Hsp90 in *Artemia franciscana* During Development and Diapause

by

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Submitted in partial fulfillment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
June 2017

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TABLE OF CONTENTS

List of Tables v

List of Figures vi

Abstract vii

List of Abbreviations and Symbols Used viii

Acknowledgements x

Chapter 1 Introduction 1

1.1 *Artemia franciscana* life history 1

1.2 Heat shock proteins 3

1.3 Stress related proteins in *A. franciscana* 4

1.4 The structure and function of Hsp90 5

1.5 Hsp90 and diapause in organisms other than *A. franciscana* 7

Chapter 2 Materials and Methods 9

2.1 Culture of *A. franciscana* 9

2.2 Hsp90 mRNA and protein detection in *A. franciscana* cysts and nauplii 9

2.3 Preparation of dsRNA and injection of *A. franciscana* females 11

2.4 Knock down of Hsp90 mRNA in *A. franciscana* nauplii and cysts 15

2.5 Knockdown of Hsp90 in *A. franciscana* cysts and nauplii 16

2.6 Phenotypic modification of *A. franciscana* cysts and nauplii upon knockdown of Hsp90 16

2.7 Viability and stress tolerance of *A. franciscana* nauplii upon knockdown of Hsp90.16

2.8 Viability and stress tolerance of *A. franciscana* cysts upon knockdown of Hsp90...17

2.9 Statistical Analysis 18

Chapter 3 Results 19

3.1 Hsp90 mRNA and protein occur in nauplii and cysts of *A. franciscana*....................19

3.2 Generation of GFP and Hsp90 dsRNA 19

3.3 Knock down of Hsp90 mRNA in *A. franciscana* cysts and nauplii 23

3.4 Knock down of Hsp90 in *A. franciscana* cysts and nauplii.......................................23

3.5 Cysts released by females injected with dsRNA for Hsp90 were metabolically active upon release from females 26

3.6 Knockdown of Hsp90 slows the development of nauplius-destined *A. franciscana* embryos 26

3.7 Hsp90 knockdown reduces nauplii survival and stress tolerance 29

3.8 Morphology of *A. franciscana* cysts and nauplii after knockdown of Hsp90 32

3.9 Cysts released from females receiving dsRNA for Hsp90 exhibited reduced stress tolerance 32

Chapter 4 Discussion 36

4.1Hsp90 is knocked down in cysts and nauplii by RNAi 36

4.2 Reduction of Hsp90 increases the time between fertilization and release of nauplii from *A. franciscana* females 37

4.3 Hsp90 contributes to survival and stress resistance of *A. franciscana* nauplii 37

4.4 Knock down of Hsp90 disrupts the development of *A. franciscana* nauplii 38

4.5 Hsp90 is required for desiccation and freezing tolerance of *A. franciscana* cysts ..39

4.6 Conclusion 40

References 41

Appendix A Supplemental Data 49

Appendix B Micropipette Preparation 52

Appendix C Solutions and Recipes 53

LIST OF TABLES

Table 1. Primers used for the production of dsRNA and for RT-qPCR 12

Table 2. Knockdown of Hsp90 increased the time to release for nauplii of *A. franciscana* 28

LIST OF FIGURES

Fig. 1. *A. franciscana* life history 2

Fig. 2. Schematic representation of Hsp90 structural changes 6

Fig. 3. Microinjection of *A. franciscana* 14

Fig. 4. Hsp90 mRNA occurs in nauplii and cysts of *A. franciscana* 20

Fig. 5. Hsp90 occurs in nauplii and cysts of *A. franciscana* 21

Fig. 6. Generation of GFP and Hsp90 dsRNAs 22

Fig. 7. Hsp90 mRNA was reduced in cysts and nauplii released by females injected with Hsp90 dsRNA 24

Fig. 8. Hsp90 was knocked down in cysts and nauplii of *A. franciscana* by RNAi 25

Fig. 9. Hsp90 knock down cysts were viable upon release from females 27

Fig. 10. Hsp90 knock down nauplii exhibited reduced survival 30

Fig. 11. Hsp90 knock down nauplii exhibit increased heat sensitivity...........................31

Fig. 12. Morphology of cysts and nauplii released by knockdown *A. franciscana* females 33

Fig. 13. Knockdown of Hsp90 does not affect the size of newly released cysts and nauplii...34

Fig. 14. Cysts containing diminished amounts of Hsp90 exhibited reduced stress tolerance 35

ABSTRACT

Females of the extremophile crustacean, *Artemia franciscana*, either release motile nauplii via the ovoviviparous pathway of development or encysted embryos (cysts) via the oviparous pathway. Cysts contain an abundant amount of the ATP-independent small heat shock protein termed p26 that contributes to stress tolerance and embryo development, however, little is known of the role of ATP-dependent molecular chaperones such as Hsp90 in stress tolerance. In other organisms Hsp90 is required for the maturation of substrate proteins, often termed clients, and is involved via a dynamic ATP-dependent cycle in ensuring protein folding, degradation and tolerance to stress. In this study, Hsp90 mRNA and protein were respectively detected in cell free extracts of *A. franciscana* nauplii and cysts by qRT-PCR and immunoprobing of western blots. RNA interference (RNAi) was then used to characterize Hsp90 in *A. franciscana* nauplii and cysts, with the latter one of the few times Hsp90 has been examined *in vivo* during diapause. The partial knock down of Hsp90 slowed the development of nauplius-destined, but not cyst-destined embryos. Hsp90 knockdown also reduced the survival and stress tolerance of nauplii newly released from *A. franciscana* females. Although reduction of Hsp90 had no effect on the development of diapause-destined embryos, the resulting cysts displayed reduced tolerance to desiccation and low temperature, two stresses normally encountered by *A. franciscana* in its natural environment. The results reveal that Hsp90 contributes to development, growth and stress tolerance of *A. franciscana*, an organism of practical importance as a feed source in aquaculture.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ATP	adenosine 5'-triphosphate
AU	arbitrary units
bp	base pairs
Ct	cycle threshold
dsRNA	double-stranded RNA
ECL	enhanced chemiluminescence
GFP	green fluorescent protein
HRP	horse radish peroxidase
HST	high salt/Tween
IgG	immunoglobulin G
kDa	kiloDaltons
mRNA	messenger RNA
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
RT-qPCR	reverse transcription quantitative PCR
SDS	sodium dodecylsulfate
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

ACKNOWLEDGEMENTS

First and foremost, sincere thanks to God and then my country, the kingdom of Saudi Arabia, for giving me this chance to continue my study as a scientist for a better future. Similar thanks to my supervisor, Dr. Thomas H. MacRae for his support from the beginning until now, for the opportunity to work on this project, his excellent guidance in my development as a scientist, kind character, providing me with professional advice and always keeping in touch even when he was suffering. Always Tom accepts dialogue and consultation, creating an excellent atmosphere that keeps me working hard in lab and enjoying scientific research. I learned from him a lot, not only as a scientist but also how to be courageous and self-confident with an optimistic view in this life.

I extend a sincere thanks to my parents, my family (brothers and sisters) and my mother in law for their prayers and continuous moral support; their love and support are integral to my success. Moreover, sincere thanks to the special person in my life, my husband, Mohammed who always does everything to support me, give me the energy, raise my spirits to make me keep going to my target. Thank you, Mohammed, for your love with enormous amounts of patience throughout this journey. Not the least, thank you to my son Ali for bearing my situation and making my life joyful.

Additionally, I would like to thank my friend Afrah Al-Othman for her always support, as well as all my lab mates, Sheethal, Jiabo, Nathan, Azzah, Hajer, Yayra, and Alicia, for their support and help, but I'm particularly grateful to thank Sheethal and Jiabo for their time and assistance on my project. I would not have been able to complete my experiments without their troubleshooting advice, suggestion and technical support.

Finally, thanks to my committee members, Dr. Sophia Stone, Dr. Arunika Gunawardena and Dr. Neil Ross for their guidance and feedback on my research and thesis work. Also, I can’t forget to thank Dr. Patrick Lett for financial assistance.

CHAPTER 1 INTRODUCTION

1.1 *Artemia franciscana* life history

Artemia franciscana, an extremophile crustacean commonly known as brine shrimp or sea monkeys, is a good model organism for basic and applied research, the former including the molecular basis of diapause and stress tolerance. *A. franciscana* is readily available and easy to grow under laboratory conditions, with adults reaching sexual maturity in approximately one month if conditions are optimal (King 2013; Gilchrist 1960). Male adults are distinguished from females by their anterior claspers and lack of an egg sac at sexual maturity (King, 2013). The female possesses an egg sac which prior to fertilization appears as two distinct lateral sacs rather than one continuous sac (King, 2013).

To survive harsh environmental conditions such as high salinity, cold, drying, UV radiation and food shortage *A. franciscana* undergo alternative pathways of development termed ovoviviparous and oviparous (Fig. 1), respectively leading to the release of free-swimming nauplii (larvae) and encysted gastrula-stage embryos (cysts) that enter diapause, a physiological state of dormancy (Liang and MacRae, 1999; King, 2013; Ma et al., 2013; De los Rios-Escalante and Salgado, 2012; Alekseev, 2010; Clegg at al., 1996; Liang and MacRae, 1999). Diapause occurs in anticipation of adverse environmental conditions and results in greatly reduced metabolic activity and enhanced stress tolerance (Alekseev, 2010; Liang et al., 1997; Poelchau et al., 2013; Fan et al., 2013). *A. franciscana* remain in diapause, even under favourable growth conditions, until experiencing a transient environmental termination signal such as cold

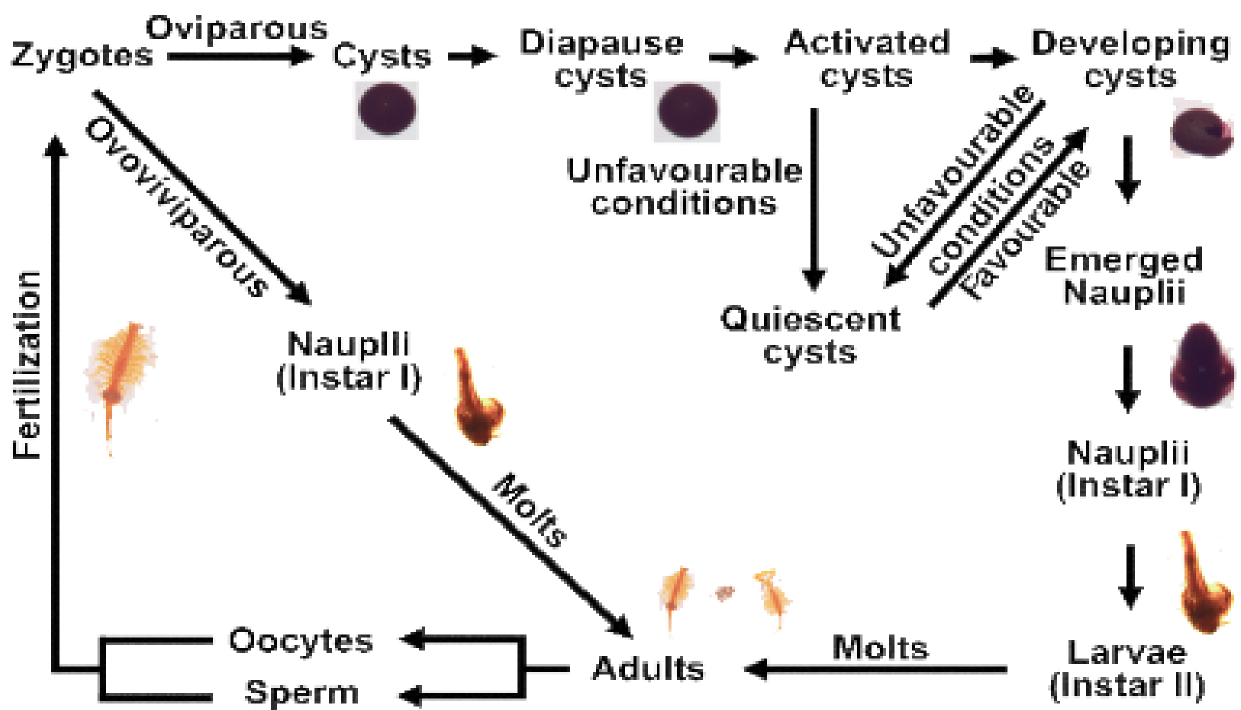

Fig 1. *A. franciscana* life history. Females release free-swimming nauplii via the ovoviviparous pathway and encysted embryos (cysts) via the oviparous pathway. The cysts enter diapause after 5 to 10 days and are maintained there until the appropriate environmental signal is received and termination occurs. If conditions are favourable the cysts hatch and produce nauplii that undergo development. If an unfavourable state prevails the cysts enter quiescence and remain there until conditions become conducive for growth (King, 2013).

and/or desiccation (Ma et al., 20; Zhu et al., 2009). Once diapause terminates cysts develop into nauplii if conditions are favourable, but if conditions are not suitable for growth the activated cysts are maintained in post-diapause quiescence until introduction to an environment suitable for development (Liang and MacRae, 1999). Both diapause and quiescence allow *A. franciscana* to survive in harsh conditions and are essential to their success but differ in that an exogenous signal is required to terminate diapause whereas quiescence terminates upon introduction to favourable growth conditions (Zhang et al., 2011). Diapause and quiescence are very important for coordinating active phases of the life cycle with times when food resources and access to mating are available (Zhang et al., 2011). As based on insects, diapause is divided into three eco-physiological phases termed pre-diapause, diapause maintenance and post-diapause which may include quiescence. In insects diapause generally occurs at a specific developmental stage that varies from one species to another, and in response to key stimuli from the environment (facultative diapause) or as an obligatory part of an animal's life history (obligatory diapause) (Lu et al., 2013). In *A. franciscana* diapause can be divided into three phases known as initiation, maintenance and termination and these share similarities to the phases seen in insects (King, 2013).

1.2 Heat shock proteins

Heat shock proteins (HSPs), also known as molecular chaperones, were discovered in *Drosophila* (Ciocca et al., 2013) and they have been found in all organisms examined. The synthesis of HSPs occurs either constitutively or in response to stress and most organisms respond to environmental, metabolic and pathophysiological stressors

by up-regulating HSPs. Based on their molecular mass, sequence and function, HSPs are classified into six major categories, which are Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small heat shock proteins (sHsps) (Fan et al., 2013). HSPs are required for the folding of nascent proteins, subcellular localization of proteins, protein degradation and the protection of proteins under stress conditions which lead to unfolding (Zhu et al., 2009; Fan et al., 2013; Qiu and MacRae, 2008).

1.3 Stress related proteins in *A. franciscana*

Three sHsps, several late embryogenesis abundant proteins (LEA proteins) and artemin, a cysteine-enriched ferritin homologue, are up-regulated during diapause of *A. franciscana*, and these proteins have a role in stress tolerance (King and MacRae, 2012). p26 may also contribute to the development of diapause-destined *A. franciscana* embryos and to diapause maintenance by regulating diapause termination (King and MacRae 2012; King et al., 2013). Although the role of ATP-independent effectors of stress tolerance such as the sHSPs has been examined in some detail in *A. franciscana* (Fan et al., 2013; Qiu and MacRae, 2008; King and MacRae, 2015), there is little information on the occurrence and activities of the ATP-dependent molecular chaperones such as Hsp90 during diapause.

1.4 The structure and function of Hsp90

Hsp90, a dimeric protein with each monomer consisting of an amino-terminal ATP-binding domain, a middle domain, and the carboxyl-terminal dimerization domain, is an ATP-dependent molecular chaperone (Fig. 2). Hsp90 has structural flexibility via a

complex mechanism of ATPase-coupled conformational changes with an open conformation when not bound to ATP and a closed conformation when bound to nucleotide, a change that mediates protein folding (Röhl et al., 2013). The amino (N)-domain contains the ATP binding site for Hsp90 and is responsible for ATP hydrolysis. The middle (M) domain binds clients and co-chaperones and influences ATP hydrolysis, whereas the carboxyl (C)-domain mediates dimerization. The N- and M-domains are connected by a charged, unstructured linker (L) domain which allows for the movement of the N- and M-domains relative to one another, this of functional importance (Li et al., 2012) (Fig 2). Hsp90 interacts with many different client proteins, especially those that are metastable. Kinases and steroid receptors are particularly important substrates of Hsp90 (King & MacRae, 2015) allowing the chaperone to influence gene regulation through intracellular signaling (Pearl & Prodromou, 2006) which may be significant during exposure to stress (Pearl & Prodromou, 2006, Röhl et al., 2013, King & MacRae, 2015).

Many co-chaperones that regulate the functions of Hsp90, such as p23 co-chaperone that facilitates binding to substrate and ATPase hydrolysis, have been identified in eukaryotic cells (Felts & Toft., 2003; Li et al., 2012). Hsp90 often interacts with co-chaperones in series with the binding of one co-chaperone leading to a change in Hsp90/co-chaperone conformation and allowing for the binding of another co-chaperone, thus establishing a progressive series of interactions that drive Hsp90 function. As one example, the co-chaperone, Hop, binds to Hsp70 during the chaperone cycle leading to a conformational state that is important for the formation of an

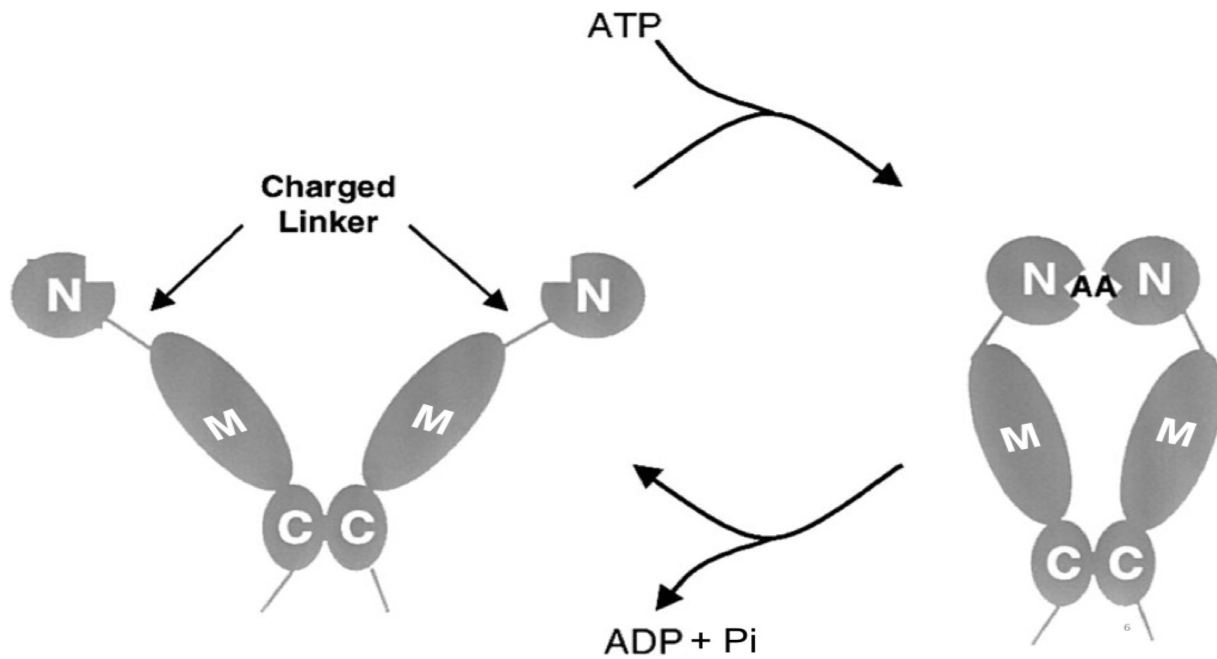

Figure 2. Schematic representation of Hsp90 structural changes. The Hsp90 chaperone cycle is initiated by ATP binding to the N-domain(s) which assists in the transient dimerization of N-domains to create a “closed’’ conformation. In the closed conformation ATP is hydrolyzed, proteins are folded and ADP + Pi is released, allowing Hsp90 to return to the open state. N, amino-domain; L, linker domain, M, middle-domain; C, carboxyl-domain; AA, ATP (Prodromou et al., 2000).

Hsp90-Hsp70 complex and transfer of clients from Hsp70 to Hsp90 (Panaretou et al., 2002; Röhl et al., 2013). Hop contains three domains for interaction with two chaperones; one domain is specific to Hsp90 while the other two domains bind to Hsp70 (Panaretou et al., 2002; Röhl et al., 2013). The function of some co-chaperones may be restricted to specific client proteins and be required for the stimulation (Aha1) or inhibition (Hop) of Hsp90 ATPase activity.

1.5 Hsp90 and Diapause in Organisms Other than *A. franciscana*

Small Hsps such as p26 were examined in *A. franciscana* and shown to have an important role in stress tolerance during diapause (Clegg et al., 1999; Qiu and MacRae, 2007, Qiu and MacRae, 2008), however the contribution of Hsp90 to stress tolerance during diapause has not yet been investigated. It is known, however, that *Hsp90* is downregulated during the pupal diapause of the flesh fly *Sarcophaga crassipalpis* (Tachibana et., 2005). Additionally, *Hsp90* increases before and after diapause in *S. crassipalpis* but the reason for this is not known (Rinehart and Denlinger, 2000). whereas *Hsp90* is unchanged quantitatively during diapause in the fruit fly *Drosophila triauraria* (Aruda et al., 2011). In the current study, Hsp90 function was examined by RNAi in *A franciscana* nauplii and cysts, the latter adding to what is known about this molecular chaperone during diapause.

Hypothesis

Hsp90 is required for the development of nauplius-destined and diapause-destined *A. franciscana* embryos, most likely through its effects on protein folding and intracellular signaling, although its role has not been investigated in this context in this study. Secondly, Hsp90 may contribute to the stress tolerance of nauplii and cysts through sequestering substrate proteins during diapause when ATP is limiting, thereby protecting them from irreversible denaturation but not immediately refolding them (MacRae, 2010; King & MacRae, 2015). This study was designed to demonstrate the presence of Hsp90 in nauplii and cysts of *A. franciscana* and to determine if Hsp90 affects their development and stress tolerance.

CHAPTER 2 MATERIALS AND METHODS

2.1 Culture of A. franciscana

A. franciscana cysts (INVE Aquaculture, Inc., Ogden, UT, USA) were hydrated in distilled water on ice, washed with cold water, and then incubated with aeration in autoclaved water from Halifax Harbour, Nova Scotia, Canada, hereafter known as sea water, at room temperature. Larvae and adults, maintained at room temperature in seawater, were fed *Isochrysis galbana* (T-iso) (Provasoli-Guillard Center for Culture of Marine Phytoplankton, West Boothbay Harbor, ME, USA). *A. franciscana* adults were mated in 6-well plates and both nauplii and cysts were collected upon release from females, usually at day 5 post-fertilization (King, 2013; Qiu and MacRae, 2007; King and MacRae, 2012).

2.2 Hsp90 mRNA and protein detection in A. franciscana cysts and nauplii

RNA was prepared from 200 cysts and nauplii with the TRIzol® Plus RNA Purification Kit (Invitrogen) prior to isolation with the RNeasy Kit (Qiagen). Single stranded cDNA was produced with Superscript III® reverse transcriptase (Invitrogen) and oligo-dT primers (Invitrogen) according to manufacturer's instructions. cDNA encoding Hsp90 was then amplified by PCR using single-stranded cDNA as template and Hsp90-specific forward (5-GTCAGTTTGGTGTGGGTTTC-3), and reverse (5-CCTTGGGTTTGTCCTCT TC-3), primers based on the sequence of *A. franciscana* Hsp90. Hsp90 cDNA was cloned from *A. franciscana* by Jiabo Tan (unpublished data). Amplification was under the following reaction conditions, 5 min at 94°C, 30 cycles of 94°C for 30 sec, 57°C for 30 sec and 72°C for 1 min, followed by 10 min at 72°C. Amplification products were resolved in

1.2% agarose gels in 0.5 x TBE buffer (50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 8.0) at 100 V, stained with Gelstar® (Lonza, Basel, Switzerland) or cybersafe (Invitrogen) and visualized in a DNR Bio-imagining Systems MF-ChemiBIS 3.2 Gel Documentation system (Montreal Biotech, Montreal, Quebec, Canada).

For protein preparation, 100 *A. franciscana* cysts or first instar nauplii obtained from females grown in the laboratory were homogenized on ice with a micropestle (Fisher Scientific) in a 1.5 ml microtube containing 25 µl treatment buffer for SDS polyacrylamide gel electrophoresis (62.5 mM Tris, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) B-mercaptoethanol, 0.05% (w/v) bromophenol blue, pH 6.8). The homogenate was placed in a boiling water bath for 5 min and centrifuged for 10 min at 10000 RPM (8600 g) in a microcentrifuge at 4°C. The PiNK Plus Prestained Protein Ladder (FroggaBio Inc., Toronto, ON, Canada), and 20 µl of each protein sample were resolved in 12.5% SDS polyacrylamide gels at 35 mA for 60 min and then transferred to nitrocellulose membranes at 100 mA overnight at room temperature (BIO-RAD Mini PROTEIN Tetra Cell, Hercules, CA, USA). Membranes were then incubated with Ponceau stain for 5 min to check protein transfer, rinsed with distilled water and blocked with 5% low fat milk solution in TBS (10 mM Tris, 140 mM NaCl, pH 7.4) at room temperature for 60 min. Washed membranes were incubated for 20 min with antibody to the Hsp90 peptide 76-CLELFEEIAEDKENYKKFYE-97 (ABBIOTEC, San Diego, CA, USA) diluted 1:1000 in TBS, then washed three times for 5 min with TBS-T (10 mM Tris, 140 mM NaCl, 0.1% Tween-20, pH 7.4) and three times for 5 min with HST (10 mM Tris, 1 M NaCl, 0.5% Tween-20, pH 7.4). The membranes were then incubated for 20 min at room temperature with HRP-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich) diluted 1:10000 in

TBS, and washed three times with TBS-T and HST as before. Following a final wash for 3 min in TBS, immunconjugates were visualized with Western Lightning Enhanced Chemiluminescence (ECL) Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA) in a DNR Bio-Imaging Systems MF-ChemiBIS 3.2 gel documentation system (Montreal Biotech). Experiments were done in triplicate.

2.3 Preparation of dsRNA and injection of A. franciscana females

Platinum Taq DNA Polymerase (Invitrogen, Burlington, ON, Canada) was used with cDNA and primers (0.2 mM) containing the T7 promoter sequence at their 5' ends (Table 1) to produce dsRNA. Amplification was under the following reaction conditions: 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, with a final 10 min at 72°C. As control, green fluorescent protein (GFP) cDNA in the vector pEGFP-N1 (Clontech, Mountain View, CA, USA) was amplified using the reaction conditions described and specific primers (Table 1).

dsRNAs for Hsp90 and GFP were generated using the MEGAscript® RNAi kit (Ambion Applied Biosystems, Austin, TX, USA) and they were resolved by electrophoresis in 1.2% agarose gels before visualization with SYBR® Safe DNA gel stain (Invitrogen) as described before. dsRNAs for Hsp90 and GFP were separately diluted with 1 volume of 0.5% phenol red in Dulbecco's phosphate buffered saline (DPBS) (Sigma-Aldridge, Oakville, ON, Canada) (King and MacRae 2012). Unfertilized adult *A. franciscana* females were placed on 1.6 % agarose at 4°C and injected by using the Nanoject II microinjector (Drummond Scientific Co., Broomall, PA, USA) and

Table 1. Primers used for the production of dsRNA and for RT-qPCR.

Primer Function	Primer sequence (5' to 3')	
	Forward	**Reverse**
dsRNA production		
Hsp90	TAATACGACTAACTATAGGG ACCATTCGACCAGACAGTGG	TAATACGACTAACTATAGGG GCGAGGTGATCTTCCCAGTC
GFP[a]	TAATACGACTCACTATAGGG AGACACATGAAGCAGCACG ACCT	TAATACGACTCACTATAGGG AGAAGTTCACCTTGATGCCC TTC
qRT-PCR		
Hsp90	GCTGACCGTGTTGTTGTCAC	ACGATCTTGGTTCCACGTCC
α-tubulin[b]	CGACCATAAAAGCGCAGTCA	CTACCCAGCACCACAGGTCTCT

All primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). Highlighted sequences indicate the T7 promoter; a, Zhao et al., 2012; b, King et al., 2013.

glass needles prepared with preset program 25 on a P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Co., Novato, CA, USA) (Fig. 3). Needles were cut at 40° angle with a clean razor blade under an Olympus SZ61 Stereomicroscope (Olympus Canada Inc., Markham, ON, Canada). For each injection 250 µl of solution containing 80 ng of dsRNA for either Hsp90 or GFP was used.

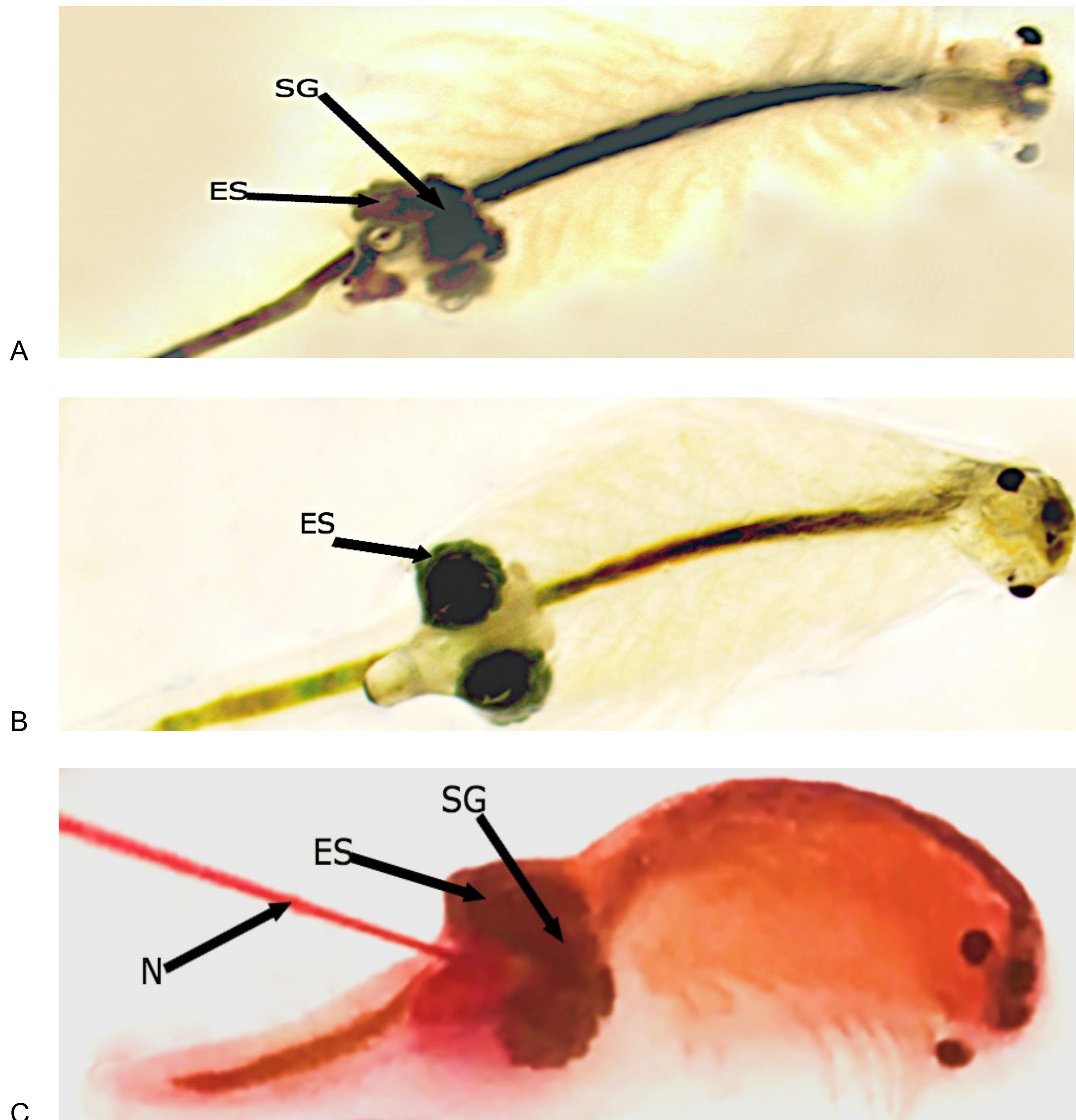

Fig 3. **Microinjection of *A. franciscana females*.** Light micrographs of *A. franciscana* adult females. A, unstained female producing cysts; B, unstained female producing nauplii; C, injected female producing cysts; N, needle; ES, egg sac; SG, shell gland.

2.4 Knock down of Hsp90 mRNA in A. franciscana nauplii and cysts

RNA was prepared from either 80 cysts or 50 nauplii immediately upon release from *A. franciscana* females injected with dsRNA for either Hsp90 or GFP, by homogenizing with a micropestle (Fisher Scientific) in a 1.5 ml microtube with 100 µl TRIzol® (ThermoFisher Scientific). cDNA was generated with the SuperScript® III First-Strand Synthesis Kit for RT-PCR (ThermoFisher Scientific) using 0.1 µg of RNA as template and oligo-dT_{20} primers. qPCR, using 1 µl of cDNA, was conducted with the QuantiTect® SYBR® Green PCR Kit (Qiagen, Mississauga, ON, Canada) in a Rotor-Gene RG-3000 (Corbett Research, Sydney, Australia) with primers for Hsp90 and tyrosinated α-tubulin (Table 1) at 10 µM. The concentration of PCR products for each template was determined by measuring absorbance at 260 nm, and copy number was calculated based on Hsp90 PCR product length. Six-fold dilution series of DNA fragments were prepared in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and 0.5 µl of each dilution was used as template for qPCR with the QuantiTect® SYBR® Green PCR Kit (Qiagen). The Ct values from the standard curve were fitted and the copy number of each cDNA was determined using rotor-gene 6 software (Corbett Research). The copy numbers of Hsp90 and GFP after knockdown were normalized against tyrosinated α-tubulin and these values were plotted. Melting curve analysis was performed for each experiment. Hsp90 and GFP mRNA were quantified after knockdown in three samples independently prepared from cysts and nauplii with each sample tested twice by qPCR.

A one-tailed student's t-test was used to compare the level of mRNA in cysts and nauplii from females injected with Hsp90 dsRNA compared to GFP dsRNA.

2.5 Knockdown of Hsp90 in A. franciscana cysts and nauplii

Eighty cysts and 50 nauplii collected from females injected with either Hsp90 or GFP dsRNA were homogenized on ice with a micropestle (Fisher Scientific) in a 1.5 ml microtube containing 20 μl treatment buffer. Proteins were resolved in SDS polyacrylamide gels and blotted to nitrocellulose as described above. The experiment was done in duplicate with tyrosinated α-tubulin as control.

2.6 Phenotypic modification of A. franciscana cysts and nauplii upon knockdown of Hsp90

Microscopic observation using Infinity capture imaging software with an Olympus SZ61 stereomicroscope was employed to determine if cysts and nauplii released from 25 females injected with dsRNA for Hsp90 were morphologically normal compared to cysts and nauplii released from 25 females injected with dsRNA for GFP. The experiment was done 4 times for both knockdowns with separate preparations of 50 cysts and 50 nauplii in each study, n=200. The time from fertilization to release was determined for cysts and nauplii that contained either normal or reduced amounts of Hsp90. Cysts were incubated in sea water for 10 days to determine if they hatched and thus failed to enter diapause in the absence of Hsp90.

2.7 Viability and stress tolerance of A. franciscana nauplii upon knockdown of Hsp90

Nauplii, released from females injected with dsRNA for either Hsp90 or GFP were incubated in sea water at room temperature and observed daily to determine the number

of dead (immobile) organisms. Dead nauplii were removed after counting. To determine stress tolerance, nauplii immediately after release from females were heat shocked at 39°C for 1 h (Liang and MacRae, 1999) and then incubated at 25°C to allow recovery. The number of dead (immobile) organisms was determined after 24 h. Three independent experiments were done with the number of nauplii ranging from 57 to 134 per experiment.

2.8 Viability and stress tolerance of A. franciscana cysts upon knockdown of Hsp90

To determine if cysts were viable upon release from females they were examined for metabolic activity by the phenol red assay (King, 2013). Ten cysts were incubated in sea water containing 0.03% phenol red at pH 8.6, and changes in absorbance, which indicates metabolic activity, were measured at 553 nm. The metabolic activities of cysts newly released from females injected with dsRNA for Hsp90 and GFP, along with commercially obtained hydrated cysts killed by heating in a boiling water bath for 10 min were determined. The experiment was done twice, each with 3 independent cyst samples comprised of 10 cysts each.

Cysts released from females injected with dsRNA for either Hsp90 or GFP were incubated in sea water for 10 days at room temperature to allow diapause entry, then placed in aluminum foil-covered weigh boats in a desiccator containing Indicating Drierite (Sigma-Aldrich) for 10 days prior to freezing at -80C for 2 months. Desiccation and freezing were required to terminate diapause and to act as stressors. The viability of cysts, and thus their stress tolerance, was determined by hatching assay (King, 2013). During

the 10 days incubation in sea water at room temperature prior to desiccation cysts were monitored microscopically for hatching, an indication, if it occurred, that they did not enter diapause. The experiment was done in duplicate with 3 separate preparations of 40 to 50 cysts in each trial.

2.9 Statistical Analysis

A one-tailed student's t-test (α= 0.05) via Two-Sample Assuming Equal Variances was used to assess whether Hsp90 mRNA was more abundant in cysts and nauplii that were released from females injected with GFP dsRNA as compared to Hsp90 dsRNA. As well a one-tailed student's t-test was used to assess differences in the survival and viability of first brood cysts and nauplii released from females injected with Hsp90 and GFP dsRNA. Results are expressed as the mean ± standard deviation of these measurements. One-way analysis of variance (ANOVA) was used to determine statistical significance of differences in cyst metabolism upon release from females ($p<0.05$). All data were expressed as the mean ± standard deviation of these measurements.

CHAPTER 3 RESULTS

3.1 *Hsp90* mRNA and protein are present in nauplii and cysts of *A. franciscana*

RT-PCR using primers specific to *Hsp90* produced *Hsp90* mRNA in nauplii and cysts of *A. franciscana* of the expected size as shown by agarose gel electrophoresis (Fig. 4). Probing of western blots containing cell free protein extracts with antibody to Hsp90 yielded immunoreactive bands of the expected size (Fig. 5).

3.2 Generation of *GFP* and *Hsp90* dsRNA

dsRNAs generated with *GFP* and *Hsp90* cDNA as templates were of the expected sizes, respectively 309 and 413 bps., Amplification products were resolved by electrophoresis in 1.5% agarose gels and DNA was visualized with SYBR® Safe DNA gel stain (Invitrogen) (Fig. 6).

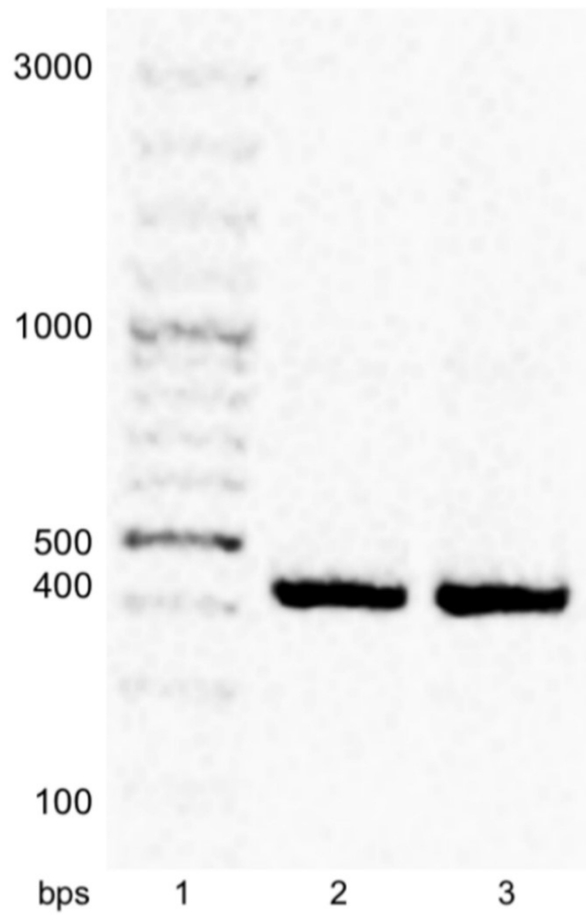

Fig. 4. ***Hsp90* mRNA in nauplii and cysts of *A. franciscana*.** *Hsp90* cDNA was prepared from RNA by RT-PCR after which amplification products were resolved by electrophoresis in 1.2% agarose gels and visualized with SYBR® Safe DNA gel stain. Lane 1, Size marker in bp; 2, nauplii; 3, cysts.

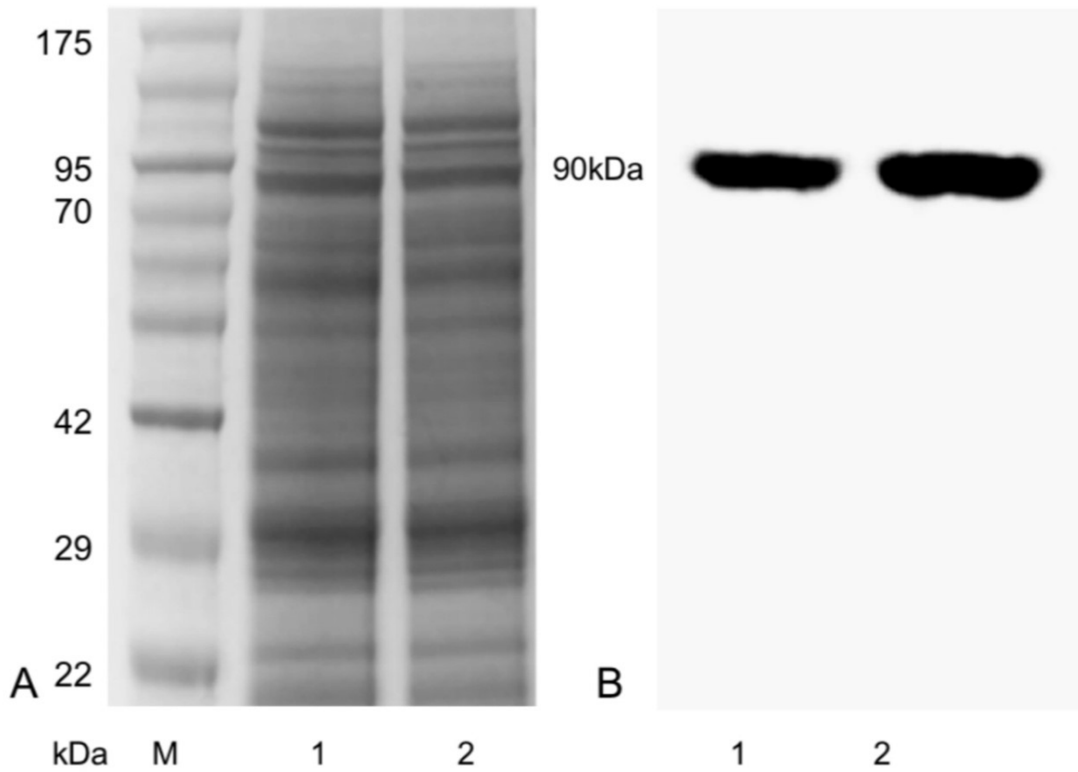

Fig. 5. **Hsp90 in nauplii and cysts of *A. franciscana*.** Protein extracts from cysts and nauplii were resolved in SDS polyacrylamide gels and either stained with Coomassie blue (A) or blotted to nitrocellulose and reacted with antibody to Hsp90 (B). Cell free protein extracts were prepared from nauplii (1) and cysts (2). M, molecular mass markers in kDa.

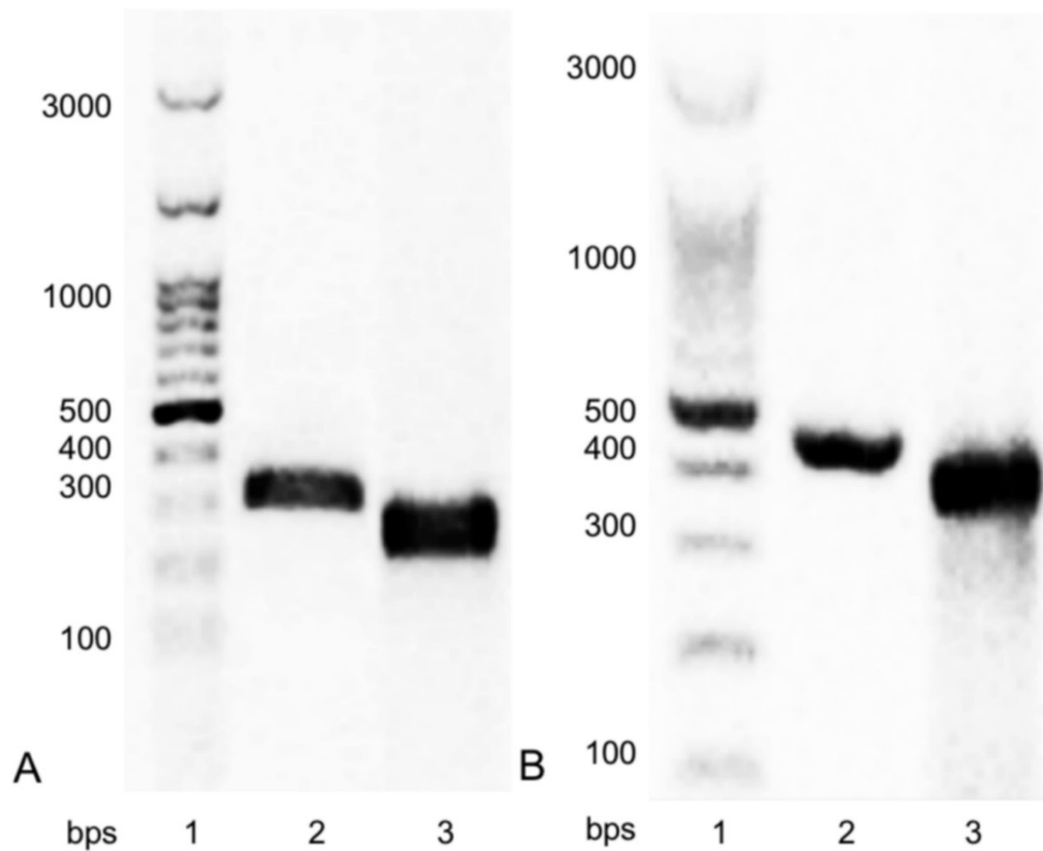

Fig. 6. **Generation of *GFP* and *Hsp90* dsRNAs**. cDNAs and dsRNAs amplified by PCR were resolved in 1.2% agarose gels and visualized by staining with SYBR® Safe DNA gel stain (Invitrogen). A, GFP; B, Hsp90. Lane 1, size marker in bps; 2, cDNA;3, dsRNA.

3.3 Knock down of *Hsp90* mRNA in *A. franciscana* cysts and nauplii

qRT-PCR, used to determine *Hsp90* mRNA and α-tubulin mRNA transcript copy number, revealed that *Hsp90* mRNA in nauplii and cysts released from females injected with dsRNA for Hsp90 was respectively reduced to 0.052 and 0.070 of the amounts in nauplii and cysts from females injected with GFP dsRNA (Fig. 7).

3.4 Knock down of Hsp90 in *A. franciscana* cysts and nauplii

Protein extracts prepared from cysts and nauplii released from females, after injection with dsRNA for HSP90, exhibited a reduced amount of Hsp90 when compared to the amount of Hsp90 in cysts and nauplii from females injected with dsRNA for GFP (Fig. 8). Immunoprobing with Anti-Y revealed that tyrosinated α-tubulin did not change during knockdown and indicated that similar amounts of protein were loaded in all lanes of the SDS polyacrylamide gels.

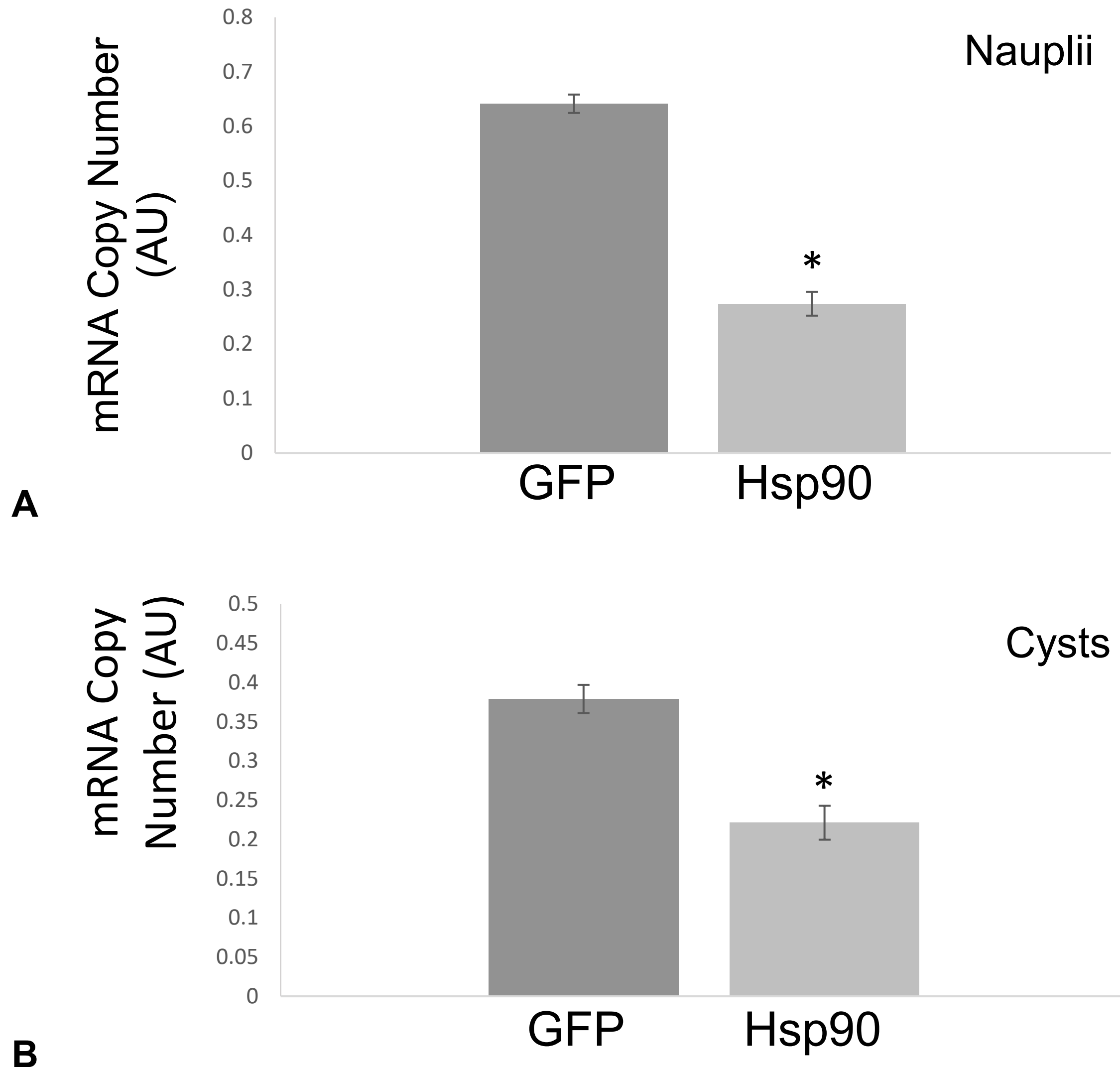

Fig. 7. *Hsp90* mRNA was reduced in cysts and nauplii released by females injected with *Hsp90* dsRNA. qRT-PCR was employed to determine the amount of *Hsp90* mRNA innauplii (A) and cysts (B) released from females injected with dsRNA for either GFP or Hsp90. *Hsp90* mRNA copy numbers were compared to α-tubulin mRNA copy numbers. Eighty cysts and nauplii were homogenized for each experiment and the experiment was done 3 times. Error bars represent standard deviation. The asterisks indicate that the amount of mRNA knockdown achieved by injecting dsRNA for GFP versus Hsp90 were significantly different ($p<0.05$)**.**

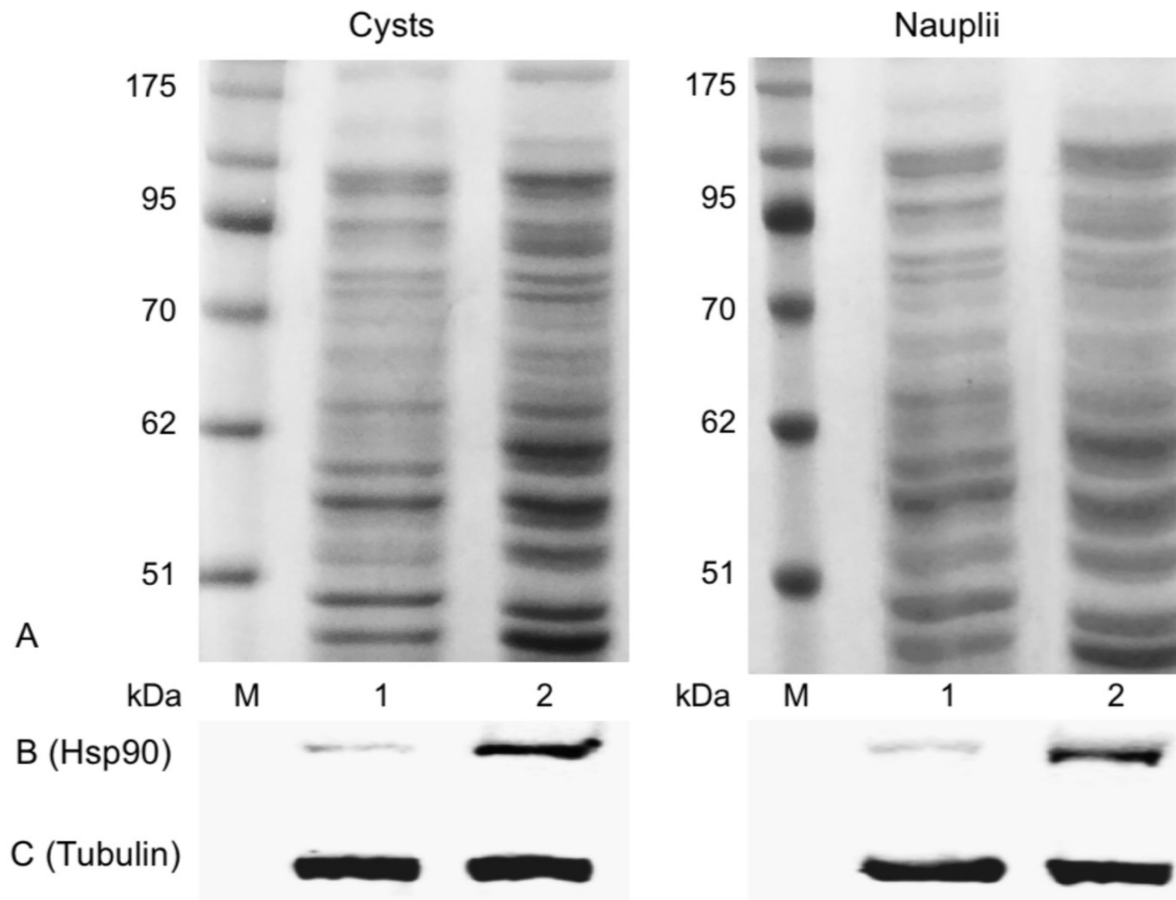

Fig. 8. Hsp90 was knocked down in cysts and nauplii of *A. franciscana* by RNAi. Protein extracts prepared from cysts and nauplii released by females injected with dsRNA for Hsp90 and GFP were resolved in SDS polyacrylamide gels and either stained with Coomassie blue (A) or blotted to nitrocellulose and reacted with antibody to Hsp90 (B) or to tyrosinated α-tubulin (C). M, Size marker (KDa); lane 1, females were injected with dsRNA for Hsp90; 2, females were injected with dsRNA for GFP.

3.5 Cysts released by females injected with dsRNA for Hsp90 were metabolically active upon release from females

Cysts released from females injected with dsRNA for either GFP or Hsp90 produced similar color changes in phenol red seawater after incubation for one day indicating they were metabolically active and thus alive (Fig. 9). Colour change of the phenol red was due to acidification of the sea water via the production of CO_2.

3.6 Knockdown of Hsp90 slows the development of nauplius-destined *A. franciscana* embryos

The time from fertilization of females to release of first broods of cysts from females injected with either GFP or Hsp90 dsRNA was approximately 5 days as was the case for nauplii from females injected with GFP dsRNA (Table 2). In contrast, the release time for nauplii from females injected with dsRNA for Hsp90 was 6.5 days, which is significantly different from nauplii released from females injected with dsRNA for GFP using t-test to determine statistical significance (Table 2).

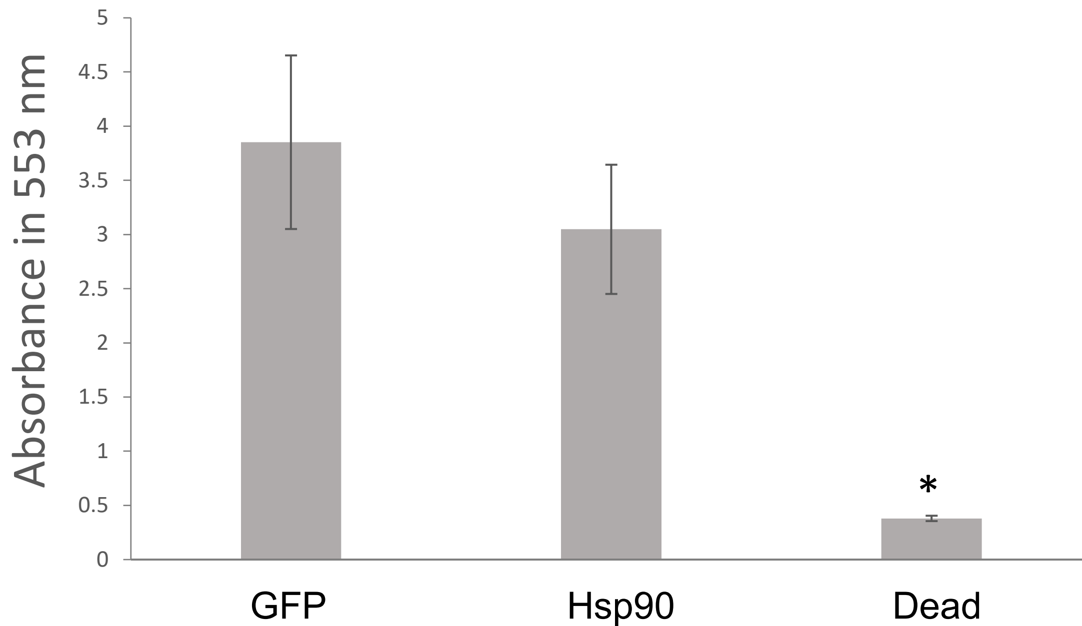

Fig. 9. Hsp90 knock down cysts were viable upon release from females. The metabolic activity of 10 cysts released by females receiving either *GFP* dsRNA or *Hsp90* dsRNA was measured immediately after release from females by colourmetric change in phenol red as described in Materials and Methods. Dead, dead cysts. Error bars show standard error. Asterisk indicates statistically significant difference via ANOVA ($p<0.05$).

Table 2. Knockdown of Hsp90 increased the time to release for nauplii of *A. franciscana*.

Time to release (Days)			
GFP±	**Hsp90±**	**(Δ Days)**	**p-value**
Cyst			
5.4±	5.5±	0.1	p>0.05
Nauplii			
5.5±	6.5±	1.0	p<0.001

(Δ Days), the difference in time from fertilization to release of cysts and nauplii. Release time was checked twice a day for first broods from 10 females injected with dsRNA for either Hsp90 or dsRNA GFP. The results were compared using t-test. The experiment was done 5 times with 50 females in total.

3.7 Hsp90 knockdown reduces nauplii survival and stress tolerance

Nauplii from females injected with dsRNA for GFP survived with only 4% mortality over 5 days, the length of the experiment. Nauplii released from females injected with dsRNA for Hsp90 began to die 2 days after release declining by 22% on day 5, $p>0.05$ (Fig. 10). Upon exposure to 39°C for 1 h and then incubation at 25°C for recovery, 87% of nauplii from females injected with dsRNA for GFP survived while only 67% of nauplii from females injected with Hsp90 dsRNA survived ($p<0,05$) (Fig. 11).

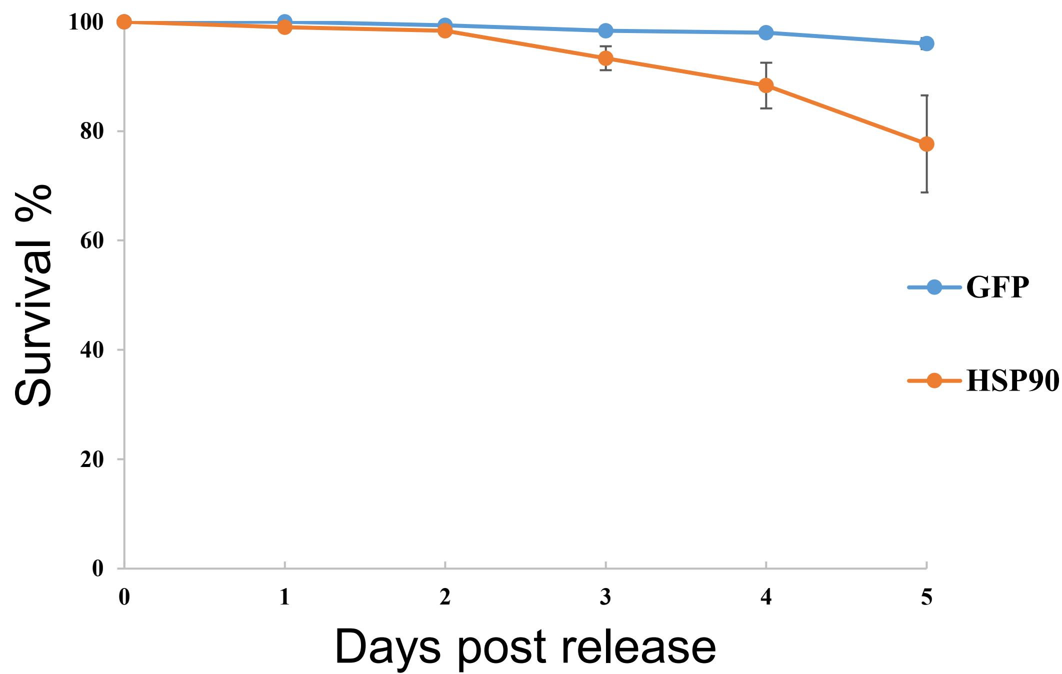

Fig. 10. Hsp90 knock down nauplii **exhibited reduced survival.** Nauplii released from 25 females injected with dsRNA for GFP (blue) and Hsp90 (orange) were incubated at room temperature and surviving (swimming) nauplii were counted daily for 5 days. T-test was used to determine statistical significance.

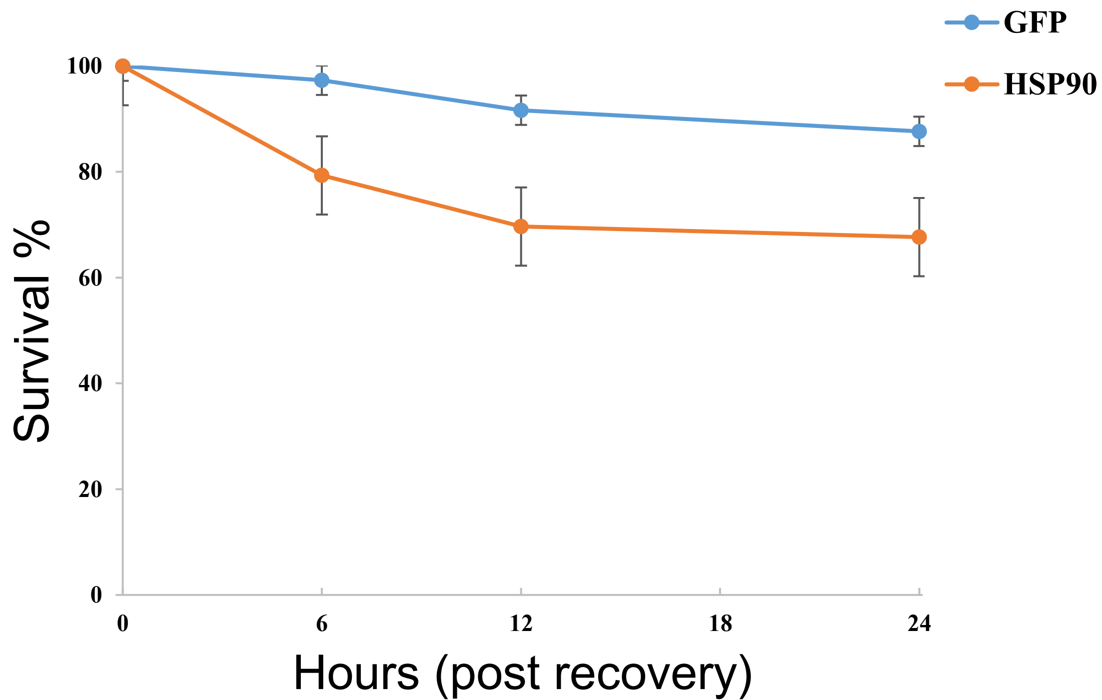

Fig. 11. Hsp90 knock down nauplii exhibited increased heat sensitivity. Nauplii released from females injected with either GFP dsRNA (blue) or Hsp90 dsRNA (orange) were heat shocked at 39°C for 1 h and then incubated at room temperature for 2 h to allow recovery (time 0). Surviving (swimming) nauplii were counted at the times indicated and compared to the initial number of viable nauplii at time 0 to give % survival. The number of nauplii ranged from 57 to 134 in each experiment and the experiment was done 3 times. The results were compared t-Test: Two-Sample Assuming Equal Variances.

3.8 Morphology of *A. franciscana* cysts and nauplii after knockdown of Hsp90.

Cysts released from females injected with either *GFP* dsRNA or *Hsp90* dsRNA were spherical with an average diameter of approximately 1 μm and brown in colour, all of which are normal characteristics of cysts (Fig. 12A, B). The swimming nauplii released from females receiving dsRNA for either GFP or Hsp90 were similar to one another in activity and shape (Fig. 12C, D) but the latter tended to be somewhat shorter than the former, although the difference was not significant (Fig. 13). Twenty-five % of females destined to produce nauplii that were injected with dsRNA for Hsp90 released broods of green, irregular shaped bodies (Fig. 12E). By comparison, 2% of females injected with dsRNA for GFP produced similar structures (Fig. 12E). The results were compared using t-test.

3.9 Cysts released from females receiving dsRNA for Hsp90 exhibited reduced stress tolerance.

Following desiccation and freezing, treatments required to terminate diapause and which served as stressors, only 5.5% of cysts from females receiving dsRNA for Hsp90 survived whereas 20% of cysts from females receiving dsRNA for GFP survived as indicated by hatching, $p < 0.05$, t-test was used to determine statistical significance (Fig. 14).

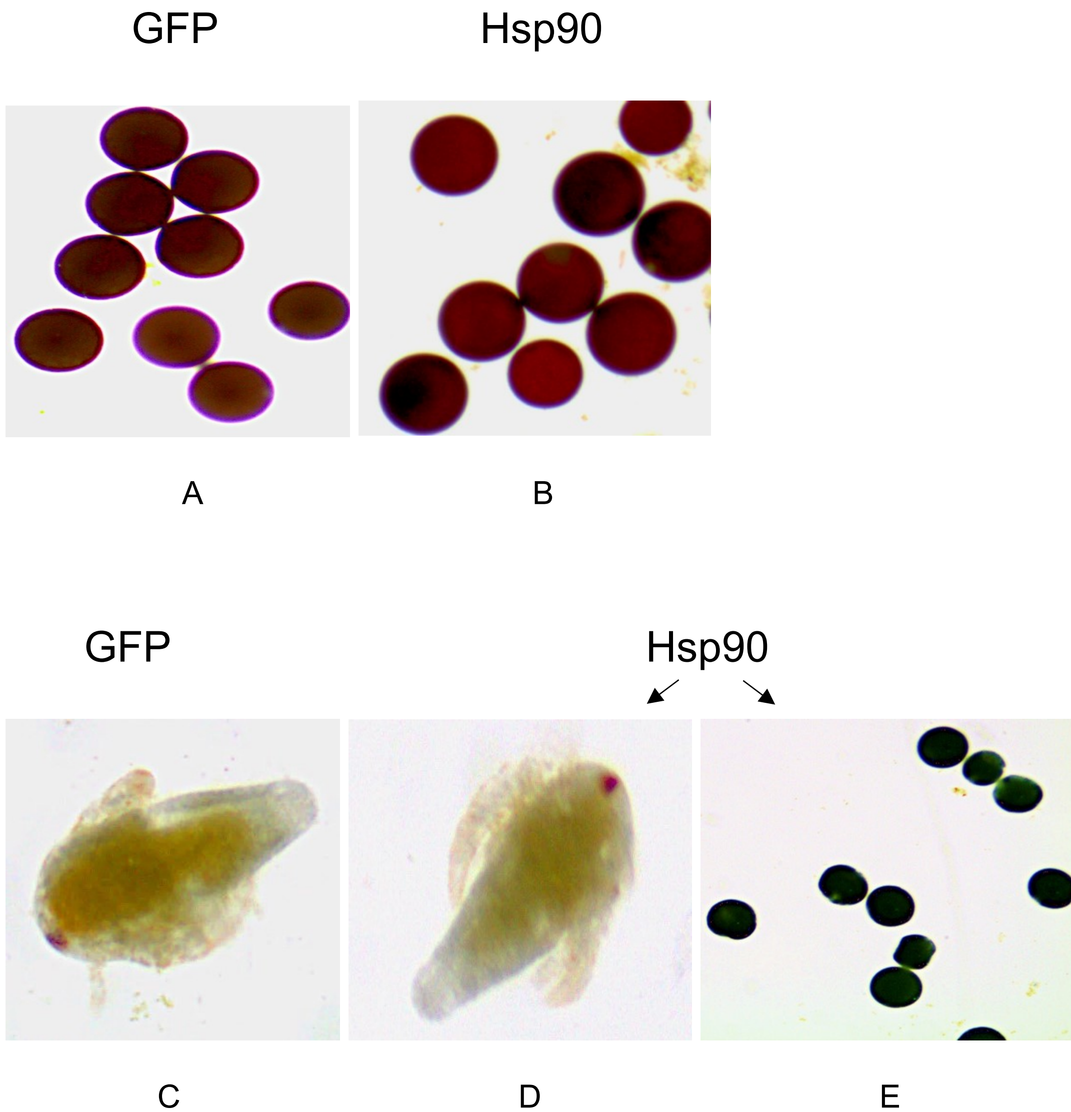

Fig. 12. Morphology of cysts and nauplii released by knockdown *A. franciscana* females. Light micrographs of *A. franciscana* cysts (A, B), nauplii (C, D) and irregular green bodies (E) from 25 females injected with GFP dsRNA (A, C) and Hsp90 dsRNA (B, D, E). n=200 from 4 studies, t-test was used to determine statistical significance $p > 0.05$.

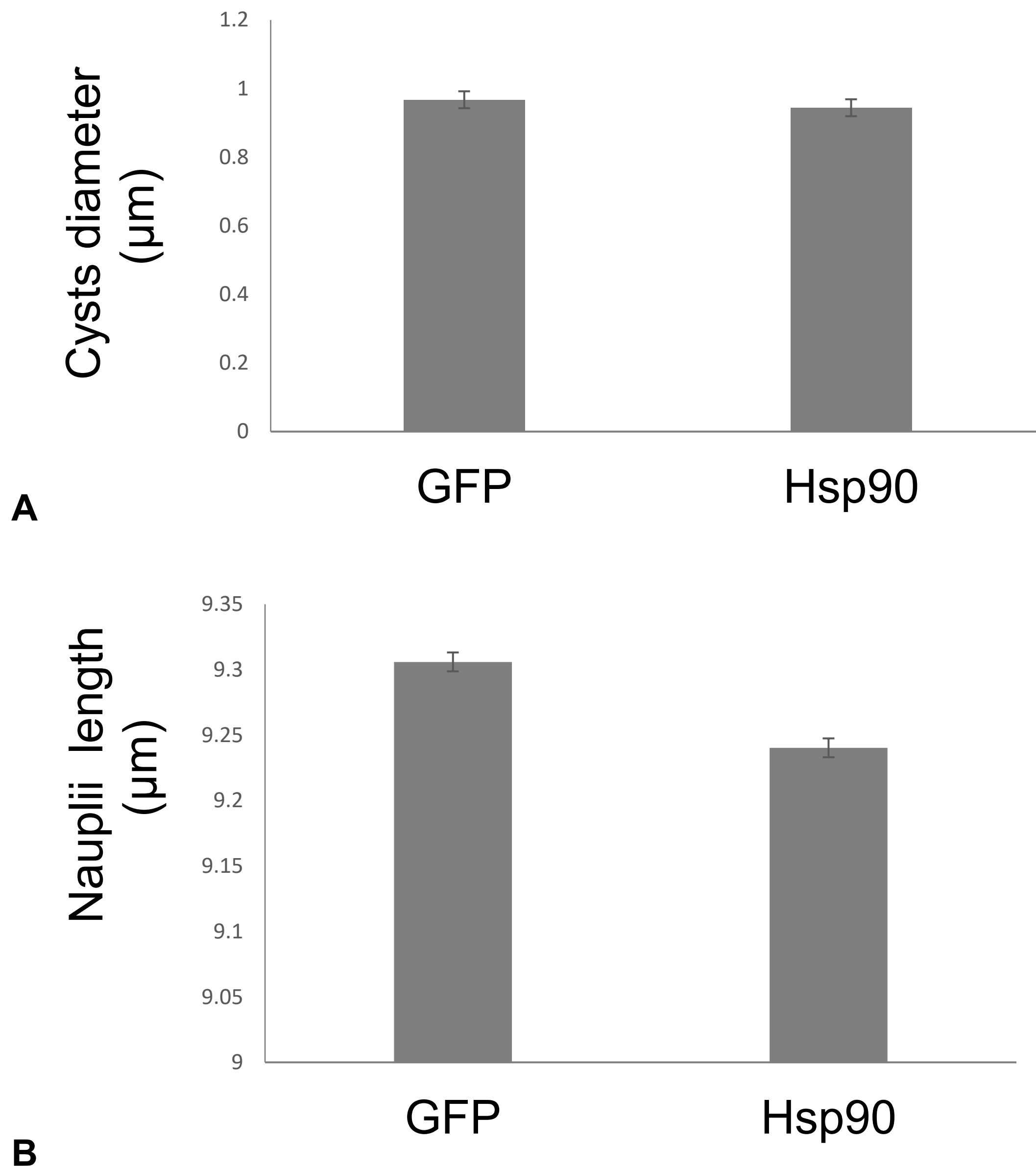

Fig. 13. Knockdown of Hsp90 does not affect the size of cysts and nauplii. The diameter of cysts (A) and the length of nauplii (B) released from females receiving dsRNA for either GFP or Hsp90 dsRNA were measured with an eyepiece micrometer. Between 45-50 cysts and nauplii were measured in each of 4 experiments. $p>0.05$ was determined by t-test for statistical significance.

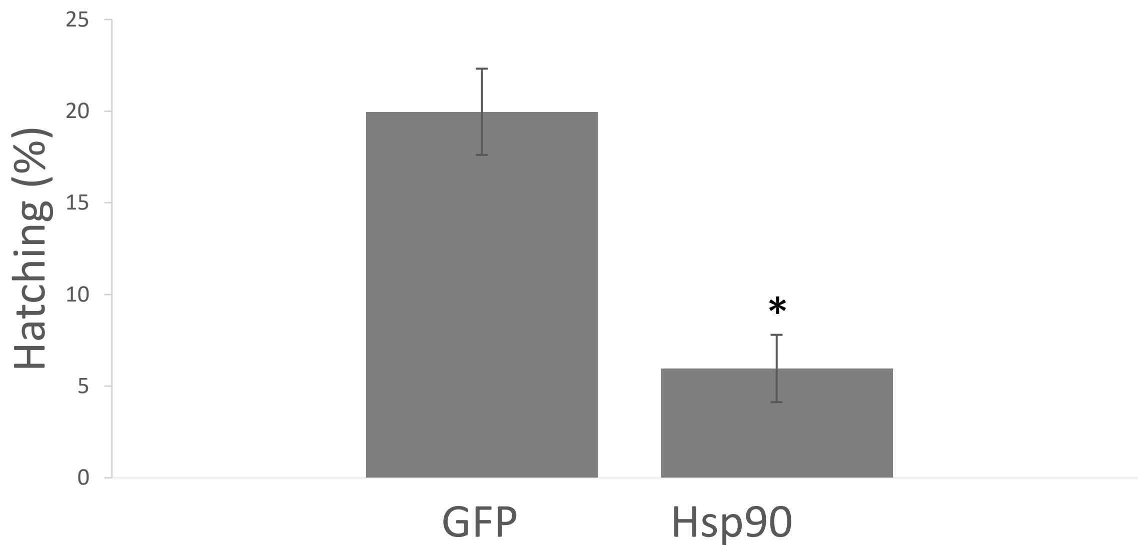

Fig. 14. Cysts containing diminished amounts of Hsp90 exhibited reduced stress tolerance. Cysts exposed to desiccation and freezing were rehydrated in sea water at room temperature and monitored for hatching. The number of hatched organisms was compared to the starting number of cysts to generate % hatching. The experiment was done in triplicate with first brood cysts. Thirty-five, 50 and 45 cysts from females receiving dsRNA for GFP and 50, 55, 45 cysts from females receiving dsRNA for Hsp90 were employed in the experiments. Error bars represent standard deviation. Asterisk indicates statistically significance difference via t-test, $p<0.05$.

CHAPTER 4 DISCUSSION

4.1 Hsp90 was knocked down in cysts and nauplii by RNAi

Hsp90, which is well characterized functionally, is synthesized in different eukaryotic cells where it is involved in protein folding, stress tolerance and the activation of signaling proteins and steroid receptors (Kadota & Shirasu, 2012; Li & Buchner, 2013). Hsp90 has often been studied *in vitro* and in transfected mammalian cells (Li & Buchner, 2013; Wu and MacRae, 2010) where it prevents protein aggregation during stress and refolds proteins (Feng et al., 2010). Hsp90 has also been examined *in vivo* in yeast, however, studies of Hsp90 in higher eukaryotes *in vivo* are limited and little is known about the role of Hsp90 during diapause. To address these issues, Hsp90 was knocked down by RNAi in *A. franciscana* during development and diapause confirming it has a role in conferring stress tolerance, possibly by preventing protein denaturation and or refolding denatured proteins.

RNAi was used herein to examine Hsp90 function in *A. franciscana* during normal growth and diapause, a procedure employed previously to examine the function of sHsps in *A. franciscana* cyst stress tolerance and embryo development (King and MacRae, 2012; King et al., 2014), and to examine the role of Hsp70 in the viability and disease resistance of nauplii (Iryani et al., 2017). In this study nauplii and cysts from *A. franciscana* females that received Hsp90 dsRNA were compared to those receiving GFP dsRNA, revealing reduced amounts of both Hsp90 mRNA and Hsp90, as shown respectively by qRT-PCR (Fig. 7) and immunoprobing of western blots (Fig. 8). The results reconfirm that RNAi is a useful technique for knocking down specific proteins in *A. franciscana* and

examining protein function in cysts and nauplii (King and MacRae, 2012; King 2013; King et al., 2014; Iryani et al., 2017).

4.2 Reduction of Hsp90 increases the time between fertilization and release of nauplii from *A. franciscana* females

The time between fertilization and release of cysts from females injected with Hsp90 and GFP dsRNA was similar, suggesting that reducing Hsp90 did not affect the development of diapause-destined embryos. In contrast, reduction of Hsp90 reduced the rate of development of nauplius-destined embryos when compared to nauplii containing unaltered amounts of Hsp90 (Table 2). This slower rate of embryo development in nauplii containing less Hsp90, as seen in *Sarcophaga crassipalpis,* may reflect a reduced requirement for Hsp90 as *A. franciscana* embryos encyst and eventually reduce their metabolic activity (Rinehart & Denlinger., 2000). In contrast, nauplius-destined embryos are actively developing and grow into swimming nauplii, events that require the chaperone activity of Hsp90.

4.3 Hsp90 contributes to survival and stress resistance of *A. franciscana* nauplii

After release from females, fewer nauplii with reduced amounts of Hsp90 survived compared to those nauplii with normal amounts of the molecular chaperone (Fig. 10), suggesting the importance of its role in maintaining biological processes such as protein folding and signaling, the latter affecting gene expression. That nauplii did not die quickly is likely due to residual Hsp90 in these animals. Previous studies indicate that other HSPs are generally present in low amounts in other animals but Hsp90 is approximately 1–2%

of total protein in many animal cell types and it increases dramatically in response to stress (Zou et al., 1998). The exact amount of Hsp90 under normal and stress conditions was not evaluated in this study, but could be determined in the future in order to more fully characterize the role of Hsp90 in the stress tolerance of *A. franciscana* nauplii and cysts. Hsp90 mRNA increases during diapause in *S. nonagrioides* and *C. suppressalis*, but it is not differentially regulated in the non-feeding diapause stage of the blow fly *Lucilia sericata*, although it increases as the transition out of diapause occurs (Sonoda et al., 2006; Tachibana et al., 2005). The results generated in this study using RNAi indicate that Hsp90 has an important protective role during stress in *A. franciscana* nauplii, either by protecting proteins from irreversible denaturation or assisting in their refolding or degradation.

4.4 Knock down of Hsp90 disrupts the development of *A. franciscana* nauplii

The knockdown of Hsp90 had no apparent effect on the development of diapause-destined embryos as cysts with reduced amounts of Hsp90 were similar in color, shape and size to those with normal amounts of Hsp90 (Fig.12 A, B). Some *A. franciscana* nauplius-destined embryos with reduced amounts of Hsp90 developed normally, yielding nauplii similar in morphology to those with typical amounts of Hsp90 (Fig.12C, D). Other nauplius-destined embryos with reduced Hsp90 developed into irregular shaped greenish bodies that eventually disintegrated without hatching (Fig.12 E). The molecular basis for the development of the irregular shaped bodies is uncertain but may reflect the disruption of cell cycle events or alterations in cell signaling pathways. Both of these possibilities are feasible based on the known roles of Hsp90 in binding signaling molecules and

transcription factors (Zuehlke & Johnson 2010; Aruda et al., 2011). It is also possible that by reducing the amount of Hsp90 there is a decrease in the efficiency of protein folding within cells of nauplius-destined embryos leading to more widespread effects in the cell and thus on the developing embryos as a whole. These results indicate the importance of further examining the role of Hsp90 during the development of other invertebrates to determine if similar changes occur.

4.5 Hsp90 is required for desiccation and freezing tolerance of *A. franciscana* cysts

The knockdown of Hsp90 reduced the ability of cysts to hatch in sea water after diapause was terminated by desiccation and freezing at -80°C for two months, indicating reduced stress tolerance in the knock down cysts (Fig. 14). Since ATP is limiting during diapause in *A. franciscana* and protein refolding by Hsp90 is ATP-dependent (King and MacRae, 2015), Hsp90 would be unable to function effectively as a molecular chaperone, possibly resulting in increased amounts of nonfunctional proteins and disruption of cell activities. The degradation of such proteins and their recovery from aggregates would also be reduced. It has been proposed that ATP-requiring molecular chaperones such as Hsp90 sequester substrate proteins in the absence of ATP as a protective method during diapause (King and MacRae, 2015) and this activity would be diminished in Hsp90 knock down cysts leading to reduced survival during diapause. Alternatively, Hsp90 has been shown in other organisms such as *Omphisa fuscidentalis* to increase in abundance as diapause terminates (Tungjitwitayakul et al., 2008) and the inhibition of this process in knock down cysts, should it occur, may adversely affect their ability to terminate diapause and resume development. In other examples, Hsp90 is down regulated during pupal

diapause of the flesh fly *Sarcophaga crassipaipis* (Tachibana et al., 2005) but was unchanged in the fruit fly *Drosophila triauraria* (Aruda et al., 2011), Hsp90 in *Belgica antarctica* and *Culex pipiens* is reduced upon dehydration and upregulated with rehydration in *S. crassipalpis* (Lopez-Martinez et al., 2009). Hsp90 is also present in eggs of *Bombyx mori* during non-diapausing and early diapausing stages and may influence embryo development, metabolism, and immune defence (Sasibhushan et al., 2012; Fan et al., 2013). Moreover, Hsp90 confers stress resistance in yeast, mammalian cells and bacteria (Wu and MacRae, 2010; Li & Buchner, 2013; Zhu & Pisetsky, 2001). In spite of these findings, the role of Hsp90 in diapausing organisms remains uncertain and the finding herein that Hsp90 confers stress tolerance during diapause is novel.

4.6 Conclusions

Hsp90 was shown by RNAi to be required for normal development of some but not all *A. franciscana* nauplius-destined embryos. This suggests that there is sufficient Hsp90 after knock down to sustain a number of of the proteins involved in these processes and thus allow for development. Additionally, Hsp90 is required for stress tolerance in nauplii and cysts potentially through ATP-dependent activities such as protein folding/refolding or independently of ATP by binding proteins and preventing their irreversible denaturation. This work sets the stage for further investigation of the role of Hsp90 during growth, development and diapause of *A. franciscana* including elucidating Hsp90 function in limiting ATP environments such as those that occur in diapausing cysts. It could be good idea in the future work to test the function of isolated Hsp90 *in vitro* to see if protein binding is affected by ATP.

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APPENDIX A SUPPLEMENTAL DATA

Figure A.1. Experimental time line for desiccation and freezing knockdown cysts released from *A. franciscana*. After microinjection of dsRNA for both GFP and Hsp90, females were mated and then released cysts were incubated in sea water for 10 days. After that desiccated at room temperature for two weeks were then transferred to the -80 freezer for 2 months equally 10 weeks to confirm the knockdown.

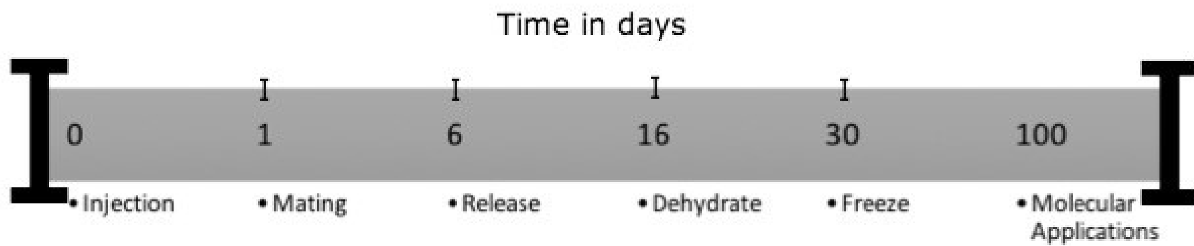
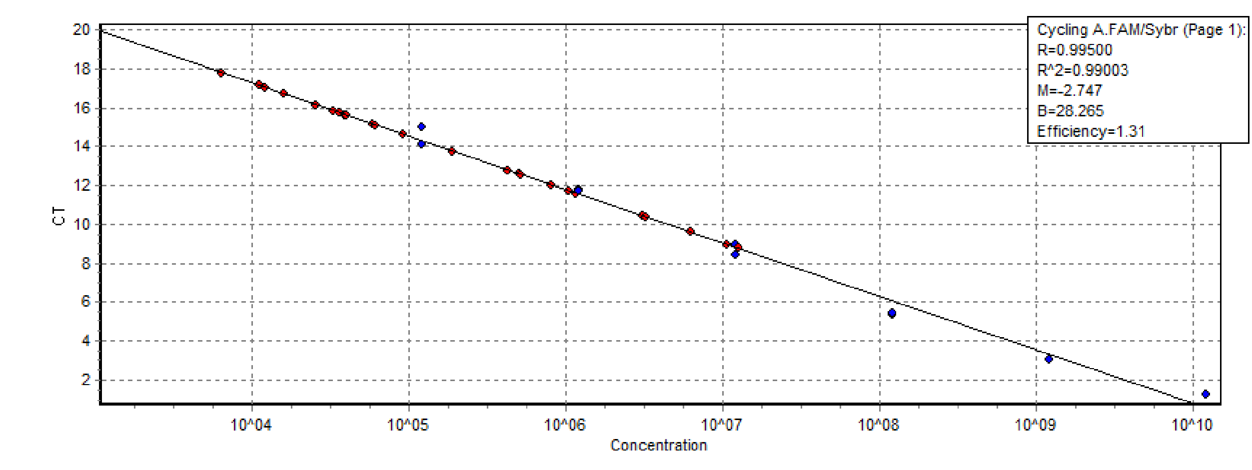


Figure A.2. qPCR Standard curves for quantifying Hsp90 mRNA and α-tubulin mRNA transcript copy number. Blot standard curves of Ct (cycle threshold) values for Hsp90 (A, R2 =0.99003) and α-tubulin (B, R2 =0.99555) as described in Materials and Methods created from duplicate known templates concentration (copy number per μl) with SYBR® Green.

A



B

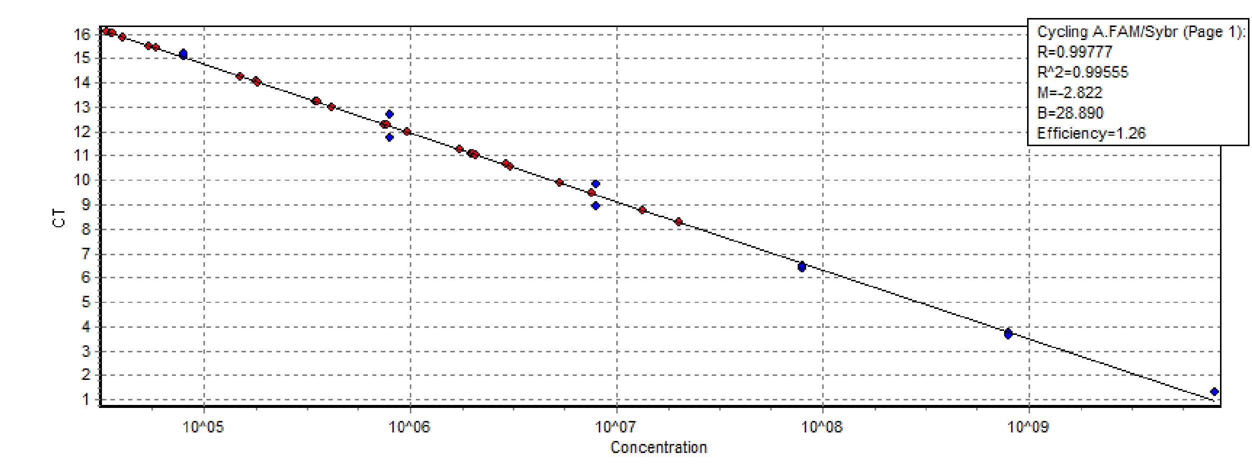


Table A.1. Co-chaperones and client that function with Hsp90 during protein folding. Several co-chaperones and clients up and down regulated during diapause were identified by selective substrate that interact with Hsp90 to activate or inhibit ATPase and effect Hsp90 function. These co-chaperones and clients identified on knockdown Hsp90 using RNAi in other species demonstrate the importance of molecular chaperone function in diapause and stress tolerance (Zuehlke & Johnson., 2010; Horikawa et al., 2015).

Protein family	**Classification**	**Function**	**Hsp90 binding site**
Hsp90	Chaperone	Supports meta-stable protein conformation in signal transduction pathway	-
Hsp70	Chaperone	Help fold nascent polypeptide chains	Interact with Hop
Hop	adaptors	Mediate interaction between Hsp70 and Hsp90	C-terminus
Aha1	Co-Chaperone	Stimulates Hsp90 ATPase activity	M-domain
P23	Co-Chaperone	Stimulates Hsp90 association with clients	C-terminus

APPENDIX B MICROPIPETTE PREPARATION

Setting for pulling micropipettes used in microinjection of *A. francsicana* 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: acylamide/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
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
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

4X Treatment Buffer (diluted four-fold for electrophoresis):

250 mM Tris, 280 mM SDS, 40% (v/v) glycerol, 20% (v/v) ù-mercaptoethanol,0.2% (w/v) bromophenol blue, pH 6.8

1.2 g	Tris
3.2 g	SDS
16 ml	glycerol
8 ml	ß-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	

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	

HST: 10 mM Tris, 1 M NaCl, 0.5% Tween-20, pH 7.4

1.21 g	Tris
58.4 g	NaCl
5 ml	Tween-20
pH 7.4	
dH2O to 1 L	