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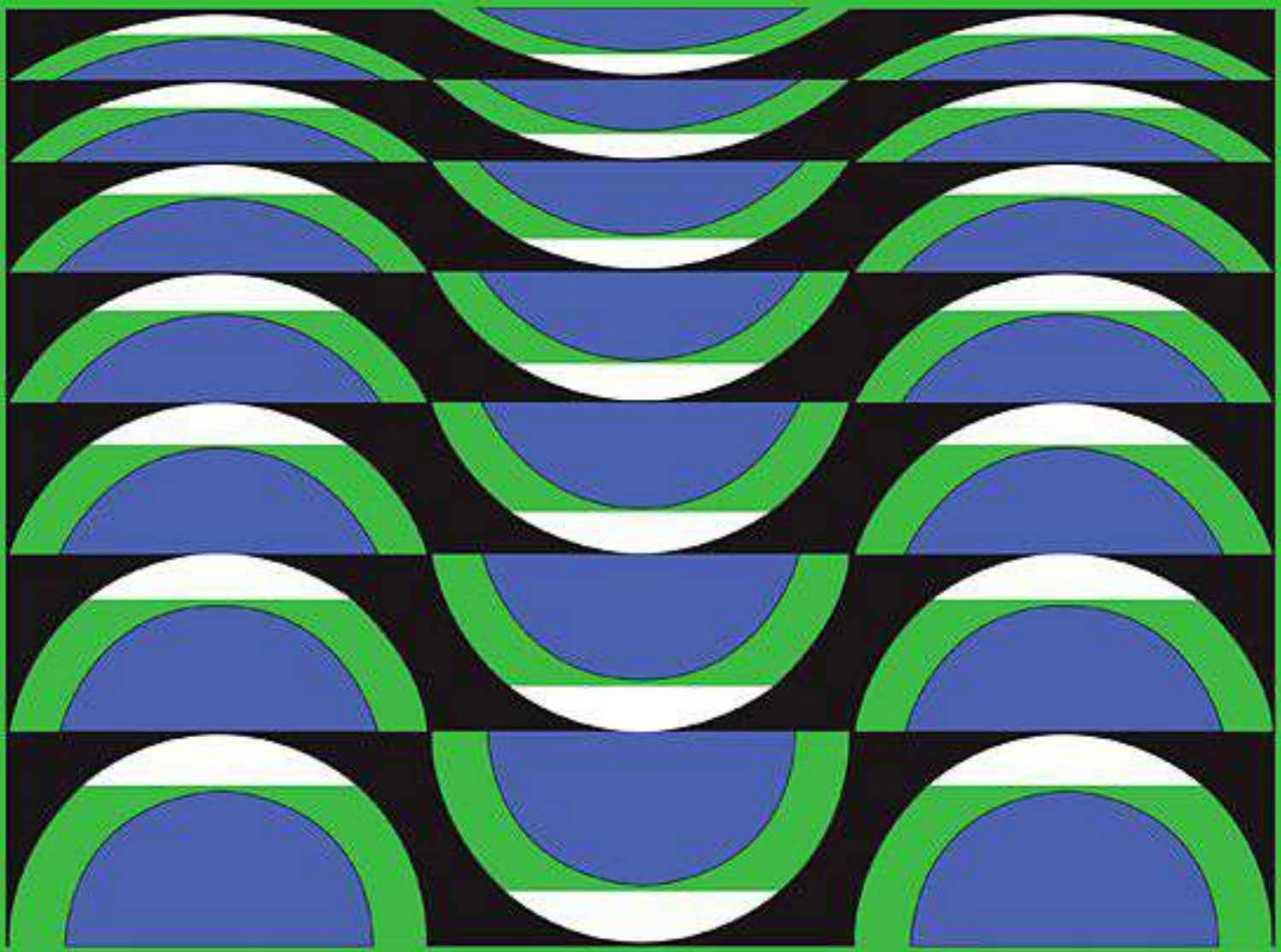

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RNA interference of Hsp70 in *Artemia franciscana* nauplii and its effect on morphology, growth, survival and immune response

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ABSTRACT

Brine shrimp *Artemia franciscana* release either encysted embryos via oviparous reproduction or motile nauplii via ovoviviparous reproduction. *Artemia* cysts contain an abundant amount of ArHsp21, ArHsp22, artemin, and p26, small heat shock proteins which regulate diapause and stress tolerance of this branchiopod crustacean. However, not much is known of the role of molecular chaperone, Hsp70 in this animal. In this study, the immunostimulatory role of Hsp70 in *A. franciscana* was investigated, work which included examining the function of this protein in embryo development, growth, survival and stress tolerance. Knockdown of Hsp70 was successfully performed by RNA interference (RNAi), with specific Hsp70 dsRNA construct delivered to female broods by microinjection to knockdown the protein in nauplii. The loss of Hsp70 neither affected the growth nor morphology as *Artemia* nauplii hatched normally and thereafter grew to adult within 28 days, observations similar to the control animals, the latter injected with GFP dsRNA. The survival of nauplii lacking Hsp70 appeared lower upon 28 days culture but the differences were insignificant when comparisons were made with the controls ($P > .05$). On other aspect, knockdown of Hsp70 reduced approximately 31% survival upon *Vibrio campbellii* challenge, indicating that Hsp70 assisted in the tolerance of *Artemia* nauplii during pathogen infection, perhaps through immune stimulation. Initial findings revealed that Hsp70 knockdown did not affect the expression of prophenoloxidase (proPO), a crucial immune protein of the shrimp innate immunity. By examining the morphology, growth, viability and molecular changes upon Hsp70 elimination, this study contributes to our understanding of the important roles of Hsp70 in *A. franciscana*, an important live food species used in aquaculture.

1. Introduction

The brine shrimp *Artemia* is a crustacean highly adapted to living in hypersaline waters of inland salt lakes, coastal lagoons and solar saltworks (Kappas et al., 2004; Persoone and Sorgeloos, 1980). *Artemia* occur in many colours, from white to pink, shadow or green with females appear pink or orange (King, 2013). Males are usually grey in color and distinguished from females based on several features, notably their anterior pincers and lack of an egg sac at sexual maturity (King, 2013). Adult *Artemia* are generally 1 cm long with females longer than males. Reproduction may proceed in two pathways: ovoviviparous or oviparous. Females release free swimming nauplii via ovoviviparous pathway, which then undergo a series of molts before reaching adulthood (Liang and MacRae, 1999). Under unfavorable or extremely critical environmental conditions, females produce a highly resistant encysted gastrula embryo, known as cyst via oviparous pathway.

Heat shock proteins (Hsp70) have been studied extensively and are associated with the protection of *Artemia* against abiotic and biotic

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stress. For example, exposure of *A. franciscana* nauplii to a non-lethal heat shock from 28 °C to 37 °C for 30 min with 6 h recovery enhances Hsp70 accumulation and promoted heat tolerance at 42 °C (Sung et al., 2008). The tolerance of *Artemia* against virulent *V. campbellii* and *V. proteolyticus* were enhanced, with survival increased two-fold when compared to the non-heated controls (Sung et al., 2007). As Hsp70 is involved in the immune response, the immunoregulatory roles of Hsp70 during shrimp disease and infection were examined. Hsp70 potentially function in shrimp immunity through activation of Toll like receptors (TLRs). As a ligand for TLRs, Hsp70 binds pathogen-associated molecular pattern (PAMP) molecules and modulates PAMP-induced Toll-like receptor (TLR) signaling, transferring signals to cells of the innate immune system and thereby promoting pathogen recognition and elimination (Sung et al., 2018; Sung and MacRae, 2011).

RNAi is a gene silencing technique in which dsRNA related in sequence to a specific mRNA causes degradation of that mRNA, enabling a specific gene to be silenced (Chopra et al., 2002). This powerful tool was first discovered during a study of the biochemical nature of inducers of gene silencing in the nematode *Caenorhabditis elegans*, mediated by dsRNA molecules (Aigner, 2006; Fire et al., 1998). To further examine the function of *Artemia* Hsp70 in stress tolerance and immune response, a protocol to knockdown this particular protein by way of RNAi was developed (Iryani et al., 2017). In this context, Hsp70 knockdown nauplii were produced by injection of Hsp70 dsRNA into the berried females of *Artemia* to eliminate both the Hsp70 mRNA and protein in nauplii released from these females. This protocol facilitated the study on the role of Hsp70 in the protection of *A. franciscana* against abiotic and biotic stressors. An established molecular techniques like RNAi makes *A. franciscana* an exceptional model organism to explore many basic studies such as biological evolution (Copf et al., 2006), gene function (King and MacRae, 2012) and stress tolerance (Iryani et al., 2017; Toxopeus et al., 2014). It was demonstrated through previous study that Hsp70 knockdown nauplii were less resistant to heat perturbation and pathogenic *Vibrio campbellii*, with survival percentage reduced approximately 41% and 34%, respectively in challenge assays (Iryani et al., 2017). The role and association of Spalt gene with Hox gene was studied using the RNAi, with results indicated that Spalt acts as a repressor of Hox gene expression and knocking down *Spalt* expression caused various segmental anomalies in *A. franciscana* (Copf et al., 2006). Gnotobiotic *A. franciscana* were used to investigate whether a phenolic compound, phloroglucinol is effective against *V. parahaemolyticus* and pretreatment of Hsp70-knockdown *Artemia* larvae with this compound indicated that phloroglucinol-induced Hsp70 mediates the survival of *Artemia* against *V. parahaemolyticus* (Kumar et al., 2018).

In this study, the introduction of Hsp70 dsRNA into the egg sacs of *Artemia* females significantly reduced Hsp70 content in the released nauplii. Protein knockdown by dsRNA microinjection was successful in *Artemia* with caudal dsRNA (Copf et al., 2004), *C. pipiens* with catalase dsRNA (Sim and Denlinger, 2011), *Artemia* females with p26 dsRNA (King and MacRae, 2012) and *Artemia* females with AfrLEA1 dsRNA (Toxopeus et al., 2014). *Artemia* embryos with reduced amounts of Hsp70 developed normally, yielding nauplii similar morphology to those with normal amounts of Hsp70. Hsp70-knockdown nauplii however, displayed slower growth during the culture period. Hsp70 was absent in the nauplii but the expression resumed upon reaching juvenile and adult stage. The survival of *Artemia* lacking Hsp70 in the absence of infection was lower when compared to the control 28 days upon culture. This result suggested that Hsp70 plays crucial roles in cell maintenance, growth and survival of *Artemia*. Real time-PCR analysis revealed that there was no apparent difference between the expression levels of proPO with the control, whereas the expression level of Hsp70 is down-regulated. The number of proPO transcript was not elevated in *Artemia* without bacterial exposure. Upon *V. campbellii* infection, survival of nauplii lacking Hsp70 decreased, indicating that Hsp70 protects *Artemia* against *Vibrio* infection. This work makes fundamental but important contributions to our understanding on the role of Hsp70 in *A. franciscana* and determining the Hsp70 function by evaluating effects of Hsp70 knockdown in nauplii could lead to the formulation of strategies to protect *Artemia* and perhaps other crustaceans against stress and disease.

2. Materials and methods

2.1. Culture of A. franciscana

A. franciscana cysts (INVE Aquaculture) were hatched in 30 mg/l of seawater with vigorous aeration and constant illumination for 24 to 48 h. Nauplii were collected with a 200 μm plankton net and grown to adult stage at 28 °C with constant aeration. Nauplii were fed daily with PKC Nutri + ®, a formulated feed developed at Universiti Malaysia Terengganu (UMT) for use in *Artemia* biomass production. Adult *Artemia* were collected 28 days after for microinjection.

2.2. Preparation of Hsp70 dsRNA

pRSET-C plasmid (Invitrogen, Burlington, ON., Canada) containing *Artemia* Hsp70 cDNA were transformed into *E. coli* competent cells (Invitrogen, USA) and colonies grown on LB-Amp agar were cultured in LB-Amp broth at 37 °C prior to extraction. Hsp70 cDNA were harvested from 5 ml overnight cultures of *E. coli* using miniprep kit (PureLink™ Quick Plasmid Miniprep Kit, Invitrogen. USA). The Hsp70 cDNA was amplified using forward (5′-TAATACGAC TCACTATAGGGATTCTCAA AGACAAGC-3′) and reverse (5′-TAATAC GACTCACTATAGGGCATAGA GCTTGGTAAT-3′) primers, each specific for Hsp70 and containing the T7 promoter, TAATACGACTCACTATAGG, at the 5′-end (Bioline, UK). PCR was performed using ImmoMix™ (Bioline, UK) at the manufacturer's suggested concentration as follows: 5 min at 94 °C followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by 10 min at 72 °C. The PCR product was resolved in 1.7% (*w/v*) agarose gels in 1× TBE (20 mM TRIS, 10 mM acetic acid, 0.5 mM EDTA, pH 8.5) at 90 V for 45 min and stained with ethidium bromide (Sigma Aldrich, USA) prior to visualization with a Gel Doc™ XR + with Image Lab™ Software (BioRad, USA). The PCR product was used as template to generate dsRNA using the MEGAscript® RNAi kit (Ambion Applied Biosystems, USA). Green fluorescence protein (GFP) dsRNA was generated as control solution using forward (5′-TAATACGACTCA CTAAGGGAGACACATGAAGCAGCACGACCT-3′) and reverse (5′-TAAT ACGACTCACTATAGGGAGAAGTTCACCTTGATGCCGTTC-3′) primers, with PCR conditions as described above except annealing temperature was at 60 °C. The GFP cDNA used as template was cloned in the vector pEGFP-N1 (Clontech, Mountain View, CA, USA) (King and MacRae, 2012).

2.3. Injection of A. franciscana females with dsRNA

Adult *A. franciscana* females with unfertilized eggs sacs and highly possible to produce nauplii were chosen for dsRNA injection (Iryani et al., 2017). They were differentiated from females destined to produce cysts by the absence of a shell gland (Liang and MacRae, 1999). dsRNA was mixed with 0.5% (*w/v*) phenol red in Dulbecco's phosphate buffered saline (DPBS) (Sigma Aldrich, USA). *Artemia* females were immobilized on cooled 3% (w/v) agar and Hsp70 dsRNA solution containing approximately 100 ng of dsRNA was injected into the egg sacs with a micromanipulator (InjectMan®, Eppendorf, Germany). Injected females were placed in 6 well plates and monitored for at least 2 h. Females exhibiting normal swimming, straight tails and dye retention were used in the experiment. Males were added to females 24 h post-injection and females were monitored with a stereomicroscope for fertilization, noted by the fusion of egg sacs (King and MacRae, 2012) and for the release of nauplii.

2.4. Detection of Hsp70 in nauplii, juvenile and adult A. franciscana

Protein were extracted from 40 nauplii released from *A. franciscana* females injected with either Hsp70 dsRNA or GFP dsRNA as described in previous study with minor modification (Sung, 2014). Sample was rinsed in cold distilled water and homogenized in cold Buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4) (Sung et al., 2007; Clegg et al., 2000) supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich Inc−P8849, USA) as recommended by the manufacturer. The mixture was centrifuged at 4 °C for 5 min and the supernatant was transferred to a new tube. For SDS polyacrylamide gel electrophoresis, aliquots of homogenate were combined with equal volumes of SDS sample buffer, vortexed and heated at 95 °C for 5 min (Laemmli, 1970). The samples was cooled and centrifuged at 2200 x*g* for 1 min. Fifteen (15) µl of each protein sample was applied per lane of 10% SDS polyacrylamide gels prior to electrophoresis at 120 V for 15 min and then at 150 V for 45 min. One gel was stained with Coomassie Biosafe (BioRad, USA) and the other transferred to polyvinylidene fluoride transfer membrane (BioRadImmun-Blot™ PVDF, USA) for antibody probing. PVDF membrane were probed in mouse monoclonal antibody to Hsp70 (SMC-164D) (StressMarq, Canada), diluted 1:5000 in Tris (pH 7.6) for 2 h. Membrane was then washed in Trissaline (pH 7.6) prior to incubation in polyclonal HRP-conjugated goat anti-mouse IgG (Bioreagent-SAB-100 J) (Stressgen, Canada) diluted 1:5000 in Tris. For detection, antibody-reactive proteins were detected with 0.7 mM diaminobenzidine tetrahydrochloride dihydrate (DAB) used as substrate in association with 0.01% (*v*/v) H_2O_2 in 0.1 M Tris-HCL (pH 7.6). Detection of Hsp70 in either juvenile or adult stage was performed as described, with extracts from 1 whole animal used for SDS-PAGE and WB.

2.5. Densitometry analysis

For densitometry analysis, blots were scanned with a GS-800 calibrated densitometer (BioRad Laboratories, USA) and quantification was performed by measuring protein bands from the control and treatments with Quantity One software (BioRad Laboratories, USA). The amount of protein were interpreted as reflective density/mm2, with the density value generated by the software.

2.6. Phenotypic modification, growth development and survival of nauplii upon knockdown

Nauplii released from females injected with Hsp70 dsRNA were observed with a stereomicroscope for 28 days to determine if they were morphologically normal compared to the nauplii released from females injected with control solution. Nauplii length was determined using a Nikon Profile Projector V−12B fixed with a Nikon Digital Counter SC-212 at 7 days intervals. Thirty nauplii were collected and measured each week.

Thirty nauplii released from females injected with dsRNA for either Hsp70 or GFP were incubated in seawater at 28 °C for 28 days. Nauplii were fed and observed daily to determine the number of surviving animals. Dead nauplii were removed after counting. The experiment was performed two times in triplicate.

2.7. Quantification of Hsp70 and ProPO mRNAs in Hsp70-knockdown nauplii

Total RNA were extracted from 20 nauplii using TRIsure™ reagent (Bioline, UK) by following the manufacturer's instructions with minor modification. Nauplii released from *A. franciscana* females injected with dsRNA specific to either Hsp70 or GFP were rinsed with ice cold distilled water, homogenized in 100 µl of TRIsure and the mixture incubated at room temperature for 5 min. Twenty (20) µl of chloroform were added to the sample, vortex vigorously for 15 s and incubated for 2 min at room temperature. The samples were centrifuged at 12,000 x*g* for 15 min at 4 °C. The upper colorless aqueous phase which contains the RNA were transferred to a fresh tube prior to RNA isolation. First strand cDNA was synthesized using Tetro cDNA Synthesis Kit (Bioline, UK). The premix was prepared on ice (Mix 1), mixed gently by pipetting and incubated at 65 °C for 10 min before placed on ice for 2 min. The second mixture (Mix 2) was added and the sample was incubated at 42 °C for 45 min followed by a 15 min incubation at 70 °C.

Table 1
Primers specific for Hsp70, Prophenoloxidase and β-actin.

Gene	Sequences of forward and reverse primers
Hsp70 (F)	5′-GATTCTCAAAGACAAGC-3′
Hsp70 (R)	5′-GCATAGAGCTTGGTAAT-3′
Prophenoloxidase (F)	5′-CGCTGGCATAAGCACATCGATG-3′
Prophenoloxidase (R)	5′-GTCATTTCTCACTGTGAAACG-3′
β-actin (F)	5′-GGTCGTGACTTGACGGACTATCT-3′
β-actin (R)	5′-AGCGGTTGCCATTTCTTGTT-3′

qPCR was performed with 2 µl of cDNA (100 ng) using 2× SensiMix SYBR No-ROX Kit (Bioline, UK) in a CFX Connect™ System (Bio-Rad, USA) and primers for the immune related genes of *A. franciscana*, Hsp70 and prophenoloxidase (Baruah et al., 2011), with β-actin served as the house-keeping gene (Jiang et al., 2007) (Table 1). Amplification was performed at 95 °C for 10 min followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. The cycle threshold (CT) values and fold difference for each immune-related genes was calculated according to the 2-ΔΔCt method provided by the CFX Manager Software (Bio-Rad, USA) (Clegg et al., 2000). Data generated in CFX Connect™ System were analyzed with the Bio-Rad CFX (Gene Expression Analysis) software and data were normalized against β-actin.

2.8. Tolerance of nauplii to Vibrio campbellii

Thirty nauplii from *Artemia* females injected with either Hsp70 dsRNA or control solution were transferred into Falcon tubes containing 30 ml of filtered, autoclaved seawater. Nauplii were challenged by immersion to *Vibrio campbellii* at 1×10^8 cells/ml (Iryani et al., 2017) and vibrio tolerance was determined 24 h after challenge by counting actively swimming nauplii. The experiment was performed two times, each in triplicate.

3. Results

3.1. Hsp70 expression in different developmental stages of A. franciscana

As revealed by SDS gel electrophoresis and immunoprobing of western blots, Hsp70 was absent in *Artemia* nauplii. The administration of Hsp70 dsRNA by injection decreased Hsp70 accumulation in *Artemia* nauplii, indicating that the synthesis of Hsp70 in *Artemia* nauplii was obscured, an expected outcome when the dsRNA used were constructed and delivered appropriately. Though Hsp70 was undetectable in nauplii, the synthesis increased in juvenile and adult stages upon 28 days, revealing that knockdown of this protein was transient (Fig. 1). The Hsp70 content in juvenile *Artemia* produced from female broodstock receiving Hsp70 dsRNA was augmented 0.3 fold over time and the adults accumulated Hsp70 levels similar to those injected with GFP dsRNA (control) upon 28 days culture (Table 2).

3.2. Morphology of Artemia Lacking Hsp70

Microscopic observation of nauplii released from females injected with Hsp70 dsRNA displayed normal morphological structure, as with those released from control females (Fig. 2). It was noted that the growth of Hsp70 knockdown nauplii was slightly slower than the control, but the differences were insignificant ($P > .05$).

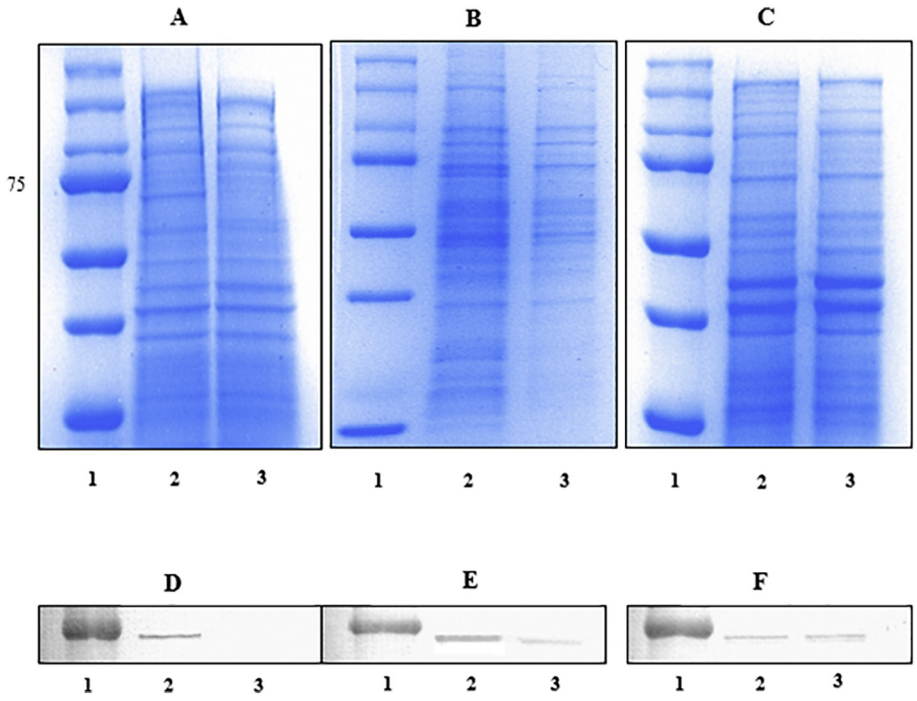

Fig. 1. Hsp70 expression in different growth stages of *A. franciscana*. Protein extracts prepared from different stages of *Artemia* were resolved by electrophoresis in SDS polyacrylamide gels and either stained with Coomassie brilliant blue or blotted to PVDF membrane and probed with antibody SMC-164. The secondary antibody was HRP-conjugated goat anti-mouse IgG. Antibody reactive protein was detected by DAB reagent. A & D, nauplii; B & E, juvenile; C & F, adult. 1, protein markers; 2, GFP dsRNA; 3, Hsp70 dsRNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Relative density of Hsp70 in different developmental stages of *A. franciscana.*

Nauplii	Adj. Vol. (RD)	Relative quantity
Control	2294.56	–
Hsp70 dsRNA	–	–
Juvenile	Adj. Vol. (RD)	Relative Quantity
Control	3124.56	1
Hsp70 dsRNA	890.80	0.3
Hsp70 dsRNA	–	–
Adult	Adj. Vol. (RD)	Relative Quantity
Control	1908.39	1
Hsp70 dsRNA	2143.09	1.1

3.3. Growth and survival of A. franciscana nauplii lacking Hsp70

Nauplii released from females receiving dsRNA for Hsp70 grown to adult and their morphology were similar to the control animals. The final length of Hsp70 knockdown *Artemia* was 11.3 ± 1.3 mm, whereas the control animals reached 12.1 ± 1.6 mm upon 28 days culture (Fig. 3). Approximately 43% of control nauplii (released from GFP dsRNA injected-females) survived 28 days upon culture whereas those lacking Hsp70 were maintained at 23% (Fig. 4).

3.4. Immune genes expression upon Hsp70 knockdown in nauplii

Hsp70 mRNA in nauplii released from *Artemia* females injected with Hsp70 dsRNA reduced approximately 4.2 fold. Prophenoloxidase gene transcripts in Hsp70 knockdown nauplii were augmented by 0.3 fold, but the differences were insignificant when compared to the controls, indicating that regulation of this immune gene was not affected by Hsp70 knockdown (Fig. 5).

3.5. Vibrio tolerance of Hsp70-knockdown nauplii

The 24 h survival of nauplii released from females injected with GFP dsRNA and subsequently challenged with 1×10^8 *V. campbellii*/ml was approximately 78%. In similar experimental conditions, the survival of nauplii lacking Hsp70 reduced to 31% (Fig. 6). The significance in survival reduction strongly indicated that Hsp70 contributes to *V. campbellii* tolerance.

4. Discussion

RNAi was used in this study to elucidate Hsp70 function in *Artemia*, with particular emphasis on morphological changes, growth and survival upon Hsp70 knockdown in nauplii-destined embryos, work which included verification of prophenoloxidase expression, an important shrimp innate immune component to battle pathogens. Administration of Hsp70 dsRNA into the egg sacs specifically knocked down Hsp70 in *Artemia* females, an outcome consistent with our previous study (Iryani et al., 2017). *A. franciscana* nauplii with reduced amounts of Hsp70 developed normally, having morphology similar to those produced from females injected with GFP dsRNA, with neither malformation nor mutation noticed. Nauplii lacking Hsp70 grew to adult stage within 12 to 14 days upon given a standardized feeding regime with PKC-Nutri +®, suggesting that Hsp70 is unessential for *Artemia* growth at early developmental stages. Nevertheless, based on the outcome of our previous study, fertilization rate and the time used to release nauplii were slightly affected by the loss of Hsp70 (Iryani et al., 2017).

Further investigation into the Hsp70 content across different stages of *Artemia* revealed that this group of stress protein, initially undetected in nauplii stage, increased in juveniles and adults upon RNAi, indicating that Hsp70 knockdown with this method is transient. To compare, knockdown of p26 diapause-destined *Artemia* embryos lasted for at least four broods from the same female but the RNAi effect does not carry over into subsequent generations (King and MacRae, 2012). Various factors influence the efficiency of RNAi. A GFP knockdown with transfection of GFP-dsRNA into the fall armyworm *Spodoptera frugiperda* cells and subsequently infected with recombinant baculovirus carrying GFP gene revealed fluorescence inhibition of 60% and 30% at day 5 and 9, respectively (Flores-Jasso et al., 2004). The reduction in the inhibition efficiency might be due to the specificity, length and quantity of dsRNA, delivery method as well as target tissue site, developmental stage and host's characteristics (Yang et al., 2018). Interestingly, RNAi in the nematode *C. elegans* lasted for generations, and this effect was most probably due to the presence of RNA-directed RNA polymerases (RdRP), a crucial enzyme required in the RNA

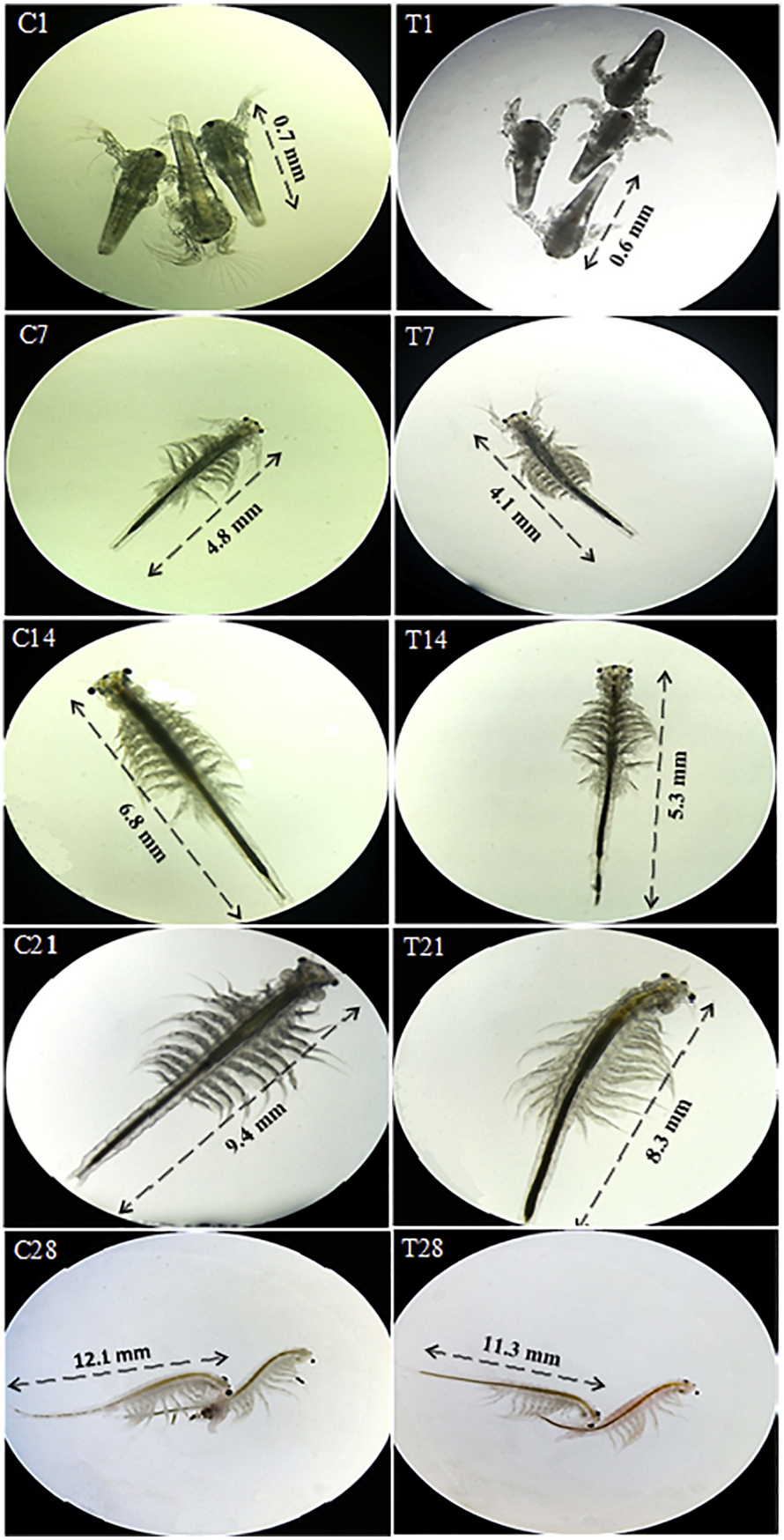

Fig. 2. Morphology of *A. franciscana* nauplii lacking Hsp70. Microscopic observation using a stereomicroscope was employed for 28 days to determine if nauplii released from females injected with dsRNA for Hsp70 were morphologically normal compared to nauplii released from females injected with dsRNA for GFP. C, GFP dsRNA; T, Hsp70 dsRNA. C1 & T1, day 1; C7 & T7, day 7; C14 & T14, day 14; C21 & T21, day 21; C28 & T28, day 28.

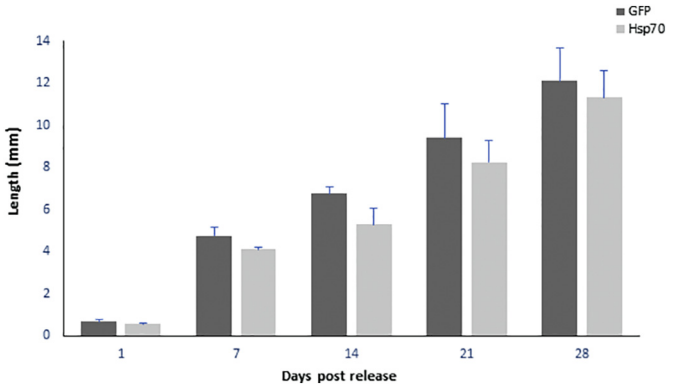

Fig. 3. Knockdown of Hsp70 does not affect *A. franciscana* growth. Size of nauplii released from females injected with dsRNA for Hsp70 and GFP were determined at 7 days intervals for 28 days. Thirty nauplii were collected and measured each week for both knockdowns. Bars represent length of *Artemia*. The error bars represent the corresponding standard deviation (SD) with ($P > .05$).

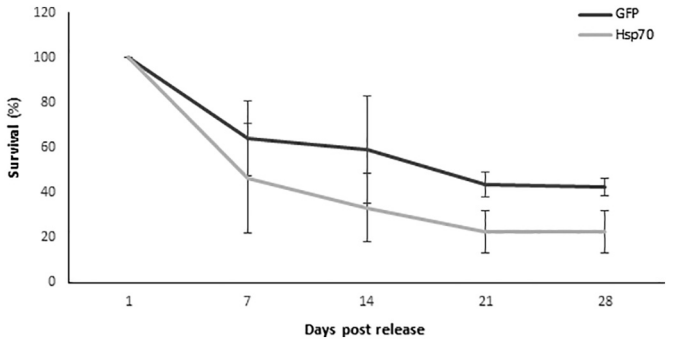

Fig. 4. Hsp70 knockdown *Artemia* exhibited reduced survival. Nauplii released from females injected with dsRNA for Hsp70 and GFP were incubated at 28 °C and surviving (swimming) nauplii were counted for 28 days. The experiment was done two times for both knockdowns with 30 nauplii in each replicate. Lines represent survival percentage of *Artemia*. The error bars represent the corresponding standard deviation (SD) from three replicates with (P > .05).

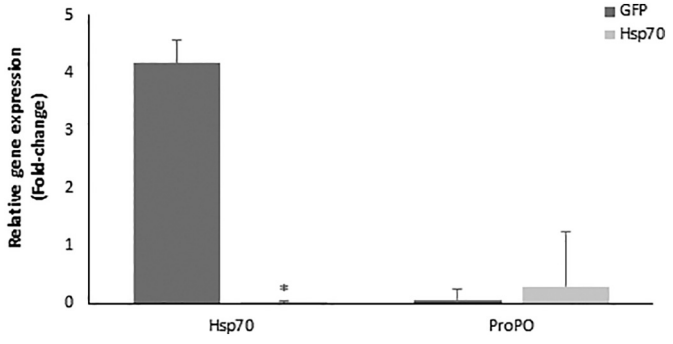

Fig. 5. The effect of Hsp70 knockdown on gene expression of *A. franciscana* nauplii. The relative gene expression of Hsp70 and proPO genes was quantified by qRT-PCR in nauplii *Artemia*. The value at each point represents the fold change of mRNA expression (normalized against internal control β-actin) relative to the value of control. The error bars represent the corresponding standard deviation (SD) from three replicates independently prepared after knockdown. Asterisks denote statistically significant differences between treatments with ($P < .05$).

silencing pathway of many invertebrates (Fire et al., 1998).

In this study, *Artemia* with reduced amounts of Hsp70 appeared slightly shorter than the control in the early developmental phase. However, the total length upon reaching 28 days culture were

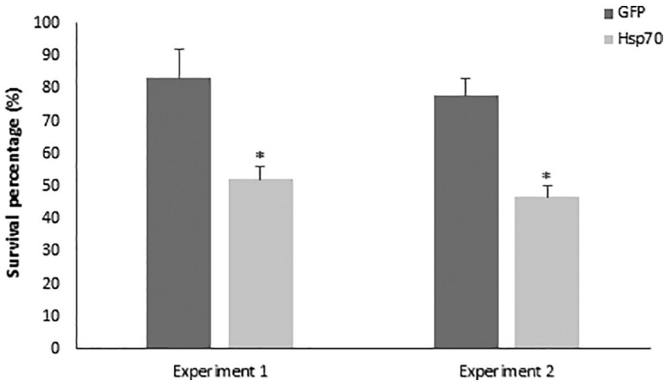

Fig. 6. Hsp70 protected *A. franciscana* nauplii against pathogenic *V. campbellii*. Nauplii released from females injected with either Hsp70 dsRNA or GFP dsRNA were exposed to 1×10^8 *Vibrio campbellii*/ml for 24 h. Vibrio tolerance was determined by counting surviving (swimming) nauplii. The experiment was done two times with 30 nauplii in each replicate. The bars in the graph represent survival percentages and the error bars represent the corresponding standard deviation (SD) from three replicates within each experiment. Asterisks denote statistically significant differences between treatments with ($P < .05$).

statistically indifferent than the controls. Knockdown of Hsp70 had no apparent effect on *Artemia* survival, though it was initially noted that the survival of *Artemia* lacking Hsp70 was lower than those accumulating normal amounts of the protein. Hsp70 are crucial in maintaining several biological processes such as protein folding and signaling which affect gene expression. Reducing the amount of Hsp70 decrease cellular protein folding efficiency and this condition may adversely affect the general well-being of *Artemia* and their tolerance against stress, resulting in low survival. Mortality was not observed in this study possibly because only one Hsp70 isotype was knockdown by the dsRNA. Several Hsp70 isotypes occur in *Artemia* and they may play a role in sustaining growth and survival (Junprung et al., 2019). Hence, it is interesting to determine the number of Hsp70 isotypes in *Artemia* and examine their function(s) in stress tolerance.

Hsp70 was linked to several pathways of the invertebrate immune system. In *Artemia*, augmentation of Hsp70 by a non-lethal heat shock protected against *V. campbellii* and *V. proteolyticus* (Sung et al., 2008, 2007). Additionally, feeding *Artemia* with either *Artemia* Hsp70 or DnaK, a bacterial Hsp70, provided similar protection, boosting survival two-fold against *V. campbellii* challenge (Sung et al., 2009). The increase in proPO mRNA led to the assumption that this immune protein was associated with the enhanced tolerance (Baruah et al., 2011). ProPO typically confers protection by melanization and coagulation, two processes crucial in pathogen elimination in invertebrates, including brine shrimp *Artemia* (Norouzitallab et al., 2016). Specifically, the activation of the proPO system by pathogen pattern-recognition proteins (PPRPs), activates a serine proteinase cascade, eventually leading to the cleavage of the inactive proPO to the active PO that functions to produce the melanin and toxic reactive intermediates against invading pathogens (Amparyup et al., 2013). Using *Artemia* as a model organism, the link between Hsp70 and ProPO in this crustacean species was examined. As revealed by qRT-PCR, the number of ProPO mRNA in Hsp70-knockdown and control nauplii were insignificantly different, revealing that Hsp70 knockdown, under the experimental condition performed in this study, does not affect proPO expression. Interestingly, it was observed that the survival of nauplii containing Hsp70 upon 24 h challenge with *V. campbellii* was significantly higher than those lacking Hsp70, indicating that protection against pathogenic Vibrio in *Artemia* is perhaps due solely to Hsp70 accumulation and/or other immune proteins which were not examined in this study. In fact, many studies have revealed that Hsp70 accumulation boosts aquatic species tolerance against disease and infection other than *Artemia*. As one example, priming of adult mussels *P. viridis* with a 30 min NLHS at 38 °C followed

by 6 h recovery increased resistance to *V. alginolyticus*. The induction of Hsp70 in parallel with enhanced thermotolerance and improved protection against *V. alginolyticus*, suggested Hsp70 functions in *P. viridis* as a molecular chaperone and as a stimulator of the immune system (Aleng et al., 2015).

Whatever the outcome, this study represents the first to demonstrate the effect of Hsp70 knockdown on morphology, growth, survival and immune response of the brine shrimp *Artemia*. Considering the massive economic losses incurred by disease outbreak in the shrimp culture industry, studies focusing on Hsps have become increasingly popular because of their putative regulatory function in the immune system. Understanding the role of Hsp70 in *Artemia* and/or other shrimp species may assist in the formulation of strategies to boost stress and disease tolerance during aquaculture.

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Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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