ArHsp40 and ArHsp40-2 contribute to stress tolerance and longevity in *Artemia franciscana*, but only ArHsp40 influences diapause entry

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Key words: Hsp40, J-domain proteins, diapause, stress tolerance, RNA interference, *Artemia franciscana*

List of abbreviations: DPSB, Dulbecco's phosphate buffered saline; dsRNA, double stranded RNA; GFP, green fluorescent protein; **GSL, Great Salt Lake;** HPD, histidine-proline-aspartic acid; miRNA, micro RNA; RNAi, RNA interference; ZBD, zinc binding domain

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Summary Statement – Two Hsp40s function differently from one another in diapause entry, stress tolerance and longevity of *Artemia franciscana*.

ABSTRACT

Embryos of the crustacean, *Artemia franciscana*, develop either ovoviviparously or oviparously, respectively yielding swimming larvae (nauplii) or encysted gastrulae (cysts). Nauplii molt several times and become adults whereas cysts enter diapause, a state of dormancy characterized by exceptionally low metabolism and high stress tolerance. Synthesis of molecular chaperones such as the J-domain proteins, ArHsp40 and ArHsp40-2 occurs during embryo development and post-diapause growth of *A. franciscana* and they influence development and stress tolerance. To further investigate J-domain protein function ArHsp40 and ArHsp40-2 were each knocked down by RNA interference. Reductions in ArHsp40 and ArHsp40-2 had no effect on adult survival, time to release of cysts and nauplii from females and first brood size. However, knockdown of both *A. franciscana* J-domain proteins reduced the longevity and heat tolerance of nauplii with the loss of ArHsp40 having a greater effect. The knockdown of ArHsp40, but not of ArHsp40-2, caused approximately 50% of cysts to abort diapause entry and hatch without exposure to an exogenous signal such as low temperature and/or desiccation. Cysts lacking ArHsp40 that entered diapause exhibited decreased stress tolerance as did cysts with reduced ArHsp40-2, the latter to a lesser degree. The longevity of nauplii hatching prematurely from cysts was less than for nauplii arising by other means. The results expand our understanding of Hsp40 function during *A. franciscana* stress tolerance and development, especially during diapause, and they provide the first example of a molecular chaperone that influences diapause entry.

INTRODUCTION

Diapause, a phylogenetically wide spread state of dormancy wherein animals gain the ability to cope with unfavourable environmental conditions, is characterized by the arrest of development, reduced metabolism and enhanced stress tolerance, these varying in intensity across species and life history stages (Denlinger, 2002; Koštál, 2006; Hahn and Denlinger, 2007; Denlinger and Armbruster, 2014; King and MacRae, 2015). Diapause is divided into phases termed initiation, maintenance and termination which may be further subdivided (Koštál, 2006; Yocum et al., 2009; Koštál et al., 2017). Diapause initiation can entail diet change, differential gene expression and protein synthesis, structural changes for tissue defense and lipid accumulation (Hayward et al., 2005; Hahn and Denlinger, 2007; MacRae, 2010; Clark et al., 2012; Tarrant et al., 2014, 2016; Romney and Podrabsky, 2017; Arezo et al., 2017). Initiation leads to maintenance where, most notably, stress tolerance and metabolic depression are maximized. Diapause termination occurs in response to environmental cues such as desiccation and temperature and to chemical agents such as hydrogen peroxide (Tachibana and Numata, 2004; Robbins et al., 2010; Terao et al., 2012). Although generally restricted to a single life history stage in each species, diapause occurs in embryos, larvae, pupae and adults of crustaceans and insects (King and MacRae, 2015).

The extremophile crustacean *Artemia franciscana* survives inhospitable habitats because it undertakes diapause as part of its life history which may follow one of two distinct developmental pathways. During ovoviviparous development *A. franciscana* fertilized eggs develop into nauplii in the female's brood sac and swimming larvae are released approximately five days post-fertilization (Liang and MacRae, 1999; MacRae, 2003). In contrast, oviparous development, also of about 5 days duration, yields embryos arrested as gastrulae and encased by a chitinous shell (Ma et al., 2013; MacRae, 2016). The encysted embryos, termed cysts, are released from females and experience severe metabolic depression as they enter diapause (Clegg et al., 1996; Clegg, 1997; Hand et al., 2013). Diapausing *A. franciscana* cysts are unusually stress tolerant, resisting temperature extremes (Clegg, 2005; King and MacRae, 2012), ultraviolet radiation (UV) (Liu et al., 2009; MacRae, 2010, 2016; Dai et al., 2011), anoxia (Clegg, 1997; Clegg et al., 2000), and desiccation (Clegg, 2005; Toxopeus et al., 2014; Janis et al., 2017). Upon diapause termination growth of embryos resumes and swimming larvae emerge from cysts to develop into adults.

Survival of *A. franciscana* cysts depends on several structural and molecular adaptations including the presence of molecular chaperones (MacRae, 2016), of which the ATP-independent small heat shock proteins (sHsps) have received the most attention (Jackson and Clegg, 1996; Liang and MacRae, 1999; Liang et al., 1997a, b; King and MacRae, 2012; King et al., 2013). ATP-dependent molecular chaperones have also been examined in *A. franciscana* and these include Hsp70 (Clegg et al., 2011; Iryani et al., 2016), a major heat shock protein (HSP) responsible for the folding of nascent proteins, promoting the assembly of protein complexes and either salvaging or destroying aberrant proteins (Mayer and Bukau, 2005; Zuiderweg et al., 2017; Żwirowski et al., 2017; Mayer, 2018). These activities depend on conformational changes in Hsp70 modulated by its ATPase activity and this is influenced by co-chaperones such as the J-domain proteins or Hsp40s (Mayer and Bukau, 2005; Craig and Marszalek, 2017; Bascos et al., 2017; Kityk et al., 2018). The J-domain proteins, characterized by a J-domain possessing a highly conserved histidine-proline-aspartic acid (HPD) motif, are divided into three main groups termed types 1, 2 and 3 or A, B and C (Craig and Marszalek, 2017). J-domain proteins deliver substrate to Hsp70, stimulate Hsp70 ATPase activity and function in the Hsp70-Hsp40-Hsp110 protein disaggregation system resulting in either the refolding or destruction of proteins (Nillegoda et al., 2015, 2017, 2018; Kityk et al., 2018).

Two J-domain proteins have been identified in *A. franciscana*, namely the type 1 ArHsp40 and the type 2 ArHsp40-2 (Jiang et al., 2016; Rowarth and MacRae, 2018). These J-domain proteins are induced by heat shock in nauplii, indicating a role in stress tolerance, and both are developmentally regulated during post-diapause development of *A. franciscana*, however their patterns of synthesis vary suggesting different functions. To further investigate J-domain proteins in *A. franciscana* ArHsp40 and ArHsp40-2 were separately knocked down in nauplii and cysts by RNA interference (RNAi). Injection with double stranded RNA (dsRNA) for either ArHsp40 or ArHsp40-2 had no apparent effect on mature females. However, nauplii lacking either J-domain protein exhibited reduced longevity and stress tolerance. Diapausing cysts with reduced amounts of J-domain proteins also had lower stress tolerance and as for nauplii the loss of ArHsp40 had a greater effect than reduction of ArHsp40-2. The knockdown of ArHsp40, but not ArHsp40-2, caused about 50% of cysts to abort diapause entry, to our knowledge the first time the loss of a molecular chaperone has been shown to have this effect.

MATERIALS AND METHODS

Culture of *A. franciscana*

A. franciscana cysts from the Great Salt Lake (GSL) (INVE Aquaculture Inc., Ogden, UT, USA) hydrated for at least 3 h on ice in distilled H_2O and collected by suction filtration were incubated at room temperature in 1 µm filtered, UV treated and autoclaved 33.0 ppt saltwater from Halifax Harbor, NS, Canada, hereafter termed seawater. Animals incubated in sea water at room temperature were fed daily with *Isochrysis galbana* from The Provasoli-Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, Maine, USA. The research described in this paper was performed in accordance with the ethical guidelines provided by the Canadian Council on Animal Care (CCAC). The University Committee on Laboratory Animals (UCLA) of Dalhousie University approved the research and assigned Protocol Number 117-36.

Injection of *A. franciscana* females with double stranded RNA (dsRNA)

To prepare dsRNA, Invitrogen™ Platinum *Taq* DNA Polymerase (Fisher Scientific, Ottawa, ON, Canada) and primers containing the T7 promoter (Table 1) were used to amplify *ArHsp40-1* (Jiang et al., 2016) and *ArHsp40-2* (Rowarth and MacRae, 2018) cDNAs by PCR. Green fluorescent protein (*GFP*) cDNA was amplified from the vector pEGFP-N1 (Clontech, Mountain View, CA, USA) (King and MacRae, 2012). The cDNAs were used as template for the generation of *ArHsp40, ArHsp40-2* and *GFP* dsRNA with the MEGAscript® RNAi kit (Ambion Applied Biosystems, Austin, TX, USA) following manufacturer's instructions. Concentrations of cDNAs and dsRNAs were determined at 260 nm and they were resolved in 1.0% agarose gels and stained with Invitrogen SYBRSafe™ gel stain (Fisher Scientific) prior to visualization in a MF-Chemi-BIS 3.2 gel documentation system (Montreal Biotech, Montreal, QC, Canada).

Mature females carrying diapause-destined or nauplius-destined unfertilized eggs were **selected from populations of animals maintained in seawater and** injected in the egg sac with either *ArHsp40, ArHsp40-2* or *GFP* dsRNA, each of which was diluted to 0.7 ng/nl with 0.5% phenol red in Dulbecco's phosphate buffered saline (DPBS) (King and MacRae, 2012). Injections were performed under an Olympus SZ61 stereomicroscope (Olympus Canada, Inc., Markham, ON, Canada) with a borosilicate micropipette pulled with a custom programmed P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Co., Novato, CA, USA) and broken at 45° using a clean razor blade. Each female was placed on 5% agar and lightly blotted with a

Kimwipe (Sigma-Aldrich, Oakville, ON, Canada) prior to injection with 175 ng of dsRNA in approximately 250 nl using the Nanoject II Microinjector (Drummond Scientific, Co., Broomall, PA, USA). Injected females were returned to sea water and observed for 3 h to ensure retention of phenol red, and thus dsRNA, and maintenance of normal behavioral characteristics (King and MacRae, 2012). Each female was mated with a male 24 h post-injection in a 6-well culture plate containing sea water. Animals were fed daily with *I. galbana*.

Knockdown of ArHsp40 and ArHsp40-2 in *A. franciscana* cysts and nauplii

To prepare RNA, 30 to 50 *A. franciscana* cysts were collected 10 d post-release from females injected with either *ArHsp40-1, ArHsp40-2, or GFP* dsRNA. Similar numbers of nauplii were collected immediately after release from females. RNA was extracted from cysts and nauplii by homogenizing flash frozen animals in 100 µl TRIzol® (Invitrogen) and cDNA was synthesized as described above. qPCR was performed in a Rotor-Gene RG-3000 system (Corbett Research, Sydney, NSW, Australia) using 0.5 µl cDNA as template (King and MacRae, 2012; King et al., 2013) and 0.4 mM primers for ArHsp40, ArHsp40-2 and α-tubulin (Table 1) under the following conditions: 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 54°C for ArHsp40 and ArHsp40-2 and 49°C for α-tubulin and 1 min at 72°C, followed by 10 min at 72°C. qPCR was conducted with a QuantiFast® SYBER® Green PCR Kit (Qiagen, Mississauga, ON, Canada). The experiment was performed in triplicate using three different preparations of cysts and nauplii and each preparation was analyzed in duplicate. cDNA copy numbers for ArHsp40 and ArHsp40-2 were determined from a standard curve of Ct values ($R^2 > 0.99$), and normalized against α-tubulin (King et al., 2013).

To prepare protein extracts, 30 to 50 cysts and nauplii were flash frozen and homogenized in 30 µl of 4 X treatment buffer (250 mM Tris, 280 mM SDS, 40% (v/v) glycerol, 20% (v/v) β-mercaptoethanol, 0.2% (w/v) bromophenol blue, pH 6.8), placed in a boiling water bath for 5 min and then centrifuged at 4°C for 10 min at 10,000g. Proteins were resolved in 12.5% SDS polyacrylamide gels, transferred to 0.2 µm nitrocellulose membranes (BioRad, Mississauga) overnight at 100 mAmps and blocked for 1 h at room temperature in 5% (w/v) Carnation low fat milk powder in TBS (10 mM Tris, 140 mM NaCl, pH 7.4). Membranes were then probed with the antibodies Anti40-type 1 (Jiang et al., 2016), Anti40-type 2 (Rowarth and MacRae, 2018) or Anti-Y (Xiang and MacRae, 1995) each diluted 1:1000 in TBS for 15 min

followed by washing in TBS-T (10 mM Tris, 140 mM NaCl, 0.1% Tween-20, pH 7.4) for 1 min, 2 min, 3 min and 4 min. Subsequent to washing, membranes were probed for 20 min with HRP-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich, Oakville, ON, Canada) diluted 1:10,000 in TBS and washed as before in TBS-T followed by TBS for 3 min. Immunoreactive proteins were visualized with Clarity™ ECL blotting substrate (Biorad, Missassauga, ON, Canada) in a MF-Chemi-BIS 3.2 gel documentation system (DNR Bio-Imaging Systems).

Female viability and embryo development after ArHsp40 and ArHsp40-2 knockdown

Females surviving successive brood releases after injection with either *ArHsp40*, *ArHsp40*-2, or *GFP* dsRNA and incubation at room temperature in sea water were counted. The number of either cysts or nauplii produced by a female after fertilization, known as the brood size, was recorded for the first release. The time from fertilization, marked by the fusion of egg sacs until the release of cysts or nauplii, was monitored at room temperature. The experiment involved 125 females of which 51 produced nauplii and 74 produced cysts.

Abortion of diapause entry by *A. franciscana* cysts deficient in ArHsp40

Cysts collected immediately after release from adult females injected with either *ArHsp40*, *ArHsp40-2* or *GFP* dsRNA were incubated at room temperature in seawater for 10 days. Emerging cysts and nauplii were counted at daily intervals. The experiment was performed 6 times with the number of animals per trial ranging from 36 to 72.

Stress tolerance of *A. franciscana* cysts and nauplii deficient in ArHsp40 and ArHsp40-2

Cysts that did not abort diapause entry were collected by centrifugation at 5000g for 1 min at room temperature 10 days after release from females injected with either *ArHsp40*, *ArHsp40-2* or *GFP* dsRNA. Seawater was removed and cysts were blotted dry prior to incubation for 4 weeks at room temperature in a desiccator containing Drierite (Sigma-Aldrich) and freezing at −20°C for 12 weeks. Cysts were then incubated in seawater at room temperature and the number of hatched nauplii, the measure of viability, was counted. Cysts were monitored for an additional 5 days after the appearance of the last nauplius to ensure no further hatching occurred. Experiments were done in triplicate with the number of animals per trial ranging from 18 to 83.

To test heat tolerance first brood nauplii collected immediately after release from females injected with either *ArHsp40*, *ArHsp40-2* or *GFP* dsRNA were incubated in 20 ml seawater in Corex tubes at 39°C for 1 h in a programmable water bath (VWR International LLC, Mississauga, ON, Canada). Heat shock was followed by recovery in sea water at room temperature for 24 h before swimming nauplii were counted. The experiment was performed in triplicate with the number of animals per trial ranging from 26 to 95.

Longevity of *A. franciscana* nauplii deficient in ArHsp40 and ArHsp40-2

Nauplii from females injected with either *ArHsp40*, *ArHsp40-2* or *GFP* dsRNA were collected immediately after release as were nauplii prematurely hatched from cysts released by females injected with *ArHsp40* dsRNA. The nauplii, incubated at room temperature in seawater, were fed daily with *I. galbana*. The number of surviving animals was recorded daily until 100% mortality was reached. The experiment was performed in triplicate with the number of animals per trial ranging from 35 to 113.

Image processing and analysis

Images of animals captured with a Nikon AZ100 microscope were prepared for publication using Photoshop (Adobe Creative Cloud; Adobe Systems Inc.). Immunoreactive proteins on western blots were quantified with Image Studio Software (Li-Core Biosciences, Lincoln, NE, USA), and band intensities for ArHsp40 and ArHsp40-2 were compared to band intensities for tyrosinated α-tubulin at each development stage examined.

Statistical Analysis

One-way ANOVA followed by a Dunnett's test was carried out to detect significant difference between means and control means. All data were plotted as means**±s.e.m**. Analyses were carried out using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Injection of *A. franciscana* females with dsRNA

dsRNAs were synthesized from *ArHsp40*, *ArHsp40-2* and *GFP* cDNAs. *ArHsp40* dsRNA was complementary to 324 bp (26.7%) of the full length *ArHsp40* mRNA and targeted the zinc binding domain (ZBD) which is lacking in ArHsp40-2. The *ArHsp40-2* dsRNA was complementary to 327 bp (30.7%) of the full length *ArHsp40-2* mRNA and targeted parts of the glycine/phenylalanine (G/F) rich region and the substrate binding region. *GFP* dsRNA is not complementary to any mRNA in the *A. franciscana* transcriptome (Zhao et al., 2012). Females destined to produce nauplii possessed green oocytes within two separated egg sacs (Fig. 1A-C) whereas females fated to produce diapause-destined embryos possessed a shell gland (Liang & MacRae, 1999). The injected dsRNA, as shown by red staining (Fig. 1D), circulated throughout the entire body. Females that exhibited normal swimming and feeding 1 day post injection were paired with a male for fertilization.

Injection of *A. franciscana* adult females with *ArHsp40* and *ArHsp40-2* dsRNA affected neither survival nor embryo development

Survival of adult *A. franciscana* females injected with either *ArHsp40*, *ArHsp40-2* or *GFP* dsRNA was similar (Fig. 2A). Regardless of the dsRNA that they received approximately 57% of females survived and released a brood of cysts or nauplii, 15% released a second brood and 5% a third brood. Post-fertilization release times from females injected with either *ArHsp40*, *ArHsp40-2* or *GFP* dsRNA for first brood cysts and nauplii was 5.0 days (Fig. 2B) and equal numbers of cysts and nauplii were released in spite of the dsRNA injected (Fig. 2C).

ArHsp40 and ArHsp40-2 were knocked down by RNAi in *A. franciscana* cysts and nauplii

ArHsp40 mRNA in first brood cysts released from females injected with *ArHsp40* dsRNA was reduced approximately 86% compared to cysts from females injected with either *ArHsp40-2* or *GFP* dsRNA (Fig. 3A). A lightly staining band of ArHsp40 was visible on western blots containing protein extracts of cysts released by females injected with *ArHsp40* dsRNA but a much stronger band was visible in extracts of cysts from females injected with either *ArHsp40-2* or *GFP* dsRNA (Fig. 3B). Scanning of western blots indicated that ArHsp40 was reduced

approximately 86% compared to cysts from females injected with either *ArHsp40-2* or *GFP* dsRNA (Fig. 3C). *ArHsp40-2* mRNA in first brood cysts released from females injected with *ArHsp40-2* dsRNA was reduced approximately 89% compared to cysts from females injected with either *ArHsp40* or *GFP* dsRNA (Fig. 3A). ArHsp40-2 in first brood cysts released from females injected with *ArHsp40-2* dsRNA was lightly stained on western blots and scanning indicated that ArHsp40-2 was reduced approximately 73% compared to cysts from females injected with either *ArHsp40* or *GFP* dsRNA (Fig. 3B, C). Similar knockdowns were observed with first brood nauplii released by females injected with either *ArHsp40* or *ArHsp40-2 dsRNA* (Fig. 4A-C).

A. franciscana cysts deficient in ArHsp40 but not ArHsp40-2 aborted diapause entry

Rather than entering diapause upon incubation in sea water, cysts deficient in ArHsp40 began hatching by 2 days post-release and after 5 days 47% of cysts had hatched and released swimming nauplii (Fig. 5 A-C). No further hatching was observed up to 10 days post-release. The resulting nauplii were morphologically normal and they reached sexual maturity. None of the cysts released from females receiving either *ArHsp40-2* or *GFP* dsRNA hatched by 10 days nor was there any hatching of these cysts after 120 days at which time observations ceased.

Knockdown of ArHsp40 and ArHsp40-2 reduced the stress tolerance of _A. franciscana_ cysts and nauplii

After release from females, knockdown cysts were incubated in sea water for 10 d at room temperature, dried, stored frozen and then incubated in seawater to determine hatching success, the indicator of viability. Fifty-seven % of cysts from females receiving *GFP* dsRNA, and thus possessing both ArHsp40 and ArHsp40-2, hatched. By comparison, 21% of cysts lacking ArHsp40 and 38% lacking ArHsp40-2 hatched (Fig. 6A). Eighty-nine % of nauplii released from *A. franciscana* females receiving *GFP* dsRNA survived after heat shock at 39 ^{o}C for 1 h and recovery for 24 h, whereas 45% and 67% of nauplii from females injected respectively with *ArHsp40* and *AHsp40-2* dsRNA were alive after recovery (Fig. 6B).

Longevity of *A. franciscana* nauplii deficient in ArHsp40 and ArHsp40-2

At 3 weeks post-release 97% of nauplii from females injected with *GFP* dsRNA survived whereas 53% and 81% of nauplii, respectively deficient in ArHsp40 and ArHsp40-2, survived (Fig. 7). Five weeks after release, when *A. franciscana* reached sexual maturity, 76% of the animals from females injected with *GFP* dsRNA were alive. In contrast, no animals lacking ArHsp40 survived for 5 weeks post-release and 53% of animals deficient in ArHsp40-2 were viable. Only 2.3% of nauplii hatching from cysts that aborted diapause survived 3 weeks and in 4 weeks all of these nauplii were dead. All animals were dead 8 weeks after release (Fig. 7).

DISCUSSION

A. franciscana females were injected with dsRNA complementary to J-domain proteins in order to study their function in active larvae as well as in diapause-destined embryos and diapausing cysts. PCR primers were designed to amplify either the ZBD of ArHsp40, which is lacking in ArHsp40-2, or the variable G/F rich-substrate binding region of ArHsp40-2. *ArHsp40* dsRNA knocked down ArHsp40 and its mRNA but had no apparent effect on ArHsp40-2, nor did injection with *ArHsp40-2* dsRNA reduce ArHsp40. Protein extracts of cysts and nauplii from females injected with *GFP* dsRNA, used as control, contained ArHsp40 and ArHsp40-2. *GFP* dsRNA was not expected to affect *A. franciscana* because the organism does not synthesize GFP (Zhao et al., 2012). RNAi was employed previously to examine sHsps, artemin and late embryonic abundant (LEA) proteins in developing embryos, cysts and nauplii of *A. franciscana* (King and MacRae, 2012; King et al., 2013, 2014; Toxopeus et al., 2014), and its use in this work was equally successful.

Injection with *ArHsp40* and *ArHsp40-2* dsRNA did not decrease the survival of adult *A. franciscana* females although in a previous study the injection of dsRNA for the sHsp ArHsp22, which is stress inducible in adults, killed females (King, 2013). The results indicate that ArHsp40 and ArHsp40-2 were not knocked down in adults by the injection of dsRNA for either protein even though they were in offspring or, if these proteins were reduced, then they were not required for the survival of adult *A. franciscana*. Additionally, the time to release and the brood size of cysts and nauplii were indifferent to *ArHsp40* and *ArHsp40-2* dsRNAs, indicating that embryo development and release are normal even though ArHsp40 and ArHsp40-2 may have been reduced or absent. This contrasts the situation for the sHsp p26, the loss of which increases

the time to release of cysts (King and MacRae, 2012) and the knockdown of artemin which increases the time for complete brood release in *A. franciscana* (King et al., 2014).

By 10 d post-release from females 47% of ArHsp40 knockdown cysts hatched yielding morphologically normal nauplii that developed into sexually mature animals, whereas no cysts with either normal amounts of ArHsp40 and ArHsp40-2 or reduced ArHsp40-2 hatched. Cysts that did not hatch had either entered diapause or died. By comparison, the knock down of the stress-induced transcription factor p8 by RNAi causes approximately 50% of *A. franciscana* cysts to hatch prematurely by 36 h after release from females (King, 2013). p8 is developmentally regulated in *Artemia* embryos and progressively accumulates in the nuclei of encysted embryos (Qiu and MacRae, 2007; Lin et al., 2016). ArHsp40 may assist in the translocation of p8 or other transcription factors into nuclei thereby promoting transcription of genes that arrest the cell cycle and depress metabolism as occurs during diapause. Conversely, transcription factors depending on ArHsp40 for transport into nuclei may inhibit expression of genes that support cell cycle progression and metabolism. Thus, in the absence of ArHsp40 cells remain active and entry into diapause is inhibited. The knockdown of ArHsp40-2 did not affect diapause entry, implying that this J-domain protein does not interact with proteins that promote diapause entry. Cysts with reduced ArHsp40 that did not hatch within the first 10 days post-release from females remained dormant for 180 d in sea water at room temperature. This result contrasts the knock down of p26 which results in almost 90% of cysts hatching spontaneously after 90 d in seawater at room temperature (King and MacRae, 2012).

Micro RNAs (miRNAs) modulate diapause in insects and crustaceans. Diapausing pupae of the flesh fly *Sarcophaga bullata* down-regulate several miRNAs, some of which shape lipid metabolism and increase the synthesis of Hsps that protect against stress (Reynolds et al., 2013, 2017). The knockdown of the miRNAs, miR-100 and miR-34 in *A. parthenogenetica* inhibits cell cycle arrest during diapause entry by down-regulating polo-like kinase 1 and activating cyclin K and RNA polymerase II (Zhao et al., 2015). In another example, the reduction of p90 ribosomal S6 kinase 2 mRNA by RNAi in *A. franciscana* and *A. parthenogenetica* embryos causes cell cycle arrest to fail resulting in the production of pseudo-diapause cysts that develop within their shells (Zhao et al., 2015; Dai et al., 2008). The molecular pathways and signaling factors that regulate diapause entry remain uncertain but J-domain proteins, among other factors, play a role in this process.

Prolonged desiccation and freezing are required to terminate diapause in *A. franciscana* cysts from GSL and this causes water loss and reactive oxygen species accumulation which leads to the denaturation and aggregation of proteins (Toxopeus et al., 2014). After desiccation and freezing approximately 20% and 40% of cysts, respectively with reduced ArHsp40 and ArHsp40-2, hatched. By comparison, 6% of cysts with reduced p26 hatch under similar conditions (King and MacRae, 2012) and 5% of cysts with reduced amounts of group 1 LEA proteins survive (Toxopeus et al., 2014). These molecular chaperones, along with ArHsp40 and ArHsp40-2, are likely to work cooperatively to promote protein homeostasis and stress tolerance during diapause. ArHsp40 and ArHsp40-2 may function as part of the Hsp110-Hsp70-Hsp40 protein disaggregase system (Nillegoda et al., 2015, 2017, 2018; Kaimal et al., 2017; Kityk et al., 2018), rescuing proteins from aggregates and refolding them which is likely to be especially important as post-diapause development occurs. The J-domain proteins may be required, in concert with Hsp70 and other molecular chaperones, to extract proteins from p26, an abundant sHsp in diapausing cysts that is thought to bind partially denatured proteins and protect them from irreversible denaturation (Liang et al., 1997a, b; Sun et al., 2006; King and MacRae, 2012). It is also possible that during diapause, ATP is limiting and J-domain proteins sequester proteins required for embryo development after diapause termination. Cysts without ArHsp40 and ArHsp40-2 may not sequester proteins as efficiently as when these proteins are present and consequently they experience higher mortality during diapause.

J-domain proteins transport substrates to Hsp70 for protection during stress which provides tolerance to heat, desiccation and oxidation (Cyr and Ramos, 2015; Glover and Lindquist, 1998). Newly released nauplii deficient in either ArHsp40 or ArHsp40-2 exhibited reduced stress tolerance as compared to nauplii from females injected with *GFP* dsRNA, with loss of ArHsp40 having a greater effect. Based on the lower survival upon knockdown, ArHsp40 may either enhance protein refolding by Hsp70 more effectively than does ArHsp40-2 or it may react with protein substrates more vital to growth.

ArHsp40 is more important for the longevity of nauplii than is ArHsp40-2, although both J-domain proteins are required for maximum longevity. Interestingly, this is apparently not true for adults because injection of dsRNA for both J-domain proteins had no effect on adult life span. Moreover, nauplii that arose from cysts upon abortion of diapause entry live shorter lives than do nauplii arising directly from females, even if the females have been injected with dsRNA

for either ArHsp40 or ArHsp40-2. Why ArHsp40 and ArHsp40-2 have divergence effects on longevity in larvae and adults and on larvae from different sources is unknown but may reflect interaction with dissimilar substrates at diverse life history stages or variation in the need for J-domain proteins for the folding of other proteins as organisms develop.

CONCLUSIONS

ArHsp40, a type 1 J-domain protein and ArHsp40-2, a type 2 J-domain protein were knocked down in *A. franciscana* cysts and nauplii by RNAi. The reduction of these J-domain proteins had no apparent effect on adult females of *A. franciscana*. Some cysts lacking ArHsp40, but not ArHsp40-2, aborted diapause entry and hatched, yielding nauplii that were morphologically normal but with reduced longevity. ArHsp40 may assist in the folding or intracellular transport of proteins required for transcription and other processes involved in diapause entry. Cysts and nauplii with reduced ArHsp40 and ArHsp40-2 exhibited lower stress tolerance, with loss of the former more damaging than loss of the latter. J-domain proteins promote protein folding and in cooperation with Hsp70 they rescue damaged proteins which may explain their role in stress tolerance. Interestingly, injection of females with *ArHsp40* and *ArHsp40-2* dsRNA did not lessen the longevity of adults but did reduce the longevity of larvae they produced with larvae arising from cysts that aborted diapause entry more severely affected than larvae arising directly from females. The results presented herein advance our appreciation of J-domain protein function during stress tolerance and demonstrate previously unrecognized roles for molecular chaperones in diapause entry and larvae longevity.

Competing Interests

The authors declare no competing interests.

Author contributions

Methodology: N.M.R., T.H.M.; Investigation: N.M.R.; Resources: T.H.M.; Writing - original draft: N.M.R.; Visualization: N.M.R., T.H.M.; Supervision: T.H.M.; Project administrator: T.H.M.; Funding acquisition: T.H.M.

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Figures

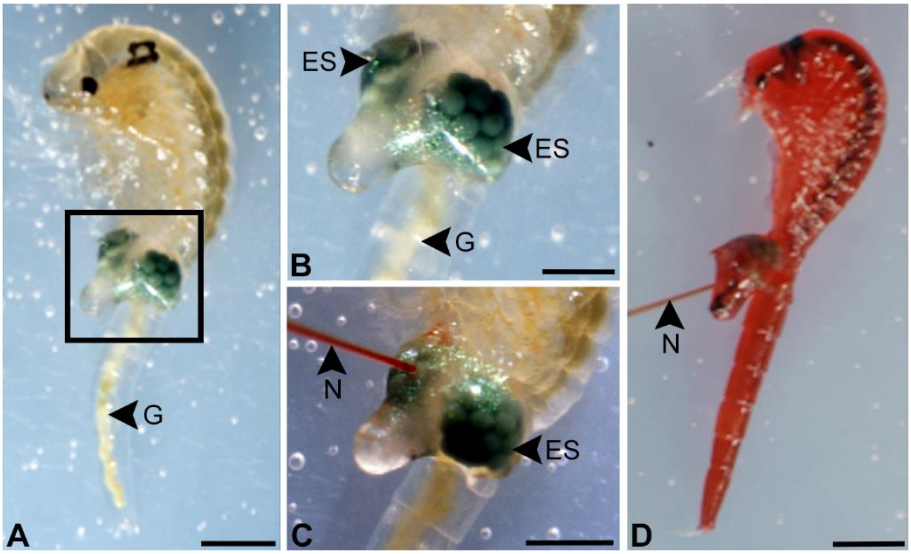

Fig. 1. Injection of *A. franciscana* females with dsRNA. A. Light micrograph of an adult *A. franciscana* female with egg sacs boxed in white. B. The boxed region was enlarged showing egg sacs with unfertilized nauplius-destined oocytes. C. Injection of an egg sac. D. A female containing phenol red. G, gut; ES, egg sac; N, needle. Scale bars, a, d, 1 mm; b, c, 0.5 mm.

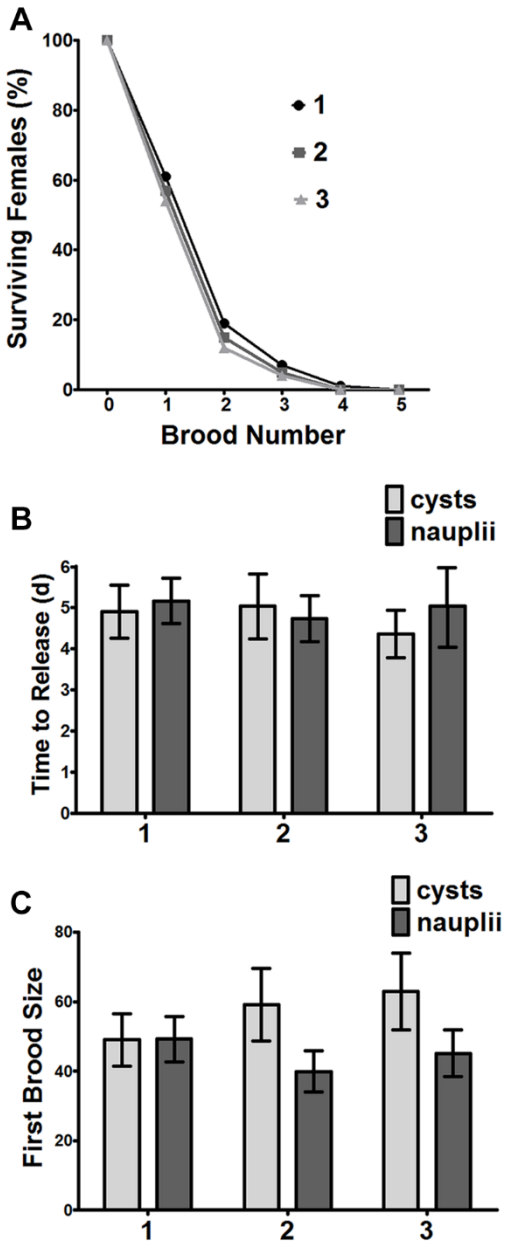

Fig. 2. dsRNAs affected neither the survival of *A. franciscana* females nor embryo development. A. Survival of *A. franciscana* females injected with *GFP* (1), *ArHsp40* (2) and *ArHsp40-2* (3) dsRNA after successive brood releases. Forty-two animals were examined for *GFP* dsRNA, 42 for *ArHsp40* and 41 for *ArHsp40-2*. B. Mean time from fertilization to release of first brood cysts and nauplii from females injected with either *GFP* (1), *ArHsp40* (2) or *ArHsp40-2* (3) dsRNA. C. Mean size of first broods (number of offspring) released by females injected with either *GFP* (1), *ArHsp40* (2) or *ArHsp40-2* (3) dsRNA**. d,** days. **One-way ANOVA, Dunnett's test,** error **means±s.e.m.** of n=12 replicates per experiment $P \geq 0.05$.

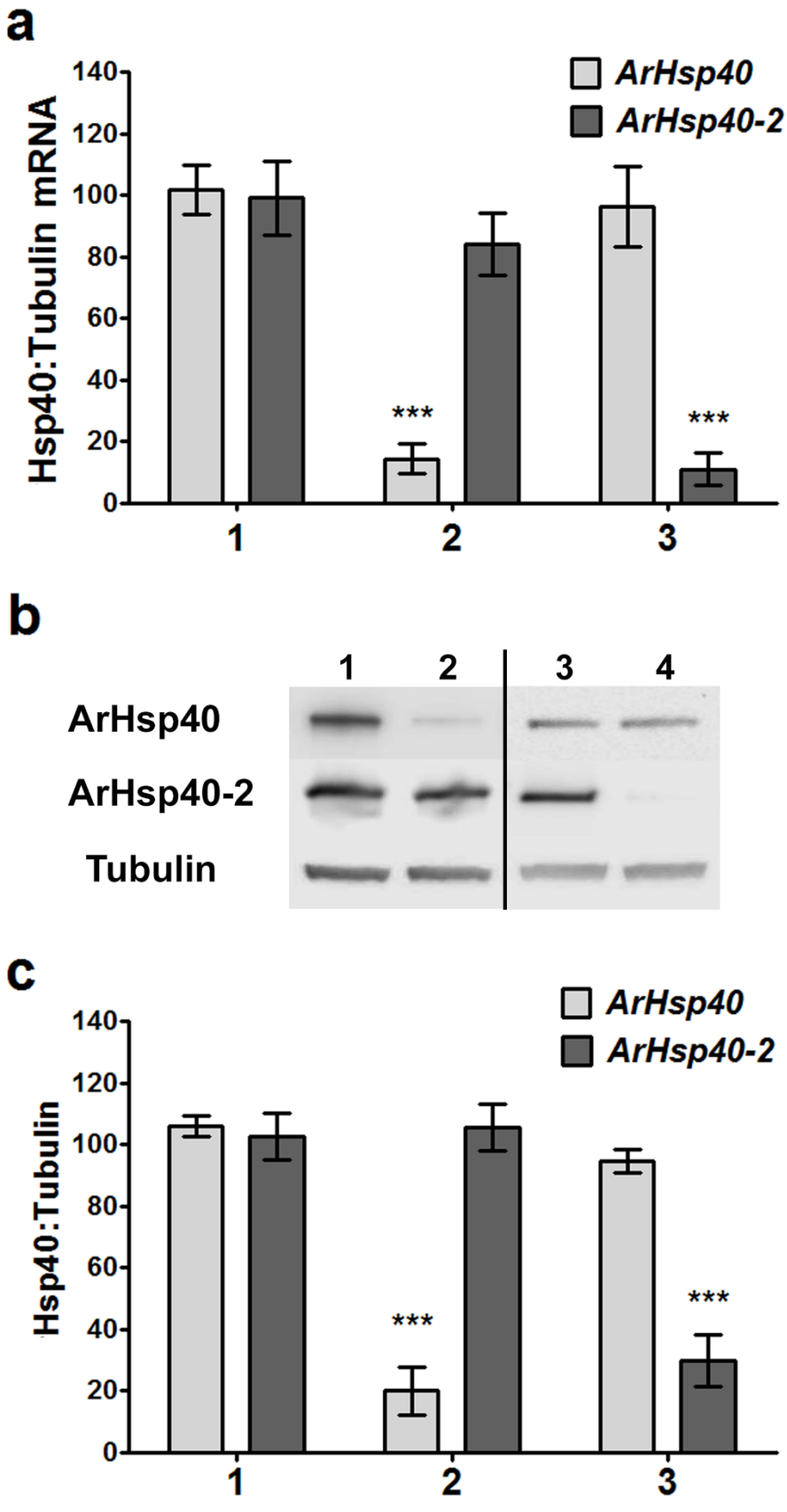

Fig. 3. Knock down of ArHsp40 and ArHsp40-2 in *A. franciscana* cysts. A. RNA recovered from cysts released by females injected with either *GFP* (1), *ArHsp40* (2) or *ArHsp40-2* (3) dsRNA was reverse transcribed and the amount of *ArHsp40*, *ArHsp40-2* and α-tubulin mRNA in each sample was determined by qPCR. The experiment was performed in duplicate with 3 replicates for each knockdown. *ArHsp40* and *ArHsp40-2* mRNAs were normalized to *α-tubulin* mRNA and averaged (n=3). Error bars represent standard error of 3 replicates per experiment. B. Protein extracts of cysts 10 d post-release from females injected with either *GFP* (1,3), *ArHsp40* (2) or *ArHsp40-2* dsRNA (4) were resolved in SDS-polyacrylamide gels, transferred to nitrocellulose membranes and probed as indicated with antibodies specific for ArHsp40, ArHsp40-2 and tyrosinated α-tubulin. The experiment was performed in triplicate. C. The antibody-reactive proteins were quantified with Image Studio Software and the ratios of ArHsp40 and ArHsp40-2 to tyrosinated α-tubulin were calculated. **One-way ANOVA, Dunnett's test,** the values for bars labeled with asterisks are significantly different from values for GFP, ***, $P<0.005$. Error bars represent **means±s.e.m.** of n=3 replicates per experiment.

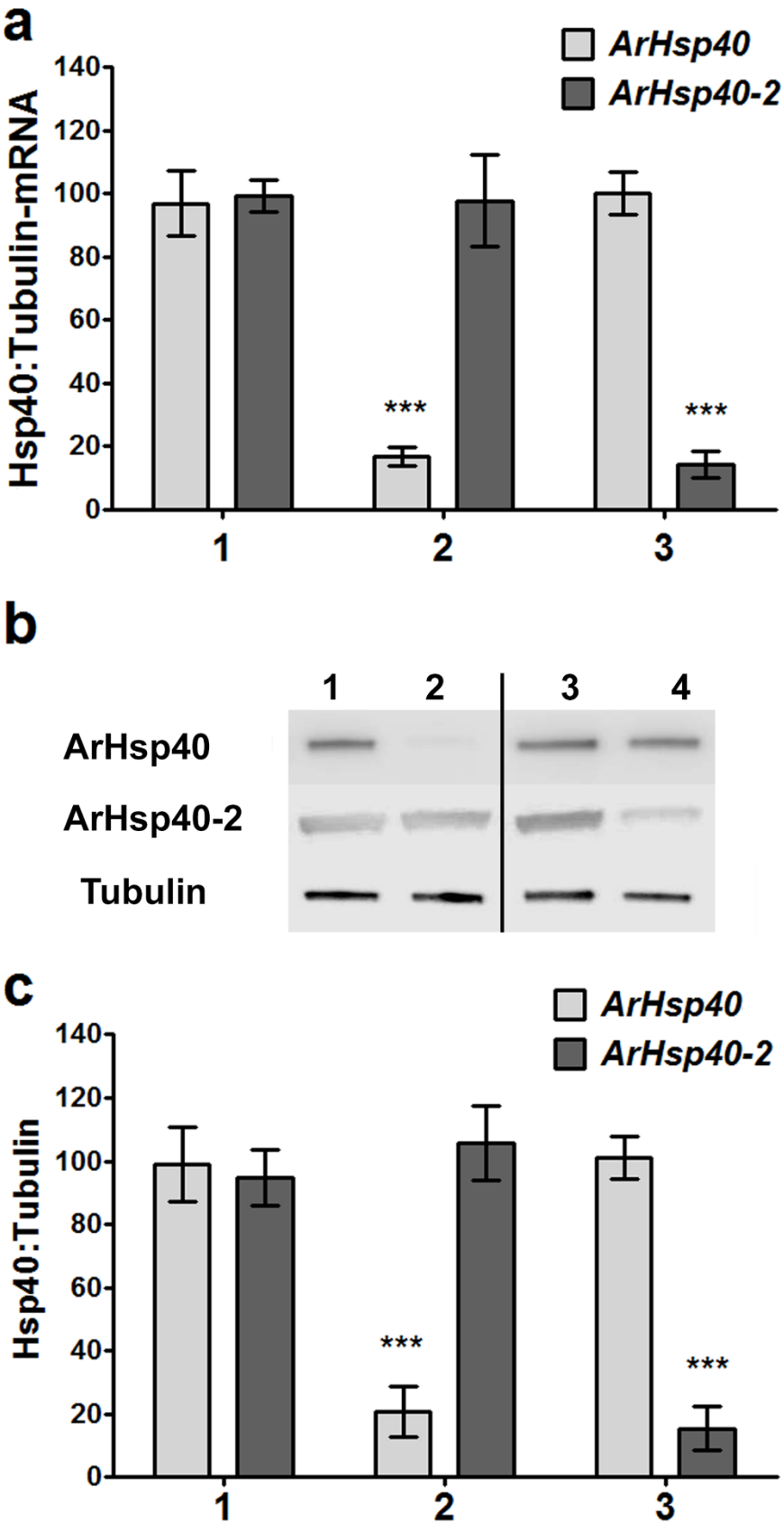

Fig. 4. Knock down of ArHsp40 and ArHsp40-2 in *A. franciscana* nauplii. A. RNA extracted from first brood nauplii collected immediately after release from females injected with either *GFP* (1), *ArHsp40* (2) or *ArHsp40-2* (3) dsRNA was reverse transcribed and the amount of *ArHsp40*, *ArHsp40-2* and α-tubulin mRNA in each sample was determined by qPCR. The experiment was performed in duplicate with 3 replicates for each knockdown. *ArHsp40* and *ArHsp40-2* mRNA were normalized to *α-tubulin* mRNA and averaged (n=3). B. Protein extracts prepared from nauplii immediately after release from females injected with either *GFP* (1,3), *ArHsp40* dsRNA (2) *ArHsp40-2* dsRNA (4) were resolved in SDS-polyacrylamide gels, transferred to nitrocellulose membranes and probed as indicated with antibodies specific for ArHsp40, ArHsp40-2 and tyrosinated α-tubulin. The experiment was performed in triplicate. C. The antibody-reactive proteins were quantified with Image Studio Software and the ratios of ArHsp40 and ArHsp40-2 to tyrosinated α-tubulin were calculated. **One-way ANOVA, Dunnett's test,** the values for bars labeled with asterisks are significantly different from the values for GFP, ***, $P<0.005$. Error bars represent **means±s.e.m.** of n=3 replicates per experiment.

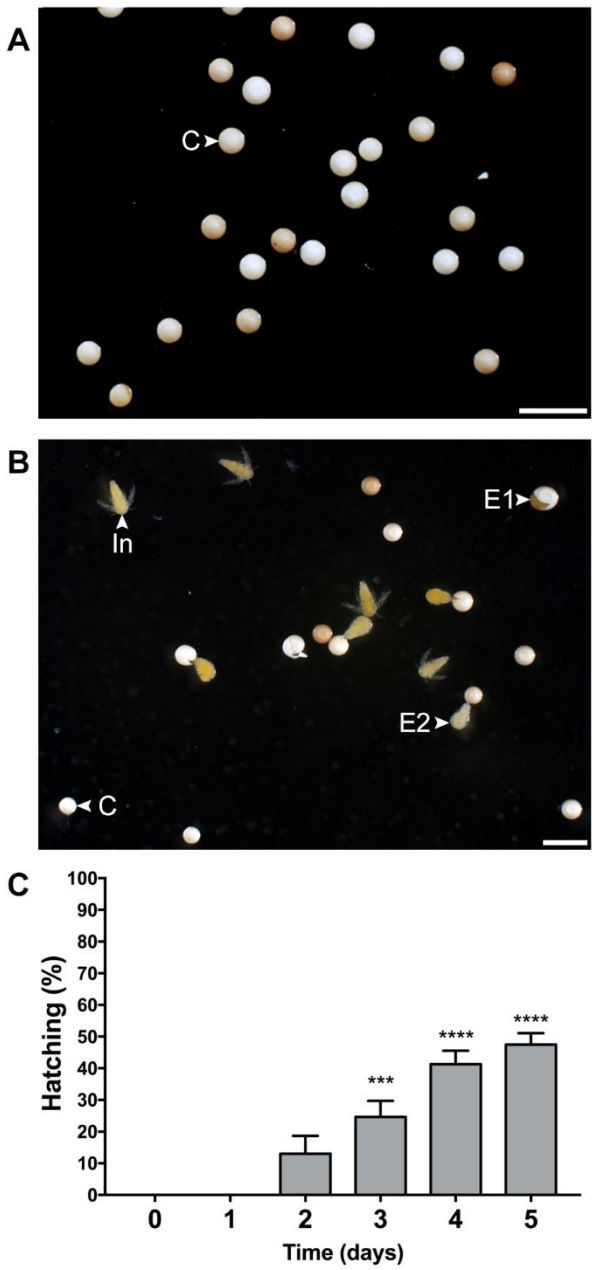

Fig. 5. *A. franciscana* cysts with reduced ArHsp40 aborted diapause entry. Light micrographs of cysts from females injected with *ArHsp40* dsRNA one day (A) and three days (B) post release. C, cysts, E1, emerged nauplius 1; E2, emerged nauplius 2; In, first instar nauplius. C. First brood cysts from females injected with dsRNA were incubated in sea water immediately upon release and the number of cysts that hatched (aborted diapause) compared to the total number of cysts released, expressed as percentage, was determined. The results were averaged for 6 broods of cysts from females injected with either *GFP* dsRNA (1), *ArHsp40*

dsRNA (2) or *ArHsp40-2* dsRNA (3). **One-way ANOVA, Dunnett's test,** the values for bars labeled with asterisks are significantly different from values for 0 days post release; ***, $P<0.005$; ****, $P<0.001$. Error bar represents **means±s.e.m.** of n=6 replicates per experiment.

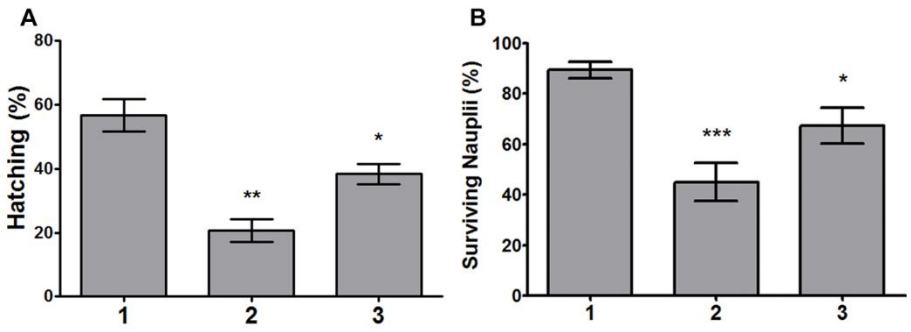

Fig. 6. ArHsp40 and ArHsp40-2 contributed to the stress tolerance of *A. franciscana* cysts and nauplii. A. First brood cysts obtained from *A. franciscana* females injected with either *GFP* (1), *ArHsp40* (2), or *ArHsp40-2* (3) dsRNA were incubated in seawater at room temperature after desiccation and freezing, the resulting nauplii were counted and compared to the initial number of cysts. Data were presented as percentages. The results were averaged (n=6) for each dsRNA. B. Newly released first brood nauplii from females injected with either *GFP* (1), *ArHsp40* (2), or *ArHsp40-2* (3) dsRNA were heat shocked at 39 °C for 1 h and then incubated for 24 h at 27 °C after which swimming nauplii **were counted and compared to the initial number of cysts** as a measure of viability. **Data were presented as percentages.** The results were averaged (n=3). **One-way ANOVA, Dunnett's test,** the values for bars labeled with asterisks are significantly different from values for GFP; *, $P<0.05$; **, $P<0.01$; ***, $P<0.005$. Error bars represent **means±s.e.m.** of n≥3 replicates per experiment.

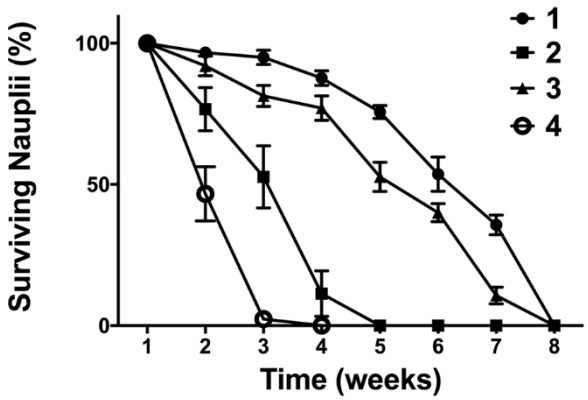

Fig. 7. Knockdown of ArHsp40 and ArHsp40-2 reduced the longevity of *A. franciscana* nauplii. Newly released first brood nauplii from females injected with either *GFP* (1), *ArHsp40* (2), or *ArHsp40-2* (3) dsRNA and nauplii prematurely hatched from cysts released by females injected with *ArHsp40* (4) dsRNA were incubated at 27 °C and swimming nauplii were counted weekly for 8 weeks as a measure of viability. The number of surviving nauplii at each time point was compared to the initial number of nauplii and the data were presented as percent. Error bars represent **means±s.e.m.** of n=3.

Table 1. Primers used for the synthesis of dsRNA and for qPCR

Primer	Sequence (5'-3')	Tm (°C)
dsRNA template[a]		
ArHsp40 Forward	TAATACGACTCACTATAGGGA GTGCATCAGTTGAGCGTCAC	54.0
ArHsp40 Reverse	TAATACGACTCACTATAGGGA TCCGGGGTCCCATGTACTTC	54.0
ArHsp40-2 Forward	TAATACGACTCACTATAGGGA GTGGGTTCTCCAGTTTTGGGG	54.0
ArHsp40-2 Reverse	TAATACGACTCACTATAGGGA ACCACGTCCGCTGGGATTTTA	54.0
GFP Forward[b]	TAATACGACTCACTATAGGGA GACACATGAAGCAGCACGACTT	53.5
GFP Reverse[b]	TAATACGACTCACTATAGGGA GAAGTTCACCTTGATGCCGTTTC	53.5
qPCR		
ArHsp40 Forward	GTGCATCAGTTGAGCGTCAC	54.0
ArHsp40 Reverse	TGCTGAACAATTCCAGGAGC	54.0
ArHsp40-2 Forward	TGACCCATTCGGTGGGTTTG	54.0

ArHsp40-2 Reverse	TCGTGTTCAATGGGTGGGTC	54.0
α-tubulin Forward[c]	CGACCATAAAAGCGCAGTCA	49.0
α-tubulin Reverse[c]	CTACCCAGCACCACAGGTCTCT	49.0

Primers were produced by Integrated DNA Technologies (IDT), Coralville, IA, USA; [a]Bold sequences, T7 promoter; [b]Zhao et al., 2013; [c]King et al., 2013.