ORIGINAL PAPER

Exposure of gnotobiotic *Artemia franciscana* larvae to abiotic stress promotes heat shock protein 70 synthesis and enhances resistance to pathogenic *Vibrio campbellii*

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Abstract Larvae of the brine shrimp *Artemia franciscana* serve as important feed in fish and shellfish larviculture; however, they are subject to bacterial diseases that devastate entire populations and consequently hinder their use in aquaculture. Exposure to abiotic stress was shown previously to shield *Artemia* larvae against infection by pathogenic *Vibrio*, with the results suggesting a mechanistic role for heat shock protein 70. In the current report, combined hypothermic/hyperthermic shock followed by recovery at ambient temperature induced Hsp70 synthesis in *Artemia* larvae. Thermotolerance was also increased as was protection against infection by *Vibrio campbellii*, the latter indicated by reduced mortality and lower bacterial load in challenge tests. Resistance to *Vibrio* improved in the face of declining body mass as demonstrated by measurement of ash-free dry weight. Hypothermic stress only and acute osmotic insult did not promote Hsp70 expression and thermotolerance in *Artemia* larvae nor was resistance to *Vibrio* challenge augmented. The data support a causal link between Hsp70 accumulation induced by abiotic stress and enhanced resistance to infection by *V. campbellii*, perhaps via stimulation of the *Artemia* immune system. This possibility is now under investigation, and the work may reveal fundamental properties of crustacean immunity. Additionally, the findings are important in aquaculture where development of procedures to prevent bacterial infection of feed stock such as *Artemia* larvae is a priority.

Keywords Temperature stress · Osmotic stress · *Artemia franciscana* · Hsp70 · Cross-protection

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Introduction

Artemia are predominantly found in extreme habitats where few animals exist (Van Stappen 2002), and the ability to tolerate environmental perturbations makes this aquatic crustacean an interesting model organism for stress response studies (Clegg and Trotman 2002). For example, numerous studies address the effects of temperature and salinity, important physical factors in the life of this organism, on the survival of *Artemia* cysts, larvae, and adults (Miller and McLennan 1988a, b; Liang and MacRae 1999; Frankenberg et al 2000; Browne and Wanigasekera 2000; Clegg et al 2000a, b). Moreover, in these studies, including the pioneering work by Miller and McLennan (1988a, b) on p68 and p89, apparent equivalents to Hsp70 and Hsp90, respectively, several *Artemia* Hsps were identified. This work is important fundamentally because it is related to

stress physiology, and *Artemia* also have an essential role in aquaculture as a component of live diets, particularly in fish and shellfish larviculture (Sorgeloos et al 1986).

Stress is a state where organismal homeostasis is either threatened or interrupted by intrinsic and/or extrinsic stimuli or stressors (Chrousos and Gold 1992; Mercier et al 2006). Aquatic organisms are regularly exposed to severe environmental and pathophysiological stresses (Song et al 2006) that induce a cascade of molecular and physiological responses (Livingstone 1985). Animals survive adverse conditions in several ways, and one well-characterized mechanism is by the induction of stress proteins, also termed heat shock proteins (Hsps). These proteins are synthesized constitutively in cells, and they are induced after exposure to stress including heat, cold, oxygen deprivation, desiccation, infection, and disease (Lindquist and Craig 1988; Steinert and Pickwell 1993; Parsell and Lindquist 1994; Jolly and Morimoto 1999; Sørensen and Loeschcke 2001). Besides cell maintenance during stress, Hsps assist in proper folding of proteins, prevention of protein aggregation, and transport of proteins across membranes (Lindquist 1992). Moreover, Hsps play essential roles in immune reactions of animals against infection and disease (Robert 2003; Pockley 2003) and cross-tolerance to environmental perturbations (DuBeau et al 1998; Todgham et al 2005).

Cross-tolerance or cross-protection is a mechanism whereby a primary stress transiently increases the resistance to other insults of the same or a different nature (Volker et al 1992), thus, allowing cells or animals to survive subsequent, more severe, stress (Jean et al 2004). In line with these observations, induced Hsp synthesis confers secondary hyperthermic stress tolerance on *Artemia* (Clegg et al 2000a; Frankenberg et al 2000). In other work, a non-lethal heat shock promoted Hsp70 expression and cross-protected *Artemia* larvae against *Vibrio* challenge (Sung et al 2007). The present study extends this focus, featuring the differential expression of Hsp70 in *Artemia* larvae upon exposure to abiotic stressors. Additionally, the effects of these stresses on weight loss, induced thermotolerance, and immune response as revealed by survival after *Vibrio* challenge were determined, revealing a potential relationship between Hsp70 and enhanced immunity in *Artemia* larvae.

Materials and methods

Maintenance of bacteria

Vibrio campbellii (LMG 21363) isolates stored in 40% glycerol at −80°C were grown at 28°C on marine agar. Marine broth 2216 (Difco Laboratories, Detroit, MI) was then inoculated with single colonies and incubated over-

night with constant shaking at 28°C to stationary phase. Bacterial cells were harvested under aseptic conditions by centrifugation at $2200 \times g$ for 15min before suspension in seawater which had been filtered and autoclaved as used throughout the study. Culture densities were determined spectrophotometrically at 550nm, and bacterial numbers were calculated according to the McFarland standard (BioMerieux, Marcy L'Etoile, France) where an optical density of 1.0 corresponds to 1.2×10^9 cells/ml.

Preparation of axenic *Artemia* larvae

Axenic *Artemia* larvae were obtained essentially as described (Marques et al 2004a). One gram of high-hatching *A. franciscana* cysts from the Great Salt Lake, Utah, USA (EG® Type, INVE Aquaculture, Belgium) was hydrated in 90ml of tap water for 1h with vigorous aeration and then transferred to a laminar flow hood for decapsulation. Fifty milliliters of cold sodium hypochlorite containing 15% (w/v) active chlorine and 3.3ml of 32% (w/v) sodium hydroxide were added to the hydrated cysts, followed after 150s by 70ml of autoclaved sodium thiosulfate pentahydrate at 10mg/l. Decapsulated cysts were washed several times with seawater and collected over 50-μm sterile sieves. A few milligrams of cysts were transferred to separate sterile 50-ml Falcon tubes containing 30ml of seawater and capped. The tubes were incubated at 28°C for 18–24h with rotation at four cycles per min and constantly exposed to incandescent light at $\pm41\mu Em^{-2}$. Subsequently, hatched larvae at stage 2 of development, when the mouth has opened and bacterial ingestion can occur, were harvested within the next 4–6h.

Thermal and osmotic stress of *Artemia* larvae

Axenically hatched larvae acclimated at 28°C were transferred to seawater at 4°C in sterile 500-ml bottles and held for 1h at 4°C with aeration before incubation at 28°C for 6h, a treatment termed CS1. Larvae incubated at 4°C were also exposed to a non-lethal heat shock of 37°C ($\Delta t = 5°C\ min^{-1}$) for 30min, followed by recovery for 6h at 28°C, a procedure termed CS2. Air used during culturing was passed through a 0.22-μm filter.

Axenically hatched *Artemia* larvae, held normally at a salinity of 30g/l, were exposed for 30min to osmotic stress including a hypotonic shock at 4g/l and hypertonic shocks at 50, 100, and 150g/l. For osmotic stress under axenic conditions, larvae acclimated at 30g/l were collected on cloth filters and transferred to 500-ml aerated bottles containing 50ml of salt solutions at 4, 50, 100, and 150g/l. Salt solutions consisted of Instant Ocean® synthetic salt, Aquarium Systems Inc., France sterilized by autoclaving and diluted with autoclaved distilled water. After stress,

larvae were acclimated to normal salinity for 6h either by adding salt to the hypotonic solution or by diluting the hypertonic solutions with distilled water. Larvae were then harvested and used for protein characterization, ash-free dry weight measurements, induced thermotolerance experiments, and *V. campbellii* challenge tests.

Protein extraction, SDS polyacrylamide gel electrophoresis and immunoprobing of Western blots

Protein extraction was performed essentially as described previously (Clegg et al 2000a; Sung et al 2007). *Artemia* larvae were collected on 50-μm sieves and rinsed with ice-cold distilled water. Two hundred milligrams per milliliter (wet weight) of tissue was homogenized in cold buffer K (150mM sorbitol, 70mM potassium gluconate, 5mM $MgCl_2$, 5mM NaH_2PO_4, 40mM HEPES, pH7.4) and supplemented with protease inhibitor cocktail (Catalogue # P8340, Sigma-Aldrich, Inc. USA) as recommended by the manufacturer. Aliquots of homogenate were combined with equal volumes of sodium dodecyl sulfate (SDS) sample buffer, vortexed, heated at 95°C for 5min (Laemmli 1970), cooled, and centrifuged at 2,200×g for 1min.

Ten microliters samples representing 1.0mg (wet weight) of animals were applied to each lane of a 10% SDS polyacrylamide gel (Frankenberg et al 2000; Clegg et al 2000a) before electrophoresis. Two gels were run simultaneously, and one was stained with Coomassie Biosafe (BioRad Laboratories, USA). Proteins in the second gel were transferred to a polyvinylidene fluoride transfer membrane (BioRad Immun-Blot™ PVDF, USA) for antibody probing. Membranes were incubated in blocking buffer [50ml of phosphate-buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin] for 60min at 25°C. Mouse monoclonal anti-Hsp70 antibody, clone 3A3 (Affinity BioReagents Inc., Golden, CO), which recognizes both constitutive and inducible Hsp70 (Sung et al. 2007), was used as primary antibody at the recommended dilution of 1:5,000. Donkey anti-mouse IgG coupled with horseradish peroxidase conjugate (Affinity BioReagents Inc.) was employed as secondary antibody at the recommended dilution of 1:2,500. Detection was performed with 0.7mM diaminobenzidine tetrahydrochloride dihydrate as substrate in association with 0.01% (v/v) H_2O_2 in 0.1M Tris–HCl (pH7.6).

Determination of larval ash-free dry weight

To determine ash-free dry weight, *Artemia* larvae were collected over a 50-μm cloth filter and washed several times with autoclaved distilled water. One hundred larvae from each treatment were placed in triplicate in separate porcelain cups cleaned previously with formic acids, dried,

and weighed. The samples were heated 4h at 103°C for dry weight measurement, then combusted at 600°C for 6h to determine ash content. The ash-free dry weight was calculated as the difference between the total dry weight and the ash weight.

## Determination of induced thermotolerance in *Artemia* larvae

After recovery from an initial stress, 50 larvae were transferred in triplicate into separate Falcon tubes containing 30ml of seawater, exposed to heat shock at 42°C for 30min (Clegg et al 2000a), and then transferred to 28°C. Induced thermotolerance was determined 12h after heating by counting actively swimming larvae. The experiments were repeated once.

V. campbellii challenge tests with *Artemia* larvae

For bacterial challenge, 50 larvae were incubated at 28°C in each of six sterile Falcon tubes containing 30ml of seawater. Three tubes received bacteria and three did not, the latter used for assessing bacterial contamination by incubating 100μl of culture medium on marine agar 2216 (Marques et al 2004a) for 5days at 28°C. If contamination was detected, the experimental results were discarded. Challenge tests were performed as described (Marques et al 2006a), except *V. campbellii* (LMG 21363), a relatively virulent pathogen for gnotobiotically grown *Artemia* larvae (Marques et al 2006a, b, c; Defoirdt et al 2006) was added to 1×10^7 cells/ml. Survival was determined 24 and 36h after challenge by collecting actively swimming larvae and fixing in Lugol's solution before counting. Survival percentage was calculated as $N_t \times 100/N_o$ where N_t and N_o are final and initial numbers of larvae, respectively.

Colonization of *Artemia* larvae by *V. campbellii*

Artemia larvae subjected to treatments CS1 and CS2 were harvested 8h after *Vibrio* challenge by sieving on sterile 150-μm pore size nylon filters and then rinsed twice with 10ml of autoclaved nine-salts solution (NSS; 17.6g/l NaCl, 1.47g/l Na_2SO_4, 0.08g/l $NaHCO_3$, 0.25g/l KCl, 0.04g/l KBr, 1.87g/l $MgCl_2$, 0.41g/l $CaCl_2$, 0.008g/l $SrCl_2$, and 0.008g/l H_3BO_3). Ten larvae, transferred to sterile plastic bags containing 10ml of NSS, were homogenized with a stomacher blender (400SN, Seward Medical, UK) for 10min. The homogenates were transferred to Falcon tubes, tenfold serial dilutions were prepared, and samples were plated on marine agar with a Spiral-plater (Spiral Systems Inc., USA) before incubation at 28°C for 24h and colony counting. The experiments were conducted in duplicate with each test performed in triplicate.
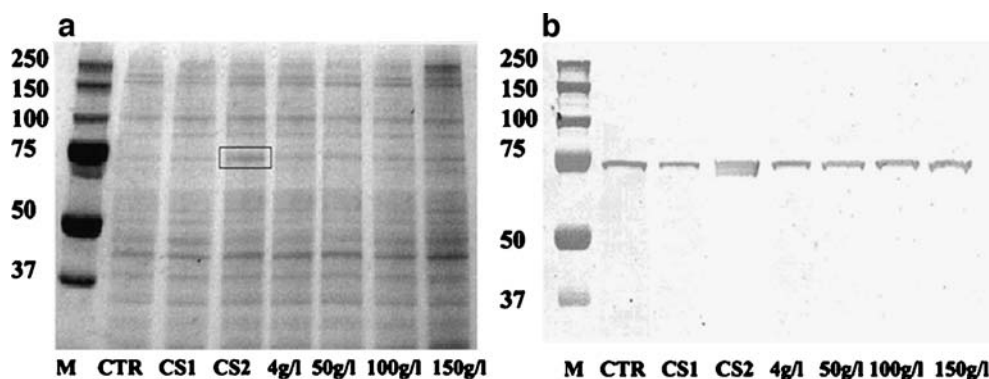
Fig. 1 **a** SDS polyacrylamide gel electrophoresis of protein samples from stressed *Artemia* larvae. Ten microliters samples containing equivalent amounts of protein were loaded in each lane. **b** Immuno-detection of Hsp70 on western blots; CTR, non-stressed larvae; CS1, cold shock at 4°C for 1 h followed by recovery at 28°C for 6 h; CS2, cold shock at 4°C followed by heat shock at 37°C for 30 min and recovery at 28°C for 6 h; 4 g/l, hypotonically stressed larvae exposed to a 4 g/l salt solution for 30 min followed by recovery for 6 h; 50 g/l, 100 g/l and 150 g/l, hypertonically stressed larvae exposed to 50, 100 and 150 g/l salt solution for 30 min followed by recovery for 6 h; *M*, protein standards in kDa. *Box*, 70-kDa protein

Statistical analyses

Values for larval survival (%) were ArcSin to satisfy normality and homocedasticity requirements whenever necessary. For *V. campbellii* load, the CFU values were log-transformed. Significant differences in larval survival and *V. campbellii* colonization were determined by performing one-way analysis of variance followed by Tukey test at a significance level of 0.05. All statistical analyses were performed with software SPSS® version 11.5 for Windows®.

Results

Synthesis of Hsp70 in stressed *Artemia* larvae

Coomassie staining of SDS polyacrylamide gels clearly demonstrated increased amounts of a 70-kDa polypeptide in CS2 samples (Fig. 1a). Immunoprobing of Western blots with a monoclonal antibody to Hsp70 revealed a single co-migrating polypeptide of approximately 70kDa, which, as shown in stained gels, increased only in CS2 samples (Fig. 1b). In contrast, CS1 treatment yielded a minor reduction in the antibody-reactive 70-kDa protein, and the protein was indifferent to osmotic stress. The same results were obtained in two independent experiments for which Fig. 1 is a representative example.

Larval ash-free dry weight is stress-dependent

Animals osmotically stressed at 100 and 150g/l and those from the CS2 treatment had lower ash-free dry weights than non-stressed larvae (Fig. 2). In contrast, no significant differences were detected in ash-free dry weight of larvae subjected to CS1 treatments and osmotic stresses at 4 and 50g/l ($P > 0.05$).

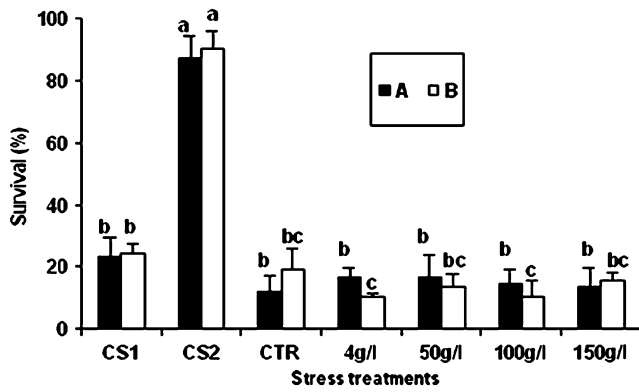#### Induced thermotolerance in larvae occurred only in response to CS2 treatment

Thermotolerance in *Artemia* larvae exposed to the CS2 treatment was enhanced, whereas larvae subjected to CS1 treatment and osmotic stress were less resistant to temperature increase (Fig. 3).

Enhanced survival of CS2 stressed larvae in *V. campbellii* challenge tests

In replicate experiments, approximately 80% of non-stressed and unchallenged larvae survived incubation at
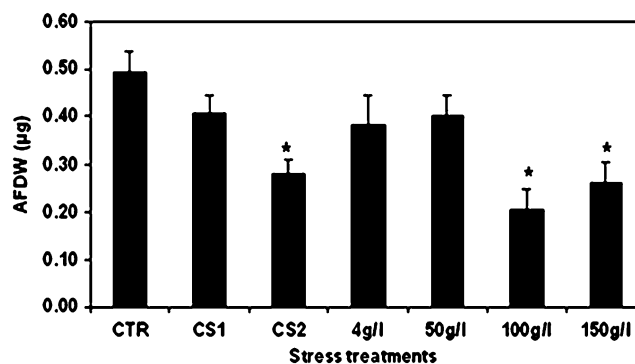
Fig. 2 Ash-free dry weight of stressed *Artemia* larvae. One hundred larvae were heated 4 h at 103°C for dry weight measurement, then combusted at 600°C for 6 h to determine ash content. The ash-free dry weight was calculated as the difference between the total dry weight and the ash weight. Refer to Fig. 1 for explanation of sample designations. *Asterisk* indicates a significant difference ($p<0.05$) between the stress treatment and control

Fig. 3 Thermotolerance induction in *Artemia* larvae. Non-stressed (*CTR*) and stressed *Artemia* larvae were exposed to heat shock at 42°C for 30 min. Induced thermal tolerance was based on larval survival after 12 h of heat shock. Experiments were repeated once with replicates indicated by a and b. Refer to Fig. 1 for explanation of sample designations. Values showing the *same superscript letter* for each experiment are not significantly different (*p>0.05*)

28°C (Fig. 4), whereas about 40% of non-stressed larvae challenged with *V. campbellii* were viable. CS2-treated larvae exhibited substantially higher survival as compared to non-stressed challenged controls, while a small but significantly reduced survival ($p < 0.05$) occurred for larvae experiencing CS1 treatment. Larvae osmotically stressed at 4, 100, and 150g/l salinity had significantly lower survival ($p < 0.05$) than non-stressed animals upon exposure to *V. campbellii*, and for the latter, viability was very low (Fig. 5). Approximately 90% and 72% of the unchallenged larvae were alive at 24 and 36h, respectively, in these experiments (not shown).

Reduced *V. campbellii* load in CS2-stressed larvae

Approximately 8.7×10^3 *V. campbellii* accumulated per non-stressed larva (Fig. 6), with higher numbers generally present after CS1 treatment, although differences were not significant. In contrast, reductions of approximately 49%

and 61% occurred in the number of *V. campbellii* per larva after CS2 treatments ($p < 0.05$).

Discussion

Exposing gnotobiotic *Artemia* larvae to 4°C for 1h followed by an abrupt non-lethal heat shock at 37°C for 30min and a 6h recovery is shown in this report to induce Hsp70 production. Conversely, a small reduction in the amount of Hsp70 was observed in larvae cooled from 28 to 4°C and then warmed to 28°C. Stress often stimulates Hsp expression in aquatic organisms with Hsp70 induced in hypoxic Nile tilapia *Oreochromis niloticus* (L.) juveniles (Delaney and Klesius 2004), Indian major carp, *Cirrhinus mrigala* (Ham.; Das et al 2005), and the tiger prawn, *P. monodon* (de la Vega et al 2006). A temperature increase from 21 to 37°C for 30min followed by a 24-h recovery strongly induced Hsp70 in *Artemia* adults (Clegg et al 2000a), and a non-lethal heat shock from 28 to 37°C for 30min with a 6-h recovery triggered Hsp70 production in gnotobiotic-grown larvae (Sung et al 2007). In contrast to the situation with thermal perturbation, neither hypotonic nor hypertonic osmotic stress for 30min induced Hsp70 production in *Artemia* larvae. Likewise, the absence of Hsp70 induction was observed in hypo-osmotic stressed *P. monodon* obtained by switching from 35 to 10g/l for 8h (de la Vega et al 2006). Although *Artemia* normally adapt readily to changing salt concentrations (Browne and Wanigasekera 2000), larvae exposed to salinities of 100 and 150g/l for 30min experienced significant weight loss. This may have resulted from reduced energy reserves as a consequence of increased metabolic activity required to cope with the imposed stress, a result seen in rats (Harris et al 1998).

Stress may compromise organismal immune response and increase vulnerability to infection. As an example,
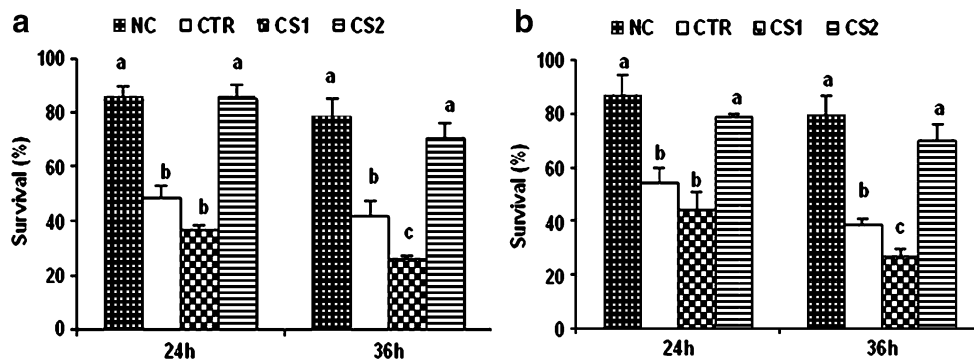
Fig. 4 Survival of *Artemia* larvae in *V. campbellii* challenge tests. After CS1 and CS2 treatments, larvae were exposed to a *V. campbellii* challenge of 1×10^7 cells/ml. Live larvae were counted 24 h and 36 h after challenge, and each experiment was repeated once, with

replicates labeled **a** and **b**. *NC* Non-stressed and unchallenged larvae, *CTR* non-stressed larvae with *V. campbellii* challenge, *CS1* and *CS2*, refer to Fig. 1 for explanation. Values in matching time columns with the *same superscript letter* are not significantly different (*p>0.05*)
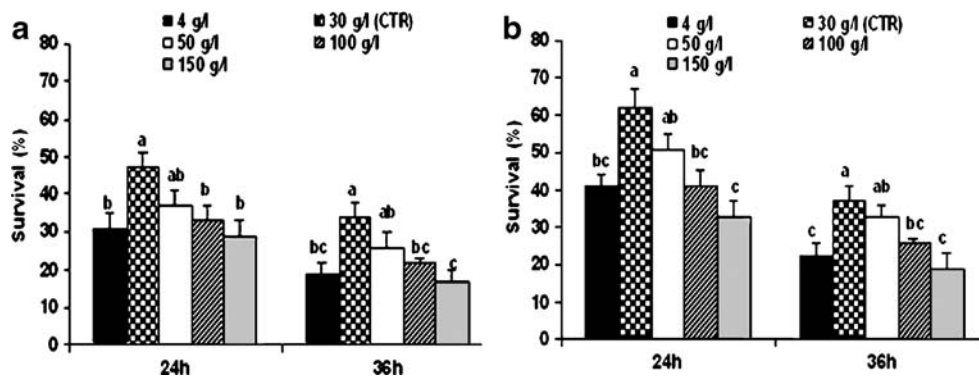
Fig. 5 Survival of osmotically stressed *Artemia* larvae after challenge tests with *V. campbellii*. Live larvae were counted 24 h and 36 h after challenge with 1×10^7 *V. campbellii* per milliliter. Each experiment was repeated once and replicates are labeled **a** and **b**. Refer to Fig. 1 for explanation of sample designations. Values in the same column for each experiment with *matching superscript letters* are not significantly different ($p > 0.05$)

temperature stress increases the susceptibility of sea bass, *Dicentrarchus labrax*, to nodavirus (Varsamos et al 2006). Additionally, thermal stress reduces resistance of the tiger shrimp *P. monodon* to *P. damselae* subsp. *damselae* (Wang and Chen 2006b), and the White shrimp *L. vannamei* is more prone to *V. alginolyticus* infection after heating (Cheng et al 2005). Transfer of *P. monodon* from 25 to 5ppt and from 15 to 35ppt salinity reduced immune capability and decreased resistance against *P. damselae* subsp. *damselae* infection (Wang and Chen 2006a). H_2O_2 accelerates the mortality of *Tenacibaculum maritimum*-infected turbot (Avendaño-Herrera et al 2006), while metal stress influences disease transmission and susceptibility of aquaculture species (Liao et al 2006). Furthermore, hypoxic-stressed *Penaeus styliriostris* are more sensitive to infection by *V. alginolyticus* (Le Moullac et al 1998). Collectively, these studies indicate that stress suppresses immunity, leading to increased vulnerability to infections and greater mortalities. In agreement with these data, gnotobiotic *Artemia* larvae experiencing CS1 treatment and exposure to high salt were less resistant to *V. campbellii* than were non-stressed animals.

Although many stresses reduce immunity, a non-lethal heat shock may cross-protect against further insult, a phenomenon usually correlated with Hsp production and defense against subsequent environmental disturbance. Thermal shock of salmon guards against a subsequent severe osmotic challenge, perhaps due to Hsp70 induction (DuBeau et al 1998), and the induced expression of Hsps in most fishes by high temperature is correlated with increased resistance to a second heat stress (Basu et al 2002). A sublethal heat shock promotes Hsp70 accumulation in adult *Artemia* and shields against exposure to lethal heat shock (Frankenberg et al 2000). In this study, stressed gnotobiotic *Artemia* larvae undergoing CS2 treatment produced Hsp70, indicating that this protein is directly involved in conferring enhanced heat tolerance. Furthermore, combined hyperther-

mic and hyperthermic stress up-regulates Hsp70, and this is associated with cross-protection against *V. campbellii*. Unexpectedly, these animals exhibited a significantly lower ash-free dry weight, although a twofold increase in larval survival was recorded after *V. campbellii* challenge. Additionally, stressed larvae failing to accumulate Hsp70 lacked protection against *V. campbellii*, agreeing with earlier results indicating a role for Hsp70 in cross-protection of *Artemia* larvae (Sung et al 2007).

On an applied note, it is common practice in commercial aquaculture to store *Artemia* larvae in the cold, thus, reducing the number of daily harvests, preventing larvae from molting, prolonging storage, and preserving biomass, all with economic advantage (Merchie 1996). The transfer of cold-stored larvae to fish tanks often involves sudden temperature increases because 28°C is an optimal rearing condition for most warm water aquaculture species. Our results suggest that such larvae have increased sensitivity to opportunistic pathogens like *Vibrio* species.
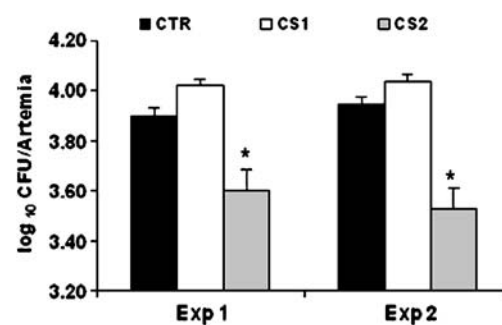
Fig. 6 *Vibrio* colonization of *Artemia* larvae. Larvae from CS1 and CS2 treatments were exposed to 1×10^7 *V. campbellii* per milliliter for 8 h before collection of 10 animals and counting of bacteria. The experiment was repeated once with replicates labeled Exp 1 and Exp 2. CTR, non-stressed larvae with *V. campbellii* challenge; CS1 and CS2; refer to Fig. 1 for explanation of sample designations. *Asterisk* indicates significant difference ($p < 0.05$) between stress treatment and control

How Hsp70 and other Hsps protect against pathogenic *V. campbellii* is unclear, but extracellular Hsps are known to regulate the innate immune response (Pockley 2003; Chen et al 1999). For instance, the heat-induced synthesis of small heat shock proteins and Hsp90 triggers *C. elegans* immunity to pathogenic *Pseudomonas aeruginosa* (Singh and Aballay 2006a). The mechanism may involve heat shock transcription factor-1 and the associated DAF2/DAF-16 pathway which regulates aging and immunity in nematodes (Singh and Aballay 2006b). Furthermore, extracellular Hsp72 robustly promotes inflammatory cytokine production (Johnson and Fleshner 2006) and may stimulate production of inducible nitric oxide synthase (Panjwani et al 2002; Campisi and Fleshner 2003), tumor necrosis factor α, interleukin-1β and IL-6 (Asea et al 2000; Campisi and Fleshner 2003), all known to modulate infection. Substantial evidence indicates that Toll-like receptors 2 and/or 4, which act as cell surface receptors for extracellular Hsp72, transduce inflammatory signals to innate immune cells such as macrophages, dendritic cells, and neutrophils (Visintin et al 2001; Vabulas et al 2002; Asea et al 2002; Ménoret 2004). Findings presented in this report support the emerging idea that Hsps activate innate immune responses in *Artemia* and other invertebrates, thereby protecting against pathogens such as *V. campbellii*. These observations are of fundamental importance in understanding invertebrate immune function, and they have significant potential for application in aquaculture.

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