

**DEVELOPMENTALLY REGULATED HSP40S MEDIATE DIAPAUSE AND
STRESS TOLERANCE OF *ARTEMIA FRANCISCANA***

by

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Dedicated to my grandparents, Mary and Michael Rowarth. Thank you for all of your love and support in helping make me the man I am today.

TABLE OF CONTENTS

LIST OF TABLES	vi
LIST OF FIGURES	vii
ABSTRACT	viii
LIST OF ABBREVIATIONS USED	ix
ACKNOWLEDGMENTS	xi
CHAPTER 1 INTRODUCTION	1
1.1 DIAPAUSE AND QUIESCENCE.....	1
1.2 <i>A. FRANCISCANA</i> , A MODEL ORGANISM FOR THE STUDY OF DIAPAUSE	3
1.3 MOLECULAR CHAPERONES	7
1.3.1 The heat shock protein network.....	7
1.3.2 Hsp40 and the co-chaperones	9
1.4 HSP SYNTHESIS DURING DIAPAUSE	11
1.4.1 Insect HSPs during diapause and stress	11
1.4.2 HSPs in <i>A. franciscana</i> during diapause and development	13
1.4.3 Hsp40 synthesis during diapause and development.....	14
1.5 Hsp40 DURING <i>A. FRANCISCANA</i> DIAPAUSE AND DEVELOPMENT	15
CHAPTER 2 MATERIALS & METHODS	17
2.1 CULTURE OF <i>A. FRANCISCANA</i>	17
2.2 CLONING AND SEQUENCING OF <i>A. FRANCISCANA</i> HSP40 cDNAs.....	17
2.2.2 Synthesis of ArHsp40 in <i>Escherichia coli</i>	19
2.2.3 Determining the specificity of antibodies to Hsp40	20
2.3 <i>ARHSP40</i> mRNA AND PROTEIN QUANTIZATION DURING POST-DIAPAUSE DEVELOPMENT OF <i>A. FRANCISCANA</i>	22
2.3.1 ArHsp40 mRNA quantization in post-diapause <i>A. franciscana</i>	22
2.3.2 ArHsp40 quantization in post-diapause <i>A. franciscana</i>	23
2.4 HEAT INDUCTION OF ARHSP40	24
2.5 KNOCKDOWN OF ARHSP40 IN <i>A. FRANCISCANA</i> CYSTS AND NAUPLII	25
2.5.1 Synthesis of double-stranded RNA.....	25
2.5.2 Injection of <i>A. franciscana</i> adult females with dsRNA	25
2.5.3 Knockdown of ArHsp40 mRNAs in <i>A. franciscana</i> cysts and nauplii.....	26
2.5.4 Knockdown of ArHsp40 in <i>A. franciscana</i> cysts and nauplii.....	27

2.6 FEMALE VIABILITY AND EMBRYO DEVELOPMENT AFTER ARHsp40 KNOCKDOWN.	27
2.7 VIABILITY AND STRESS TOLERANCE OF <i>A. FRANCISCANA</i> NAUPLII DEFICIENT IN ARHsp40	28
2.7.1 Viability of nauplii released from adult females.....	28
2.7.2 Heat tolerance of nauplii deficient in ArHsp40	28
2.8 STRESS TOLERANCE AND DEVELOPMENT OF <i>A. FRANCISCANA</i> CYSTS DEFICIENT IN ARHsp40	28
2.8.1 Metabolism of cysts	28
2.8.2 Monitoring development and diapause entry of cysts deficient in ArHsp40...	29
2.8.3 Viability of <i>A. franciscana</i> cysts deficient in ArHsp40.....	29
2.9 IMAGE PROCESSING AND ANALYSIS.....	30
2.10 STATISTICAL ANALYSIS	30
CHAPTER 3 RESULTS	31
3.1 CLONING OF <i>ARHSP40</i> cDNAs	31
3.2 ANTI40-TYPE1 AND ANTI40-TYPE2 IMMUNOREACTIVITIES	31
3.3 THE POST-DIAPAUSE SYNTHESIS OF <i>ARHSP40-1</i> AND <i>ARHSP40-2</i> WAS DEVELOPMENTALLY REGULATED.....	40
3.4 ARHsp40-1 AND ARHsp40-2 WERE STRESS INDUCIBLE.....	40
3.5 INJECTION OF <i>A. FRANCISCANA</i> FEMALES WITH DSRNAs	40
3.6 INJECTION OF <i>A. FRANCISCANA</i> FEMALES WITH ARHsp40 DSRNA AFFECTED NEITHER THEIR SURVIVAL NOR EMBRYO DEVELOPMENT	49
3.7 ARHsp40-1 AND ARHsp40-2 WERE KNOCKED DOWN BY RNAi IN <i>A. FRANCISCANA</i> CYSTS AND NAUPLII	52
3.8 ARHsp40 KNOCKDOWN REDUCED THE VIABILITY OF <i>A. FRANCISCANA</i> NAUPLII	52
3.8.1 ArHsp40-1 and ArHsp40-2 knockdown reduced survival of developing nauplii	52
3.8.2 ArHsp40-1 and ArHsp40-2 knockdown reduced the heat tolerance of nauplii	59
3.9 CYSTS DEFICIENT IN ARHsp40-1 SPONTANEOUSLY ABORTED DIAPAUSE ENTRY ...	59
3.10 LOSS OF ARHsp40 LOWERED THE STRESS TOLERANCE OF CYSTS	59
CHAPTER 4 DISCUSSION.....	67
4.1 TYPE 1 AND TYPE 2 Hsp40 cDNA FROM <i>A. FRANCISCANA</i>	67
4.2 ARHsp40s ARE DEVELOPMENTALLY REGULATED AND HEAT INDUCIBLE POST-DIAPAUSE	68

4.3 ARHsp40-1 AND ARHsp40-2 dsRNAs KNOCKED DOWN CORRESPONDING PROTEINS	69
4.4 LACK OF ARHsp40s DID NOT ALTER THE SURVIVAL OF <i>A. FRANCISCANA</i> FEMALES OR THE DEVELOPMENT OF EMBRYOS	70
4.5 ARHsp40s AID THE POST-DIAPAUSE DEVELOPMENT OF <i>A. FRANCISCANA</i> NAUPLII AND PROTECT AGAINST HEAT	70
4.6 CYSTS DEFICIENT IN ARHsp40-1 SPONTANEOUSLY ABORT DIAPAUSE ENTRY	72
4.7 ARHsp40s PROVIDE STRESS TOLERANCE TO CYSTS.....	73
4.8 CONCLUSIONS AND FUTURE WORK.....	75
REFERENCES	77
APPENDIX A SUPPLEMENTAL DATA.....	88
APPENDIX B MICROPIPETTE PREPERATION	95
APPENDIX C SOLUTIONS AND RECIPES	96
APPENDIX D COPY RIGHT RELEASE LETTERS	98

LIST OF TABLES

Table 1. Primers used for PCR	19
Table A1. ArHsp40-2 shares sequence identity with animal type 2 Hsp40s.....	88

LIST OF FIGURES

Figure 1. Post-diapause life history stages of <i>A. franciscana</i>	4
Figure 2. ArHsp40-1 sequence	32
Figure 3. ArHsp40-2 sequence	34
Figure 4. Sequence alignment of ArHsp40-1 and ArHsp40-2.....	36
Figure 5. Specificity of antibodies raised to ArHsp40-1 and ArHsp40-2.....	38
Figure 6. <i>ArHsp40-1</i> and <i>ArHsp40-2</i> mRNA were developmentally regulated in post-diapause <i>A. franciscana</i>	41
Figure 7. ArHsp40-1 and ArHsp40-2 were developmentally regulated in post-diapause <i>A. franciscana</i>	43
Figure 8. ArHsp40-1 and ArHsp40-2 were induced by heat shock in post-diapause larvae of <i>A. franciscana</i>	45
Figure 9. Injection of <i>A. franciscana</i> females with dsRNAs	47
Figure 10. Injection of <i>A. franciscana</i> females with ArHsp40 dsRNAs affected neither their survival nor embryo development	50
Figure 11. Knock down of ArHsp40-1 and ArHsp40-2 in <i>A. franciscana</i> cysts	53
Figure 12. Knock down of ArHsp40-1 and ArHsp40-2 in <i>A. franciscana</i> nauplii.....	55
Figure 13. The knockdown of ArHsp40-1 and ArHsp40-2 reduced the viability of <i>A. franciscana</i> nauplii.....	57
Figure 14. The knockdown of ArHsp40-1 and ArHsp40-2 reduced heat tolerance of <i>A. franciscana</i> nauplii.....	61
Figure 15. Cysts lacking ArHsp40-1 aborted diapause entry	63
Figure 16. ArHsp40-1 and ArHsp40-2 contributed to the stress tolerance of <i>A. franciscana</i> cysts.....	65
Figure A1. Standard curves for quantifying copy numbers of <i>ArHsp40s</i> and <i>α-tubulin</i> mRNA transcripts in post-diapause life-history stages.....	89
Figure A2. Standard curves for quantifying copy numbers of <i>ArHsp40s</i> and <i>α-tubulin</i> mRNA transcripts in knockdown cysts and nauplii.....	91
Figure A3. Metabolic activities of cysts lacking ArHsp40-1 remained elevated	93

ABSTRACT

Female *Artemia franciscana* release either swimming nauplii or cysts, the latter entering a reversible state termed diapause, characterized by developmental arrest, reduced metabolism and increased stress tolerance. That cysts survive diapause is due in part to heat shock proteins (HSPs), which protect proteins from irreversible denaturation and assist in their folding, intracellular translocation or degradation. This study examines how Hsp40 affects development, diapause and stress tolerance of *A. franciscana*. Two Hsp40 cDNAs, ArHsp40-1 and ArHsp40-2, were cloned. The synthesis of both Hsp40s was regulated during post-diapause development of larvae and induced by stress. The ArHsp40s were individually knocked down by RNAi and cysts lacking either ArHsp40 exhibited reduced stress tolerance. Many cysts deficient in ArHsp40-1 aborted diapause and resumed growth, demonstrating a role in diapause entry. Knowing how Hsp40s influence *A. franciscana* development and diapause has implications in aquaculture as this organism is a common feed for commercially important species.

LIST OF ABBREVIATIONS USED

aa	amino acid
<i>ArHsp40-1</i>	<i>Artemia franciscana</i> type 1 Hsp40 gene
<i>ArHsp40-2</i>	<i>Artemia franciscana</i> type 2 Hsp40 gene
AU	arbitrary units
bp	base pairs
DPBS	Dulbecco's phosphate buffered saline
dsRNA	double-stranded RNA (ribonucleic acid)
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
g	acceleration due to gravity, 9.81 m/s ²
GFP	green fluorescent protein
GSL	Great Salt Lake
h	hour
HRP	horse radish peroxidase
IgG	immunoglobulin G
kDa	kiloDaltons
LEA	late embryonic abundant protein
min	minute
mRNA	messenger RNA (ribonucleic acid)
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
qRT-PCR	reverse transcription quantitative PCR

SDS-PAGE	sodium dodecylsulfate - polyacrylamide gel electrophoresis
siRNA	short interfering RNA
sHSP	small heat shock protein
TBE	Tris, boric acid, EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline Tween
TE	Tris, EDTA
Tris	tris-(hydroxymethyl) aminomethane
Tween	polyoxyethylene sorbitan monolaurate
UV	ultraviolet

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CHAPTER 1 INTRODUCTION

1.1 Diapause and Quiescence

Diapause is a class of dormancy that allows animals to enter a physiological state wherein they are able to cope with unfavourable environmental conditions (Denlinger & Armbruster, 2014; Denlinger, 2002; King & MacRae, 2014; Košťál, 2006). Endogenous signals control diapause leading to the arrest of development and the cell cycle, the limitation of metabolism, and enhancement of stress tolerance (Hahn & Denlinger, 2007; MacRae, 2010). The degree to which these characteristics are imposed varies across species and life history stage (Renfree & Shaw, 2000; Lopes et al., 2004; Renfree & Shaw, 2014). Many phyla of animals possess species that undergo diapause at one or multiple stages of their life history (King & MacRae, 2014; Musolin & Numata, 2003; Ragland et al., 2011).

In many phyla, diapause is facultative and initiation, regulated by hormones in response to external cues such as gradual changes in daylight length, feeding or crowding and temperature modifications, occurs in anticipation of future stress. In some animals, diapause is obligatory and it occurs at particular life history stages regardless of external conditions and cues (Denlinger et al., 2012; House, 1967; Wipking, 1988). Both facultative and obligate diapause are strategies to overwinter until favourable growth conditions return. Although restricted to a single life history stage in each species, insects and crustaceans have the potential for diapause to occur in embryos, multiple larval stages, pupae and adults (King & MacRae, 2014).

Diapause is divided into three phases termed initiation, maintenance and termination, and in some cases these divisions are further subdivided (Košťál, 2006;

Shiomi et al., 2015; Yocum et al., 2009). Diapause initiation is characterized by diet change, differential gene expression and protein synthesis, structural changes for cell or tissue defense, and lipid accumulation (Hahn & Denlinger, 2007; Hayward et al., 2005; MacRae, 2010). Initiation leads to maintenance where cell cycle and division cease, metabolism is limited and programmed cell death inhibited, but most notably where stress tolerance and metabolic depression are maximized. The termination phase of diapause can be induced by different factors in insects and crustaceans. Some insects require a temperature or photoperiod change to exit diapause while some require acute stress (Tachibana & Numata, 2004; Terao et al., 2012). Diapause termination is mediated by the absence of ecdysteroids as well as hexane treatment in insects like the flesh fly *Sarcophoga crassipalpis* (Fujiwara & Denlinger, 2007) and by external stress such as desiccation, freezing or exposure to H₂O₂ in the crustacean *Artemia franciscana* (Robbins et al., 2010). So far, physical, chemical, hormonal and environmental conditions are known to induce diapause induction and termination yet the molecular mechanisms that control diapause remain unknown.

Another state of dormancy distinguishable from diapause is quiescence. The onset and termination of quiescence is triggered by external cues such as anoxia, freezing or desiccation. The termination of quiescence is in response to favourable growth conditions unlike diapause which is programmed with a fixed period of dormancy (Denlinger et al., 2012; Hayward et al., 2005). Subsequent to diapause and quiescence, organisms continue development and growth or they can re-enter quiescence (Bagshaw et al., 1986; MacRae, 2016), remaining dormant and stress tolerant, until temperature, oxygen, photoperiod, humidity, pH and/or salinity are suitable.

Entry into diapause allows animals to avoid environmental conditions that are non-optimal for life processes while favouring the synchronization of reproduction. Diapause occurs in almost all animal phyla but predominately in insects where ecologically relevant pollinators, pests and pathogen vectors rely on diapause to survive winter and then continue their lives when food is abundant and other conditions are favourable in the summer. Diapause enhances the impact of many animals on the world's ecology and economy, thus studying the critical mechanisms that control diapause initiation, maintenance and termination has important implications for aquaculture, agriculture and medicine where diapause contributes to the resistance of pest organisms to eradication.

1.2 *A. franciscana*, a Model Organism for the Study of Diapause

The *Artemia* spp. is widely distributed and populates hypersaline lakes and ponds across all continents except Antarctica, with *A. franciscana* the dominant species (Muñoz et al., 2008; Pacios & Muñoz, 2010). *Artemia* develop by two distinct pathways, either ovoviviparously or oviparously (King et al., 2014; Liang & MacRae, 1999). During the former, fertilized eggs develop into nauplii in the female's brood sac and approximately five days post-fertilization the swimming larvae are released from the mother (Fig. 1 e, f) (Liang & MacRae, 1999; MacRae, 2003). Under optimal growing conditions larvae go through several molts and develop into sexually mature adults in approximately 4-5 weeks. The development mode shifts with changing environmental parameters such as photoperiod and temperature, and as for insects, female *Artemia* detect these changes in

Figure 1. Post-diapause life history stages of *A. franciscana*

Light micrographs of *A. franciscana* obtained with a Nikon AZ100 microscope. **(a)** Cysts prior to emergence (0 h). **(b)** Emerged nauplius 1 (E1). **(c)** Emerged nauplius 2 (E2). **(d)** Emerged nauplius 3 (E3). **(e)** Instar 1 nauplius. **(f)** Early instar 2 nauplius. Early and late (not shown) instar 2 nauplii are morphologically similar. ES, eye spot. Scale bars: 102 μm (a), 250 μm (b, c), 140 μm (d), 107 μm (e), 115 μm (f).



cues that forecast adverse conditions and favor the initiation of diapause. In the oviparous pathway, embryo development is arrested at the gastrula phase comprised of approximately 4000 cells and these embryos are coated with a chitinous shell that is impermeable to non-volatile compounds (Ma et al., 2013; MacRae, 2016).

The encysted embryos, termed cysts (Fig. 1a), are released from the brood sac of the mother and after 6-10 days enter diapause exhibiting a high level of metabolic depression (Clegg et al., 1996; Hand et al., 2013; Warner et al., 2010). *Artemia* cysts resist extreme temperature stress (King et al., 2013; Toxopeus et al., 2014), ultraviolet (UV) radiation (Liu et al., 2009; MacRae, 2010, 2016), anoxia (Clegg, 1997; Clegg et al., 2000), and desiccation (Clegg, 2005; Toxopeus et al., 2014). Once diapause terminates the embryo resumes growth, a nauplius emerges from the cyst (Fig. 1b-e) and development continues if environmental conditions are favourable. The specific environmental cues that terminate diapause vary between *Artemia* species and between populations of an ecotype (Drinkwater & Crowe, 1987; Robbins et al., 2010). In the laboratory, desiccation and freezing, but not desiccation alone, terminate diapause in *A. franciscana* cysts from the Great Salt Lake (GSL), Utah (King & MacRae, 2012; Toxopeus et al., 2014). In contrast, *A. franciscana* cysts from the San Francisco Bay, California only require desiccation without freezing to terminate diapause (Clegg et al., 2001). Hydrogen peroxide (H₂O₂) terminates diapause in embryos of many populations of *A. franciscana* (Robbins et al., 2010; Veeramani & Baskaralingam, 2011). The ability of *A. franciscana* to survive extreme environmental insults makes them one of the most stress tolerant metazoans (MacRae, 2016).

1.3 Molecular Chaperones

1.3.1 The heat shock protein network

Molecular chaperones, or heat shock proteins (HSPs) as they are known, are important contributors to stress tolerance, functioning within networks of complementary proteins to fold, transport and degrade proteins in the cell. The molecular chaperones consist of several ubiquitous families of proteins and they are divided into groups based on sequence, molecular mass and function, including Hsp100, Hsp90, Hsp70, Hsp60, and the small heat shock proteins (sHSPs) (Kampinga et al., 2009; Lindquist & Craig, 1988; Wang et al., 2004, Fan et al., 2013; Haslbeck et al., 2005; Jakob et al., 1993; Sun & MacRae, 2005). The main role of the sHSPs, all of which are characterized by an α -crystallin domain, is to bind client proteins independently of ATP and prevent their irreversible degradation (Mounier & Arrigo, 2002; Sun & MacRae, 2005; Villeneuve et al., 2006). The sHSPs require an energy dependant chaperone such as Hsp70 to release and refold client proteins successfully (Haslbeck et al., 2005).

The ATP-dependent molecular chaperones promote protein folding and localization, mediate protein degradation and contribute to the disaggregation of intracellular toxic protein aggregates. The collection of HSPs and their co-chaperones form synergistic networks to maintain protein homeostasis and in turn influence important cell processes such as signalling, transcription, and metabolism (Calderwood et al., 2007; Cheng et al., 1989; Goldberg, 2003; Morimoto, 1998; Storey & Storey, 2011). Hsp100, the largest of the ATP-dependent HSPs, functions as a nucleotide exchange factor (NEF) for Hsp70 and as a chaperone in the Hsp70 protein disaggregation system, either refolding or destroying proteins recovered from aggregates (Parsell et al., 1994;

Shorter, 2011). Hsp90, functioning as a dimer, aids the folding of regulatory proteins such as kinases and receptors and influences gene expression and intracellular signalling (Neckers, 2007; Whitesell & Lindquist, 2005). Hsp60 forms a complex multimeric ring, that folds newly synthesized nascent proteins, often in concert with Hsp70, and has an important role in protein transport into mitochondria (Bukau & Horwich, 1998). The best-studied and the most conserved ATP-dependent HSP is Hsp70, a family of proteins, the members of which are either synthesized constitutively or up-regulated in response to stressors. Depending on their modulation by co-chaperones, Hsp70s assists in the folding of nascent and denatured proteins and targets damaged proteins for degradation (Mayer & Bukau, 2005; Shorter, 2011; Young, 2010).

Because sHSPs are energy independent they are suitable for protein protection when cell metabolism and ATP are limited, such as during diapause. sHSP monomers range in mass from 15-42 kDa but they can form large oligomers. All sHSPs contain a conserved α -crystallin domain that allows the oligomers to bind to the exposed hydrophobic regions of denaturing proteins, thereby preventing their irreversible denaturation and aggregation (Basha et al., 2012; de Jong et al., 1993; Liang & MacRae, 1999; Sun & MacRae, 2005).

The HSP network of molecular chaperones works in concert with co-chaperones to maintain cell homeostasis through protein folding, protection, localization and degradation of proteins. Not only do HSPs properly fold damaged proteins but they ensure the folding of nascent peptides to achieve tertiary structure, therefore affecting cell transcription, growth, apoptosis, signalling, cell cycle and metabolism (Hartl, 1996, 2002).

1.3.2 Hsp40 and the co-chaperones

Molecular chaperones are required to produce native proteins and to refold or degrade denatured proteins and thus maintain cell homeostasis. Hsp70 is a major cytosolic HSP responsible for the folding of proteins and it also expands the proteome by promoting the assembly of complex proteins that cannot form without chaperone assistance. Conformational changes in Hsp70 to either bind, refold, release or target proteins for degradation are modulated by Hsp70 ATPase activity which is influenced by co-chaperones (Cyr, 2008; Mayer & Bukau, 2005).

The molecular co-chaperones modulate the ATPase activity of Hsp70 and thus the activity of Hsp70 in protein refolding by affecting the hydrolysis of bound ATP or acting as NEFs which replace bound ADP with ATP. The BAG-1 protein inhibits Hsp70 ATPase during the refolding of denatured proteins *in vitro* and directs Hsp70 bound proteins to proteasomes for degradation (Lüders et al., 2000; Nollen et al., 2000; Takayama et al., 1997). The Hsp70 interacting protein (Hip) and the Hsp70 organizing protein (Hop) both strengthen Hsp70-substrate binding by inhibiting NEFs from removing bound ADP (Chen & Smith, 1998; Johnson et al., 1998; Li et al., 2013). Hip and Hop allow further protein folding by Hsp70, while Hop mediates Hsp90-Hsp70-substrate binding promoting higher order protein stabilization (Odunuga et al., 2004). To release folded proteins Hsp70 NEFs are required to promote the exchange of bound ADP for ATP. Hsp110 is the major NEF for Hsp70 in the cells of metazoans (Andréasson et al., 2008; Mogk et al., 2015).

The Hsp40 family, the major focus of this thesis, was initially recognized as a group of Hsp70 co-chaperones (Glover & Lindquist, 1998; Hennessy et al., 2005).

Hsp40s, known as J-domain proteins, facilitate the delivery of non-native proteins to the Hsp70 substrate binding domain for refolding and by enhancing Hsp40 ATPase activity they accelerate protein refolding within cells (Fan et al., 2003; Hernández et al., 2002). Hsp40s constitute a widely diverse chaperone family and they are divided into three main groups all containing a J-domain characterized by a highly conserved HPD motif between α -helix II and III which interacts with Hsp70 and activates its ATPase (Hennessy et al., 2005; Lee et al., 2002).

Following in order from the amino-terminal, type 1 J-domain proteins possess an amino-terminal J-domain, a G/F rich region that recognizes subsets of client proteins and facilitates Hsp70 binding, a zinc-binding domain for polypeptide substrate interaction and a carboxyl-terminal domain for dimer formation with other J-domain proteins. Type 2 Hsp40s are similar to Type 1 Hsp40s but lack a zinc-binding domain. Type 3 Hsp40s possess a J-domain found anywhere in the protein sequence, but lack a G/F rich region and a zinc-binding domain, and each is limited in its range of clients (Cyr & Ramos, 2015; Ramos et al., 2008).

J-domain proteins possess chaperone activity in their own right and they aid in the Hsp70-Hsp40-Hsp110 disaggregation system and the refolding of proteins, processes that involve the stimulation of ATPase in Hsp70 and Hsp110 (Nillegoda et al., 2015; Shorter, 2011). *In vitro* studies show that the efficiency of the Hsp70-Hsp100 refolding system, using heat aggregated luciferase as substrate, is greater with equal amounts of type 1 and type 2 Hsp40s present rather than one of them alone (Nillegoda & Bukau, 2015).

The J-proteins and other co-chaperones play a crucial role in synergistically working with Hsp70 to rapidly recruit and refold aggregated and damaged proteins. The

abundance and ratios of co-chaperones in response to stress influence Hsp70 performance and protein quality control.

1.4 HSP Synthesis During Diapause

1.4.1 Insect HSPs during diapause and stress

During diapause organisms exhibit arrested development, metabolic depression and high stress tolerance. Moreover, HSPs are synthesized differently to protect the organism from stress exposure and prepare for periods of low metabolism. Insects are the best studied organisms for investigating HSP synthesis in preparation for diapause and post-diapause development because many of them over-winter and/or survive periods of low nutrient and ATP availability in diapause (King & MacRae, 2015; Rinehart et al., 2007).

Diapause destined embryos of the cricket *Allonemobius socius* down-regulate *Hsp20.7* and *Hsp90* mRNA while the amounts of *Hsp70* transcripts remain unchanged (Reynolds & Hand, 2009). The larvae of the corn stalk borer, *Sesamia nonagrioides* up-regulate *Hsp90* and *Hsc70* mRNA and down-regulate *SnoHsp20.8* and *Hsp70* mRNA during diapause, then accumulate *Hsp70* transcripts after diapause termination (Gkouvtis et al., 2009; Gkouvtis et al., 2008). Proteomic studies show that the synthesis of Hsp90 down-regulates and Hsp20 up-regulates during diapause of the parasitic wasp larvae *Nasonia vitripennis* and this may be associated with the depression of metabolism and limited availability of ATP (Wolschin & Gadau, 2009). By comparison, diapausing larvae of the blow fly *Calliphora vicina* up-regulate *hsp23*, *hsp24*, and *hsp70* transcripts (Fremdt et al., 2013). In larvae of the blow fly *Lucilia*

sericata transcripts encoding *Hsp23*, *Hsp70* and *Hsp90* do not change in amounts during transition into diapause but at termination *Hsp90* mRNA up-regulates upon the resumption of development and metabolism (Tachibana et al., 2005). Clearly, there is no conserved pattern of HSP gene expression or synthesis in preparation for diapause in insects. The coordinated synthesis of HSPs and sHSPs upon diapause entry implies their role in protecting proteins during diapause when metabolism is limited.

RNA interference (RNAi) experiments have been used with insects to test the roles of HSPs in diapause regulation and stress tolerance. The knockdown of sHSP mRNAs decreases the cold recovery of *Drosophila melanogaster* (Colinet et al., 2010) and the heat resistance of leaf beetle *Gastrophysa actrocyanea* adults, implying a role for the sHSPs in protection against temperature stress (Atungulu et al., 2006). Reduction of *Hsp70* by RNAi in the linden bug *Pyrrhocoris apterus* (Košťál & Tollarová-Borovanská, 2009) and the flesh fly *S. crassipalpis* (Rinehart et al., 2007) lowers their ability to survive after cold and heat stress. The knockdown of *Hsp23* and *Hsp70* in *S. crassipalpis* pupae during diapause decreases their resistance to cold and heat but does not alter their ability to enter diapause (Rinehart et al., 2007).

Several proteomic and genomic studies have shown that insects synthesize diapause specific HSPs. The degradation of insect HSPs by RNAi demonstrates that they protect protein integrity during temperature stress in diapausing embryos, pupae and adults. However, knockdown of insect HSPs has not been shown to interrupt entry into or termination of diapause, albeit only a limited number of experiments have been performed.

1.4.2 HSPs in A. franciscana during diapause and development

Molecular chaperones interact with denatured or nascent proteins and either fold them into their native state or protect them from irreversible aggregation. The sHSPs form oligomers, binding to proteins and protecting them from degradation or aggregation without the need for ATP. The sHSPs are tightly associated with diapause and development in insects and also in *A. franciscana*. In preparation for diapause and quiescence *A. franciscana* cysts up-regulate the sHSPs p26, ArHsp22, and ArHsp21 and the ferritin homologue artemin, of which p26 and artemin are thought to sequester denaturing proteins when ATP concentration is low and ATP-dependent chaperones mostly non-functional (King et al., 2013; King & MacRae, 2014). p26 is only synthesized in diapause destined embryos and constitutes about 7% of the soluble protein in cysts. p26 translocates to the nuclei of diapause cells during heat, anoxia and pH stress. RNAi experiments have shown that p26 plays an important role in protecting diapausing cysts from stress imposed by heat, desiccation and freezing. Knockdown of p26 also leads to spontaneous termination of diapause and allows nauplii to emerge successfully after approximately 90 days incubation in sea water at room temperature (King & MacRae, 2012). These results show that p26 provides stress tolerance and regulates maintenance/termination of diapause in *A. franciscana* cysts.

ATP-dependent HSPs such as Hsp70 and Hsp90 are synthesized in developing *A. franciscana* and *A. sinica* embryos that are destined for diapause but their role in stress tolerance and diapause mediation is undetermined (Chen et al., 2003; Clegg, 2000; MacRae, 2010; Zhou et al., 2008). HSPs also play a role in *A. franciscana* nauplii development. Embryos destined for nauplii development up-regulate transcripts for

Hsp60, Hsp70, Hsp90 and Hsp110 (Chen et al., 2003). Hsp70 is up-regulated in nauplii under heat stress and it plays a role in resisting *Vibrio campbellii* infection (Baruah et al., 2012; Norouzitallab et al., 2015).

As shown for non diapausing and diapausing insects (Fan et al., 2013; Saravanakumar et al., 2008; Sasibhushan et al., 2013), nauplius-destined and diapause-destined embryos of *A. franciscana* synthesize different HSPs. *A. franciscana* embryos destined for diapause synthesize the sHSPs ArHsp21, ArHsp22, and p26, the latter contributing to stress tolerance during diapause (King & MacRae, 2012; Qiu & MacRae, 2008; Sun & MacRae, 2005). Developing nauplii synthesize ATP-dependent HSPs such as Hsp70 either constitutively or in response to stress presumably in order to maintain cell homeostasis (Clegg et al., 2000; Willsie & Clegg, 2001). Other than the detection of Hsp60 and Hsp70 in *Artemia* (Wang et al., 2007; Zhou et al., 2008), little is known about how the ATP-dependent HSPs and co-chaperones contribute to development and stress tolerance during cyst diapause.

1.4.3 Hsp40 synthesis during insect diapause and development

The synthesis of *Hsp40* mRNA is heat inducible in the leaf miner *Liriomyza sativa* and cold inducible in *Drosophila* (Colinet et al., 2010; Huang et al., 2009). Under oxidative stress, *Hsp40* mRNA is up-regulated in *S. crassipalpis* and *Drosophila* adults (Liu et al., 2006; Michaud et al., 2011). Hsp40s are developmentally regulated in insects preparing for diapause. Larvae of the gall fly *Eurosta solidaginis* synthesize Hsp70 during diapause and Hsp40 under freezing stress, while sHSPs, Hsp60 and Hsp110 are up-regulated during recovery from freezing (Zhang et al., 2011). A comparative analysis

between diapause and non-diapause destined embryos of *B. mori* revealed that *Hsp70*, *Hsc70*, and *Hsp40* mRNA increases upon diapause entry and metabolic depression (Hwang et al., 2005).

J-domain proteins work with other co-chaperones to accelerate ATP hydrolysis by Hsp70 and promote substrate binding and refolding. Hsp40s may be up-regulated in diapausing organisms such as insects, which favours substrate binding to Hsp70 as metabolism declines and free ATP becomes scarce. In the early diapause of *S. crassipalpis* Hsp40 is up-regulated and hyper-phosphorylated in the brain (Pavlidis et al., 2011; Ragland et al., 2010), but how this contributes to diapause is unknown. There have been no experiments investigating how J-proteins contribute to diapause regulation by loss of function in insects or crustaceans such as *A. franciscana*.

1.5 Hsp40 Function during *A. franciscana* Development and Diapause

In this investigation two Hsp40 cDNAs termed ArHsp40-1 and ArHsp40-2 were cloned and sequenced from *A. franciscana*. Immunoprobings of western blots and qRT-PCR revealed that ArHsp40 is developmentally regulated during post-diapause growth of nauplii emerging from cysts. In addition to being developmentally regulated, ArHsp40s were induced by heat stress in 1st instar nauplii before returning to basal levels during recovery. The function of Hsp40 in *A. franciscana* cysts and nauplii was investigated by RNAi. Ovoviviparously produced nauplii deficient in *ArHsp40s* exhibited shorter life spans and were less heat tolerant than control groups. Cysts deficient for *ArHsp40s* were less viable after desiccation and freezing. Cysts deficient in *ArHsp40-1* spontaneously aborted diapause entry 2-5 days post release from females and hatched to yield nauplii

which developed normally into adults. The abortion of diapause by cysts lacking ArHsp40-1 provides a foundation for future proteomic and genomic studies of the molecular mechanisms controlling diapause entry. Moreover, from a practical perspective this study provides information on J-proteins, diapause regulation and stress tolerance in insects and other arthropods that impact aquaculture, agriculture, forestry and human health.

CHAPTER 2 MATERIALS & METHODS

2.1 Culture of *A. franciscana*

A. franciscana cysts from the Great Salt Lake (GSL) (INVE Aquaculture Inc., Ogden, UT, USA), hydrated for at least 3 h on ice in distilled H₂O and collected by suction filtration, were incubated at room temperature in 1 µm filtered, UV treated and autoclaved 33.0 ppt saltwater from Halifax Harbor, NS, Canada, hereafter termed seawater. Cultured animals were fed *Isochrysis sp.* (clone synonym T-Iso) from The Provasoli-Guillard National Centre for Culture of Marine Phytoplankton (West Boothbay Harbour, Maine, USA). Animals were collected for mRNA and protein preparation following hydration (cysts), after 5 h at 27 °C (cysts), after 10 h at 27 °C (emerged E1) and as emerged, membrane-enclosed E2/E3 nauplii. Collected animals were homogenized (Fig. 1). After hatching of E2/E3 nauplii, 1st instar larvae (Fig. 1) were collected by photo-taxis (Langdon et al., 1991; Jiang et al., 2016) and either homogenized immediately or incubated at 27 °C for either 16 h or 26 h to respectively generate early and late 2nd instar larvae.

2.2 Cloning and Sequencing of *A. franciscana* Hsp40 cDNAs

One mg of 5 h cysts (Fig. 1b) was harvested from seawater on 5 µm nylon mesh filters (Spectrum Labs Inc., Rancho Dominguez, CA, USA), flash frozen in liquid nitrogen and then homogenized in 500 µl of TRIzol® (Invitrogen, Burlington, ON, Canada). RNA was extracted according to manufacturer's instructions. Single-strand cDNA was synthesized with the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Burlington, ON, Canada). RNA was quantified by measuring the

absorbance at 260 nm and 0.1 µg mRNA was used as a template for synthesizing cDNA with SuperScript® III First-Strand Synthesis Kit for RT-PCR (Invitrogen, Burlington, ON, Canada) and oligo dT₂₀ following the manufacturer's instructions. All RNA samples had corresponding negative controls prepared lacking reverse transcriptase to confirm the absence of genomic DNA.

To obtain partial *Hsp40 type 1* and *type 2* cDNAs from *A. franciscana*, the NCBI Expressed Sequence Tag (EST) database was searched for *Hsp40 type 1* and *type 2* sequences using *Daphnia pulex* (Accession number EFX77852.1) and (Accession number EFX80146.1) sequences respectively as references. The sequences obtained were then BLASTED to the *A. franciscana* genome Online Resource for Community Annotation of Eukaryotes (ORCAE) database for likely Hsp40 cDNAs (Sterck et al., 2012). Based on sequence comparisons, primers containing restriction enzyme sites for the amplification of full length *A. franciscana Hsp40* cDNAs were prepared (Integrated DNA Technologies, Coralville, IA, USA) (Table 1) and used to amplify PCR products from cyst, E2/E3, and 1st instar cDNAs. The products, resolved in 1.0 % agarose gels and purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), were then ligated into TA vectors (TOPO TA Cloning Kit, Life Technologies, Burlington, ON, Canada) and used to transform competent TOP10 *Escherichia coli* (Invitrogen, Burlington, ON, Canada). Recombinant TA vectors containing cDNA inserts of the appropriate size were isolated with the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Oakville, ON, Canada), and inserts were sequenced (DNA Sequencing Facility, Centre for Applied Genomics, Hospital for Sick Children, Toronto, ON, Canada) revealing *Hsp40-1* and *Hsp40-2* cDNAs (Jiang et al., 2016; Qiu & MacRae, 2007).

Table 1. Primers used for PCR.

Primer function Gene	Primer sequence (5' to 3')	
	Forward	Reverse
dsRNA template production^a		
ArHsp40-Type 1	TAATACGACTCACTATAGGGA GTGCATCAGTTGAGCGTCAC	TAATACGACTCACTATAGGGA TCCGGGGTCCACATGTACTTC
ArHsp40-Type 2	TAATACGACTCACTATAGGGA GTGGTTCTCCAGTTTTGGGG	TAATACGACTCACTATAGGGA ACCACGTCCGCTGGGATTTTA
GFP ^b	TAATACGACTCACTATAGGGA GACACATGAAGCAGCAGACTT	TAATACGACTCACTATAGGGA GAAGTTCACCTTGATGCCGTTTC
Full Length PCR*		
ArHsp40-Type 1	CGATGGGGATCCATGCCA AAGGAAACAGGGTTGTATG	TACCAGCTGCAGCTACTG GGTTGGACATTGAACTCC
ArHsp40-Type 2	CGATGGGGATCCATGGGA AAGGATTTCTATAAAATAC	GCCGGATCAAGCTTCA ATTAGGCAAGGCATCACG
qRT-PCR		
ArHsp40-Type 1	GTGCATCAGTTGAGCGTCAC	TGCTGAACAATTCCAGGAGC
ArHsp40-Type 2	TGACCCATTGCGGTGGGTTTG	TCGTGTTCAATGGGTGGGTC
α -tubulin ^c	CGACCATAAAAGCGCAGTCA	CTACCCAGCACCACAGGTCTCT

Primers were produced by Integrated DNA Technologies (IDT), Coralville, IA, USA.

a Bolded sequences indicate the T7 promoter region

b Zhao et al. 2012

c King et al. 2013

* Underlined sequences indicate restriction enzyme sites

2.2.2 Synthesis of ArHsp40 in *E. coli*

Full-length cDNAs for *ArHsp40-1* and *ArHsp40-2* were generated by PCR using 0.5 µg of *A. franciscana* cyst cDNA as template. Platinum *Taq* Polymerase (Invitrogen, Burlington, ON, Canada) and full-length primers containing restriction enzyme sites (Table 1) at 0.2 mM were used to amplify *ArHsp40-1* and *ArHsp40-2* using the following reaction: 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 54°C, and 1 min at 72°C, with a final 10 min at 72°C. The full length cDNAs were digested with restriction enzymes at 37 °C overnight and then purified with Wizard® SV PCR Clean-Up System (Promega, Madison, WI, USA). The digested cDNAs were cloned into the His-tagged prokaryotic expression vector pRSET C (Invitrogen). The recombinant plasmids were transformed into *E. coli* BL21(DE3) pLysS (Invitrogen). The synthesis of ArHsp40-1 and ArHsp40-2 was induced with 1 mM isopropyl thio-β-D-galactoside for 6 h at 37 °C and each recombinant ArHsp40 was purified from cell free extracts of *E. coli* with the MagneHis™ Protein Purification System (Promega, Madison, WI, USA) by following the manufacturer's instructions.

2.2.3 Determining the specificity of antibodies to Hsp40

One-hundred mg each of 0 h cysts and 1st instar nauplii (Fig. 1) was recovered from seawater on 5 µm nylon mesh filters (Spectrum Labs Inc., Rancho Dominguez, CA, USA), flash frozen and then homogenized on ice in 100 µl of Pipes buffer (100 mM Pipes, 1 mM MgCl₂, 1 mM EGTA, pH 7.4) with proteolytic enzyme inhibitors (1:100 to homogenate - Halt Protease Cocktail, #87,786, Pierce Biotechnology, Rockford, IL, USA) and centrifuged at 12,000g for 10 min at 4°C. After determination of protein

concentration by the Bradford assay (Bradford, 1976) samples of extracts from 0 h cysts and 1st instar nauplii and purified His-tagged recombinant ArHsp40-1 and ArHsp40-2 were diluted in 4 X treatment buffer (250 mM Tris, 280 mM SDS, 40% (v/v) glycerol, 20% (v/v) β -mercaptoethanol, 0.2% (w/v) bromophenol blue, pH 6.8), placed in a boiling water bath for 5 mins and centrifuged for 10 min at 10,000g at 4 °C.

Ten μ g of protein from each supernatant and 1 μ g of purified recombinant ArHsp40-1, 1 μ g of purified recombinant ArHsp40-2, and 5 μ l Pink Plus Prestained Protein Ladder (FroggaBio Inc., Toronto, ON, Canada) were resolved in 12.5 % SDS polyacrylamide gels at 250 V for 30 min, transferred to 0.2 μ m nitrocellulose membranes (BioRad, Mississauga, ON, Canada) over night at 100 mAmps and blocked for 1 h at room temperature in 5% (w/v) Carnation low fat milk powder in TBS (10 mM Tris, 140 mM NaCl, pH 7.4). Subsequent to blocking, membranes were probed for 15 min at room temperature with commercially obtained polyclonal antibodies (Abiocode, Agoura Hills, CA, USA) raised to either ArHsp40-1 peptide 331-VKFPDVINPALIPQLE-346 (Anti40-type 1) or ArHsp40-2 peptide 277-DALCGTKVDVPTLSGE-292 (Anti40-type 2) diluted 1:1,000 in TBS. Membranes were washed after incubation in primary antibody for 1, 2, 3 and 4 min in TBS-T. Subsequent to washing, membranes were incubated with HRP-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich, Oakville, ON, Canada) diluted 1:10,000 in TBS for 20 min and then washed for 1, 2, 4 and 4 min in TBS-T followed by a final 3 min wash in TBS. Subsequent to the final membrane washing, antibody-reactive proteins were visualized with Clarity ECLTM Western Blotting Substrate (BioRad, Mississauga, ON, Canada) and a MF-Chemi-BIS 3.2 gel documentation system (DNR Bio-Imaging Systems, Neve, Israel).

2.3 *ArHsp40* mRNA and Protein Quantization during Post-Diapause Development of *A. franciscana*

2.3.1 *ArHsp40* mRNA quantization in post-diapause *A. franciscana*

One mg of each of seven different *A. franciscana* life history stages (Fig. 1) were recovered from seawater on 5 µm nylon mesh filters (Spectrum Labs Inc), flash frozen and homogenized in 500 µl of TRIzol® (Invitrogen) prior to RNA extraction following the manufacturer's instructions. RNA was quantified as before and 0.1 µg mRNA was used as a template for synthesizing cDNA with the SuperScript® III First-Strand Synthesis Kit for RT-PCR (Invitrogen) as described above.

For qPCR, forward and reverse primers (Table 1) were used to amplify an *ArHsp40-1* cDNA fragment of 194 bp, an *ArHsp40-2* cDNA fragment of 144 bp, and an *α-tubulin* cDNA fragment of 276 bp, the latter as internal standard. Primer amplification targets for *ArHsp40-1* and *ArHsp40-2* are illustrated in Figs. 2 and Fig. 3, respectively. Primers were at 1 mM and 0.5 µl of cDNA was employed as template using the following reaction: 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 54°C for *ArHsp40-1* (54°C for *ArHsp40-2* and 49°C for *α-tubulin*), and 1 min at 72°C, with a final 10 min at 72°C in a Rotor-Gene RG-3000 system (Corbett Research, Sydney, NSW, Australia). qPCR was conducted with a QuantiFast® SYBR® Green PCR Kit (Qiagen, Mississauga, ON, Canada). Melt curve analysis were calculated by Rotor-Gene 6 Software, qPCR experiments with an efficiency of 90% or greater were accepted for analysis (Corbett Research, Sydney, NSW, Australia).

The mRNA copy numbers for *α-tubulin*, *ArHsp40-1* and *ArHsp40-2* were calculated by the use of standard curves, $R^2 > 0.99$ (Appendix A, Fig. A1) (King et al.,

2013). Standard curves were generated by PCR using 0.5 μ l cDNA with Platinum PCR supermix (Qiagen) and 0.4 mM primers for *ArHsp40-1*, *ArHsp40-2* and *α -tubulin* (Table 1). The PCR product concentrations were determined by measuring the absorbance at 260 nm, and the copy number calculated based on the length of the PCR products using a base pair mass of 650 Da (<http://cels.uri.edu/gsc/cdna.html>). The cDNA standards were diluted in a 10-fold series with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and 0.5 μ l of each dilution was used as template with the QuantiTect® SYBR® Green PCR Kit (Qiagen). All cDNA samples were assayed in duplicate. The Ct values employed to create the standard curves were fitted by linear regression for each of *ArHsp40-1*, *ArHsp40-2* and *α -tubulin* (Appendix, Fig. A1)

2.3.2 *ArHsp40* quantization in post-diapause *A. franciscana*

One-hundred mg of *A. franciscana* in each life history stage shown in Fig. 1 was recovered from seawater on 5 μ m nylon mesh filters (Spectrum Labs Inc), flash frozen in liquid nitrogen and homogenized on ice in 100 μ l of Pipes buffer with proteolytic enzyme inhibitors (Halt Protease Cocktail, Pierce Biotechnology) in a 1:100 ratio (v/v) with homogenate, and centrifuged at 12,000g for 10 min at 4°C. After determination of protein concentration by the Bradford assay (Bradford, 1976) protein samples were diluted in 4 X treatment buffer, placed in a boiling water bath for 5 min and centrifuged for 10 min at 10,000g at 4 °C.

Forty μ g of protein from each supernatant was resolved in 12.5 % SDS polyacrylamide gels at 250 V for 30 min and either stained with Colloidal Coomassie Blue (10% (w/v), ammonium sulfate, 0.1% (w/v), Coomassie G-250, 3% (v/v),

phosphoric acid, 20% (v/v), ethanol) (Candiano et al., 2004) or transferred to 0.2 μm nitrocellulose membranes (BioRad, Mississauga) overnight at 100 mAmps and blocked for 1 h at room temperature in 5% (w/v) Carnation low fat milk powder in TBS. Subsequent to blocking, membranes were probed for 15 min at room temperature with either Anti40-type 1, Anti40-type 2 or a polyclonal antibody to tyrosinated α -tubulin (Anti-Y) (Xiang & MacRae 1995) diluted 1:1,000 in TBS. Membranes were washed after incubation in primary antibody for 1, 2, 3 and 4 min in TBS-T. Subsequent to washing membranes were incubated with HRP-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich) 1:10,000 in TBS for 20 min and then washed for 1, 2, 4 and 4 min in TBS-T followed by a 3 min wash in TBS. Subsequent to the final membrane washing, antibody-reactive proteins were visualized with Clarity™ ECL Western Blotting Substrate (BioRad) and a MF-Chemi-BIS 3.2 gel documentation system (DNR Bio-Imaging Systems).

2.4 Heat Induction of ArHsp40

First instar *A. franciscana* nauplii grown at 27 °C in seawater were collected 4 h after hatching when they contained reduced amounts of ArHsp40-1 and ArHsp40-2, and then incubated in 20 ml seawater in Corex tubes at 39 °C for 1 h in a programmable water bath (VWR International LLC, Mississauga, ON, Canada) (Liang & MacRae 1999). Heat shocked animals were either then harvested from seawater on 5 μm nylon mesh filters (Spectrum Labs Inc.), flash frozen, and homogenized on ice 1:1 (w/v) with proteolytic enzyme inhibitors in Pipes buffer as before or allowed to recover at 27 °C for 2, 4, 6, and 8 h and then collected and homogenized. Forty μm of each cell-free protein

homogenate was separated in SDS polyacrylamide gels, transferred to nitrocellulose, blocked and probed with antibodies Anti40-type1, Anti40-type2 or Anti-Y prior to washing and imaging as described above.

2.5 Knockdown of ArHsp40 in *A. franciscana* Cysts and Nauplii

2.5.1 Synthesis of double-stranded RNA

Platinum *Taq* DNA Polymerase (Invitrogen) and primers containing the T7 promoter sequence (Table 1) were used to amplify *ArHsp40-1* and *ArHsp40-2* by PCR. Additionally, green fluorescent protein (*GFP*) cDNA (control) was amplified from the commercial vector pEGFP-N1 (Clontech, Mountain View, CA, USA) (King & MacRae, 2012; Zhao et al., 2012). *Hsp40-1*, *Hsp40-2* and *GFP* PCR products were resolved in 1.0% agarose gels to determine size and purity. PCR products were used as template for the generation of *ArHsp40-1*, *ArHsp40-2* and *GFP* dsRNA with the MEGAscript® RNAi kit (Ambion Applied Biosystems, Austin, TX, USA). The dsRNAs were resolved by electrophoresis in agarose gels and the dsRNA concentration was determined by measuring absorbance at 260 nm. For the injection of females, each of *Hsp40-1*, *Hsp40-2*, and *GFP* dsRNA was separately diluted to 0.7 ng/nl with 0.5% phenol red in Dulbecco's phosphate buffered saline (DPBS) (King & MacRae, 2012).

2.5.2 Injection of *A. franciscana* adult females with dsRNA

Mature females carrying diapause-destined or nauplius-destined unfertilized eggs were injected in the egg sac with either *ArHsp40-1*, *ArHsp40-2* or *GFP* dsRNA mixed with 0.5% phenol red in DPBS (Sigma-Aldrich, Oakville, ON, Canada) (King & MacRae

2012). Females destined to produce cysts were identified by the presence of a shell gland (Liang & MacRae 1999). Injections were performed under an Olympus SZ61 stereomicroscope (Olympus Canada, Inc., Markham, ON, Canada) with a borosilicate micropipette pulled with a custom programmed P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Co., Novato, CA, USA) and broken at 45° using a clean razor blade (Copf et al., 2004). Each female was placed on 5% agar and lightly blotted with Kimwipes (Sigma-Aldrich) prior to injection with 175 ng of dsRNA in approximately 250 nl using the Nanoject II Microinjector (Drummond Scientific, Co., Broomall, PA, USA). Females were returned to sea water and observed for 3 h to ensure retention of injected dsRNA and maintenance of normal behavioral characteristics (King & MacRae, 2012). Each female was mated with a male 24 h post-injection in a 6-well culture plate containing sea water and they were fed daily.

2.5.3 Knockdown of ArHsp40 mRNAs in A. franciscana cysts and nauplii

Thirty to 50 *A. franciscana* cysts were collected 10 d post-release from females injected with either *ArHsp40-1*, *ArHsp40-2*, or *GFP* dsRNA. Similar amounts of nauplii were collected immediately after release. RNA was extracted by homogenizing flash frozen animals in 100 µl TRIzol® (Invitrogen), and cDNA was synthesized as described above. qPCR was conducted using 0.5 µl cDNA as template (King et al., 2013; King & MacRae, 2012) and 0.4 mM primers for *ArHsp40-1*, *ArHsp40-2* and *α-tubulin* (Table 1) with the same reaction cycle conditions as described above. The experiment was performed in triplicate using three different preparations of cysts or nauplii; each preparation was analyzed in duplicate. cDNA copy numbers were determined from a

standard curve of Ct values ($R^2 > 0.99$), and normalized against α -tubulin (King et al. 2013) (Appendix, Fig. A2).

2.5.4 Knockdown of *ArHsp40* in *A. franciscana* cysts and nauplii

Thirty to 50 *A. franciscana* cysts were collected 10 d post-release from females injected with either *ArHsp40-1*, *ArHsp40-2*, or *GFP* dsRNA. Similar amounts of nauplii were collected immediately after release. Animals were flash frozen and homogenized in 30 μ l of 4 X treatment buffer, placed in a boiling water bath for 5 min and then centrifuged for 10 min at 10,000g at 4 °C. Protein preparations were resolved in 12.5% SDS polyacrylamide gels at 250 V for 30 mins, transferred to 0.2 μ m nitrocellulose membranes (BioRad, Mississauga) overnight at 100 mAmps and blocked for 1 h at room temperature in 5% (w/v) Carnation low fat milk powder in TBS. Subsequent to blocking, membranes were probed either Anti40-type 1, Anti40-type 2 or Anti-Y, washed and imaged as described above.

2.6 Female Viability and Embryo Development After *ArHsp40* Knockdown

The number of females surviving successive brood releases was documented after injection with either *ArHsp40-1*, *ArHsp40-2*, or *GFP* dsRNA. Brood size for the first release was recorded. The time from fertilization, marked by the fusion of egg sacs, until the release of cysts or nauplii was monitored for each brood release. One-hundred sixty-four females were incubated at room temperature for the experiments.

2.7 Viability and Stress Tolerance of *A. franciscana* Nauplii Deficient in ArHsp40

2.7.1 Viability of nauplii released from adult females

Thirty-five to 113 first brood 1st instar nauplii were collected immediately after release from females injected with either *ArHsp40-1*, *ArHsp40-2* or *GFP* dsRNA. The nauplii, incubated at room temperature in seawater contained in hexagonal weigh boats, were fed daily. The number of surviving animals was recorded until 100% mortality was reached. The experiment was performed in triplicate.

2.7.2 Heat tolerance of nauplii deficient in ArHsp40

Thirty-six to 83 first brood 1st instar nauplii, collected immediately after release from females injected with either *ArHsp40-1*, *ArHsp40-2* or *GFP* dsRNA, were incubated in 20 ml seawater in Corex tubes at 39 °C for 1 h in a programmable water bath (VWR International LLC). Nauplii were then allowed to recover in sea water at room temperature for 24 h before counting surviving nauplii. Live nauplii were actively swimming. The experiment was performed in triplicate.

2.8 Stress Tolerance and Development of *A. franciscana* Cysts Deficient in ArHsp40

2.8.1 Metabolism of cysts

Metabolic activity of cysts was determined by monitoring growth medium acidification (Yang & Balcarcel, 2003). Ten cysts either deficient in or containing *ArHsp40-1* and *ArHsp40-2* were incubated immediately after release from females in 100 µl of “medium solution” (seawater containing 1000 U penicillin, 100 µg/ml streptomycin sulfate and 0.03% phenol red at pH 8.5) in Parafilm sealed Costar 96 well UV plates

(Corning Inc., Corning, NY, USA). Other wells contained test solution only, test solution with 10 commercially obtained cysts, or 10 commercially obtained cysts killed by boiling in water for 10 min (INVE Aquaculture, Inc.). The A_{553} of test solutions was measured at 24 h intervals after addition of cysts to wells using a SPECTRAMax PLUS microplate reader (Molecular Devices, Sunnyvale, CA, USA). To measure A_{553} , the test solution from each well, excluding cysts, was transferred to a fresh 96 well plate. After A_{553} measurement residual solution was removed from the original wells containing cysts and fresh test solution was added for the next 24 h incubation. The assay was optimized previously in King & MacRae, 2012. The experiment was performed in triplicate.

2.8.2 Monitoring development and diapause entry of cysts deficient in *ArHsp40*

Thirty-six to 72 cysts were collected immediately after release from adult females injected with either *ArHsp40-1*, *ArHsp40-2* or *GFP* dsRNA. Cysts were either incubated at room temperature in hexagonal weigh boats containing seawater for up to 10 days to allow diapause entry before starting stress tolerance tests (see below) or for up to 120 days to check for spontaneous termination of diapause, which was observed previously in cysts lacking p26 (King & MacRae, 2012). The experiment was performed 6 times.

2.8.3 Stress tolerance of *A. franciscana* cysts deficient in *ArHsp40*

Cysts were collected 10 days after release from females injected with either *ArHsp40-1*, *ArHsp40-2* or *GFP* dsRNA by centrifugation for 1 min at 5000g at room temperature. Seawater was removed with a Pasteur pipette and cysts were blotted dry prior to incubation for 4 weeks at room temperature in a desiccator containing Drierite

(Sigma-Aldrich) and then freezing in 1.5 ml microtubes at $-20\text{ }^{\circ}\text{C}$ for 12 weeks. Cysts were then incubated in seawater at room temperature and the number of hatched swimming nauplii, a measure of viability, was determined. Cysts were monitored for an additional 5 days after the appearance of the last nauplius to ensure no further hatching occurred. Experiments were done in triplicate with 18 to 83 cysts in each replicate.

2.9 Image Processing and Analysis

Images of all developing animals were captured using a Nikon AZ100 microscope. All images and figures were prepared for publication using Photoshop (Adobe Creative Cloud; Adobe Systems Inc.). When required to improve image quality, modifications to brightness, contrast and colour were made evenly. Bands of immunoreactive proteins on western blots were quantified with Image Studio Software (Li-Core Biosciences, Lincoln, NE, USA), and band intensities for ArHsp40-1 and ArHsp40-2 were compared to band intensities for tyrosinated α -tubulin at each development stage examined.

2.10 Statistical Analysis

One-way ANOVA followed by a Dunnett's test was carried out in order to detect significant difference between all means from control means. All data were plotted as means \pm SE unless otherwise stated. Analyses were carried out using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA).

CHAPTER 3 RESULTS

Some data contained in Figures 2, 6, 7 and 8 appear in:

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

3.1 Cloning of *ArHsp40* cDNAs

cDNAs from 5 h cysts (Fig. 1b) were cloned by RT-PCR and sequenced. The ArHsp40-1 cDNA contained an open reading frame (ORF) of 1212 bp which encoded a 404-amino acid polypeptide with a predicted molecular mass of 45.0 kDa and a theoretical pI of 6.73 (Fig. 2). The ArHsp40-2 cDNA contained a 1065 bp ORF which encoded a 354-amino acid polypeptide with a predicted molecular mass of 38.9 kDa and a theoretical pI of 8.99 (Fig. 3). Both proteins contained a conserved N-terminal J-domain with a HPD motif, followed by a Gly/Phe (G/F) rich domain with a conserved DIF motif. Sequence alignment showed that the two ArHsp40s possessed 64% similarity and 36% identity. Only ArHsp40-1 possessed a true zinc binding domain (ZBD) of 4 CXXCXGXG motifs with lysine replacing glycine in the final position of the 4th motif (Fig. 4).

3.2 Anti40-type1 and Anti40-type2 Immunoreactivities

Anti40-type 1 antibody reacted with purified recombinant ArHsp40-1 but not with purified recombinant ArHsp40-2 (Fig. 5). Conversely, Anti40-type2 antibody reacted

Figure 2. ArHsp40-1 sequence

Nucleotide and amino acid sequences for the full length open reading frame of ArHsp40-1. Accession #AKH4096.1 (Jiang et al., 2016). The J-domain (underlined in red), HPD motif (boxed in red), G/F rich region (underlined in blue), the DIF motif (boxed in blue), Zinc-binding domain (underlined in black) containing 4 CXXCXGXG repeats (boxed in black), and the C-terminal region (underlined in green) are indicated. The *ArHsp40-1* full length forward and reverse primers are complementary to the red dash-boxed region, the dsRNA forward and reverse primers are dash-boxed in red, and the RT-qPCR reverse primer dash-boxed in blue (the dsRNA forward primer and RT-qPCR forward primer share the same sequence). The Anti40-type1 peptide target is dash-boxed in purple.

<u>atgccaaggaacagggttgtatgatg</u> <u>tccttggtcttagtcctagctgtaccctgat</u>	60
M P K E T G L Y D V L G L S P S C T P D	20
gaattgaagaaagcctacagaaaattggctttaaagtaccaccagataaaaaatccaaat	120
E L K K A Y R K L A L K Y H P D K N P N	40
gaaggagagaagtttaagcaaatatcatttgcatatgaagtactttccaatcctgaaaag	180
E G E K F K Q I S F A Y E V L S N P E K	60
cgggaaatztatgacaaatattggcgaacaaggcctaaaagaaggtggcgggtggaggtggt	240
R E I Y D K Y G E Q G L K E G G G G G G	80
ggaggttttctactccccatggatatttttgagatgtttttgggtgggtgatgggtggc	300
G G F H S P M D I F E M F F G G G M G G	100
agaggaagacgagaaagaagaggggaaggacata <u>gtgatcagttgagcgtcac</u> <u>ccttggga</u>	360
R G R R E R R G K D I V H Q L S V T L G	120
gaattgtataaaggatgtactagaaaattagccctacaaaagaatgttgtatgttcgaaa	420
E L Y K G C T R K L A L Q K N V V C S K	140
tgtgaaggtcgtggaggtaagaaggggtgctactcagaaatgtgtcagatgtcgaggctct	480
C E G R G G K K G A T Q K C V R C R G S	160
ggtatgcaggtgaggattcagcagctagctcctggaatggttcagcaattcagagcgtt	540
G M Q V R I Q Q L A P G M V Q Q I Q S V	180
tgtcacgattgttctggacaagggcaggttattgacgccaaagataaagtgcaaaaattgt	600
C H D C S G Q G E V I D A K D K C K N C	200
gaagggaaaaagattattagggagaagaaaatctta <u>gaagtacatgtggaccccgaatg</u>	660
E G K K I I R E K K I L E V H V D P G M	220
gttgatgggtcaaaagattacttttagtggagagggggatcaagaaccaggacttgaacct	720
V D G Q K I T F S G E G D Q E P G L E P	240
ggagatatcatcatagttcttgacgaaaaagagcatgcaatgtataaacgcgtagataga	780
G D I I I V L D E K E H A M Y K R V D R	260
agtgatttgttgacggttatggatctggaacttgttgaagctctctgtggattccaaaag	840
S D L L T V M D L E L V E A L C G F Q K	280
gttctcaaacacttgatgatagaaatcttgtgctcacatgtattccaggcgaaaatatac	900
V L K T L D D R N L V L T C I P G E N I	300
aagcaggatcgactgaaatgtatttataatgaaggaatgccccattacaagaatcccatg	960
K Q D R L K C I Y N E G M P H Y K N P M	320
gagaaaggaaaattaataatcaagtttaacgtgaaattcccggatgtaattaatcctgct	1020
E K G K L I I K F N V K F P D V I N P A	340
ttgattcctcagcttgaagcatgtcttccccctaggacagaagaaatgattccttggtgac	1080
L I P Q L E A C L P P R T E E M I P G D	360
gccgaagaagtaatgcttcaggacttcgacccagaaatacacagaaggcagcaaagagcg	1140
A E E V M L Q D F D P E I H R R Q Q R A	380
caaagacctacttatgatgactcagacgatgaaggtgggtgctgggccaagaggagttaa	1200
Q R P T Y D D S D D E G G A G P R G V Q	400
<u>tgtccaaccagtag</u>	1215
C P T Q -	404

Figure 3. ArHsp40-2 sequence

Nucleotide and amino acid sequences for the full length open reading frame of ArHsp40-2, with highlighted motifs shown. The J-domain (underlined in red), HPD motif (boxed in red), G/F rich region (underlined in blue), the DIF motif (boxed in blue), and the C-terminal region (underlined in green) are indicated. The *ArHsp40-2* full length forward and reverse primers are complementary to the black dash-boxed region, the dsRNA forward and reverse primers (red dash-boxed), and the qPCR forward and reverse primers (blue dash-boxed). The Anti40-type2 peptide target (purple dash-boxed).

atgggaaaggatttctataaaatacttggaatatccaaaggtgcatcagatgatgaata 60
M G K D F Y K I L G I S K G A S D D E I 20
aagaaagcctacaggaatttagcattgaaataccaccagataagaacaagaaaaggga 120
K K A Y R K L A L K Y H P D K N K E K G 40
gcagaagaaaggttcaaagaaatagcagaagcctatgaaattttaagtgacaaaaagaag 180
A E E R F K E I A E A Y E I L S D K K K 60
agagacatttttgaccaatatggtgaagaaggacttaaaggctgctgcaggagcacctgga 240
R D I F D Q Y G E E G L K G A A G A P G 80
ggaggaggagctggtggtccagagttttcatatgcattccatggtgatccaagagcaacg 300
G G G A G G P E F S Y A F H G D P R A T 100
tttgctcagttctttggatctgcaaatccatttgaggccttctttaattttggtggtcca 360
F A Q F F G S A N P F E A F F N F G G P 120
ggtggtcaggaagaacacatggaaacagatgacccattcggtaggtttggtggccttgg 420
G G Q E E H M E T D D P F G G F G G L G 140
gggttctccagttttgggggtccaggcgttggacgatcacagtcgtttaatgttcaagga 480
G F S S F G G P G V G R S Q S F N V Q G 160
atgagaggcaaagaaagaggacccatcacccaagacccacccattgaacacgatcttcca 540
M R G K E R G P I T Q D P P I E H D L P 180
ttgtctttggaagaaattatgaaaggctgtactaagcgaatgaaaatctctcgaaaaatt 600
L S L E E I M K G C T K R M K I S R K I 200
tacaggagagtaggtactgaagacagaataactaaagaagacaaaatactgactataaac 660
Y R R V G T E D R I T K E D K I L T I N 220
gttaagccggggtggaagcaggcactaaaataacatttgccaaggaaggagatcaacta 720
V K P G W K A G T K I T F A K E G D Q L 240
ccaaatataaatcccagcggacgtggttttcgcatcaaggacaaacctcatcctactttc 780
P N K I P A D V V F V I K D K P H P T F 260
aaaagagatggctctgatatccgctatactgcaaaaatatcgctaagagatgctctctgt 840
K R D G S D I R Y T A K I S L R D A L C 280
ggtaccggggtcgatgtccctactctatctggggagataatacgtattgatacaagcagc 900
G T R V D V P T L S G I E I I R I D T S S 300
gaaatagttaaaccaaccactcaacgtaaggcatcgggctatggacttccctatccaag 960
E I V K P T T Q R K A S G Y G L P Y P K 320
gagcctaatcgtcgaggatctcatcatcactttcgatattaagttcccagatcacttg 1020
E P N R R G D L I I T F D I K F P D H L 340
ccgcaaactgtagagaagttttatcgtgatgccttgcctaattga 1065
P Q T V R E V L R D A L P N - 354

Figure 4. Sequence alignment of ArHsp40-1 and ArHsp40-2

The amino acid sequences of ArHsp40-1 and ArHsp40-2 were compared by CLUSTAL OMEGA (<http://www.ebi.ac.uk/clustalw2>). J-domain (red line), HPD motif (red box), G/F region (blue line), DIF motif (blue box), zinc binding motif (contained in type 1 only, black line), CXXCXGXG motifs (black boxes), C-terminal substrate binding domain (green line). Identical amino acid residues are indicated by an asterisk, similar amino acid residues are indicated by a colon, semi-conserved amino acid residues by a period, and no amino acid residues by a dash. The number of residues in each sequence is indicated on the right side of the figure.

J-domain

```

ArHsp40-1  MPKETGLYDVLGLSPSCTPDELKKAYRKLALKYHPDKNPNEG--EKFKQISFAYEVLNSP  58
ArHsp40-2  --MGKDFYKILGISKGASDDEIKKAYRKLALKYHPDKNKEKGAEERFKEIAEAYEILSDK  58
               ..:*.::*: * ..: ***** ::* *:*:*: *:*:*:

```

G/F Region

```

ArHsp40-1  EKREIYDKYGEQGLKEGGGGGGGFH-----SPMDIIFEMFFGGG-----MG  99
ArHsp40-2  KKRDIIDQYGEGLKGAAGAPGGGGAGGPEFSYAFHGDPRATFAQFFGSANPFEAFFNFG  118
               :*:*:*:*:*:* * ..: * * * * * . * * * * * :*

```

```

ArHsp40-1  GRGRERRRGKDIVHQLSVTLGELYKCTRKLALQKNVCSKCEGRGSKK--GATQKCVRC  157
ArHsp40-2  GPGGQEEHME-----TDDPFGGFGGLGG-----FSSFGGPGVGRSQSFNVQGMRG  163
               * * :*. : : . : * . * * : . . : : *

```

Zinc Binding Domain

```

ArHsp40-1  RGSQMQRVRIQQQLAPGMVQQIQSVCHDCSGQCEVIDAKDKCKNCEGKKI  217
ArHsp40-2  KERGPITQDPPIEHDLPLSLEIIMKGCTKRMKI-SRKIYRRVGTEDRIKEDKILTINVK  222
               : * . : : . : . : : : . * : . : * : * . * * : * .

```

```

ArHsp40-1  PGMVDGQKITFSGEGDQEPGLEPGDIIIVLDEKEHAMYKRVDSDLLTVMDELVEALCG  276
ArHsp40-2  PGWKAGTKITFAKEGDLPNKIPADVVFVIKDKPHPTFKRD-GSDIRYTAKISLRDALCG  281
               ** * ****: **** * . *.:::***: * * :** **: . . : * :****

```

Substrate Binding Domain

```

ArHsp40-1  FQKVLKTLDDRNLVLTCPGENIKQDRLKCIYNEGMPHYKNPMEKGLIIFNVKFPDVI  336
ArHsp40-2  TRVDVPTLSGEIIRIDT-SSEIVKPTTQRKASGYGLPYKPNRRGDLIITFDIKFPDHL  340
               : : **... : : . * : * : . * : * : * . * . * . * . * :

```

```

ArHsp40-1  NPALIPQLEACLPPRTEEMIPGDAEEVMLQDFDPEIHRROQRAQRPTYDSDDEGGAGPR  387
ArHsp40-2  PQTVREVLRLDALPN-----  354
               : : * . . **

```

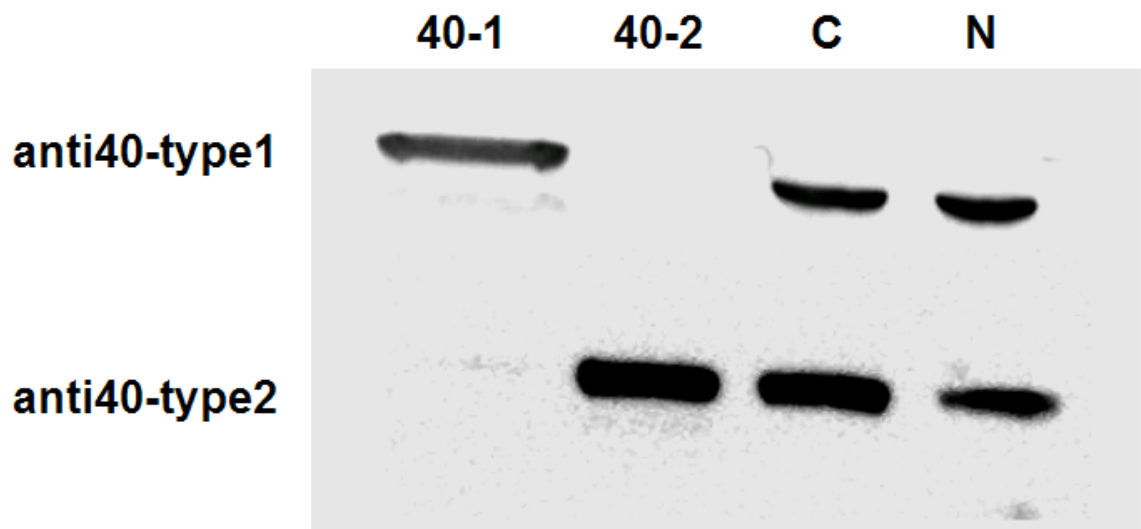
```

ArHsp40-1  GVQCPTQ  404
ArHsp40-2  -----

```


Figure 5. Specificity of antibodies raised to ArHsp40-1 and ArHsp40-2

Western blot containing *A. franciscana* protein extracts and recombinant ArHsp40s that were resolved in SDS polyacrylamide gels. **40-1**, 1 µg of purified recombinant ArHsp40-1; **40-2**, 1 µg purified recombinant ArHsp40-2; **C**, 20 µg of protein extract from *A. franciscana* cysts; **N**, 20 µg of protein extract from *A. franciscana* instar 1 nauplii. Blots were probed with antibodies specific for either ArHsp40-1 (**Anti40-type1**) or ArHsp40-2 (**Anti40-type2**).



with purified recombinant ArHsp40-2 but not with purified recombinant ArHsp40-1 (Fig. 5). Anti40-type 1 recognized only a 49 kDa protein in extracts from *A. franciscana* cysts and nauplii while Anti40-type 2 recognized only a 39 kDa protein (Fig. 5).

3.3 The Post-Diapause Synthesis of *ArHsp40-1* and *ArHsp40-2* Was Developmentally Regulated

qRT-PCR and immunoprobings of western blots using α -tubulin mRNA and tyrosinated α -tubulin as internal controls respectively showed that the amounts of *ArHsp40-1* mRNA (Fig. 6) and protein (Fig. 7) remained relatively constant during early post-diapause development, until decreasing in 1st instar nauplii and then decreasing significantly further in 2nd instar nauplii. *ArHsp40-2* mRNA (Fig. 6) and protein (Fig. 7) increased significantly in 5 h cysts and E1 as compared to 0 h cysts and then decreased significantly in 2nd instar nauplii to levels similar to 0 h cysts for mRNA. There were no significant changes in tyrosinated α -tubulin used as loading control.

3.4 ArHsp40-1 and ArHsp40-2 Were Stress Inducible

Immunoprobings of western blots showed that ArHsp40-1 and ArHsp40-2 both increased almost 10-fold in 1st instar nauplii that were heat shocked at 39°C for 1 h and that they remained high for the first 2 h of recovery, before declining to basal levels 8 h after heat shock (Fig. 8).

3.5 Injection of *A. franciscana* Females with dsRNAs

dsRNAs synthesized from *ArHsp40-1*, *ArHsp40-2* and *GFP* cDNA migrated to their expected positions in 1.0% agarose gels (Fig. 9a). The *ArHsp40-1* dsRNA is

Figure 6. *ArHsp40-1* and *ArHsp40-2* mRNA were developmentally regulated in post-diapause *A. franciscana*

The amounts of *ArHsp40* mRNA normalized to α -*tubulin* were determined by qRT-PCR in seven post-diapause life history stages of *A. franciscana*. Lane **1**, 0 h cysts; **2**, 5 h cysts; **3**, 10 h cysts/E1 nauplii; **4**, E2/E3 nauplii; **5**, instar 1 nauplii; **6**, early instar 2 nauplii; **7**, late instar 2 nauplii. Copy numbers of *ArHsp40* and α -*tubulin* cDNAs determined by Roto-Gene 6 Software (Corbett Research), were compared to each other. The experiment was performed in duplicate with RNA samples from 3 replicates of each life history stage. Means of copy number of *ArHsp40*: α -*tubulin* mRNA copy numbers represented by different asterisks are significantly different from the means of *ArHsp40*: α -*tubulin* mRNA copy numbers in 0 h cysts; **, $P < 0.01$; ***, $P < 0.005$. Error bars represent standard error of n=3 replicates per experiment.

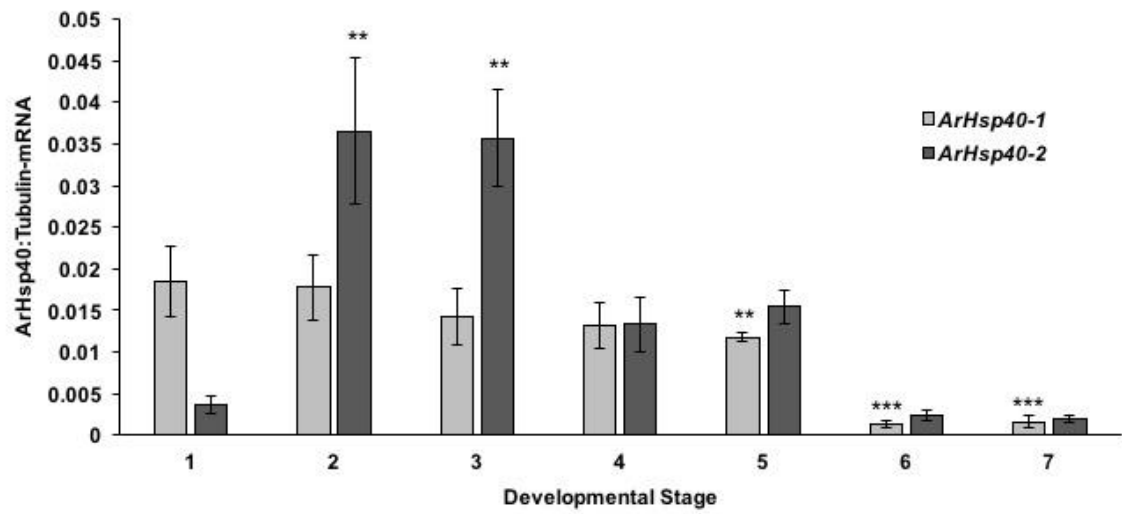


Figure 7. ArHsp40-1 and ArHsp40-2 were developmentally regulated in post-diapause *A. franciscana*

The amounts of ArHsp40 were normalized to tyrosinated α -tubulin by immunoprobings samples resolved in SDS polyacrylamide gels and blotted to nitrocellulose from seven post-diapause life history stages of *A. franciscana*. Cell-free homogenates were resolved in SDS-polyacrylamide gels and either stained with Colloidal Coomassie blue (**a**) or blotted to nitrocellulose and immunoprobed with Anti40-type1 (**b**) or Anti40-type2 (**c**) or anti-Y (**d**). The antibody-reactive protein bands were quantified with Image Studio Software and the ratio of ArHsp40:tyrosinated α -tubulin was calculated (**e**). Lane **1**, 0 h cysts; **2**, 5 h cysts; **3**, 10 h cysts/E1 nauplii; **4**, E2/E3 nauplii; **5**, instar 1 nauplii; **6**, early instar 2 nauplii; **7**, late instar 2 nauplii. Means of ArHsp40:tyrosinated α -tubulin represented by different asterisks are significantly different from the means of ArHsp40:tyrosinated α -tubulin in 0 h cysts; ***, $P < 0.005$. Error bars represent standard error of $n=3$ replicates per experiment.

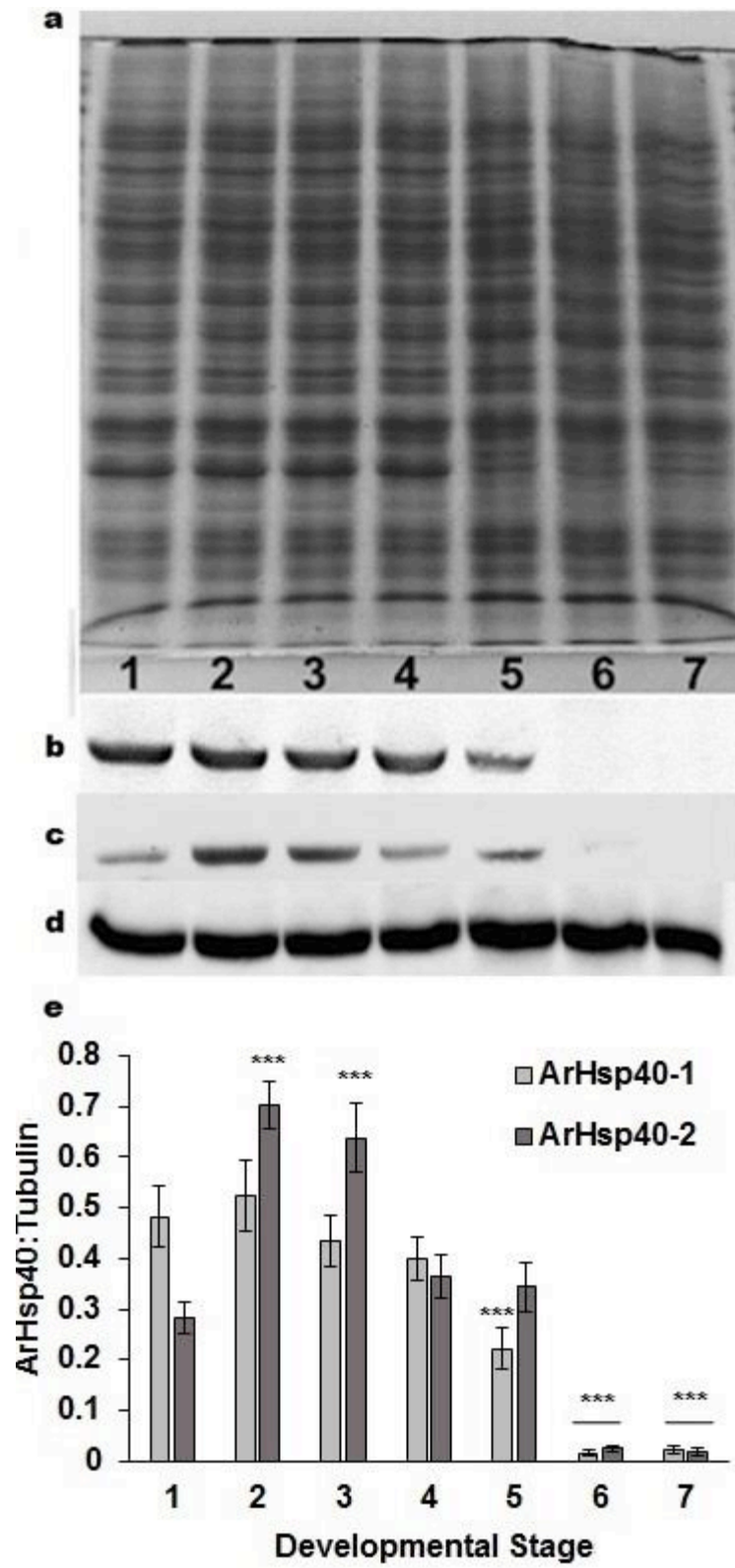


Figure 8. ArHsp40-1 and ArHsp40-2 were induced by heat shock in post-diapause larvae of *A. franciscana*

Cell-free homogenates from heat shocked instar 1 nauplii were resolved in SDS-polyacrylamide gels and either stained with Colloidal Coomassie blue (**a**) or blotted to nitrocellulose and immunoprobed with either Anti40-type1 (**b**) Anti40-type2 (**c**) or anti-Y (**d**). The antibody-reactive protein bands were quantified with Image Studio Software and the ratio of ArHsp40:tyrosinated α -tubulin was calculated (**e**). Lane **C**, no heat shock; **HS**, 1 h at 39 °C; **2R**, **4R**, **6R**, **8R** recovery at 27 °C for 2, 4, 6 and 8 h respectively. Means of ArHsp40:tyrosinated α -tubulin represented by different asterisks are significantly different from the means of ArHsp40:tyrosinated α -tubulin in respective control samples; *, $P < 0.05$; ***, $P < 0.005$. Error bars represent standard error of $n = 3$ replicates per experiment.

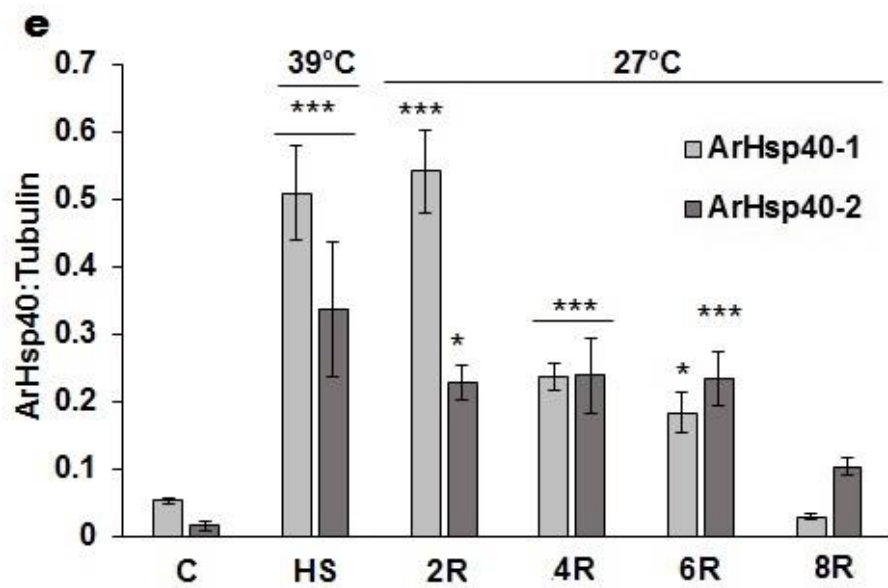
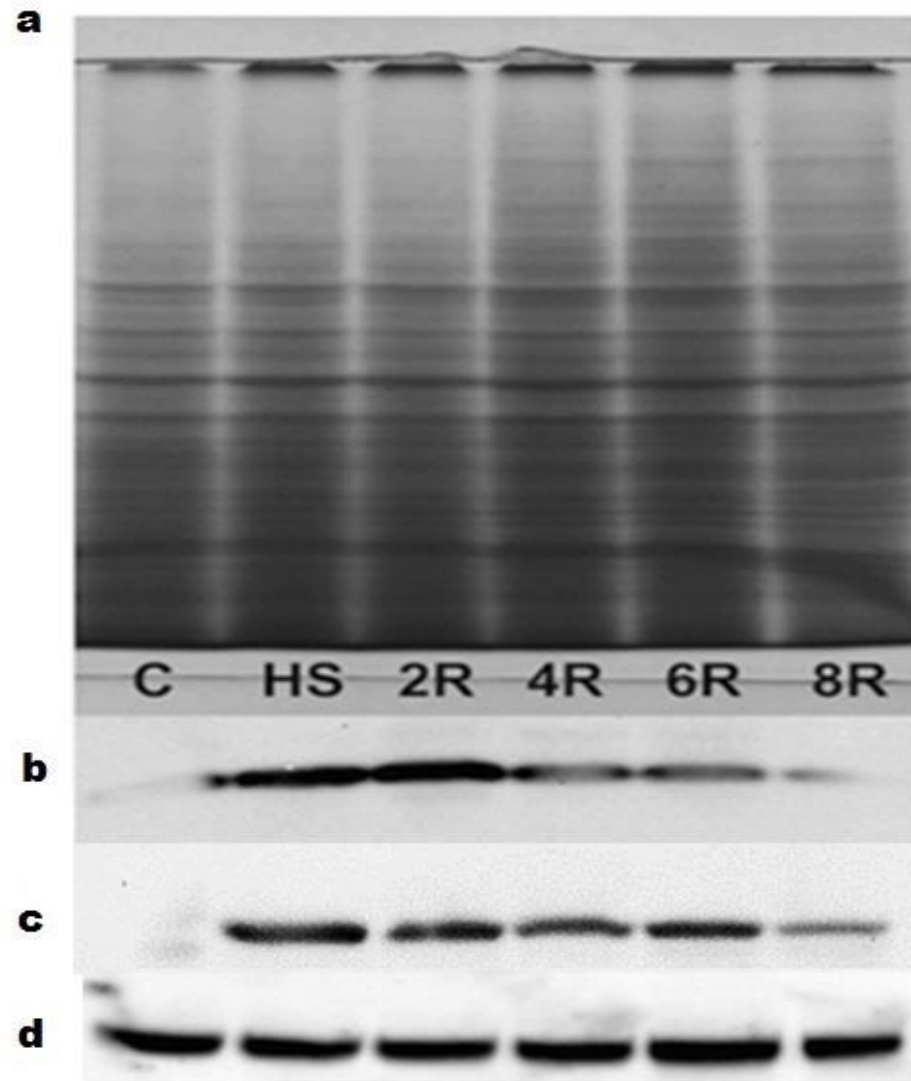


Figure 9. Injection of *A. franciscana* females with dsRNAs

(a) Complementary dsRNAs for *ArHsp40-1*, *ArHsp40-2* and *GFP* (Lane 3, 5, 7)

synthesized from their respective cDNAs (Lane 2, 4, 6) using primers containing the T7 promoter were resolved in 1.0% agarose gels; **bp**, base pairs; Lane 1, Gene Ruler 100 bp

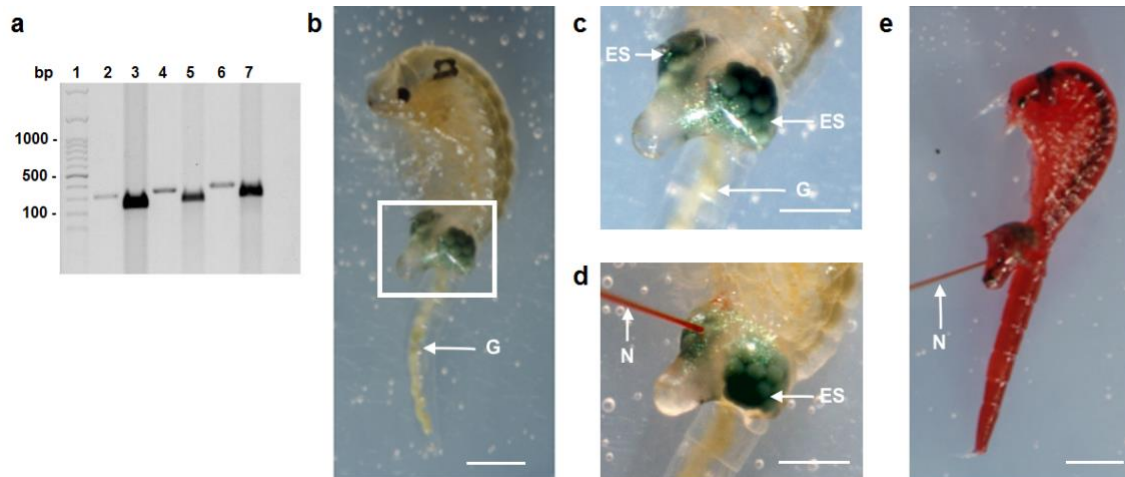
DNA Ladder (Thermo Scientific). (b) Light micrograph of an *A. franciscana* adult female

with the ovasac boxed in white. (c) The boxed region of **b** was enlarged showing an

ovasac containing nauplius-destined unfertilized oocytes. (d) injection of dsRNA into the

egg sac; (e) an injected female containing phenol red and thus dsRNA. Scale bars: 1 mm

(b, e), 0.5 mm (c, d). **G**, gut tract; **ES**, embryo sac; **N**, micro injector needle.



complementary to 324 bp (26.7%) of the full length *ArHsp40-1* mRNA and targets the ZBD to reduce the off targeting of other ArHsp40 isotypes. The *ArHsp40-2* dsRNA is complementary to 327 bp (30.7%) of the full length *ArHsp40-2* mRNA and targets the highly variable C-terminal binding region to reduce the off targeting of other ArHsp40s. *GFP* dsRNA is not complementary to any *ArHsp40* mRNA or any other native mRNA recorded in the *A. franciscana* transcriptome (Zhao et al., 2012).

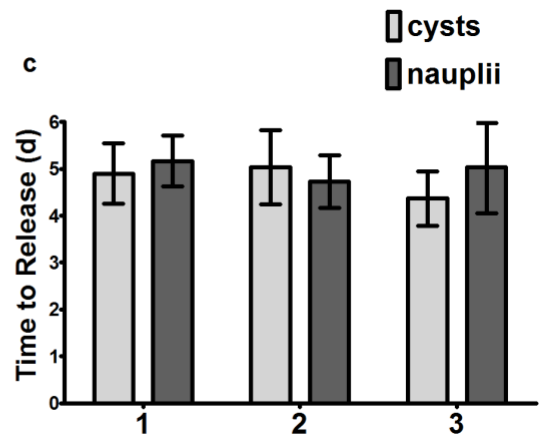
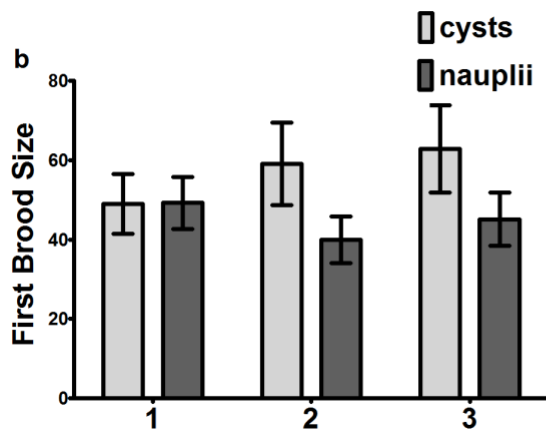
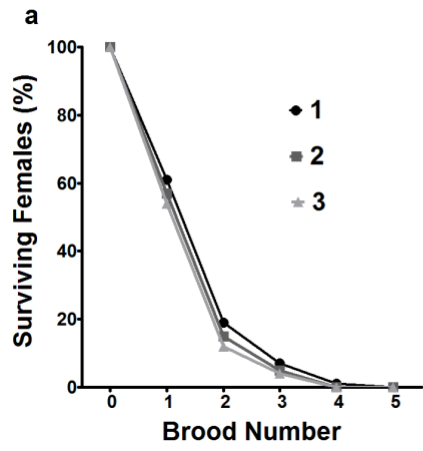
Prior to injection and fertilization females destined to produce nauplii possessed green oocytes within two separated egg sacs (Fig. 9b-d). Those females destined to produce diapause-destined embryos possessed a shell gland (Liang & MacRae 1999). The injected dsRNA was retained in the female for at least 2 h 95% of the time and circulated through the entire body. Females 1 d post injection that exhibited normal swimming and feeding were paired with a male for fertilization.

3.6 Injection of *A. franciscana* females with ArHsp40 dsRNA Affected Neither Their Survival nor Embryo Development

Survival of adult females injected with either ArHsp40-1, ArHsp40-2, or GFP dsRNA and cultured at room temperature was similar for 3 successive broods (Fig. 10a). Approximately 57% of females survived incubation and released a 1st brood of nauplii or cysts, but only 15 % of females survived to release a 2nd brood, and 5% a 3rd brood. Considering only the 1st brood, females injected with *ArHsp40-1*, *ArHsp40-2* or *GFP* dsRNA released statistically equal amounts of cysts (40-70 individuals) or nauplii (32-58) (Fig. 10b). Post-fertilization release times from females injected with *ArHsp40-1*, *ArHsp40-2* or *GFP* dsRNA for 1st brood cysts and nauplii was 5.0 days (Fig. 10c).

Figure 10. Injection of *A. franciscana* females with ArHsp40 dsRNAs affected neither their survival nor embryo development

(a) Survival of 42 females injected with *GFP* dsRNA (1), 42 females injected with *ArHsp40-1* dsRNA (2) and 41 females injected with *ArHsp40-2* dsRNA (3) after successive brood releases. (b) Mean 1st brood sizes released by females destined to release cysts or nauplii and injected with either *GFP* (1), *ArHsp40-1* (2) or *ArHsp40-2* (3) dsRNA. (c) The mean time from fertilization to release of 1st brood cysts or nauplii from females injected with either *GFP* (1), *ArHsp40-1* (2) or *ArHsp40-2* (3) dsRNA. Means of first brood size (b), or time to release (c) represented by different asterisks are significantly different from the means respective control samples. Error bars represent standard error of n=3 replicates per experiment. d, days. $P>0.05$.



3.7 ArHsp40-1 and ArHsp40-2 Were Knocked Down by RNAi in *A. franciscana* Cysts and Nauplii

ArHsp40-1 mRNA in 1st brood cysts released from females injected with *ArHsp40-1* dsRNA was reduced approximately 86% compared to cysts from females injected with either *ArHsp40-2* or *GFP* dsRNA. *ArHsp40-2* mRNA in 1st brood cysts released from females injected with *ArHsp40-2* dsRNA was reduced approximately 89% compared to cysts from females injected with either *ArHsp40-1* or *GFP* dsRNA (Fig. 11a).

ArHsp40-1 was not detected in western blots of protein extracts of cysts released by females injected with *ArHsp40-1* dsRNA but was detected in cysts from females injected with either *ArHsp40-2* or *GFP* dsRNA (Fig. 11b). ArHsp40-1 was reduced approximately 86% compared to cysts from females injected with either *ArHsp40-2* or *GFP* dsRNA (Fig. 11c). ArHsp40-2 in 1st brood cysts released from females injected with *ArHsp40-2* dsRNA was reduced approximately 73% compared to cysts from females injected with either *ArHsp40-1* or *GFP* dsRNA (Fig. 11b, c). Similar knockdowns were observed with 1st brood nauplii released by females injected with either *ArHsp40-1*, *ArHsp40-2* or *GFP* dsRNA (Fig. 12b, c).

3.8 ArHsp40 Knockdown Reduced the Viability of *A. franciscana* Nauplii

3.8.1 *ArHsp40-1* and *ArHsp40-2* knockdown reduced survival of developing nauplii

Ninety-seven % of nauplii released from females injected with *GFP* dsRNA survived for 3 weeks whereas 81% of nauplii deficient for ArHsp40-2 and 53% deficient for ArHsp40-1 survived (Fig. 13). Five weeks after release, when *A. franciscana*

Figure 11. Knock down of ArHsp40-1 and ArHsp40-2 in *A. franciscana* cysts

RNA and protein were extracted from first brood cysts 10 d post-release from females that had been injected with either *GFP* (1), *ArHsp40-1* (2), or *ArHsp40-2* (3) dsRNA. (a) Extracted RNA from 50-80 cysts was reverse transcribed and copy numbers quantified by qPCR to determine *ArHsp40-1*, *ArHsp40-2* and α -*tubulin* mRNA copy number in each sample. The experiment was performed in duplicate with RNA samples from 3 replicates of each knockdown cyst brood. Copy numbers for *ArHsp40-1* and *ArHsp40-2* mRNA were normalized to α -*tubulin* mRNA copy numbers and averaged for cysts from females injected with *ArHsp40-1* dsRNA (n=3), *ArHsp40-2* dsRNA (n=3) and *GFP* dsRNA (n=3). (b) Protein extracts from 50-80 cysts 10 d post-release from females injected with *GFP* dsRNA (1,3), *ArHsp40-1* dsRNA (2), or *ArHsp40-2* dsRNA (4) were resolved in SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies specific for ArHsp40-1, ArHsp40-2 or tyrosinated α -tubulin. The experiment was performed in triplicate. (c) The antibody-reactive protein bands were quantified with Image Studio Software and the ratio of ArHsp40:tyrosinated α -tubulin was calculated. Error bars represent standard error of *ArHsp40*: α -*tubulin* mRNA copy numbers (a) or protein levels (c). Means of *ArHsp40*: α -*tubulin* mRNA copy numbers (a) or ArHsp40:tyrosinated α -tubulin (c) represented by different asterisks are significantly different from the means in respective *GFP* dsRNA samples; ***, $P < 0.005$.

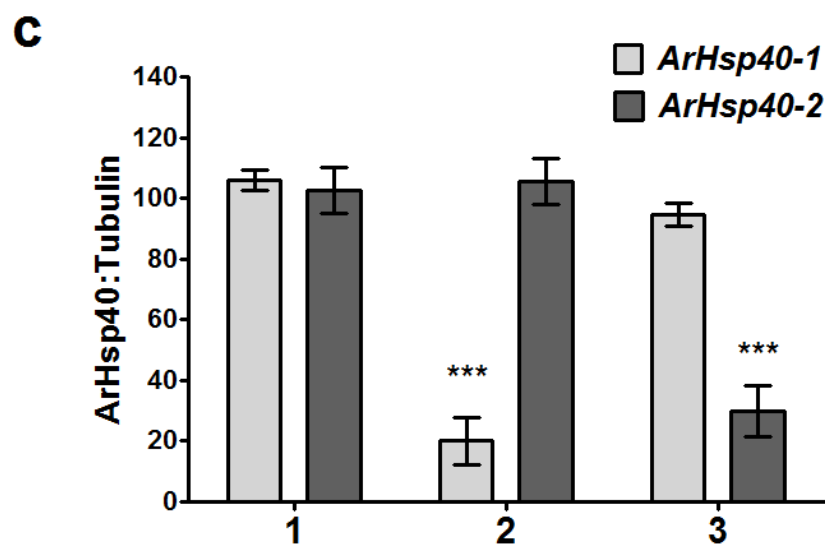
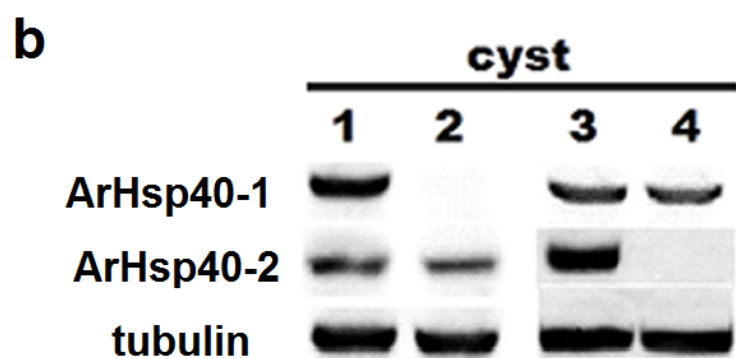
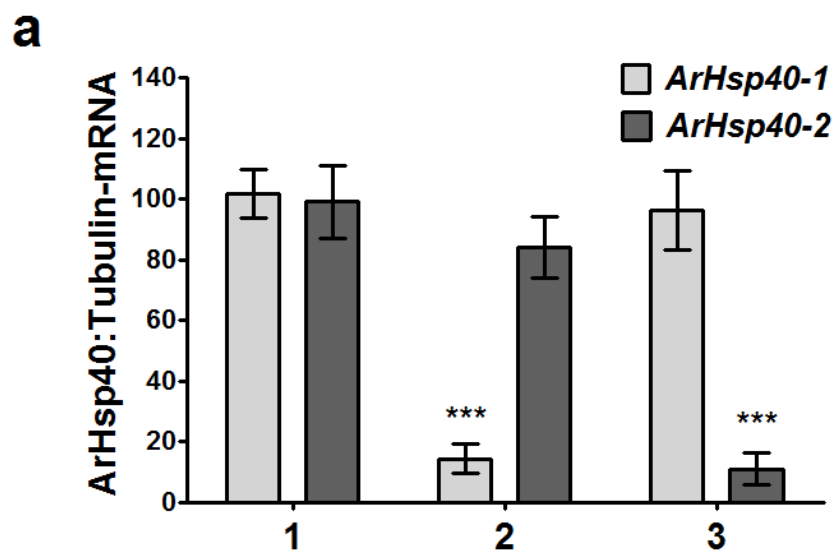


Figure 12. Knock down of ArHsp40-1 and ArHsp40-2 in *A. franciscana* nauplii

RNA and proteins were prepared from first brood nauplii immediately after release from females injected with either *GFP* (1), *ArHsp40-1* (2), or *ArHsp40-2* (3) dsRNA. (a) RNA extracted from 50-80 nauplii was reverse transcribed and copy numbers of *ArHsp40-1*, *ArHsp40-2* and α -*tubulin* mRNA were determined in each sample by qPCR. The experiment was performed in duplicate with RNA samples from 3 replicates of each knockdown nauplii brood. Copy numbers for *ArHsp40-1* and *ArHsp40-2* mRNA were normalized to α -*tubulin* mRNA copy numbers and averaged for nauplii from females injected with either *ArHsp40-1* dsRNA (n=3), *ArHsp40-2* dsRNA (n=3) or *GFP* dsRNA (n=3). (b) Protein extracts prepared from 34-65 nauplii immediately after release from females injected with either *GFP* dsRNA (1,3), *ArHsp40-1* dsRNA (2), or *ArHsp40-2* dsRNA (4) were resolved in SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies specific for either ArHsp40-1, ArHsp40-2 or tyrosinated α -tubulin. The experiment was performed in triplicate. (c) The antibody-reactive protein bands were quantified with Image Studio Software and the ratio of ArHsp40:tyrosinated α -tubulin was calculated. Error bars represent standard error of *ArHsp40*: α -*tubulin* mRNA copy numbers (a) or of protein levels (c). Means of *ArHsp40*: α -*tubulin* mRNA copy numbers (a) or ArHsp40:tyrosinated α -tubulin protein (c) represented by different asterisks are significantly different from the means in respective *GFP* dsRNA samples; ***, $P < 0.005$.

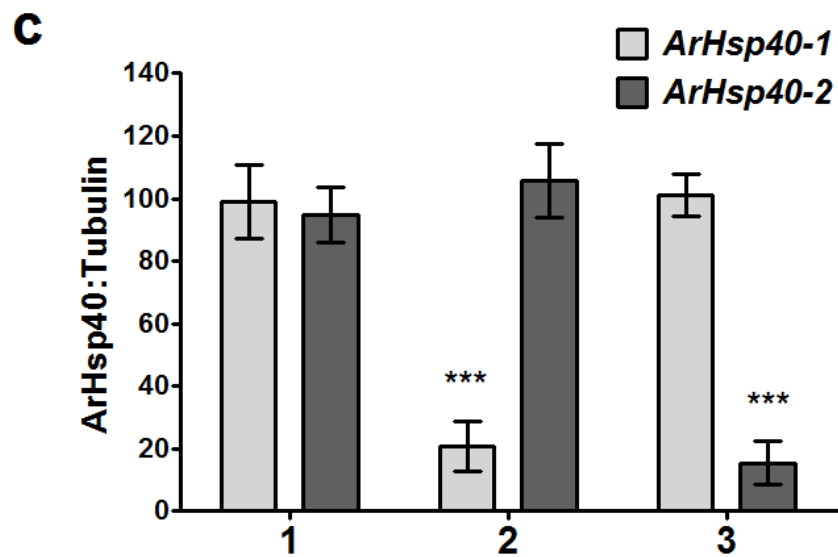
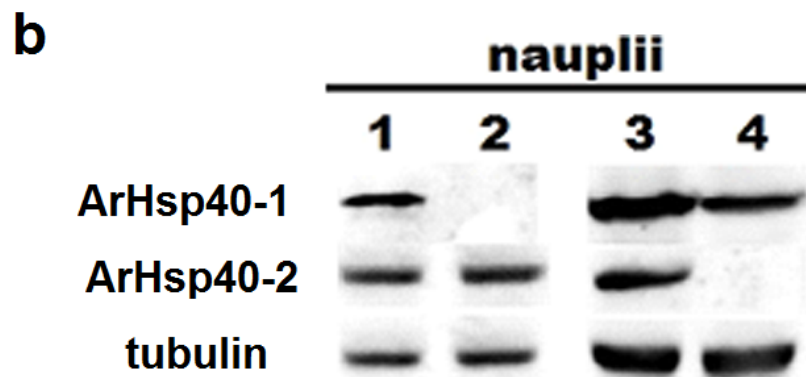
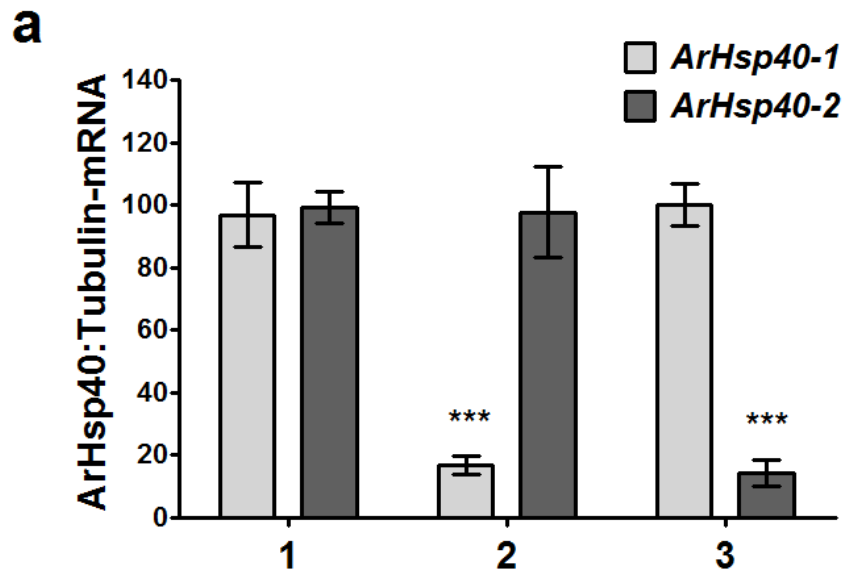
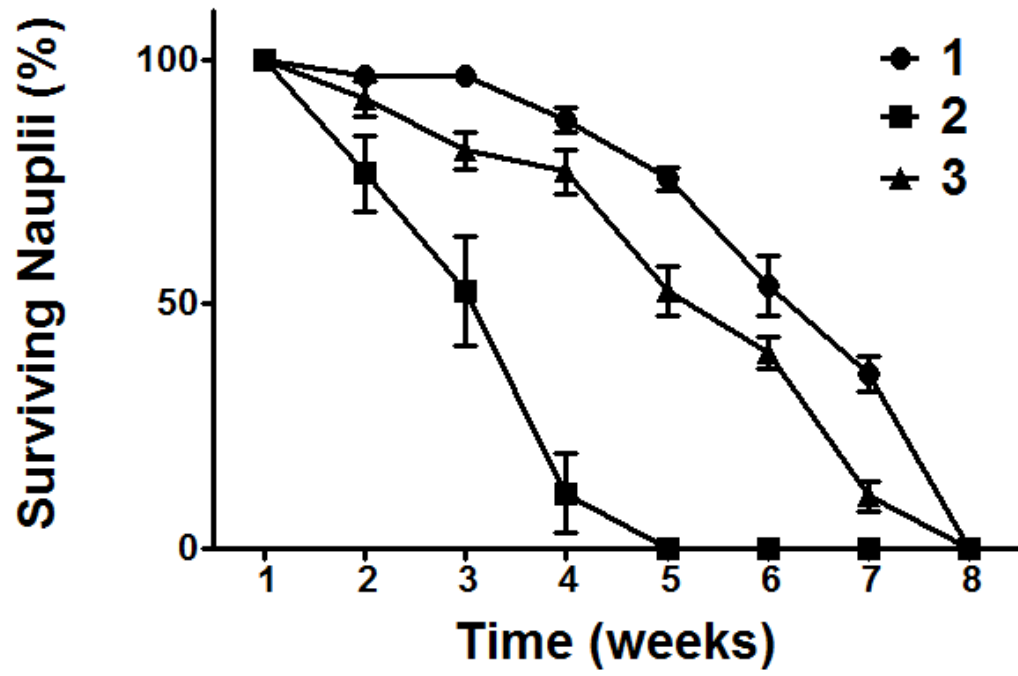


Figure 13. The knockdown of ArHsp40-1 and ArHsp40-2 reduced the viability of *A. franciscana* nauplii

The short-term survival of newly released nauplii from females injected with either *GFP* (1), *ArHsp40-1* (2) or *ArHsp40-2* (3) dsRNA was determined. The experiment was performed in triplicate with 1st brood nauplii. *GFP* dsRNA, n = 35; 56, 43; *ArHsp40-1* dsRNA, n = 38, 53, 60; *ArHsp40-2* dsRNA, n = 46, 113, 92. Error bars represent standard error.



typically reach sexual maturity under the culture conditions used in this study, 76% of nauplii from females injected with GFP dsRNA survived whereas 53% of animals deficient in ArHsp40-2 and 0% of animals deficient in ArHsp40-1 survived. By week 8 after release all animals in the experimental groups were dead (Fig. 13).

3.8.2 ArHsp40-1 and ArHsp40-2 knockdown reduced the heat tolerance of nauplii

Eighty-nine % of nauplii released from *A. franciscana* females injected with GFP dsRNA survived after heat shock at 39 °C for 1 h and recovery for 24 h at room temperature whereas 45% and 67% of nauplii released from females injected respectively with dsRNA for *ArHsp40-1* and *AhHsp40-2* survived under the same conditions (Fig. 14).

3.9 Cysts Deficient in ArHsp40-1 Spontaneously Aborted Diapause Entry

Knockdown cysts incubated in seawater were monitored 10 days for diapause entry and metabolic activity (Appendix, Fig. A3). Three days after release from females, 47% of cysts deficient in ArHsp40-1 hatched and released swimming nauplii (Fig. 15a). Cysts released from females injected with either *ArHsp40-2* or *GFP* did not hatch prematurely even 120 days after release from females (Fig. 15b).

3.10 Loss of ArHsp40 Lowered the Stress Tolerance of Cysts

Released knockdown cysts incubated in sea water for 10 d at room temperature then desiccated for 4 weeks over Drierite and stored at -20 °C for 12 weeks were

incubated in seawater to determine hatching success, used as a measure of viability. Fifty-seven % of cysts from females receiving GFP dsRNA, and thus possessing both ArHsp40-1 and ArHsp40-2, hatched. In contrast, 38% of cysts lacking ArHsp40-2 and 21% lacking ArHsp40-1 hatched (Fig. 16).

Figure 14. The knockdown of ArHsp40-1 and ArHsp40-2 reduced heat tolerance of *A. franciscana* nauplii

Newly released 1st brood nauplii from females injected with dsRNA were heat shocked at 39 °C for 1 h and then allowed to recover for 24 h at 27 °C after which survival was measured. Active swimming nauplii after recovery were considered to be alive, while motionless or degraded nauplii were considered to be dead. The experiment was performed in triplicate. *GFP* dsRNA (**1**), n = 55; 66, 53; *ArHsp40-1* dsRNA (**2**), n = 38, 53, 60; *ArHsp40-2* dsRNA (**3**), n = 36, 83, 72. Error bars represent standard error. Means of survival represented by different asterisks are significantly different from the means of survival in respective *GFP* dsRNA samples; *, $P < 0.05$; ***, $P < 0.01$.

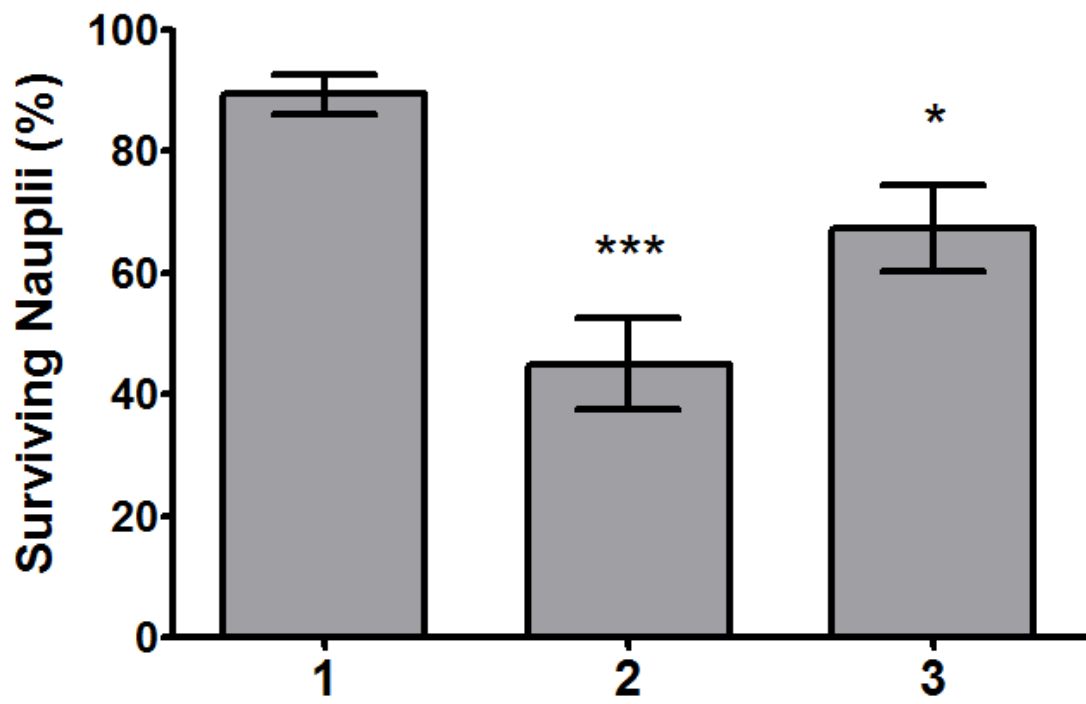
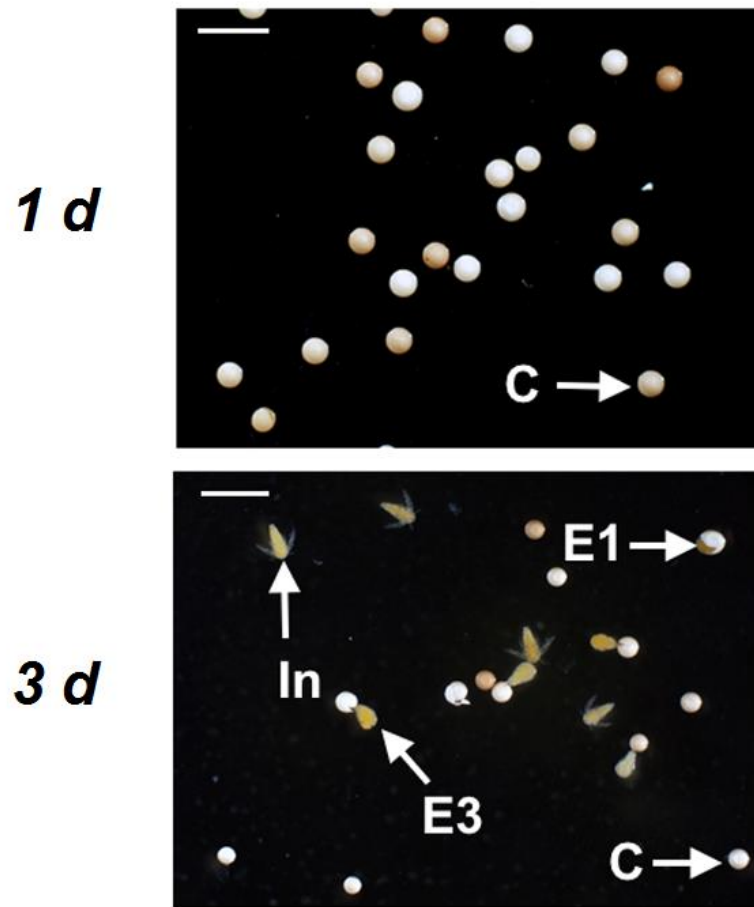


Figure 15. Cysts lacking ArHsp40-1 aborted diapause entry

(a), Light micrographs of cysts from females injected with *ArHsp40-1* dsRNA **1 d** and **3 d** post release from females. *C*, cysts, *E1*, emerging nauplius, *E3*, emerged nauplius 3, In 1st instar 1 nauplius. (b), The % of knockdown cysts that successfully hatched and emerged after release from females. Swimming nauplii were counted for 5 d after the last emerging animal. Cysts from females injected with *GFP* or *ArHsp40-2* dsRNA did not exhibit premature hatching. The experiment was performed in triplicate for 1st brood cysts from females injected with: *GFP* dsRNA (**1**), n = 55; 66, 53; *ArHsp40-1* dsRNA (**2**), n = 38, 53, 63; *ArHsp40-2* dsRNA (**3**), n = 36, 83, 72. Error bars represent standard error. Means of hatching % represented by different asterisks are significantly different from the means of hatching % in respective *GFP* dsRNA samples; ***, $P < 0.005$.

a



b

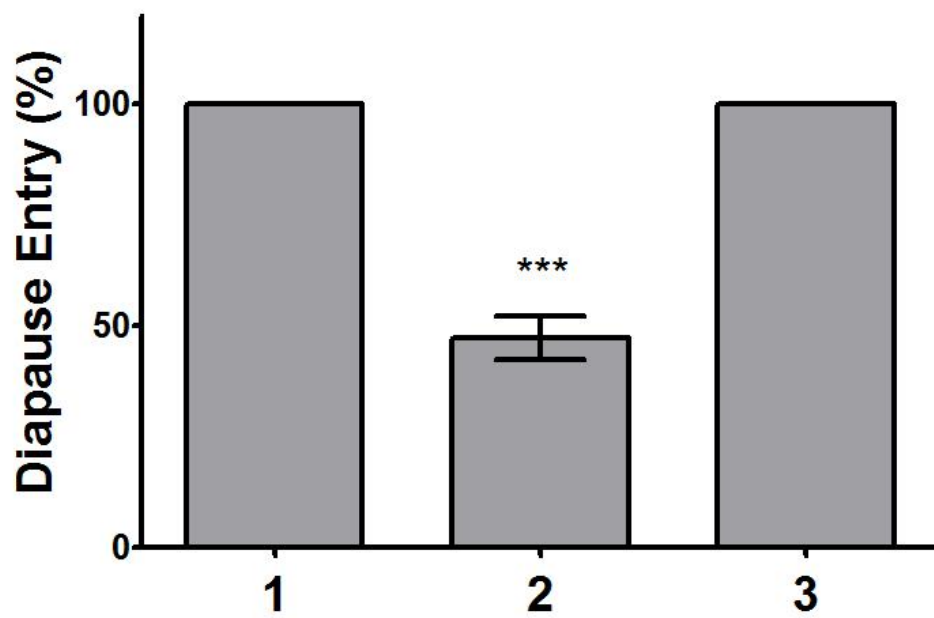
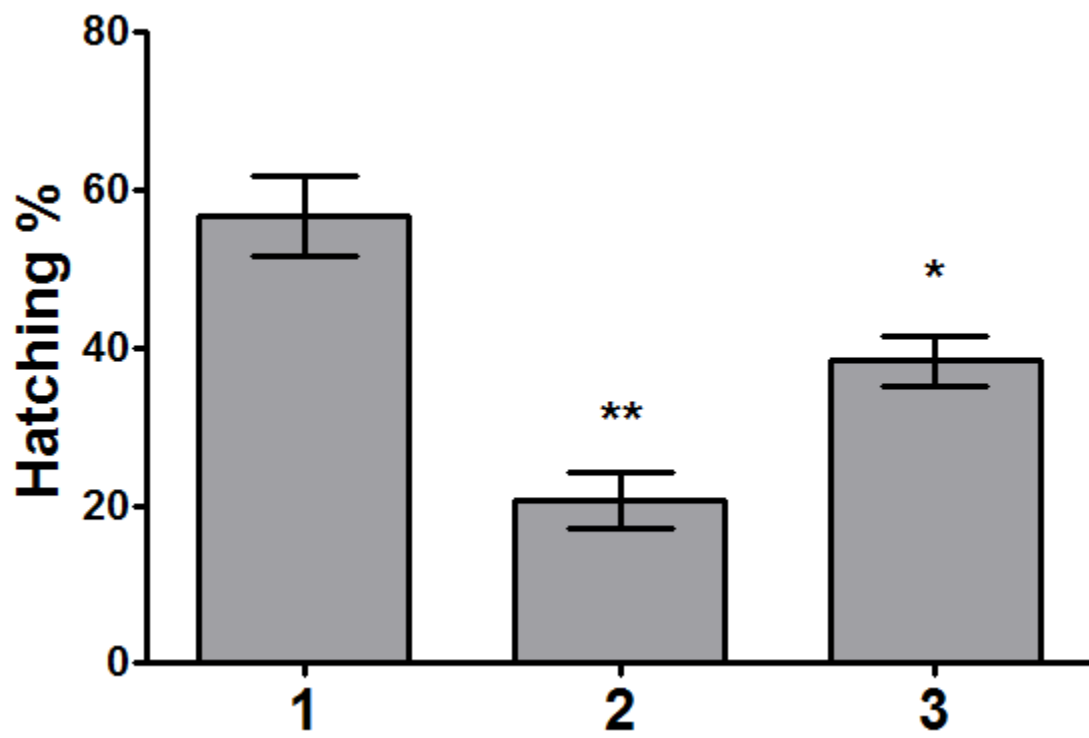


Figure 16. ArHsp40-1 and ArHsp40-2 contributed to the stress tolerance of *A. franciscana* cysts

Knockdown cysts were incubated in seawater at room temperature after desiccation and freezing to break diapause after which emerging and hatched swimming nauplii were counted. The experiment was performed in triplicate for 1st brood cysts from females injected with: *GFP* dsRNA (**1**), n = 55; 66, 53; *ArHsp40-1* dsRNA (**2**), n = 18, 26, 31; *ArHsp40-2* dsRNA (**3**), n = 36, 83, 72. Error bars represent standard error. Means of hatching % represented by different asterisks are significantly different from the means of hatching % of cysts from females receiving *GFP* dsRNA; *, $P < 0.05$; **, $P < 0.01$.



CHAPTER 4 DISCUSSION

4.1 Type 1 and Type 2 Hsp40 from *A. franciscana*

Hsp40, the major Hsp70 co-chaperone is well characterized structurally and functionally in bacteria, insects and mammals but not in crustaceans. Cloning of the *A. franciscana* ArHsp40-1 and ArHsp40-2 cDNAs from 5 h cysts was performed by RT-PCR using primers based on an eukaryotic annotation data base search (Sterck et al., 2012) with Hsp40 cDNAs of *Daphnia pulex* as references. Consistent with other J-proteins, ArHsp40s from *A. franciscana* possess an N-terminal J-domain, a conserved HPD motif, a G/F-rich region, and a C-terminal substrate binding region. Type 1 Hsp40 cDNA contained a ZBD composed of four CXXCXGXG motifs, where the fourth motif ended in K rather than G, a feature seen occasionally in type 1 Hsp40s from other types of animals (Martinez-Yamout et al., 2000). The type 2 Hsp40 cDNA did not contain a true ZBD, as is typical of type 2 Hsp40s from other species.

The amino acid sequence alignment of ArHsp40-1 and ArHsp40-2 showed similarity to other eukaryotic type 1 and type 2 Hsp40s, suggesting similar *in vivo* function (Jiang et al., 2016; Appendix, Table A1). The J-proteins transport protein substrates to Hsp70 for refolding or destruction, two processes important in protein turnover and cell function, and regulated by the influence of Hsp40 on Hsp70-ATPase activity. Differences in Hsp40 function are based on their variation in molecular mass and sequence, which may dictate the size range of clients that each type of Hsp40 binds and transports to Hsp70 (Fan et al., 2003; Nillegoda et al., 2015).

4.2 ArHsp40s are Developmentally Regulated and Heat Inducible Post-Diapause

The immunoblotting of western blots revealed that ArHsp40-1 and ArHsp40-2 were present in all life history stages of *A. franciscana* examined. Protein and mRNA for ArHsp40-2 peaked between 5 h and 10 h of cyst development while protein and mRNA for ArHsp40-1 remained at intermediate levels through development before both ArHsp40s declined to barely detectable levels by the 2nd instar nauplius stage. The synthesis of both ArHsp40s was induced during heat stress of 1st instar larvae before returning to basal levels upon recovery at room temperature. The results indicate that ArHsp40s influence growth and post-diapause development when cysts become metabolically active after diapause and that they protect the animal from thermal stress, most likely in concert with Hsp70.

Although Hsp40s have been identified in crustaceans (Lee et al., 2012; Zhang et al., 2013), these results are the first to indicate a developmental role for Hsp40s in crustaceans. In insects, the differential expression of several Hsp40 genes occurs within *B. mori* diapause-induced embryos (Li et al., 2016; Sirigineedi et al., 2014), while Hsp40 and Hsp70 are up-regulated during diapause in the oriental fruit moth larvae *Grapholita molesta* before being down-regulated upon post-diapause development to adults (Zhang et al., 2016). During post-diapause development, *A. franciscana* resumes metabolism and protein synthesis (Zhu et al., 2009). ArHsp40s may be regulated along with Hsp70 and other HSPs as ATP becomes abundant after diapause termination, and this may occur in the apple fly maggot *Rhagoletis pomonella* (Ragland et al., 2011). The coordinated synthesis of Hsp70, Hsp40s and other HSPs is also induced in cold-acclimated larvae of *E. solidaginis* and cold stressed *D. melanogaster* (Colinet et al., 2010; Zhang et al.,

2011). These molecular chaperones may protect proteins from thermal stress in overwintering diapause embryos and promote their repair in larvae recovering from thermal stress.

4.3 ArHsp40-1 and ArHsp40-2 dsRNAs Knocked Down Corresponding Proteins

RNAi was previously used to study sHSPs and late embryonic abundant (LEA) proteins in developing embryos and cysts of *A. franciscana* (King & MacRae, 2012; Toxopeus et al., 2014). Consequently, RNAi was employed herein to examine how the loss of ArHsp40-1 and ArHsp40-2 from *A. franciscana* affected cysts and nauplii. Adult females prior to fertilization were injected with dsRNA complementary to either ArHsp40-1, ArHsp40-2 or GFP mRNA, the latter not expected to affect *A. franciscana* (Zhao et al., 2012), because the animal does not synthesize GFP.

Cysts and nauplii released from female *A. franciscana* injected with dsRNA exhibited reduced amounts of ArHsp40-1 or ArHsp40-2 without discernible off target effects. PCR primers were designed to amplify either the ZBD region of ArHsp40-1 or the G/F rich region of ArHsp40-2. More than one protein can be knocked down by a single dsRNA, this occurring in previous *A. franciscana* RNAi experiments (Toxopeus et al., 2014). dsRNA complimentary to ArHsp40-1 in this experiment had no apparent effect on ArHsp40-2 mRNA, and vice-versa, but whether degradation of other *A. franciscana* J-proteins occurred was not examined. Western blots containing extracts of cysts and nauplii from females injected with *GFP* dsRNA contained both ArHsp40-1 and ArHsp40-2.

4.4 Lack of ArHsp40s Did Not Alter the Survival of *A. franciscana* Females or the Development of Embryos

HSPs and their co-chaperones refold denatured proteins and mediate the folding of nascent proteins thereby affecting development, as well as stress tolerance (Hayashi et al., 2006; Zhu et al., 2010). Secondary effects from injected dsRNAs may cause stress in adult females and, in previous studies, the injection of ArHsp22 dsRNA caused high mortality, killing females before they could be fertilized and produce embryos (King et al., 2013). In contrast, ArHsp40-1 and ArHsp40-2 dsRNA injected into adult females at the concentrations used in this study did not reduce their survival, nor was there any effect on the time to release and the brood size of cysts and nauplii. These results indicate ArHsp40s do not influence embryo development or release, unlike either the sHSP p26 that affects time to release of cysts after fertilization (King & MacRae, 2012) or artemin, the loss of which increases the length of time for brood release from the female (King et al., 2014).

4.5 ArHsp40s Aid the Post-Diapause Development of *A. franciscana* Nauplii and Protect Against Heat

Acting as either monomers or dimers, J-proteins transport subsets of clients to Hsp70 for protection during stress or recovery from stress, which provides tolerance to thermal insults, desiccation and oxidation (Cyr & Ramos, 2015; Glover & Lindquist, 1998). Newly released nauplii deficient in either ArHsp40-1 or ArHsp40-2 exhibited reduced survival as compared to nauplii from females injected with *GFP* dsRNA, with loss of ArHsp40-1 having a more drastic effect. Both ArHsp40s are up-regulated in

response to heat shock at 39 °C, as is true for Hsp70 in nauplii exposed to 40 °C (Clegg et al., 2008), and they are likely to maintain the integrity of proteins under thermal stress and thus maximize viability. These results suggest that Hsp40s protect *A. franciscana* from thermal stress by promoting the refolding of other proteins by Hsp70 and that each J-protein plays different roles in this process. Based on the greater reduction in survival, ArHsp40-1 may contribute to the efficiency of protein refolding by Hsp70 more effectively than does ArHsp40-2 or it may recruit more vital protein substrates.

Nauplii deficient in ArHsp40s were more likely to die during development when compared to nauplii from females injected with dsRNA for GFP and they failed to reach sexual maturity. In T-cells the overexpression of J-protein DnaJB6 promotes the nuclear accumulation of Schlafen 1 leading to enhanced cell cycle arrest and suppression of proliferation (Zhang et al., 2008). The overexpression of Hsp70 in *Drosophila* larvae slows development by delaying cell cycle arrest (Feder et al., 1992; Krebs & Feder, 1997). It is still unclear if Hsp40s or other HSPs play a direct role in mediating cell cycle progression in eukaryotic cells. Emerging cysts are capable of re-entering quiescence and halting development if environmental conditions become non-optimal (Bagshaw, 1986; MacRae, 2016). If ArHsp40s play a role in inhibiting cell cycle progression rather than promoting growth, then it would help explain why these proteins down-regulate once *A. franciscana* has emerged and reached the nauplius stage, where they are incapable of re-entering quiescence.

4.6 Cysts Deficient in ArHsp40-1 Spontaneously Abort Diapause Entry

Without ArHsp40-1, cysts began hatching spontaneously 3 d after release from females and by 10 d 47.1% had hatched successfully, whereas no cysts lacking ArHsp40-2 hatched. Nauplii that hatched spontaneously exhibited normal morphological characteristics, grew and reached sexual maturity, while cysts that did not prematurely hatch presumably entered diapause or were dead.

There are a limited number of other studies showing spontaneous abortion of diapause entry or termination of diapause maintenance in *Artemia* cysts. Knock down of the stress-induced transcription factor p8 by RNAi causes approximately 50% of newly released *A. franciscana* cysts to hatch prematurely 36 h after release from females (King, 2013). p8 is developmentally regulated in *Artemia* embryos and progressively accumulates in the nuclei of encysted embryos (Lin et al., 2016; Qiu & MacRae, 2007). In *A. parthenogenetica* cysts, the knockdown of p8 inhibits autophagy before diapause entry (Lin et al., 2016). ArHsp40-1 may assist in the translocation of p8 to nuclei promoting transcription of genes that facilitate cell cycle arrest and metabolic depression, while the absence of ArHsp40-1 allows cells to remain active and represses entry into diapause. The knockdown of ArHsp40-2 did not affect the entry of cysts into diapause, implying that ArHsp40-1 recruits and binds specific proteins that promote diapause entry, whereas ArHsp40-2 does not. It has not been determined if ArHsp40-1 dsRNA affects the accumulation of other Hsp40s in developing *A. franciscana* cysts, leading to this unusual phenotype. Cysts lacking ArHsp40-1 that did not hatch remained dormant after 180 d of incubation in sea water at room temperature (data not shown). This result is in contrast to the knock down of p26 which causes almost 90% of cysts to spontaneously hatch after 90

d in seawater at room temperature (King & MacRae, 2012). These results show that HSPs differentially maintain *A. franciscana* diapause but how this occurs is unknown.

Micro RNAs (miRNAs) are key regulators of diapause entry and maintenance in insects and crustaceans. Diapausing pupae of the flesh fly *Sarcophaga bullata* down-regulate several miRNAs, some of which influence lipid metabolism and increase the synthesis of HSPs and other stress protectors (Reynolds et al., 2013, 2017). In *Artemia* the knockdown of miRNAs miR-100 and miR-34 leads to inhibition of cell cycle arrest during diapause entry by down-regulating polo-like kinase 1 (PLK1) and activating cyclin K and RNA polymerase II (RNAP II). The reduction of p90 ribosomal S6 kinase 2 (RSK2) mRNA by RNAi in *A. franciscana* and *A. parthenogenetica* embryos causes cell cycle arrest to fail and produces pseudo-diapause cysts that develop within their shells (Zhao et al., 2015; Dai et al., 2008). The molecular pathways and key signaling factors that control diapause regulation are not identified but ArHsp40-1 and other type 1 J-proteins may contribute to controlling cell cycle arrest during diapause entry.

4.7 ArHsp40s Provide Stress Tolerance to Cysts

Following diapause termination by desiccation and freezing 21% of cysts lacking ArHsp40-1 and 38% of cysts lacking ArHsp40-2 hatched whereas 58% of cysts released by females injected with GFP dsRNA hatched. These results show ArHsp40s contribute to cyst stress tolerance during diapause and ArHsp40-1 plays a larger role than ArHsp40-2. Only cysts that did not hatch prematurely after 10 d post release, which represented 51% of the released cysts lacking ArHsp40-1, were used for viability tests. Only 6% of cysts lacking p26 hatched after desiccation and freezing (King & MacRae, 2012) while

cysts lacking group 1 LEA proteins were less than 5% viable (Toxopeus et al., 2014).

These molecular chaperones, along with ArHsp40s, likely work together, but in different ways, to promote protein homeostasis during diapause.

Similar to previous studies of diapause termination using *A. franciscana* (King & MacRae 2012; King et al., 2013), 50-60 % cysts from females injected with GFP dsRNA and frozen for 8 weeks hatched after diapause termination, which was higher than the amount of hatching after shorter periods of freezing (Toxopeus et al., 2014). It is unknown why longer periods of freezing increase hatching, as the molecular pathway of diapause termination is not understood. However, cysts in native habitats that do not break diapause until winter or long periods of stress is over would be at an advantage.

Prolonged desiccation and freezing stress is thought to cause water loss, reactive oxygen species (ROS) accumulation and glass formation within cyst cells (Toxopeus et al., 2014), yielding an environment in which proteins denature and aggregate. ArHsp40s may function as part of the Hsp110-Hsp70-Hsp40 disaggregate system (Kaimal et al., 2017; Shorter, 2011), rescuing proteins from aggregates and refolding them when ATP is available, as cysts navigate diapause. During diapause, ATP is limited and ArHsp40s are unlikely to cooperate with Hsp70 to actively fold proteins, but they may bind and sequester vital proteins that mediate embryo development after diapause. Cysts without ArHsp40s are likely not able to perform protein refolding or sequestering efficiently and thus they experience higher rates of mortality during diapause related stress. *In vitro* chaperone assays using ArHsp40-1, ArHsp40-2, Hsp70 would be beneficial in determining if ATP affects the chaperoning activity of Hsp40s in *A. franciscana*.

4.8 Conclusions and Future Work

J-proteins constitute a family of co-chaperones that promotes protein folding and the rescue of damaged proteins by Hsp70. Type 1 and type 2 Hsp40s are the major co-chaperones of Hsp70 and their synthesis in diapausing insects is well documented. In *A. franciscana* the amounts of ArHsp40-1 change little during post-diapause development until they down-regulate upon reaching the 1st instar nauplius stage. ArHsp40-2 is up-regulated 5-10 h into post-diapause development when the nauplius begins to emerge from the cyst before down-regulating significantly as the nauplii develop further. ArHsp40s are also up-regulated in heat stressed nauplii. The ArHsp40s may work individually or in concert with other molecular chaperones to play multiple roles in stress tolerance and development of early post-diapause *A. franciscana* nauplii.

When ArHsp40-1 and ArHsp40-2 were knocked down the resistance of cysts to desiccation and freezing was reduced, with loss of ArHsp40-1 having a greater effect than loss of ArHsp40-2 perhaps because the type 1 Hsp40 contributes more to protein protection when ATP is limited. Future work should address if Hsp40s work individually or with other molecular chaperones such as p26 and LEA proteins which provide significant desiccation and freeze stress tolerance for *Artemia* cysts. Some of the cysts lacking ArHsp40-1 spontaneously aborted diapause entry and hatched, yielding typical developing nauplii, suggesting that ArHsp40-1 prevents cysts from escaping diapause entry. The molecular pathway that leads to this phenotype is not understood but may result because ArHsp40-1 assists in the folding of important proteins, required, for example, in transcription.

In conclusion, ArHsp40s contribute to the development of nauplii and their protection from thermal stress. ArHsp40-1 and ArHsp40-2 play a role in the high stress tolerance of diapausing *A. franciscana* cysts, while ArHsp40-1 mediates diapause entry but not termination. The abortion of diapause by cysts lacking ArHsp40-1 provides a foundation for future proteomic and genomic studies for uncovering the molecular mechanisms controlling diapause entry. These results contribute to the understanding of co-chaperone function during development and diapause, the latter a physiological process used by many metazoans such as crustaceans and insects to survive stress. *A. franciscana* is a popular feed in aquaculture, while diapause enhances the effects of pest insects that challenge the agriculture, forestry and medical industries. Uncovering key signals and molecular pathways that regulate how these organisms enter and exit diapause therefore has significant economic implications as well as scientific interest.

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Appendix A SUPPLEMENTAL DATA

Table A1. ArHsp40-2 shares sequence identity with animal type 2 Hsp40s.

The % identity between ArHsp40-2 and other type 2 Hsp40s from other organisms was calculated by BLASTP at <http://www.ncbi.nih.gov>.

Species	Class	Identity (%)	Accession Number
<i>Caenorhabditis elegans</i>	Chromadorea	54	NP_496468.1
<i>Aedes aegypti</i>	Insecta	58	XP_001658074.1
<i>Culex quinquefasciatus</i>	Insecta	58	XP_001845463.1
<i>Daphnia pulex</i>	Branchiopoda	64	EFX80146.1
<i>Acyrtosiphon pisum</i>	Insecta	60	XP_001949061.2
<i>Megachile rotundata</i>	Insecta	62	XP_003707298.1
<i>Dendroctonus ponderosae</i>	Insecta	65	XP_019765112.1
<i>Tribolium castaneum</i>	Insecta	63	XP_008200761.1
<i>Helobdella robusta</i>	Citellata	52	XP_009022917.1
<i>Biomphalaria glabrata</i>	Gastropoda	55	XP_013081596.1
<i>Homo sapiens</i>	Mammalia	58	NP_008965.2
<i>Saccoglossus kowalevskii</i>	Enteropneusta	58	XP_002732367.1
<i>Branchiostoma floridae</i>	Leptocardii	52	XP_002601425.1

Figure A1. Standard curves for quantifying copy numbers of *ArHsp40s* and *α-tubulin* mRNA transcripts in post-diapause life-history stages.

qPCR was performed in duplicate on templates of known concentration (copy number per μl) with SYBR® Green to generate standard curves of Ct (cycle threshold) values for *ArHsp40-1* (**a**, $R^2 = 0.99079$), *ArHsp40-2* (**b**, $R^2 = 0.99801$), and *α-tubulin* (**c**, $R^2 = 0.99565$) as described in Materials and Methods.

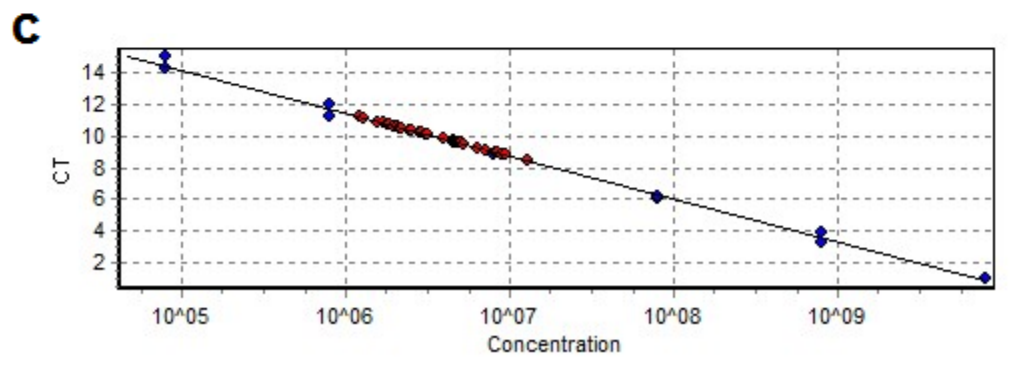
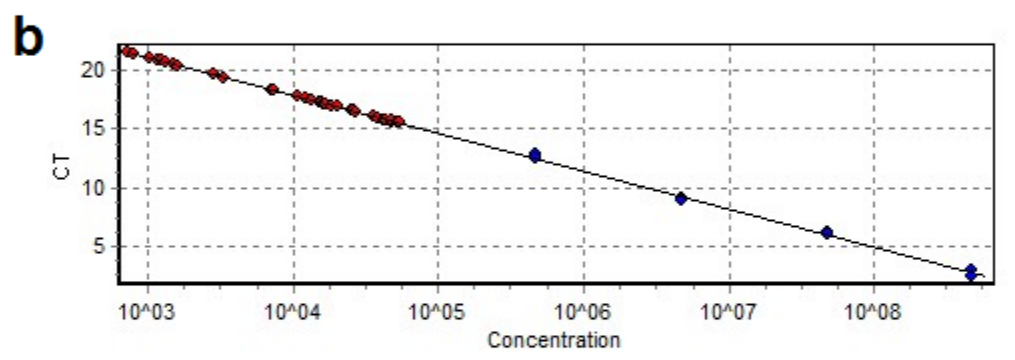
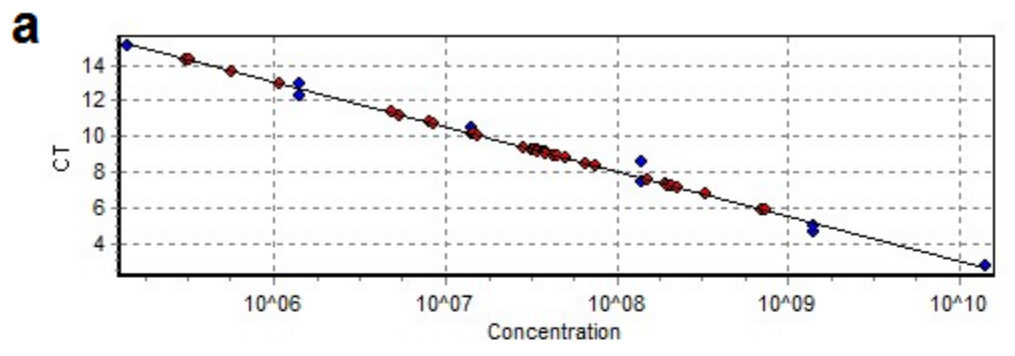


Figure A2. Standard curves for quantifying copy numbers of *ArHsp40s* and α -*tubulin* mRNA transcripts in knockdown cysts and nauplii.

qPCR was performed in duplicate on templates of known concentration (copy number per μ l) with SYBR® Green to generate standard curves of Ct (cycle threshold) values for *ArHsp40-1* (**a**, $R^2 = 0.99886$), *ArHsp40-2* (**b**, $R^2 = 0.99900$), and α -*tubulin* (**c**, $R^2 = 0.99894$) as described in Materials and Methods.

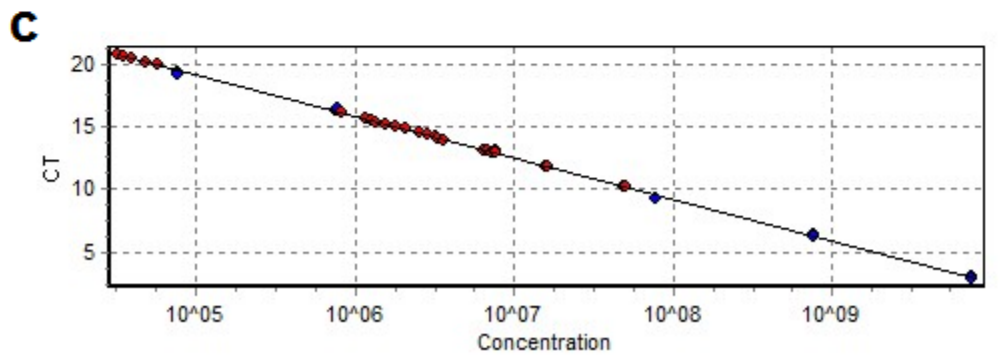
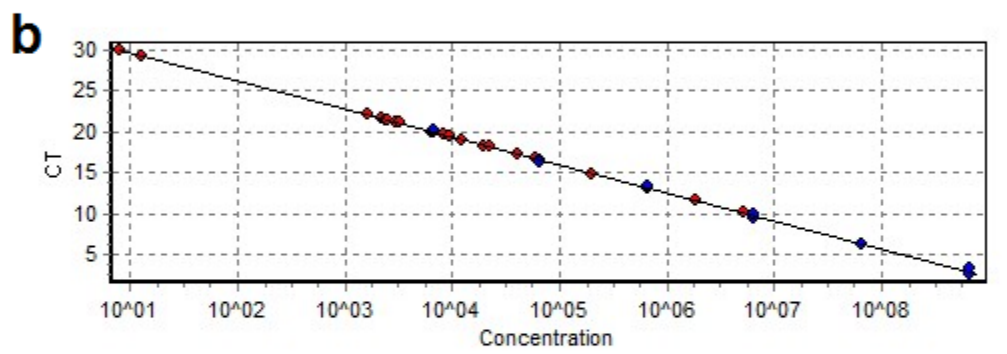
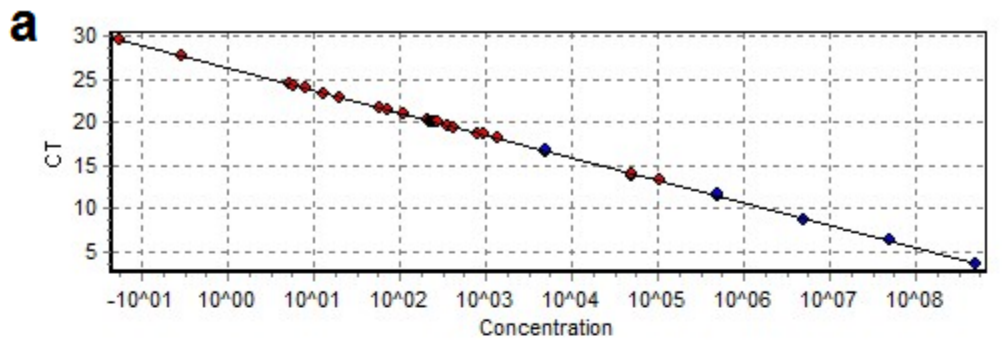
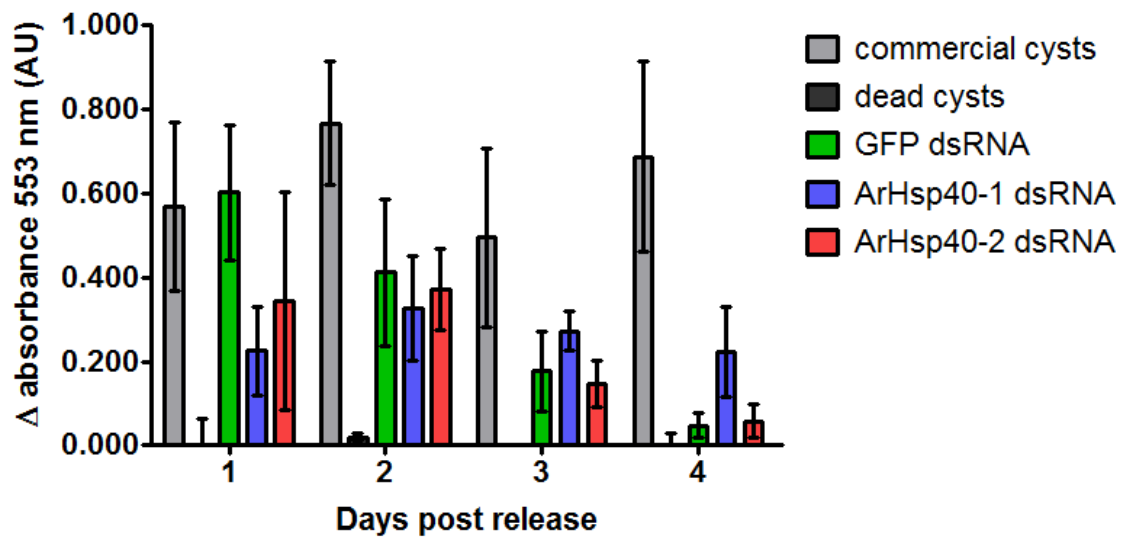


Figure A3. Metabolic activities of cysts lacking ArHsp40-1 remained elevated

The metabolic activity of cysts from females injected with *GFP* (green), *ArHsp40-1* (blue), or *ArHsp40-2* (red) dsRNA were similar to metabolically active quiescent cysts (gray) days 1-3 post release from injected females. By day 4 cysts from females injected with *GFP* (green) and *ArHsp40-2* (red) dsRNA had similar metabolic activity to dead cysts (black) while cysts from females injected with *ArHsp40-1* dsRNA had maintained similar metabolic activity to quiescent cysts (gray). Ten 1st brood cysts were incubated in 0.03% phenol red diluted in seawater, pH 8.6, the absorbance change was measured at 553 nm, and absorbance change between 24 h intervals was a measure of metabolic activity. *AU*, arbitrary units. Error bars represent *standard error*.



Appendix B MICROPIPETTE PREPERATION

Parameters for pulling micropipettes used in microinjection of adult *A. franciscana* females:

Heat:	560
Pull:	150
Velocity:	100
Time:	150
Pressure:	300

Appendix C SOLUTIONS AND RECIPES

Arranged by the order in which they appear in the Materials and Methods.

Solutions for Agarose Gel Electrophoresis

5 X TBE (diluted 10-fold for electrophoresis)

54 g Tris
27.5 g boric acid
20 ml 0.5 M EDTA, pH 8.0
dH₂O to 1 litre

Solutions for SDS-Polyacrylamide Gel Electrophoresis

1A: acrylamide/bis-acrylamide, 37.5:1 in dH₂O

1B: 1.5 M Tris (pH 8.8) with 0.5% (w/v) SDS

18.3 g Tris-base
2.5 ml 20% (w/v) SDS
pH 8.8
dH₂O to 100 ml

1C: 0.2% (v/v) TEMED

100 µl TEMED
dH₂O to 50 ml

1D: 5% (w/v) ammonium persulfate

50 mg ammonium persulfate
dH₂O to 10 ml

2B: 0.5 M Tris (pH 6.8) with 0.5% (w/v) SDS

6.0 g Tris-base
2.5 ml 20% (w/v) SDS
pH 6.8
dH₂O to 100 ml

2C: 2% (v/v) TEMED

100 µl TEMED
dH₂O to 5 ml

12.5% SDS Polyacrylamide Running Gel

1A acrylamide/bis-acrylamide, 37.5:1 in deionized H₂O) 5.0 ml, 1B 4.0 ml, 1C 2.0 ml, dH₂O 3.0 ml, 1D 2.0 ml

SDS Polyacrylamide Stacking Gel

1A 1.0 ml, 2B 2.5 ml, 2C 1.25 ml, dH₂O 4.0 ml, 1D 1.25 ml

4 X Treatment Buffer (diluted four-fold for electrophoresis): 250 mM Tris, 280 mM SDS, 40% (v/v) glycerol, 20% (v/v) β -mercaptoethanol, 0.2% (w/v) bromophenol blue, pH 6.8

1.2 g Tris
3.2 g SDS
16 ml glycerol
8 ml β -mercaptoethanol
0.08 g bromophenol blue
pH 6.8
dH₂O to 40 ml

Running Buffer: 25 mM Tris, 200 mM glycine with 0.04% (w/v) SDS

12.0 g Tris
57.6 g glycine
8.0 ml 20% (w/v) SDS
dH₂O to 4 L

Solution for Staining SDS-PAGE Gels

Colloidal Coomassie Blue Stain: 10% (w/v) ammonium sulfate, 0.1% (w/v) Coomassie G-250, 3% (v/v) phosphoric acid, 20% (v/v) ethanol

10 g ammonium sulfate
0.1 g Coomassie G-250
3 ml phosphoric acid
20 ml ethanol
dH₂O to 100 ml

Solutions for Western Blotting

Transfer Buffer: 25 mM Tris, 200 mM glycine in 20% (v/v) methanol

800 mL methanol
12.0 g Tris
57.6 g glycine
dH₂O to 4 L

TBS: 10 mM Tris, 140 mM NaCl, pH 7.4

1.21 g Tris
8.18 g NaCl
pH 7.4
dH₂O to 1 L

TBS-T: 10 mM Tris, 140 mM NaCl and 0.1% Tween-20, pH 7.4

1.21 g Tris
8.18 g NaCl
1 mL Tween-20
pH 7.4
dH₂O to 1 L

Appendix D COPY RIGHT RELEASE LETTERS

B.1 Copyright Release for Chapter 3

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