THE EFFECT OF COLD STRESS ON HEAT SHOCK PROTEINS IN NAUPLII (LARVAE) OF THE BRINE SHRIMP, *ARTEMIA FRANCISCANA*

By

Yayra Aku Gbotsyo

A Thesis Submitted to

Saint Mary's University, Halifax, Nova Scotia

in Partial Fulfillment of the Requirements for

the Bachelor of Science Degree, Biology Honours.

April 2017, Halifax, Nova Scotia

Approved: Dr. Thomas MacRae

_____

Supervisor 1

Approved: Dr. Laura K. Weir

_____

Supervisor 2

Approved: Dr. Genlou Sun

_____

Thesis Reader

Date: April 21st, 2017.

THE EFFECT OF COLD STRESS ON HEAT SHOCK PROTEINS IN NAUPLII (LARVAE) OF THE BRINE SHRIMP, *ARTEMIA FRANCISCANA*

by Yayra Aku Gbotsyo

ABSTRACT

Heat shock proteins (HSPs) are highly conserved and present in organisms under normal physiological conditions and function in protein folding, degradation and localization. HSPs are also synthesized in response to environmental stressors such as heat, anoxia, desiccation and cold. These molecular chaperones protect other proteins from irreversible denaturation during stress, thereby maintaining proteostasis and allowing cells to survive unfavorable conditions. *Artemia franciscana,* commonly known as brine shrimp, are micro-crustaceans that live in harsh aquatic environments and experience high salinity, extreme temperatures, variable water levels, and hypoxia. In anticipation of stressful conditions, *A. franciscana* alter their life cycle by releasing cysts that enter diapause. Nauplii (larvae) emerge after diapause termination or when favorable conditions return. The effect of cold shock on three HSPs, HSP90, HSP70 and HSP40 was studied in the nauplii of *A. franciscana* during 6 h of cold-shock at 1^{o}C and 6 h of recovery from cold shock at 27^{o}C. Immunoprobing of western blots containing cell-free protein extracts of stressed nauplii showed that the amounts of HSP90 and HSP40 decreased and increased, respectively, during recovery from cold shock, while the level of HSP70 was not altered in response to cold stress. The changes in HSP90 and HSP40 suggest their role in cold stress tolerance, however additional research, such as the use of RNA interference (RNAi), is required to explore how these proteins protect *A. franciscana* during cold stress. This study is important because it contributes to the understanding of stress physiology and how organisms cope with cold.

April 21st, 2017.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I thank my supervisor, Dr. Thomas MacRae, for his tremendous support and encouragement throughout my thesis project. Giving me an opportunity to work in his lab as a volunteer and an Honours student piqued my interest in molecular biology. A great amount of appreciation goes to Dr. Laura K. Weir, my second supervisor who has been a great role model to me. Her time and dedication have shaped me into becoming a better writer and presenter. Nathan Rowarth, for taking me under his wing, helping me with my experiments and teaching me all the lab techniques I needed. I would also like to thank the wonderful and amazing people in the MacRae Lab for all their help and support. A great amount of thanks goes to Dr. Anne Dalziel, and the Biology Honours Class 2017 for their valuable feedback on my thesis and for making my Honours year a remarkable one. To my best friends, Arve Cooper and Alexis Chute, thank you for believing in me and for keeping things brighter in stressful times. I cannot end without thanking the shrimpees for their awesome tips on how to cope with stress.

CHAPTER 1 - INTRODUCTION

1.1 Thermal Extremes and The Stress Response

Many organisms are faced with environmental stressors such as severe temperatures, low oxygen levels, desiccation and exposure to harmful chemicals and metals [1,2]. Responses to such stressors involve changes in behaviour, physiology and/or internal cellular processes. Temperature is one of the most important factors that influences an organism's distribution and success in its environment [3,4]. Adaptations to temperatures vary within and between vertebrates and invertebrates, as well as between ectotherms and endotherms [5,6]. Endothermic homeotherms, such as polar bears, penguins and walruses, regulate their body temperatures by using their fur, feathers, secretory glands or by changing their skin thickness [7–9]. Invertebrates, most of which are ectotherms, generally rely on physiological and biochemical changes to deal with the effects of temperature rather than behavioral adaptations [5,6]. Physiological and biochemical changes occur mainly to preserve cellular homeostasis until external conditions improve [4]. These may include altering cell membrane fluidity, maintaining ionic balance, suppressing the rate of metabolism and monitoring cell cycle progression to keep a stable internal environment and uphold the integrity of the cell [4,10].

1.2 Heat Stress and Adaptations

High temperatures can negatively impact cellular functions [4]. The cell membrane, which is responsible for the interaction between cells and their external environment,

becomes disorganized under the influence of heat. The phospholipid bilayer breaks down, disrupting transport, signalling and ion exchange across the membrane [11,12]. At high temperatures, proteins fold incorrectly and are prone to denature, aggregate and overcrowd cell space [11,12]. Enzymes commonly assume incorrect ligand-binding configurations, halting important cellular processes [13]. Protein synthesis slows down or is halted in response to heat stress [11,12]. However, the synthesis of heat shock proteins (HSPs), which aid in protein folding, increases [14]. This increase is predictable based on the role of HSPs in cellular stress response.

1.3 Cold Stress and Adaptations

Organisms are faced with stress when exposed to temperatures below their optimal growth ranges. Cold, especially during winter seasons, has severe effects on the physiology and metabolism of organisms [3]. Freezing temperatures interrupt vital processes such as heartbeat and breathing, cause cell shrinkage, alter osmolality and induce mechanical damage to tissues [15]. To survive freezing or cold, some arthropods and mammals enter a state of dormancy and metabolic suppression characterized by low energy demand [3]. This is true of insects including the fruit fly, *Drosophila melanogaster* [16] and the mosquito, *Culex pipiens* [17], crustaceans such as the brine shrimp, *Artemia* [18], and mammals such as bats and bears [19]. Many ectotherms become freeze-tolerant by producing glycerol, antifreeze proteins, and ice-nucleating agents to prevent ice formation in their body fluids [20–22].

As with heat stress, extreme cold changes internal cellular processes and organisms must be equipped with mechanisms to respond to these changes [4]. While heat stress responses have been studied extensively, cold stress responses have garnered attention only recently [12,23,24]. Biological activity slows down during cooling [25]. In the bacterium, *Escherichia coli*, a 10°C decrease in temperature slows the rate of biochemical reactions by a factor of ~ 2 [25]. Membrane fluidity also decreases at low temperatures because of a decrease in the proportion of unsaturated fatty acids and shortening of the length of fatty acid chains in membrane lipids [25]. Unique to cold stress, RNA and DNA structures become overly stabilized, which impedes transcription and ribosomal movements during translation [26]. Cold shock proteins, which have only recently been discovered, function as RNA chaperones that destabilize RNA secondary structures and translation [25,26]. HSPs, which are typically associated with heat stress, also respond to protein denaturation during cold stress [12,27].

1.4 HSPs and Their Role in Temperature Tolerance

Under normal cellular conditions, proteins often self-assemble into multimeric structures that carry out biological and biochemical activities of the cell. Thermal stress causes protein subunits to assemble improperly and causes large proteins to disassemble, making them unstable and prone to degradation [28,29].

HSPs are molecular chaperones that are constantly present in cells to correctly fold proteins involved in routine cellular processes such as translocation, cell-signalling and

metabolism [23,30,3123,32]. However, HSPs become abundant in most organisms in response to protein denaturation caused by environmental, metabolic and pathological stress [33,34]. For example, insects such as the leaf miner, *Liriomyza hudobrensis*, the onion maggot, *Delia antiqua,* and the gall fly, *Eurosta solidaginis,* increase the production of HSPs to augment survival in cold environments [35,36]. Different HSPs increase heat and cold tolerance during diapause and other life history stages in various species of *Drosophila* [37]. HSPs are either up- or down-regulated when heat shock factors (HSFs) bind to promoter regions called heat shock elements (HSEs) of HSP genes [38]. First identified in the fruit fly, *Drosophila melanogaster* [39], HSPs are classified into five major groups based on molecular mass (kDa), sequence and function. These groups are the ATP-dependent HSPs which include HSP100, HSP90, HSP70 and HSP60, and the ATP-independent HSPs which include HSP40 or DnaJ and small HSPs that have an α-crystallin domain [40].

HSP90, abundant in most eukaryotic organisms, is a large ATP-dependent HSP that assists in the folding of nascent proteins, the re-folding of non-native proteins and protein degradation [40–42]. In *Drosophila, Arabidopsis* and other organisms, HSP90 functions in cell-cycle control and signalling, protein trafficking, and protein degradation [41,43–45]. HSP90 is co-chaperoned by at least 20 other proteins that oversee the proper substrate binding and ATPase activity of HSP90 [44,45]. As well, HSP90 stabilizes and aids in the recovery of cytoskeletal elements which are thermally sensitive and important for maintaining cell structure [42].

The family of HSP70, aside from their role in protein folding and degradation, facilitate the movement of cellular proteins across the mitochondrial and endoplasmic reticulum membranes [46]. HSP70 complexes with other HSPs or works independently to stabilize newly synthesized polypeptide chains until translation terminates, thereby reducing the aggregation of non-native proteins in the cell [44,45]. As part of their chaperoning role, HSP100 and HSP70 cooperate in re-solubilizing and refolding proteins [47]. The HSP40s are J-domain proteins that function independently as chaperones and co-chaperone other ATP-dependent HSPs [48]. For example, HSP40 co-chaperones HSP70 and is essential for stabilizing the interaction between HSP70 and its substrates. Together, HSP70 and HSP40 assist in protein translation, folding, unfolding and degradation of proteins [48,49].

HSP60, also known as chaperonin, exists in two families, the GroEL group found in bacteria, mitochondria and chloroplast, and the CCT chaperonins found in the cytosol of Archaea and Eukarya [50]. HSP60s have a double ring structure and play a crucial role in translocation of proteins, as well as proper protein folding [50].

sHSPs, the monomers of which range from 14 kDa to about 40 kDa, exist as dimers or oligomers and function independently of ATP. The sHSPs, all of which contain an α-crystallin domain, bind to denaturing proteins to prevent irreversible denaturation and subsequently deliver them to ATP-requiring HSPs for refolding or degradation [48,51].

1.5 The Life History and Environmental Distribution of Artemia franciscana

Artemia franciscana, brine shrimp, are small branchiopod crustaceans native to the Great Salt Lake in Utah, San Francisco Bay in California and other locations worldwide [52,53]. *A. franciscana* is a good model species for studying environmental stress responses because they are extremophiles that survive in harsh aquatic environments of high salinity, low oxygen levels and extreme temperatures. These environmental parameters are characteristic of the Great Salt Lake [54]. The relative salt content in the Great Salt Lake is 27 parts per thousand (ppt) and temperatures fluctuate annually with average temperatures of 0.5°C in January (winter) and 25.5°C in August (summer) [55]. Female *A. franciscana* give birth to swimming nauplii (larvae) ovoviviparously if environmental conditions are favourable or, in anticipation of unfavourable conditions, release encysted gastrulae which enter diapause (Fig. 1.1) [56]. Diapausing gastrulae have very low metabolic activity and in this encysted form, embryos are protected from environmental stressors [18,56]. When favorable conditions return, diapause is terminated and nauplii emerge, moulting several times until they become adults [56,57] (Fig. 1.1).

1.6 HSPs in Artemia

A. franciscana, the species chosen for this study, is one of 6 bisexual species of *Artemia* [58]. Males are usually bluish or whitish in colour and possess relatively large anterior mating claspers whereas females are pink or reddish in color with a prominent ventral egg sac [59]. Adult *A. franciscana* are approximately 1 cm in length and are capable of sexual reproduction within three weeks following birth or hatching, with females releasing nauplii or cysts every five to six days. Nauplii, which are widely used as food in

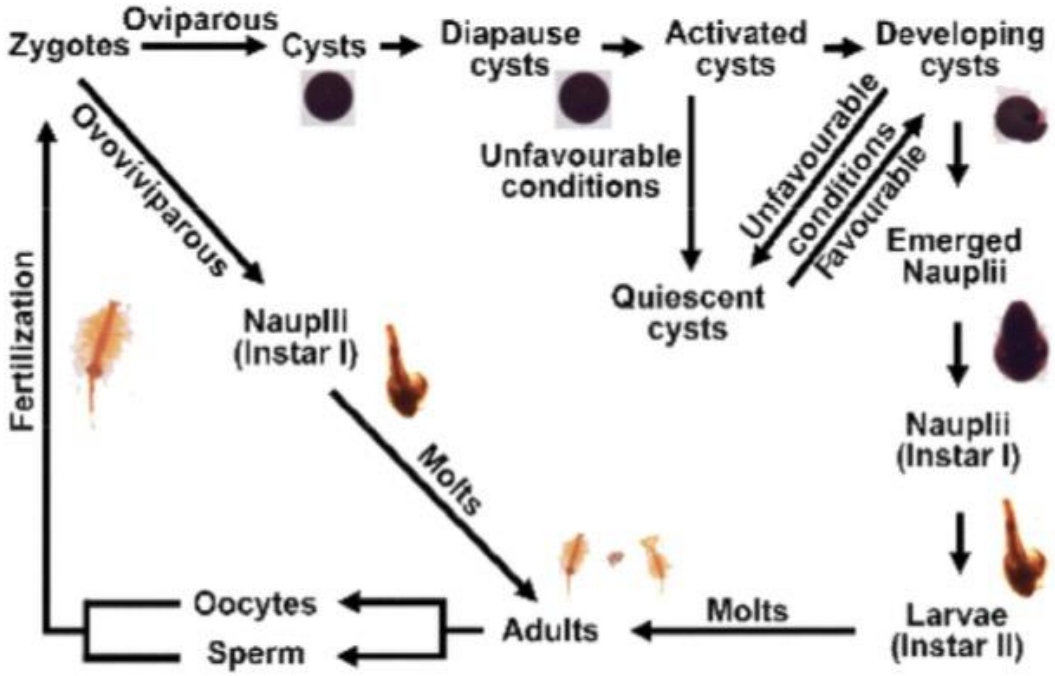

Figure 1.1. The life history of *A. franciscana*. Under favorable conditions, live nauplii emerge from the egg sac of female *A. franciscana*, undergo several moults and become adults, this known as the ovoviviparous life cycle. Alternatively, in anticipation of unfavorable conditions, oviparously-developing embryo are released from the female as cysts which enter diapause. When diapause terminates, nauplii emerge from cysts and moult to become adults. Image modified from Liang & MacRae, 1999 [56]

fish and shell-fish larviculture, are bright orange in color due to large amounts of carotenoid pigment, and are approximately 0.4 mm in length [58,59]. Because of its robust nature, the life cycle of *A. franciscana* has piqued an interest in stress tolerance at both molecular and ecological levels, with a focus on the induction of HSPs. The synthesis of HSPs in *A. franciscana* has been studied extensively in relation to stress tolerance during diapause. During diapause, metabolic suppression occurs and genes encoding sHSPs are expressed [60]. Three sHSPs, *ArHsp21, ArHsp22* and p26, together with several late embryogenesis abundant (LEA) proteins and artemin are upregulated in response to heat, cold and desiccation during diapause of *A. franciscana* [47,48,61–63]. The sHSP, p26, helps *A. franciscana* survive long periods of anoxia and facilitates development of diapause-destined embryos [61,64]. ArHsp40, which is the equivalent of HSP40 in *A. franciscana*, is synthesised in response to heat stress in cysts and in nauplii, and is present in other life stages [49]. Thus far, only one study has examined the response of two HSPs, HSP90 and HSP70 to long-term cold stress in adult *A. franciscana* [65]. In this study HSP90 was synthesized during 2-8 h of cold shock but not after 12-22 h; and HSP70 was not induced by cold shock in adults [65]. There is a rising interest in how juveniles *A. franciscana* survive during stress.

1.7 Objectives

Most research describing stress tolerance in *A. franciscana* has focussed on the role of sHSPs during diapause [47,49,63,64,66,67]. However, populations *of A. fransiscana* include nauplii (larvae) and adults. Unlike cysts, nauplii are not encased in hard shells and are

therefore prone to damage from harsh environmental conditions. Other mechanisms must exist to allow nauplii to survive in extreme conditions of heat and cold, and understanding these responses highlights the importance of this study. In addition, the roles of ATP-dependent HSPs in cold stress tolerance of *A. franciscana* have not yet been studied extensively in nauplii, even though it is assumed that HSPs are essential for cold stress tolerance. Studies on the role of HSP90 during diapause, and in response to heat stress, cold stress and desiccation are currently on-going.

The overall goal of this research is to examine the effect of cold on the synthesis and accumulation of HSPs in *A. franciscana* nauplii. The objectives are to: 1) determine whether three HSPs, HSP90, HSP70 and HSP40 are synthesized in *A. franciscana* nauplii during cold shock and recovery from cold, and 2) quantify the amounts of HSP90, HSP70 and HSP40 during cold shock and recovery of nauplii. I hypothesized that if HSPs play a role in cold stress, then the levels of HSP90, HSP70 and HSP40 in *A. franciscana* nauplii will change in response to cold shock.

CHAPTER 2 – MATERIALS & METHODS

2.1 Hydration, Incubation and Hatching of Artemia franciscana Cysts

Twenty g of desiccated *A. franciscana* cysts from the Great Salt Lake, Utah (INVE Aquaculture, Inc., Ogden, UT, USA) were hydrated in 600 mL of double distilled water (ddH_20) with constant aeration at 4°C overnight (El-Magsodi et al., 2014). After hydration, cysts were collected by suction using a mini vacuum pump and an EMD Millipore glass filter with 47 mm disc filters. Five g of cysts (wet weight) were transferred to 600 mL of filtered, autoclaved sea water (Halifax Harbour, Nova Scotia, Canada), hereafter termed sea water, and incubated in the dark at 27°C, 150 RPM for 19 h.

Following incubation, newly emerged instar 2 larvae were separated from shells and unhatched cysts using a separatory funnel, followed by exposure to light to induce phototaxis in swimming nauplii [49]. Briefly, a mixture of unhatched and emerged nauplii, empty shells and sea water was incubated for 20 min without agitation, allowing empty shells to float to the top. Unhatched and emerged nauplii that respectively sank or swam to the bottom of the separatory funnel were collected in a Pyrex bowl (Fig. 2.1). The Pyrex bowl was covered with aluminum foil, leaving only a small vertical slit through which light from a lamp penetrated. Opaque objects were placed on either side of a light source to ensure that light was directed toward the side of the Pyrex bowl. Prior to collection, a glass rod was used to stir the mixture, which concentrated all animals to the centre of the bowl. After stirring, the mixture was left for 20 min in a dark room with the light source left on [49]. Because *A. franciscana* nauplii are positively phototactic, nauplii swim and gather in a small area and are easy to collect [49]. Swimming nauplii were

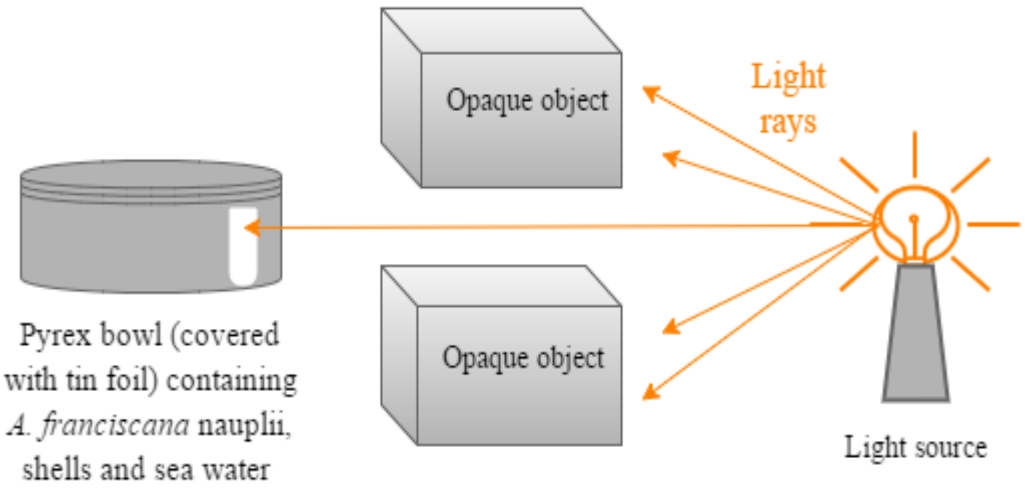

Figure 2.1. Collection of first instar *A. franciscana* nauplii by phototaxis. Instar 2 nauplii were separated from shells and unhatched cysts by shining light through a thin slit in tin foil covering the Pyrex bowl. Opaque objects were placed on either side of the light source to ensure that light was directed toward the slit on the Pyrex bowl.

collected from the position in the bowl located near the light source using a plastic pipette.

2.2 Cold Shock and Recovery of A. *franciscana Nauplii*

2.2.1 Control Samples

Following the collection of nauplii, 30 mL of early instar 2 nauplii in sea water was collected directly from the Pyrex bowl; these nauplii were 20 h old (Fig. 2.2) [49]. This sample, which was not exposed to cold shock, was the 'control' sample (Fig. 2.2), and was immediately processed for protein extraction. The remaining nauplii were used for cold shock treatment followed by a recovery phase, both of which are described in detail below.

2.2.2 Cold Shock Treatment

Nauplii and seawater were immediately transferred from the Pyrex bowl to a 500 mL sterilized Erlenmeyer flask submerged in a VWR water bath (VWR International LLC, Mississauga, ON, Canada) at 1°C with constant aeration. A thermometer was secured in the flask for constant temperature monitoring. Time 0 for the experiment was when the sea water containing swimming nauplii reached 1°C, thus cold shock began when instar 2 nauplii were 20 h old. Approximately 30 mL samples were collected from the flask after 3 h (3CS, 23 h old; Fig. 2.2) and 6 h (6CS, 26 h old; Fig. 2.2) of cold shock. All samples contained between 0.10 g and 0.15 g nauplii. Immediately after sample collection, nauplii were collected by suction as described in section 2.1, using 7 µm mesh filters, washed with cold PIPES buffer (100 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.4),

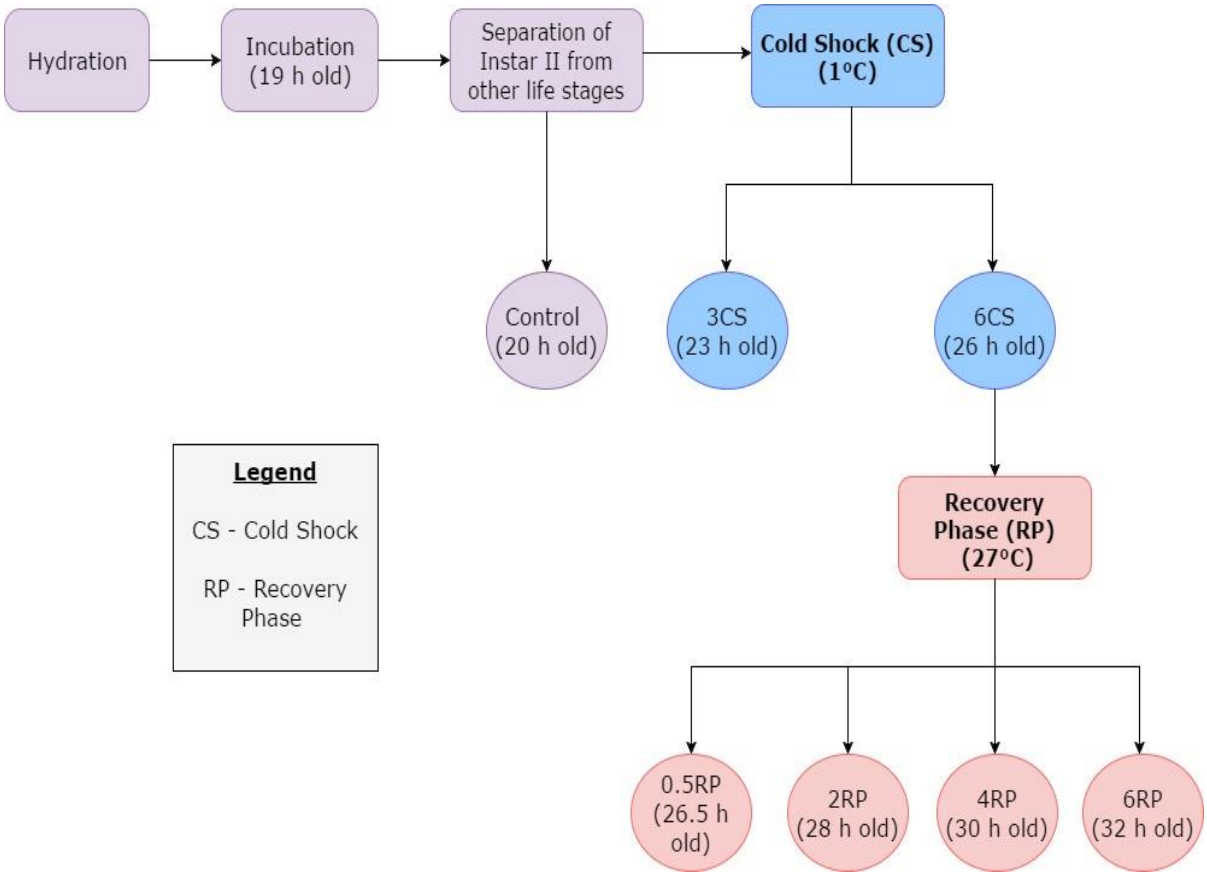

Figure 2.2. Overview of the experimental procedure. Instar 2 nauplii of *A. franciscana* collected for control (20 h old), 3 h (3CS, 23 h old), 6 h (6CS, 26 h old) in cold shock, 30 min (0.5RP, 26.5 h old), 2 h (2RP, 28 h old), 4 h (4RP, 30 h old) and 6 h (6RP, 32 h old) in recovery.

weighed and frozen in liquid nitrogen. Protein samples that were not processed immediately were stored at -80°C. After sampling, the sea water in the Erlenmeyer flask was adjusted back to 500 mL by adding 30 mL of sea water at 1°C.

2.2.3 Recovery Phase

Following 6 h of cold shock, nauplii remaining in the Erlenmeyer flask were transferred to 27°C for recovery. Recovering nauplii were constantly aerated and 30 mL samples were collected after 30 min (0.5RP, 26.5 h old), 2 h (2RP, 28 h old), 4 h (4RP, 30 h old) and 6 h (6RP, 32 h old; Fig. 2.2). After each sample collection, the volume of the flask was adjusted to 500 mL by adding approximately 30 mL of sea water at 27°C. Nauplii were collected by suction as described in section 2.1, weighed and immediately frozen in liquid nitrogen.

The entire experiment, from cyst hydration to the recovery phase was repeated using two separate preparations of nauplii to obtain two replicate sample sets.

2.3. Protein Extraction, Western Blotting and Quantification of HSP90, HSP70 & HSP40

2.3.1 Protein Extraction from Frozen Nauplii

Frozen tissues in Eppendorf tubes were diluted 1:1 w/v with cold PIPES Buffer and 1:100 w/v Halt™ Protease Inhibitor Cocktails (Thermo Scientific, Rockford, IL, USA) before cell lysis and homogenization on ice using a micro-pestle for 60 s. Cell-free homogenates were centrifuged at 10,000 x g, 4°C for 15 min to obtain the protein

supernatant for analysis [49]. Fat that floated to the top and cell residue that accumulated at the bottom of the tubes were discarded.

2.3.2 Protein Separation via SDS-PAGE and Western Blotting

A Bradford assay [69] was used to quantify the amount of protein in each sample of nauplii using a Pierce™ Coomassie (Bradford) Protein Assay Kit, and Bovine Serum Albumen (BSA) standards (2.0 mg/mL in 0.9% NaCl) with BSA concentration ranging from 0 µg/µL to 2 µg/µL. A standard curve of absorbance was derived using Softmax pro software (Molecular Devices Corporation, California) and, based on the results of a trial experiment, 30 µg/µL of each sample was chosen as the final amount of protein loaded per well of SDS polyacrylamide gels.

Thirty µg of cell free extract protein for each sample was mixed with 10 µL of 4 X loading buffer, placed in a boiling water bath for 5 min, centrifuged at 10,000 x g for 10 min at 27**°**C and resolved in 10% SDS polyacrylamide gels in Running Buffer (25 mM Tris, 200 mM glycine with 0.04% (w/v) SDS). Gels were loaded with protein extracts from both cold shock and recovery treatments, and run for 1 h and 45 min at 35 mAmp [66]. GeneDireX BLUelf Prestained Protein Ladder (Froggabio, Toronto, ON, Canada) was used as a molecular weight marker. Two replicate SDS gels were run simultaneously with one stained with Colloidal Coomassie Blue (10% $(NH_4)_2SO_4$, 0.1% of 5% Coomassie G-250 stain, 3% Phosphoric acid, 20% Ethanol) [70] and the other transferred to nitrocellulose membrane overnight at 100 mAmp in Transfer Buffer (25 mM Tris, 200 mM glycine in 20% (v/v) methanol).

2.3.3 Staining and Immunoprobing of Western Blots to Detect HSPs

To ensure that proteins were transferred from the gel, nitrocellulose membranes were stained with Ponceau (0.1% (w/v) Ponceau, 1.5% (w/v) trichloroacetic acid) for 2 min to reveal protein bands and then rinsed in dH_2O for approximately 5 mins to remove stain [71]. For HSP90, HSP70 and HSP40 detection, nitrocellulose membranes were blocked in 5% non-fat milk in Tris Buffered Saline (TBS; 10 mM TRIS, 140 mM NaCl, pH 7.4) for 1 h at 27°C [64].

Blocked membranes were incubated in antibodies diluted (Table 1) in TBS or PBS (PBS; 10mM Na_2HPO_4, 137mM NaCl, 2.7mM KCl, pH 7.4;) for 20 min at 27**°**C. Membranes were washed once for 1 min and three times for 5 min in TBS-Tween (TBST; 10 mM TRIS, 140 mM NaCl, 0.1% Tween 20, pH 7.4) followed by HST (10 mM TRIS, 1M NaCl, 0.5% Tween 20, pH 7.4) for HSP90 and HSP70 blots [71]. HSP40 blots were washed with PBS-Tween (10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, pH 7.4) using the same wash protocol. Blots were then incubated with HRP-conjugated goat-anti-rabbit IgG (Life Technologies, Burlington, ON, Canada) diluted 1:5000 in TBS for HSP90 detection and 1:5000 in PBS for HSP40 detection (Table 2.1). HRP-conjugated goat-anti-mouse IgG (Life Technologies) was diluted 1:5000 in TBS for HSP70 blots (Table 2.1). The wash steps were repeated as described above and antibody reactive proteins were visualized using Criterion® ECL western blotting detection reagents (GE Healthcare, Baie d'Urfé Quebec, Canada) and a DNR Bio-Imaging Systems MF-ChemiBIS 3.2 gel documentation system (Montreal Biotech).

Table 2.1. Antibodies used for the immunostaining of *A. franciscana* molecular chaperones (modified from Rowarth, 2015).

Target protein	Antibody	Antibody dilution	Supplier
Primary Antibodies			
ArHsp90	rabbit anti ArHsp90	1:2000	Abbiotec
HSP70	mouse anti Hsp70	1:5000	StressMarq SMC-164
HSP40	rabbit anti Hsp40	1:5000	Abbiocode H2-32
Secondary Antibodies			
Rabbit IgG	goat anti-rabbit IgG	1:5000	Life Technologies [72]
Mouse IgG	goat anti-mouse IgG	1:5000	Life Technologies [73]

2.3.4. Quantification of Western Blots and Statistical Analysis

Protein bands on the western blots were quantified using Image Studio Lite Version 5.2 and the control sample was used as the standard for comparing the intensity of protein bands for cold shock and recovery samples. Probing of western blots detected two immunoreactive protein bands for HSP90 in *A. franciscana* nauplii; an upper band with a molecular mass of 90 kDa and a lower band of approximately 75 – 80 kDa, both of which were present in all samples examined (Fig. 3.2 & 3.3). These bands were termed HSP90A and HSP90B, respectively. Relative to the protein band for the control sample, the levels of HSP90A, HSP90B, HSP70 and HSP40 for the cold shock and recovery samples were calculated and averaged between the two replicate sample sets. Graphs were plotted with standard deviations to represent the relative HSP levels for each HSP. A one-way analysis of variance (ANOVA) test was used to test the effect of cold shock and recovery treatments and Tukey post-hoc tests were used to identify significant differences between specific treatments.

CHAPTER 3 - RESULTS

3.1 Qualitative Assessment of SDS Polyacrylamide Gel Electrophoresis and Western Blotting

Coomassie blue staining after electrophoresis showed, as indicated by the Bradford assay for determining protein concentration, that all the lanes in the SDS polyacrylamide gels received equal amounts of protein for all cold shock and recovery samples (Fig. 3.1a). Western blots stained with Ponceau revealed successful transfer of proteins from SDS gels to nitrocellulose membranes (Fig. 3.1b).

3.2 HSP90 During Cold Shock and Recovery

There was an overall decrease in the amount of HSP90A during the 12 h experimental period (one-way ANOVA: $F_{5,11} = 11.54$, p=0.005; Fig. 3.2a; Appendix B), with a marked difference in the amounts of HSP90A at 3 h of cold shock (3CS) and 6 h of recovery (6RP) when there was almost no HSP90A (6RP; Tukey post-hoc test, T = -6.13, p = 0.007, Fig. 3.2a; Appendix B).

In contrast with HSP90A, there was no significant difference in HSP90B levels over the 12-hour experimental period (one-way ANOVA: $F_{5,11} = 2.31$, p=0.169; Fig 3.3; Appendix B) in *A. franciscana* nauplii. However, there was a trend towards an increase at 6 h of recovery (6RP) at 27°C (Fig.3.3).

3.3 HSP70 during Cold Shock and Recovery

Relative to the control, cold shock at 1°C and recovery at 27°C had no significant effect on the amount of HSP70 in *A. franciscana* nauplii (one-way ANOVA: $F_{5,11} = 0.89$; p

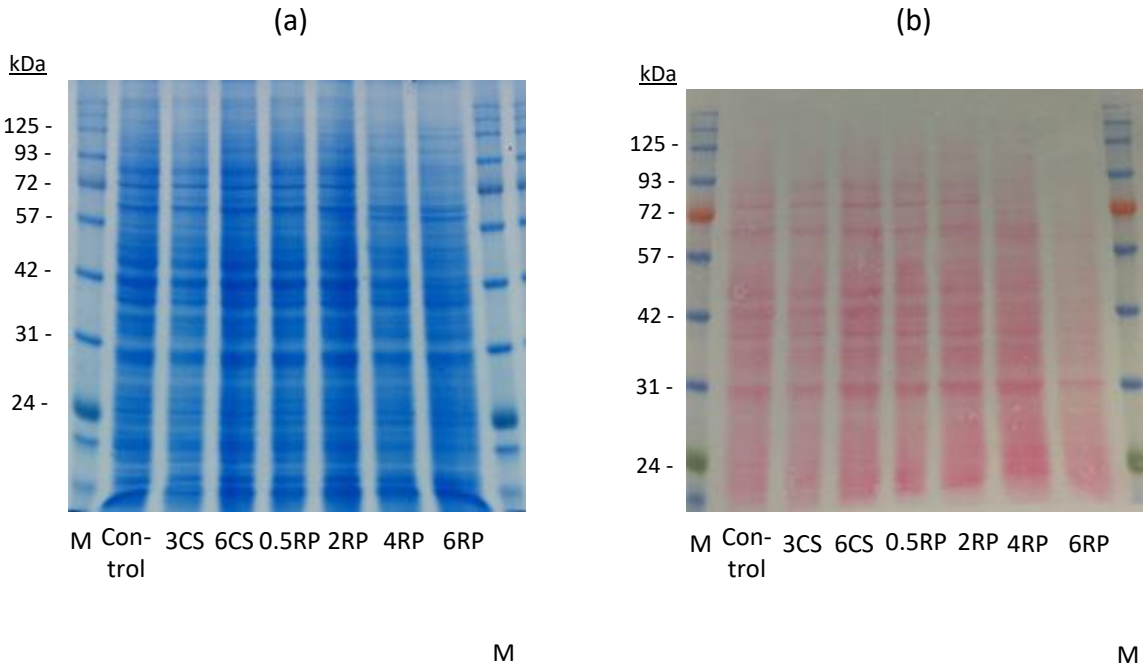

Figure 3.1. SDS polyacrylamide gels and western blots of protein extracts from *A. franciscana* nauplii. (a) Protein extracts from *A. franciscana* nauplii were resolved in SDS polyacrylamide gels and stained with Coomassie. Lane M, molecular mass markers in kDa; Control, no cold shock; 3CS, 3 h cold shock; 6CS, 6 h cold shock; 0.5RP, 0.5 h recovery; 2RP, 2 h recovery; 4RP, 4 h recovery; 6RP, 6 h recovery. All lanes received 30 µg of protein. (b) Ponceau stain of a western blot obtained by transfer of proteins from an SDS polyacrylamide gel. Labels are as for panel A. All lanes received 30 µg of protein.

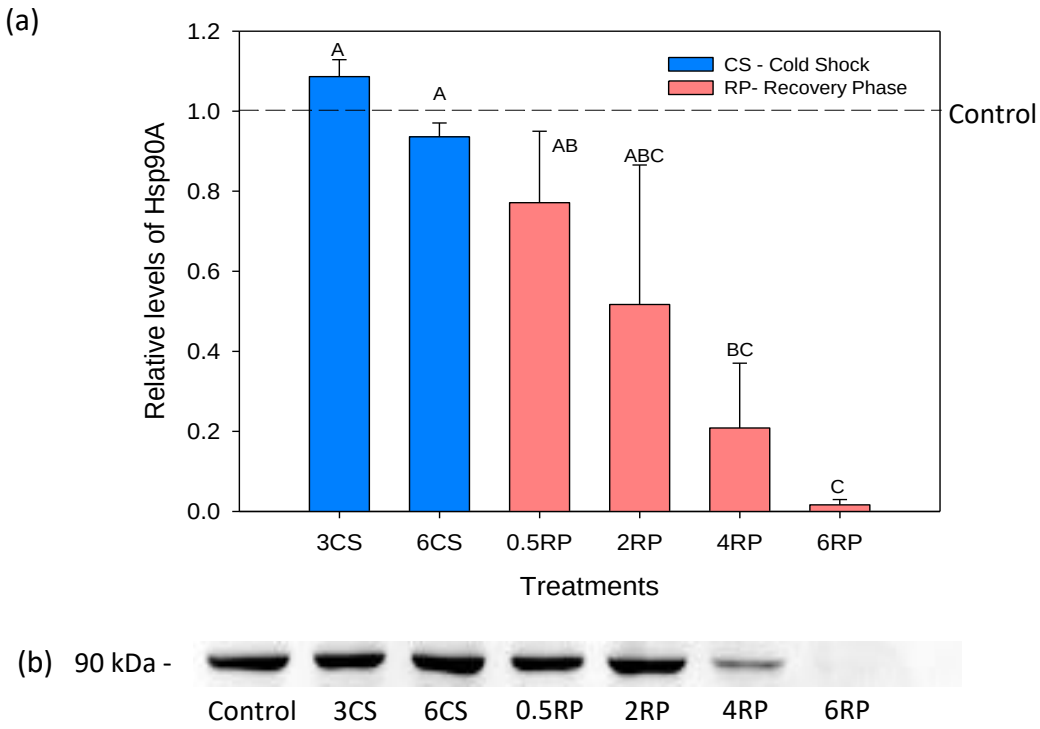

Figure 3.2. HSP90A in *A. franciscana* nauplii during cold shock and recovery. (a) Mean levels of HSP90A relative to control in *A. franciscana* nauplii during cold shock (CS) and recovery phase (RP). Relative HSP90 levels were averaged for two different blots. The control value was set at 1.0 and is represented as the dashed line. Numbers associated with treatment names indicate time in h. Error bars depict standard deviations. Different Letters above bars indicate significant differences among treatment (Tukey pot-hoc tests). (b) A representative immunoblot of protein levels during cold shock and recovery.

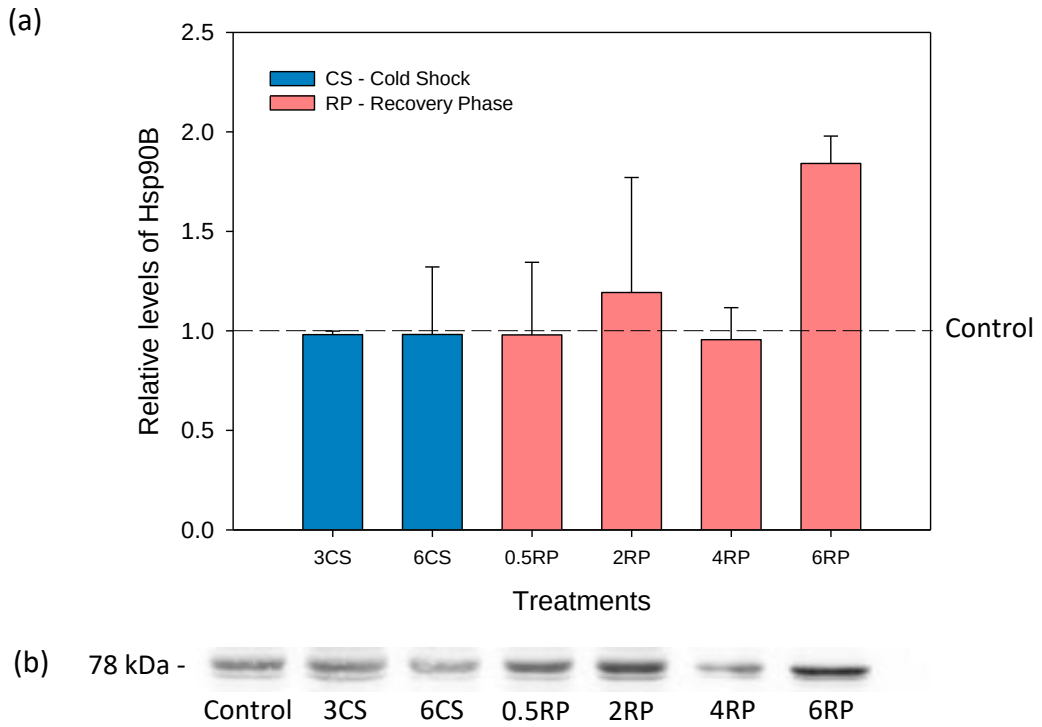

Figure 3.3. HSP90B in *A. franciscana* nauplii during cold shock and recovery. (a) Mean levels of HSP90B relative to control in *A. franciscana* nauplii during cold shock (CS) and recovery phase (RP). Relative HSP90 levels were averaged for two different blots. The control value was set at 1.0 and is represented as the dashed line. Numbers associated with treatment names indicate time in h. Error bars depict standard deviations. (b) A representative immunoblot of protein levels during cold shock and recovery.

= 0.543; Fig. 3.4; Appendix B). A slight increase in the amount of HSP70 was detected in the 6RP treatment compared to the other cold shock and recovery treatments, but the difference was not significant (Fig. 3.4).

3.4 HSP40 during Cold Shock and Recovery

There was a significant increase in the amount of HSP40 during recovery from cold shock (one-way ANOVA: $F_{5,11} = 5.74$; $p = 0.028$; Fig. 3.5; Appendix B). The increase was influenced by a large difference between 3 h of cold shock (3CS) and the 6 h of recovery (6RP) treatments (Tukey post-hoc test, $T = 4.44$, $p = 0.031$; Fig. 3.5; Appendix B), with the most prominent increase in HSP40 recorded after 6 h of recovery (Fig. 3.5).

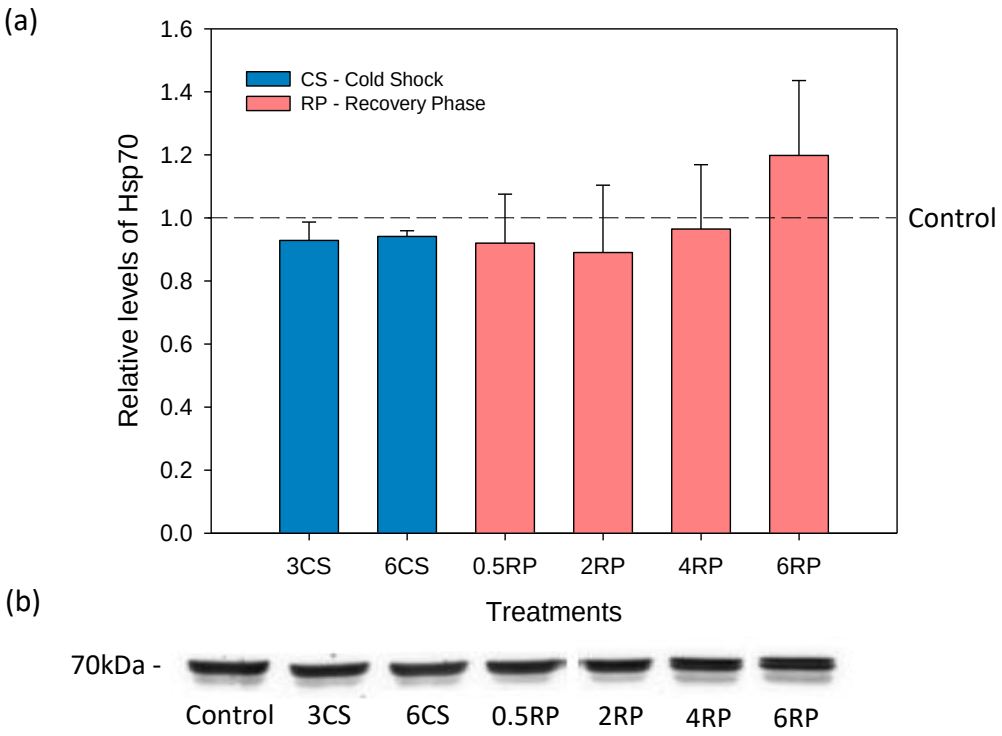

Figure 3.4. HSP70 in *A. franciscana* nauplii during cold shock and recovery. (a) Mean levels of HSP70 relative to control in *A. franciscana* nauplii during cold shock (CS) and recovery phase (RP). Relative HSP90 levels were averaged for two different blots. The control value was set at 1.0 and is represented as the dashed line. Numbers associated with treatment names indicate time in hours. Error bars depict standard deviations. (b) A representative immunoblot of protein levels during cold shock and recovery.

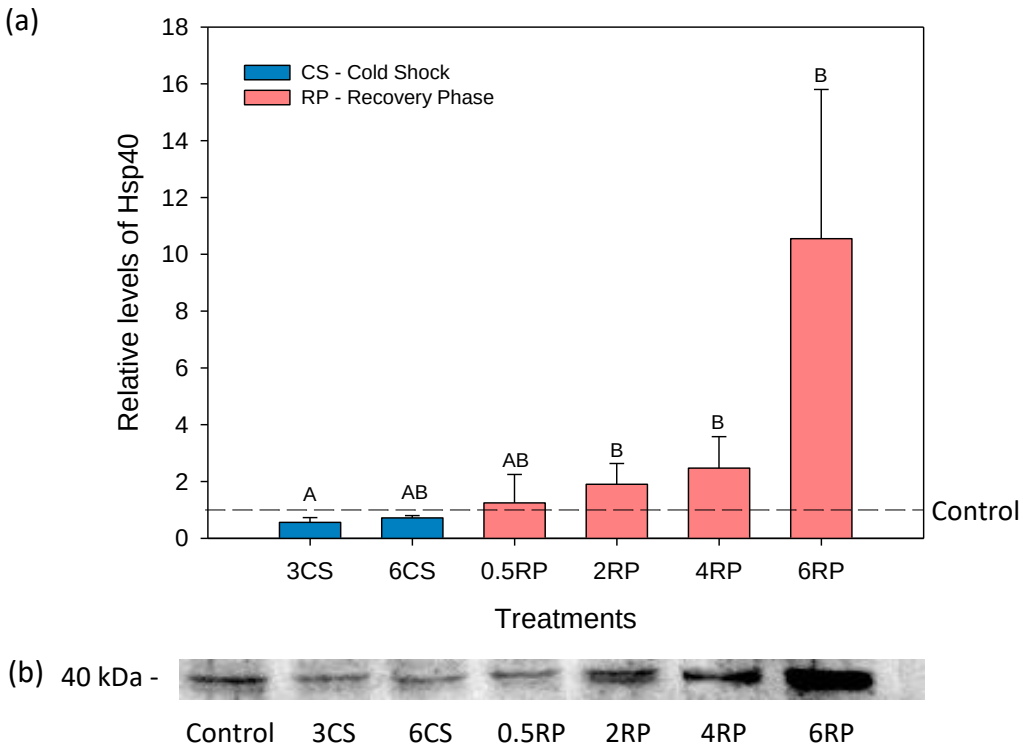

Figure 3.5. HSP40 in *A. franciscana* nauplii during cold shock and recovery. (a) Mean levels of HSP40 relative to control in *A. franciscana* nauplii during cold shock (CS) and recovery phase (RP). Relative HSP40 levels were averaged for two different blots. The control value was set at 1.0 and is represented as the dashed line. Numbers associated with treatment names indicate time in hours. Error bars depict standard deviations. Different Letters above bars indicate significant differences among treatment (Tukey pot-hoc tests). (b) A representative immunoblot of protein levels during cold shock and recovery.

CHAPTER 4 - DISCUSSION

The results of this study showed that HSP90A and HSP40 respond to cold in *A. franciscana* nauplii suggesting that they play a role in the survival of larvae during low temperature stress. HSPs have been studied extensively in many organisms, including the diapause and adult stages of *A. franciscana* during heat stress [14,35,57,74–77]. However, HSP synthesis has been understudied in relation to cold stress in the nauplii stage of *A. franciscana* and hence the importance of this study.

Confirming the hypothesis, HSP90, HSP70 and HSP40 were synthesized in varying amounts in response to cold shock in *A. franciscana* nauplii, giving an indication of the different cellular functions of HSPs during stress (Fig. 3.2 – 3.4). In their natural habitats such as the Great Salt Lake, *A. franciscana* deal with fluctuating temperatures and experience near freezing temperatures in the fall and the winter [55]. Not many organisms inhabit the Great Salt Lake, but *A. franciscana* have thrived there despite the harsh conditions [78]. Previous studies showed that *Artemia,* like other arthropods, synthesize varying amounts of HSPs in response to thermal stress [61,64,66]. As molecular chaperones, HSPs function in many cellular processes under normal physiological conditions, however when required they become stress proteins, protecting cells from adverse conditions.

There was a significant decrease in the amount of HSP90A, during recovery from cold shock demonstrating that cold shock influenced HSP90A synthesis. HSP90B, the lower HSP90 band seen on western blots, did not change, suggesting that it was indifferent to cold shock. The results indicate that HSP90 may occur in different forms in *A.*

franciscana, hence two bands were observed, although it is not possible to rule out the possibility of shared epitopes by dissimilar proteins. HSP90 is the most abundant heat shock protein found in all organisms, from bacteria to humans [42]. Under normal conditions, HSP90 is constitutively expressed in the cell and interacts with other proteins, such as cell-signaling proteins, cytoskeletal elements and protein kinases to regulate cell functions [79]. HSPs are upregulated to enhance stress tolerance in many organisms [80]. For example, HSP90 is up-regulated in the onion maggot, *Delia antiqua*, upon exposure to cold and heat stress during diapause [35,57,67]. However, HSP90 is down-regulated when favorable conditions return, and development reinitiates [57]. By contrast, HSP90 is down-regulated during diapause in the pupae of *Sarcophaga crassipalpis* when cold-stressed and up-regulated when normal conditions return [77]. When *A. franciscana* larvae were exposed to extreme cold, HSP90A remained constant in amount but it was down-regulated during recovery at normal room temperature. The different amounts suggest that HSP90 may respond differently to various stressors, and can either be up-regulated or down-regulated in different species, depending on the role it plays during stress. At very low temperatures, HSP90 may decrease thus arresting cellular processes, but if increased, HSP90 would promote cold tolerance by being available to protect proteins from denaturation.

The HSP70 family is very large and occurs in all organisms. In eukaryotes, there are more than a dozen different HSP70s present in the cytosol, mitochondria, endoplasmic reticulum and other cell compartments [42]. Some forms of HSP70 are constitutive, while others are stress-inducible [32]. HSP70 is up-regulated in the salivary glands of *D.*

melanogaster during both heat and cold stress [14,67,81]. This suggests that HSP70 plays a role in thermal acclimation and survival. Contrary to this, the levels of HSP70 did not change during either cold shock or recovery in *A. franciscana* nauplii. This finding is consistent with studies that investigated the responses of HSP70 to cold stress in other organisms [27,80,82]. In addition to its role in protein folding, HSP70 is associated with protein translocation from the cytosol to the mitochondria during which HSP70 has weak ATPase activity [32]. However, when associated with J-domain proteins, such as HSP40[83], the ATPase activity of HSP70 increases, allowing it to efficiently bind to substrates [32,42,83]. The constant levels of HSP70 observed during cold and recovery of *A. franciscana* nauplii (Fig. 3.4) may reflect the constitutive synthesis of HSP70. Thus, because HSP70 is already synthesized under optimal conditions, its concentration in the cell need not be increased during cold stress. Rather, the cold stress response may be reflected in an increase in the levels of its co-chaperones, which was the case for HSP40. While this study only considered the response of one HSP70, other isotopes of HSP70 may be modified during cold stress in *A. franciscana* nauplii during cold stress.

HSP40 is an HSP that falls under the J-domain group of proteins. While HSP40 is a molecular chaperone in its own right, it associates with and affects the activity of other ATP-dependent proteins such as HSP70 [32,42,84]. HSP40 is up-regulated during recovery from cold stress in *D. melanolgaster*[84], and in the cold-adapted gall fly, *Eurosta solidaginis* [36,37,49]. In *A. franciscana* nauplii, the amount of HSP40 did not increase during cold stress but rather during the recovery stage. The up-regulation of HSP40 in these arthropods, during and following cold stress, shows that HSP40 synthesis is influenced

by cold but not until the animal begins to recover from this stress. HSP40 may prevent irreversible denaturation of proteins during cold stress and in concert with other proteins binds to nascent proteins, preventing their premature folding and targeting them to HSP70 [42].

Most HSPs, while stress-inducible, are involved in cellular processes such as differentiation, signal transduction, metabolism and proliferation [23]. These processes occur during development, which in itself is stressful. While this study focussed on the effects of cold stress on HSPs, it would be interesting to contrast the results from this study with changes in HSP levels during ontogeny in *A. franciscana*. This will confirm whether the changes observed in HSP90A and HSP40 are crucial to cold stress tolerance or if they are related to developmental changes. In addition, the quantification of mRNA for the three HSPs would allow for an estimate of levels of gene expression, and indicate whether differential gene transcription and post-transcriptional changes influence HSP synthesis during stress. Future studies should examine the effects of using RNAi to eliminate the mRNAs encoding the HSPs and the effect of their absence on the survival of nauplii.

Overall, the results of this study indicate that HSPs are synthesized differently in response to cold stress in *A. franciscana* larvae compared to other life stages. The diverse levels of HSP90, HSp70 and HSP40 in cold-shocked and recovering nauplii suggest that these HSPs play different roles as *A. franciscana* nauplii adapt and survive to cold stress. As molecular chaperones, HSPs likely maintain the integrity of cellular proteins in nauplii, and promote proper functioning of cells during cold stress.

Environmental stressors, such as extreme cold and global changes in climate affect organisms across their entire life history and thus, it is important to understand how organisms respond to extreme temperature fluctuations.

APPENDIX: ANOVA & TUKEY POST-HOC TESTS

One-way ANOVA: Rel. Hsp90B levels versus Treatments

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatments	5	1.7574	0.35147	11.54	0.005
Error	6	0.1827	0.03045		
Total	11	1.9400			

S	R-sq	R-sq(adj)	R-sq(pred)
0.174487	90.58%	82.74%	62.34%

Pooled StDev = 0.174487

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatments	N	Mean	Grouping
3CS	2	1.0864	A
6CS	2	0.9358	A
0.5RP	2	0.771	A B
2RP	2	0.517	A B C
4RP	2	0.209	B C
6RP	2	0.01644	C

Means that do not share a letter are significantly different.

One-way ANOVA: Rel. Hsp90B levels versus Treatments

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatments	5	1.2066	0.2413	2.31	0.169
Error	6	0.6271	0.1045		
Total	11	1.8337			

S	R-sq	R-sq(adj)	R-sq(pred)
0.323291	65.80%	37.30%	0.00%

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatments	N	Mean	Grouping
6RP	2	1.8413	A
2RP	2	1.193	A
6CS	2	0.982	A
3CS	2	0.9808	A
0.5RP	2	0.980	A
4RP	2	0.956	A

Means that do not share a letter are significantly different.

One-way ANOVA: Rel. Hsp70 levels versus Treatments

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatments	5	0.1267	0.02534	0.89	0.543
Error	6	0.1717	0.02862		
Total	11	0.2984			

S	R-sq	R-sq(adj)	R-sq(pred)
0.169164	42.46%	0.00%	0.00%

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatments	N	Mean	Grouping
6RP	2	1.198	A
4RP	2	0.965	A
6CS	2	0.9414	A
3CS	2	0.9286	A
0.5RP	2	0.920	A
2RP	2	0.890	A

Means that do not share a letter are significantly different.

One-way ANOVA: Rel. Hsp40 levels versus Treatments

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatments	5	145.35	29.070	5.74	0.028
Error	6	30.36	5.061		
Total	11	175.72			

S	R-sq	R-sq(adj)	R-sq(pred)
2.24962	82.72%	68.32%	30.88%

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatments	N	Mean	Grouping
6RP	2	10.55	A
4RP	2	2.472	A B
2RP	2	1.904	A B
0.5RP	2	1.245	B
6CS	2	0.7203	B
3CS	2	0.560	B

Means that do not share a letter are significantly different.

REFERENCES

1. Duman, J. G. Antifreeze and ice nucleator proteins in terrestrial artrhopods. *Annu. Rev. Physiol.* **63,** 327–57 (2001).

2. Storey, K. B. & Storey, J. M. Heat shock proteins and hypometabolism: adaptive strategy for proteome preservation. *Res. Rep. Biol.* **2,** 57–68 (2011).

3. Haynes, T. M. The effects of temperature on living organisms. *Am. Biol. Teach.* **26,** 511–514 (1964).

4. Lepocky, J. R. & Borrelli, M. J. How do cells respond to their thermal environment? *Int. J. Hyperth.* **21,** 681–687 (2005).

5. Tattersall, G. J. *et al.* Coping with thermal challenges: Physiological adaptations to environmental temperatures. *Compr. Physiol.* **2,** 2151–2202 (2012).

6. Ramløv, H. Aspects of natural cold tolerance in ectothermic animals. *Hum. Reprod.* **15,** 26–46 (2000).

7. Scholander, P. F., Hock, R., Walters, V. & Irving, L. Adaptation to cold in Arctic and Tropical mammals and birds in relation to body temperature, insulation, and basal metabolic rate. *Biol. Bull.* **99,** 259–271 (1950).

8. Le Maho, Y. The emperor penguin: A strategy to live and breed in the cold. *Am. Sci.* **65,** 680–693 (1977).

9. Rausch, R. L. *et al.* Effect of climatic warming on the Pacific walrus , and potential modification of its helminth fauna. *J. Parasitol.* **93,** 1247–1251 (2007).

10. Jeffery, C. J. Moonlighting proteins. *TIBS* **24,** 8–11 (1999).

11. Lee, G. J., Roseman, A. M., Saibil, H. R. & Vierling, E. A small heat shock protein stably binds heat denatured model substrates and can maintain a substrate in a folding competent state. *EMBO J.* **16,** 659–671 (1997).

12. Richter, K., Haslbeck, M. & Buchner, J. The heat shock response: Life on the verge of death. *Mol. Cell* **40,** 253–266 (2010).

13. Cooper, A. Heat capacity effects in protein folding and ligand binding: A re-evaluation of the role of water in biomolecular thermodynamics. in *Biophys. Chem.* **115,** 89–97 (2005).

14. Stetina, T., Kostal, V. & Korbelova, J. The role of inducible Hsp70, and other heat shock proteins, in adaptive complex of cold tolerance of the fruit fly (*Drosophila melanogaster*). *PLoS One* **10,** 1–22 (2015).

15. Storey, K. B. & Storey, J. M. Molecular biology of freezing tolerance. *Compr. Physiol.* **3,** 1283–1308 (2013).

16. Baker, D. A. & Russell, S. Gene expression during *Drosophila melanogaster* egg

development before and after reproductive diapause. *BMC Genomics* **10,** 242 (2009).

17. Robich, R. M., Rinehart, J. P., Kitchen, L. J. & Denlinger, D. L. Diapause-specific gene expression in the northern house mosquito, *Culex pipiens L*., identified by suppressive subtractive hybridization. *J. Insect Physiol.* **53,** 235–245 (2007).

18. Robbins, H. M. *et al.* Diapause termination and development of encysted *Artemia* embryos: roles for nitric oxide and hydrogen peroxide. *J. Exp. Biol.* **213,** 1464–1470 (2010).

19. Renfree, M. B. & Shaw, G. Diapause. *Annu. Rev. Physiol.* **62,** 353–75 (2000).

20. Cannon, R. & Block, W. Cold tolerance of microarthropods. *Biol. Rev.* **63,** 23–77 (1988).

21. Elderidge, B. Environmental control of ovarian development in mosquitoes of the *Culex pipiens* Complex. *Science* **151,** 826–828 (2008).

22. Bale, J. S. Insects and low temperatures: from molecular biology to distributions and abundance. *Biol. Sci.* **357,** 849–862 (2002).

23. Lindquist, S. The heat-shock response. *Ann. Rev. Biochem.* **55,** 1151–91 (1986).

24. Parsell, D. A. & Lindquist, S. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* **27,** 437–496 (1993).

25. Inouye, M. & Phadtare, S. Cold shock response and adaptation at near-freezing temperature in microorganisms. *Sci. STKE* **2004,** pe26 (2004).

26. Jiang, W., Hou, Y. & Inouye, M. CspA, the major cold-shock protein of *Escherichia coli*, is an RNA chaperone. *J. Biol. Chem.* **272,** 196–202 (1997).

27. Takle, H., Baeverfjord, G., Lunde, M., Kolstad, K. & Andersen, Ø. The effect of heat and cold exposure on HSP70 expression and development of deformities during embryogenesis of Atlantic salmon (*Salmo salar*). *Aquaculture* **249,** 515–524 (2005).

28. Rothman, J. E. Polypeptide chain binding proteins: Catalysts of protein folding and related processes in cells. *Cell* **59,** 591–601 (1989).

29. Dobson, C. M. Experimental investigation of protein folding and misfolding. *Methods* **34,** 4–14 (2004).

30. Hendrick, J. P. & Hartl, F.-U. Molecular chaperone functions of heat shock proteins. *Ann. Rev. Biochem.* **62,** 349–384 (1993).

31. Dobson, C. Protein folding and misfolding. *Am. Sci.* **90,** 445–453 (2002).

32. Hartl, F. U. Molecular chaperones in cellular protein folding. *Nature* **381,** 571–579

(1996).

33. Thomas, P. J., Qu, B.-H. & Pedersen, P. L. Defective protein folding as a basis of human disease. *Trends Biochem. Sci.* **20,** 456–459 (1995).

34. Kaufmann, S. H. . Heat shock proteins and the immune response. *Immunol. Today* **11,** 129–136 (1990).

35. Chen, B., Kayukawa, T., Monteiro, A. & Ishikawa, Y. The expression of the HSP90 gene in response to winter and summer diapauses and thermal-stress in the onion maggot, *Delia antiqua*. *Insect Mol. Biol.* **14,** 697–702 (2005).

36. Zhang, G., Storey, J. M. & Storey, K. B. Chaperone proteins and winter survival by a freeze tolerant insect. *J. Insect Physiol.* **57,** 1115–1122 (2011).

37. Colinet, H., Lee, S. F. & Hoffmann, A. Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult *Drosophila melanogaster*. *FEBS J.* **277,** 174–185 (2010).

38. Iwama, G. K., Thomas, P. T., Forsyth, R. B. & Vijayan, M. M. Heat shock expression in fish. *Rev. Fish Biol. Fish.* **8,** 35–51 (1998).

39. Ritossa, F. A new puffing pattern induced by temperature shock and DNP in drosophila. *Experientia* **18,** 571–573 (1962).

40. Richter, K. & Buchner, J. Hsp90: Chaperoning signal transduction. *J. Cell. Physiol.* **188,** 281–290 (2001).

41. Young, J., Moarefi, I. & Hartl, U. Hsp90: A specialized but essential protein-folding tool. *J. Cell Biol.* **154,** 267–273 (2001).

42. Fink, A. L. Chaperone-mediated protein folding. *Physiol. Rev.* **79,** 425–49 (1999).

43. Pratt, William, B., Priti, K. & Olsen, Laura, J. Hsp90-binding immunophilins in plants: the protein movers. *Trends Plant Sci.* **6,** 54–58 (2001).

44. Frydman, J. Folding of newly translated proteins in vivo: the role of molecular chaperones. *Annu. Rev. Biochem.* **70,** 603–647 (2001).

45. Queitsch, C., Sangster, T. A. & Lindquist, S. Hsp90 as a capacitor of phenotypic variation. *Nature* **417,** 618–624 (2002).

46. Chirico, W. J., Waters, M. G. & Blobel, G. 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature* **332,** 805–810 (1988).

47. Warner, A. H., Brunet, R. T., MacRae, T. H. & Clegg, J. S. Artemin is an RNA-binding protein with high thermal stability and potential RNA chaperone activity. *Arch. Biochem. Biophys.* **424,** 189–200 (2004).

48. Qui, Z. Molecular analysis of encystment and diapause in *Artemia*: Gene expression and stress response. *Published Honors Thesis,* Dalhousie University,

(2006).

49. Jiang, G., Rowarth, N. M., Panchakshari, S. & MacRae, T. H. ArHsp40, a type 1 J-domain protein, is developmentally regulated and stress inducible in post-diapause *Artemia franciscana*. *Cell Stress Chaperones* **21,** 1077–1088 (2016).

50. Ranson, N., White, H. & Saibil, H. Chaperonins. *Biochem. J.* **333,** 233–242 (1998).

51. Lee, G. J. & Vierling, E. A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. *Plant Physiol.* **122,** 189–198 (2000).

52. Bowen, S. T. *et al.* Ecological isolation in *Artemia*: Population differences in tolerance of anion. *J. Crustac. Biol.* **5,** 106–129 (1985).

53. Kellogg, V. L. A new *Artemia* and its life conditions. *Sci. New Ser.* **24,** 594–596 (1906).

54. Dai, L. *et al.* Extracellular matrix peptides of *Artemia* cyst shell participate in protecting encysted embryos from extreme environments. *PLoS One* **6,** 1–9 (2011).

55. Crosman, E. T. & Horel, J. D. MODIS-derived surface temperature of the Great Salt Lake. *Remote Sens. Environ.* **113,** 73–81 (2009).

56. Liang, P. & Macrae, T. H. The synthesis of a small heat shock/-crystallin protein in *Artemia* and its relationship to stress tolerance during development. *Dev. Biol.* **207,** 445–456 (1999).

57. MacRae, T. H. Molecular chaperones, stress resistance and development in *Artemia franciscana*. *Semin. Cell Dev. Biol.* **14,** 251–258 (2003).

58. Ruebhart, D. R., Cock, I. E. & Shaw, G. R. Brine Shrimp Bioassay: Importance od Correct Taxonomic Identification of Artemia (Anostraca) Species. *Environ. Toxicol.* **23,** 555–560 (2008).

59. Gilchrist, B. M. & Green, J. The pigments of *Artemia*. *Biol. Sci.* **152,** 118–136 (1960).

60. MacRae, T. H. Gene expression, metabolic regulation and stress tolerance during diapause. *Cell. Mol. Life Sci.* **67,** 2405–2424 (2010).

61. Clegg, J. S., Willsie, J. K. & Jackson, S. A. Adaptive significance of a small heat shock / α-Crystallin protein (p26) in encysted embryos of the brine shrimp , *Artemia franciscana*. *Am. Zool.* **39,** 836–847 (1999).

62. Warner, A. H. *et al.* Evidence for multiple group 1 late embryogenesis abundant proteins in encysted embryos of *Artemia* and their organelles. *J. Biochem.* **148,** 581–592 (2010).

63. King, A. M., Toxopeus, J. & Macrae, T. H. Artemin, a diapause-specific chaperone,

contributes to the stress tolerance of *Artemia* cysts and influences their release from females. *J. Exp. Biol.* **217,** 1719–1724 (2014).

64. King, A. M. & MacRae, T. H. The small heat shock protein p26 aids development of encysting *Artemia* embryos, prevents spontaneous diapause termination and protects against stress. *PLoS One* **7,** 1–10 (2012).

65. Rowarth, N. M. Cold induction of molecular chaperones in *Artemia franciscana* and their role in stress tolerance. *Published Honors Thesis,* Dalhousie University, (2015).

66. Toxopeus, J., Warner, A. H. & MacRae, T. H. Group 1 LEA proteins contribute to the desiccation and freeze tolerance of *Artemia franciscana* embryos during diapause. *Cell Stress Chaperones* **19,** 939–48 (2014).

67. King, A. Elucidating the functions of proteins up-regulated during diapause in *Artemia franciscana* using RNAi. *Published Honors Thesis,* Dalhousie University, (2013).

68. El-Magsodi, M. O., Bossier, P., Sorgeloos, P. & Van Stappen, G. Hatching and nutritional quality of *Artemia* cysts progressively deteriorates as a function of increased exposure to hydration/dehydration cycles. *Aquac. Int.* **22,** 1515–1532 (2014).

69. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254 (1976).

70. Dyballa, N. & Metzger, S. Fast and sensitive colloidal coomassie G-250 staining for proteins in polyacrylamide gels. *J. Vis. Exp.* **30,** 2–5 (2009).

71. Xiang, H. & MacRae, T. H. Production and utilization of detyrosinated tubulin in developing *Artemia* larvae: evidence for a tubulin-reactive carboxypeptidase. *Biochem. Cell Biol.* **73,** 673–85 (1995).

72. Koyasu, S. *et al.* Two mammalian heat shock proteins, HSP90 and HSP100, are actin-binding proteins. *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8054–8058 (1986).

73. Warrick, J. M. *et al.* Suppression of polyglutamine-mediated neurodegeneration in *Drosophila* by the molecular chaperone HSP70. *Nat. Genet.* **23,** 425–428 (1999).

74. King, A. M., Toxopeus, J. & Macrae, T. H. Functional differentiation of small heat shock proteins in diapause-destined *Artemia* embryos. *FEBS J.* **280,** 4761–4772 (2013).

75. King, A. M. & MacRae, T. H. Insect heat shock proteins during stress and diapause. *Annu. Rev. Entomol.* **60,** 59–75 (2015).

76. Rinehart, J. P. *et al.* Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 14223–14227

(2006).

77. Rinehart, J. P. & Denlinger, D. L. Heat-shock protein 90 is down-regulated during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, but remains responsive to thermal stress. *Insect Mol. Biol.* **9,** 641–645 (2000).

78. Clegg, J. J. S. *et al.* Thermal resistance, developmental rate and heat shock proteins in *Artemia franciscana*, from San Francisco Bay and southern Vietnam. *J. Exp. Mar. Bio. Ecol.* **252,** 85–96 (2000).

79. Bose, S., Weikl, T., Bügl, H. & Buchner, J. Chaperone function of Hsp90-associated proteins. *Science* **274,** 1715–1717 (1996).

80. Rinehart, J. P. *et al.* Up-regulation of heat shock proteins is essential for cold survival during insect diapause. *Proc. Natl. Acad. Sci.* **104,** 11130–11137 (2007).

81. Ritossa, F. Discovery of the heat shock response. *Cell stress & chaperones* **1,** 97–98 (1996).

82. Laios, E., Rebeyka, I. M. & Prody, C. A. Characterization of cold-induced heat shock protein expression in neonatal rat cardiomyocytes. *Mol. Cell Biochem.* **173,** 153–159 (1997).

83. Ngosuwan, J., Wang, N. M., Fung, K. L. & Chirico, W. J. Roles of cytosolic Hsp70 and Hsp40 molecular chaperones in post-translational translocation of presecretory proteins into the endoplasmic reticulum. *J. Biol. Chem.* **278,** 7034–7042 (2003).

84. Neal, S. J. *et al.* Thermoprotection of synaptic transmission in a *Drosophila* heat shock factor mutant is accompanied by increased expression of Hsp83 and DnaJ-1. *Physiol Genomics* **25,** 493–501 (2006).