Elucidating the Functions of Proteins Up-regulated During Diapause in
***Artemia franciscana* Using RNAi**

by

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Dedicated to my Father Rupert King: Who's memory has gotten me through many a storm

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Abstract

Diapause embryos of the animal extremophile *Artemia franciscana*, a crustacean, are metabolically dormant and exceptionally tolerant to stressors such as heat and anoxia, characteristics thought to depend on the protective activity of molecular chaperones. RNAi methodology was developed and used to knock down individual molecular chaperones which are normally up-regulated during *Artemia* diapause. DsRNA and siRNAs injected into females were effective in knocking down proteins in embryos into the fifth release. Five proteins were knocked down including the α-crystallin related small heat shock proteins (sHsps) p26, ArHsp21 and ArHsp22, artemin, a species-specific chaperone as well as p8 a transcription co-factor. The individual sHsps, artemin and p8 exhibited different roles during cyst development and diapause with only some of these proteins contributing to stress tolerance. p26, for example, enhances stress resistance in *Artemia* embryos, facilitates embryo development and prevents diapause termination indicated by spontaneous hatching. ArHsp21, another α-crystallin type small heat shock protein contributes only slightly to freezing and desiccation stress and is not protective during heat stress. DsRNA specific to ArHsp22 is lethal to both male and female adults. Artemin contributes to stress tolerance but to a lesser extent than p26. Artemin also extended the period of time over which cysts were released. Cysts that did not contain p8 were also less stress resistant than those that did contain p8 and hatched upon release 10% for the time, suggesting an important role in diapause. By revealing separate and novel roles for molecular chaperones this work contributes substantially to our understanding of diapause, an important, phylogenetically widespread developmental process.

List of Abbreviations and Symbols Used

ATP	adenosine 5'-triphosphate
com1	candidate of metastasis 1
DMSO	dimethylsulfoxide
dsRNA	double stranded RNA
ECL	enhanced chemoluminescence
EDTA	ethylenediaminetetraacetic acid
GelDoc	Gel documentation system
GFP	green fluorescence protein
h	hour
HMG	high mobility group
Hsp	heat shock protein
Hsp21	ArHsp21
Hsp22	ArHsp22
HRP	horseradish peroxidase
HST	high salt tween
IgG	immunoglobulin G
Kda	kilodalton
min	minute
mRNA	messenger RNA
NUPR1	Nuclear protein 1
ORF	open reading frame
PAGE	poly acrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RITS	RNAi-induced transcriptional silencing complex
RNA	ribonucleic acid

RNAi	RNA interference
RPM	rotations per minute
RSC	RNAi induced silencing complex
RT-PCR	reverse transcription polymerase chain reaction
shRNA	short hairpin RNA
siRNA	short interfering RNA
SDS	sodium dodecyl sulfate
sHsp	small heat shock protein
TBE	Tris/Borate/EDTA
TBS	Tris buffered saline
Tris	Tris-(hydromethyl)aminomethane
Tween	polyoxyethylene sorbitan monolaurate

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Chapter 1: Introduction

1.1 *Artemia franciscana*

Artemia franciscana are crustaceans commonly known as brine shrimp or more colloquially as 'sea monkeys'. Members of the genus are distributed globally (Muñoz and Pacios, 2010), except for Antarctica, and depending on the area they colonize, can be considered an invasive species because *A. franciscana* out competes native species of *Artemia* (Amat et al., 2007; Muñoz et al., 2009). Adults of the species are approximately 1 cm long and they reach sexual maturity in about 3 weeks under laboratory conditions (Gilchrist, 1960). Once sexually mature, females produce several broods of offspring, typically releasing a brood every 5 days (Browne, 1980). Male adult *Artemia* are distinguished from females based on a number of features, notably their blue color, anterior pincers and lack of an egg sac at sexual maturity (Fig. 1). The female appears pink or orange when compared to the male and is often longer. A female is unfertilized if the egg sac consists of two distinct lateral sacs rather than one continuous sac. Between the male and female are several encysted embryos called cysts (Fig. 1). *A. franciscana* embryos may develop ovoviviparously, yielding free-swimming nauplii upon release from females. Or, embryos develop oviparously producing encysted gastrulae known as cysts, of approximately 4000 cells possibly forming syncytia, which are

Figure 1. Male and female adult *A. franciscana.* The female is pictured on the left and the male on the right in this light micrograph. Animals were fixed for 24 hours in 5% paraformaldehyde and imaged using an infinity 1.1 camera mounted on an olympus stereomicroscope. G, gut, H, head, C, claspers, E, egg sac containing cysts, O, developing oocytes.

2.5 mm
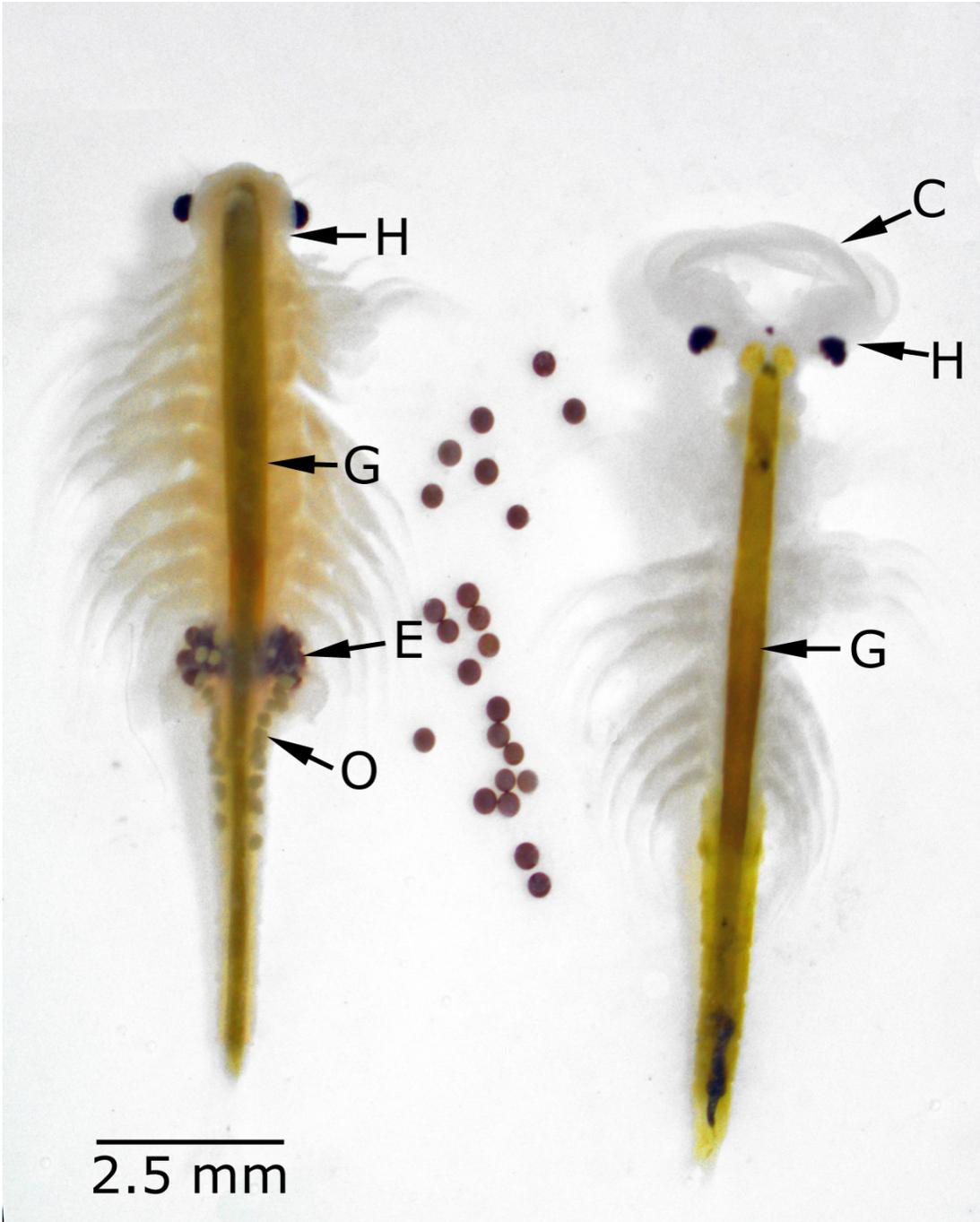
encased by a protective shell (Clegg, 1974) (Fig. 2). Embryos produced oviparously enter diapause, a physiological state of dormancy characterized by enhanced stress tolerance (Clegg, 1997; MacRae, 2005). The relatively easy culturing conditions, large output of experimental material and small foot print make *Artemia* an extremely good model organism to study the molecular basis of diapause and stress tolerance.

1.2 Diapause, a state of dormancy and enhanced stress tolerance

Diapause is a widely distributed physiological state of developmental delay, characterized by dormancy and enhanced stress tolerance in organisms ranging from crustaceans to mammals, and it promotes survival under adverse conditions (Denlinger, 2002; MacRae, 2005, 2010; Koštál, 2006; Hahn and Denlinger, 2011; Ptak et al., 2012).

Enhanced stress resistance is closely associated with diapause but the degree to which diapause is a protective state varies widely. For example *Drosophila tiauraria* exhibits limited stress resistance during diapause and its cold tolerance increases only 0.7°C (Goto et al., 1998; Goto and Kimura, 2004). In contrast, encysted embryos of *A. franciscana* display tremendous stress tolerance while in diapause and quiescence, withstanding repeated freezing and thawing, desiccation, heat and years of anoxia (Clegg, 1994, 1997, 2005, 2007;

Figure 2. *A. franciscana* life history. Females release free swimming nauplii via the ovoviviparous pathway which then undergo a series of molts before reaching adulthood. Alternatively, females release stress tolerant encysted gastrulae via the oviparous pathway which enter diapause. Once diapause is terminated cysts develop into nauplii if conditions are favorable. If conditions are not favorable activated cysts are maintained in post-diapause quiescence until introduction to an environment conducive to development (adapted from Liang and MacRae, 1999).
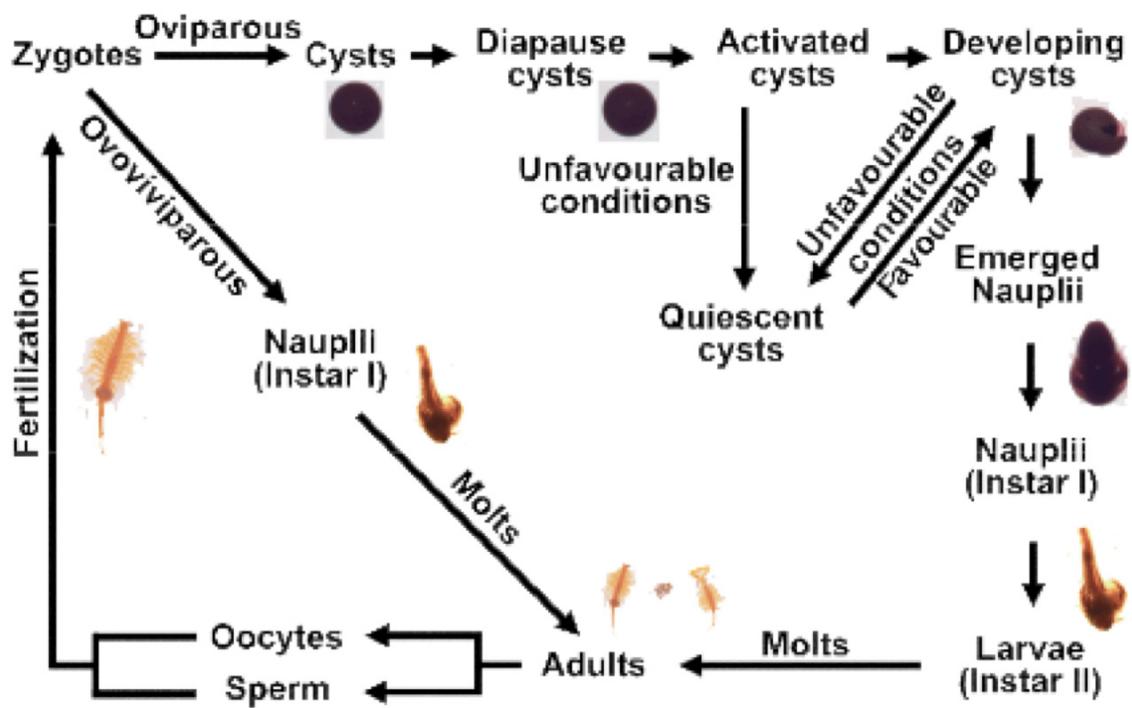
Clegg et al., 1996, 2000). The degree of metabolic suppression during diapause is variable with some organisms remaining active and continuing to feed (Pasternak et al., 2009), while metabolism in embryos of *Artemia* is virtually halted (Clegg, 2011). Certain aspects of diapause including stress resistance and metabolic depression are more universal than others such as behavioral modifications in the diapause organism or changes in expression of common genes (MacRae, 2010). In some organisms, like the stem borer *Chilo suppressalis* (Xiao et al., 2010), diapause is facultative, induced by environmental changes such as photoperiod, temperature and crowding.   In others, like *Artemia spp.* diapause is obligate and occurs as part of the life cycle regardless of other factors (Muñoz et al., 2010).

Diapause can be divided into three potentially overlapping phases known as initiation, maintenance and termination, although other sub-divisions of the process have been proposed with useful implications (Koštál, 2006). During initiation, organisms prepare for diapause by increased feeding, altered gene expression and changes in protein synthesis and accumulation. Initiation leads to maintenance which may include inhibition of DNA replication (Denlinger, 2002), metabolism (Reynolds and Hand, 2009), cell division (Tammariello and Denlinger, 1998) and inhibits apoptosis (Villeneuve et al., 2006). It is during the maintenance phase of diapause that individuals achieve maximum stress tolerance and metabolism is suppressed most dramatically.   The last phase of diapause is termination, a signal mediated process.   The termination signals include eytosteroids and juvenile hormone in some insects such as the flesh fly

Sarcophaga crassipalpis (Fujiwara and Denlinger, 2007), but the molecular mechanisms regulated by these hormones are unknown. In *A. franciscana* cold, dehydration and H_2O_2 are used independently or jointly to terminate diapause by an undetermined mechanism under laboratory conditions (Robbins et al., 2010; Van Stappen et al., 1998).

Upon diapause termination organisms resume development if conditions are favorable or they enter quiescence where they remain dormant until encountering circumstances appropriate for growth (Koštál, 2006); these include temperature, aeration and hydration for *Artemia* (Sorgeloos, 1979). Stress tolerance persists during quiescence in *A. franciscana* (Clegg et al., 2000).

Diapause occurs in many distantly related families of organisms (Renfree and Shaw, 2000). The molecular mechanisms controlling diapause have not been elucidated for any organism and diapause in different species may be controlled by similar or different processes. If these regulatory mechanisms differ significantly in unrelated organisms exhibiting diapause, it suggests that the trait is convergent and has evolved independently in many different organisms to deal with stressful situations. Diapause occurs in embryos of *A. franciscana* (Clegg, 1997), larvae of the moth *Sesamia nonagrioides* (Gkouvitsas et al., 2009a, b), pupae of *S. crassipalpis* (Li et al., 2007) and adults of the mosquito *Culex pipiens* (Kim et al., 2010). This temporal plasticity makes convergent evolution of diapause much more likely than divergent evolution from a common ancestor (Alekseev and Starobogatov, 1996) meaning that dormancy and enhanced stress tolerance to harsh conditions evolved many times independently.

Though diapause is phylogenetically wide spread it is most common in insects. Diapauses of many beneficial insects such as pollinators depend on environmental cues, namely temperature and day length (Kemp and Bosch, 2001), and may be altered by ongoing global climate change. Because diapause allows many pest organisms to over-winter and survive efforts by humans directed at their eradication an understanding of this developmental/physiological process has important repercussions for agriculture, forestry and medicine.

1.3 Classes of Molecular Chaperones

Newly synthesized proteins were classically thought to spontaneously attain proper tertiary structure within the cell and then maintain that structure until degradation. More recently molecular chaperones were demonstrated to assist in attaining or maintaining the tertiary structures of client proteins in the crowded cellular environment and during times of stress. There are several large classes of more or less ubiquitous molecular chaperones, sometimes termed heat shock proteins (HSPs) or stress proteins, and examples of species specific molecular chaperones such as artemin in *A. franciscana.*

The HSPs can be subdivided into at least 6 groups. The largest HSPs fall into the Hsp110 group and they function as nucleotide exchange factors for Hsp70, as well as operating as chaperones (Vos et al., 2008). A second group of high molecular weight HSPs makes up the Hsp104 family; these HSPs rescue unfolded proteins from aggregates and either help them to refold or target them

for destruction in an energy dependent manner (Bösl et al., 2006). The ATP-dependent, dimeric Hsp90 aids in protein folding and stability, often of regulatory proteins, and has roles in modifying gene expression and signaling (Wandinger et al., 2008; Hahn, 2009; Retzlaff et al., 2009). Members of the ATP dependent Hsp70 family are often up-regulated in response to stress where they re-fold damaged proteins but Hsp70 also folds nascent chains and like Hsp90 functions in protein degradation (Vos et al., 2008; Daugaard et al., 2007; Meimaridou et al., 2009). Hsp60, the structurally most complex chaperone consisting of two multimeric protein rings arranged back to back and requiring ATP for its function, folds newly made amino acid chains into functional proteins (Hartl and Hayer-Hartl, 2009). The sHSPs, a major focus of this thesis, prevent irreversible protein degradation in an energy independent fashion and have roles in protein degradation, stabilizing the cytoskeleton and preventing apoptosis (Laksanalamai and Robb, 2004; Sun and MacRae, 2005; Mchaourab et al., 2009)

Molecular chaperones function as networks of complementary proteins, each either performing a discrete function or co-operating with one or more other chaperones to ensure proper protein folding, protection, localization and degradation (Pratt et al., 2010). Nascent polypeptides are assisted by molecular chaperones in order to attain proper tertiary structure and prevent undesired interactions with other proteins which may lead to aggregation. Rather than guiding folding current models suggest that molecular chaperones allow unfolded polypeptides to experiment with different conformations in order to reach the

native structure based on the lowest potential energy as compared with all other possible structures (Hartl et al., 2011).

1.3.1 sHSPs in Diapause-destined *Artemia* Embryos

In addition to their role in folding nascent proteins, molecular chaperones are strongly up regulated in response to many stressors including cold in *Drosophila* (Colinet et al., 2010), desiccation in snails (Mizrahi et al., 2010) and handling in sharks (Heberer et al., 2010). Molecular chaperones bind proteins that are partially denatured due to stress and either assist in their refolding or prevent irreversible protein degradation until the stress has passed. The sHSPs bind substrate proteins independent of a need for ATP, but require an energy dependent chaperone such as HSP70 for protein refolding to occur. Alternatively protein refolding after stress may occur spontaneously (Haslbeck, 2002). Molecular chaperones, including the sHSPs, are closely integrated with diapause in many different species (MacRae, 2010), as is the case in *A. franciscana*. Several sHSPs are up regulated during *Artemia* diapause including the α-crystallin sHSPs, p26, ArHsp21 and ArHsp22 and the species specific ferritin homologue artemin. All four proteins have chaperone activity in *in vitro* turbidimetric assays (Qiu and MacRae, 2008 a, b; Villeneuve et al., 2006; Hu at al., 2011) and they show similar expression profiles during diapause.

sHSP monomers, which range from 15 to 42 kDa in molecular mass and tend to form oligomers, consist of an amino-terminus, a conserved α-crystallin domain and a carboxy-terminal extension (Sun and MacRae, 2005; Haslbeck et

al., 2005; Mchaourab et al., 2009; Basha et al., 2012). Dimerization, oligomerization and chaperone activity depend on the highly conserved α-crystallin domain (Mchaourab et al., 2009; Hilario et al., 2011), while the amino- and carboxy-domains are required for maximum efficiency (Wu and MacRae, 2010; Chen et al., 2010; McDonald et al., 2012). In addition, the amino- and carboxy-terminal domains, the latter generally containing an I/V-X-V/I motif, contribute to substrate binding, subunit dynamics and solubility (Liao et al., 2009; Treweek et al., 2010; Jehle et al., 2011; McDonald et al., 2012). sHSP oligomers from some organisms exhibit identical quaternary structure (Kim et al., 1998; van Montfort et al., 2001; Kennaway et al., 2005), but in most cases oligomer structure is heterogeneous (Benesch et al., 2010). Each sHSP oligomer is capable of interacting with several partially denatured proteins without consuming ATP and this ability enables sHSP function in physiological situations of low energy characteristic of diapause. Binding of denatured proteins to sHSPs is promoted by temperature dependent increases in surface hydrophobicity which likely favors interaction with the newly exposed hydrophobic regions of unfolding client proteins (Lee et al., 1997; van Montfort et al., 2001; Stromer et al., 2004, Ghosh et al., 2005, 2006). Substrate interaction is thought to require rearrangement and/or disassembly of oligomers in some if not all species (Tiroli-Cepedaa and Ramos, 2010; McDonald et al., 2012).

1.3.2 *A*rtemin, a ferritin homologue with chaperone activity

Artemin is an ATP independent molecular chaperone (Chen et al., 2007) which comprises 10-15% of the post-ribosomal protein in diapause cysts of *Artemia* (Warner et al., 2004; Tanguay et al., 2004) and confers stress tolerance in transfected mammalian cells (Chen et al., 2007).   Artemin is a ferritin homologue (De Graaf et al., 1990) that does not bind iron because the carboxy terminal extension of the protein fills the cavity where iron typically resides and artemin is missing all but one of the residues in the di-iron ferroxidase center that allow iron binding in ferritin (Chen et al., 2003, 2007; Andrews, 2010).   Artemin monomers associate in groups of 24 to form oligomers of approximately 600 kDa. Artemin, like ferritin, is extremely thermotolerant. Native artemin remains in solution when heated to 75 ^{o}C for 13 minutes and when bound to RNA stability is maintained for 30 minutes at 90°C (Hu et al., 2011; Warner et al., 2004).

Molecular modeling suggests that monomers of artemin contain five alpha helices, four in a parallel bundle and a shorter helix perpendicular to the bundle (Chen et al., 2003).   Modification by site-directed mutagenesis indicates that unstructured loops of the protein, thought to aid in oligomerization, are important in thermostability and that surface hydrophobicity is important in chaperoning but not stability (Hu et al., 2011).   The high degree of thermostability along with demonstrated *in vitro* chaperoning activity and abundant accumulation in cysts make artemin a good candidate for increasing stress resistance during the maintenance phase of diapause.

Although artemin exhibits 60% amino acid conservation with ferritin (Hu et al.*,* 2011), artemin does not, as mentioned previously, bind metals. The ferritin

monomer consists of four alpha helices and the monomers oligomerize to form a hollow protein sphere critical for iron binding and storage (Chriton and Declercq, 2010). Like artemin, ferritin exhibits chaperone function *in vitro* (Chen et al., 2007). Analysis of virtually all ferritin sequences indicates that two of its helices, C and D, are the result of an internal duplication of helices A and B (Andrews, 2010). Ferritin monomers associate to form oligomers of 12 subunits found only in prokaryotes or 24 monomers in eukaryotes (Arosio et al., 2009). It is possible that ferritin protects cells from oxidative damage by storing iron in the cavity of the protein oligomer.

1.4 The Transcription Cofactor p8

The transcription cofactor p8 was first characterized when it was discovered to be strongly up regulated during acute pancreatitis (Mallo et al., 1997). p8, also known as nuclear protein 1 (NUPR1) and candidate of metastasis 1 (com1), shows a moderate degree of similarity (35%) to the high mobility group (HMG) proteins (Hoffmeister et al., 2002) but the AT hook motif is not conserved (Encinar et al., 2001). The members of the HMG family and p8 share basic structural properties as well as a bipartite nuclear localization signal (NLS) (Vasseur, 1999a). p8 is likely regulated by the ubiquitin/proteasome pathway as it contains a PEST region in the N-terminus (Goruppi and Iovanna, 2010). p8 is a basic helix-loop-helix type protein (Mallo et al., 1997) which ranges from 69 amino acids in the fruit fly to 82 amino acids in humans (Vasseur

et al., 1999a, b) and it binds DNA (Garcia-Montero et al., 2001). Nuclear localization, and the fact that p8 is a target of acetylation, ubiquitination, and sumoylation, indicates the protein regulates transcription (Qiu and MacRae, 2007; Garciá-Montero et al., 2001; Hoffmeister et al., 2002; Quirk et al., 2003; Goruppi et al., 2007; Carracedo et al., 2006a, b). p8 is expressed in the diapause destined embryos of *A. franciscana* first appearing at two days post fertilization and disappearing shortly after diapause ends making it a potential regulator of the synthesis of proteins involved in the downstream cascade of diapause-related events (Qiu and MacRae, 2007).

1.5 HSP Gene Expression during Stress

Virtually all organisms show altered gene expression during heat stress and begin to produce HSPs (Åkerfelt et al., 2010) and these HSP genes may be induced by stressors other than heat. The potato beetle *Leptinotarsa decemlineata* up regulates Hsp70 in response to cold shock (Lyytinen et al., 2012) demonstrating that synthesis of the protein is modulated by cold as well as heat. Desiccation leads to the up regulation of many HSPs in the land snails *S. zonata* and *S. cariosa* (Mizrahi et al., 2010) but no changes in expression are detected for either Hsp70 or Hsp90 genes in the dehydrated tardigrades, *Bertolanius volubilis* and *Ramazzottius oberhaeuseri* (Altiero et al., 2012). HSPs are up regulated in response to many different chemical stressors such as curcumin in frogs (*Xenopus laevis*) (Khan and Heikkila, 2011) or in response to

anthracene, cadmium and chloridazone in the alga *Desmodesmus subspicatus* (Tukaj and Tukaj, 2010). HSPs are up regulated by a number of other stressors including radiation in *C. elegans* (Bertucci et al., 2009), handling in the copepod *Calanus finmarchicus* (Aruda et al., 2011) and viral infection in Vero E6 cells (Yu et al., 2009). Given the wide range of conditions that induce HSP production it is possible that almost any perturbation an organism experiences stimulates HSP production and it is useful to consider the HSPs more generally as stress proteins.

The diverse conditions that induce synthesis and accumulation of HSPs in different organisms show it is possible to deal with many stressors by increasing HSPs. The same stressor can have very dissimilar effects on HSPs in different organisms; for example land snails up regulate one Hsp90, two Hsp70s and two sHSPs in response to desiccation (Mizrahi et al., 2010) and at least one member of each of these families is expressed during desiccation in the nematode, *Plectus murrayi* (Adhikari et al., 2009). However, no change is seen in Hsp70 or Hsp90 family members during desiccation of tardigrades (Altiero et al., 2012). Variations in HSP synthesis occur in response to cold stress in different organisms. For example, the fruit fly, *D. melanogaster,* up regulates at least one member of the sHSP, Hsp40, Hsp60 and Hsp70 families, but not other Hsp60 and Hsp70s (Colinet et al., 2010). The fungus *Tuber melanosporum*, on the other hand, differentially regulates only two members of the sHSP family (Zampieri et al., 2011) whereas porcine fibroblast cells appear to up regulate only Hsp90 family members in response to cold stress (Li et al., 2012).   Clearly, the

expression of HSPs in response to a given stressor is a common occurrence but there is wide variation in the number and types of HSPs up regulated upon exposure to the same stressor.   This plasticity, along with the diversity of stressors that trigger HSP expression may allow for the functional overlap of HSPs during stress. If varying HSP families are turned on in different organisms encountering the same stress then it is possible that each performs the same function or that they work together cooperatively to perform a common function.

1.6 HSP Gene Expression during Diapause

Diapause is employed across species to deal with harsh conditions and presumably a wide variety of stressors. Some diapauses allow organisms such as the copepod *Calanoides acutus* to overwinter (Pond et al., 2012) while others help insects such as the cabbage beetle *Colaphellus bowringi* to avoid hot summer conditions (Kuang et al., 2011). Diapause in *A. franciscana* is especially protective probably due to the harsh conditions that encysted embryos encounter in their natural environment. Embryos are likely to wash up on shore where they are subject to freeze-thaw cycles, extreme heat or cold, high salt, desiccation and UV radiation.   Embryos may sink and become buried in sediment and therefore they must be able to survive anoxic environments. HSPs are induced in response to many of these stressors and it would be beneficial to express them in advance of a period of potentially extreme stress like diapause.   It has long been hypothesized that HSP synthesis in diapause organisms prevents

permanent protein damage at high and low temperatures but due to the many stressors that cause their up regulation, HSPs undoubtedly protect against environmental parameters other than temperature.

Usually more than one chaperone is up regulated in response to a given stress or during diapause, likely because chaperones function together in networks (Haslbeck, 2002).   sHSPs, Hsp70, Hsp90 and HSP60 are often up regulated during diapause (MacRae, 2010) and may co-operate in protein refolding once diapause terminates. sHSPs bind partially denatured proteins through hydrophobic interactions and deliver them, often via co-factors, to Hsp70 which is responsible for their ATP-dependent re-folding. Hsp90 may interact with Hsp70 leading to much greater folding efficiency and both chaperones may sequester proteins, especially in the absence of ATP. Differentially regulating the synthesis of proteins in the sHSP/Hsp70/Hsp90/HSP60 chaperone network in the low ATP conditions characteristic of organisms during diapause may maximize protein protection by sequestering proteins in association with these chaperones. The differential chaperone accumulation seen in several diapausing species has the potential to effectively stall the protein refolding machinery, maximizing the number of damaged proteins which are sequestered and ensuring that limited cell resources are not wasted by repeatedly folding, releasing and re-folding the same proteins.

Many organisms up regulate HSPs during diapause presumably to enhance stress resistance. The flesh fly *S. crassipalpis* up regulates Hsp70 and several sHSP family members during diapause, but the expression of Hsp90 is

unaffected (Li et al., 2007). The reverse is true during diapause in the rice stem borer *Chilo suppressalis* where Hsp70 expression is constant and Hsp90 is up regulated (Sonoda et al., 2006), whereas in the closely related corn stem borer, *Sesamia nonagrioides*, Hsp70 and Hsp90 are both up regulated (Gkouvitas et al., 2008; 2009a,b).   Diapausing *A. franciscana* embryos up regulate three sHSPs, ArHsp21, ArHsp22 and p26 (Liang and MacRae, 1999; Qiu and MacRae, 2008 a, b; Villeneuve et al., 2006) and a species specific molecular chaperone termed artemin (Chen et al., 2003).

1.7 Protein Function and RNA Interference (RNAi)

The expression of particular genes can be turned on and off as needed yielding proteins that are the functional workhorses of the cell. Proteins do most of the mechanical work of the cell, acting as gate keepers, engaging in local and long distance communication and participating in virtually all metabolic reactions. As such, elucidating protein function is vital to understanding cell activities and this task can be challenging (Whisstock and Lesk, 2003). Organisms often contain similar proteins, but these proteins may serve very dissimilar functions in diverse organisms or even within cells of the same organism (Lai and Gallo, 2009).   To elucidate function, proteins are cloned into other organisms, often *Escherichia coli*, in order to study them *in vitro*. While useful insights are gleaned from this process it may not be a true measure of how a protein functions in the organism from which it was originally obtained.   Proteins, much like organisms,

evolved to fill a certain niche, and similar proteins may function very differently when cloned into a different species or when purified and studied *in vitro* as is the case with p26.

Using RNAi technology specific mRNA transcripts can be targeted for degradation generating an organism without a particular protein/s. The resulting organism displays a loss-of-function phenotype and it may be possible to determine the function of the missing protein *in vivo* (Siomi and Siomi, 2009). RNAi can reveal the functions of newly identified proteins, show new functions for characterized proteins and identify new proteins involved in previously studied functions (Bellés, 2010). The knock down of proteins by RNAi is generally achieved by using long double stranded RNA (dsRNA) especially in non-mammalian systems, short interfering RNA (siRNA) and short hairpin RNA (shRNA) (Jinek and Doudna, 2009). Within cells, RNAi, discovered by Fire et al. (1998), is thought to be a nearly ubiquitous general antiviral response (Haasnoot et al., 2007; Li and Ding, 2006). If the dsRNA introduced into the cell is long, as in this study, it first interacts with the enzyme dicer (Bernstein et al., 2001) which, as its name suggests, cuts the dsRNA into short segments of 21-22 nucleotides called siRNAs. siRNAs may be introduced into the cell directly as done in this study. Intracellular siRNAs are key to generating the Argonaute based RNAi induced silencing complex (RISC) which targets and destroys the endogenous mRNA transcript matching the siRNAs (Hutvagner and Simard, 2008). Additionally, some organisms stop gene expression by enhanced nucleosome formation, thus reducing access of the transcriptional machinery to the gene

targeted by RNA within the nucleus (Schramke et al., 2005), a process involving the RNA-induced transcriptional silencing complex (RITS) which contains a specialized Argonaute family member (Grewal and Elgin, 2007).

In this study RNAi methodology was developed and employed in *A. franciscana* to study the function of several proteins previously shown by subtractive hybridization to be up regulated during diapause (Qiu and MacRae, 2007). These proteins include the three α-crystallin type sHSPs ArHsp21, ArHsp22 and p26 as well as a species specific molecular chaperone, artemin, all of which appear during post-fertilization development in diapause destined embryos. These proteins are not synthesized in embryos that develop directly into swimming nauplii. Additionally, the transcription co-factor, p8 was targeted using RNAi. p8, which is stress inducible, is up regulated during *Artemia* diapause and first detectable at two days post-fertilization. It was hypothesized when the work was initiated that p8 controls the expression of genes, such as those encoding HSPs, during diapause. RNAi was used in this study to knockdown proteins by injecting the egg sacs of unfertilized females with dsRNA. Specific knockdown of mRNA and proteins in diapause-destined *Artemia* embryos was respectively confirmed by reverse transcriptase PCR (RT-PCR) and immunoprobing of western blots. Females and resulting embryos were monitored for developmental and phenotypic changes as compared to control organisms injected with either dsRNA specific to green florescent protein (GFP) or control solution lacking dsRNA.

Chapter 2: Materials and Methods

2.1 *Artemia* culture and handling

Dehydrated *A. franciscana* cysts from the Great Salt Lake, Utah, USA (INVE Aquaculture, Salt Lake City, UT) were aerated for 48 h in 1 l Erlenmeyer flasks each containing 1 l of filtered, autoclaved sea water from Halifax Harbor (sea water). Upon termination of aeration cultures were left undisturbed for 30 min, cyst shells were removed from the surface and hatched nauplii were incubated at room temperature with light aeration in small aquaria containing sea water. The *Artemia* were grown to maturity by daily feeding of *Isochrysis sp*. algae obtained from The Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP).

Mature males and females were maintained in different 1 l containers. For fertilization and embryo production females were placed in 6-well plates (Liang and MacRae, 1999) and males were introduced. Adults were handled using a modified disposable 10 ml pipette that had the narrow end removed and filed so adults were not damaged. Mating pairs were fed daily and fecal material was removed. Dead adults and those showing signs of infection or illness were discarded. Encysted embryos (cysts) were collected upon release from females and incubated for at least 10 days in sea water in small hexagonal weigh boats covered in foil to ensure metabolic arrest and initiation of diapause. Weigh boats were used to culture animals for molecular techniques and hatching experiments.

To follow their development into adulthood and examine subsequent cyst production, nauplii arising from females in culture were incubated in hexagonal weigh boats at room temperature and fed daily until sexual maturity. To prevent evaporation, weigh boats were topped with un-stretched Parafilm (Fisher Scientific, Mississauga, Ontario) containing a hole to allow light penetration. To terminate diapause, cysts were dried in a desiccator over Dryrite (Dryrite, Nashville, TN, USA) for at least 4 weeks, followed by transfer to -20°C for at least 8 weeks.

2.2. Development of a microinjection procedure for adult *Artemia*

To optimize the procedure for delivery of dsRNA to *Artemia* the number of animals that succumbed to injection was initially determined in the absence of dsRNA by injecting groups of approximately 12 organisms with control solution not containing dsRNA. Adult males and females were injected. After discarding animals that were morphologically or behaviorally abnormal the groups were monitored for 3 days to determine when and how many animals died in comparison to a similar group that was not injected.

In order to determine if knockdown of molecular chaperones had an effect on females or offspring mated pairs were observed daily using a stereo microscope. The time of fertilization, type of embryo produced, appearance and time to release of cysts and nauplii, and the day the egg sac emptied, were recorded for each injected adult. Whether translucent cysts were empty or

contained an embryo was determined by breaking the cyst shell with a sharp dissecting probe.   If embryos were present cyst contents streamed into the surrounding medium when the shell was broken. Observations were made to assess protein function such as time between fertilization and release of cysts from females and to monitor cysts for abnormalities.

2.3 Protein Knockdown by RNAi

2.3.1 Preparation of dsRNAs and siRNAs

pRSET C plasmids (Invitrogen, Burlington, Ont., Canada) containing cDNA for p26, ArHsp21, ArHsp22 and p8 were harvested from overnight cultures of BL21(DE3)pLysS *E. coli* (Invitrogen) (Liang and MacRae, 1999; Qiu and MacRae, 2007; Qiu and MacRae, 2008a, b). Artemin cDNA was harvested from *E. coli* BL21PRO (Clontech Laboratories, Inc., Mississauga, ON) transformed with the prokaryotic expression vector pPROTet.E133 using a miniprep kit (Sigma-Aldrich, Oakville, Ont., Canada) (Chen et al., 2003). The DNA was harvested from overnight cultures as above. These cDNAs were amplified using primers obtained from Integrated DNA Technologies (IDT), Coralville, IA, USA, and under the reaction conditions described in Table 1.

The T7 promoter sequence (TAATACGACTCACTATAGG) was added to the 5' end of all primers used for dsRNA production. PCR was carried out with Platinum® Taq DNA polymerase (Invitrogen) using cDNA from mini-preps according to manufacturer's instructions.

Table 1. Primers used to generate dsRNA for molecular chaperones up regulated during diapause. The T7 promoter sequence is highlighted in blue.

Chaperone	Forward primer	Reverse primer	Reaction conditions
p26	5'-TAATACGACTCACTATAGGGAGACCACTCCCAGAACATGTCAAACCA-3'	5'-TAATACGACTCACTATAGGGAGACCACTGCACCTCCTGATCTTGTTG-3'	5 min at 94°C, 30 cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 1 min, followed by 10 min at 72°C
ArHsp21	5'-TAATACGACTCACTATAGGGAGACAAAGCGTTACCATCGTCCT-3'	5'-TAATACGACTCACTATAGGGAGAGTTTTCTGCTCGCACTTTCC-3'	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
ArHsp22	5'-TAATACGACTCACTATAGGGAAAGCCTGGTAGTTGCATGG-3'	5'-TAATACGACTCACTATAGGGAGAAAAGCCTGGTAGTTGCATGG-3'	5 min at 94°C, 30 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min, followed by 10 min at 72°C
artemin	5'-TAATACGACTCACTATAGGGAGATGCATCGGTTCACAAGGA-3'	5'-TAATACGACTCACTATAGGGAGATCAAGGCACGATTGATGG-3'	5 min at 94°C, 30 cycles of 94°C for 30 sec, 61°C for 30 sec and 72°C for 1 min, followed by 10 min at 72°C

The PCR products were resolved by electrophoresis in 1.5% agarose gels in 0.5 x TBE buffer (50 mM TRIS, 50 mM boric acid and 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). The PCR products described above were employed as templates in a 3 h incubation to generate dsRNA using the MEGAscript® RNAi kit (Ambion Applied Biosystems, Austin, TX, USA), which exploits the attached T7 promoter sequence, according to manufacturer's instructions. The dsRNA was resolved by agarose gel electrophoresis as described.

As a control for RNAi procedures dsRNA specific to green fluorescence protein (GFP) was generated using the forward (5'-TAATACGACTCACTAAG GGAGACACATGAAGCAGCACGACTT-3') and reverse (5'-TAATACGACTCAC TATAGGGAGAAGTTCACCTTGATGCCGTTC-3') primers from IDT (Zhao et al., 2012). The GFP cDNA used as template was cloned in the vector pEGFP-N1 (Clonetech, Mountain View, CA, USA). The conditions and materials described above were used to produce dsRNA for GFP except that the annealing temperature was 60°C and the MgCl$_2$ concentration was 2.25 mM.

Several attempts were made to produce dsRNA specific to p8 using 12 different primer pairs with the T7 promoter sequence attached but none of these was successful. siRNAs for p8 were designed using Ambion's siRNA target finder software. The program returned 31 potential siRNAs which were then BLAST searched in NCBI databases to select sequences with the least potential

for off target effects. Five p8 siRNAs were selected that lacked matches to sequences in genes from *Artemia* and those organisms closely related to *Artemia.*

2.3.2 Injection of *A. franciscana* with dsRNA and siRNA

dsRNA and elution solution from the dsRNA kit were mixed separately in a 1:1 ratio (v/v) with 0.5% phenol red in Dulbecco's phosphate buffered saline (DPBS) (Sigma-Aldrich). The mixture of elution solution and phenol red lacking dsRNA was termed control solution. Two hundred and fifty nl of dsRNA and control solution were introduced individually into the egg sacs of different mature *Artemia* females, or the ocular extension in males, using a Nanoject II microinjector (Drummond Scientific Co., Broomall, PA, USA). Needles were prepared with preset program 33 on a p97 Flaming/Brown Micropipette Puller (Sutter Instrument Co., Novato, CA, USA) and broken at 45° using fine forceps under an Olympus SZ61 stereomicroscope (Olympus, Markham, Ontario, Canada) (Copf et al., 2004). Females destined to produce cysts and nauplii, as indicated by the presence or absence of a shell gland and the coloration of oocytes (Anderson et al., 1970; Liang and MacRae, 1999), were immobilized using a cooled 3% agar bed, gently blotted with Kimwipes and injected. The injected females were rinsed off the agar plates with sea water into six well plates. siRNAs were injected in the same way, first individually for assessment of

knockdown using immunoprobing, and then the three siRNAs showing the greatest degree of knockdown were pooled and microinjected.

Adults were monitored for at least 3 h after injection and only those that returned to normal morphology by straightening their tails and assumed typical swimming and feeding behavior were used in experiments. Females were monitored for retention of inoculum for 3 h, and those females losing the dye within this time were discarded. Females were observed with an Olympus SZ61 stereomicroscope to determine the time of fertilization which was marked by fusion of lateral egg sacs after introduction of a male. Embryo development and other morphological or physiological changes were also observed by microscopy. Hatched nauplii were raised to sexual maturity in small hexagonal weigh boats as described above. Females were observed for 5 fertilizations and embryo releases or until death.

2.4 Knockdown of mRNA and protein by RNAi

2.4.1 Evaluation of mRNA Knockdown by RT-PCR

Knockdown of mRNA was assessed using RT-PCR. Hydrated cysts from females receiving either dsRNA specific to the target protein or GFP purified with the RNAeasy kit (Quiagen, Mississauga, Ontario), or control solution were homogenized with a microfuge pestle (Fisher) in 600 μl of RLT reagent from the RNAeasy kit and passed through a 20 gauge needle 10 times. The mixture was centrifuged in a microcentrifuge for 1 min at 500 RPM (500 g) and the

supernatant was transferred to a fresh tube. Following manufacturer's instructions the purified mRNA was reverse transcribed with the Superscript III first strand synthesis system for RT-PCR (Invitrogen) using oligo-dT supplied in the kit. The cDNA generated in this reaction was then amplified using forward and reverse primers from IDT and Taq polymerase (Fermentas) under the conditions listed (Table 2) using 4 μl of cDNA from the first strand reaction. In order to determine if the RNA samples used in these experiments contained contaminating genomic DNA the reverse transcriptase reaction was omitted prior to amplification. The final RT-PCR products were resolved in 2% agarose gels in 0.5 x TBE buffer at 100 V, stained with Sybersafe (Invitrogen) and visualized in a DNR Bio-Imaging Systems MF-ChemiBIS 3.2 gel documentation system.

Table 2. Primers and reaction conditions for RT-PCR confirmation of mRNA knockdown

Target protein	[1]Number of cysts	Forward primer	Reverse primer	Reaction conditions
p26	50	5'-TAATACGACTCACTATAGGGAGACCACTCCCAGAACATGTCAAACCA-3'	5'-TAATACGACTCACTATAGGGAGACCACTGCACCTCCTGATCTTGTTG-3'	5 min at 94°C, 40 cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 1 min, followed by 10 min at 72°C
ArHsp21	58	5'-AGACCACAATCCCGTCACCTGTTT3'	5'-ATCAATCTTGACGTCCTCGGGCTT3'	5 min at 94°C, 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, followed by 10 min at 72°C
artemin	34	5'-TTCTATGCCAGACGCCAAGGCTGCCGTGG-3'	5'-CCACGGCAGCCTTGGCGTCTCTGGCATAGAA-3'	5 min at 94°C, 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, followed by 10 min at 72°C
p8	81	5'-TCGGAAGTCCGGGACCTATAGAAT-3'	5'-ACATGATGTTTGAGGTTTTG-3'	5 min at 94°C, 40 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min, followed by 10 min at 72°C

1, Number of cysts homogenized for each PCR reaction.

2.4.2 Evaluation of Protein Knockdown by Immunoprobing of Western Blots

Protein extracts from cysts obtained from females injected with either dsRNA specific to the target protein, dsRNA for GFP or control solution were resolved in SDS polyacrylamide gels. Prior to electrophoresis cysts at room temperature were transferred to ice cold distilled water and collected by centrifugation for 1 min at 500 RPM (20 g) using a microcentrifuge. The supernatant was discarded and each tube received 10 μl of sample buffer and 10 μl of ice cold Pipes buffer (100 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.4) and the cysts were homogenized using a microfuge pestle. The homogenate was placed in a boiling water bath for 5 min. All tubes were centrifuged for 10 min at 10000 RPM (8600 g) in a microcentrifuge at 4°C and 15 μl of each protein sample was resolved in 12.5% SDS polyacrylamide gels using a constant current of 35 mA for approximately 45 min. Proteins were then transferred to nitrocellulose membranes at 100 mA overnight at room temperature. Fermentas plus pre-stained protein ladders (Fermentas, Glen Burnie, MD, USA) were used as molecular mass markers.

Membranes with cyst proteins were incubated in 8% Carnation non fat milk solution for 1 h followed by exposure for 20 min at room temperature to antibody raised against the target protein diluted as indicated (Table 3) in 10 mM TRIS containing 140 mM NaCl, pH 7.4 (TBS). The membranes were washed 3 times for 5 min in 10 mM TRIS containing 140 mM NaCl and 0.1% Tween 20, pH 7.4

Table 3. Conditions for immunoprobing of western blots to confirm knockdown of target proteins

Target protein	[1]Number of cysts	Antibody name	Antibody dilution	Reference
p26	25	Rabbit anti p26	1:10000	Liang and MacRae, 1999
ArHsp21	55	Rabbit anti ArHsp21	1:7500	Qiu and MacRae, 2008a
ArHsp22	88	Rabbit anti ArHsp22	1:2000	Qiu and MacRae, 2008b
artemin	42	Rabbit anti artemin	1:20000	Chen et al., 2007
p8	53	Rabbit anti p8	1:2000	Qiu and MacRae, 2007

1. Number of cysts used to prepare protein extract for each lane of a SDS polyacrylamide gel.

(TBS-Tween), and then 3 times for 5 min in 10 mM TRIS containing 1M NaCl, 0.5% Tween 20, pH 7.4 (HST). The membranes were incubated in HRP-conjugated goat anti-rabbit IgG (Sigma-Aldrich) diluted 1:10000 in TBS.   The membranes were washed as above in TBS-Tween and HST and antibody-reactive proteins were visualized with ECL plus western blotting detection reagents (GE Healthcare, Baie d'Urfe Quebec, Canada) and a DNR Bio-Imaging Systems MF-ChemiBIS 3.2 gel documentation system.

2.5 Determining the effect of protein knockdown on *Artemia* cysts

2.5.1 Assessment of cyst metabolic activity

Metabolic activity was evaluated using an assay modified from Yang and Balcarcel (2003). Ten cysts, either with or without p26, were incubated immediately after release from females in 100 μl of test solution consisting of seawater containing 1000 U penicillin, 100 μg/ml streptomycin sulfate (Warner et al., 1979) and 0.03% phenol red at pH 8.5 in covered Costar 96 well UV plates (Corning Inc., Corning, NY, USA). Other wells contained test solution only or test solution with 10 commercially produced quiescent cysts (INVE Aquaculture, Inc.). The absorbance of test solutions at 553 nm (A_{553}) was determined with a SPECTRAmax PLUS microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 24 h intervals after addition of cysts to wells. To measure A_{553} the test solution from each well, excluding cysts, was transferred to a fresh well. Absorbance values were corrected for changes that occurred in test solutions in

the absence of added cysts. After A_{553} determination residual liquid was removed with autoclaved Q-tips from the original wells containing cysts and 100 μl of fresh test solution was added for the next 24 h incubation.

2.5.2 Establishing the effect of protein knockdown on the rate of embryo development

Once females had been mated with males and placed into 6-well plates they were observed daily using a stereomicroscope. The date of fertilization, denoted by egg sac fusion, release and type of embryos were recorded. Six-well plates were housed in an Adaptis chamber (Conviron, Winnipeg, MB, Canada) set to a 16:8 hour light:dark cycle at 18 and 16°C respectively when plates were not being observed. Results were then subjected to statistical analysis inluding t-tests.

2.5.3 Determining the effect of protein knockdown on diapause termination

Cysts collected from female *A. franciscana* exposed to either dsRNA specific to the target protein, dsRNA for GFP or control solution were incubated in 15 ml of sea water for at least 90 days at room temperature in sealed 15 ml conical centrifuge tubes. The cysts were flushed into a hexagonal weigh boat by spraying sea water from a wash bottle into the tube and then observed for two weeks to determine if hatching occurred.

2.5.4 Ascertaining the effect of protein knockdown on the ability of *Artemia* cysts to tolerate stress associated with diapause breakage

Cysts collected from female *A. franciscana* exposed to either dsRNA targeting a specific protein, GFP or control solution were incubated in sea water for 10 days at room temperature in hexagonal weigh dishes covered with aluminum foil (Clegg and Conte, 1980). To break diapause the cysts were then dried in a desiccator over Dryrite for at least 4 weeks, followed by transfer to -20°C for at least 8 weeks. To quantify diapause breakage as indicated by hatching, cysts processed as just described were incubated in sea water at room temperature during which nauplii were counted and removed. Counting continued for at least 10 days and ceased 5 days after the last nauplius emerged.

2.6 Determining the effect of protein knockdown on heat tolerance of *Artemia* cysts

To generate a standard curve establishing the effect of temperature on hatching, 100 commercially obtained cysts were hydrated at 4°C for at least 4 h in 500 μl of seawater. The cysts were then incubated in a water bath for 30 min at temperature increments of 2°C followed by incubation in 25 ml of sea water at room temperature during which nauplii were counted and removed as described

above. The experiment was done in triplicate and stopped when no nauplii had hatched for 5 days.

Laboratory produced cysts lacking p26 that hatched spontaneously and commercially obtained cysts possessing p26 were incubated either at room temperature or at 41°C for 30 min and their hatching was assessed as described previously.

With the exception of experiments examining p26, dehydrated and frozen cysts, prepared as described above, were obtained from *A. franciscana* females either injected with dsRNA specific to the target protein, dsRNA for GFP or control solution. The broods were divided, with half of the cysts heated at 41°C for 30 min and the remainder kept at room temperature. After heating the cysts were incubated in sea water and hatching was quantified by counting nauplii. The experiment was performed in triplicate.

2.7 Determining the effect of dsRNA specific to ArHsp22 on adult *Artemia*

In order to determine the effects of dsRNA specific to ArHsp22 groups of 12 animals were injected with either control solution or dsRNA specific to ArHsp22. This experiment was performed in triplicate with groups of males and females. After discarding animals that were morphologically or behaviorally abnormal the groups were then monitored for 4 days to determine when and how many animals succumbed to injection of dsRNA specific to ArHsp22 in comparison to a group that was injected with control solution only.

Chapter 3: Results

3.1 Injection of *Artemia* adults with dsRNA

Prior to fertilization female *Artemia* destined to produce cysts possessed green oocytes similar in colour to those pictured in Liang and MacRae, 1999. positioned in two egg sacs separated by a brown shell gland. (Figs. 3A, B). Injection of an egg sac with either dsRNA specific to the target protein or control solution, both containing phenol red, stained the entire female, demonstrating that inoculum migrated readily throughout the animal (Figs. 3C, D). Approximately 90% of females exhibited phenol red staining 2 h post-injection but the dye disappeared by the next day. Appendages normally visible on adult *Artemia* and poorly resolved in the upper third of Fig. 3B are obscured at the lower magnifications (Figs.3 A, C, D) because animals were immobilized on the surface of cold agarose and partially dried by blotting with a Kim Wipe prior to injection. Males were injected in the unprotected area of the cephalon where claspers originate.

Fig. 3. Injection of a female primed to produce cysts. All light micrographs are of the same animal. A. *Artemia* female prior to fertilization with the shell gland and egg sacs boxed; B. boxed region of Fig A enlarged; C, injection of dsRNA into the egg sac; D, an injected female which has retained phenol red for 2 h and assumed normal morphology. G, gut; ES, egg sac; M, microneedle SG, shell gland.

A   B   C   D
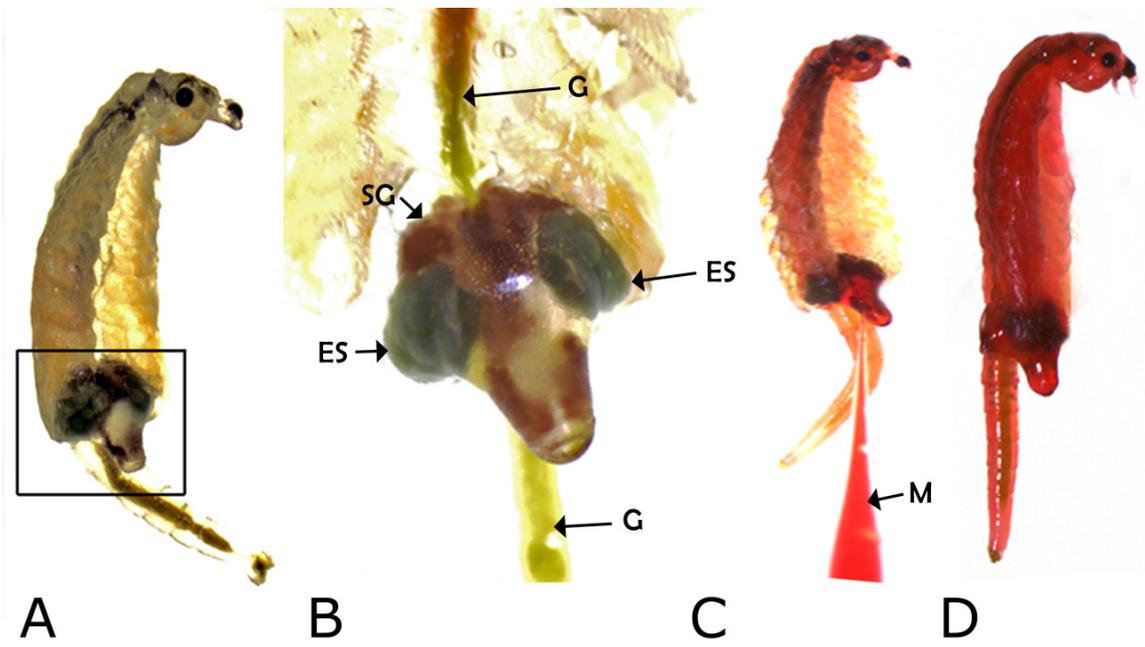
3.2 p26 has multiple roles in *Artemia* embryos

3.2.1 p26 dsRNA specifically knocked down p26 mRNA and protein

p26 dsRNA used for injection of *Artemia* females migrated in agarose gels at the expected size, slightly larger than the cDNA from which it originated (Fig. 4A). Electrophoresis of RT-PCR products yielded a band in agarose gels stained with gelstar (Lonza, Switzerland) when mRNA from cysts produced by A*rtemia* females injected with control solution was used for amplification (Fig. 4B). When the mRNA was from cysts generated in females injected with p26 dsRNA the PCR product was greatly diminished (Fig 4B). Omission of reverse transcriptase revealed the essential absence of genomic DNA contamination in samples used for PCR (Fig. 4B). To ensure that cyst count was an appropriate measure for determining the amount of sample in experiments hydrated cysts were measured (n = 1426) revealing an average diameter of 242 μm with a standard deviation of 12 μm. Over 95% of the cysts measured were between 218 μm and 266 μm (Appendix 1).

As revealed by immunoprobing of western blots, p26 occurred in cysts released from females injected with control solution and GFP, the latter not shown, but the protein was not detectable in cysts from females receiving p26 dsRNA even when blots were over-exposed (Fig. 4C). ArHsp21 and ArHsp22 (Fig. 4C), sHSPs similar to p26 and synthesized in diapause-destined *Artemia* embryos, were unaffected by p26 dsRNA specific to p26.

Fig. 4: Knock down of p26.   Panel A, production of p26 dsRNA as described in Materials and Methods. Amplification products were resolved by electrophoresis in 1.2% agarose gels and stained with Gelstar®. Lane 1, size markers; 2, p26 cDNA; 3, p26 dsRNA. Marker size in base pairs is on the left of the figure.   Panel B, amplification of p26 mRNA by RT-PCR using RNA from cysts released by females injected with either p26 dsRNA (1, 3) or control solution (2, 4). Lanes 1 and 2, RNA samples were incubated with reverse transcriptase; 3 and 4, RNA samples were not incubated with reverse transcriptase. Electrophoresis was in 1.2% agarose and gels were stained with Gelstar®.   Panel C,   Protein extracts from 25 cysts (26) and 40 cysts (21, 22) produced by females injected with either p26 dsRNA (1) or control solution (2) were resolved in 12.5% SDS polyacrylamide gels and blotted to nitrocellulose. The blots were reacted with antibody specific to p26 (26), ArHsp21 (21) and ArHsp22 (22), followed by HRP-conjugated goat anti-rabbit IgG antibody. Proteins were visualized by chemiluminescence.
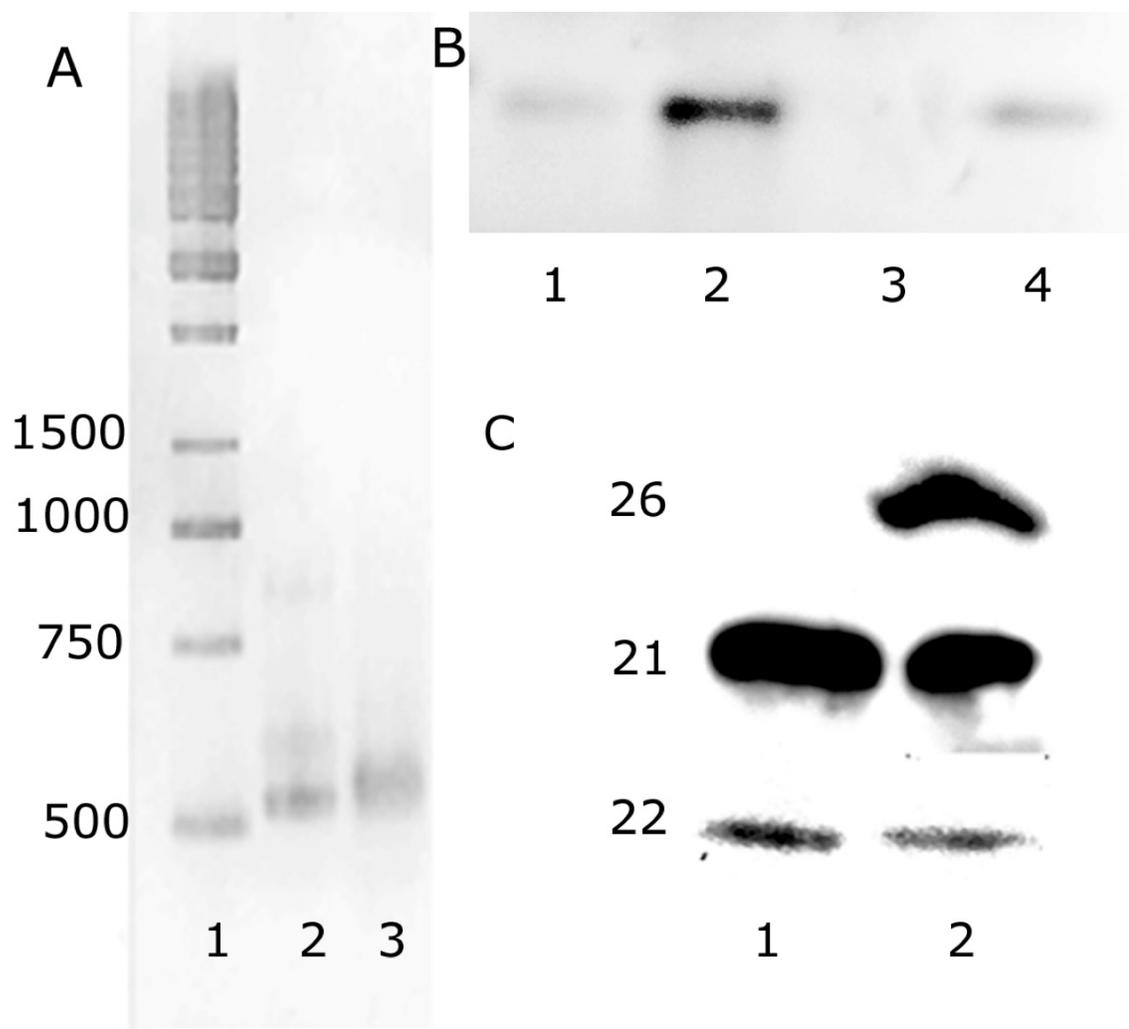
3.2.2 Determination of metabolic activity in newly released cysts

Groups of ten cysts that contained p26 (blue), cysts that did not contain p26 (red) and quiescent cysts that hatched into nauplii (green) were incubated in seawater containing phenol red for four days and monitored daily for colour changes. Cysts containing and lacking p26 showed a similar initial metabolism and a similar slowdown in metabolism after release, while hatching cysts showed increasing metabolism over two days and then lowered metabolism because they were not being fed (Fig. 5).

3.2.3 Loss of p26 slows the development of diapause-destined *Artemia* embryos

The time from fertilization to release of either cysts or nauplii (time to release) was approximately 5 days for first broods from females injected with control solution, this was comparable to the time to release of nauplii from females injected with p26 dsRNA (Table 4) and for nauplii and cysts from non-injected females. In contrast, injection of females with p26 dsRNA increased the time to release for first brood cysts to approximately 7 days (Table 4). The developmental delay imposed by elimination of p26 persisted for at least 3 additional broods from the same female, the maximum number observed (Fig 6). No p26 was detectable by immunoprobing of western blots containing protein

Fig. 5. The metabolic activities of p26 knock down and control cysts are similar. The metabolic activity of cysts containing p26 (blue) and those that do not (red) as well as commercially obtained cysts (green) are shown. Ten cysts were incubated in sea water that contained 0.03% phenol red, pH 8.6, and the change in absorbance was measured at 553 nm. AU, arbitrary units. Colour change over 24 hours indicated level of metabolism. Error bars show standard error.
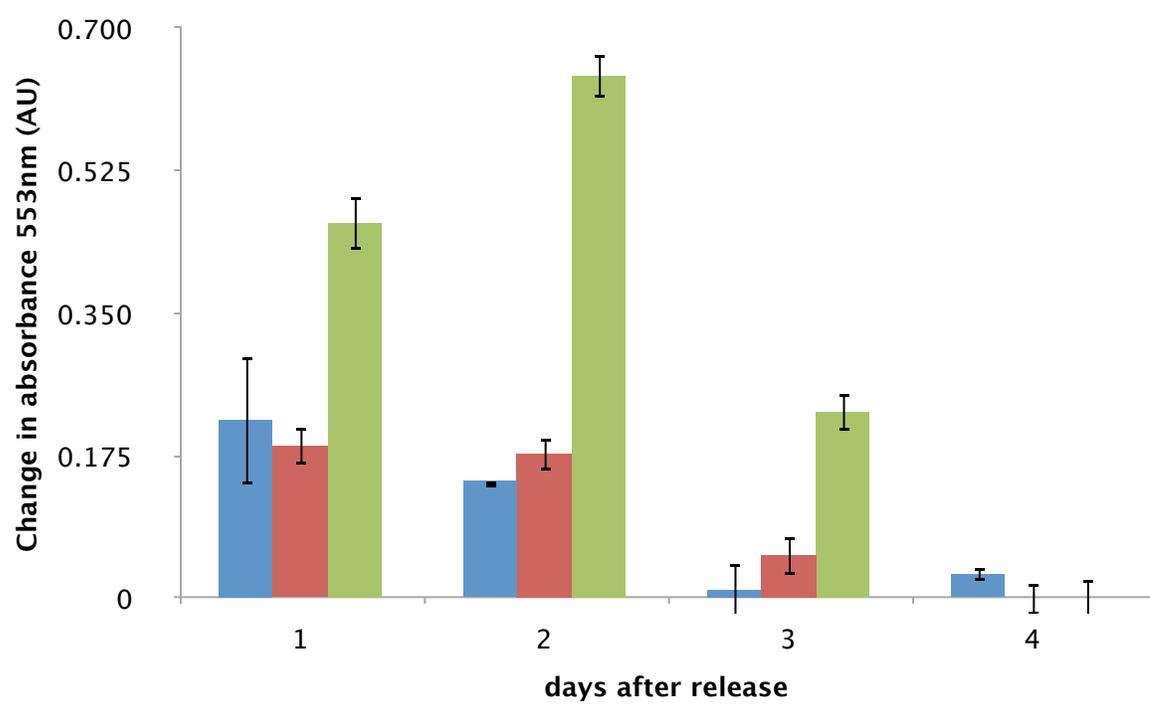
Table 4. Elimination of p26 slows *Artemia* embryo development. The change in time from fertilization to release of cysts and nauplii (Δ Days) for first broods from females injected with either dsRNA or control solution was compared using a two sided t test.

	Time to release (Days)		Δ Days	p-value
	-p26	+p26		
Cysts	7.6	4.7	2.9	0.0313
Nauplii	4.7	4.2	0.5	0.3878

Fig. 6. The knockdown of p26 persisted through 4 broods. The time between fertilization and release (time to release) for nauplii from females injected with control solution (blue), nauplii from females injected with p26 dsRNA (red), cysts from females injected with control solution (green), cysts from females injected with dsRNA (purple) is shown with error bars representing standard error (n= 3 - 12). The inset shows a western blot of protein extract from 15 cysts in four successive broods (lanes 1 - 4) obtained from females injected with p26 dsRNA and probed with antibody to p26 (top) and ArHsp21 (bottom). Lane C, protein extract from commercial cysts containing p26 and ArHsp21.
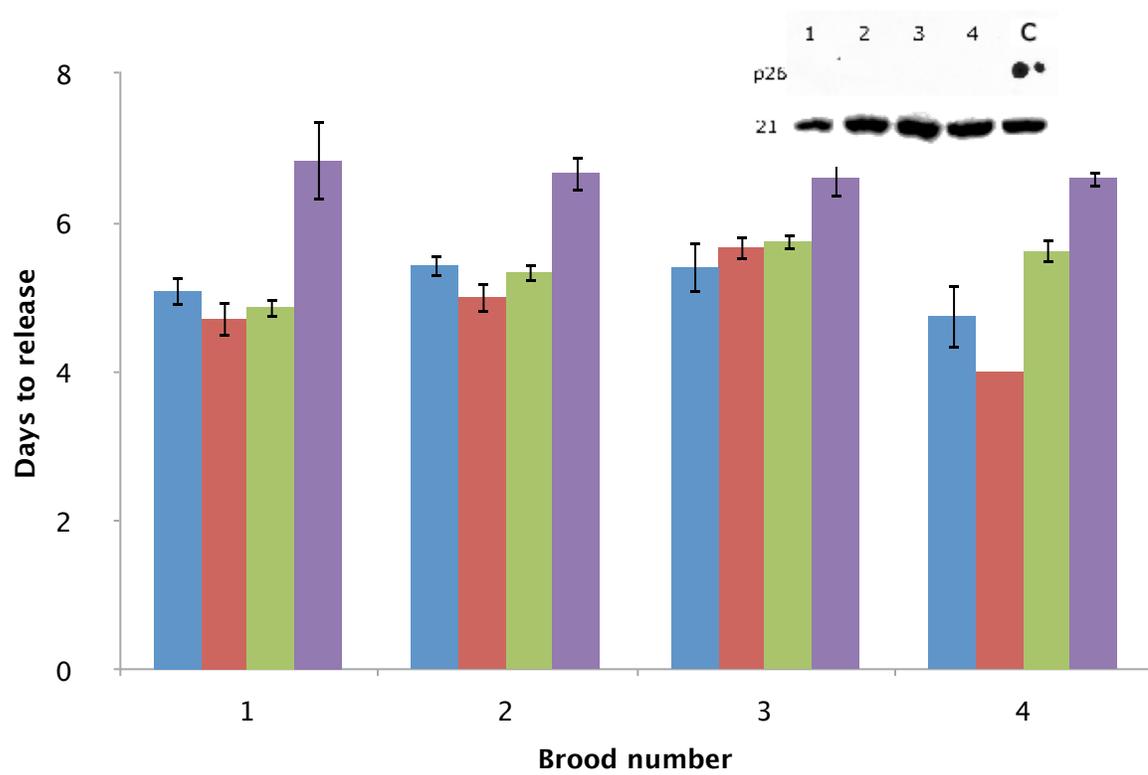
from cysts experiencing developmental delays although stripping of blots and probing with antibody to ArHsp21 gave strong bands (inset, Fig 6). Delays in embryo development did not extend to subsequent generations of females arising from cysts produced by animals injected with dsRNA and these females produced cysts with normal amounts of p26.

3.2.4 Loss of p26 leads to spontaneous termination of diapause

After incubation in sea water at room temperature for at least 90 days approximately 90% of cysts lacking p26 hatched upon transfer to weigh boats, indicating diapause had terminated (Fig. 7, A-D). Under the same experimental conditions cysts containing p26 did not hatch (Fig. 7 E, F). This experiment was performed in triplicate.

3.2.5 p26 contributes to the stress tolerance of *Artemia* cysts

Diapause was terminated in laboratory generated cysts by 4 weeks of desiccation followed by 8 weeks at -20°C. Hatching success varied with this procedure and in the example shown 58% of cysts containing p26 survived diapause termination and hatched as opposed to only 6% of cysts lacking p26 (Fig. 8). Hatching data after diapause termination by desiccation and freezing

Fig. 7. p26 prevents spontaneous diapause termination. A. Cysts lacking (-p26) and containing (+p26) p26 were incubated without agitation in sealed tubes for at least 90 days, transferred to weigh boats and observed with a dissecting microscope for 5 days after the last cyst hatched. Error bars represent standard error. B – F. Light micrographs showing the development, or absence thereof, for cysts lacking (B - D) and containing (E, F) p26. c, cyst; e, emerged cyst; es, eye spot. The nauplii shown in Fig D originated from commercial cysts and they are representative of nauplii obtained from laboratory reared cysts lacking p26.
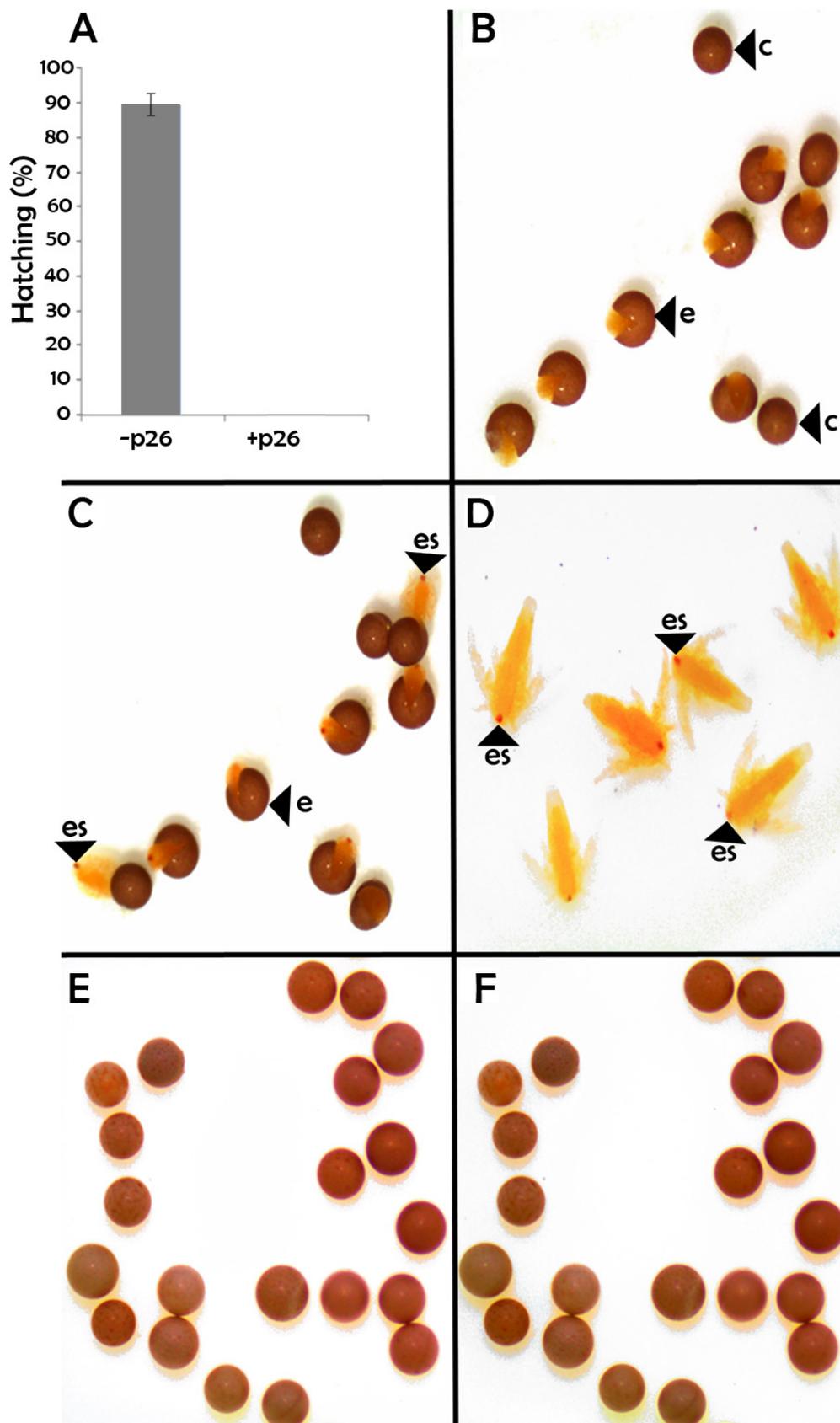
Fig. 8. p26 enhances the stress tolerance of *Artemia* cysts. Diapause of *Artemia* cysts either containing (+p26) or lacking (-p26) p26 was terminated by desiccation and freezing. The cysts were then incubated in sea water at room temperature and hatched nauplii were counted and removed. The results are given as the percentage of cysts that hatched with hatching an indicator of diapause termination and cyst viability. Error bars indicate standard error.
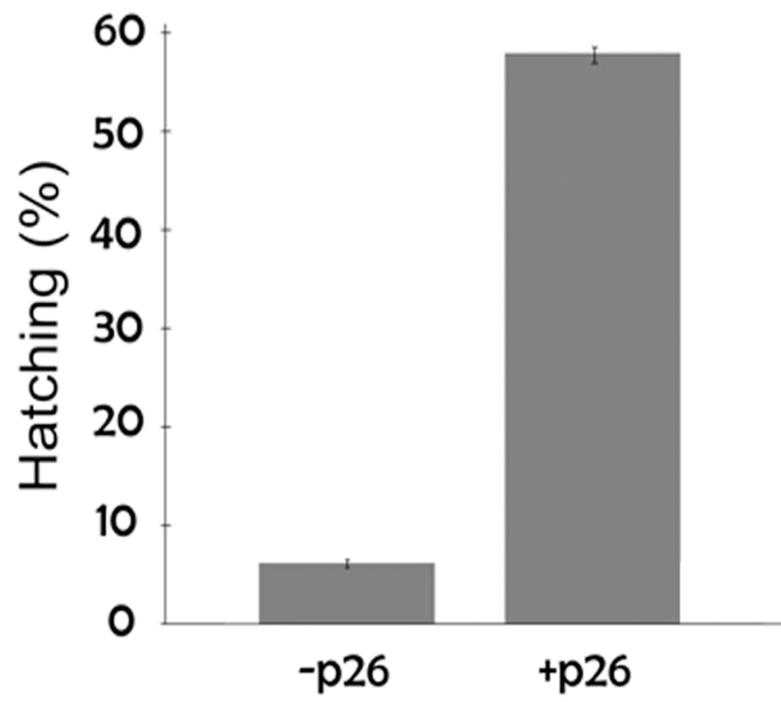
indicated that p26 contributes to stress protection and to test this idea, cysts were heat shocked. Generation of a standard curve demonstrated that 56% of cysts survived exposure to 41°C for 30 min and these conditions were chosen for subsequent heat shock experiments (Fig. 9, top).

Hatching of cysts exposed to temperatures higher than 35°C was delayed when compared to the hatching of cysts incubated at lower temperatures. The duration of emergence, that is, the time taken for a membrane-enclosed nauplius to completely exit the cyst, was also longer as stress increased (data not shown). Groups of commercial cysts containing p26 and broods of laboratory reared cysts lacking p26 which terminate diapause without desiccation and freezing (Fig. 9, bottom), were divided in half and transferred to weigh boats either after incubation at room temperature or heating at 41°C for 30 min. For p26 containing cysts 91% and 52% respectively of unheated and heated cysts hatched whereas when p26 was absent 71% of unheated and 30% of heated cysts hatched (Fig. 9, bottom). Cysts from different sources were compared because terminating diapause in laboratory produced cysts required exposure to stress, namely desiccation and freezing. The findings demonstrate that p26 increased cyst tolerance to heat shock.

Fig. 9. Heat tolerance of *Artemia* cysts. Groups of 100 hydrated cysts were placed in 200 μl of sea water and heated for 30 min at the indicated temperatures (top). The cysts were then incubated at room temperature and the hatched nauplii were counted and removed. Hatching continued until no nauplii emerged for 5 consecutive days. The experiment was performed in triplicate and the error bars represent standard error. The bottom panel shows maximum heat tolerance of cysts depends on p26. Cysts containing (+p26) and lacking (-p26) p26 were either held at room temperature (a) or heated at 41°C for 30 min (b), cooled on ice, incubated in sea water and observed for hatching (heated). Results are presented as the hatching percentage with hatching equivalent to viability. Error bars represent standard error.
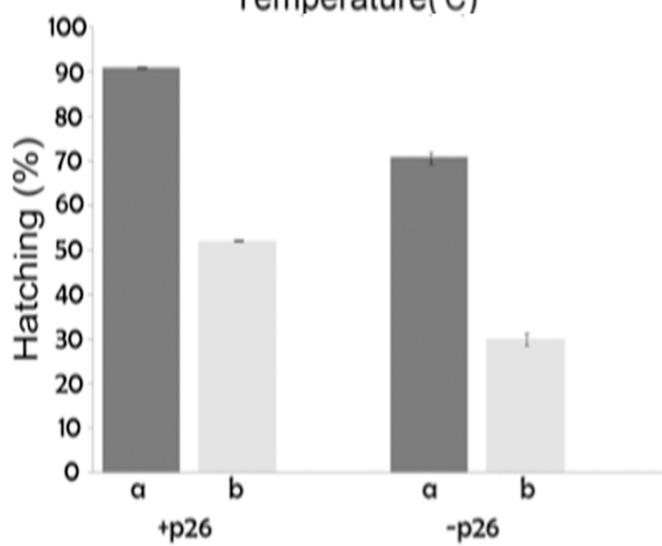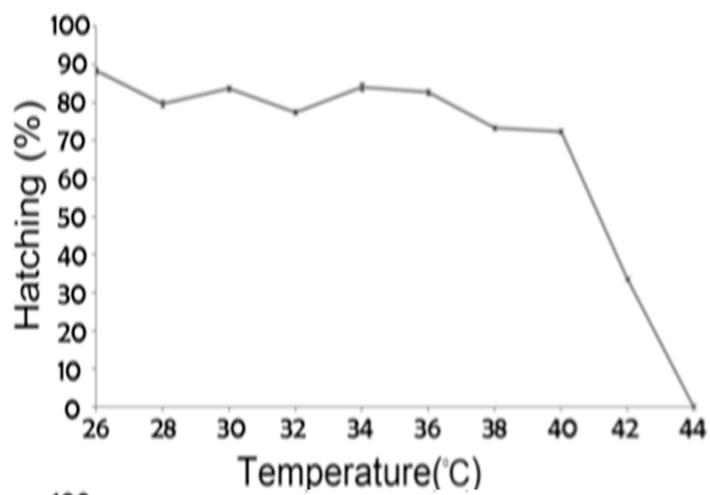
3.3 ArHsp21 is not required for completion of diapause.

3.3.1Generation of ArHsp21 dsRNA and injection of *Artemia* females

When resolved in agarose gels ArHsp21 cDNA and dsRNA yielded bands of the expected size, with the dsRNA slightly larger than the cDNA (Fig. 10A). dsRNA representing ArHsp21 and GFP and control solution, each containing phenol red, were injected separately into the egg sacs of unfertilized adult *A. franciscana* females. Those animals retaining dye and resuming normal behavior 3 h after the injection were used in subsequent experiments.

3.3.2 Knockdown of ArHsp21 mRNA and protein in *Artemia* embryos

As determined by RT-PCR the injection of *Artemia* females with dsRNA completely knocked down ArHsp21 mRNA (Fig. 10B). There was no genomic DNA contamination in the mRNA prepared from cysts employed in these experiments. The ArHsp21 protein was knocked down by injection of females with dsRNA for ArHsp21 (Fig. 10C). The knock down was specific because injection of females with control solution (Fig. 10C) and dsRNA for GFP did not affect ArHsp21expression. Elimination of ArHsp21 did not effect the expression of ArHsp22.

Fig. 10. Injection of females with dsRNA for ArHsp21 specifically knocks down ArHsp21. Panel A. Lane 1, Generuler 1 kb DNA size markers; 2, cDNA used to generate dsRNA; 3, dsRNA specific to ArHsp21 produced with the MEGAscript RNAi kit. Electrophoresis was in 1.2% agarose and RNA was visualized with Gel star. The size of markers is given in bp on the left side of the gel. Panel B, amplification was performed by RT-PCR using RNA from cysts released by females injected with either ArHsp21 dsRNA (1, 3) or control solution (2, 4). Lanes 1 and 2, RNA samples were incubated with reverse transcriptase; 3 and 4, RNA samples were not incubated with reverse transcriptase. Electrophoresis was in 1.2% agarose and gels were stained with Sybersafe®. Panel C, protein extracts from 60 cysts (21 and 22) produced by females injected with either ArHsp21 dsRNA (1) or control solution (2) were resolved in 12.5% SDS polyacrylamide gels and blotted to nitrocellulose. The blots were reacted with antibody specific to ArHsp21 (21) then stripped and re-probed with antibody specific to ArHsp22 (22) followed in each case by HRP-conjugated goat anti-rabbit IgG antibody. Proteins were visualized by chemiluminescence.
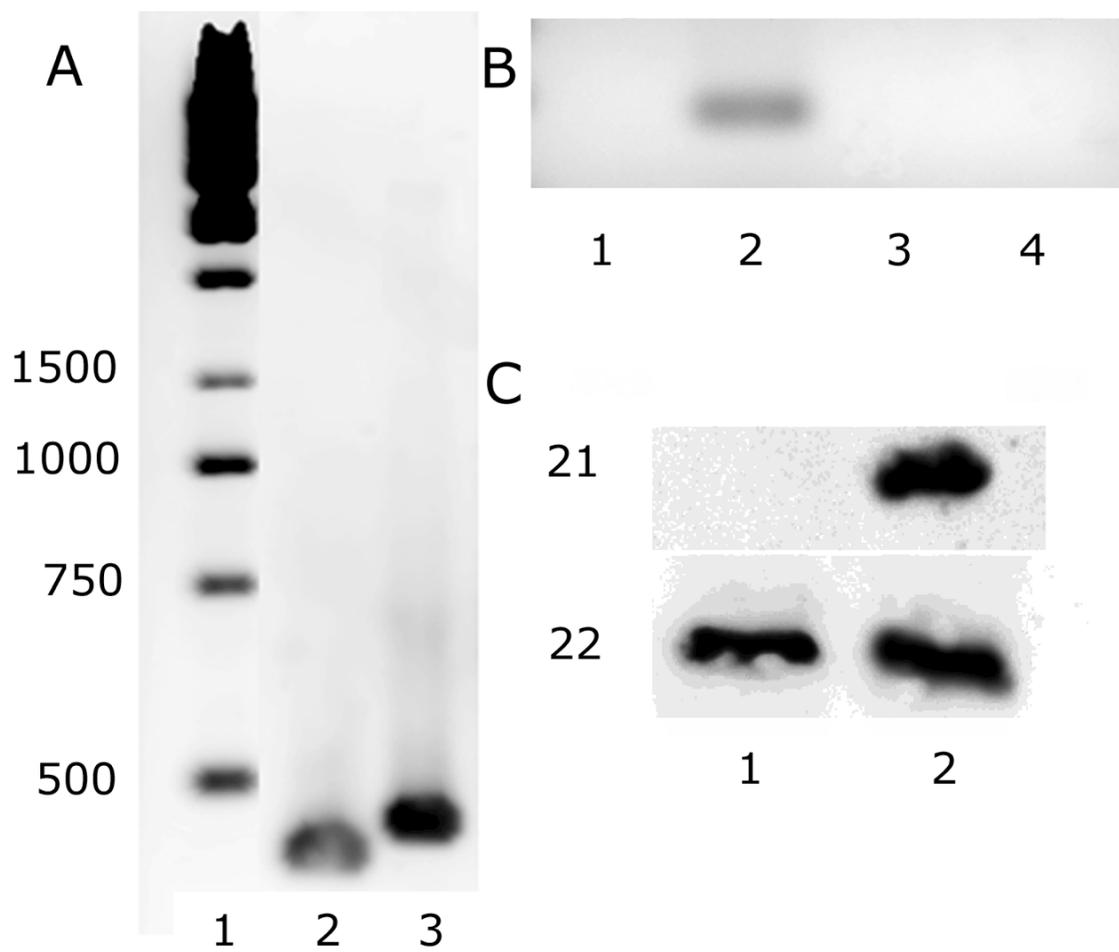
3.3.3 Knocking down ArHsp21 had no effect on the development of diapause-destined embryos

Artemia females injected with dsRNA specific to ArHsp21 behave normally and survive injection as well as those females injected with either dsRNA for GFP or control solution, and they were fertilized when introduced to males. The rate of embryo development was not significantly different between diapause-destined embryos that contained ArHsp21 and those that did not (P-value =0.86)

3.3.4 Cysts lacking ArHsp21 fail to spontaneously break diapause

Cysts released from females injected with dsRNA specific to ArHsp21 were placed in seawater in 15 ml conical centrifuge tubes and incubated for at least 90 days at room temperature. When flushed into hexagonal weigh boats the cysts lacking ArHsp21 did not hatch spontaneously as occurred for cysts lacking p26 (Not shown).

3.3.5 Cysts lacking ArHsp21 retain stress tolerance

After diapause termination by desiccation and freezing, 54% and 47% respectively of cysts containing and lacking ArHsp21 hatched, a difference that was significant but varied by only 7% (Fig. 11). The resulting nauplii grew to sexual maturity and reproduced normally. Cysts lacking ArHsp21

Fig. 11. The elimination of ArHsp21 does has a minor effect on cyst tolerance to desiccation and freezing. Diapause of *Artemia* cysts either lacking (-ArHsp21) or containing (+ArHsp21) was terminated by desiccation and freezing. The cysts were then incubated in sea water at room temperature and hatched nauplii were counted and removed. The results are given as the percentage of cysts that hatched with hatching an indicator of diapause termination and cyst viability. Error bars indicate standard error.
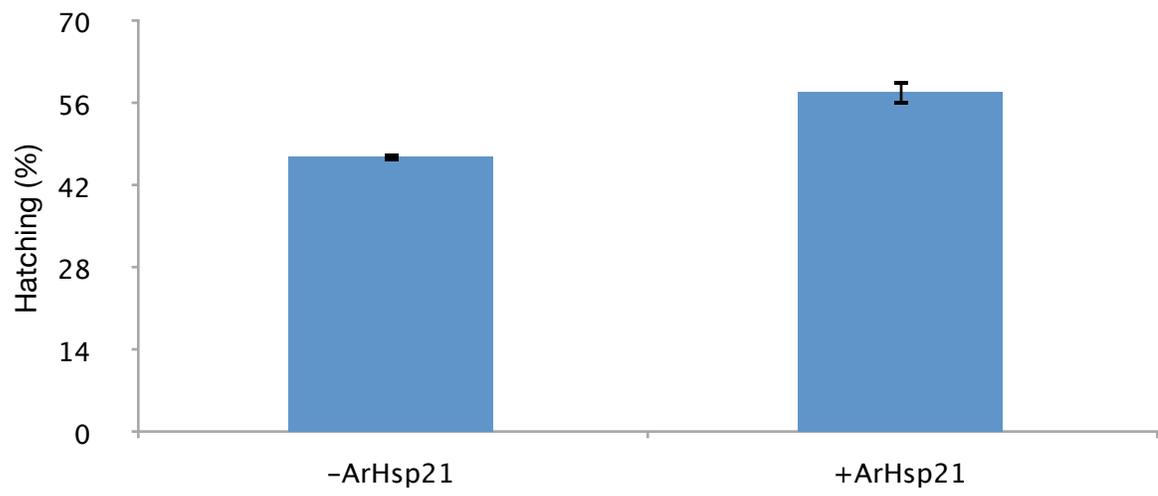
were equally resistant to heat stress (Fig. 12). Overall, knocking down ArHsp21 had no detectable effect on *Artemia* adult females and cysts when analyzed by the procedures employed herein.

Fig. 12. ArHsp21 had no effect on the heat tolerance of *Artemia* cysts. Diapause was terminated in broods of cysts that did not contain ArHsp21 and then the broods were divided in half. Half of a brood was incubated at 41°C for 30 min and the other half was not heat stressed. Nauplii were then hatched at room temperature. Error bars show standard error. The experiment was done in triplicate with different broods of cysts.
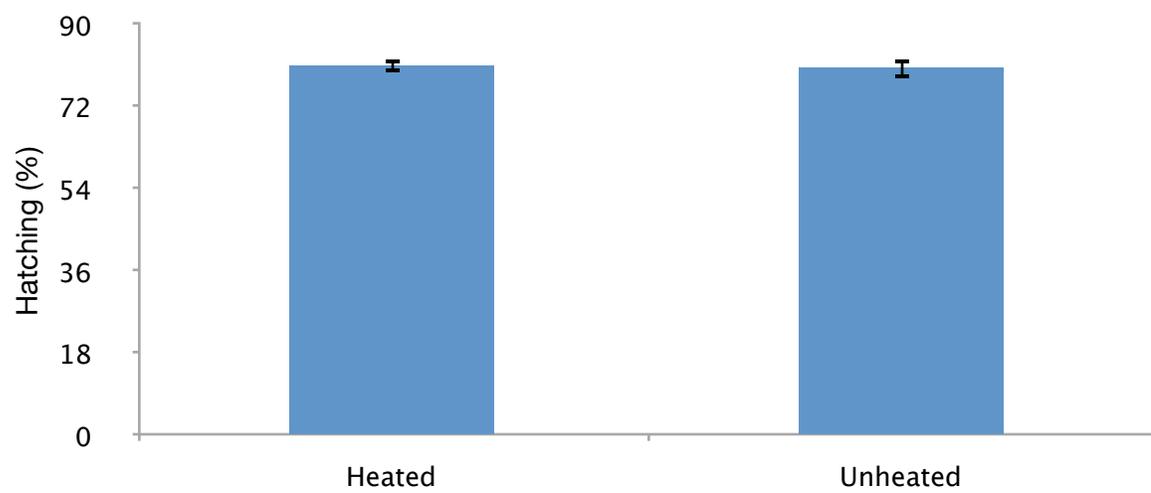
3.4 dsRNA for ArHsp22 is lethal to adult *Artemia*

3.4.1 Generation of ArHsp22 dsRNA and injection of *Artemia* females

When resolved in agarose gels ArHsp22 cDNA and dsRNA yielded bands of the expected size, with the dsRNA slightly larger than the cDNA (Fig. 13). dsRNA for ArHsp22 and GFP and control solution were injected separately into the egg sacs of unfertilized adult *A. franciscana* females and those animals retaining dye and resuming normal behavior 3 h after the injection were used in subsequent experiments.

3.4.2 Injection of ArHsp22 dsRNA kills adult *Artemia*

The initial recovery of *Artemia* females injected with dsRNA specific to ArHsp22 was similar to the recovery of animals injected with control solution or dsRNA specific to other proteins including sHSPs, artemin, p8 and GFP. Normal swimming behavior, filtering, as indicated by moving appendages and extension of the tail occurred at the same time and frequency as for females injected with dsRNA for p26 and ArHsp21. However, only 50% of females survived one day after receiving dsRNA specific to ArHsp22 whereas typically 90% of females receiving control solution or dsRNA for other proteins survived injection for one day. After the first day post-injection adult females generally died within the next 2 days (Fig. 14). The lateral appendages of *Artemia* that are near death as a consequence of injection with dsRNA for ArHsp22 assume a feathery

Fig. 13. Generation of dsRNA specific to ArHsp22. Lane 1, Generuler 1 kb DNA size markers; 2, cDNA used to generate dsRNA; 3, dsRNA specific to ArHsp22 produced with the MEGAscript RNAi kit. Electrophoresis was in 1.2% agarose and RNA was visualized with Gel star. The size of markers is given in bp on the left side of the gel.

1500

1000

750

500

1   2   3

Fig. 14. Injection with dsRNA specific to ArHsp22 kills *Artemia* adults. Females were injected with control solution (blue) and dsRNA specific to ArHsp22 (red). The experiment was repeated with males which received dsRNA specific to ArHsp22 (purple) and control solution (green). The experiment was performed in duplicate with approximately 10 animals in each group. Error bars represent standard error.
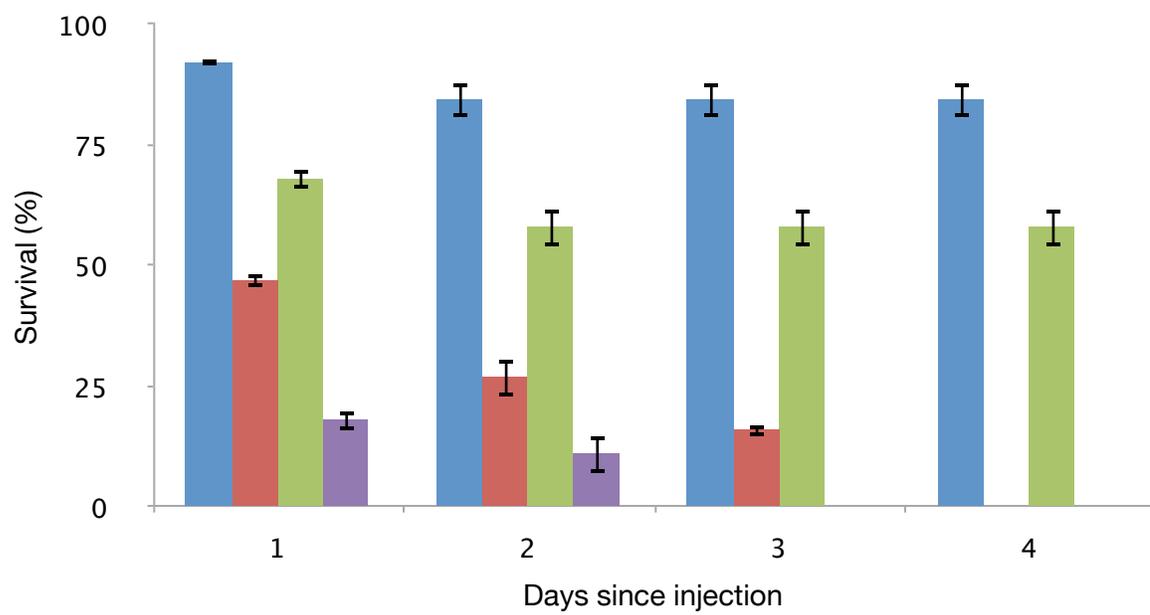
appearance and death ensues shortly after this morphological change (Fig. 15). The egg sacs of *Artemia* females tended to empty near to or soon after death and this occurred when females were injected with ArHsp22 dsRNA. Male *Artemia* were killed by injection with ArHsp22 dsRNA and they tended to die more rapidly than females (Fig .14). Due to the death of females it was not possible to determine if ArHsp22 was knocked down in cysts by injection of ArHsp22 dsRNA.

Fig. 15. Appendage morphology changed when adult *A. franciscana* were near death*.* The animals were viewed with an Olympus microscope (SZ61) and an infinity 1 lumenera camera was used to take the light micrograph. A, appendages, G, gut, H, head, E, egg sac containing cysts, O developing oocytes. Pictured on the right is a healthy animal and on the left a female near death.
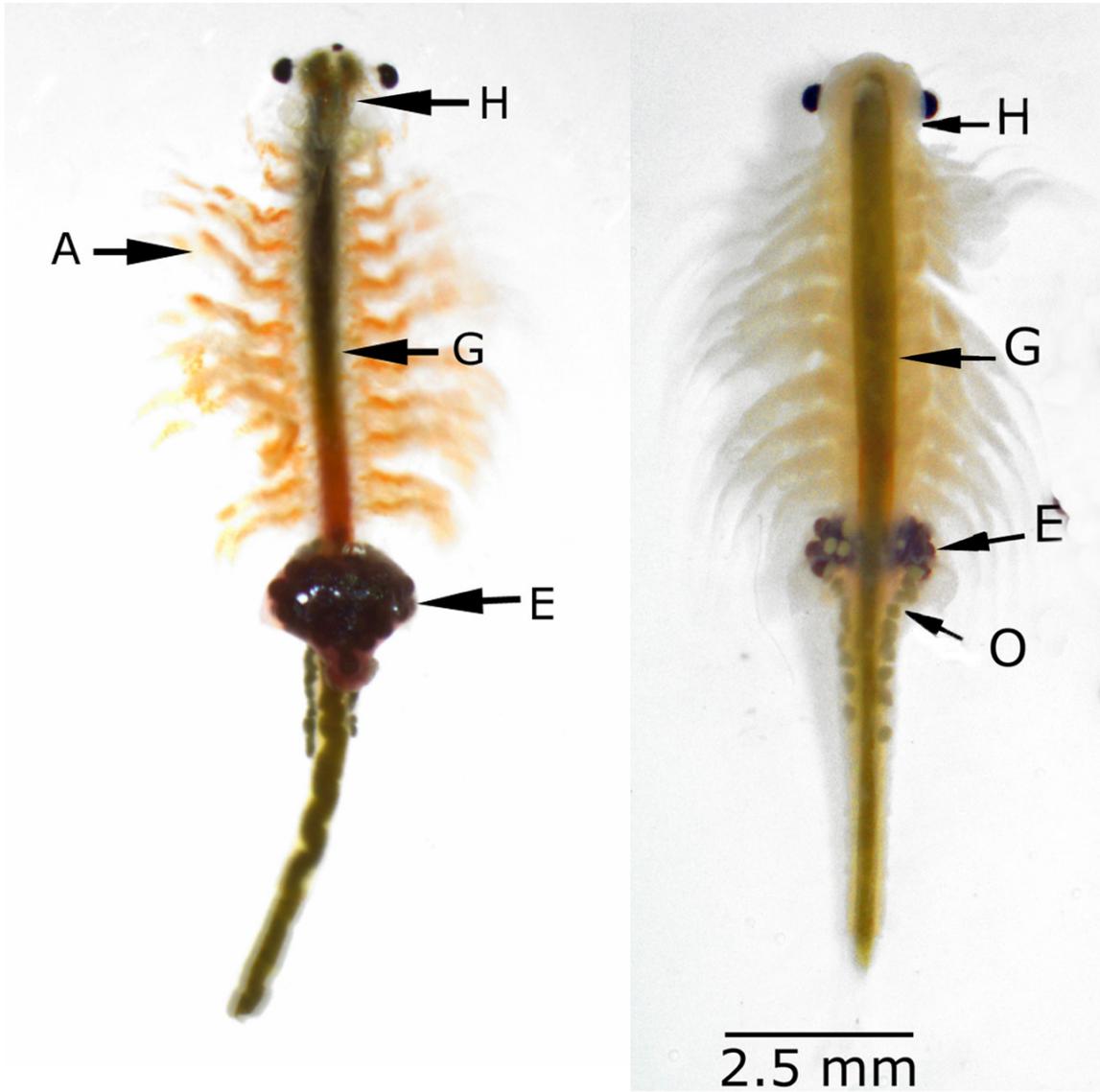
3.5 The knockdown of artemin had adverse effects on *Artemia* embryos

3.5.1 Generation of artemin dsRNA and injection of *Artemia* females

When resolved in agarose gels artemin cDNA and dsRNA yielded bands of the expected size, with the dsRNA slightly larger than the cDNA (Fig. 16A). dsRNAs representing artemin and GFP and control solution were injected separately into the egg sacs of unfertilized adult *A. franciscana* females and those animals retaining dye and reassuming normal behavior 3 h after the injection were used in subsequent experiments.


3.5.2 Knockdown of artemin in *Artemia* embryos

As determined by RT-PCR the injection of *Artemia* females with dsRNA completely knocked down artemin mRNA (Fig. 16B). Knock down of artemin protein by dsRNA was almost complete but some of the protein remained detectable on western blots (Fig. 16C). There was no genomic DNA contamination in the mRNA prepared from cysts employed in these experiments. The knock down was specific because injection of females with control solution and dsRNA for GFP did not affect artemin RNA and protein (not shown).

3.5.3 The time for complete release of a brood of cysts from females lacking artemin was extended. *Artemia* females injected with dsRNA specific to artemin

Fig. 16. Knockdown of artemin by dsRNA. Panel A, dsRNA specific to ArHsp22 was produced with the MEGAscript RNAi kit, resolved by electrophoresis in 1.2% agarose gels and visualized with Gel star.   Lane 1, Generuler 1 kb DNA size markers, Lane 2, cDNA used to generate dsRNA; 3, dsRNA generated specific to ArHsp21.   Marker size is given in bp on the left side of the gel in panel A. Panel B, Knockdown of artemin mRNA by injection of *Artemia* females with artemin dsRNA. Amplification was performed by RT-PCR using RNA from cysts released by females injected with either artemin dsRNA (1, 3) or control solution (2, 4). Lanes 1 and 2, RNA samples were incubated with reverse transcriptase; 3 and 4, RNA samples were not incubated with reverse transcriptase. Electrophoresis was in 1.2% agarose and gels were stained with Sybersafe®. Panel C, knockdown of artemin protein by injecting *Artemia* females with artemin dsRNA. Protein extracts from 35 cysts produced by females injected with either dsRNA for artemin or GFP (1) or control solution (2) were resolved in 12.5% SDS polyacrylamide gels and blotted to nitrocellulose. The blots in panel art and GFP were reacted with antibody specific to artemin followed by HRP-conjugated goat anti-rabbit IgG antibody. Proteins were visualized by chemiluminescence.
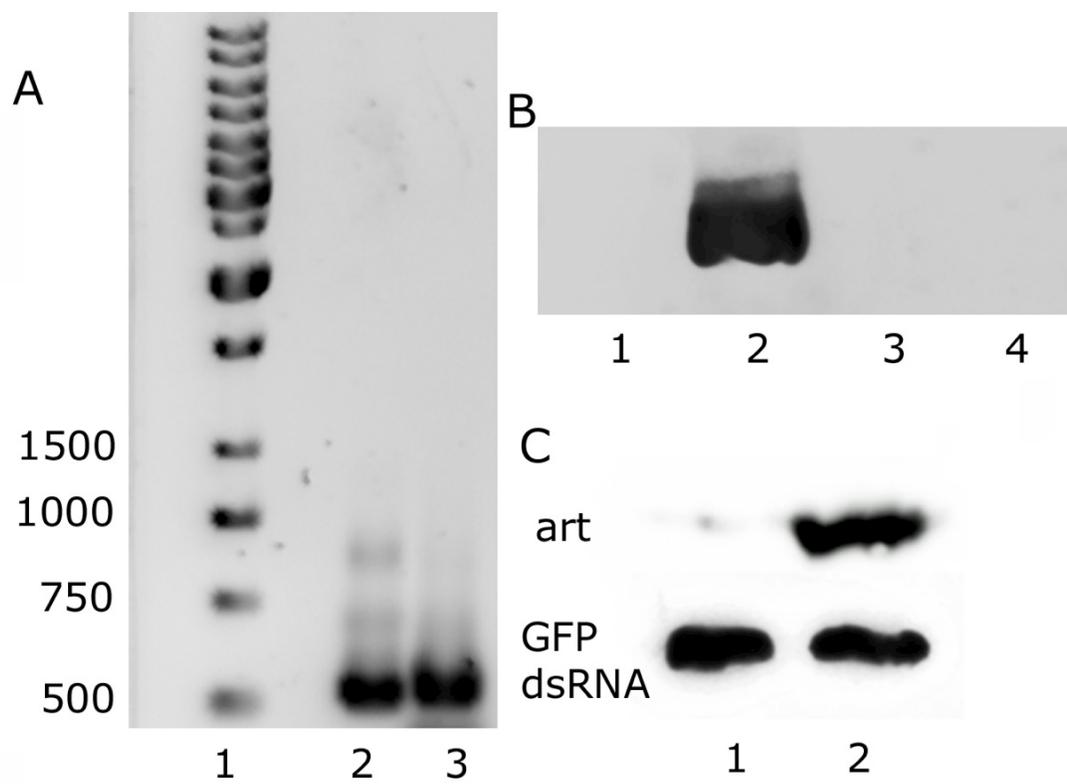
behaved normally and were fertilized when introduced to males. Broods of cysts are normally completely released within 2 hours but when artemin was knocked down females retained cysts in the egg sacs for up to two days after the initiation of release (Fig. 17). However, unlike the situation with p26, the rate of development (time to release) was not significantly different between embryos that either contained or lacked artemin (P value 0.94) (not shown). That is, the release of cysts was initiated at day 5 after fertilization. The release extension is most pronounced in the first brood release after injection and diminishes with each successive brood.

3.5.4 Cysts lacking artemin have reduced stress tolerance

After diapause termination by desiccation and freezing, 73% and 43% respectively of cysts containing and lacking artemin hatched (Fig. 18). The resulting nauplii grew to sexual maturity and reproduced normally.

3.5.5 Cysts lacking artemin do not spontaneously break diapause

When cysts released from females injected with dsRNA specific to artemin were incubated 90 days in seawater in 15 ml conical centrifuge tubes at room

Fig. 17. Release from females is slowed for cysts lacking artemin. Brood release was tracked for several releases after injection with dsRNA specific to artemin. The first appearance of cysts was noted as was the day that the brood sac was empty. The data from 5 females were pooled and the average release time by brood number graphed. Error bars indicate standard error.

Fig. 18. The elimination of artemin significantly reduced the stress tolerance of *Artemia* cysts. Diapause of *Artemia* cysts either lacking (-artemin) or containing (+artemin). was terminated by desiccation and freezing. The cysts were then incubated in sea water at room temperature and hatched nauplii were counted and removed. The results are given as the percentage of cysts that hatched with hatching an indicator of diapause termination and cyst viability. Error bars indicate standard error.
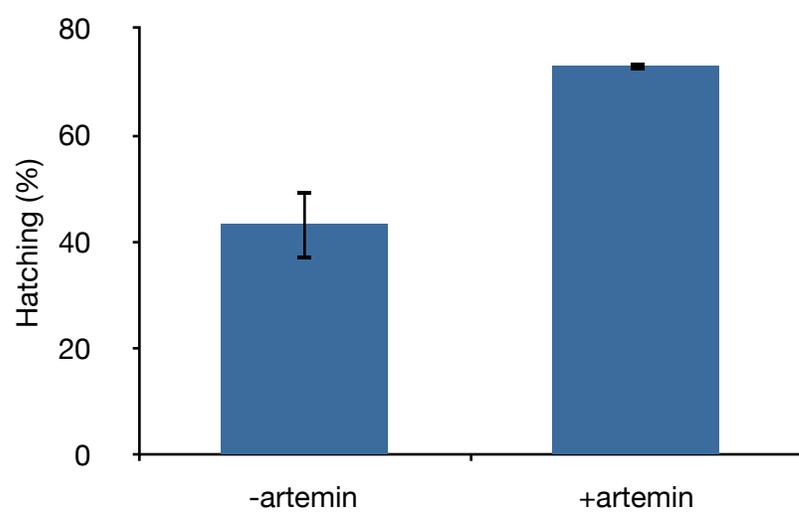
temperature, then flushed into hexagonal weigh boats, the cysts lacking artemin did not hatch spontaneously as occurred for cysts lacking p26 (not shown).

3.6 Knock down of the transcription cofactor p8

3.6.1 Pooled p8 siRNAs knocked down p8 mRNA

As determined by RT-PCR the injection of *Artemia* females with pooled siRNAs completely knocked down p8 mRNA (Fig. 19A). RNA preparations from cysts that received control solution (1) or pooled siRNAs for p8 (2) were subjected to RT-PCR. In lane 3 reverse transcriptase was omitted from the amplification reaction showing no genomic RNA contamination. siRNAs specific to p8 are effective in knocking down p8 mRNA.

3.6.2 p8 does not regulate the synthesis of diapause-specific sHSPs

Cysts from females receiving pooled siRNAs or control solution were homogenized, resolved in a 12.5% SDS polyacrylamide gels and transferred to nitrocellulose. Immunoprobing of membranes with antibodies demonstrated that knocking down p8 had no effect on the amount of p26, ArHsp21, ArHsp22, or artemin in *Artemia* cysts (Fig. 19B).

Fig. 19. Molecular chaperones are unaffected by knock down of p8 in cysts. Panel A. Amplification of p8 mRNA was by RT-PCR using RNA from cysts released by females injected with either control solution (2) or p8 siRNA (3) and Lane 1, Generuler 1 kb DNA size markers. Electrophoresis was in 1.2% agarose and gels were stained with Sybersafe®. Panel B, protein extracts from 91 cysts produced by females injected with either pooled siRNAs specific to p8 (1) or control solution (2) were resolved in 12.5% SDS polyacrylamide gels and blotted to nitrocellulose. The blots were reacted with antibody specific to p8 (p8), p26 (26), ArHsp21 (21), ArHsp22 (22) and artemin (A) followed by HRP-conjugated goat anti-rabbit IgG antibody. Proteins were visualized by chemiluminescence.
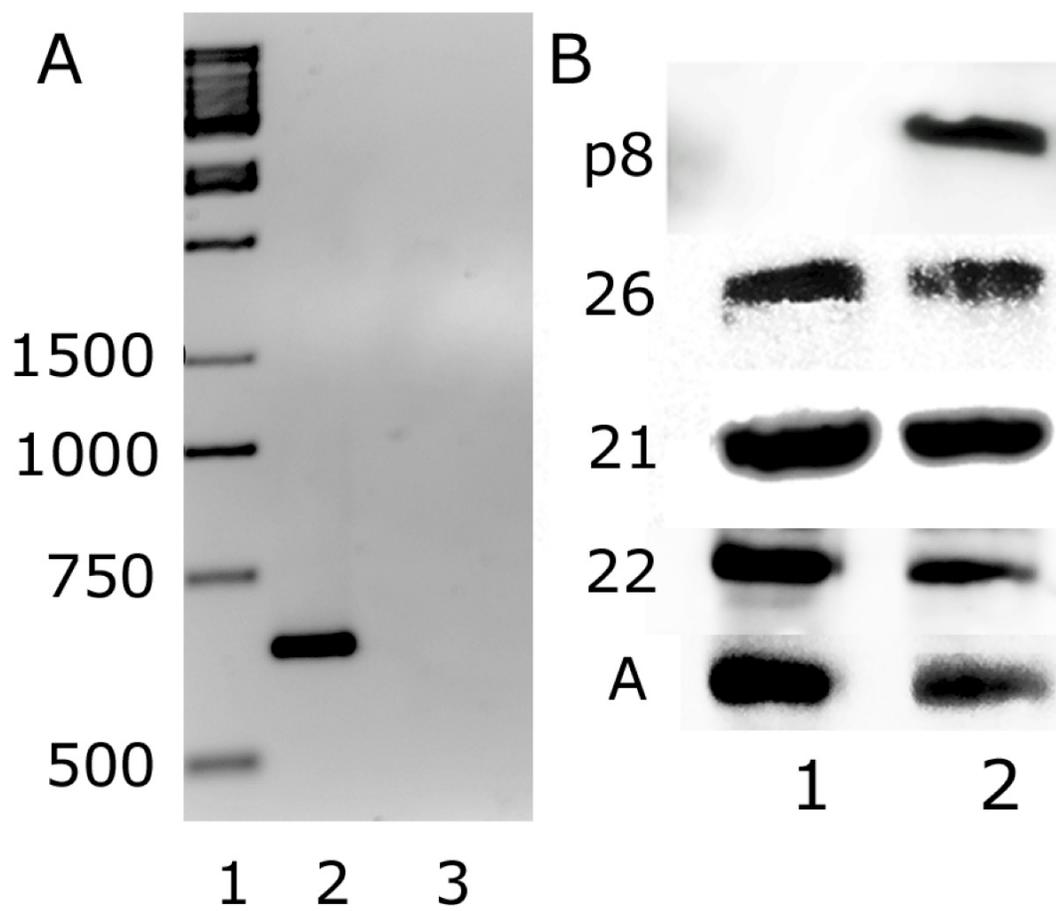
3.6.3 Cysts lacking p8 exhibit reduced stress tolerance

After diapause termination by desiccation and freezing, 76% and 44% respectively of cysts containing and lacking p8 hatched (Fig. 20). The resulting nauplii grew to sexual maturity and reproduced normally.

3.6.4 Cysts lacking p8 are capable of spontaneous diapause termination at the time of release

Cysts lacking p8 occasionally hatched within 36 h of release from females. Hatching occurred in 8% of broods released and approximately 50% of cysts in those broods hatched (Fig. 21). The resulting nauplii did not show any morphological abnormalities, grew to sexual maturity and reproduced normally.

3.6.5 Cysts lacking p8 did not spontaneously break diapause after incubation in seawater for 90 days

Cysts released from females injected with pooled siRNAs specific to p8 were incubated in seawater in 15 ml conical centrifuge tubes for at least 90 days at room temperature. When flushed into hexagonal weigh boats the cysts lacking p8 did not hatch spontaneously as occurred for cysts lacking p26 (not shown).
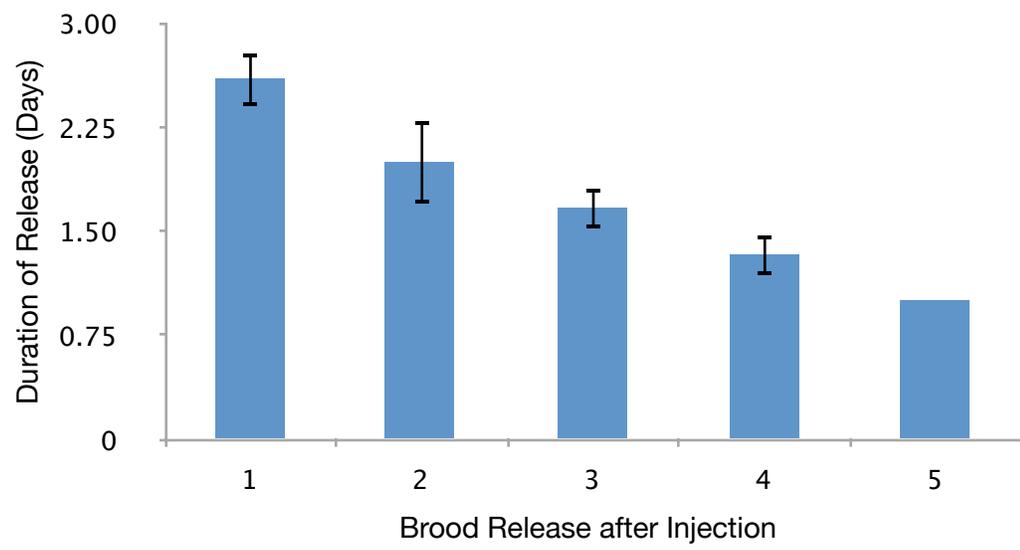
Fig. 20. The elimination of p8 significantly reduced the stress tolerance of *Artemia* cysts. Diapause of *Artemia* cysts either lacking (-p8) or containing (+p8) p8 was terminated by desiccation and freezing. The cysts were then incubated in sea water at room temperature and hatched nauplii were counted and removed. The results are given as the percentage of cysts that hatched with hatching an indicator of diapause termination and cyst viability. Error bars indicate standard error.
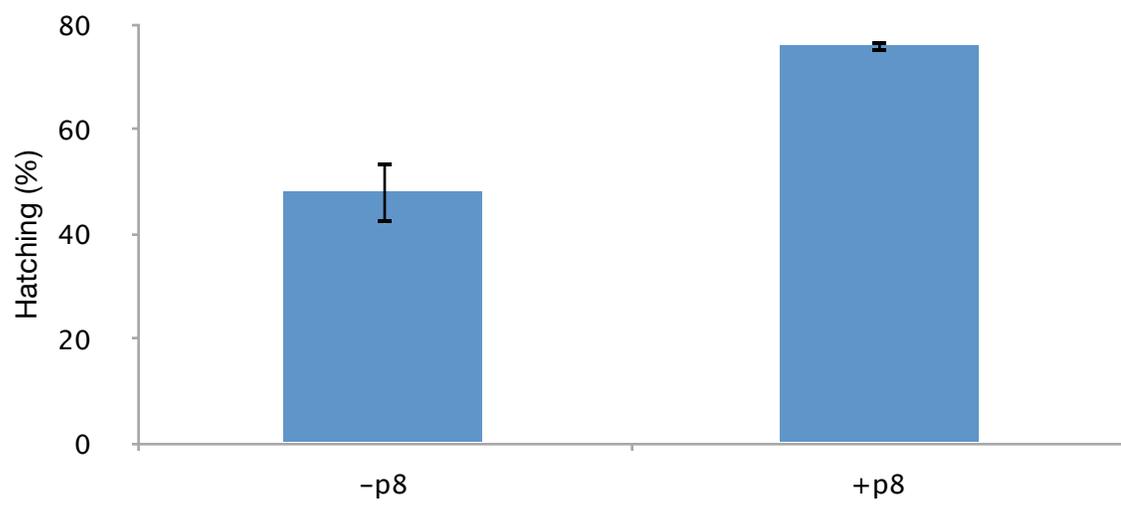
Fig. 21. Cysts lacking p8 occasionally hatch upon release from females. Panel A, after incubation at room temperature for thirty six hours after release of cysts from the female that lack p8, nauplii were visible (red arrows) as were unhatched cysts (blue arrows). Fecal matter is green in color. Panel B. Cysts that lack p8 were imaged as described below were imaged at a higher magnification. Shown is an unhatched cyst (FS), a shell from a hatched nauplius (ES) and an emerged nauplius (N) are visible. Light micrographs were obtained with an Olympus SZ61 microscope and infinity 1 lumenera camera.
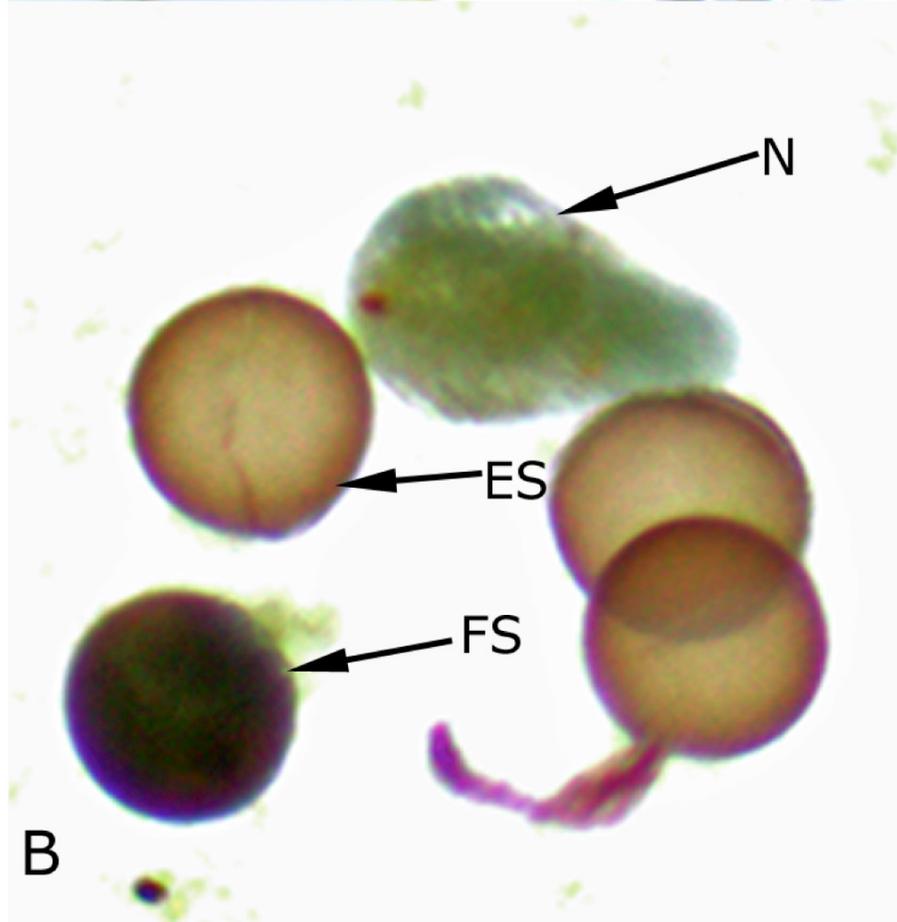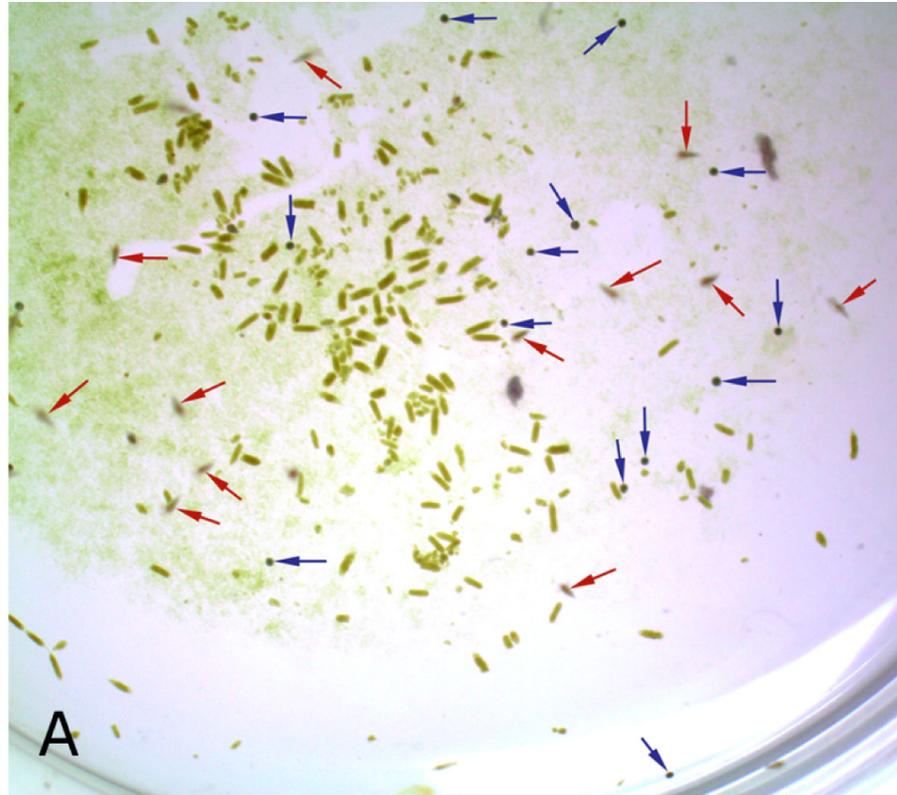
Chapter 4: Discussion

4.1 RNAi permits the study of *Artemia* proteins *in vivo*

4.1.1 Injection of adult females with dsRNA and siRNA knocked down targeted proteins in *Artemia* embryos

RNAi was used to elucidate protein function in *Artemia* embryos and cysts by examining morphological, developmental and molecular changes that occurred upon elimination of specific proteins. Protein function is often studied *in vitro* (Wu and MacRae, 2010) in transfected mammalian cells. While elucidating protein function in transfected cells has produced important functional information it is generally more informative to determine a protein's function *in vivo.* A protein in transfected cells may show a function different from that initially determined by other procedures or a function may be overlooked.   For example this study confirmed roles for two proteins, p26 and artemin in stress resistance as shown in *in vitro* studies and also revealed developmental roles for these proteins.

RNAi has been employed in *Artemia* to show caudal gene function in axis elongation and segmentation (Copf et al., 2004) and the repression of Hox genes (Copf et al., 2006). A role for p90 ribosomal S6 kinase in the termination of cell cycle arrest as post-diapause *Artemia* embryos resume development was indicated by the use of RNAi (Dai et al, 2008), as is the requirement for shell gland-specifically expressed genes SGEG1 (previously SGEG) and SGEG2 as integral components of stress resistance attributed to the cyst shell (Liu et al., 2009; Dai et al., 2011). To the best of my knowledge this is the first time that the

injection of females with dsRNA and siRNA has been demonstrated to knock down proteins in embryos of *Artemia*.

The techniques employed in the study knock down mRNAs and the corresponding proteins in embryos in a very specific manner which allows the study of protein function in *Artemia* embryos undergoing diapause. Once developed, the technique proved relatively easy to employ and sufficient amounts of experimental material were obtained for analyses. The RNAi effect in *Artemia* is long lasting knocking down p26 in embryos for at least four broods from the same female, which facilitates collection of experimental materials. However, the RNAi effect does not carry over into subsequent generations.

Injection of the females is monitored by the addition of phenol red to the inoculum which allows visualization of entry and retention of injected material. The appearance of the female after injection indicates the quality of the injection. A successfully injected animal is red, swims with a straight tail and no phenol red appears in the culture medium for at least two h. Females not injected successfully may swim abnormally and/or they exhibit bent tails two hours post-injection. Alternatively the injection solution may not be retained and animals appear normally colored after injection and the sea water in which they are swimming becomes reddish. The success of injection depends largely on the quality of the needle which requires breakage at an angle of 45° and in such a way that the end is very thin. Additionally, the bore of the opening must be large enough to draw in sufficient solution when filling the needle.

dsRNA specific to GFP as well as sham injections were used as controls to examine the effects of dsRNA introduction and injection (Dai et al., 2011). Generally 80% of females survive the injection procedure for dsRNAs. It was not possible to generate dsRNA for p8 so custom designed siRNAs were used. This method was much less labour intensive but was considerably more expensive than generation of dsRNA. p8 was knocked down using siRNAs showing that siRNAs are effective in *Artemia*. Clearly, using siRNAs is a practical approach for knocking down proteins for which dsRNAs cannot be produced. Generating dsRNA requires gene specific primers with T7 promoters attached which requires very strong primer interaction with cDNA templates. When the gene sequence is short or lacks sites for primer binding it may be prudent to use siRNA instead. Generating dsRNA takes two days plus lag time on primer design while custom designed siRNA takes about two weeks.

4.2 p26 has multiple functions in *Artemia* embryos

4.2.1 Elimination of p26 increases the time between fertilization and release of *Artemia* embryos

The increase in time to release of cysts from females injected with p26 dsRNA versus control solution and dsRNA for GFP revealed that the loss of p26 slowed the development of diapause-destined embryos or, in other words, p26 is required to attain the maximum rate of development of diapause-destined embryos. How this occurs has yet to be determined. Moreover, these data imply

that p26 does not arrest DNA replication and the cell cycle during diapause, as suggested for sHSPs in other organisms where their accumulation correlates with reduced growth (Yocum et al., 1998; Clegg et al. 1999; Li et al., 2007). That another sHSP such as ArHsp21 or ArHsp22 (Qiu and MacRae, 2008a, b) impedes DNA replication and/or cell division in diapause-destined *Artemia* embryos is possible.

4.2.2 Cysts lacking p26 terminate diapause spontaneously

The absence of p26 allows for spontaneous diapause termination in cysts after long term incubation in sea water at room temperature. The molecular mechanism explaining this result may originate in the ability of p26, as a molecular chaperone, to sequester signaling proteins critical to diapause termination or to counter the effects of reactive oxygen species (ROS) (Robbins et al., 2010; MacRae, 2010). ROS often play a role in signaling and may be involved in the signaling pathways of diapause in some species (Jovanović-Galović et al., 2004). If p26 is not present these signaling molecules may not be sequestered and they are available to provide the cue necessary to terminate diapause.

 The bottom layers of conical tubes in which the cysts were stored likely became anoxic over three months and when the cysts were transferred to hatch medium oxygen became available and the primed cysts developed. It is possible that cysts lacking p26 are unable to maintain diapause which would result in

termination. When p26, an abundant molecular chaperone in diapause cysts is absent proteins normally bound to p26 would be free in the cells and thus functional. It is possible that another molecular chaperone sequesters proteins normally held by p26 but that does not appear to be the case in *Artemia* cysts as knocking down other chaperones did not cause spontaneous diapause termination. The absence of p26 potentially leads to disruption of diapause maintenance which allows cysts to hatch spontaneously.

Termination is often not possible for a period of time in organisms that exhibit diapause (Roach and Adkisson, 1971: Tauber and Tauber, 1976). Data suggest that *A. franciscana* cannot hatch for a duration after entering diapause (Robbins, unpublished data)*.* Diapause termination by H_2O_2 increases after the cysts are released from females and peaks at three months post release (Robbins, unpublished data). Cysts hatch spontaneously upon transfer to weigh boats after three months and not at the time of release when stored under the same conditions. The spontaneous hatching seen when p26 is knocked down indicates that cyst hatching may not be possible for a period of time after entering diapause and that p26 has a role in preventing diapause termination.

Damage by ROS during diapause is a common issue in many species. The mitochondria in Killifish *Austrofundulus limnaeus* are not primed to make ATP but rather to minimize the proton motive force, likely to avoid damage by ROS (Duerr and Podrabsky, 2010). The caterpillar *Chlosyne lacinia* controls ROS by maintaining 6-phosphate dehydrogenase activity throughout diapause (Moreira et al., 2012). The antioxidants catalase and sod-2 aid in survival during

diapause of the mosquito *Culex pipiens* (Sim and Denlinger, 2011). The three examples discussed are diverse and indicate that ROS are a widespread challenge across species during diapause. ROS may also have a role in diapause termination in *A. franciscana* (Robbins et al., 2010).

The ability to interrupt diapause has practical implications. Many pest species use the enhanced stress resistance diapause confers to escape harsh conditions which would otherwise be fatal during normal growth and development. Diapause termination must coincide with environmental conditions favorable for growth and development if the emerging organism is to survive. These observations suggest that it is possible to use RNAi to cause early emergence from diapause in pest species subjecting them to potentially fatal conditions as a means of management. While at an early stage it is possible that directing RNAi targeting sHSPs may prove effective in pest management. T h i s study introduced dsRNA by microinjection which would not be feasible in pest management. Other studies have introduced dsRNA to organisms in more practical ways. dsRNA can be introduced by feeding in the Apple moth, *Epiphyas postvittana* (Turner et al., 2006) and so could possibly be administered to pest insects orally in feed containing dsRNA. dsRNA can also be administered by soaking in *C. elegans* (Maeda et al., 2001). In aquatic environments dsRNA could be introduced by addition directly to the water. More work remains to be done on employing dsRNA, possibly directed at small heat shock proteins, in the field but this study suggests that it may be an effective strategy for pest management.

4.2.3 p26 contributes to stress resistance

As evidence that p26 has a role in stress resistance only 6% of cysts lacking p26 hatch after diapause breakage by desiccation and freezing, both very stressful conditions, compared with 58% of cysts from females receiving control solution and therefore containing p26. Cysts lacking p26 and which hatched spontaneously were more sensitive to heat shock than cysts containing p26 demonstrating that p26 has a role in stress resistance. p26 confers stress tolerance on bacteria (Liang MacRae, 1999) and mammalian cells (Wu and MacRae, 2010), but this is the first time p26 dependent stress tolerance has been demonstrated *in vivo*.

These findings indicate that during diapause, p26 may protect proteins from irreversible denaturation. sHSPs are promiscuous with one sHSP oligomer typically binding many different proteins and preventing their irreversible denaturation, thus accounting for their protective effects during stress (Picard, 2002). sHSPs interact with microfilaments, intermediate filaments (Lavoie et al., 1993) and microtubules suggesting that p26 may protect the structural proteins of these filaments and enhance stress resistance (Liang and MacRae, 1997). sHSPs usually experience a temperature dependent increase in abundance and surface hydrophobicity, either by disassembly or structural rearrangement of oligomers (Bagnéris et al., 2009) which is thought to promote interactions with partially denatured proteins (Van Montfort et al., 2001). The N-terminal and α-

crystallin domains of sHSP monomers are primarily responsible for binding of denaturing proteins (Lee et al., 1997; Stromer et al., 2004; Ghosh et al., 2005, 2006). It is likely that p26 protects proteins during diapause in the manner just described. The stresses associated with diapause necessitate the protection of many different proteins. Thus, association with sHSPs, like p26, provides a way to store proteins and prevent irreversible damage of proteins, some of which are necessary for resuming growth once post-diapause development resumes, while interaction with cytoskeletal elements may preserve cell structure and shape. In addition, p26 inhibits apoptosis (Villeneuve et al., 2006;) and may protect cells by preventing death.

It has long been hypothesized that p26 contributes to stress tolerance during diapause. Cysts are most stress resistant during the maintenance phase of diapause and it was thought that p26 was crucial in providing stress resistance during this phase.   While confirming the role in stress tolerance, this study revealed novel roles for p26 in development of embryos and possibly in maintaining or preventing the termination of diapause. This finding illustrates the value of *in vivo* procedures for studying protein function revealing as it did that p26 has multiple roles in diapausing cysts as is possible for sHSPs up regulated during diapause in other species. For example, a 23 kD sHSP is up regulated during the diapause of *S. crassipalpis* (Li et al., 2007; Yocum et al., 1998) and may have multiple roles during diapause. The corn steam borer *S. nonagrioides* differentially regulates a 20.8 kD sHSPs during diapause, down regulating it during deep diapause but up regulating it upon termination (Gkouvitsas et al.,

2008).   sHSPs differentially regulated during diapause might share developmental roles or fulfill diverse roles in different species.

4.3 ArHsp21 is non-essential during *Artemia* diapause

4.3.1 Embryos lacking ArHsp21 hatch normally after desiccation and freezing

ArHsp21 is one of the three known sHSPs up regulated during the development of diapause-destined *Artemia* embryos, and it is present in lower amounts than p26 (manuscript in preparation). The message is detectable at two days post fertilization in embryos entering diapause and those undergoing direct development, though to a much lesser extent in the latter (Qiu and MacRae, 2008a). ArHsp21 protein is first detectable at four days post fertilization and only in diapause-destined embryos.   Like p26, ArHsp21 is not heat inducible in larval and adult *Artemia* (Qiu and MacRae, 2008a).

The numbers of cysts either containing or lacking ArHsp21 that hatched when diapause was broken by desiccation and freezing were statistically different but only varied by 7% suggesting that ArHsp21 contributes minimally, at best, to stress tolerance during diapause. This conclusion is further supported by the statistically similar response of cysts containing ArHsp21 and those that did not to thermal stress.   No other effects on cysts and females were noted when ArHsp21 was knocked down suggesting that the protein plays only a minor role in stress tolerance, if any, in diapause and/or that its function(s) are assumed by another sHSP when it is eliminated.

Many organisms up regulate multiple HSPs during diapause and it has been theorized that these proteins confer stress resistance during diapause (Yocum et al., 1998, MacRae, 2010). The results with ArHsp21 suggest that this is not always the case as ArHsp21 aids in stress resistance minimally. sHSPs and molecular chaperones in other species up regulated during diapause may have principal roles other than stress resistance as hypothesized, such as the developmental role revealed for p26.

4.4 ArHsp22 may protect *Artemia* adults from stress.

4.4.1 Injection with dsRNA specific to ArHsp22 kills adult *Artemia*

ArHsp22 is expressed in diapause-destined embryos with the mRNA first detectable at three days post fertilization and the protein at four days post fertilization. Neither the message nor the protein is detectable in embryos undergoing direct development into nauplii (Qiu and MacRae, 2008a). Furthermore, ArHsp22 is the only known heat inducible sHSP in *Artemia* first appearing two hours after heat shock of adult*s*. These data indicate that ArHsp22 functions in both embryos and adults of *Artemia*.

Unlike other dsRNAs employed herein, ArHsp22 dsRNA is lethal to male and female adult *Artemia*. It is possible that either microinjection and/or handling constitute stresses that induce ArHsp22 synthesis. Consequently, if ArHsp22 dsRNA is injected into adults ArHsp22 mRNA produced in response to stress is destroyed and ArHsp22 is not produced, thus lowering stress tolerance. Though

the handling stress is not readily quantifiable adults are partially dried and placed on a cold agar plate for approximately 5 min during injection, perhaps sufficient to elicit a stress response. The injection itself may be sufficiently stressful to cause a classical heat shock response. This is to my knowledge the first work to suggest that injection leads to a stress response and that it is a factor to be considered when injecting animals with dsRNAs specific to HSPs.

That injection constitutes a stress was examined by determining the amount of ArHsp22 by immunoprobing of western blots in cell free extracts of adult males four hours after injection. This result must be further investigated especially as it is known that stressors other than heat illicit a classical heat shock response. These stresses include cold in *Drosophilla* (Colinet et al., 2010), desiccation in snails (Mizrahi et al., 2010) and handling in sharks (Heberer et al., 2010). When RNAi was used in *Drosophilla* to knock down either Hsp22 or Hsp23 flies were significantly less likely to survive being held at 0°C for 12 hours. This suggests that both of these stress inducible proteins function in stress resistance to harsh environmental conditions (Colinet et al., 2010).

In other species a heat shock response is elicited from seemingly everyday occurrences when stress is seemingly absent. For example in the mosquito *Aedes aegypti* simply ingesting hot blood increases production of Hsp70. When RNAi is used to target Hsp70 and the mosquito is fed hot blood digestion of the meal is impaired and subsequent egg production is lowered (Benoit et al., 2011). Up regulation of HSPs like ArHsp22 in *A. franciscana* may

not be a part of a stress response but rather part of a standard organismal function.

4.5 Artemin knockdown disrupts the normal behavior of *Artemia* embryos and cysts

4.5.1 Artemin contributes to stress tolerance in *Artemia*

Artemin is an abundant soluble protein in the cells of diapause destined embryos and is not present in embryos that undergo direct development (Chen et al., 2003), indicating a role in diapause. After diapause breakage by desiccation and freezing cysts lacking artemin hatched in lower amounts than those containing artemin, suggesting that this protein has a role in stress resistance during diapause. As cyst survival is better than when p26 is eliminated the results indicate *Artemia* has a lesser role in stress resistance than does p26 even though they function equally in *in vitro* assays (Chen et al., 2007; Sun et al., 2005). In concert with the analysis of ArHsp21, the data on p26 and artemin indicate the value of *in vivo* experiments to determine the relative importance of molecular chaperones in stress tolerance. Moreover, these data suggest that p26, ArHsp21 and artemin work in concert to protect cells from stress, possibly by binding partially denatured proteins. Artemin may back up p26 or chaperone a different set of proteins that are less critical for surviving stress.

Artemin may protect cells against oxidative damage (Chen et al., 2003) thereby preventing cell death during diapause. Or, stated in another way,

decreased hatching in cysts that do not contain artemin may result because cells are irrevocably damaged by reactive oxygen species during diapause. Hatching in cysts that do not contain artemin is reduced by approximately half compared to those containing the protein, whereas knock down of p26 almost eliminates hatching after stress exposure. The differences in hatching between cysts that did not contain artemin and those that did not contain p26 after freezing and desiccation suggests that hatching is affected in each case in a different way. It is also possible that cysts that do not contain artemin are damaged over time by the buildup of ROS and unable to hatch.

4.5.2 Artemin lengthens the time for cyst release from females

A brood of cysts is normally completely released from the female in a matter of hours, however when artemin is eliminated release extends over days, with cysts being released each day. As indicated by the error bars in Fig. 16 there is a significant difference and clear downward trend in release time between successive broods. The knockdown of artemin was not as complete as for the other proteins with some protein still detectable on western blots. The shortening of release time over successive broods likely indicates that the RNAi effect is wearing off. It is possible that the cysts are maturing at different rates and are released when they are matured. Typically cysts mature in the female over five days with the shell appearing on the third or fourth day (Liang and MacRae, 1999). Developing cysts not containing artemin look no different than

those with artemin and shells are deposited on the expected day. The staggered release data indicate that the loss of artemin delays embryo development in a non-uniform way with cysts possibly released as they mature.

While the release time is extended, the initiation of embryo release is not delayed as seen with p26. The beginning of cyst release is typically on the fifth day which is normal for cysts and p-values do not show a significant delay as is the case with cysts lacking p26. Previous studies of *Artemia* have not suggested developmental roles for molecular chaperones in diapause nor does this appear to be common for other species, although it has been little investigated. Artemin binds non-polyadenlated RNA (Warner et al., 2004) and it is possible that RNA usually bound to artemin is free in the cytosol when artemin is absent. If artemin is not present this RNA may interfere with translation decreasing the amounts and types of proteins usually present during diapause. When artemin is absent it is possible that regulatory RNAs are not sequestered and are destroyed during diapause.   These regulatory RNAs may have a role in either interrupting metabolism or in co-ordinating the return to normal growth and development. The absence of artemin may cause differential delay of cyst maturation, depending on which proteins are missing from embryos, but the molecular mechanism by which artemin extends release time has yet to be elucidated.

4.6 The role of the transcription cofactor p8 in *Artemia* development

4.6.1 p8 does not regulate the expression of sHSPs or artemin

p8 is a stress induced transcription co-factor (Encinar et al., 2001) that shows peak expression in diapause-destined *Artemia* embryos two days after fertilization (Qiu and MacRae, 2007) while the accumulation of up regulated diapause-specific molecular chaperones peaks four days after fertilization (Liang and MacRae, 1999; Qiu and MacRae, 2008 a, b; Qiu et al., 2006). Based on the early expression of p8 it was hypothesized that this transcription cofactor controls the diapause cascade of molecular chaperone expression seen later in diapause initiation. When p8 is knocked down immunoprobing of western blots reveals that p26, ArHsp21, ArHsp22 and artemin are synthesized equally when compared to cysts that contain p8. While p8 does not regulate the molecular chaperones tested in this study it may activate or repress the expression of other proteins. p8 potentially down regulates proteins that function in growth and development or up regulates proteins that aid in cell cycle arrest (Carracedo et al., 2006). When p8 is knocked down in mammalian cells there is an increase in basal autophagy markers and a decrease in cell viability (Kong et al., 2010). It is possible that p8 participates in a pathway that prevents destructive autophagy during diapause in *A. franciscana.* preventing cell death.

4.6.2 p8 may differentially regulate proteins during diapause

The elimination of p8 from *Artemia* cysts reduces hatching by about 50% when diapause is terminated by desiccation and freezing. p8 thus has a role in stress tolerance and since it is a transcription co-factor it may affect the

expression of one or more other proteins yet to be identified. For example, p8 may aid or prevent the transcription of genes during diapause which leads to a decrease in hatching as indicated by decreased stress tolerance. If p8 turns on genes that lead to cell cycle arrest, then when it is absent these genes are not activated and the cell cycle does not shut down, the cells remain active and may be damaged by the desiccation and freezing required for diapause termination. If cell cycle arrest is not occurring this explains why cysts hatch spontaneously after release from females, which happens occasionally. Alternatively p8 may be inhibiting the expression of genes that function in growth and development or metabolism.   If these genes are not turned off cells are not able to attain the shutdown necessary for diapause which could lead to decreased hatching. In future studies it will be interesting to use either selective subtractive hybridization or proteomics to examine the differences in gene expression/protein synthesis of cysts that do and do not contain p8.

Broods of cysts that do not contain p8 occasionally hatch 24 – 36 h after release from the female. Commercially available cysts typically hatch in 24 - 36 hours after being hydrated. Hatching upon release from females shows that at least some cysts lacking p8 are capable of spontaneous diapause termination or they never enter diapause.   The spontaneous termination seen when p8 is knocked down is different from that seen when p26 is eliminated because it occurs immediately upon release from the female possibly indicating that the initiation or maintenance phase is interrupted. In contrast, cysts that lack p26 do not hatch when released from the female but rather after being incubated in sea

water for three months.   Further indicating that p8 does not have a role similar to p26 in diapause termination is that cysts not hatching upon release from the female do not hatch after incubation in sea water for three months.   This indicates that the molecular mechanism that leads to spontaneous hatching when p8 is knocked down is different from that for p26 knockdown.

p8 is quickly up regulated in response to stress in the amphioxus (*Branchiostoma belcheri tsingtaunese*) when in a starved state (Liu et al., 2009). p8 is also strongly up regulated in the human pancreas after injury and notably during the acute phase of pancreatitis (Mallo et al., 1997). The up regulation of p8 in response to starvation and injury implies a role in stress resistance for the protein in other species beyond *A. franciscana*.

4.7 Conclusions

RNAi can be used in *Artemia* to study protein function *in vivo* in embryos. For example when p26 is knocked down embryos are less stress resistant and the time between fertilization and release is significantly extended showing a role for this sHSP in development. Moreover, cysts that do not contain p26 terminate diapause spontaneously suggesting another developmental role for p26.

Females injected with dsRNA specific to ArHsp21, as well as cysts released by these females were unaffected by knockdown of this chaperone. Cysts lacking ArHsp21 and experiencing the stresses of freezing and desiccation, hatched at only a slightly reduced rate but were equally heat

resistant.   When dsRNA specific to ArHsp22 was injected into females death ensued, the same fate experienced by males. This unusual result may involve disruption of the ArHsp22 stress response in adults, a proposal currently under study.

Injection of dsRNA specific to artemin resulted in staggered release of embryos within a brood, stretching the exit of a single brood over several days. Females receiving control solution released all embryos within a brood in a matter of hours.   Artemin also contributes to stress tolerance but less so than p26.

p8 is a stress-related transcription cofactor with expression highest at about two days post-fertilization, just as other diapause up-regulated proteins start to appear. siRNAs for p8 were injected into adult *Artemia* females and the protein was knocked down in embryos, but there was no effect on the accumulation of the four molecular chaperones examined in this study during embryogenesis. This suggests that p8 aids in stress resistance in a way other than inducing the synthesis of molecular chaperones.

This work demonstrates that heat shock proteins up regulated during diapause have varying roles in stress tolerance, even though they possess similar chaperone activity in *in vitro* assays.   Novel developmental effects were discovered for two molecular chaperones, p26 and artemin, but not for ArHsp21 or ArHsp22 during diapause.   For example, cysts not containing p26 hatch spontaneously suggesting that the protein has a crucial role in diapause maintenance and/or termination. Overall this work makes fundamental

contributions concerning the role of molecular chaperones in diapause. On a more applied basis the work contributes to our understanding of the role molecular chaperones play during diapause in *A. franciscana*, an important feed source in aquaculture. The research adds to the fields of medicine, forestry and agriculture, as many pest and disease vectors, such as mosquitoes and ticks, exhibit diapause. Interrupting diapause by either making it permanent or by disruption, as done in this work, could result in eliminating pesticide use to control pest species and lowering transmission rates of insect-transmitted disease.

Appendix 1: Supplemental Figures

Figure A.1. Cysts vary little in diameter. The diameters of 1426 cysts hydrated for

3 h were measured with a stereomicroscope.
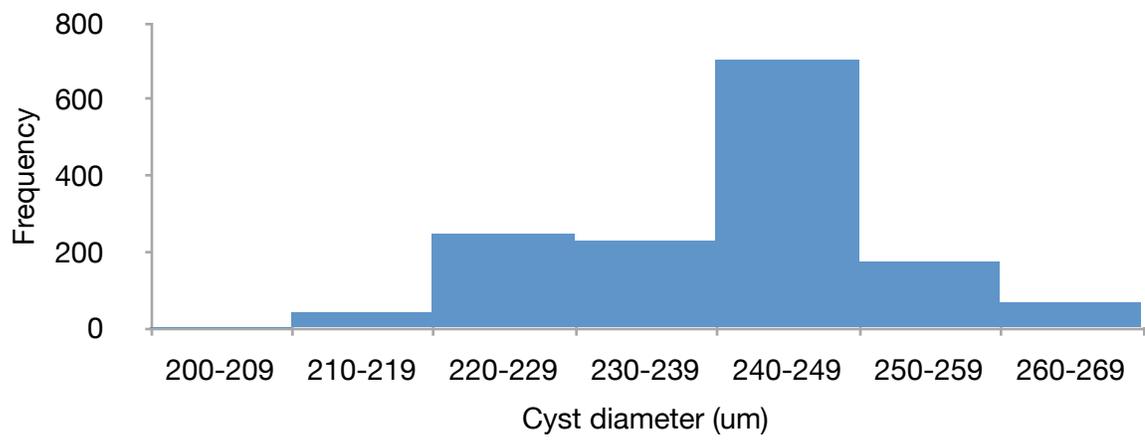
Figure A.2. Experimental time line for confirming knockdown in *A. franciscana.*

After microinjection of dsRNA specific to a particular protein females were mated

and once cysts were released they were incubated in sea water for 10 days.

After two weeks of desiccation at room temperature cysts were then transferred

to the freezer for an additional 11 to 12 weeks before knockdown was confirmed

by western blotting and RT-PCR.

Time in Days

0 1   6   16   30   105

Injection
Mating
Release
Dehydrate
Freeze
Molecular
Applications
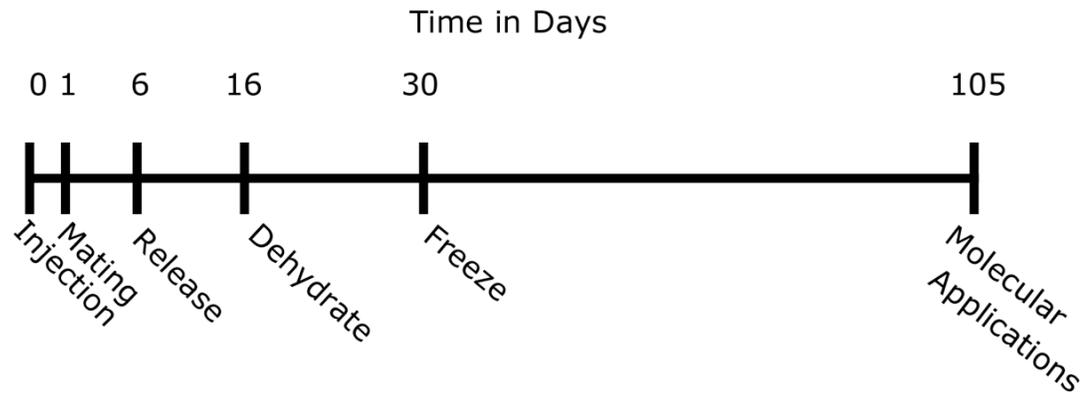
Table A.1. Genes up regulated during diapause. Several genes up regulated during diapause were identified by selective subtractive hybridization (Qiu and MacRae, 2007). Four of those identified and another species specific molecular chaperone were targeted for knockdown using RNAi.

Category	Gene Name	Function
Cellular processes	p26	sHSP, Molecular Chaperone
Cellular processes	p26-like (hsp22)	sHSP, Molecular Chaperone
Cellular processes	hsp16-like (hsp21)	sHSP, Molecular Chaperone
Environmental information processing	p8	Transcription co-factor

Appendix 2: Solutions and Recipes

Solutions for SDS-polyacrylamide gel electrophoresis

Pipes Buffer (1l)

        100 mM Pipes (30.24 g)
        1 mM MgCl$_2$ (0.31 g)
        1 mM EGTA (0.38 g)
            pH 6.5

1B   18.3 g Tris
            Ph 8.8
        2.5 ml 20% SDS stock solution
        Add ddH$_2$O to 100 ml

1C   0.1 ml TEMED
        Add ddH$_2$O to 50 ml

1D   50 mg Ammonium persulfate
        Add ddH$_2$O to 10 ml

2B   6 g Tris
            pH 6.8
        2.5 ml 20% SDS stock solution
        Add ddH$_2$O to 100 ml

2C   0.2 ml TEMED
        Add ddH$_2$O to 10 ml

SDS Stock Solution   20 g SDS
        100 ml ddH$_2$O

10% Running gels   1A (Accrylimide) 4.0 ml, 1B 4.0 ml, 1C 2.0 ml, ddH$_2$O 4.0 ml and 1D 2.0 ml

12.5% Running gels   1A (Accrylimide) 5.0 ml, 1B 4.0 ml, 1C 2.0 ml, ddH$_2$O 3.0 ml and 1D 2.0 ml

Stacking gels   1A (Accrylimide) 1.0 ml, 2B 2.5 ml, 2C 1.25 ml, ddH$_2$O 4.0 ml and 1D 1.25ml

4X Treatment buffer (20 ml)	0.6 g Tris 1.6 g SDS 8.0 ml Glycerol 4.0 ml 2-mercaptoethanol 0.2% Bromophenol blue pH 6.8
Running buffer (4l)	12.0 g Tris 57.6 g Glycine 8.0 ml 20% SDS stock solution
Coomassie Blue staining solution (2l)	10 g Coomassie brilliant blue 140 ml Acetic acid 800 ml Methanol Stir one hour and filter through miracloth
Destaining solution (2l)	400 ml Methanol 140 ml Acetic acid 100 ml Glycerol

Solutions for western blotting

Blot electrode buffer (4l)	12.0 g Tris 56.7 g Glycine 800 ml Methanol
TBS buffer (1l)	1.12 g Tris 8.18 g NaCl pH 7.4
TBS-Tween buffer (1l)	Add 1.0 ml of Tween 20 to 1l of TBS buffer
HST buffer (1l)	1.21 g Tris 58.40 g NaCl pH 7.4 5.0 ml Tween 20
2% Ponceau S solution	2.0 g Ponceau S 30.0 g Trichloroacetic acid Add ddH$_2$O to 100 ml Dilute 1:9 in ddH$_2$O for staining blots
ECL Stripping buffer	100 mM 2-mercaptoethanol 2% SDS 62.5 mM Tris-HCl pH 6.7

Solutions for agarose gel electrophoresis

6X DNA loading buffer	0.25% Bromophenol blue 0.25% Xylwne cynol 15% Ficoll (type 400)
50X TBE	242 g Tris 57.1 ml Acetic acid 100 ml 0.5 M EDTA (pH 8.0) Add ddH$_2$O to 1l

Solutions for RNAi

Injection solution	100 ul dsRNA in elution solution 100 ul 0.5% phenol red in Dulbecco's phosphate buffered saline
Control Solution	100 ul elution solution 100 ul 0.5% phenol red in Dulbecco's phosphate buffered saline

Other solutions

Solution A	10 mg Leupeptin 10 mg Soybean trypsin inhibitor Dissolved in 10 ml Pipes buffer
Solution B	10 mg Pepstatin 20 mg PMSF Dissolved in 10 ml 95% ethanol

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