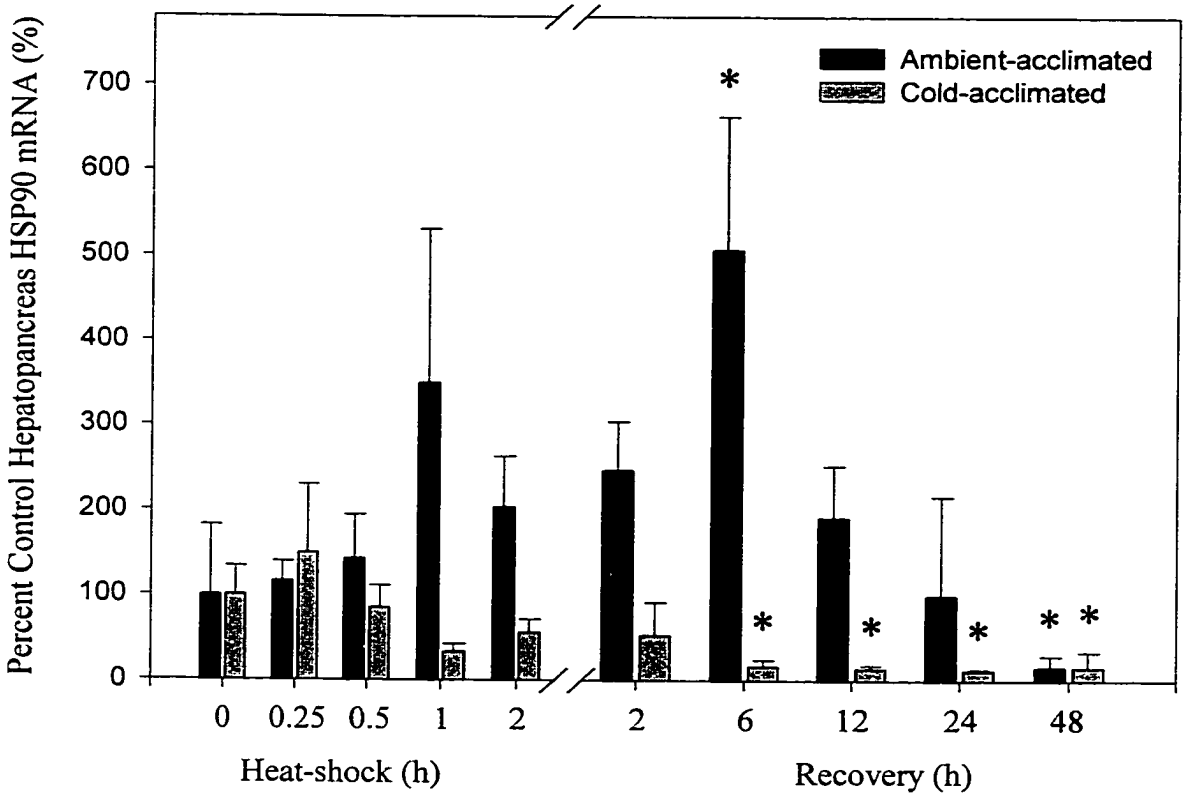


Fig. 4. Comparisons of A) HSP70, B) HSP90, and C) polyubiquitin mRNA levels in hepatopancreas of ambient- and cold-acclimated lobsters during heat-shock and recovery. Data are normalized against actin levels (to control for equal loading) and presented as percent control expression levels. N=3 for all time points. Error bars represent one S.D. of the mean. Asterisks denote statistical significance from control (no heat-shock) mRNA levels within an acclimation group (* $p<0.05$).

A) Hepatopancreas HSP70 mRNA levels



B) Hepatopancreas HSP90 mRNA levels



C) Hepatopancreas polyubiquitin mRNA levels

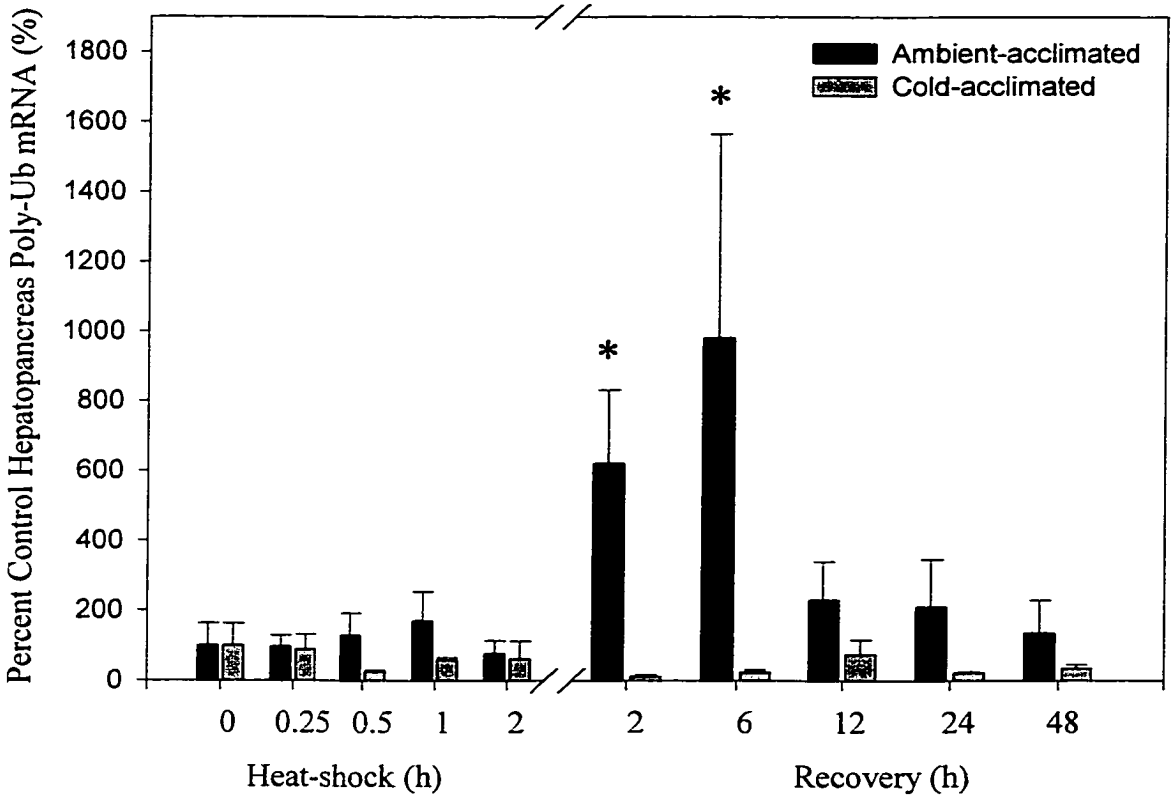


Fig. 5. Comparison of polyubiquitin mRNA levels in hepatopancreas and abdominal muscle of ambient-acclimated lobsters during heat-shock and recovery. Data are normalized against actin (to control for equal loading) and presented as percent control expression levels. N=3 for all time points. Error bars represent one S.D. of the mean. Asterisks denote statistical significance from control (no heat-shock) mRNA levels within an acclimation group (* $p<0.05$).

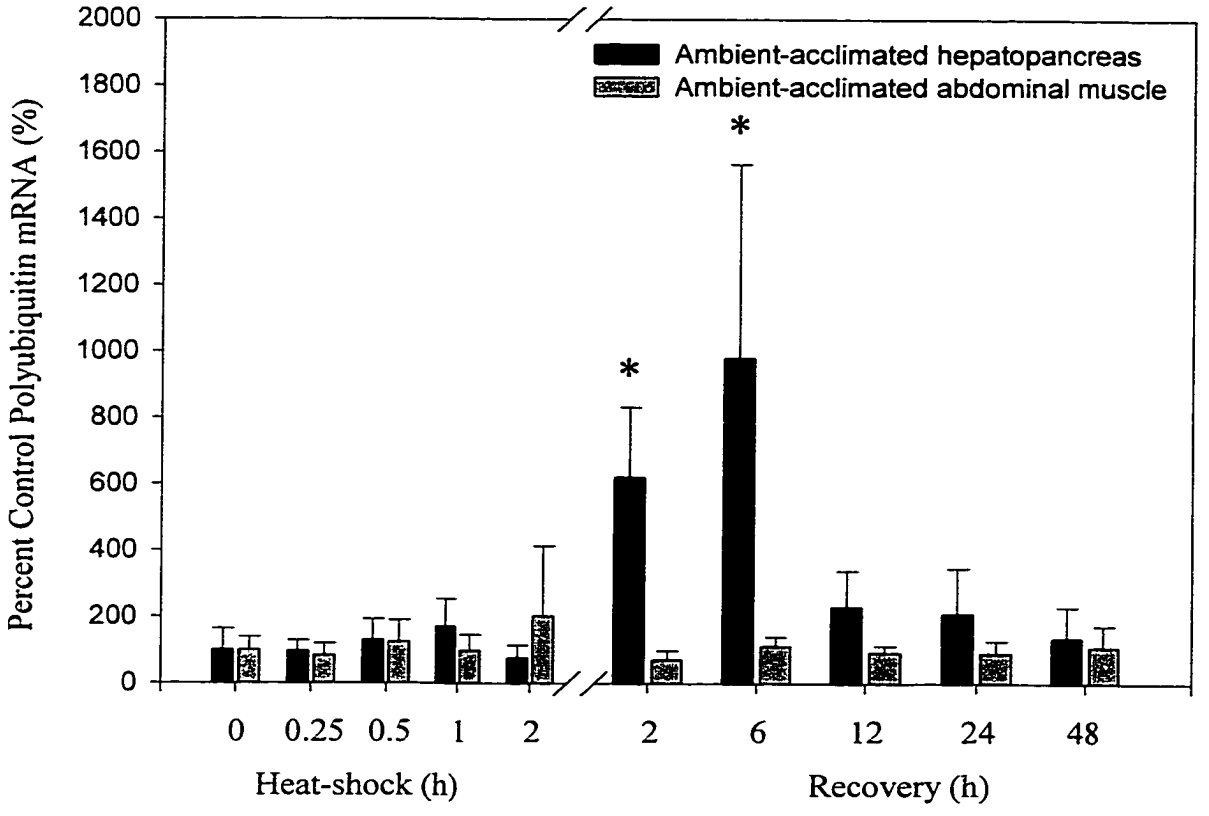
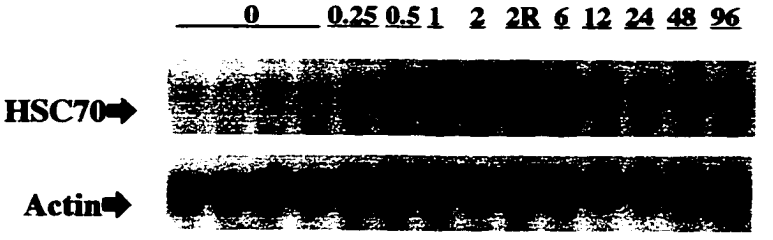
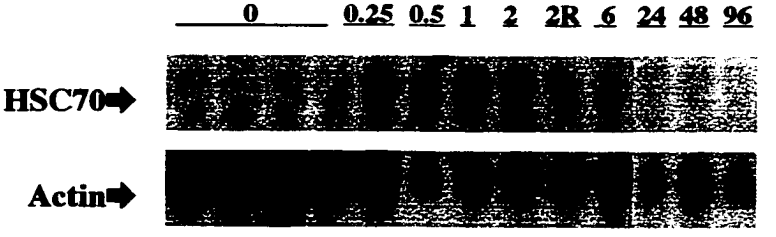


Fig. 6. Northern analysis of HSC70 expression in A) ambient- and B) cold-acclimated abdominal muscle and C) ambient- and D) cold-acclimated hepatopancreas during heat-shock and recovery. Heat-shock times are indicated in hours (0.25, 0.5, 1, 2). A 2 h heat-shock followed by a 2 h recovery is indicated by 2R. Subsequent times (6, 12, 24, 48, 96) are hours of recovery following the 2 h heat-shock. Any missing time points were excluded due to degradation of RNA.

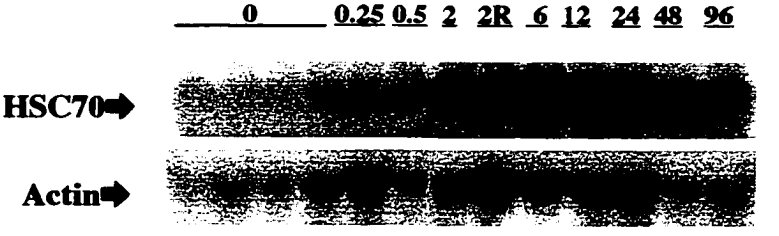
A) Ambient-acclimated abdominal muscle



B) Cold-acclimated abdominal muscle



C) Ambient-acclimated hepatopancreas



D) Cold-acclimated hepatopancreas

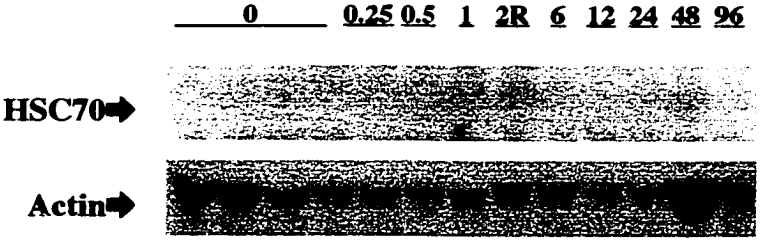
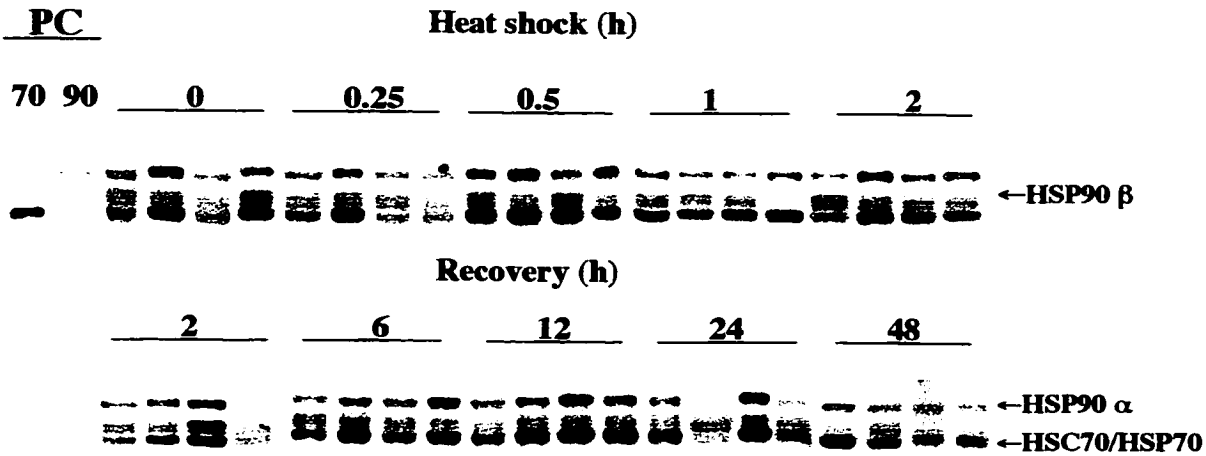


Fig. 7. Representative western immunoblot of abdominal muscle HSC70/HSP70 (bottom band), HSP90α (top band), and HSP90β (middle bands) protein levels in A) ambient- and B) cold-acclimated lobsters during an acute heat-shock and recovery. 70=HSP70 positive control (PC) 90=HSP90 positive control (see statistical analysis in Results).

A) Ambient-acclimated abdominal muscle



B) Cold-acclimated abdominal muscle

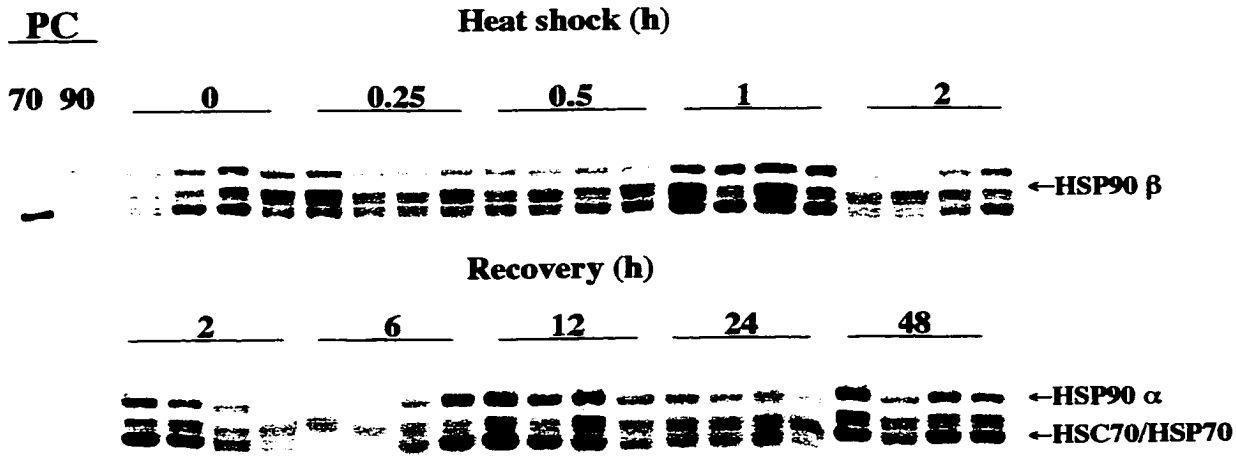
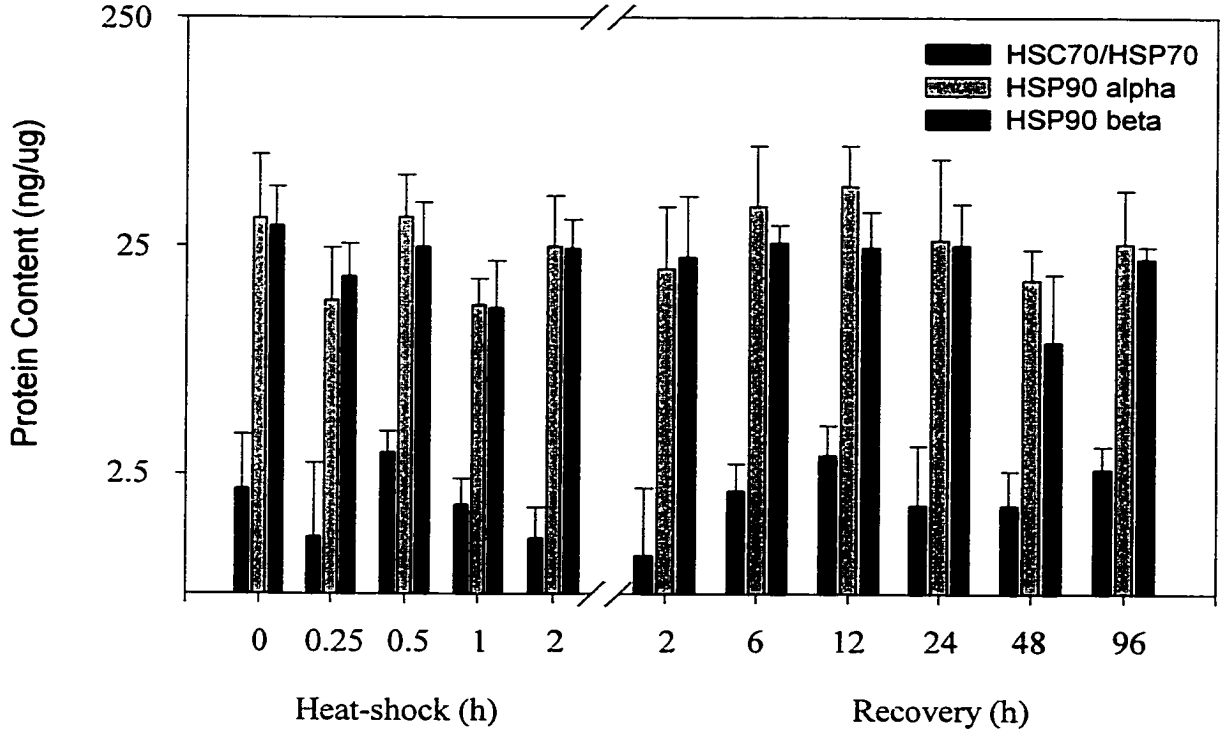
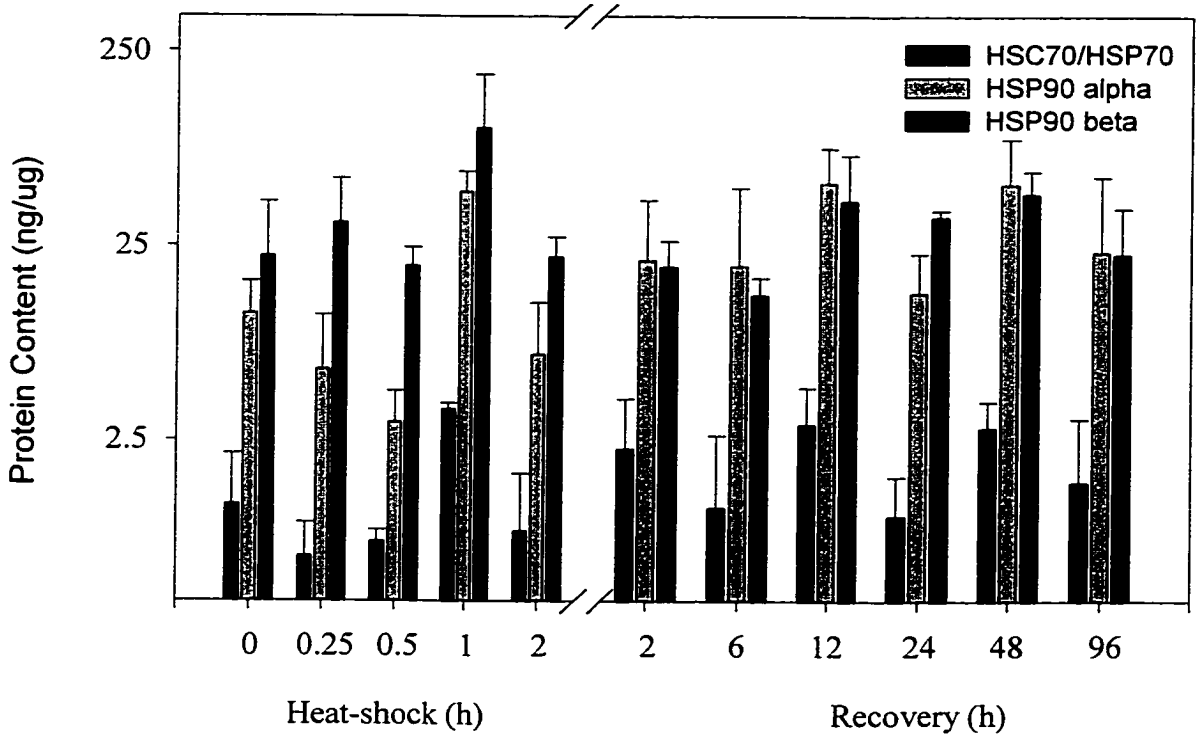


Fig. 8. Molecular chaperone protein content (ng per ug of protein) from A) ambient- and B) cold-acclimated adominal muscle during heat-shock and recovery. N=4 for all time points. Note log scale. Control levels did not differ significantly between ambient- versus cold-acclimated animals (see statistical analysis in Results).

A) Ambient-acclimated abdominal muscle molecular chaperone content



B) Cold-acclimated abdominal muscle molecular chaperone content



Chapter Two

Osmotic and thermal stress interactively alter the expression of stress-responsive genes in the American lobster, Homarus americanus

Abstract

Responding to multiple environmental stressors is critical for cellular and organismal survival. The American lobster, Homarus americanus, can encounter both osmotic and thermal stress throughout its life-cycle. To understand the molecular basis of osmotic and thermal stress responses, I used homologous cDNA probes to first characterize mRNA patterns for lobster HSP90 and polyubiquitin during hypo- and hyper-osmotic stress in abdominal muscle and hepatopancreas (a digestive tissue). I then examined mRNA levels for both genes after a 2 h osmotic stress immediately followed by a 2 h thermal stress (and recovery). Hypo- and hyper-osmotic stress induced HSP90 mRNA levels in abdominal muscle. Only hyper-osmotic stress induced HSP90 mRNA in hepatopancreas. In contrast, polyubiquitin mRNA levels were not induced by either osmotic treatment for abdominal muscle, and were reduced by hyper-osmotic stress in hepatopancreas. Two-way ANOVA detected significant interactive effects of osmotic and thermal stress on the mRNA levels of HSP90 and polyubiquitin in abdominal muscle and HSP90 in hepatopancreas. Interactive effects on stress-responsive gene expression are discussed in terms of biochemical economy, "buffering" against future environmental stress, and transcriptional regulation.

Introduction

Organisms in nature survive by responding to multiple stressors. Environmental perturbations are often experienced against a shifting background of numerous abiotic variables. Marine animals such as the American lobster, Homarus americanus, can experience osmotic and thermal stress concurrently or in close temporal proximity. The lobster life-cycle includes several stages that experience both osmotic and thermal stress. First, as pelagic larvae, suspended at the surface of the water column, near-shore larval lobsters encounter hypo-osmotic stress during periods of rainfall, and thermal stress at the ocean surface (Ennis, 1995). Lobster larvae (zoeal stages I-III) can tolerate reduced salinities. Tolerance to low salinities decreases as development proceeds to stages IV and V (Charmantier et al., 1988). Following metamorphosis and settlement, intertidal juvenile lobsters can experience osmotic shifts due to freshwater run-off and increased temperatures in shallow rocky areas. Migrating seasonally into coves and estuaries to reproduce, adult lobsters may endure osmotic stress in brackish waters that can additionally possess elevated thermal profiles relative to cooler offshore ocean temperatures. In contrast to pelagic larval life stages, juvenile and adult lobsters (primarily benthic organisms) are stenohaline and described as poor osmoregulators (Dall, 1970).

In support of a thriving fishery, early studies of lobster environmental physiology were primarily concerned with the determination of optimal conditions for maintaining lobsters in captivity. McLeese (1956) found that the survival of adult H. americanus was decreased by various levels of hypo-osmotic and thermal stress. By acclimating lobsters

to several reduced salinities, he determined that there were interactive effects of low salinity and temperature on survival (McLeese, 1956). Gonzalez and Bradley (1994) observed both up- and down-regulation in the synthesis of specific proteins in the copepod, Eurytemora affinis, in response to low and high salinity stress. Concurrent heat stress was shown to modify these patterns.

To examine the osmotic stress response and to determine whether multiple stressors could have interactive effects at the level of gene transcription, I used several homologous molecular probes now available for stress-responsive genes in H. americanus (Shean and Mykles, 1995; Chang et al., 1999; Spees et al., unpublished). As indexes of lobster stress in vivo, I examined mRNA levels of the molecular chaperone HSP90 (=90 kD heat-shock protein) and polyubiquitin. Well-characterized and pervasive "chaperones" of nascent peptides during protein synthesis, HSPs also help to re-fold denatured proteins resulting from environmental perturbation of cells (reviewed in: Morimoto et al., 1990; Hartl, 1996; Nover and Scharf, 1997; Bukau and Horwich, 1998; Feder and Hofmann, 1999). HSP90 is one of the most abundant proteins in eukaryotic cells, accounting for as much as 1-2% of total cytosolic protein even in the absence of stress (Welch and Feramisco, 1982; Parsell and Lindquist, 1993; Jakob and Buchner, 1994). Despite its abundance, HSP90 has historically received less attention in the literature than the HSP70 family of chaperones and many of its intracellular folding targets (substrates) remain enigmatic.

Some HSP90 chaperoning functions have been demonstrated in vivo. HSP90 does not appear to be a "chaperoning generalist" like HSP70; it chaperones specific

proteins, many of which require its presence for final maturation (Nathan et al., 1997). Classical examples of HSP90 substrates include a variety of steroid hormone receptors, the aryl hydrocarbon receptor, kinases, the heat-shock transcription factor, and the tumor-suppressor protein p53 (Buchner, 1999). HSP90 plays dual roles in environmental stress responses (Buchner, 1999) and cellular signal transduction (Pratt, 1997, 1998), and has potential as an anti-tumor target (Whitesell et al., 1994; Stebbins et al., 1997; Schulte and Neckers, 1998; Neckers et al., 1999). Rutherford and Lindquist (1999) have shown HSP90 to be a "capacitor for morphological evolution", capable of altering the developmental program by networking signal transduction and environmental stress response pathways. In Drosophila, environmental stress may act to titrate HSP90 away from its target proteins in signal transduction pathways, thereby allowing the expression of cryptic trait variants and potentially providing a mechanism for evolutionary change. HSP90 is likely to regulate the crustacean ecdysteroid (steroid molting hormone) receptor as demonstrated in Drosophila (Arbeitman and Hogness, 2000). In the absence of environmental stress, lobster HSP90 mRNA may be induced by injections of ecdysteroid (20-hydroxyecdysone; Chang et al., 1999) and is significantly induced in claw muscle during premolt atrophy (an ecdysteroid-dependent process) (Spees, et al., unpublished).

During cell stress, damaged proteins that can not be salvaged by chaperones are no longer functional, and are potentially cytotoxic (Feder, 1999). The process of destroying proteins and the recycling of useful peptides and amino acids occurs via the ubiquitin/proteasome pathway. Ubiquitin (a 76 AA peptide) is bound to targets through a multi-step enzyme cascade. The resulting pattern of moieties signals a degradation

complex (26S proteasome) to remove targeted proteins or regulatory subunits from the cell (Varshavsky, 1997). To expedite the production of ubiquitin, eukaryotic genetic coding regions for multiple ubiquitin molecules are linked and transcribed as a single "polyubiquitin" transcript. The resulting polypeptide is post-translationally cleaved into functional ubiquitin units. In mussels (Mytilus trossulus) conjugation of ubiquitin has been used to quantify irreversible protein damage in nature (Hofmann and Somero, 1995). Polyubiquitin mRNA levels increase several fold in both Manduca sexta (Myer and Schwartz, 1996) and Drosophila (Lee et al., 1988) following thermal stress. Quantification of polyubiquitin mRNA has also been used to examine the degradation process during premolt claw atrophy in the land crab, Gecarcinus lateralis (Shean and Mykles, 1995).

Because osmotic and thermal stress can interactively affect survival of H. americanus (McLeese, 1956), and osmolarity and temperature may both influence the stability of proteins, I hypothesized that osmotic and thermal stress could interactively alter the expression of HSP90 and polyubiquitin, stress-responsive genes that are transcriptionally induced by the perturbation of protein structure.

Materials and Methods

Animal Care

Lobsters were reared from larvae hatched in a flow-through aquaculture system at the Bodega Marine Laboratory (BML), Bodega Bay, CA. Detailed descriptions of larval-rearing and the aquaculture system at BML are available elsewhere (Chang and Conklin,

1993; Conklin and Chang, 1993). Juvenile lobsters (intermolt, mean wet weight 80.25±13.44 g) were used for all experiments. Animals were molt-staged based upon microscopic examination of pleopod cuticular development (Aiken, 1973). Prior to the osmotic and thermal stress experiments (8 weeks), seawater temperatures were (14.9±0.7°C) with a stable salinity (32 ppt.).

Osmotic Stress

Two-liter jars were fitted with air stones and filled with either 1.0 L of 100% seawater (controls; 32 ppt.), 50% seawater (0.5 L seawater and 0.5 L distilled and millipore-filtered freshwater; 16 ppt.), or 150% seawater (1.0 L total volume with 16 g additional dissolved sea salts; 48 ppt.; Sigma). Salinity was determined with a portable refractometer. The jars were thermally equilibrated by partial submersion in the flow-through system (15°C). Lobsters (n=4 for all treatments) were removed from their compartments and placed directly into the jars where they remained for 0.5, 1.0, or 2.0 h. During these time points we have previously measured elevated levels of HSP90 mRNA in lobsters exposed to acute thermal stress (Spees et al., unpublished). Controls (100% seawater) were incubated for 2.0 h in the same manner.

Following all of the treatments (100% survival), animals were quickly removed from the jars and sacrificed. Samples of abdominal muscle and hepatopancreas (midgut gland) were dissected, frozen in liquid N_2, and stored at -70°C.

Osmotic and Thermal Stress

To examine the effects of osmotic stress on the ability of lobsters to mount an acute thermal stress response, juvenile lobsters were removed from the aquaculture

system and placed into aerated 2 L jars containing 50% or 150% seawater as above. An acute thermal stress was administered to lobsters immediately following 2 h of osmotic stress by quickly moving them to new aerated 2 L jars (100% seawater) previously equilibrated ،ر 28°C in a temperature-controlled (±1.0°C) incubator. After a 2 h thermal stress, animals recovered for 3 h (100% seawater) to allocate time for a transcriptional response to the thermal stress. Previous work (Chang et al., 1999; Spees et al., unpublished) indicated that a 3 h recovery time was appropriate to examine a robust induction in HSP90 gene expression following an acute thermal stress of similar magnitude. There was 100% survival following the 2 h acute thermal stress and 3 h recovery. Samples of abdominal muscle and hepatopancreas were dissected, frozen in liquid N_2, and stored at -70°C.

Northern Analysis

Total RNA was isolated from the abdominal muscle and hepatopancreas samples (RNAgents kit, Promega), quantified with a spectrophotometer, and equally loaded (15 μg abdominal muscle total RNA; 25 μg hepatopancreas total RNA) onto denaturing 1% agarose gels. These gels were washed (15 min, DEPC H_2O) and blotted overnight onto nylon membranes (Magnagraph, MSI). Following UV cross-linking, blots were pre-hybridized (2 h) in 5x SSPE buffer, 50% (w/v) formamide, 5x Denhardt's, 1% SDS, and 100 μg/ml denatured sheared salmon sperm DNA. A partial lobster HSP90 clone (350 bp, cloning described in Chang et al., 1999) was ^{32}P-labeled (Prime-It RmT, Stratagene), added directly to the prehybridization solution, and allowed to hybridize overnight at 42°C. Following hybridization, the blots were washed twice with 2x SSPE and placed on

film for overnight at -70°C.

Following exposure of the film, the blots were stripped with several washes (0.1x SSC, 0.1% SDS, 65°C) until background was minimal and prehybridized, hybridized, and washed as above, except that a partial lobster polyubiquitin cDNA probe was added (690 bp fragment from Acc I digest of clone; cloning of lobster polyubiquitin described in Shean and Mykles, 1995). To check for equal loading of RNA, the blots were probed with a partial lobster actin cDNA (700 bp, Harrison and El Haj, 1994). Films were scanned on a high resolution scanner, and densitometry was performed with NIH Image software. The signals from these blots were quantified, normalized against the actin signal to control for equal loading of RNA, and expressed as percent control mRNA level. All data were $\log_{10}$-transformed to normalize variance prior to statistical analysis. Two-way ANOVA (Sokal and Rohlf, 1981) was used to examine interactions between 1) salinity treatment and treatment duration and 2) osmotic and thermal stress. Following significant ANOVA results, multiple pairwise comparisons were made by the Student-Newman-Keuls method. Student's t-test was used to compare individual group means to controls.

Results

Representative Northern blots from analysis of total RNA are shown for lobster abdominal muscle (Fig. 1A) and hepatopancreas (Fig. 1B) from animals that received hyper-osmotic stress (150% seawater).

HSP90 Gene Expression in Response to Osmotic Stress

HSP90 expression in abdominal muscle was significantly affected by the 50% or 150% seawater treatments (two-way ANOVA, p=0.003; Fig. 2A). Multiple pairwise comparisons indicated that salinity effects on abdominal muscle HSP90 expression differed from one another at 0.5 h of incubation (p=0.017) and at 1 h of incubation (p=0.048); in both cases mRNA levels were higher in the hypo-osmotic treatment. HSP90 mRNA levels were significantly higher than control levels in all of the 50% seawater treatments: 0.5 h, p=0.017; 1 h, p=0.025; 2 h, p=0.008 (Student's t-test). For those animals exposed to 150% seawater, a significant increase in HSP90 mRNA levels relative to control levels was not detected until 2 h of incubation (Student's t-test, p=0.01). No significant interaction was detected between treatment duration and salinity exposure for abdominal muscle HSP90 gene expression (two-way ANOVA, p=0.636; Fig. 2A).

Patterns of hepatopancreas HSP90 gene expression in response to osmotic stress differed from those observed for abdominal muscle (compare Fig. 2A to 2B). While there was also a significant effect of osmotic state on HSP90 expression (two-way ANOVA, p=0.001; Fig. 2B), pairwise multiple comparisons indicated significant differences between hypo- and hyper osmotic stress at the 2 h incubation point alone (p<0. 001). Furthermore, unlike abdominal muscle in which HSP90 mRNA levels were most responsive to hypo-osmotic stress, hepatopancreas HSP90 mRNA levels were most responsive to hyper-osmotic stress. Hepatopancreas HSP90 mRNA levels were significantly induced relative to control levels by 2 h of hyper-osmotic stress (Student's t-

test, p=0.008), while none of the hypo-osmotic treatments increased HSP90 gene expression relative to controls. Contrary to abdominal muscle responses, hepatopancreas did show a significant interaction between treatment duration and salinity exposure (two-way ANOVA, p=0.016; Fig. 2B).

Polyubiquitin Gene Expression in Response to Osmotic Stress

Polyubiquitin gene expression in abdominal muscle was not affected by either hypo- or hyper-osmotic stress. No significant differences were detected between treatment times (two-way ANOVA, p=0.797; Fig. 3A) or salinity exposures (two-way ANOVA, p=0.179; Fig. 3A). Additionally, none of the osmotic treatment polyubiquitin mRNA levels were significantly higher than control levels (Student's t-test). No significant interaction was detected between treatment duration and salinity exposure (two-way ANOVA, p=0.428; Fig. 3A).

Unlike abdominal muscle, hepatopancreas polyubiquitin mRNA levels were significantly affected by osmotic stress (two-way ANOVA, p=0.046; Fig. 3B). Pairwise comparisons revealed significant inhibitory effects of hyper-osmotic stress relative to hypo-osmotic effects at 0.5 h (p=0.008) and 1 h (p=0.013) of exposure. Hypo-osmotic stress had no significant effect on polyubiquitin expression, but hyper-osmotic stress led to significant inhibition of polyubiquitin expression at 0.5 h of exposure (Student's t-test, p=0.037). A significant interaction between treatment duration and osmotic stress was also detected (two-way ANOVA, p=0.003; Fig. 3B). Hepatopancreas polyubiquitin expression in the hyper-osmotic treatment returned to normal levels by 2 h (Fig. 3B).

Interactive effects of Osmotic and Thermal Stress on HSP90 Gene Expression

The effects of acute thermal stress on abdominal muscle HSP90 gene expression were dependent on the previous osmotic state of the lobster. While heat-shock had a significant effect on HSP90 mRNA levels following osmotic stress (two-way ANOVA, $p=0.006$; Fig. 4A), this effect was significant for the hyper-osmotic treatments only (Student-Newman-Keuls, $p=0.002$), and not for the hypo-osmotic treatments (Student-Newman-Keuls, $p=0.523$). A significant interaction between thermal stress and osmotic stress was detected for abdominal muscle HSP90 gene expression (two-way ANOVA, $p=0.032$; Fig. 4A).

Hepatopancreas HSP90 mRNA levels following heat-shock were also dependent on the previous osmotic state of the organism. Acute thermal stress had a significant effect on HSP90 expression after osmotic stress (two-way ANOVA, $p<0.001$; Fig. 4B), but this significance was due to the hypo-osmotic treatment (Student-Newman-Keuls, $p<0.001$), and not the hyper-osmotic treatment (Student-Newman-Keuls, $p=0.189$). Note that this is opposite from the pattern of hyper- but not hypo-osmotic stress effecting HSP90 expression in the abdominal muscle tissues from the same animals (Fig. 4A). Absolute levels of HSP90 mRNA following induction by thermal stress did not differ between the hepatopancreas of hypo- and hyper-osmotically-stressed animals (Student-Newman-Keuls, $p=0.729$; Fig. 4B). Significant interactive effects of thermal stress and osmotic stress on hepatopancreas HSP90 mRNA levels were also detected (two-way ANOVA, $p=0.002$; Fig. 4B).

Interactive effects of Osmotic and Thermal Stress on Polyubiquitin Gene Expression

A significant interaction was detected between thermal stress and osmotic stress in abdominal muscle (two-way ANOVA, $p=0.041$; Fig. 5A). Thermal stress following the hypo-osmotic stress treatment induced polyubiquitin expression while thermal stress following the hyper-osmotic treatment did not (Fig. 5A).

Hepatopancreas polyubiquitin mRNA levels were significantly induced by heat-shock after the osmotic stress (two-way ANOVA, $p<0.001$; Fig. 5B). Pairwise comparisons revealed that both hypo- and hyper-osmotically-stressed animals responded to acute thermal stress by significantly increasing polyubiquitin gene expression (50% seawater, $p<0.001$; 150% seawater, $p<0.001$). Osmotic and thermal stress did not significantly interact to influence hepatopancreas polyubiquitin mRNA levels (two-way ANOVA, $p=0.523$; Fig. 5B).

Discussion

The results of this study demonstrate that 1) hypo- and hyper-osmotic stress can both significantly induce lobster HSP90 gene expression in vivo, 2) osmotic effects on HSP90 expression may differ depending on duration of stress and tissue, 3) lobster polyubiquitin gene expression is not significantly induced by whole animal incubation in either 50% or 150% seawater (hypo- or hyper-saline conditions) in abdominal muscle or hepatopancreas (up to a 2 h exposure, without thermal stress), 4) hepatopancreas polyubiquitin expression can be significantly inhibited by hyper-osmotic stress, and 5) a lobster's previous osmotic state can significantly influence transcriptional responses to

acute thermal stress (osmotic and thermal stress interactively alter gene expression in vivo). Additionally, initial results using an HSP70 cDNA probe on the blots from these studies indicate similar gene expression patterns for HSP70 as those observed for HSP90.

Osmotic induction of stress-responsive gene expression

Tissue-specific patterns of lobster HSP90 expression were observed following osmotic challenge. While lobster abdominal muscle HSP90 expression was significantly increased in all hypo-osmotic treatments, hepatopancreas expression was unaffected by any duration of hypo-osmotic stress up to 2 h. Both tissues significantly induced HSP90 mRNA levels in response to a 2 h hyper-osmotic shock. A significant interaction between treatment duration and salinity was found for hepatopancreas, but not for abdominal muscle. As the composite protein pools of abdominal muscle and hepatopancreas may differ considerably, variation in expression patterns may be due to differential stability of proteins arising from ionic or other solute interactions.

Na^+ and K^+ are compatible solutes at physiological concentrations but can perturb cellular function when present at very low or high (non-physiological) concentrations. Low availability of ions is thought more likely to perturb cells by affecting enzyme-ligand interactions rather than by altering enzyme conformations substantially (protein structure) (Somero and Yancy, 1997). High ionic concentrations can inhibit enzyme function (e.g. lowering the K_M). The cations Na^+, K^+, Mg^{+2}, Ca^{+2}, and the anion Cl^- are all present in sea water and are known to destabilize proteins at excessive concentrations (>1 M) (Somero and Yancey, 1997). In vitro results from 3T3 and SV-3T3 (rat) cells in culture show that presence of the osmolyte betaine (N-trimethylglycine) reduces the

induction of HSP70 during hypertonic (0.5 osM) incubation (Petronini et al., 1993). Betaine also inhibits HSP70 protein inductions in Madin-Darby canine kidney (MDCK) cells during thermal stress (Sheikh-Hamad et al., 1994), suggesting that some form of protein destabilization may occur during osmotic stress as is commonly observed during thermal stress. Interestingly, the heat-shock transcription factor (HSF1) can be activated by either hyper- or hypo-osmotic stress in mammalian (HeLa) cells, but does not induce HSP70 mRNA, indicating that it may play a different role in regulating osmosensing pathways or osmotic stress proteins (Caruccio et al., 1997).

Because the hypo- and hyper-osmotic conditions used in this study may not generate ionic conditions in vivo that have been observed to denature proteins in vitro, it is important to consider alternative hypotheses concerning osmotic stress and the production of HSPs. In the rat kidney, both the mRNA and protein levels of HSP72 and HSP25/27 increase steeply along the corticopapillary axis in a pattern that matches tissue solute levels in the concentrating region of the kidney. It is believed, however, that hypertonicity rather than hyperosmolality per se is actually responsible for these patterns because increased HSP synthesis correlates with the addition of relatively membrane-impermeant substances (NaCl) and not the addition of membrane-permeant substances (urea). Thus, alterations in membrane fluidity or shrinking and swelling may influence HSP expression in some cellular systems without protein denaturation (reviewed in Beck et al., 2000).

Protein synthesis, rather than degradation, is another possible reason for HSP induction during osmotic stress. Mammalian cell surface receptors for epidermal growth

factor (EGF-R), interleukin 1 (IL1-R), and the receptor for tumor necrosis factor (TNF-R) are known to aggregate, internalize, and activate a gene induction response through the c-Jun amino-terminal protein kinase cascade (JNK) in response to high osmolarity. This effect occurs in the absence of receptor ligands and is also thought to result from a perturbation of membrane structure or conformational changes in membrane proteins (Rosette and Karin, 1996). The enteric bacterium, Escherichia coli, responds to high osmolality by inducing both the σ^{32} and the σ^{E} stress regulons. The *rpo*H gene product (σ^{32}) and the *rpo*E gene product (σ^{E}) are two sigma factors that are activated by hyper-osmotic stress and operate by directing RNA polymerase to distinct sets of heat-shock promoters. The σ^{32} stress regulon includes the DnaK-DnaJ-GrpE as well as the GroEL-GroES chaperoning teams in addition to small HSPs. DnaK and GroEL are bacterial homologues of HSP70 and HSP60, respectively. It appears that this is an emergency response required to aid in the repair of misfolded proteins, to promote the proper folding of proteins that are rapidly synthesized following loss of turgor, and to increase σ^{S} activity (the primary stress factor involved in E. coli osmoadaptation) (Bianchi and Baneyx, 1999).

Increased HSP90 mRNA levels may be required in a particular tissue for signal transduction and/or osmolyte responses to intracellular ionic changes. While the present study is the first to report HSP90 induction by hypo-osmotic stress, Pan et al. (2000) examined salmon HSP90 mRNA levels in branchial lamellae and found them inducible by hyper-osmotic stress both in vitro and in vivo, but not in kidney. As cortisol is believed to govern osmoregulatory capacity in salmon by influencing chloride cell

differentiation and ATPase activity in the gill, and HSP90 is known to regulate glucocorticoid receptors, they hypothesized that HSP90 might be playing a specific role in signal transduction during osmotic stress.

While somewhat elevated in abdominal muscle for some of the hyper-osmotic treatments, lobster polyubiquitin gene expression was not significantly induced by osmotic stress for either abdominal muscle or hepatopancreas. It is possible that some protein degradation could proceed by conjugation of pre-existing or newly-translated ubiquitin peptides in the absence of polyubiquitin transcription, although my results imply that little irreversible protein damage occurred. Hyper-osmotic stress (0.5 and 1 h treatments) actually led to reduced hepatopancreas polyubiquitin mRNA levels relative to control levels, returning to control levels by 2 h.

Lobster hepatopancreas HSP90 and polyubiquitin mRNA levels are induced by thermal stress alone (Chang et al., 1999; Spees et al., unpublished) and by osmotic stress immediately followed by thermal stress in this study. Abdominal muscle appears to be a more thermally stable tissue than hepatopancreas (in vivo expression of polyubiquitin does not occur in abdominal muscle from the same lobsters which do express it in the hepatopancreas during and after a 13 °C acute heat-shock; Spees et al., unpublished). In the present study, however, abdominal muscle does increase expression of polyubiquitin when heat-shock follows a hypo-osmotic stress. Abdominal muscle also exhibits increased HSP90 mRNA levels when thermal stress follows a hyper-osmotic stress treatment.

Interactive effects of osmotic and thermal stress on gene expression

This study demonstrates a significant interaction between osmotic and thermal stress quantified by the in vivo mRNA levels of HSP90 and polyubiquitin. Aside from hepatopancreas polyubiquitin expression, which was significantly induced in similar fashion by thermal stress following either hypo- or hyper-osmotic stress, interactive effects of osmotic state and thermal stress were detected in both abdominal muscle and hepatopancreas for HSP90 expression, and in abdominal muscle for polyubiquitin expression. Ecologically, this indicates that the ability of lobsters (and likely most organisms) to respond to environmental stress is highly dependent on the prevailing milieu of abiotic conditions, and on the types of stressors they experienced in the recent past.

Abundant protective molecules such as HSPs that are induced by multiple stressors may provide a buffer against damage by future environmental stress. Both the existence of "inducible thermotolerance" and "cross-tolerant" stressors support this hypothesis. Inducible thermotolerance occurs when organisms previously exposed to a sublethal thermal stress can subsequently survive exposure to a thermal stress that would normally be lethal (Schlesinger et al., 1982; Lindquist and Craig, 1988; Clegg et al., 1998). Cross-tolerant stressors (Santos et al., 1998) demonstrate that previous stress exposures known to denature proteins and increase HSP production (e.g. chemical stress, heavy metals) often protect organisms against ordinarily lethal thermal stress.

The HSP90 gene expression data in this study also support the "buffering hypothesis". Consistently for both abdominal muscle and hepatopancreas, the 2 h

osmotic stress that induced the lowest amount of HSP90 gene expression in the initial treatment subsequently exhibited the highest relative increase in expression following thermal stress, while the 2 h osmotic stress that resulted in the highest HSP90 mRNA level exhibited the least relative increase in expression post thermal stress. Induction of abdominal muscle HSP90 mRNA was most sensitive to hypo-osmotic stress, but an additional acute thermal stress produced a larger increase in HSP90 mRNA levels for the hyper-osmotic treatment ($p=0.002$) compared with the hypo-osmotic treatment ($p=0.523$; Fig. 4A). For hepatopancreas, hyper-osmotic stress induced a substantial increase in HSP90 mRNA levels, while hypo-osmotic stress had no effect for any treatment duration examined. Subsequently, the hypo-osmotic stress treatment had the highest relative levels of hepatopancreas HSP90 mRNA post acute thermal stress ($p<0.001$) compared with the hyper-osmotic treatment ($p=0.189$; Fig. 4B). Previous induction of HSPs by osmotic stress may avert their induction to protect against thermal stress if they and their chaperoning abilities are already available to the cell.

Coupling responses to distinct stresses that share the feature of destabilizing protein structure is also possible through auto-regulation of the heat shock response (reviewed in Morimoto, 1998). Both HSP70 (Mosser et al., 1993; Shi et al., 1998) and HSP90 (Ali et al., 1998; Zou et al., 1998) are known to transcriptionally regulate HSP levels by repression of HSF1 (heat-shock transcription factor). Monitoring of free intracellular HSP70 and HSP90 concentrations provides biochemical mechanisms to prevent resource expenditure (nucleic and amino acids, ATP, etc.) during stress in animals that have recently experienced environmental stress sufficient to denature

proteins. During thermal stress, the amount of HSP70 synthesized is known to depend on the magnitude of the stress, and the initial levels of HSP70 in the cell (Mizzen and Welch, 1988). The ability of HSPs to directly modify their own expression provides an economical means to buffer but not over-buffer against environmental stress.

Studies examining stress-responsive gene expression or protein synthesis in the field may benefit from multiple regression or multi-variate analyses using plausible interacting stressors as independent variables. This could be especially important when examining molecules such as HSPs or polyubiquitin that are known to be influenced by multiple stressors, some of which in combination may interactively alter gene expression (e.g. osmotic and thermal stress in the present study). Evaluating the responses of organisms adapted to different field conditions must be done carefully to determine a causal mechanism for HSP or ubiquitin induction. Tomanek and Somero (2000) recently found that aerial versus aquatic exposure during thermal stress significantly affected the subsequent in vitro synthesis of multiple HSPs in Tegula brunnea but not in T. funebralis, congeneric marine snails adapted to different areas of the intertidal zone. Because they are responsive to multiple environmental stressors, and serve a more generalized function in cross-protection, HSPs may play a substantial role in determining the fundamental niches of organisms in nature.

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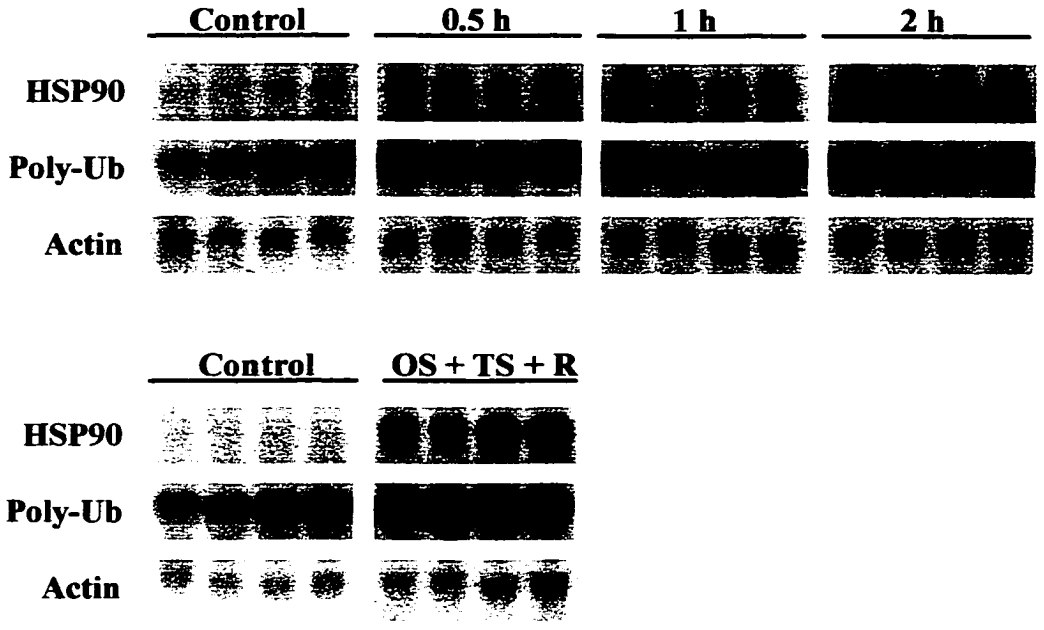
Fig. 1. Northern analysis of total RNA showing HSP90 and polyubiquitin gene expression in response to hyper-osmotic stress or hyper-osmotic and thermal stress in H. americanus A) abdominal muscle and B) hepatopancreas. The upper panel shows the effects of a 0.5, 1 , and 2 h hyper-osmotic stress (150% seawater) on HSP90 mRNA levels. The lower panel shows the effects of a hyper-osmotic stress (150% seawater, 2 h; OS) followed immediately by an acute thermal stress (13.0°C temperature increase, 100% seawater, 2 h; TS) and a recovery (ambient temperature, 100% seawater, 3 h; R). The upper and lower panels each represent single Northern blots that were serially probed with ^{32}P-labeled partial cDNAs for lobster HSP90, polyubiquitin, and actin (indicator of equal loading).

A) Abdominal Muscle (150% Seawater)



B) Hepatopancreas (150% Seawater)

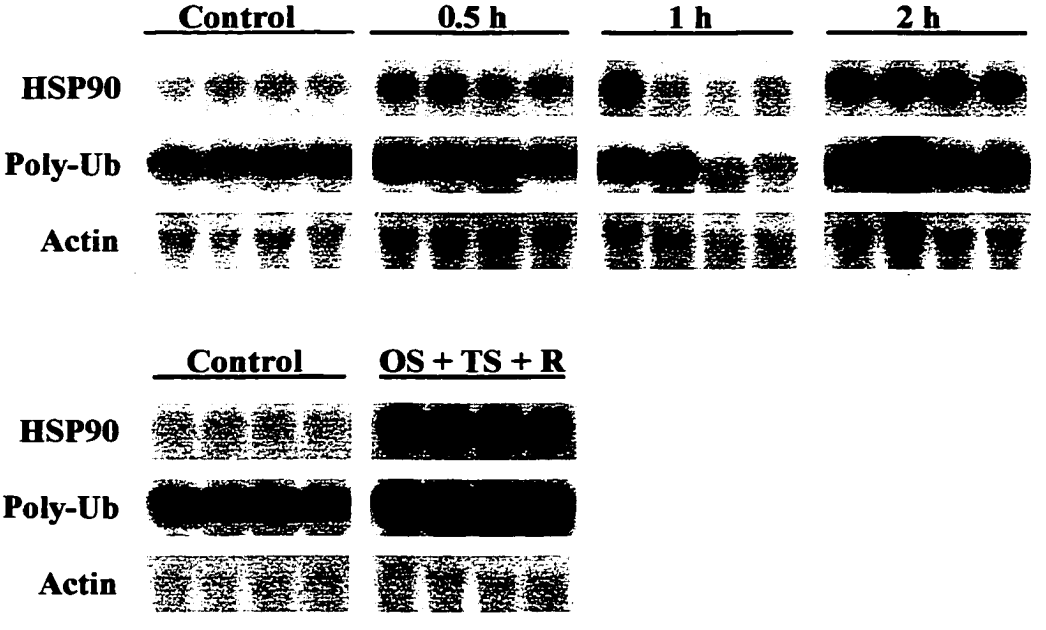
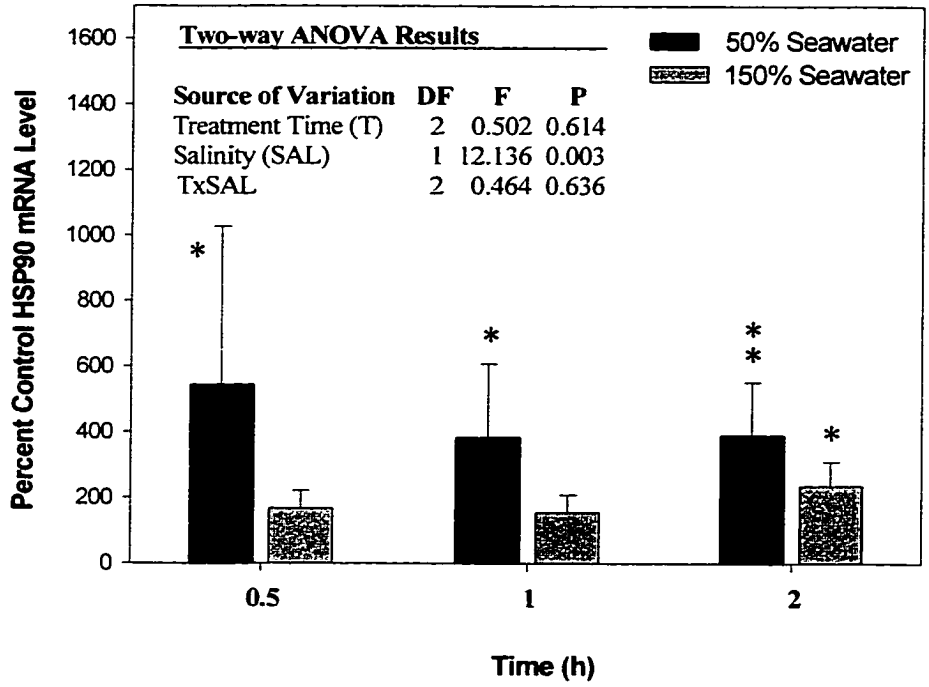


Fig. 2. Comparison of HSP90 gene expression during hypo- and hyper-osmotic stress (50% and 150% seawater) for 0.5, 1, and 2 h in A) abdominal muscle and B) hepatopancreas. Data are normalized against the actin signal (indicator of equal loading) and expressed as percent control mRNA level. N=4 for all time points. Error bars represent one S.D. of the mean. Results of two-way ANOVA are presented in upper left corner. Significance between treatment and control HSP90 mRNA levels is indicated: * $p \leq 0.05$, ** $p \leq 0.01$.

A) Abdominal Muscle HSP90 Expression



B) Hepatopancreas HSP90 Expression

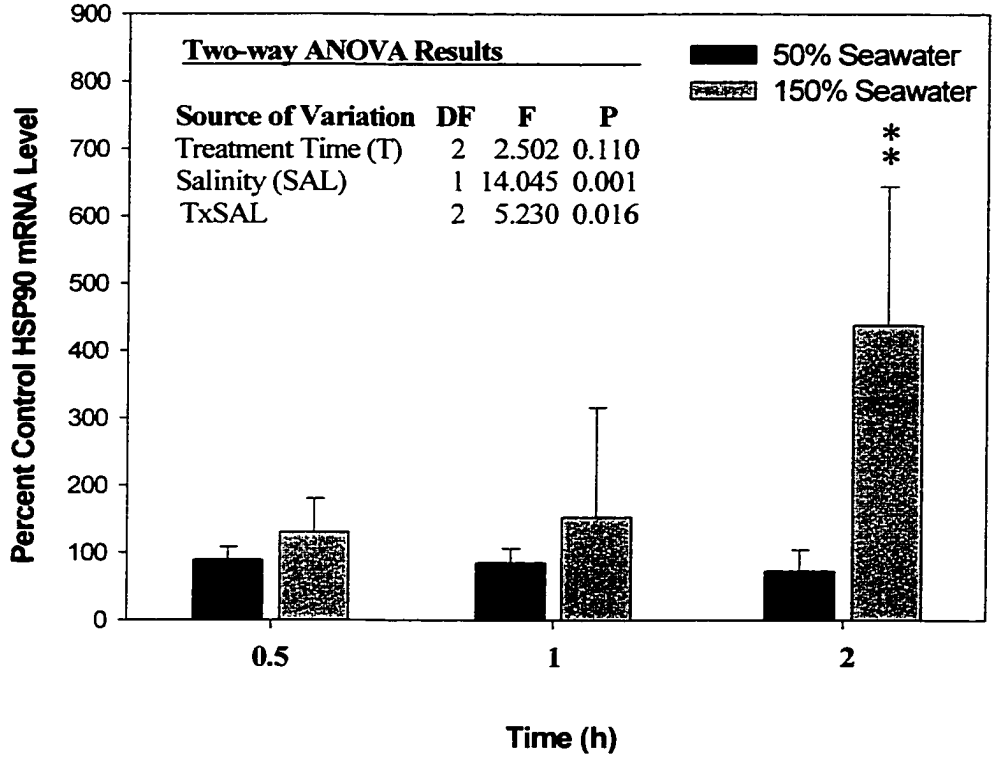
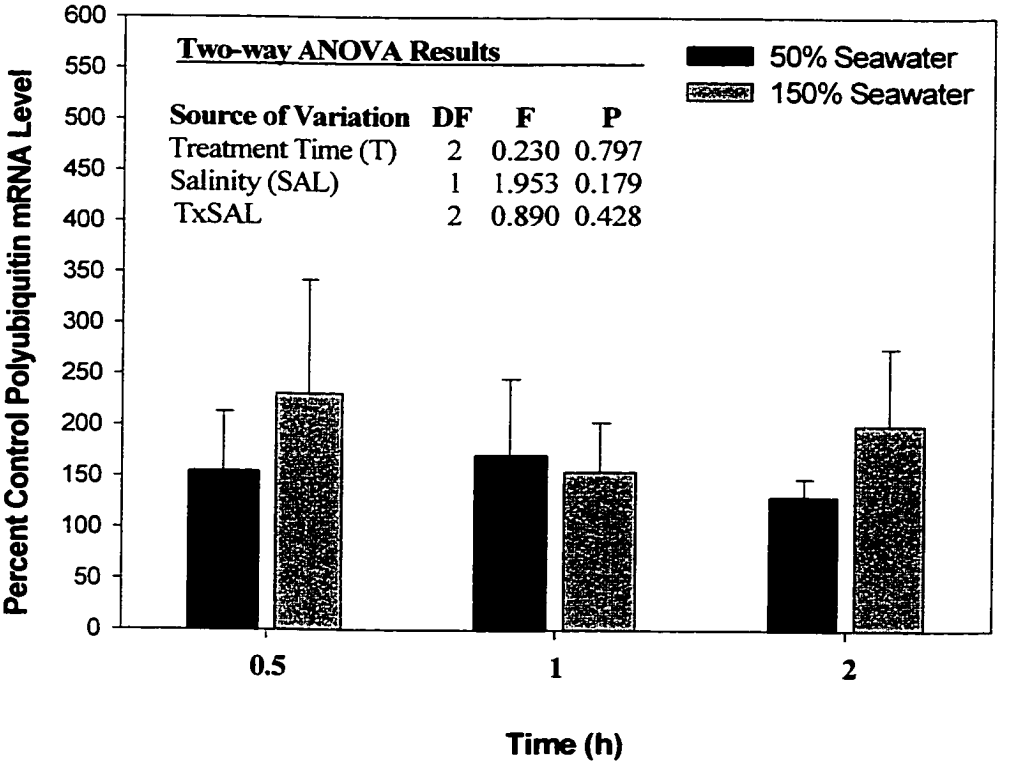


Fig. 3. Comparison of polyubiquitin gene expression during hypo- and hyper-osmotic stress (50% and 150% seawater) for 0.5, 1, and 2 h in A) abdominal muscle and B) hepatopancreas. Data are normalized against the actin signal (indicator of equal loading) and expressed as percent control mRNA level. N=4 for all time points. Error bars represent one S.D. of the mean. Results of two-way ANOVA are presented in upper left corner. Significance between treatment and control polyubiquitin mRNA levels is indicated: * $p \leq 0.05$.

A) Abdominal Muscle Polyubiquitin Expression



B) Hepatopancreas Polyubiquitin Expression

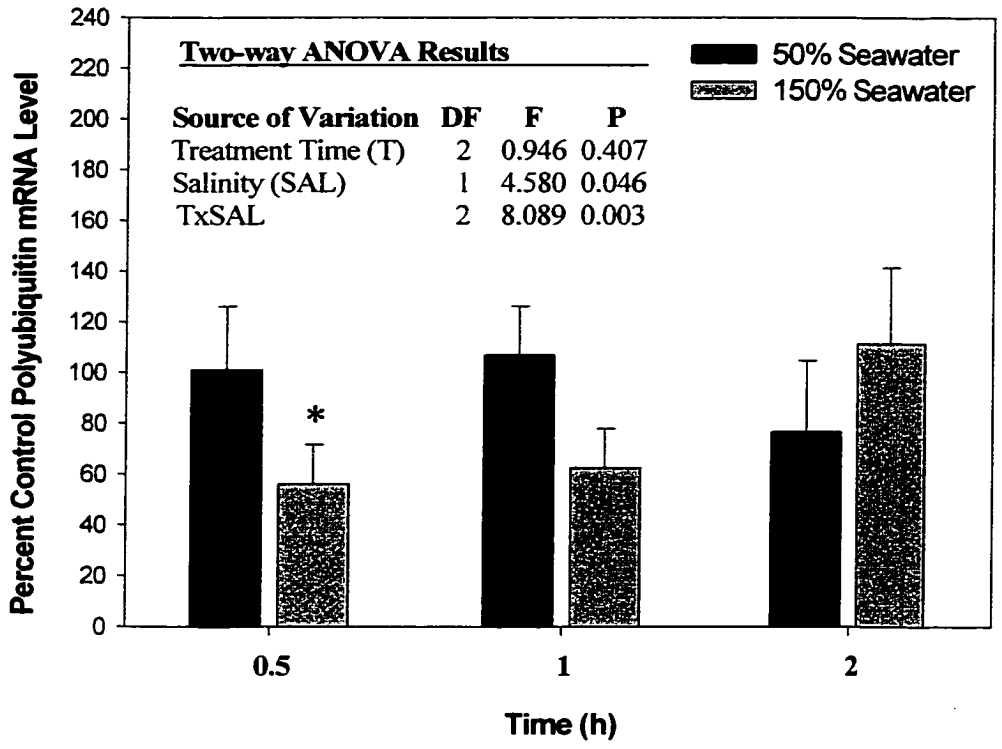
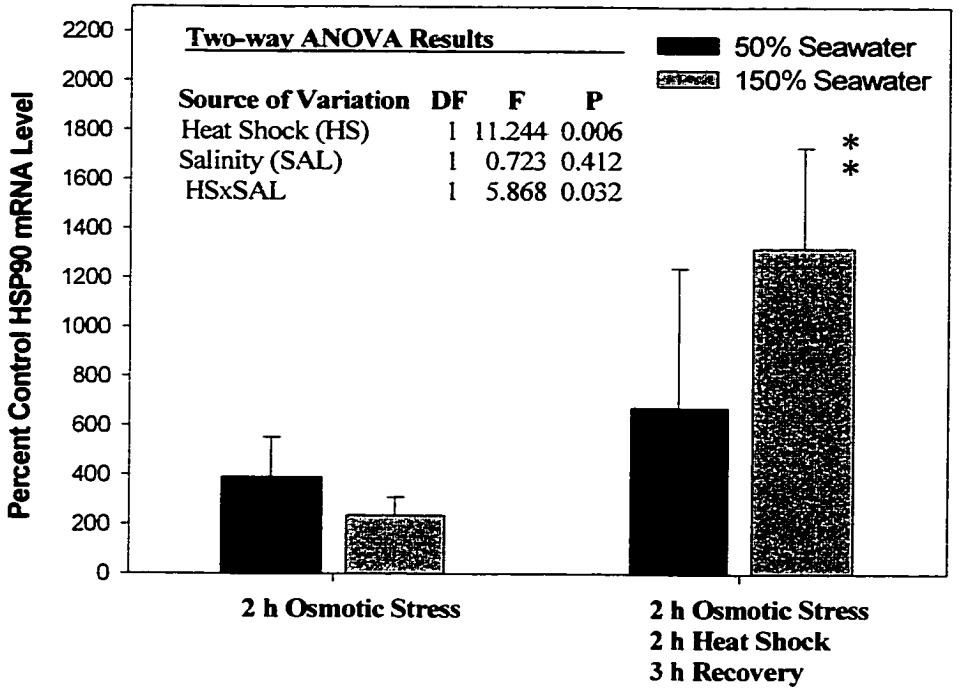


Fig. 4. Comparison of A) abdominal muscle and B) hepatopancreas HSP90 gene expression in lobsters experiencing multiple stresses. Animals were exposed to either a hypo- or hyper-osmotic stress (50 or 150% seawater, 2 h), or a hypo- or hyper-osmotic stress (50 or 150% seawater, 2 h) immediately followed by an acute thermal stress (13.0°C temperature increase, 100% seawater, 2 h) and recovery (ambient temperature, 100% seawater, 3 h). Data are normalized against the actin signal (indicator of equal loading) and expressed as percent control mRNA level. N=4 for all time points. Error bars represent one S.D. of the mean. Results of two-way ANOVA are presented in upper left corner. Significance differences for HSP90 mRNA levels between osmotic stress treatment and osmotic and thermal stress with recovery are indicated: ** $p \leq 0.01$.

A) Abdominal Muscle HSP90 Expression



B) Hepatopancreas HSP90 Expression

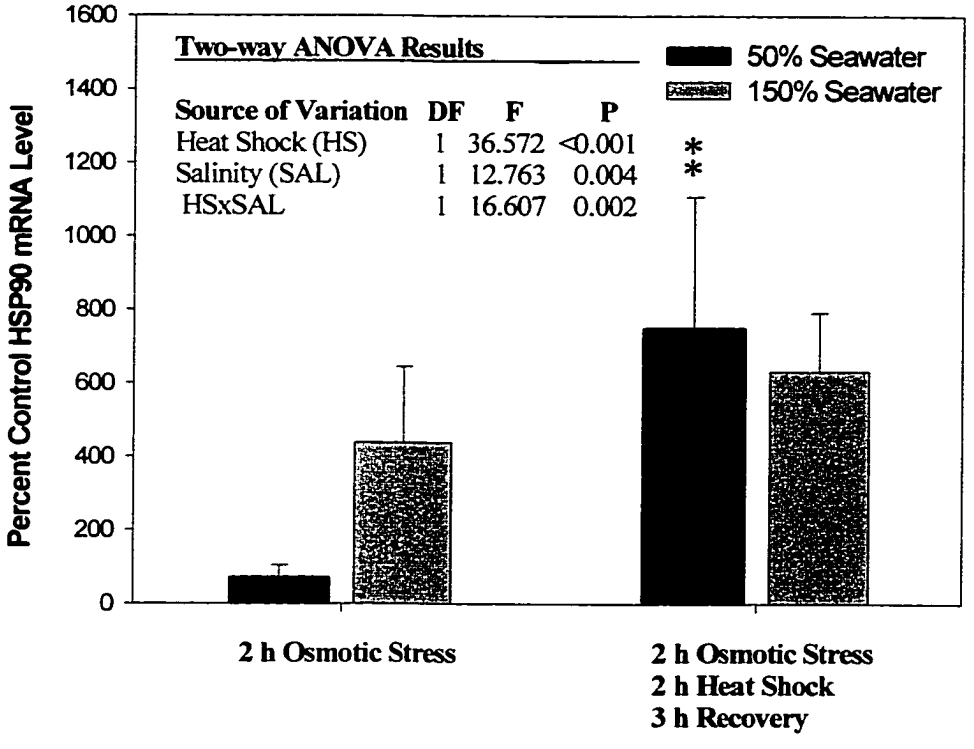
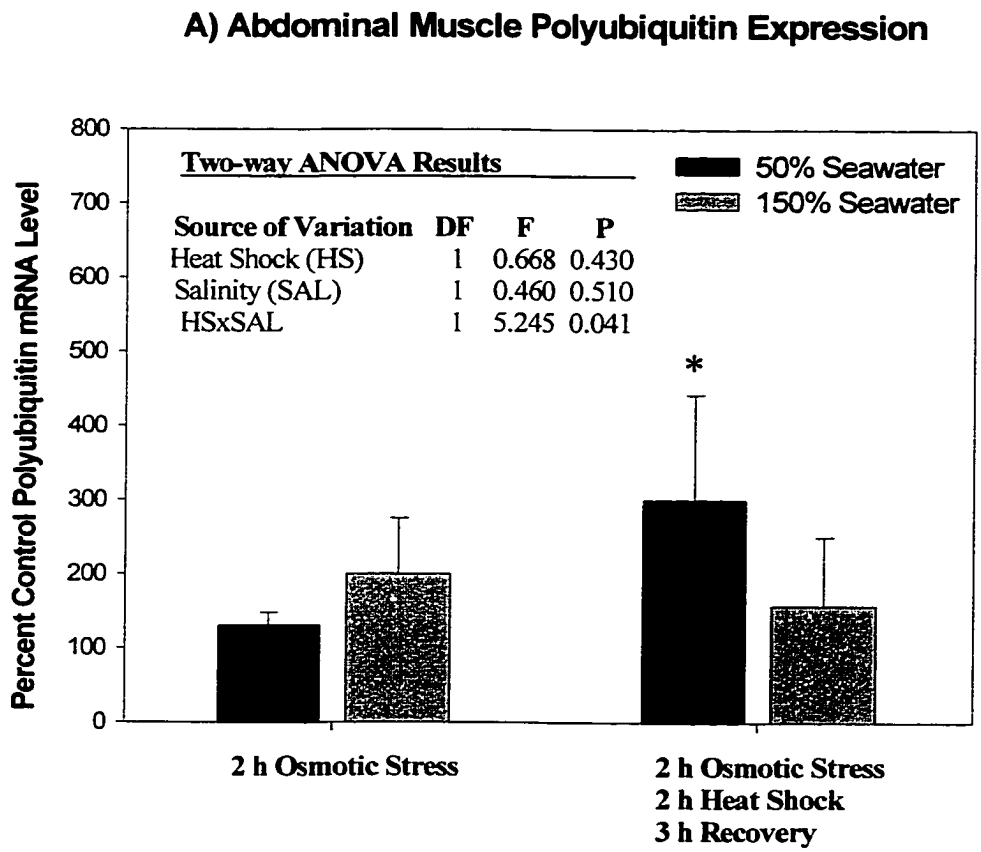
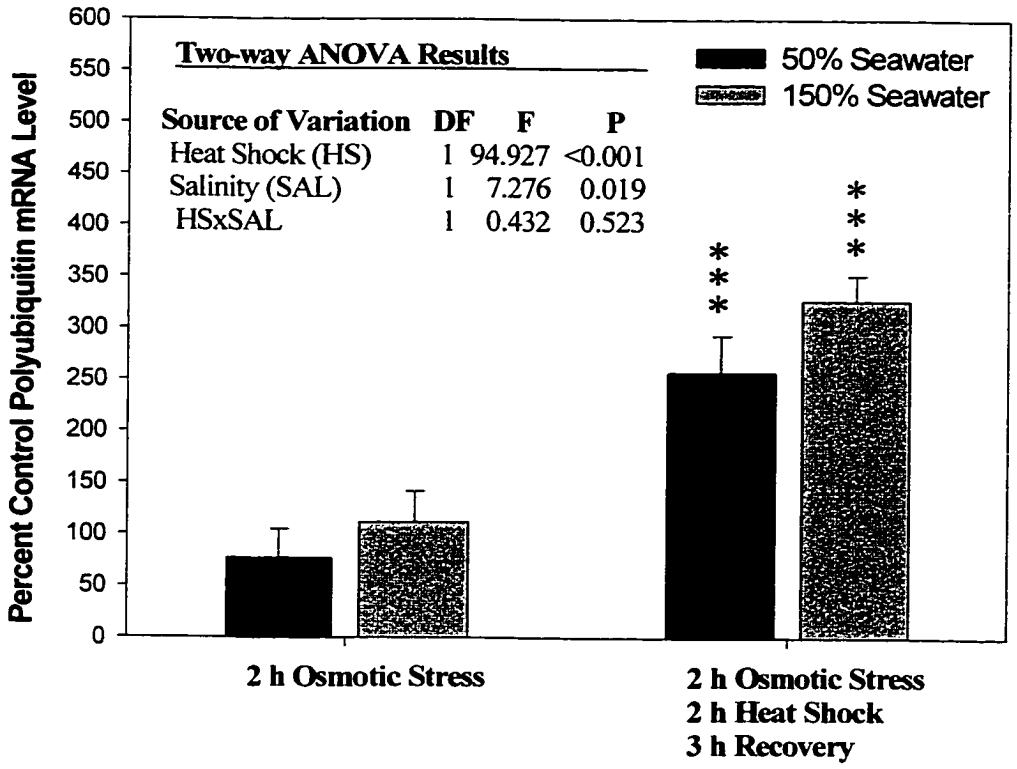


Fig. 5. Comparison of A) abdominal muscle and B) hepatopancreas polyubiquitin gene expression in lobsters experiencing multiple stresses. Animals were exposed to either a hypo- or hyper-osmotic stress (50 or 150% seawater, 2 h), or a hypo- or hyper-osmotic stress (50 or 150% seawater, 2 h) immediately followed by an acute thermal stress (13.0°C temperature increase, 100% seawater, 2 h) and recovery (ambient temperature, 100% seawater, 3 h). Data are normalized against the actin signal (indicator of equal loading) and expressed as percent control mRNA level. N=4 for all time points. Error bars represent one S.D. of the mean. Results of two-way ANOVA are presented in upper left corner. Significance differences for polyubiquitin mRNA levels between osmotic stress treatment and osmotic and thermal stress with recovery are indicated: * $p \leq 0.05$, ** $p \leq 0.01$, ** $p \leq 0.001$.



B) Hepatopancreas Polyubiquitin Expression



Chapter Three

Molt cycle-dependent molecular chaperone and polyubiquitin gene expression in lobster

Abstract

Lobster claw muscle undergoes atrophy in response to increasing ecdysteroid (steroid molting hormone) titers during premolt. In vivo molecular chaperone (HSC70, HSP70, HSP90) and polyubiquitin mRNA levels were examined in claw and abdominal muscle from premolt or intermolt individual lobsters. HSPs/HSCs are known to regulate mammalian steroid hormone receptors as well as the Drosophila ecdysteroid receptor. Polyubiquitin gene expression was quantified as a marker for muscle atrophy. Both HSC70 and HSP90 mRNA levels were significantly induced in premolt relative to intermolt lobster claw muscle, while HSP70 mRNA levels were not. HSP90 gene expression was significantly higher in premolt claw muscle versus abdominal muscle. Polyubiquitin mRNA levels were substantially elevated in premolt versus intermolt claw muscle and significantly elevated relative to premolt abdominal muscle.

Introduction

Crustaceans shed their protective exoskeletons many times during their lives in order to grow. The American lobster, Homarus americanus, undergoes 20-25 molts from the time of hatching to sexual maturity (about 500 g) (Hughes et al., 1972). Lacking a terminal molt, 15-20 kg lobsters are found in nature that are estimated to be 50-70 years old (Herrick, 1895; Cooper and Uzmann, 1980).

While possession of a protective exoskeleton has proven to be an extremely successful evolutionary strategy for marine crustaceans, the protection it affords does come with some tradeoffs. For H. americanus, molting (ecdysis) generally lasts 10-20 minutes. Pulling through and shedding the exoskeleton, they are capable of only limited movement and are vulnerable. The simultaneous release of molting fluid containing lubricants, numerous proteases, and other peptide molecules is a potent attractant for predators. Immediately after ecdysis the new exocuticle is soft and pliable, offering little protection. Accordingly, the tips of the chelipeds, or large front claws and the cutting and tearing edges of the mandibles and maxillipeds are first to harden on the new exoskeleton; they are critical for defense and feeding (Waddy et al., 1995).

Although magnificent adaptations for predation and protection, the lobster's large claws pose an anatomical dilemma for molting. The diameter of the proximal leg segments and the exoskeletal openings at the base of the claws (the basi-ischial joints) are too narrow to allow the large front claw muscles to pass through. Juvenile and adult lobsters therefore risk losing these crucial structures every time they molt. This predicament is solved hormonally. In response to increasing titers of ecdysteroids

(steroid molting hormones) during premolt, intracellular hormone receptors in claw muscles initiate a cascade of poorly understood events that culminate in the selective degradation of myofilaments in claw muscle fibers (Mykles, 1997). This process does not occur in the walking leg muscles (Mykles and Skinner, 1982) or in the lobster's large abdominal (tail) muscle, both of which fit through their designated openings in the exoskeleton. Originally identified over 30 years ago in the land crab, Gecarcinus lateralis (Skinner, 1966), molt-induced claw muscle atrophy is now understood to involve Ca^{2+}-dependent enzymatic proteolysis (Mykles, 1992; Beyette and Mykles, 1997) as well as the ATP/ubiquitin-dependent degradation system (Shean and Mykles, 1995; reviewed in Mykles, 1998).

Despite remarkable atrophy during the crustacean premolt stages (30-60% of the claw muscle protein content in the land crab; Skinner, 1966), protein synthesis rates are actually elevated in premolt vs. intermolt claw muscle tissues (G. lateralis, Mykles, 1992; H. americanus, El Haj et al., 1996; El Haj and Whiteley, 1997). This increase in protein synthesis is attributed to the synthesis of proteinases (Mykles and Skinner, 1990), and facilitating myofilament rearrangments (Mykles, 1997), although the end result is a net loss of muscle mass due to subsequent proteolysis and myofibrillar degradation.

Molecular chaperones (heat shock proteins [HSPs] and constitutive heat shock proteins [HSCs]) that aid in folding nascent proteins at the ribosome may be involved in crustacean muscle atrophy. Chaperones provide a favorable folding environment while preventing aggregation by binding to exposed hydrophobic residues on newly synthesized proteins (Feder and Hofmann, 1999). Frequently studied because of their

induction during cellular perturbations resulting in protein denaturation, HSCs and HSPs are also involved in many cellular processes including mitochondrial and endoplasmic recticular transport (Elston, 2000), the cell cycle (Milarski and Morimoto, 1986; Taira et al., 1997; Helmbrecht et al., 2000), and chaperoning of steroid hormone receptors (Pratt, 1997, 1998).

Regarding the elevated protein synthesis rates observed in premolt versus intermolt claw muscle and the regulation of steroid hormone receptors by HSPs, I hypothesized that molecular chaperones (HSC70, HSP70, and HSP90) would be expressed over the molt-cycle and in particular during premolt atrophy. To address this question, I examined mRNA levels of molecular chaperones and polyubiquitin, a marker for protein degradation and premolt muscle atrophy (Shean and Mykles, 1995). For comparison, the mRNA levels for each of these genes were analyzed in abdominal muscle from the same animals. Hypothetically, abdominal muscle would not induce molecular chaperone or polyubiquitin expression during premolt since it does not undergo premolt atrophy.

Materials and methods

Juvenile lobsters (siblings, males) reared at the Bodega Marine Laboratory (BML) were molt-staged by recording the timing of several prior molts, and by examining the degree of cuticular separation and setal development in the pleopods (Aiken, 1973). Lobsters were sacrificed and crusher claw and abdominal muscle samples were dissected, frozen with liquid N_2, and stored at -70°C. Prior to their selection, lobsters were

maintained in a flow-through aquaculture system supplied directly from the Pacific Ocean. They were held in individual compartments and fed shrimp 3 times weekly. Detailed descriptions of the aquaculture system and lobster culture techniques at BML are described elsewhere (Chang and Conklin, 1993; Conklin and Chang, 1993).

Northern analysis

Total RNA was isolated from the claw and abdominal muscle samples (RNAgents kit, Promega), quantified with a spectrophotometer, and equally loaded (15 μg total RNA) onto denaturing 1% agarose gels. These gels were washed (15 min, DEPC H_2O) and blotted overnight onto nylon membranes (Magnagraph, MSI). Following UV cross-linking (UV Stratalinker 1800), blots were pre-hybridized (2 h) in 5x SSPE buffer, 50% (w/v) formamide, 5x Denhardt's, 1% SDS, and 100 μg/ml denatured sheared salmon sperm DNA. A partial lobster HSP70 clone (499 bp) was ^{32}P-labeled (Prime-It RmT, Stratagene), added directly to the prehybridization solution, and allowed to hybridize overnight at 42°C. Following hybridization, the blots were washed twice with 2x SSPE and placed on film overnight at -70°C.

Following exposure of the film, the blots were stripped with several washes (0.1x SSC, 0.1% SDS, 65°C) until background was minimal and prehybridized, hybridized, and washed as above, except that a partial lobster HSC70 (600 bp, mostly 3' untranslated region), HSP90 (350 bp), or polyubiquitin (690 bp; Shean and Mykles, 1995) cDNA probe was added. The blots were then probed with a partial lobster actin cDNA to check for equal loading of RNA (700 bp; Harrison and El Haj, 1994). RT-PCR and cloning of the HSC70, HSP70, and HSP90 probes is described in previous work (Chang et al., 1999;

Spees et al., 2001). Films were scanned on a high resolution scanner and densitometry was performed with NIH Image software.

Results

HSP90 gene expression was significantly induced in premolt versus intermolt claw muscle (Mann-Whitney Rank Sum Test; p=0.002; Fig. 1A, B). There was no significant difference between intermolt and premolt HSP70 mRNA levels in claw muscle (Student's t-test; p=0.969; Fig. 1A, B). Unlike HSP70, HSC70 mRNA levels were significantly higher in premolt versus intermolt claw tissues (Mann-Whitney Rank Sum Test; p=0.004; Fig. 1A, B). Polyubiquitin mRNA levels, although higher in premolt relative to intermolt samples, were not significantly different (Student's t-test; p=0.067; Fig. 1A, B).

There were no significant differences in molecular chaperone expression between premolt versus intermolt abdominal muscle (Student's t-test; HSP90, p=0.058; HSP70, p=0.256, HSC70, p=0.0534; Fig. 2A, B). There was, however, a significant difference in polyubiquitin mRNA levels. In contrast to claw muscle expression, abdominal muscle showed significantly higher polyubiquitin mRNA levels in intermolt rather than premolt tissues (Mann-Whitney Rank Sum Test; p=0.002; Fig. 2A, B).

Comparison of premolt claw and abdominal muscle gene expression profiles from the same animals revealed significant differences. HSP90 mRNA levels were significantly higher in premolt claw versus premolt abdominal muscle (Student's t-test; p=0.01; Fig. 3A, B), although neither HSP70 (p=0.764) nor HSC70 (p=0.729) mRNA

levels were significantly different between tissues. Differences in polyubiquitin expression were highly significant between muscle types: premolt claw muscle had higher polyubiquitin mRNA levels than premolt abdominal muscle (Student's t-test; p=0.002; Fig. 3A, B).

Discussion

This study demonstrates significant in vivo differences in molecular chaperone and polyubiquitin mRNA levels for lobster claw and abdominal muscle types at different molt stages. These changes in expression were evident both among and between tissues. Fundamental physiological changes required for molting such as premolt-driven claw muscle atrophy are likely to account for some of the differences observed. Molt cycle-dependent muscle atrophy is a novel example of molecular chaperone (HSP90 and HSC70) mRNA induction in a differentiated somatic tissue that is not undergoing environmental stress. In the absence of protein-denaturing stress such as heat shock or transition metal exposure, human HSP70 may be induced by oncogenes and the synthetic phase of the cell cycle (Milarski and Morimoto, 1986; Taira et al., 1997; Helmbrecht et al., 2000). During Drosophila development, pulses of edysteroids are known to induce the expression of heat shock genes such as HSP70 and HSP22 (White et al., 1999).

Arbeitman and Hogness (2000) have recently shown that both HSC70 and HSP90 complex with and are required for activity of the Drosophila ecdysteroid receptor in vivo. I found significant induction of both HSC70 and HSP90 mRNAs in premolt (stage D_2) versus intermolt (stage C) lobster claw tissues. Ecdysteroid receptor (EcR)

immunoreactivity has been previously demonstrated in both walking leg extensor muscle and eyestalk neural tissues of premolt H. americanus using antisera against the Drosophila EcR (El Haj et al., 1994). Ecdysteroid titers quantified by radioimmunoassay (RIA) of hemolymph from premolt H. americanus rise during stages D_1 and D_2 and fall during D_3 and D_4 (immediately before ecdysis) (Chang and Bruce, 1980; Snyder and Chang, 1991a). Because premolt claw muscle atrophy occurs in response to increasing titers of ecdysteroids, the data in this study imply that the significant induction of HSC70 and HSP90 mRNA may be involved in the regulation of ecdysteroid receptors in claw muscle cells responsive to molting hormones. No such induction of HSC70 or HSP90 gene expression was observed in premolt abdominal muscle samples from animals undergoing claw muscle atrophy. The cells of abdominal muscle tissues may thus express lower concentrations or different isoforms of ecdysteroid receptors than claw muscles. Functional differences have been found between isoforms of the Drosophila EcR, and tissues expressing different EcR isoforms have been shown to exhibit distinct metamorphic responses to ecdysone (Talbot et al., 1993; Bender et al., 1997).

HSC70 and HSP90 mRNA levels for lobster claw muscle were induced during atrophy while the HSP70 mRNA level was not. This may also indicate a specialized need for particular molecular chaperones in receptor regulation versus a more generalized function such as chaperoning during protein synthesis. In vivo rates of protein synthesis are significantly elevated in premolt leg, claw, and abdominal muscle relative to intermolt or postmolt tissues (El Haj et al., 1996). Increased protein synthesis rates in claw muscle undergoing atrophy are believed to result from enhanced synthesis of myofibrillar

degradation enzymes and to facilitate rearrangements in myofilament packing (Mykles, 1997). Despite possible changes in protein synthesis rates over the molt cycle (and levels of chaperoning for protein synthesis), there are not significant differences in HSP70 mRNA expression between intermolt and premolt claw or abdominal muscle. Additionally, no significant difference was found between HSP70 or HSC70 mRNA levels for premolt claw versus abdominal muscle from the same individuals.

Polyubiquitin mRNA levels were significantly higher in premolt claw muscle versus abdominal muscle and premolt claw also exhibited substantially greater levels of polyubiquitin gene expression relative to intermolt claw tissues. My data are in agreement with those of Shean and Mykles (1995) who similarly found elevated polyubiquitin mRNA levels in premolt claw tissues of G. lateralis. I expected increased polyubiquitin expression in premolt claw muscle because of protein degradation during muscle atrophy. Unexpectedly, I found significant differences in polyubiquitin mRNA levels between intermolt versus premolt abdominal muscle. Opposite to lobster claw muscle, intermolt abdominal muscle had significantly higher levels of polyubiquitin mRNA than premolt muscle. Although I do not have data for ubiquitin conjugation, reduced polyubiquitin gene expression in premolt versus intermolt abdominal muscle hypothetically indicates reduced protein turnover in premolt abdominal muscle. Increased polyubiquitin expression and ubiquitin production occurs in mammalian skeletal muscle in a variety of catabolic states (for review see Attaix and Taillandier, 1998). With elevated protein synthesis rates in premolt claw and abdominal muscles and a potential reduction in protein turnover in abdominal muscle, this should correspond to

an overall deposition of muscle mass in premolt abdominal muscle versus an overall loss of muscle mass in premolt claw muscle. El Haj et al. (1996) discuss muscle-specific mass increases in preparation for the molt as indicated by a lengthening in both premolt leg and abdominal muscles.

Chung et al. (1998a) reported the cloning of crustacean ecdysteroid receptor gene homologues from the fiddler crab, Uca pugilator. These transcripts were significantly elevated in premolt (stage D_1-D_4) samples of muscle from the large cheliped relative to intermolt samples. The increases in receptor mRNA correlate with premolt peaks in hemolymph ecdysteroid levels as quantified by RIA (Chung et al., 1998b). The significant increases in lobster claw muscle mRNAs for HSC70 and HSP90 I observed during the same period in the molt cycle may also correspond with the enhanced transcription of a lobster ecdysteroid receptor isoform.

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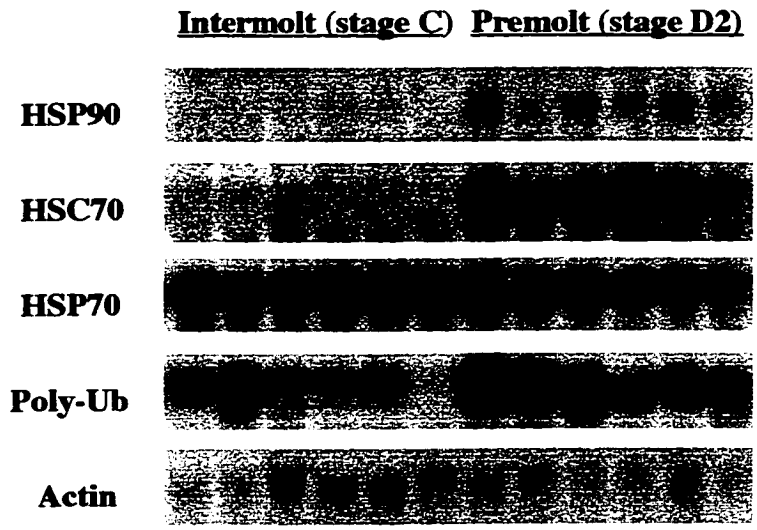
Fig. 1. A) Northern analysis of molecular chaperone and polyubiquitin mRNA levels in intermolt (stage C; lanes 1-6) and premolt (stage D_2; lanes 7-12) claw muscle from juvenile lobsters. Each lane represents tissue from a single animal. Actin mRNA levels are shown as an indicator of equal loading. Data are derived from a single blot that was serially hybridized with ^{32}P-labeled cDNA probes. B) Relative mRNA levels for molecular chaperones and polyubiquitin in intermolt versus premolt claw muscle. Data from (A) were normalized against the actin signal. N=6 for each bar. Absolute expression levels for one transcript should not be compared to any other because of potential differences in probe strength and film exposure. Significant difference between the intermolt and premolt stages is indicated; ** $p \leq 0.01$.

A) Intermolt and premolt claw muscle



B) Intermolt and premolt claw muscle

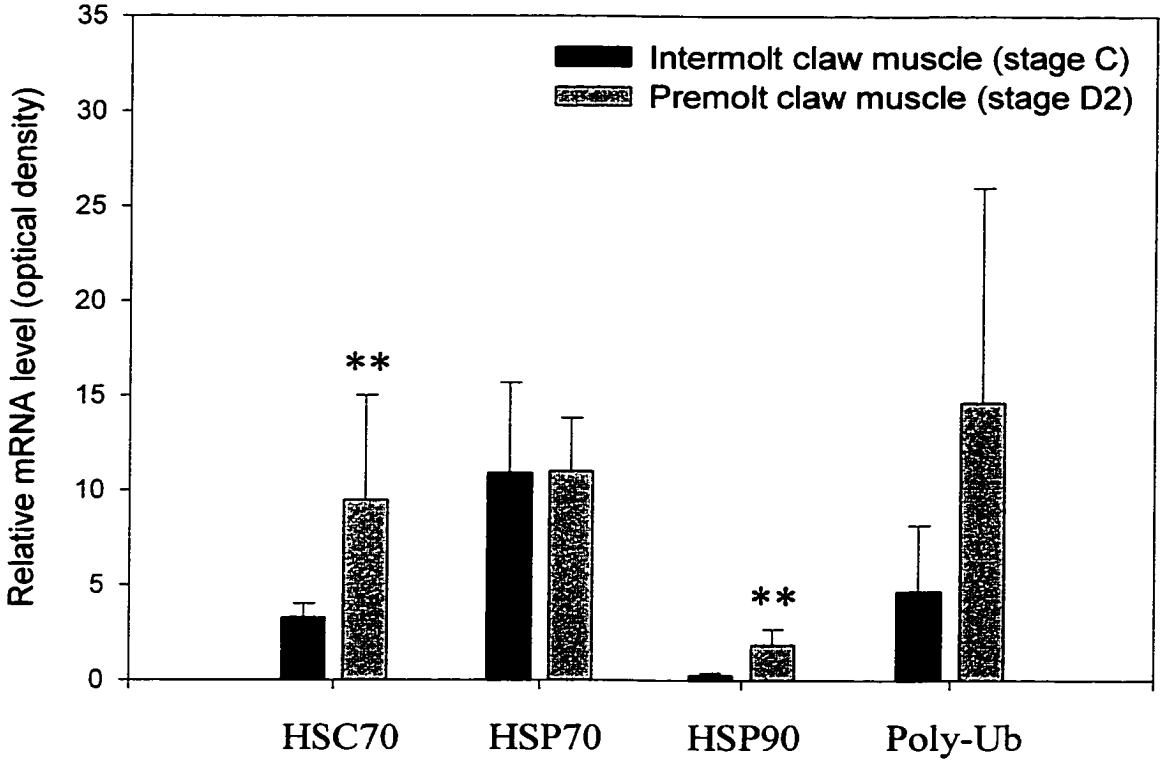
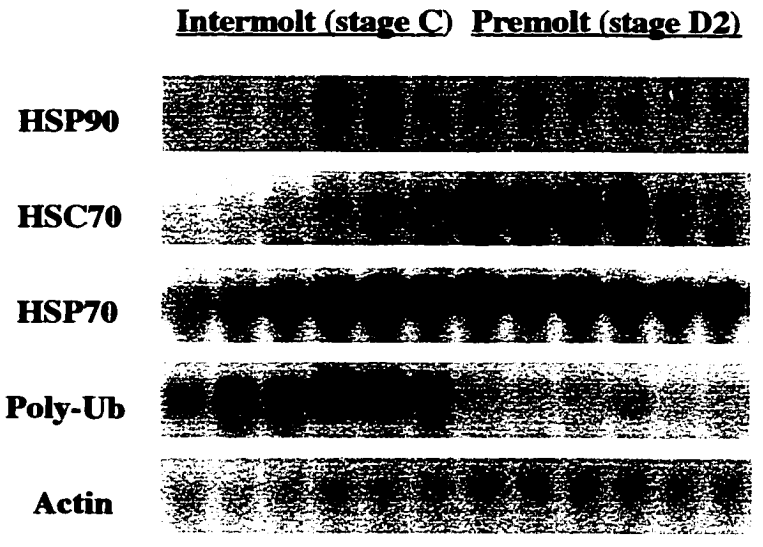


Fig. 2. A) Northern analysis of molecular chaperone and polyubiquitin mRNA levels in intermolt (stage C; lanes 1-6) and premolt (stage D_2; lanes 7-12) abdominal muscle from juvenile lobster. Each lane represents tissue from a single animal. Actin mRNA levels are shown as an indicator of equal loading. Data are derived from a single blot that was serially hybridized with ^{32}P-labeled cDNA probes. B) Relative mRNA levels for molecular chaperones and polyubiquitin in intermolt versus premolt abdominal muscle. Data from (A) were normalized against the actin signal. N=6 for each bar. Absolute expression levels for one transcript should not be compared to any other because of potential differences in probe strength and film exposure. Significant difference between the intermolt and premolt stages is indicated; ** $p \leq 0.01$.

A) Intermolt and premolt abdominal muscle



B) Intermolt and premolt abdominal muscle

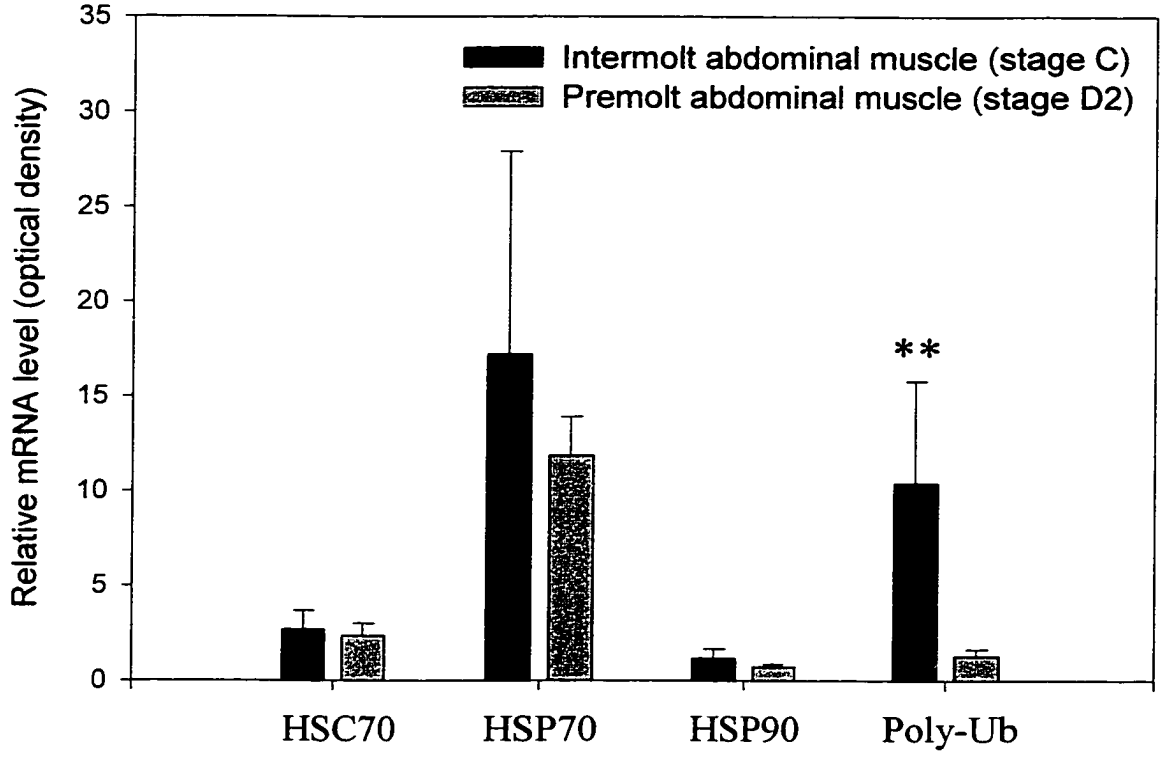
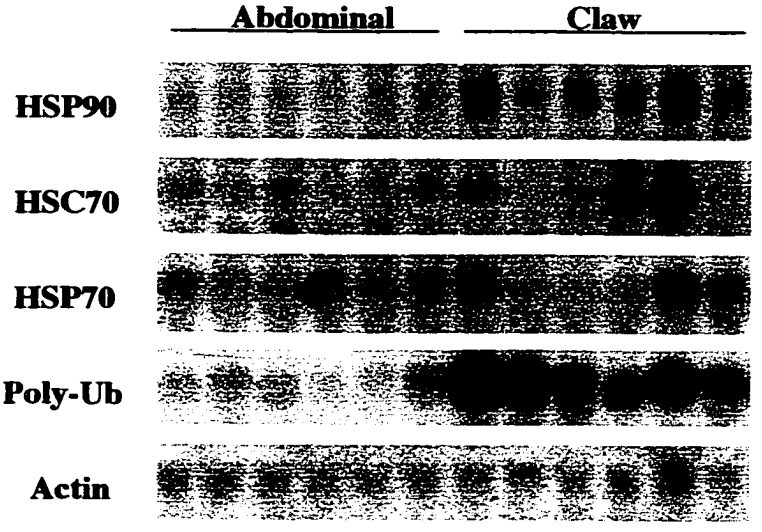


Fig. 3. A) Northern analysis of molecular chaperone and polyubiquitin mRNA levels in premolt abdominal muscle (stage D_2; lanes 1-6) and premolt claw muscle (stage D_2; lanes 7-12) from juvenile lobster. Abdominal and claw muscle samples were dissected from the same animals (i.e. lanes 1 and 7 represent RNA isolated from abdominal and claw muscles of the same lobster). Actin mRNA levels are shown as an indicator of equal loading. Data are derived from a single blot that was serially hybridized with ^{32}P-labeled cDNA probes. B) Relative mRNA levels for molecular chaperones and polyubiquitin in intermolt versus premolt abdominal muscle. Data from (A) were normalized against the actin signal. N=6 for each bar. Absolute expression levels for one transcript should not be compared to any other because of potential differences in probe strength and film exposure. Significant difference between the intermolt and premolt stages is indicated; ** $p \leq 0.01$.

A) Premolt abdominal and claw muscle



B) Premolt abdominal and claw muscle