RESPONSE TO HEAT STRESS IN THE PORCELAIN CRAB *PETROLISTHES CINCTIPES*

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By
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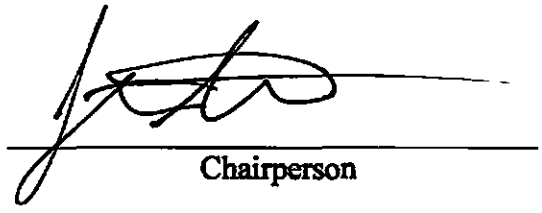
We certify that we have read this thesis and that, in our opinion, it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Zoology.

THESIS COMMITTEE



Chairperson

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ABSTRACT

The response to heat stress in the intertidal porcelain crab, *Petrolisthes cinctipes*, was investigated at the gene expression level. A 13,824 cDNA library was constructed and subsequently used to profile transcriptome changes in crabs during recovery from heat stress. 187 transcripts were either upregulated or downregulated in heat stress versus control groups 1h-30h into recovery from heat stress. Gene expression changes following heat stress involved genes from a wide variety of biological processes, including protein/folding and degradation, cytoskeletal activity, detoxification, energy generation, and lipid metabolism. The majority of expression patterns could be captured by sampling 2h and 18h following heat stress, information important for designing future experiments aiming to determine the effect seasonal changes or latitudinal differences have on the response to heat stress. The present study sets the stage for addressing broader questions relating to how global climate change might affect organisms (i.e. species distribution).

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CHAPTER 1. INTRODUCTION

Temperature is a critical abiotic factor affecting organisms at the ecological through to fundamental cellular and molecular levels (Hochachka and Somero, 2002). Generally, all organisms experience temperature change (i.e. daily, seasonal scales), at least to some extent, in their natural habitat. There is strong evidence that global temperatures have been increasing in the past century and that it will continue to rise (Karl and Trenberth, 2003). The question of how organisms might be affected by global climate change has been posed (Sagarin et al., 1999; Hughes, 2003; Stillman, 2003). Species distributions limits are likely to change in response to global warming, for example through the upward shift of southern species of the intertidal zone northward as was found over a 60 year period in Monterey, California (Sagarin et al., 1999). Understanding aspects of thermal physiology first, as a means to describe the impact global climate change may have on organisms, can be useful (Stillman, 2003).

It has been noted that the upper and lower thermal tolerance limits (CT_{max} and CT_{min}) of an organism can change with acclimation temperature (Cuculescu et al., 1998; Herrera et al., 1998; Stillman, 2004). For instance, thermal maxima and minima for post-larvae and juveniles of the prawn *Macrobrachium rosenbergii* increased at least by 4°C with increased acclimation temperatures ranging from 20°C-32°C (Herrera et al., 1998). Thermal tolerance as well as the plasticity thereof, is often dependent on thermal characteristics related to natural habitat conditions (i.e., average or maximal/minimal temperature, temporal scale thermal variability) (Cuculescu et al., 1998; Stillman, 2002). For instance, the thermal maximum of *Carcinus maenas*, a crab that experiences a wider

range of temperatures in nature than its congener *C. parurus*, exceeds that of its stenothermic congener.

Using a comparative approach, Stillman (2003) showed it may be the case that warm adapted species are less able to increase their CT_{max} in response to increased temperatures, whereas the opposite holds for cold adapted species. Thus, warm adapted species may be more affected by global warming (Stillman, 2003).

Intraspecific, latitudinal studies may also be informative for assessing the ecological consequences of climate change and, additionally, may assist in explaining the bases for organismal distribution limits. Will global warming affect populations of a species in a latitude-dependent fashion? A critical aspect for this area of study, however, is the assessment of thermal stress levels and responses characteristic of organisms in their natural habitat. Since thermal phenotype is influenced by temperature conditions, and environmental temperatures for many organisms change across seasons, it would be valuable to know the level of acclimatization occurring between seasons.

There is evidence of seasonal variation in organismal thermal phenotype both at the overall physiological level and at the molecular level (Dietz and Somero, 1992; Hofmann and Somero, 1995; Cuculescu et al., 1998). For instance, thermal maxima of field caught crabs *C. maenaas* and *C. pagurus* were significantly lower in the winter than in the summer (Cuculescu et al., 1998). Furthermore, summer-acclimatized gobies of the species *Gillichthys mirabilis* were different at the molecular level compared to winter-acclimatized gobies, showing higher levels of 90-kDa heat shock protein (Hsp90) but less prominent Hsp90 induction (Dietz and Somero, 1992). Does the response to heat stress change in winter versus summer?

Heat shock response

The response to heat stress is a complex process, first noted in *Drosophila* and notably characterized by the preferential synthesis of heat shock proteins of various sizes (e.g., Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, small heat shock proteins) (Ritossa, 1962; Tissieres et al., 1974; Parsell and Lindquist, 1993). In general, heat shock proteins synthesized in the "heat shock response" act on nascent or newly synthesized proteins by binding to exposed hydrophobic regions (exposure can be caused by heat), and may require ATP (e.g., Hsp60, Hsp70) (Fink, 1999). Regulation of heat shock proteins may involve autoregulatory processes, at least in the case of Hsp70, where interaction with heat shock factor (HSF), a transcription factor that binds to upstream heat shock elements or HSEs in response to heat stress, represses its own transcription (Shi et al., 1998).

The heat shock response is far from straightforward and may vary in timing of initiation/termination or in intensity, may rely preferentially on different Hsps in different organisms, and may involve the induction of many genes aside from those encoding Hsps (Parsell et al., 1993; Hofmann and Somero, 1996; Tomanek and Somero, 1999; Tomanek and Somero, 2000; Causton et al., 2001; Sorensen et al., 2005).

Microarray technology

Microarray technology has been used to capture gene expression changes on a broad scale to obtain a more comprehensive view of the heat shock response (Gasch et al., 2000; Causton et al., 2001; Birch-Machin et al., 2005; Sorensen et al., 2005). Gene expression profiling for *Drosophila* following heat stress indicated differential expression of 1222 genes including early-up (e.g., genes for chaperoning, glutathione transferase activity, gluconeogenesis, protein kinase cascades), early-down (e.g., peptidases,

hydrolases, genes for catabolism and glucose transport), and late-upregulated groups (e.g., peptidases, genes for catabolism and transport activity) (Sorensen et al., 2005). In heat stressed yeast, 854 genes were differentially expressed and included the induction of genes for molecular chaperoning, protein degradation, defense against oxidizing agents, metal transport, and aerobic energy generation, and the repression of genes for translation and protein synthesis (Causton et al., 2001).

Research focused on broad changes involved in the heat shock response have been limited to prokaryotes or model organisms such as *Drosophila* and yeast (Causton et al., 2001; Gao et al., 2004; Sorensen et al., 2005). What kinds of changes might be occurring in other organisms following heat stress?

Study organism

The porcelain crab, *Petrolisthes cinctipes*, serves as an interesting model for investigations related to thermal physiology. This species is one of 100 belonging to the genus *Petrolisthes* (Decapoda: Anomura: Porcellanidae) and lives in the North Temperate region of the Eastern Pacific (Haig, 1960). Although water temperatures in this region range from 8-18°C, *P. cinctipes*, as an intertidal inhabitant, experiences temperatures from 4°C to 25°C during winter and summer low tide emersion, respectively (Figure 1.1), (Stillman, 2004). High temperatures have been measured at 31°C (Stillman and Somero, 1996). Data collected from a HOBO U12 temperature data logger in the upper intertidal zone of Cape Arago (June 2004-2005) indicate temperatures ranging from 8-28°C in the summer to 3-17°C in the winter. Average summer temperature was 14°C while average winter temperature was 10°C.

Thermal limits of *P.cintipes* change during thermal acclimation

Heat and cold thermal limits of cardiac function in porcelain crabs are determined (Figure 1.2) during thermal ramps that simulate changes in temperature crabs would experience on a thermally extreme day (Stillman, 2003). The thermal limits of *P.cintipes* have been shown to change with statistical significance following acclimation to 8°C and 18°C as shown in figure 1.3 (Stillman, 2004). After 1 month, the upper and lower thermal limits of *P.cinctipes* significantly differs by more than 1°C. These differences, however, become apparent much earlier, with changes in thermal limits occurring even after several days. Thus, it is clear that physiological changes following acclimation do occur; however, it is also important to consider the organism in the context of its natural habitat. Are physiological changes observed across seasons in acclimatized crabs? Do the thermal limits of *P. cinctipes* differ depending on season? How does the response to heat stress change in winter versus summer?

Thesis focus

In order to begin addressing how the response to heat stress, especially at the molecular level, may vary in *P. cinctipes* within its natural habitat, it is first necessary to establish what kinds of gene expression changes occur and in what time frame these changes take place. Particularly, what are the kinetics of the heat stress response in *P. cinctipes*? This is the main question addressed in the present thesis.

Before the kinetics of the heat stress response could be monitored on a broad level, it was necessary to construct a cDNA library from which microarrays could be fabricated. Chapter 2 of this thesis describes the construction and annotation of a 13,824 cDNA library made from heart, nerve, whole crab, hepatopancreas, gill, and claw tissue of *P. cinctipes*. I played a major role in the construction of the library, generating 8,832

of the cDNA clones, performing preliminary sequencing of random clones, PCR amplifying all clones, and characterizing cDNA clones via gel electrophoresis. The cDNA library made possible the work in Chapter 3 in which the kinetics of the heat stress response in *P. cinctipes* was investigated. It was hypothesized that during recovery from heat stress, genes for protein chaperoning/folding and for protein degradation would increase in expression while genes for energy generation would decrease in expression for heat stressed versus control groups. Also, it was expected that genes for a wide range of biological processes (e.g., not just heat shock protein genes) would show differential expression following heat stress and that patterns of expression would not be the same for all genes.

The research described above is an instrumental component for planning seasonal as well as latitudinal studies aimed at uncovering the physiological bases important for setting species distribution limits and assessing the effects of global climate change. Futhermore, the knowledge gained from the present thesis work may also set the stage for elucidating fundamental molecular bases behind thermal tolerance and plasticity. Using the microarray method as done here has promise for inspiring novel hypotheses of how gene expression changes are involved in defining the thermal boundaries of an organism. Efforts to link organismal physiology to its molecular underpinnings serve as important steps towards developing our understanding of biology as a whole.

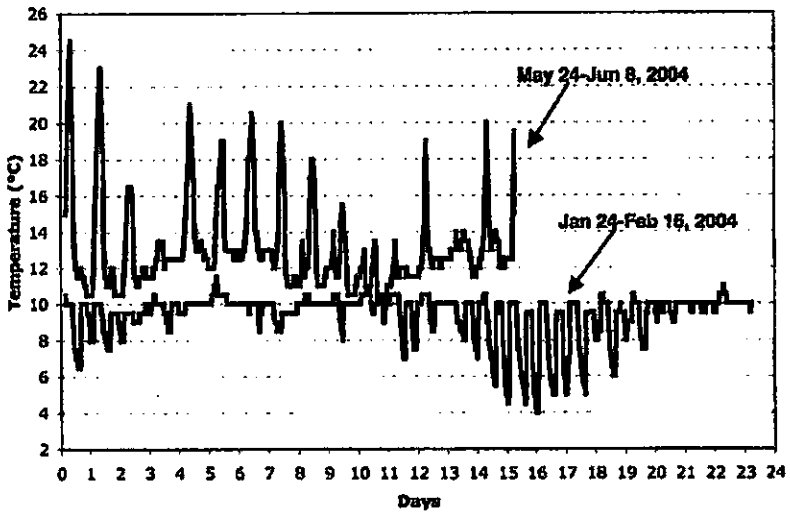

Figure 1.1. Temperature of *P.cinctipes*' microhabitat in winter and summer. The temperature shown was recorded at Cape Arago, OR, using n=2(summer) n=3(winter) ibutton temperature data loggers. ibuttons were placed underneath identical stones in the winter and summer of 2004 . Data are the maximum recorded temperatures for summer and minimum recorded temperatures for winter at each measurement timepoint. (Stillman, 2004)

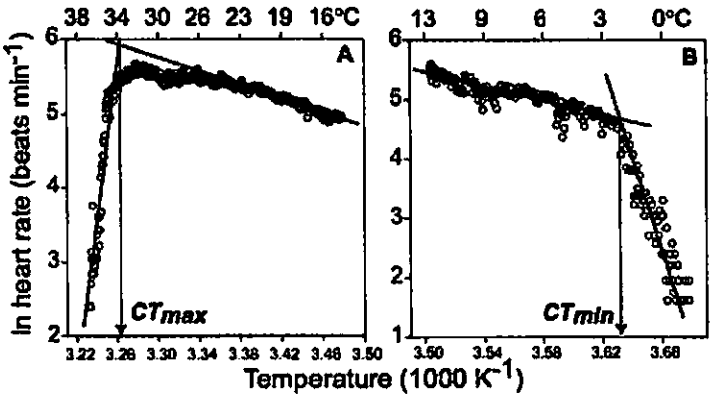

Figure 1.2. Arrhenius plots for determination of thermal limits of cardiac function in *P. cinctipes*. Arrhenius break temperatures of heart rate (CT_{max} and CT_{min}) are shown as the intersection between regression lines fitted before and after the onset of heart failure (indicated by the downward transition in slope). (Stillman, 2003)

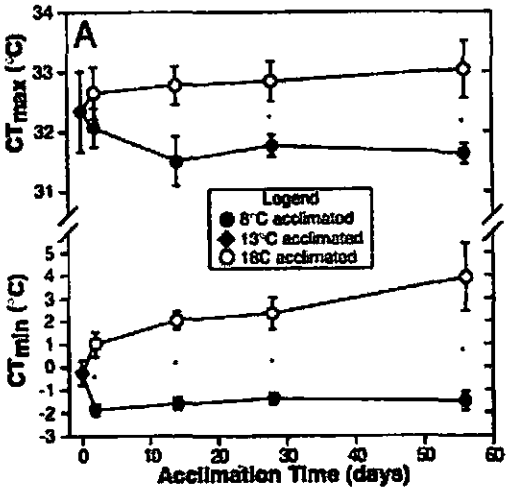

Figure 1.3. Thermal limits following acclimation of *P. cinctipes* to 8°C and 18°C. Error bars represent ±1S.D. (n=4 to 6). (Stillman, 2004)

CHAPTER 2. CONSTRUCTION AND CHARACTERIZATION OF EST LIBRARIES FROM THE PORCELAIN CRAB, *PETROLISTHES CINCTIPES*

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Synopsis

The thermal phenotype of an organism (heat and cold tolerance, thermal range, and thermal plasticity) is an essential feature of how the organism performs across thermal environments and in response to thermal stress. Porcelain crabs are of interest in addressing questions of thermal phenotype because of their high species diversity and the large variation in thermal phenotype among species, as well as the biogeographic patterning of these crabs along environmental stress gradients. We are studying the cellular bases of thermal phenotype and physiological responses to environmental stress using a functional genomics cDNA microarray approach. To do this, we have isolated total RNA from a range of tissues from one species of porcelain crab (*Petrolisthes cinctipes*) exposed to a suite of thermal conditions, and have used this RNA to construct a 13,824-clone EST library. Here, we describe construction, EST sequencing, assembly and clustering, and results of BLASTx homology search for our initial 13,824-clone library. From 12,060 usable ESTs, 6717 consensus sequences were identified, and roughly 50% of these have homology to known proteins. At present, an additional 50-75,000-clone library of *P. cinctipes* ESTs is being generated, with the aim of developing a library with near-complete coverage of the transcriptome. The libraries and sequence information that will be generated as a result of this project should be of value for crustacean biologists working across a broad range of scientific disciplines (e.g., physiology, developmental biology, biological rhythms, ecology, fisheries biology), as well as in studies of molecular evolution and phylogeography.

Introduction

Genomics-based investigative approaches have assumed a major role in advances across a wide range of disciplines, from the biomedical field to ecology, evolution, and comparative physiology (Alizadeh et al., 2000; Feder and Mitchell-Olds, 2003; Gracey and Cossins, 2003; Hofmann et al., 2005). Sequencing genomes from model organisms such as *C. elegans* and *D. melanogaster* set the stage for rapid progress in characterizing gene function and regulation (Consortium, 1998; Adams et al., 2000). It is not, however, the general case that these model organisms are necessarily the select study systems for the comparative physiologist (Gracey and Cossins, 2003).

The marine environment provides an interesting setting for addressing ecological and comparative physiological questions such as how ecosystems or organisms and their distribution limits might be affected by global environmental change (Stillman, 2003; Hofmann et al., 2005). At present, full genome sequence information for marine organisms like *Fugu rubripes* (Japanese pufferfish) (Aparicio et al., 2002) and *Ciona intestinalis* (sea squirt) (Dehal et al., 2002) are available. Additionally, many laboratories have opted to construct Expressed Sequence Tag (EST) libraries from marine study systems for use in addressing problems spanning ecotoxicology [e.g., mussels (Venier et al., 2003) and copepods as pollution bioindicators (Lee et al., 2005)], immunology [i.e., disease resistance in shrimp (Supungul et al., 2002), immune response in oysters (Gueguen et al., 2003)], muscle physiology (e.g., muscle growth in scallops (Roberts and Goetz, 2003)], and pharmacology [e.g., cone snail conotoxins (Pi et al., 2006)]. The range of organisms being studied at the gene expression level provides fruitful grounds for comparative work as well.

Recently, a hub for EST information and microarray data specific to marine organisms was developed by the Marine Genomics project (McKillen et al., 2005). Databases for over 19 marine species are accessible or underway (http://www.marinegenomics.org) and include those for 7 crustaceans *Callinectes sapidus* (Blue crab, 1742 ESTs), *Homarus americanus* (American Atlantic lobster, 5043 ESTs), *Litopenaeus setiferus* (White shrimp, 1041 ESTs), *Litopenaeus stylirostris* (Blue shrimp, 227 ESTs), *Litopenaeus vannamei* (White shrimp, 13704 ESTs), *Palaemonetes pugio* (Daggerblade grass shrimp, 8821 ESTs), and *Calanus finmarchicus* (North Atlantic copepod, 309 ESTs) (McKillen et al., 2005).

Here, we present our efforts for developing an EST database for the porcelain crab, *Petrolisthes cinctipes*, for use in comparative functional genomic analyses of mechanistic bases of thermal adaptation as well as patterns of thermal stress responses across ecological gradients. Development of this porcelain crab genomics resource allows us to take advantage of the great biological and ecological diversity of the porcelain crabs, and builds on a wealth of comparative thermal physiology studies in these organisms. Porcelain crabs of the genus *Petrolisthes* (Decapoda: Anomura: Porcellanidae) comprise over 100 species spanning a wide latitudinal range and includes species inhabiting discrete vertical zones within geographical ranges including the North Temperate, Northern Gulf of California, Tropical, and South Temperate regions of the Eastern Pacific coast (Haig, 1960; Stillman, 2002). A phylogeny of porcelain crabs from the Eastern Pacific has been conducted allowing for comparative analyses to be made in an evolutionary context (Stillman and Reeb, 2001). *P. cinctipes* has been an effective model for research in thermal physiology (Stillman, 2002; Stillman, 2003). This species

inhabits the mid to upper intertidal zone in the northeastern Pacific and experiences temperatures as high as 31°C and as low as 4°C during low tide emersion in the summer and winter respectively (Stillman, 2002; Stillman, 2004). We have constructed from *P. cinctipes* a cDNA library for use in studying the mechanisms, at the gene expression level (i.e. using microarray analyses), that underlie organismal thermal phenotypes.

Here, we present the methodology used, and results of our intial library construction, sequencing and annotation. Then, we describe our present efforts, in conjunction with the Joint Genome Institute, to expand the *P. cinctipes* EST library to cover as much of the transcriptome as possible.

Methods

Specimen Collection and Handling

Our cDNA libraries were constructed from specimens of one species of porcelain crab (*Petrolisthes cinctipes*) that were subjected to a wide array of experimental conditions before sacrifice. In our experiments, we collected crabs from across latitudinal and seasonal gradients and exposed these animals to acute heat and cold shocks, generally to ~30°C and ~0°C, respectively. We also acclimated crabs to a wide range of thermal conditions that elicit changes in whole organism thermal phenotype, and exposed these animals to acute heat and cold shocks. Acclimation temperatures ranged from 8°C to 25°C, and acclimation duration ranged from 2 days to 2 months. From hundreds of individual crabs used across two years of experiments, we preserved RNA by snap-freezing whole crabs by freeze-clamp, or by dissecting fresh tissues into 1ml Trizol

reagent (Invitrogen) and then storing frozen until RNA extraction. RNA degradation was not generally observed in Trizol preserved samples.

RNA extraction

Total RNA was extracted by either powdering tissue under liquid N_2 and then thawing the powder in Trizol (whole crabs), or by homogenizing dissected tissues (heart, gill, nerve axons from walking legs (including both sensory and motor neurons), hepatopancreas, and claw muscle tissues) in 1ml Trizol using a powered rotor-stator homogenizer. Homogenates were allowed to sit at room temperature for 15 min to ensure dissociation of nucleoprotein complexes, and centrifuged to remove cellular debris. Supernatants (up to 1ml volume) were removed to fresh microcentrifuge tubes, mixed with 200µl chloroform by vortex for 30s, and centrifuged for 15 min at 16,000x*g*. The aqueous (top) layer was removed, mixed with 250µl isopropanol and 250µl high salt precipitation solution (0.8M NaCitrate, 1.2M NaCl), and incubated at –20°C overnight. Precipitated RNA was pelleted by centrifugation at 16,000x*g* for 45 min at 4°C. Pellets were washed in 60% EtOH and resuspended in 1mM NaCitrate, pH 6.4. RNA concentration and purity were determined spectrophotometrically. Generally, A_{260}/A_{280} ratios were ≥ 1.9, and concentrations ranged from 1 to 1000ng/µl, depending on tissue type and starting tissue quantity. Following quantification of total RNA, we mixed equal amounts of RNA from each tissue type of each individual together to make 7 pooled RNA samples: 2 from heart (from different sets of RNA extracts), and 1 each from gill, nerve, hepatopancreas, muscle, and whole crabs.

cDNA library construction

From each of the 7 pooled RNA samples, we constructed a cDNA library using the BD Clontech SMART cDNA library construction kit. First strand synthesis of cDNA, long-distance PCR (LD-PCR) for synthesis of full-length ds cDNA, and *SfiI* digestion were conducted according to BD Clontech Protocol#PT3000-1, version#PR15738 except for the following: 1) PCR cleanup using Qiagen's QIAquick PCR Purification Kit (QIAquick Spin Handbook, 7/2002) replaced the proteinase K digestion step and 2) 1% xylene cyanol dye was not added immediately after *SfiI* digestion. LD-PCR was generally stopped at 14-16 cycles, about 2 cycles before saturation of PCR product amplification (as analyzed by agarose gel electrophoresis). Following *SfiI* digestion, samples were ethanol precipitated (2 volumes 100% ethanol, 1/10 volume 3M NaAcetate pH 5.2), resuspended in 25ul Qiagen buffer EB (10mM Tris-Cl, 1mM EDTA, pH 8.0), and run out on a 0.75% agarose gel. cDNAs ranging from 500-5000bp (most PCR products were 0.5-2kb) were extracted using Qiagen's QIAquick Gel Extraction Kit (QIAquick Spin Handbook 7/2002). Purified cDNAs were directionally cloned into the *SfiI* sites of either pTriplEx vector (Clontech, GenBank Accession #U39779), or a modified pTrueBlue vector, pTB (Genomics One TBP0527, a gift from Dr. Andrew Gracey) in a ligation reaction using 0.5µl T4 DNA ligase, 0.5µl T4 DNA ligase buffer (New England BioLabs M0202S), 15ng-40ng cDNA, and 0.5µl empty vector in 5µl at 16°C overnight. The ligation reaction was precipitated with 200µl isobutanol, placed on ice for 2h, washed with 70% ethanol, and resuspended in 5µl water.

Transformation of 1µl of the ligation reaction into ElectroMAX DH10B *E.coli* competent cells (Invitrogen, Cat No. 18290-015) was accomplished by electroporation.

Following 1h growth in SOC medium at 37°C, cells were plated on selective blue-white screening LB-agar medium with X-gal, IPTG, and carbenicillin overnight at 37°C. Individual colonies were hand picked with sterile wooden toothpicks into wells of Nunc 384-well plates each containing 50 µl of selective LB with ampicillin. A total of 36 384-well plates (13,824 total colonies) were picked, and this number was divided by library as in Table 2.1.

Characterization of cDNA libraries

PCR analysis of cloned cDNAs:

PCR was used to amplify cDNAs from each colony using vector specific primers to confirm that a single EST was cloned and for purposes of microarray printing. Primers used for pTriplEx were: 5'CTCGGGAAGCGCGCCATTGTGTTGGT (forward) and 5'ATACGTCTCACTATAGGGCGAATTGGCC (reverse), and for pTB were: 5'ACAGGAGCAAAAACCATGGTCG (forward) and 5'CGGGCTCTAGATCCGGAGT (reverse). Overnight bacterial cultures were used to seed 30µl PCRs in 384-well PCR plates using an MJ-research DNA engine thermal cycler. PCR conditions were 94°C for 5min, followed by 40 cycles of 92°C for 15sec, 54°C for 30 sec, and 71.5°C for 1min, with an additional 7min at 71.5°C at the end of the program for any incomplete PCR products to be finished. The 13,824 PCR products were analyzed by 1% agarose gel electrophoresis (Fig. 2.1).

Sequencing of cloned cDNAs:

Each cloned EST was sequenced by the Joint Genome Institute (JGI) 2006 Community Sequencing Program (CSP). Sequences were generated from overnight

cultures using rolling circle amplification with TempliPhi and sequenced on an ABI3730xl following standard JGI internal protocols (http://www.jgi.doe.gov/sequencing/protocols/prots_production.html). Every clone was sequenced from both the 3' and 5' ends using one of the appropriate PCR primers (above). Some clones were sequenced more than once. Raw sequence traces automatically enter the JGI EST Pipeline, as described in the following 3 paragraphs.

The JGI EST Pipeline begins with the cleanup of DNA sequences derived from the 5'and 3' end reads from a library of cDNA clones. The Phred software (Ewing and Green, 1998) is used to call the bases and generate quality scores. Vector, linker, adapter, poly-A/T, and other artifact sequences are removed using the Cross_match software (Ewing and Green, 1998), as well as a short pattern finder developed internally at JGI. Low quality regions of the read are identified using JGI software that masks regions with a combined quality score of less than 15. The longest high quality region of each read is used as the EST. ESTs shorter than 150 bp, or containing common contaminants (e.g., rRNA, mitochondrial DNA, *E. coli*, common vectors, and sequencing standards) are also removed from the data set.

EST Clustering is performed ab-initio, based on alignments between each pair of ESTs. Pair-wise EST alignments are generated using the Malign software (Chapman et al., unpublished), a modified version of the Smith-Waterman algorithm (Smith and Waterman, 1981), which was developed at the JGI for use in whole genome shotgun assembly. ESTs sharing an alignment of at least 98% identity, and 150 bp overlap are assigned to the same cluster. All alignments generated by Malign are restricted such that they will always extend to within a few bases of the ends of both ESTs. These are

relatively strict clustering cutoffs, and are intended to avoid placing divergent members of gene families in the same cluster. However, these clustering cutoffs could also have the effect of separating splice variants into different clusters. Optionally, ESTs that do not share alignments can be assigned to the same cluster, if they are derived from the same cDNA clone.

For each cluster of EST sequences, cluster consensus sequences are generated by running the Phrap software (Ewing and Green, 1998; Ewing et al., 1998) on the ESTs comprising each cluster. This matches well with the directed sequencing assumptions underlying the Phrap algorithm, as each cluster comprises a clean 'tiling path', which can be easily assembled. Additional improvements were made to the Phrap assemblies by using the 'forcelevel 4' option, which decreases the chances of generating multiple consensi for a single cluster, where the consensi differ only by sequencing errors (P. Brokstein, pers. obs.). Protein homology for each consensus sequence was determined using BLASTx to search against three different databases: GenBank non-redundant (nr), Swiss-Prot, and the Gene Ontology database (GO). BLASTx was run using NCBI Blast 2.2.6. In order to ensure consistency of BLASTx e-value between BLASTx runs on databases of varying size, the -Y parameter was set to 1.75e12. Data used for nr and Swiss-Prot BLAST were downloaded from GenBank on 2005-09-27, and the GO representative sequence file (go_200510-seqdb.fasta) was downloaded from ftp://ftp.geneontology.org/pub/go/godatabase/archive/full/latest. Classification of GO terms (Figure 2.2) was done using basic MS Excel search functions.

Results and Discussion

Insert size characterization

Overall, the smallest PCR products were about 250bp, the largest were about 2000 bp, and the average size was 1000 bp (Figure 2.1). Approximately 13,271 (96%) of the clones yielded a single PCR product, about 300 of the 13,824 clones did not produce a PCR product, and about 235 of the 13,824 clones produced multiple PCR products.

Sequence analysis

From the 13,824 clones picked from the seven libraries that were constructed (Table 2.1), a total of 35,232 sequencing reactions were performed and run on the ABI sequencer. Following quality control assessment, 22,463 sequences of high quality, representing 12,062 out of 13,824 clones, were obtained (Figure 2.1). For 1,762 clones, sequence quality was either poor (e.g., failed sequencing reactions, or the 235 cases where no or multiple PCR bands were observed, Figure 2.1), or represented empty vector or a cloned bacterial gene. There was no further processing of sequence data from these clones.

The 22,463 high quality sequences were found to represent 6,717 consensus sequences. Thus 48.6% (6717/13824) of the cDNA library represented unique cDNAs. This was moderately comparable to EST libraries for other crustaceans including the intertidal harpacticoid copepod *Tigriopus japonicus* (262/686 = 38.2% non-overlapping ESTs) and the green shore crab *Carcinus maenas* (1928/5362 = 36.0% unique cDNAs from a normalized cDNA library), and was middle range when compared to EST libraries for the waterflea *Daphnia* (*pulex*)(12600/71000 = 17.7% unique genes in a unidirectional cDNA library) and the American lobster *Homarus americanus* (3773/4604 = 82.0% unique cDNAs from a normalized cDNA library) (Colbourne et al., 2005; Lee et al., 2005; Towle, 2005).

The 6,717 consensus sequences fell into 5,078 different clusters (Table 2.2). There were 4,024 clusters that were only represented by a single clone (i.e., 4,024 singlets), and there were two clusters that represented the most redundant transcripts, one with 473 clones and the other with 2,137 clones (Table 2.2). The largest cluster contains 194 different consensus sequences, representing 102 different cDNAs.

Sequence similarity of consensus sequences or cluster sequences using a translated query (i.e., BLASTx) resulted in the greatest percentage of matches from the nr database (Table 2.3) and the lowest percentage of matches from the Swiss-Prot database (Table 2.3). BLASTx hits were considered a strong match if the expect score was $< 1e^{-4}$, a weak match if $1e^{-1} \geq$ expect $\geq 1e^{-1}$ and no match if expect $> 1e^{-1}$. In general, about 23-30% of the consensus sequences or clusters had strong matches, 20-22% had weak matches, and 50-55% did not match any known sequence (Table 2.3).

Of the strong matches, different transcripts were predominant in the different tissue libraries (Table 2.4). Anti-lipopolysaccharide factor (Anti-LPS) was most abundant in heart and whole crab libraries. Anti-LPS is an anticoagulating agent and would be expected to be highly expressed in the heart; its predominance in the whole crab library might be explained by expression in hemocytes. Genes for mitochondrial proteins were most abundant in the nerve library, which likely reflects high nerve ATP generation, and the fact that most of the nerve tissue used was axon and not cell bodies. Proteases were most common in the hepatopancreas library, which was anticipated due to the digestive role of this tissue. Cuticle protein was an abundant transcript in the gill library, and reflects the large cuticular surface of this tissue. Lastly, as anticipated, muscle proteins were most abundant in the claw muscle library (Table 2.4).

A large number of transcripts were found in only one or two of the tissue libraries (Appendix), although we should not necessarily attempt to confer function from this since our library is far from comprehensive for each tissue. Many transcripts that were observed in specific tissues have interesting biological function and warrant further discussion. For example, in heart libraries an EST encoding the iron-binding protein transferrin was cloned (Appendix). However, further analysis is required to determine whether this EST represents *transferrin* or one of the mRNAs that encode the large proteinase inhibitor, pacifastin (Liang et al., 1997).

Examination of the two largest clusters (Table 2.2) reveals that the clustering algorithm has grouped many different genes together. The 102 different cDNAs from the largest cluster (above, Table 2.2) included 4 separate cDNAs each for two proteins (anti-lipopolysaccharide factor and trypsin) and 2 separate cDNAs each for 3 proteins (tropomyosin, cytochrome b, cytochrome oxidase subunit I); the remaining 88 cDNAs in the largest cluster represented different proteins. The second largest cluster (above, Table 2.2) contains 44 different consensus sequences that encode a total of 10 different proteins, including 4 cDNAs for arginine kinase, 3 cDNAs for alpha actin, and 2 cDNAs for beta actin. In both of these large clusters, there were also a number of consensus sequences that did not match any known proteins. This result is likely reflective of the challenges of developing clustering algorithms that work every time; these challenges are being actively pursued at JGI, and refinements to clustering algorithms are forthcoming.

Gene ontology (GO) terms for each cluster were categorized by cellular compartment (Figure 2.2A) and molecular function (Figure 2.2B). Information for biological process was not available for most of the clusters, and thus these data are not

shown here. Some clusters had GO terms for more than one cellular compartment or more than one molecular function.

Construction of the second EST library for *Petrolisthes cinctipes* will be performed at the JGI using RNA derived from crabs exposed to a wide array of stressors, thermal conditions, developmental states, and physiological states (Table 2.5). For this library, we have omitted most of the hepatopancreas tissues because of the very high redundancy of trypsin in this tissue (Table 2.4). Stressors include temperature, chemicals, heavy metals, high and low salinity (osmotic), and hypoxia. Thermal conditions include warm and cold acclimation, as well as acclimation to warm-cold fluctuating temperatures, and crabs acclimatized to the species' range of natural habitat conditions. By sampling larval and freshly molted crabs, we hope to capture transcripts unique to processes occurring during those times. Total RNA has been pooled from all of the tissue samples indicated in Table 2.5, and will be used by JGI to develop and sequence a library of 50,000-75,000 clones. At the completion of that project, we hope to have captured as much of the *P. cinctipes* transcriptome as possible. The final analyzed set of ESTs will be submitted to GenBank dbEST, and a "unique" gene set will be generated for use in printing cDNA microarrays to be used for functional genomics analyses.

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Table 2.1. Library composition by tissue type.

Tissue	**# clones**	**% total of library**
Heart (2)[1]	4992	36.1%
Nerve	768	5.6%
Whole crabs	2304	16.7%
Hepatopancreas	2304	16.7%
Gill	2304	16.7%
Claw muscle	1152	8.3%

1. Two libraries were made from different sets of heart tissue RNA extracts.

Table 2.2. Summary of phrap assembly and clustering.

	# cDNAs	# Clusters	# Clones
	1	4,024	4,024
	2-5	840	2,138
	6-10	114	883
	11-20	61	851
	21-30	18	439
	31-68	13	538
	70-185	6	593
	473	1	473
	2,137	1	2,137
TOTAL:	**12,062**	**5078**	**12,062**

Table 2.3. Results of BLASTx Analysis of 12060 ESTs

	DATABASE		
	nr[1]	**Swiss-Prot**[2]	**GO**[3]
	By cluster (5078 total)		
Strong Match[4]	1597 (31.5%)	1187 (23.4%)	1289 (25.4%)
Weak Match[5]	1055 (20.8%)	1195 (23.5%)	1178 (23.2%)
No Match[6]	2426 (47.7%)	2696 (53.1%)	2611 (51.4%)
	By consensus sequence (6717 total)		
Strong Match[4]	2023 (30.1%)	1523 (22.7%)	1638 (24.4%)
Weak Match[5]	1321 (19.7%)	1482 (22.0%)	1443 (21.5%)
No Match[6]	3373 (50.2%)	3712 (55.3%)	3636 (54.1%)

1. nr: All non-redundant GenBank CDS translations+PDB+Swiss-Prot+PIR+PRF

2. Swiss-Prot: the last major release of the SWISS-PROT protein sequence database

3. GO: the Gene Ontology database

4. Strong Match: Expect $< 1e^{-4}$

5. Weak Match: $1e^{-1} \geq$ Expect $\geq 1e^{-4}$

6. No Match: Expect $> 1e^{-1}$

Table 2.4. Most represented cDNAs[1] in each tissue library

Heart:
Anti-lipopolysaccharide factor gi113657 *Tachypleus tridentatus* 4e-17 **(159)**[2]
ATP lipid-binding protein gi|18700491| *Marsupenaeus japonicus* 2e-30 **(42)**
ATP synthase epsilon chain gi|21297384| *Anopheles gambiae* 6e-12 **(12)**
Caspase recruitment domain family 6 gi|28481366| *Mus musculus* 3e-06, **(19)**
Carcinin-like protein gi|51890388| *Carcinus maenas* 1e-08 **(13)**
Cytochrome c oxidase subunit 1 gi|63003725| *Marsupenaeus japonicus* 1e-160 **(71)**
Cytochrome c oxidase subunit 2 gi|7542363| *Pagurus longicarpus* 5e-94 **(20)**
Cytochrome c oxidase subunit 3 gi|15150786| *Pagurus longicarpus* 2e-90 **(12)**
Cytochrome c oxidase subunit 7c gi|51011612| *Ixodes pacificus*, 3e-15 **(21)**
Glutathione S-transferase gi|2842718| *Anopheles gambiae* 3e-61 **(15)**
Putative antimicrobial peptide gi|17223036| *Litopenaeus vannamei* 2e-12 **(19)**
Zinc finger protein gi|55649977| *Pan troglodytes* 3e-10 **(24)**
Nerve:
Cytochrome b gi|62161334| *Pseudocarcinus gigas* 1e-148 **(32)**
Cytochrome oxidase subunit 1 gi|63003725| *Marsupenaeus japonicus* 1e-160 **(74)**
Cytochrome oxidase subunit 2 gi|7542363| *Pagurus longicarpus* 5e-94 **(38)**
Whole Crab:
Anti-lipopolysaccharide factor gi|113657| *Tachypleus tridentatus* 4e-17 **(73)**
Slow tropomyosin isoform gi|2660866| *Homarus americanus* 1e-124 **(27)**
Putative antimicrobial peptide gi|17223036| *Litopenaeus vannamei* 2e-12 **(27).**
Hepatopancreas:
Trypsin gi|785035| *Litopenaeus vannamei* 1e-132 **(703)**
Chitinase gi|55239200| *Anopheles gambiae* 3e-05 **(63)**
Collagenolytic serine protease gi|18266071| *Paralithodes camtschaticus* 1e-127 **(72)**
Zinc proteinase Mpc1 gi|19774211| *Paralithodes camtschaticus* 1e-111 **(42).**
Gills:
Arthrodial cuticle protein AMP8.1 gi|54042606 *Callinectes sapidus* 2e-17 **(23)**
60S ribosomal protein L40 gi|56417572| *Aedes albopictus* 6e-64 **(21)**

ATP lipid-binding protein like protein gi|18700491| *Marsupenaeus japonicus* 2e-30 **(19)**
Alpha actin gi|15148888| *Homarus americanus* 8e-41 **(19)**
Metallothionein-1 gi|102749| *Homarus americanus* (6e-21) **(16)**
Translationally controlled tumor protein gi|56199605| *Fenneropenaeus merguiensis* (7e-67) **(14)**
Ubiquitin/ribosomal protein S30e fusion protein gi|69608642| *Hister* 4e-23 **(12).**
Claw Muscle:
Myosin 1 light chain gi|48141152| *Apis mellifera* 1e-49 **(105)**
Putative muscle actin gi|53830696| *Oncometopia nigricans* (0.0) **(50)**
Alpha actin gi|15148888| *Homarus americanus* 8e-41 **(23)**
Troponin I fast skeletal muscle gi|102756| *Astacus astacus* 4e-34, **(20)**
Slow tropomyosin isoform gi|2660866| *Homarus americanus* 1e-124 **(19)**
Sarcoplasmic calcium-binding protein I gi|134309| *Pontastacus leptodactylus* 8e-92 **(17)**
LIM protein gi|50982101| *Apriona germari* 4e-21 **(15)**
Slow muscle myosin S1 heavy chain gi|37925239| *Homarus americanus* 1e-133 **(14).**

1. From strong matches (Table 2.3)

2. Organization: **name of gene** genbank accession # *species* e-value **(# clones)**

(www.ncbi.nlm.nih.gov/BLAST/)

Table 2.5. RNA samples from *Petrolisthes cinctipes* used in phase II library construction

Tissues	Treatment (sampling points)
Heart Gill Crab "remains" (after heart, gill, and hepatopancreas removed).	Heat 30°C, 4h (2h, 4h, and 6h 15°C recovery) Cold 2°C, 4h (2h, 4h, and 6h 15°C recovery) H_2O_2, 0.5 mM (18h) $CdCl_2$, 50 μM (24h) Selenate, 50 μM (24h) Selenite, 50 μM (24h) Hypersalinity, 54□ (18h) Hyposalinity, 13‰ (18h) Desiccation (24h) Hypoxia, 2h (20 min normoxia recovery) Starvation, 15d (2h postprandial) Insecticide, 1spray Pyrethrin/200ml, 5min (4h recovery)
Larvae, Freshly Molted Whole Crabs	Acclimated for 1-7 days in San Francisco Bay water (salinity 25-32‰)
Whole Crabs	Acclimated for 1 month to 8°, 15°, 18°, & 25°C
Heart, Hepatopancreas	Field acclimatized, north-south, winter-summer
Heart, Gill, Claw	Acclimated for 1 month to 7°, 19°C.
Heart, Nerve, Gill, Claw	Acclimated to 1 month in a thermally fluctuating condition (8:18°C, 12h:12h)

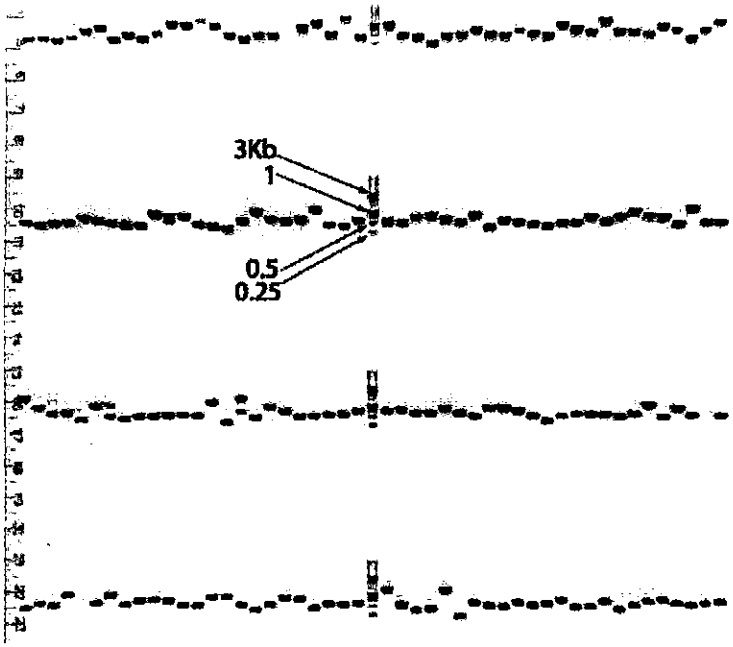


Figure 2.1. One representative gel showing ethidium bromide stained PCR products separated by 1% agarose gel electrophoresis. On this gel there are 192 PCR products, representing half of one of the heart plates. Each row on the gel contains a single lane of 1Kb ladder (sizes indicated in the second row on the gel). This is one of 72 such gels that were run to characterize the library.

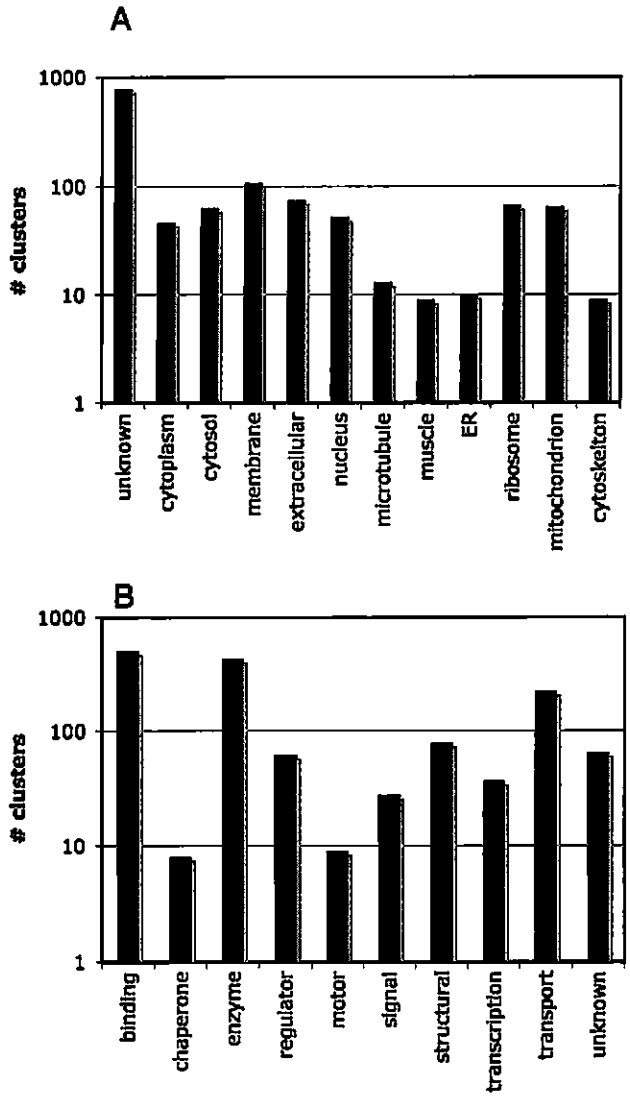


Figure 2.2. Categorization of gene ontology (GO) terms for cellular compartment (A) and molecular function (B) based on 1289 strong matches of clusters (Table 2.3). For each, "unknown" category includes clusters that had GO matches that stated "unknown" as well as clusters for which there was no GO term stated for compartment or function.

CHAPTER 3. THE KINETICS OF THE HEAT STRESS RESPONSE IN THE PORCELAIN CRAB, *PETROLISTHES CINCTIPES*

Abstract

The response of *Petrolisthes cinctipes*, an intertidal porcelain crab, to heat stress was characterized at the gene expression level. Statistically significant gene expression changes in heat stress versus control groups following 0.5h, 1h, 2h, 4h, 6h, 12h, 18h, 24h, and 30h following 4h @ 30°C (heat stress group) were monitored during recovery at 11°C. Broad scale differential expression was assessed using microarrays printed from a 13,824 cDNA library. Generally, upregulation (120 transcripts) occurred earlier and involved higher magnitudes of differential expression compared to downregulation (67 transcripts). Diverse biological processes were responsive to heat stress including protein folding/chaperoning, protein degradation, and gluconeogenesis which increased in expression and detoxification, oxygen transport, oxidative phosphorylation, and lipid metabolism which generally decreased in expression. Most transcripts were significantly expressed 18h into recovery although expression initiated as early as 1h and as late as 30h.

Introduction

Temperature is a critical abiotic factor affecting organisms on ecological, organismal, through to the cellular and molecular levels (Hochachka and Somero, 2002). For many organisms, environmental temperature does not remain constant throughout life but may fluctuate yearly, seasonally, or daily, thus raising the question of how it is that

organisms contend with long term or acute temperature change (Williams and Somero, 1996; Sagarin et al., 1999; Hochachka and Somero, 2002).

Research investigating the mechanisms behind response to acute heat stress began with the observation that heat shock induced characteristic puffs in salivary gland chromosomes in *Drosophila.* (Ritossa, 1962). It was then demonstrated that these chromosomal puffs were correlated to RNA synthesis and the production of heat shock proteins (Tissieres et al., 1974). It is now well known that heat stress causes a response termed the "heat shock response" in virtually all organisms and involves preferential synthesis of a conserved group of heat shock proteins (Lindquist, 1986).

The heat shock proteins can be grouped by size (e.g., Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small heat shock protein families) and function as molecular chaperones, assisting in proper protein folding and preventing the formation of protein aggregates within a cell (Parsell and Lindquist, 1993). In general, heat shock proteins act on nascent or newly synthesized proteins by binding to exposed hydrophobic regions, and may function in an ATP-dependent (e.g., Hsp60, Hsp70) or ATP-independent (small heat shock proteins) manner (Fink, 1999).

Studies focused on elucidating the regulatory network of heat shock proteins have made headway, revealing that induction of Hsps involve the binding of heat shock factor (HSF) to cis-regulatory heat shock element (HSE) regions (Morimoto, 1998; Morimoto and Santoro, 1998). Additionally, autoreguation may occur at least in the case of Hsp70, where interaction with HSF can repress its own transcription (Shi et al., 1998).

Research has shown that the response to heat stress is not straightforward. The heat shock response may vary in timing of initiation/termination or in intensity, may rely

preferentially on different Hsps in different organisms, and may involve the induction of many genes aside from those encoding Hsps (Parsell et al., 1993; Hofmann and Somero, 1996; Tomanek and Somero, 1999; Tomanek and Somero, 2000; Causton et al., 2001; Sorensen et al., 2005). In *Drosophila*, heat shock protein synthesis occurs during heat exposure whereas in the mussel *Mytilus trossulus*, it is only after the stress is removed that heat shock proteins are synthesized (DiDomenico et al., 1982; Hofmann and Somero, 1996). *Drosophila* thermotolerance is largely attributed to Hsp70 whereas Hsp104 maintains a dominant influence for thermotolerance in yeast (Parsell et al., 1993). It is likely that a broad spectrum of proteins are involved in response to heat as indicated by faint SDS-PAGE bands shown in addition to those from Hsps in heat stressed mussels, marine snails, and yeast (Lindquist, 1981; Hofmann and Somero, 1996; Tomanek and Somero, 2000). Furthermore, recent work on *Drosophila* and yeast has revealed that the binding targets of HSF are not limited to those for heat shock proteins but include targets for approximately 3% of genomic loci (Hahn et al., 2004; Birch-Machin et al., 2005).

With genomics tools becoming increasingly available, there has been interest in characterizing the heat shock response by monitoring gene expression on a broad scale using microarray technology (Gasch et al., 2000; Causton et al., 2001; Birch-Machin et al., 2005; Sorensen et al., 2005). Although the level of fold change in gene and protein expression is not necessarily correlated, and some proteins (e.g., elongation factors) regulated at the translational level show increased protein levels without increased gene expression, increased gene expression generally does correlate with increased protein expression (Suzuki et al., 2006). Furthermore, research on *Drosophila*, demonstrated a

tight link between genomic and metabolomic profiles following heat stress (Malmendal et al., 2006).

Gene expression profiling for *Drosophila* indicated differential expression of 1222 genes during 64 hours following recovery from heat stress (Sorensen et al., 2005). Most genes could be placed into early-up (e.g., genes for chaperoning, glutathione transferase activity, gluconeogenesis, protein kinase cascades), early-down (e.g., peptidases, hydrolases, genes for catabolism and glucose transport), and late-upregulated groups (e.g., peptidases, genes for catabolism and transport activity) (Sorensen et al., 2005). In yeast, during heat stress, 854 genes were differentially expressed (Causton et al., 2001). Induced genes included those functioning in molecular chaperoning, protein degradation, defense against oxidizing agents, metal transport, and aerobic energy generation, while repressed genes included those for translation and protein synthesis (Causton et al., 2001).

To date, studies focused on broad changes involved in the heat shock response have been limited to work on prokaryotes or model organisms such as *Drosophila* and yeast (Causton et al., 2001; Gao et al., 2004; Sorensen et al., 2005). What kinds of changes might be occurring in other organisms following heat stress?

Organisms inhabiting the marine intertidal habitat environment are exposed to a wide range of physical challenges, especially with regard to temperature (Hofmann et al., 2002; Somero, 2002; Stillman, 2002). Specifically, intertidal organisms may be frequently challenged by increased body temperatures during low tide emersion (Hofmann and Somero, 1996; Tomanek and Somero, 1999). For example, it was determined that on nearly half of the 26 days monitored in March-April, midday low tide

body temperature (≥27°C) of the intertidal marine snail, *Tegula funebralis*, was high enough to induce the onset of Hsp70 and Hsp90 synthesis (Tomanek and Somero, 1999). Interestingly, heat stress similar to that from a midday low tide (2.5h @ 30°C) in *T. funebralis* induced heightened Hsp70, Hsp38, Hsp90, and Hsp77 expression that declined to normal levels after 6h, 30min., 6h, and 14h, indicating that *T. funebralis* may be able to complete most of the stress response during immersion between two low tides (Tomanek and Somero, 2000). In addition to upregulated Hsp levels that assist in coping with reversible protein denaturation, mechanisms handling irreversible denaturation via protein degradation are also present following heat stress in the intertidal zone (Hofmann and Somero, 1996). In the intertidal mussel *M. trossulus*, a 2-fold increase in the level of ubiquitin conjugates was observed within 30 minutes of recovery from low tide emersion (Hofmann and Somero, 1996). Studies on the heat shock response in intertidal species have demonstrated the involvement of Hsps (e.g., Hsp70, Hsp90) and protein degradation, as described above, but what other mechanisms might be involved?

The intertidal porcelain crab, *Petrolisthes cinctipes*, has provided an effective study system for answering questions in the thermal physiology field (Stillman, 2002; Stillman, 2003). *Petrolisthes* is a speciose genus of more than 100 species inhabiting the North Temperate, Northern Gulf of California, Tropical, and South Temperate regions of the Eastern Pacific coast and occurring within varied vertical zones at each latitude (Haig, 1960; Stillman, 2002).

P. cinctipes resides under rocks in the mid-upper intertidal zone and experiences large fluctuations in temperature with the tidal cycle; temperatures in summer and winter can range from ~10°C-12°C during immersion to as far as 31°C and -1.27°C (J.

Stillman, Pers. Comm.) during low tide emersion respectively (Stillman and Somero, 1996; Stillman, 2004). How does *P. cinctipes* respond to acute temperature increases similar to that experienced during low tide emersion in the summer? Specifically, what genes might be upregulated/downregulated in response to thermal stress and how do these changes vary over time?

Here we explored at the transcriptome level, using cDNA microarray technology, the response to thermal stress over time in *P. cinctipes*. Gene expression levels of crabs ramped from 11°C to 30°C over a 4h period were monitored during recovery for up to 30h and compared to controls. Heat shock protein genes as well as genes for protein degradation were expected to be induced, and because a considerable energy demand may be exacted by Hsp synthesis/function and protein degradation (as suggested by Hofmann and Somero, 1996), genes for ATP generation were also expected to elevate in expression. Since studies on yeast and *Drosophila* indicate transcriptome changes on the order of a hundred to a thousand genes are involved in the heat stress response, it was anticipated that at least a few hundred genes would be up- or downregulated in response to heat, some exhibiting expression changes early and others later into recovery.

Materials and Methods

Animals

Porcelain crabs, *P. cinctipes*, were collected at 8am on a slightly foggy day in June 2005 during low tide (T_{water}=11.4°C, T_{air}=12.5°C) in Cape Arago, Oregon (43°18'N, 124°24'W), and brought back to the Oregon Institute of Marine Biology (OIMB, transport time ~30 minutes). Crabs were placed in temperature controlled coolers at 11°C (control group) or were thermally ramped from 11°C to 30°C (heat stress group)

over a four hour period, a stress similar to what *P. cinctipes* would experience during a midday summer low tide. Following this, crabs were placed in a common recovery aquarium at 11°C and sampled at 9 timepoints including 0.5, 1, 2, 4, 6, 12, 18, 24, 30 hours into recovery. During each recovery timepoint sampling, hepatopancreas tissue from n=5 crabs from each group was dissected out, placed into RNA*later* RNA stabilization reagent (Qiagen #76106), and stored for <1 month at -20°C.

RNA extraction, cDNA labeling

Hepatopancreas samples were thawed and RNA from each sample was extracted according to Qiagen's RNeasy Mini kit protocol (RNeasy Mini Handbook, 6/2001). The homogenization step for RNA extraction was conducted by adding a 3mm diameter stainless steel ball (McMaster-Carr, Cat#1598K23) to the sample in Qiagen buffer RLT for 4 minutes at 20/s. Samples of RNA were spectrophotometrically quantified (NanoDrop, ND-1000). A_{260}/A_{280} ratios were generally 2.00 for RNA samples, and RNA yields were from 14-207 μg.

Microarray printing

Microarrays generated from 13,824 clones of a *P. cinctipes* cDNA library (see previous chapter) were printed in a 24x24 spot grid pattern on lysine-coated glass slides at the University of California San Francisco's Genomics and Proteomics Core Facility (http://derisilab.ucsf.edu/core/). Preparation of poly-lysine coated slides and post-processing of arrays were performed using protocols outlined in http://schoolniklab.stanford.edu/protocols/protocols.html with slide centrifugation conducted at 500xg for 2 minutes.

Microarray hybridization

mRNA from samples were reverse transcribed into amino-allyl cDNA (5 µg total RNA used per sample hybridized) using Powerscript reverse transcriptase (Clontech Cat#639501, 1ul for 20ulrxn, incubated at 42°C for 2hrs.) and a dNTP mix of 10mM dATP, 10mM dCTP, 10mM dGTP, 4mM dTTP, 6mM 5-(3-aminoallyl)-dUTP (Ambion Cat#8439). Cy3 or Cy5 fluorescent dyes (Amersham Biosciences#PA23001,PA25001, resuspended dye in 100ul DMSO, used 5ul dye/rxn) were used for labeling. Samples were hybridized two at a time to each microarray slide for 12 h at 65°C in a dye swap fashion. Each individual was hybridized twice (once with each dye) and hybridizations always had one sample from control and one from the heat stress group. N=5 individuals were used for all timepoints except for cases of limited RNA as in 1h and 6h timepoints where n=4 and at 4h where n=3. Washing of hybridized arrays was accomplished following protocols from http://schoolniklab.stanford.edu/protocols/protocols.html.

Hybridized slides were scanned at 10µm resolution (Axon Instruments, GenePix Personal 4100A) using the automatic pmt adjustment function. Fluorescence data from each spot was extracted using Axon GenePix Pro v.6.0.

Statistical analysis

Median fluorescence intensity data was imported into MAANOVA software (http://www.jax.org/staff/churchill/labsite/software/Rmaanova/) in the 'R' statistical environment (http://www.r-project.org/). Data was $\log_2$ transformed in order to adjust for the high variance associated with higher fluorescence intensities. A lowess normalization was conducted on $\log_2$ transformed data using the joint lowess or "rlowess" function which adjusts for bias in dyes with respect to intensity and spatial levels. Log_2 transformed, lowess normalized data was analyzed for statistically

significant gene expression differences between control and treatment groups according to the Fs statistic that uses overall gene expression variation to calculate a gene-specific variance (Wu et al., 2002; Cui, 2004). To keep the number of false positives to alpha=0.05, a false discovery rate adjustment was used (Reiner et al., 2003). This was done for each timepoint. Genes that were statistically significant in expression between heat stress and control groups in at least one timepoint were tallied and grouped according to fold expression (up- vs. downregulation) by ranking M values (M = $\log_2$(treatment expression/control)). M values were calculated "$\log_2$ (heat) – $\log_2$ (control)" in excel from $\log_2$ (heat) and $\log_2$ (control) expression values obtained from R/MAANOVA output. Genes were grouped by function (BLAST matches were from the non-redundant database). Treeview software version 1.60 (http://rana.lbl.gov/EisenSoftware.htm) was used to generate heat maps.

Results and Discussion

Statistically significant changes in gene expression between heat stressed *P. cinctipes* compared to controls were monitored during recovery. Overall, 187 out of 13,824 cDNA clones significantly changed in expression during recovery, involving the up- and downregulation of 120 and 67 transcripts respectively (Figures 3.1, 3.2). The number of transcripts differentially expressed contrasts with a recent study on *Drosophila* in which genome-wide expression profiling following heat stress uncovered expression changes in 1222 out of 13,000 genes (Sorensen et al., 2005). This difference may be due to the fact that the *P. cinctipes* cDNA library currently represents only 6700 unique cDNAs, likely just a third to a half of the full transcriptome. There may be many more heat responsive genes whose transcripts are not included in the current *P. cinctipes*

library. We are presently planning to expand the library dramatically to 100,000 clones so that a majority of the *P. cinctipes* transcriptome may be captured.

Generally, upregulation of genes occurred earlier and involved higher magnitudes of differential expression compared to downregulated genes. Fold expression of significant genes ranged from 4-fold repression (hemocyanin at 24h) to 25-fold induction (hsp70 at 1h). Elevated levels of fold expression in upregulated compared to downregulated genes have also been observed in *Drosophila* in which 3-fold (lysosomal activity protein gene) repression to 91-fold (hsp23) induction occurred after heat stress (Sorensen et al., 2005). Delayed repression relative to induction is a phenomenon unique to this study and has not been observed in gene profiling work on *Drosophila*, yeast, and human cell lines following heat stress (Gasch et al., 2000; Causton et al., 2001; Murray et al., 2004; Sorensen et al., 2005). Perhaps in the porcelain crab 1) immediate repression of protein activity required following heat stress is primarily accomplished at the translational or post-translational level, and/or 2) repression of genes exists as a downstream effect of some of the products of the early recovery upregulated genes.

No differential expression between heat stress and control groups was found at 0.5h, 4h, and 6h recovery timepoints whereas at the 1h, 2h, 12h, 18h, 24h, and 30h timepoints, 56, 62, 79, 97, 52, and 37 clones were significant. In *M. trossulus*, heat shock protein synthesis following metabolic repression during heat stress is minimal during 0.5h into recovery (Hofmann and Somero, 1996). It is possible that at the 0.5h recovery stage, *P. cinctipes*' transcriptional machinery is not at full capacity. This would only explain the lack of significant differences in expression in heat stress versus control crabs at the earliest timepoint (0.5h) and fails to account for the 4h and 6h timepoint results.

The lack of statistically significant genes at the 0.5h, 4h, and 6h timepoints could indicate one of several scenarios 1) high variation in gene expression among replicates (e.g., due to experimental error) 2) an increase/decrease in gene expression of heat responsive genes in the control group to match that in the heat stress group 3) an increase/decrease in gene expression of heat responsive genes in the heat stress group to match the that in the control group. In order to determine what occurred at the 0.5h, 4h, and 6h timepoints, fluorescence intensity data (log_2 transformed and lowess normalized intensity values) was plotted for genes showing highest fold induction (hsp21.4, hsp70) and repression (hemocyanin, ATP synthase) during recovery as well as for a gene (trypsin) not changed in any timepoint (Figure 3.3). Larger standard error was noted at 0.5h, 4h, and 6h compared to the other timepoints, especially for induced genes hsp21.4 and hsp70. In looking closely at the variation among replicates within and between heat stress and control groups, a systematic pattern was identified. For each of the 0.5h, 4h, and 6h timepoints, there were two cases in which the heat stress and the control group hybridized on the same slide represented values that appeared switched. This could have been caused by incorrect sample labeling or hybridization of samples to a microarray slide intended for a different hybridization.

Up- (Figure 3.1) and downregulated (Figure 3.2) genes in heat stress vs. control groups following heat stress were visualized using heat maps and manually categorized by biological process or function. Genes encoding proteins spanning a wide range of functions (e.g., protein folding/chaperoning, protein synthesis, detoxification, oxidative phosphorylation) were changed following heat stress and are described below.

Protein folding/chaperoning

As expected, genes for molecular chaperones and protein folding were upregulated in heat stress compared to control groups. Chaperonin, hsp70, hsp90, hsp21.4, x-box binding protein, and protein disulfide isomerase (PDI) transcripts were induced as early as 1h and remained upregulated as late as 30h into recovery. Maximum levels of hsp70 (25-fold) and hsp90 (5-fold) differential expression were observed 1h into recovery while small heat shock protein transcript hsp21.4 attained a maximum 21-fold induction at 30h. Chaperonin, x-box binding protein, and PDI transcripts were induced less than 2-fold. Differences in fold changes and varying temporal dynamics of gene expression for different molecular chaperone genes were expected. At the protein level, for instance, varied expression patterns of molecular chaperones have been shown in heat stressed *T. funebralis* in which Hsp70, Hsp90, and Hsp 77 were induced ~4-5 fold and remained induced as late as 14h into recovery while Hsp38 showed less than 2-fold induction and declined to normal levels after 30 minutes (Tomanek and Somero, 2000). Furthermore, differences in molecular chaperone fold changes at the gene expression level have been observed in *Drosophila* in which Hsp22, Hsp23, and Hsp68 were induced following heat stress by 32-54 fold while Hsp83 was induced less than 2-fold (Sorensen et al., 2005).

Protein synthesis

Overall protein synthesis during heat stress is known to be repressed in model organisms like *D. melanogaster* and less studied organisms like the mussel *M. trossulus* for which clear changes occur in heat stressed versus control protein profiles (DiDomenico et al., 1982; Parsell and Lindquist, 1993; Hofmann and Somero, 1996). In yeast, translation machinery is repressed at the genomic level during heat stress as well as

other forms of stress (e.g. oxidative, high/low pH, osmotic) (Causton et al., 2001). Measurements of overall protein synthesis during recovery from heat stress has been reported in *Drosophila* in which protein synthesis slowly (within 8h) attains but does not exceed pre-stress levels (Bell et al., 1988). In *P. cinctipes*, genes for protein synthesis, specifically for ribosomal protein, were briefly upregulated (up to 3-fold) and induced early (generally 1h-2h) during recovery from heat stress. It is uncertain why ribosomal protein was upregulated, but it may indicate that in *P. cinctipes* 1) overall protein synthesis increases following removal of heat stress or 2) ribosomal proteins may be more heat sensitive and prone to heat denaturation, necessitating replacement during recovery from heat stress.

Protein degradation

Increased protein degradation is known to occur as a response to irreversible protein damage following heat stress (Parsell and Lindquist, 1993). In *P. cinctipes*, genes for ubiquitin, a protein used as a tag for protein degradation, and masquerade, a serine-like protease, were induced following heat stress as early as 1h and exhibited upregulation as late as 30h. Genome-wide expression studies on yeast and human cell lines following heat stress also show increases in transcripts for protein degradation especially for ubiquitin-proteasome degradation (Causton et al., 2001; Murray et al., 2004). Thus, the upregulation of protein degradation genes in *P. cinctipes* is in accordance with the established idea that demands for protein degradation increase in response to heat stress. Furthermore, it may be said that *P. cinctipes* incurs irreversible protein damage following temperatures similar to midday low tide period in its natural habitat.

Detoxification

Heat stress is thought to induce oxidative stress originating from the electron transport chain (Davidson and Schiestl, 2001). In yeast, heat stress resulted in an upregulation of genes for detoxification of reactive oxygen species (Causton et al., 2001). Genes for antioxidant activity, including glutathione-S-transferase which was induced up to 4-fold, were also upregulated following heat stress in *Drosophila* (Sorensen et al., 2005). In *P. cinctipes*, however, selenoprotein, glutathione-S-transferase, and metallothionein, genes for detoxification and antioxidant activity (Chen et al., 2006; Hawse et al., 2006), were repressed (1h-24h into recovery) and, in the case of metallothionein, were later (30h) induced over 2-fold. As described below, genes for energy generation, including genes in the electron transport chain, were downregulated following heat stress. It is possible that oxidative stress due to heat stress was delayed via repression of genes from the transport chain.

Protein transport

Protein transport has been shown to be upregulated according to yeast gene expression profiles following heat stress (Causton et al., 2001). In *P. cinctipes*, a gene for protein transport, similar to sec61, was induced late (30h) into recovery. Sec61 is a channel required for the retrograde transport (ER to cytosol) of misfolded secretory/transmembrane proteins destined for degradation (Romisch, 1999). It makes sense that sec61 would be induced because during heat stress misfolded proteins accumulate and must be degraded (Morimoto and Santoro, 1998). Perhaps the delayed induction of the gene for protein transport is indicative of the time required to distinguish between irreversibly damaged proteins in the ER.

Gluconeogenesis

Genes for gluconeogenesis, including phosphoenolpyruvate carboxykinase (PEPCK), were upregulated almost 2-fold within 4h following heat stress in *Drosophila* (Sorensen et al., 2005). In *P. cinctipes*, a similar patter was observed in which the PEPCK gene was induced nearly 2-fold as early as 1h into recovery. It is not known why gluconeogenesis is upregulated following heat stress. Perhaps gluconeogenesis occurs in some tissues (i.e., hepatopancreas of crab) to supply energy in the form of glucose to more vital tissues (i.e., nerve, heart tissue) following increased energy demands due to heat stress.

Cytoskeleton

Actin was induced 2-fold at 2h, 12h, and 30h into recovery. Actin upregulation was unexpected since actin is thought to be relatively stable in expression and is sometimes used for normalization Northern and Western blots (Hauser et al., 1998; Ying et al., 2005). The reason for increased actin levels remain unclear, but may be an indication of tissue-specific cytoskeletal organization. In the goby *Gillichthys mirabilis*, heat stress induced genes cytoskeletal structural genes actin, tubulin, myosin, and keratin in gill tissue but not in muscle tissue (B. Buckley, Pers. Comm.).

Oxygen transport, Phosphagen recycling, Oxidative phosphorylation

Since increased protein degradation and heat shock protein synthesis/function following heat stress exacts large demands for ATP, genes for oxygen transport and energy generation (e.g., phosphagen recycling, oxidative phosphorylation) were expected to increase (Parsell and Lindquist, 1993; Hofmann and Somero, 1996). Instead, genes for oxygen transport and energy generation were downregulated in *P. cinctipes* following

heat stress. Specifically, hemocyanin (oxygen transport), arginine kinase (phosphagen recycling), and oxidative phosphorylation genes cytochrome c oxidase, NADH dehydrogenase, and ATP synthase, were downregulated (4-fold maximum repression) as early as 2h and as late as 30h into recovery. It is unclear why mechanisms for oxygen transport and energy generation were repressed. The observed repression could account for the delayed need for antioxidant activity (see detoxification section above), via reduction in electron transport chain activity as a means to reduce heat stress induced oxidative stress. Perhaps the reduction in energy production, particularly through reduced oxidative phosphorylation, serves as a previously undescribed mechanism for contending with consequences (e.g., oxidative stress) of heat stress. In any case, maintenance of adequate energy in the face of reduced ATP output and increased ATP demand for protein degradation and heat shock protein synthesis/function could require a compensatory decrease in normal cellular activity for certain tissues (e.g., digestive function for hepatopancreas).

Lipid metabolism

The capacity to respond to acute heat stress has been linked to lipid metabolism, particularly in yeast for which translation of heat shock proteins requires the ability to synthesize sphingoliplids (Meier et al., 2006). Thus, it was expected that genes of lipid metabolism would change following heat stress. Acyl-CoA binding protein, which functions in lipid biosynthesis and breakdown, was downregulated slightly (less than 2-fold) 24h into recovery from heat stress. In the *G. mirabilis*, several lipid metabolism genes were induced while others including fatty acid synthase and ceramide kinase were repressed in gill and muscle tissue respectively following heat stress (B. Buckley, Pers.

Comm.). A comprehensive explanation for the direction of fold-change and the specific genes involved in response to acute heat stress remain to be described.

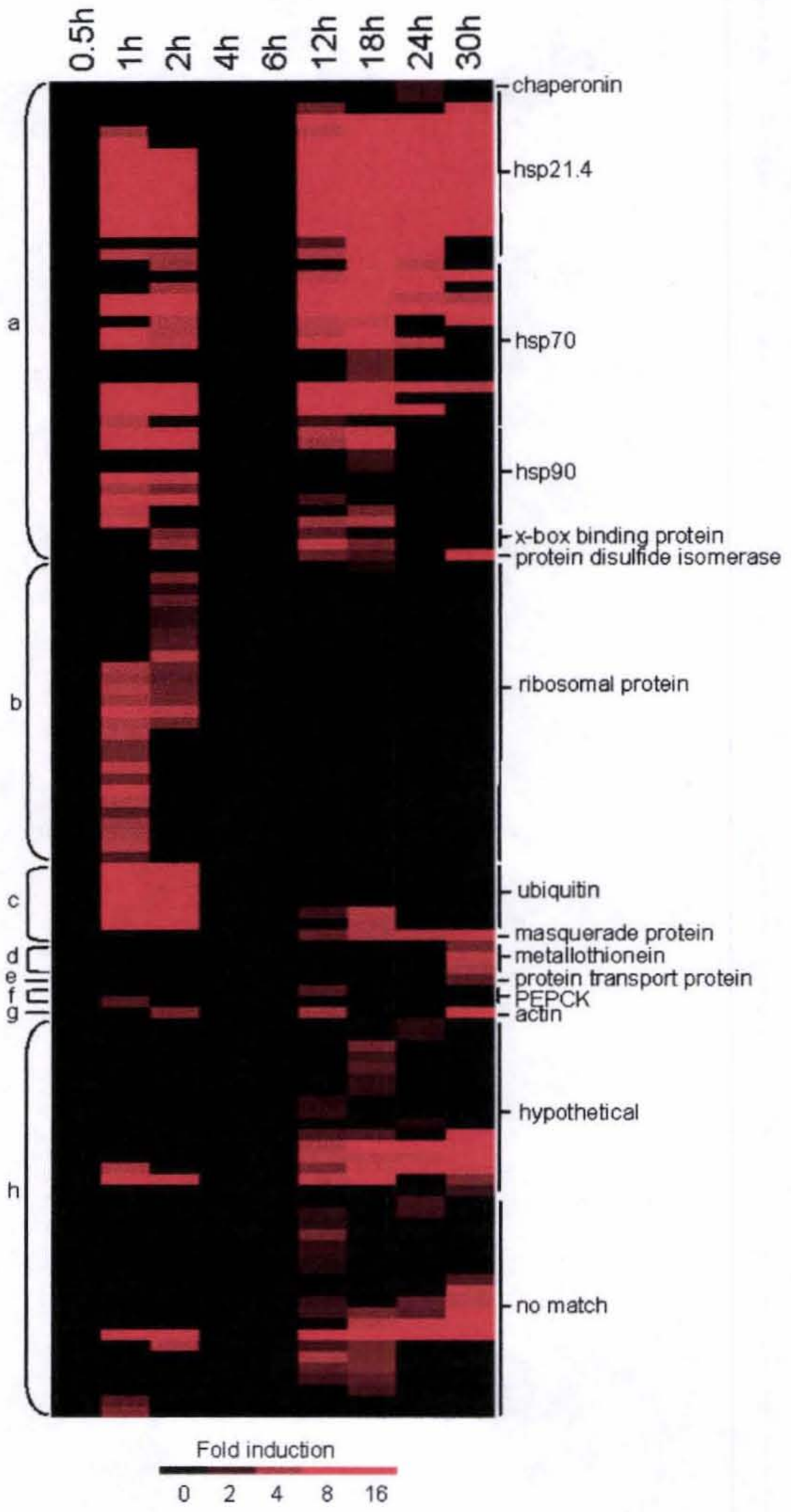
Unknown proteins

A large number of transcripts encoding unknown proteins were upregulated (20 of 36 possessed empty matches in the non redundant database) and downregulated (5 of 11 were not found in the non redundant database) in heat stress versus control groups during recovery. Spot checking unknown proteins by searching in other NCBI databases (refseq database), updating BLAST searches in the non redundant database, or searching using tblastx (translated query vs. translated database) instead of blastx (translated query vs. protein database), did not result in improved identification of unknown transcripts. The expression patterns of up- and downregulated unknown proteins were generally similar to identified up- and downregulated proteins. For instance, as observed in identified genes, unknown genes demonstrated earlier onset and higher magnitude changes in induction (ranging from 1h-30h; 5-fold maximum induction) compared to repression (ranging from 12h-24h, 3-fold maximum repression). Differential expression of genes with unknown function were expected. In yeast, essentially half of the 854 genes altered in expression following heat did not have known functions. The fact that 44 of the 187 heat-responsive transcripts in *P. cinctipes* were of unknown function demonstrates that for non-model and model organisms alike, the room for understanding how organisms respond to heat is expansive.

In summary, microarray profiling following heat stress in *P. cinctipes* confirmed expectations of increased expression for protein folding/chaperoning and protein degradation, while demonstrating unexpected increases in ribosomal expression and

decreased energy generation expression. Perhaps reduction in electron transport activity provides a mechanism for mitigating oxidative stress in *P. cinctipes*. Furthermore, an interesting overall delay in repression was observed in this study, possibly indicating that the crabs may rely heavily on translational or post-translational levels for repression. Diverse biological processes (e.g., lipid metabolism, gluconeogenesis, cytoskeletal activity) were affected by heat stress and suggest that *P. cinctipes* experiences significant genomic perturbations in nature.

While many transcripts were differentially expressed as early as 1h into recovery, most (97) transcripts were expressed at the 18h stage. The 2h timepoint was the earliest period in recovery for which genes were significantly repressed. Sampling at 2h and 18h during recovery in future experiments (e.g., of varying levels of heat stress) might be useful for capturing stress profiles of early and late recovery expression patterns. Assessing how these patterns change with different degrees of heat stress would be an informative step for investigating differences in response to acute heat stress of *P. cinctipes* from populations spanning varying latitudes. Do crabs show a gradient in their ability to respond to acute heat stress depending on latitudinal range? If, so this might offer clues into what populations of organisms could be most affected by global climate change. It is first important, however, to grasp what types of changes in response to acute heat stress occur in *P. cinctipes* populations naturally, particularly with relation to seasonal changes. Specifically, what kinds of changes in the ability to respond to acute heat stress does *P. cinctipes* implement in winter versus summer? The present kinetics study, through identifying the types and time span of gene expression changes that occur following heat stress, has set the stage for addressing these kinds of questions.


0.5h
1h
2h
4h
6h
12h
18h
24h
30h
a
b
c
d
e
f
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chaperonin
hsp21.4
hsp70
hsp90
x-box binding protein
protein disulfide isomerase
ribosomal protein
ubiquitin
masquerade protein
metallothionein
protein transport protein
PEPCK
actin
hypothetical
no match
Fold induction
0 2 4 8 16

Figure 3.1. Heat map of induced genes. Fold expression of induced genes in heat stress vs. control groups for timepoints 0.5h to 30h (columns) are shown. Each row represents a cDNA clone and is labeled by gene (right). Genes were categorized (left) by the following functions: a) protein folding/chaperoning b) protein synthesis c) protein degradation d) detoxification e) protein transport f) gluconeogenesis g) cytoskeleton h) unknown.

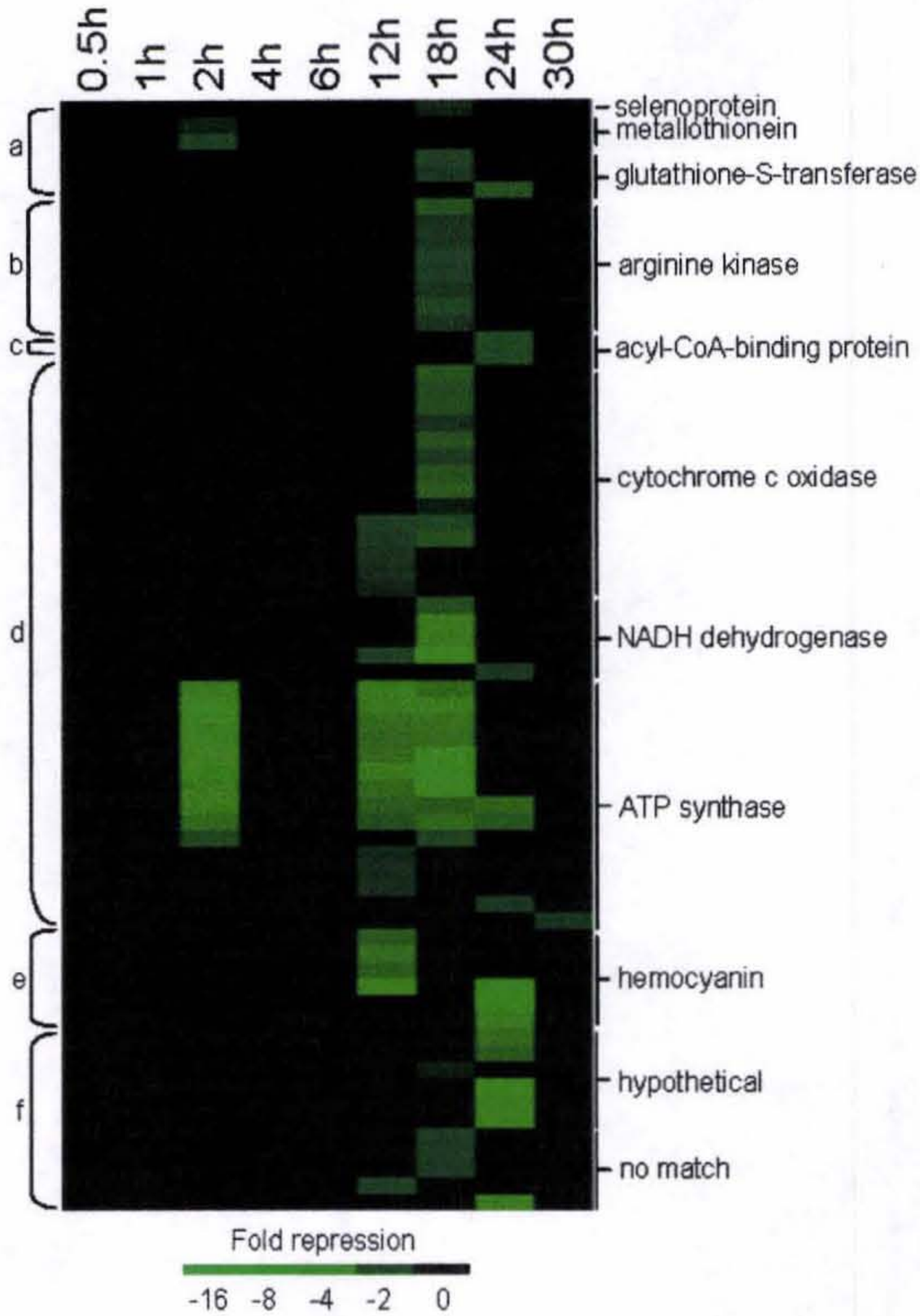
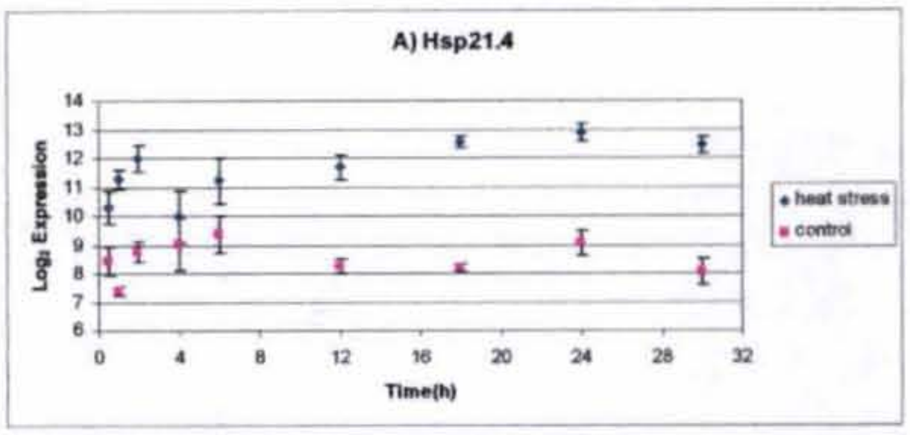
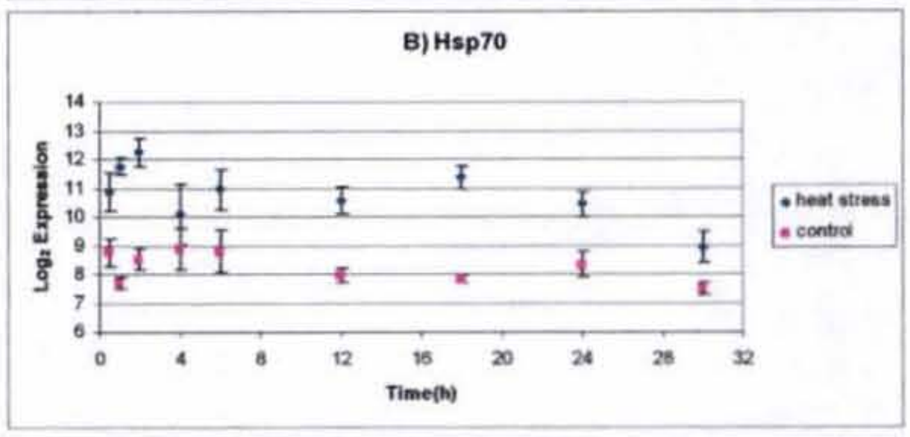
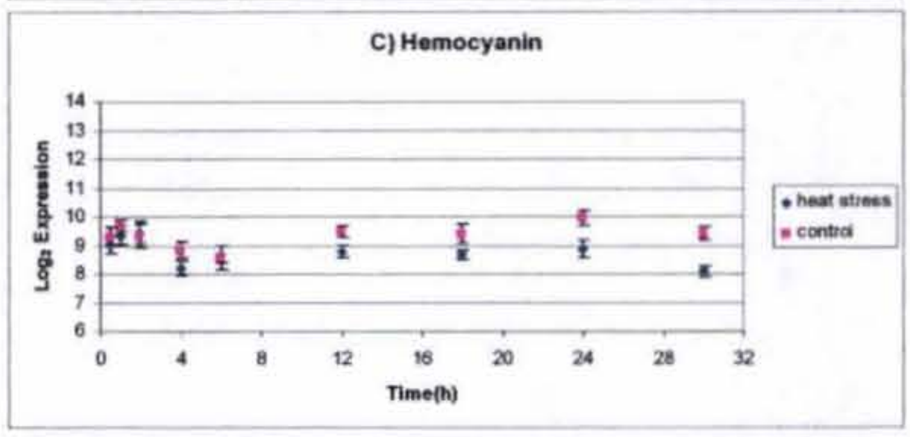
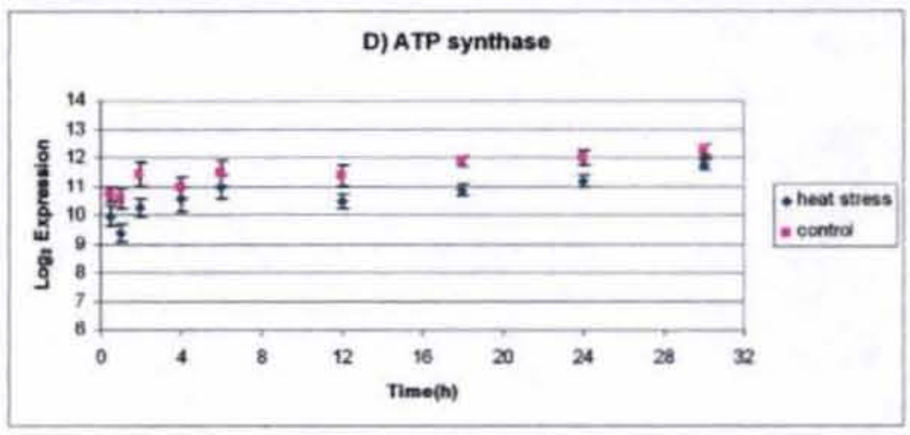


Figure 3.2. Heat map of repressed genes. Fold expression of repressed genes in heat stress vs. control groups for timepoints 0.5h to 30h (columns) are shown. Each row represents a cDNA clone and is labeled by gene (right). Genes were categorized (left) by the following functions: a) detoxification b) phosphagen recycling c) lipid metabolism d) oxidative phosphorylation e) oxygen transport f) unknown function.









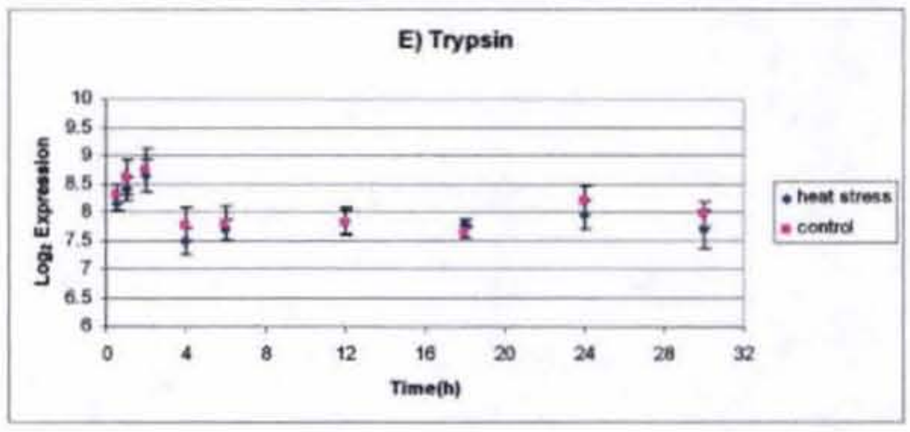


Figure 3.3. Expression levels for select genes. Log_2 transformed and loess adjusted raw intensity data for genes encoding A)Hsp21.4, B)Hsp70, C)hemocyanin, D)ATP synthase, and E)trypsin was plotted (±1S.E.) for heat stress and control groups against time in hours during recovery. Note change in Y-axis scale for trypsin.

CHAPTER 4. SUMMARY

Current global temperatures have exceeded that of natural variation and evidence for global climate change is now indisputable (Karl and Trenberth, 2003). A critical challenge for understanding organismal response to habitat temperature changes exists and is central to grasping the effects global climate change may have on life at scales ranging from the molecular and cellular level through to organismal and ecological levels. Porcelain crabs of the genus *Petrolisthes* are speciose, inhabiting wide latitudinal ranges and a diversity of thermal habitats, and have proven to function as a good study system for ecological physiology (Stillman and Somero, 1996; Stillman, 2002; Stillman, 2003). The work on the intertidal porcelain crab, *P. cinctipes*, described in this thesis, particularly the cDNA library construction and the monitoring of the kinetics of the response to heat stress, sets the stage for addressing questions relevant to thermal physiology and serves as a step toward teasing out answers relating to the effects of global climate change.

Prior to the study on the kinetics of the heat stress response, definitive knowledge of what kinds of genes changed in expression and in what time frame expression patterns occurred (i.e., what sampling timepoints might be most informative) following heat stress in *P. cinctipes* was unknown. Constructing a cDNA library was a sizable but crucial step before microarray technology could be used as a means for monitoring gene expression on a broad scale for capturing a large breadth of information on what occurs in the crabs following heat stress. Microarray analyses revealed that while many transcripts were differentially expressed as early as 1h into recovery from heat stress, most (97) transcripts were expressed at the 18h stage. Repressed genes exhibited expression changes as early

as 2h into recovery. Perhaps the 2h and 18h timepoint sampling during recovery could be adequate for capturing a descriptive stress profile in future experiments, for instance that involving different degrees of heat stress. Knowledge of stress profiles for varying levels of stress would contribute to assessing relationships between organismal differences in response to stress from populations of *P. cinctipes* at various latitudes. An investigation of heat stress responses from crabs inhabiting different latitudes could reveal clues relating to how a species' distribution range may be affected by global climate change. Before the effects of climate change on species distributions can be speculated on, it is first crucial to know what types of changes in response to acute heat stress occur naturally within a population. Does the response to acute heat stress differ in *P. cinctipes* differ seasonally, specifically in winter versus summer? The fine points, particularly what recovery timepoints could be informative and what types of gene expression changes to expect, in beginning to answer this question have been addressed in the present study.

Transcriptome profiling following heat stress similar to what *P. cinctipes* experiences in its natural habitat confirmed expectations of increased expression for protein folding/chaperoning and protein degradation and suggested that crabs in their intertidal habitat experience levels of heat stress able to irreversibly denature proteins. Decreased expression in genes for energy production and detoxification following acute heat stress was unexpected but might demonstrate that a reduction in electron transport activity provides a mechanism for mitigating heat induced oxidative stress in *P. cinctipes*. Furthermore, an interesting overall delay in gene repression was observed in this study, possibly indicating that the crabs may rely heavily on translational or post-translational

levels for immediate protein repression. Diverse biological processes (e.g., lipid metabolism, gluconeogenesis, cytoskeletal activity) were affected by heat stress and suggest that *P. cinctipes* experiences significant genomic perturbations in nature.

Many genes responsive to heat stress in *P. cinctipes* were of unknown function, indicating that understanding the full breadth of the heat shock response is far from complete. The 13,824 cDNA library constructed for *Petrolisthes cinctipes* was a valuable tool for exploring the response to heat stress, but the library represents only a third to a half of the estimated transcriptome. There are likely many genes responsive to heat stress that are not included in this library. Our laboratory will eventually use an expanded library, constructed in conjunction with the Joint Genome Institute, able to capture the majority of the *P. cinctipes* transcriptome changes.

Overall, the work conducted in the present thesis, specifically cDNA library construction and documentation of gene expression changes following heat stress, provides essential resources and information for beginning to address important questions in thermal physiology including that related to the effects of global climate change.

APPENDIX

Appendix. Tissue specific transcripts (nr database)

Gene name	gi #	Species	e value
Heart			
Carboxypeptidase, vitellogenic-like	62857515	*Xenopus tropicalis*	2e-67
Cytochrome P450 CYP4	18032259	*Cherax quadricarinatus*	8e-14
NADH dehydrogenase subunit 4	46575798	*Euphausia superba*	3e-71
Phospholipase/Carboxylesterase	50737613	*Apis mellifera*	2e-21
Zinc finger, CCHC domain	27754058	*Mus musculus*	5e-13
NADH dehydrogenase subunit 1	15150779	*Marsupenaeus japonicus*	5e-98
Collagen alpha 2(IV) chain precursor	115347	*Ascaris suum*	1e-112
Transferrin	22597202	*Mastotermes darwiniensis*	9e-46
Galactokinase	1730187	*Homo sapiens*	3e-93
Vacuolar protein sorting protein	73920458	*Danio rerio*	6e-48
Protein disulfide isomerase	12025459	*Bombyx mori*	2e-15
Eukaryotic translation initiation factor 3 subunit 2	50759828	*Gallus gallus*	1e-48
Ring finger protein 26	27661650	*Rattus norvegicus*	4e-13
Nuclear transport factor 2-like export factor 2	57530164	*Homo sapiens*	2e-25
Enolase	1311141	*Anopheles gambiae*	6e-11
Ficolin 4	14349161	*Halocynthia roretzi*	7e-25
Succinyl-CoA:3-ketoacid-coenzyme A transferase 1	2492998	*Homo sapiens*	6e-42
Outer membrane receptor proteins (Fe transport)	42628903	*Haemophilus influenzae*	7e-6
Serine kinase SRPK2-alternatively spliced form	3406051	*Homo sapiens*	1e-80
X-box binding protein	13898897	*Xenopus laevis*	2e-16
Small zinc finger-like	5107178	*Ciona intestinalis*	1e-24
Cytohesin 1-like protein	46310219	*Danio rerio*	3e-82
26S protease regulatory subunit 7	51262008	*Xenopus tropicalis*	8e-43
Ubiquitin activating enzyme	2706522	*Drosophila melanogaster*	1e-41
Transglutaminase	14579327	*Pacifastacus leniusculus*	4e-26
Kinesin light chain	1170680	*Loligo pealei*	1e-66
Elongation factor 1 delta	12328436	*Bombyx mori*	6e-42
Pontin	12004636	*Xenopus laevis*	1e-106
SH3 domain-binding glutamic acid-rich protein	66513595	*Apis mellifera*	6e-26
Sphingomyelin phosphodiesterase, acid-like 3B	50759633	*Gallus gallus*	2e-45
Glyceraldehyde-3-phosphate dehydrogenase	31338868	*Procambarus clarkii*	1e-175
Extracellular superoxide dismutase precursor	4585366	*Pacifastacus leniusculus*	7e-57

Serine (or cysteine) proteinase inhibitor	61873714	*Bos taurus*	3e-13
Iron-sulfur cluster binding protein	67932841	*Solibacter usitatus*	3e-74
Ribulose-5-phosphate-3-epimerase	37747988	*Danio rerio*	7e-25
Endothelial cell growth factor 1	19923857	*Mus musculus*	6e-45
Proteasome subunit alpha type 7	41351173	*Danio rerio*	2e-94
26S protease regulatory subunit 8	1709799	*Manduca sexta*	1e-115
ATP-dependent 26S proteasome regulatory subunit	29825445	*Apis mellifera*	2e-69
Thioredoxin-dependent peroxide reductase	2507170	*Bos taurus*	3e-76
Heat shock cognate 70	23193450	*Chironomus tentans*	3e-25
Aminotransferase	33286231	*Drosophila melanogaster*	7e-67
GTPase	23171521	*Drosophila melanogaster*	5e-11
Isocitrate dehydrogenase	48476117	*Crassostrea gigas*	2e-83
Ubiquinone biosynthesis protein COQ7-like protein	47157064	*Apis mellifera*	3e-69
26S protease regulatory subunit 4	30581054	*Drosophila melanogaster*	1e-124
UDP-glucose ceramide glucosyltransferase-like 1	50751921	*Gallus gallus*	6e-75
Cold shock domain protein A long isoform	47059495	*Mus musculus*	1e-15
Protein phosphatase 1M regulatory subunit	45382609	*Gallus gallus*	6e-06
S-adenosylmethionine synthetase	452838	*Drosophila melanogaster*	1e-128
Putative γ-aminobutyric acid receptor beta subunit	386138	*Drosophila melanogaster*	1e-114
Splicing factor, arginine/serine-rich 7	22122585	*Mus musculus*	2e-41
Glyceraldehyde-3-phosphate dehydrogenase	6016080	*Procambarus clarkii*	1e-175
Proteasome (prosome, macropain) subunit, α type, 4	28279709	*Danio rerio*	1e-101
Leukotriene A-4 hydrolase	68358508	*Danio rerio*	1e-18
NADH dehydrogenase subunit 1	15150779	*Marsupenaeus japonicus*	5e-98
Proteasome 26S subunit, non-ATPase	37681913	*Dario rerio*	1e-42
Nerve			
Similar to apoptosis-linked gene 2	48094929	*Apis mellifera*	4e-69
Putative phosphoglycerate mutase	52630953	*Toxoptera citricida*	1e-113
U3 small nucleolar ribonucleoprotein	13528759	*Homo sapiens*	7e-42
20-beta-hydroxysteroid dehydrogenase	66517758	*Apis mellifera*	4e-33
Methylmalonate semialdehyde dehydrogenase	50748470	*Gallus gallus*	2e-76
3-hydroxyacyl-CoA dehydrogenase	2078327	*Homo sapiens*	1e-101
Cathepsin L-like cysteine protease precursor	16304178	*Delia radicum*	2e-13
Translation initiation factor eIF-2B α subunit	72014480	*Strongylocentrotus purpuratus*	9e-68
Mitochondrial carrier protein	72010168	*Homo sapiens*	6e-73

Gill			
Thioredoxin reductase 1	51704106	*Xenopus laevis*	1e-64
Aldehyde dehydrogenase (mitochondrial)	55638955	*Pan troglodytes*	2e-38
Proteasome activator subunit 3 isoform 1	30410794	*Homo sapiens*	6e-69
Enoyl Coenzyme A hydratase, short chain 1	40555865	*Rattus norvegicus*	5e-67
Glyceraldehyde-3-phosphate dehydrogenase	31338868	*Procambarus clarkii*	1e-175
Chaperonin containing TCP1, subunit 5 (epsilon)	39850245	*Xenopus tropicalis*	1e-126
Defender against cell death 1 (DAD-1)	20138077	*Drosophila melanogaster*	3e-43
Glutamate dehydrogenase, short peptide	458803	*Drosophila melanogaster*	2e-58
Phosphoserine aminotransferase 1	39795813	*Danio rerio*	1e-44
26S proteasome regulatory chain 4	345717	*Drosophila melanogaster*	1e-124
NADH dehydrogenase (ubiquinone) Fe-S protein 4	4505369	*Homo sapiens*	3e-44
Acyl-CoA-binding protein homolog	1168274	*Rana ridibunda*	7e-30
ADP-sugar pyrophosphatase (Nudix motif 5)	72005173	*Strongylocentrotus purpuratus*	3e-24
Methionine-R-sulfoxide reductase (Selenoprotein R)	34922549	*Drosophila melanogaster*	8e-44
Sodium-dependent dicarboxylate transporter 2	68369674	*Dario rerio*	3e-41
Zinc finger, matrin type 5	57524615	*Dario rerio*	1e-12
putative salivary sulfotransferase	67083857	*Ixodes scapularis*	0.27
NADH-ubiquinone oxidoreductase acyl-carrier subunit	1653987	*Drosophila melanogaster*	4e-23
Glutamate-ammonia ligase	543538	*Panulirus argus*	1e-109
DNA methyltransferase 1-associated protein 1	72022174	*Strongylocentrotus purpuratus*	6e-15
X-box binding protein	13898897	*Xenopus laevis*	2e-16
Vacuolar ATP synthase subunit E, putative	15222641	*Arabidopsis thaliana*	2e-31
Transient receptor potential (channel nanchung)	31745595	*Drosophila melanogaster*	1e-10
Na+/K+-exchanging ATPase beta chain	84610	*Artemia franciscana*	7e-22
Metabotropic glutamate receptor	1834427	*Drosophila melanogaster*	3e-36
Putative epidermal growth factor receptor	13445276	*Anophales gambiae*	7e-64
Ecdysteroid receptor	13677226	*Celuca pugilator*	1e-106
Hepatopancreas			
Trypsin	*785035*	*Litopenaeus vannamei*	1e-132
Trypsin	27373057	*Aplysina fistularis*	1e-21
Collagenolytic serine protease	18266071	*Paralithodes camtschaticus*	3e-84
Duplex-specific nuclease	26892281	*Paralithodes camtschaticus*	1e-102

Transcriptional coactivator tubedown-100	22035307	*Homo sapiens*	1e-13
Carboxylesterase	66560187	*Apis mellifera*	1e-10
beta 1,4-endoglucanase	5020110	*Cherax quadricarinatus*	6e-08
Cathepsin l	1483570	*Litopenaeus vannamei*	5e-79
Dimeric dihydrodiol dehydrogenase	5766901	*Canis familiaris*	1e-57
Dipeptidyl-peptidase I precursor	50731191	*Gallus gallus*	6e-46
Carboxypeptidase B	115881	*Astacus astacus*	2e-70
Guanine nucleotide exchange factor for Rho/Rac/Cdc42-like GTPases	72005061	*Anopheles gambiae*	2e-48
Ruvbl2-prov protein	29126859	*Xenopus laevis*	1e-125
DNA topoisomerase II, α isozyme isoform 3	73966011	*Canis famliaris*	9e-80
Cystatin B	23344732	*Theromyzon tessulatum*	1e-12
Low-density lipoprotein receptor (LDL receptor)	126074	*Oryctolagus cuniculus*	2e-42
Calcitonin gene-related peptide-receptor component	55628662	*Pan troglodytes*	2e-28
Succinate dehydrogenase (ubiquinone)	67043769	*Lysiphlebus testaceipes*	1e-8
destabilase I	1255718	*Hirudo medicinalis*	1e-18
RNA-binding protein	23613209	*Plasmodium falciparum*	3e-9
Cysteine dioxygenase, type I	37748497	*Dario rerio*	6e-22
Ornithine aminotransferase	790956	*Drosophila ananassae*	2e-63
Serine proteinase inhibitor	33590491	*Procambarus clarkii*	9e-19
Claw			
70 kDa heat shock protein	55233307	*Anopheles gambia*	3e-21
Histone deacetylase 11	50754303	*Gallus gallus*	3e-26
Ficolin	47523126	*Sus scrofa*	9e-20
Ornithine decarboxylase	1200128	*Panagrellus redivivus*	3e-15
Mitochondrial short-chain enoyl-coenzyme A hydratase 1 precursor	12707570	*Homo sapiens*	1e-23
Chaperonin subunit 8 theta	44969706	*Gallus gallus*	2e-74
Eukaryotic translation elongation factor 1 beta 2	47940399	*Dario rerio*	9e-50
Cystathionine gamma-lyase	1705787	*Caenorhabditis elegans*	2e-22
Basic transcription factor 3-like 4	41152344	*Danio rerio*	1e-33
Transcription elongation factor B (SIII), polypeptide 1	63100863	*Xenopus tropicalis*	4e-27
Whole Crab			
Putative helicase	37535038	*Oryza sativa*	3e-13
Tenascin-R	1617316	*Homo sapiens*	5e-45
Site-specific recombinase	30248840	*Nitrosomonas europaea*	2e-35
Tissue factor pathway inhibitor precursor (TFPI) (Lipoprotein-	401174	*Rattus norvegicus*	7e-38

associated coagulation inhibitor) (LACI)			
Fasciclin IV precursor	160845	*Schistocerca americana*	2e-72
Prophenoloxidase activating factor	66513601	*Apis mellifera*	2e-34
U2 small nuclear ribonucleoprotein 35 kDa subunit	2833265	*Homo sapiens*	0.038
Activating signal cointegrator 1 complex subunit 1	50749326	*Gallus gallus*	2e-61
Zinc-binding dehydrogenase	56118580	*Xenopus tropicalis*	2e-28
Zinc finger CCCH-type, antiviral 1	61098418	*Gallus gallus*	1e-14
Multisubstrate deoxyribonucleoside kinase	28629060	*Anopheles gambiae*	3e-49
Guanosine monophosphate reductase 2	19527300	*Mus musculus*	5e-27
Glycine cleavage system H protein	67465060	*Mus musculus*	5e-40
Na^+/K^+ ATPase alpha subunit	23380400	*Homarus americanus*	1e-149

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