# Non-lethal heat shock protects gnotobiotic *Artemia franciscana* larvae against virulent Vibrios

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## Abstract

Brine shrimp *Artemia* were exposed under gnotobiotic conditions to a non-lethal heat shock (NLHS) from 28 to 32, 37 and 40 °C. Different recovery periods (2, 6, 12 and 24 h) and different heat-exposure times (15, 30, 45 and 60 min) were tested. After these NLHS, *Artemia* was subsequently challenged with *Vibrio*. Challenge tests were performed in stressed and unstressed nauplii at concentrations of $10^7$ cells ml$^{-1}$ of pathogenic bacteria, *Vibrio campbellii* and *Vibrio proteolyticus*. A NLHS with an optimal treatment of 37 °C for 30 min and a subsequent 6 h recovery period resulted in a cross-protection against pathogenic *Vibrio*. A 100% increase in the larval survival ($P < 0.05$) was observed. We have also demonstrated by Western blot that a NLHS increases the expression of HSP-70 in heat-shocked (HS) treated animals. This report is the first to reveal a cross protection of a NLHS against deleterious bacterial challenges in living crustaceans. The putative role of heat shock proteins (HSPs) in this process is discussed.
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## 1. Introduction

Over the years, the brine shrimp *Artemia* has gained popularity as the most widely used live diet in larviculture of fish and shellfish. Different species of *Artemia* are found in a variety of harsh environments worldwide [1−3] and have proven to be useful model organisms for studies on ecological aspects of stress response [4]. The wide range of stress tolerance has made this unique genus to be termed recently as the '*animal extremophile*' [5].

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Heat shock (HS) involves the sudden exposure of cells, tissues and organisms to a temperature well above the ambient temperature but still within the physiological range of the organism [6]. In almost all organisms, heat shock will activate the transcription and synthesis of a specific group of proteins known as the heat shock proteins [7]. Some of these heat shock proteins are thought to act as molecular chaperones in assisting the recovery of misfolded or aggregated proteins and are postulated to protect organisms against extreme forms of stress [8–10]. HSPs are not induced solely by heat shock. Expression of these proteins is also generally up-regulated by various physiological perturbations or stressors such as exposure to oxidative stress, nutritional deficiencies, ultraviolet radiation, chemicals, ethanol, viral infection and anoxia [6,11].

Until recently, most of the research on stress response aimed at understanding the localization, structures and specific functions of intracellular HSPs [8,10,12]. HSPs are also postulated to be involved in cross-protection or cross-tolerance in animals and plants, i.e. a general stress response and a transient increase in the resistance to a second heterologous physiological and environmental insult [13]. Just to select a few important reviews from a massive body of literature, an initial stress by a NLHS was demonstrated to confer thermal resistance [14–17], protection against osmotic stress [13,18–21], prevention of oxidative toxicity and damage [20,22,23] and desiccation tolerance [24]. All these reviews clearly illustrate that a NLHS can protect an organism against further and eventually more severe environmental insults. However, there is limited information on the effect of a NLHS on the subsequent resistance of the host against pathogens, probably because it is experimentally difficult to apply a NLHS to a host without affecting the associated microbial community either in its composition or its physiology.

The *Artemia* gnotobiotic experimental system is an excellent model system to control any interference of microbes during a NLHS and thus offering full experimental control [25]. The present study aims at investigating the relationship between a NLHS and its subsequent cross protection against pathogenic bacteria. Gnotobiotic *Artemia* nauplii were exposed to several heat shock conditions and subsequently challenged with *Vibrio campbellii* (LMG21363) and *Vibrio proteolyticus* (CW8T2). The *Vibrio* strains were previously described as relatively strong pathogenic bacteria for *Artemia* [26–28]. Detection of HSP-70 expression at different NLHS temperatures was performed using SDS-PAGE and Western immunoblotting.

## 2. Materials and methods

### 2.1. Bacteria culture

Isolates of the bacterial strains, *Vibrio campbellii* (LMG21363) and *Vibrio proteolyticus* (CW8T2), previously stored in 40% glycerol at −80 °C, were aseptically inoculated and grown in Petri dishes containing marine agar 2216. A colony was subsequently transferred and grown to stationary phase in marine broth 2216 (Difco Laboratories, Detroit, Mich.) by incubation overnight at 28 °C with constant agitation. Bacterial suspensions were then transferred to centrifugation tubes and centrifuged at 2200 g for 15 min. The supernatant was discarded and pellets were resuspended in filtered autoclaved sea water (FASW). The bacterial densities were determined spectrophotometrically at an optical density of 550 nm according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an optical density of 1.000 corresponds to $1.2 \times 10^9$ cells $ml^{-1}$.

### 2.2. Axenic Artemia culture

Axenic *Artemia* were obtained following decapsulation according to Sorgeloos et al. [29] and hatching procedures described by Marques et al. [25]. Bacteria-free cysts and nauplii were obtained via decapsulation whereby the hard shell called chorion that encysts the dormant *Artemia* embryo is completely removed by short-term exposure to a hypochlorite solution. High-hatching cysts of *Artemia franciscana*, originating from the Great Salt Lake, Utah, USA (EG® Type, INVE Aquaculture, Belgium) were used. About 1 g of cysts were hydrated in 90 ml tap water for 1 h with strong aeration in non-axenic conditions. The recipient with the cysts was then transferred to a laminar flow hood where decapsulation procedures were performed. All equipment was previously sterilized and autoclaved at 120 °C for 20 min prior to use. A 0.22 μm-filtered aeration was provided to avoid bacterial contamination. Then, 50 ml of cold sodium hypochlorite ($1.22$ kg $l^{-1}$ and 15%w/v active chlorine) and 3.3 ml of sodium hydroxide ($1.34$ kg $l^{-1}$) were added to the hydrated cysts. The reaction was stopped after 150 s by adding 70 ml of autoclaved

sodium thiosulphate pentahydrate (10 mg l$^{-1}$). Decapsulated cysts were washed several times with FASW and collected over a 50 μm sterile sieve. A few mg of these cysts was subsequently transferred to separate, sterile 50 ml Falcon tubes containing 30 ml of FASW and carefully capped. For hatching incubation, the tubes were placed on a rotor at 4 cycles per min and constantly exposed to incandescent light (±41 μEm$^{-2}$) at 28 °C for 18–24 h. Consequently, hatched nauplii that developed into stage II within the next 4–6 h were used in the experiments (only in stage II, the nauplii's mouth is opening, allowing ingestion of *Vibrio*).

### 2.3. Heat shock preparation and Artemia larvae collection

For hyperthermic treatment, *Artemia* nauplii kept in 200 ml of FASW at 28 °C with an approximate density of 5 animals ml$^{-1}$ were exposed to a NLHS at a Δ*t* rate of 2 °C min$^{-1}$ in a preheated and controlled water bath system with thermostat heaters accurate at ±0.5 °C. Heat-shocked *Artemia* were slowly acclimatised back to a water temperature of 28 °C (*t* = 0.5 °C min$^{-1}$). Various recovery periods were tested prior to pathogen challenge. Nauplii were then picked and transferred into a Petri dish by using sterile Pasteur pipettes. Fifty (50) stage II *Artemia* nauplii were subsequently counted and picked into sets of Falcon tubes, which were previously filled with 30 ml FASW and incubated at 28 °C. Three replicates were prepared for each treatment and bacteria free status was verified in the blanks. Larvae kept at 28 °C were used as controls in all experiments. All procedures were aseptically performed in a laminar flow hood.

### 2.4. Axenity verification

Methods used to verify axenity of the *Artemia* culture were conducted following Marques et al. [25] by using a combination of plating and live counting techniques. Bacterial contamination in the control tubes was checked by plating 100 μl of the culture medium in marine agar. Plates were then incubated for 5 days at 28 °C. Live counting involved staining of the blanks with tetrazolium salt MTT (-3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, 0.5% w/v) in a sterile recipient (1 part of MTT to 9 parts of sample) incubated at 30 °C for 30 min. Live bacterial detection and counting were performed using a light microscope at 1000× magnification. If contaminated control tubes were found, all results of that experiment were discarded.

### 2.5. Experimental design and challenge test

In the first experiment, the intensity of the NLHS was tested by exposing axenically-hatched *Artemia* nauplii to a series of different HS temperatures from 28 to 32, 37 and 40 °C for 30 min, with a subsequent recovery period of 6 or 24 h. In all experiments, non-HS nauplii were used as control. Challenge tests performed in this experiment were conducted following a small modification of the technique described by Marques et al. [28] i.e. the bacterial suspensions containing isolates of pathogenic *Vibrio campbelii* were added at concentrations of 10$^7$ cells ml$^{-1}$. All tubes were incubated under constant agitation and light. Nauplii were not fed throughout the experiment. *Artemia* survival was determined after 24 h of exposure by counting the remaining live nauplii. The best NLHS temperature (with the highest *Artemia* larval survival) was selected to perform the subsequent experiments.

Experiment 2 involved testing the effect of different recovery periods in the *Vibrio* challenge test. Axenically-hatched *Artemia* nauplii were given a NLHS at 37 °C (best temperature selected from previous experiments) for 30 min. The recovery periods tested were 2, 6, 12 and 24 h of incubation at 28 °C before starting the challenge test. The optimal recovery period was chosen and used in the subsequent experiment. In experiment 3, the duration of NLHS was investigated namely 15, 30, 45 and 60 min, respectively. The animals were given a 6 h recovery period (best recovery period selected from experiment 2) and subsequently challenged with 10$^7$ cells ml$^{-1}$ of *Vibrio campbellii*. Larval survival was determined 24 h after challenge.

In the last experiment, *Artemia* nauplii exposed to optimal NLHS conditions were challenged with 10$^7$ cells ml$^{-1}$ of *V. campbelli* and *V. proteolyticus*. All experiments were performed twice to verify the reproducibility of the results and each treatment was performed in three replicates.

## 2.6. Protein extraction, SDS-PAGE and Western blot

HSP-70 was analysed in the controls and in heat-shocked nauplii under optimal NLHS conditions. Protein extraction was performed essentially as described by Frankenberg et al. [14]. HS and non-HS treated *Artemia* nauplii were collected on cloth filters and rinsed with ice-cold distilled water to remove external sea water. About 200 mg wet weight tissue ml$^{-1}$ (equal amount of animals in all treatments) were then homogenised in cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM MgCl$_2$, 5 mM NaH$_2$PO$_4$, 40 mM HEPES, pH 7.4), pre-added with a protease inhibitor cocktail (Sigma-Aldrich, Inc. USA) at recommended level of 1:10 of extracts. Aliquots of homogenate were combined with equal volumes of 2 times SDS sample buffer, vortexed and heated at 95 °C for 5 min [30]. Samples were cooled and centrifuged at 1630 g for 3 min to remove chitinous exoskeleton fragments that prevented accurate pipetting. Protein concentrations of each sample were measured according to the Bradford method. Protein concentration was calculated and equal amounts of protein were loaded on the gels enabling direct comparison between different samples in the gel and Western blot. Western blot on equal aliquots was tested as well.

Protein extracts were electrophoresed on 15% poly-acrylamide gels. Two gels were run simultaneously: one was stained with Coomassie brilliant blue and the other transferred to a polyvinylidene fluoride transfer membrane (BiotraceTM PVDF, TALL, Gelman Laboratory, USA) for Western immunoblot analysis. The membrane was blocked overnight at 4 °C using blocking buffer (phosphate buffer saline + Tween-20 + 5% bovine serum albumin) and subsequently incubated with mouse monoclonal anti-HSP-70 antibody, clone 3A3 (Affinity BioReagents Inc., Golden, CO) at recommended dilutions of 1:5000. This antibody detects both constitutive and inducible isoforms of the HSP-70 family. Polyclonal rabbit anti-mouse IgG (Dako®, Denmark) with horseradish peroxidase conjugate was used as secondary antibody at recommended dilutions of 1:1000. For detection, 0.7 mM diaminobenzidinetetrahydrochloride dihydrate (DAB) was used as a substrate in association with 0.01% (v/v) H$_2$O$_2$ in 0.1 M Tris−HCl (pH 7.6).

## 2.7. Data collection and analysis

After a 24 h challenge test, larval survival was determined in all experiments by counting the actively swimming animals. Live nauplii were picked and fixed in Lugol's solution to facilitate counting. Values of larval survival (%) were ArcSin-transformed to satisfy normal distribution and homocedasticity. Differences between HS and non-HS treated larval survival in the *Artemia* challenge tests were investigated by performing analysis of variances (ANOVA) using statistical analysis software SPSS® version 11.5 for Windows®.

## 3. Results

### 3.1. Heat shock temperatures

Axenic *Artemia* nauplii that were given a NLHS followed by a recovery period of 6 h at 28 °C performed better in the challenge test (Table 1). Survival of nauplii was significantly higher ($P < 0.05$) in all NLHS treatments, namely HS32, HS37 and HS40, as compared to the controls. However, the HS40 treatment in the absence of challenge caused

Table 1
Average survival (%) of *Artemia* nauplii after 24 h challenge test using $10^7$ cells ml$^{-1}$ of *Vibrio campbellii* in relation to different heat shock (HS) temperature for 30 min exposure with subsequent 6 h and 24 h recovery periods (Experiment 1)

| HS treatments (°C) | A | | B | |
|---|---|---|---|---|
| | Survival (%) | | Survival (%) | |
| | 6 h recovery | 24 h recovery | 6 h recovery | 24 h recovery |
| CTR 28 | 36 ± 4[a] | 14 ± 5[a] | 38 ± 6[a] | 17 ± 6[a] |
| HS 32 | 65 ± 2[b] | 17 ± 4[a] | 63 ± 5[b] | 18 ± 5[a] |
| HS 37 | 70 ± 7[b] | 28 ± 9[a] | 71 ± 2[b] | 24 ± 5[a] |
| HS 40 | 68 ± 8[b] | 27 ± 10[a] | 63 ± 1[b] | 21 ± 8[a] |

For each average, the respective standard deviation is added (mean ± S.D.). Each experiment was repeated twice: A and B. CTR − control without heat treatment (28 °C); HS − heat shock. Values in the same column (for each experiment) showing the same superscript letter are not significantly different ($p > 0.05$).

Table 2
Average survival (%) of *Artemia* nauplii after 24 h challenge test using $10^7$ cells ml$^{-1}$ of *Vibrio campbellii* in function of different recovery periods with non-lethal heat shock temperature of 37 °C for 30 min (Experiment 2)

| Recovery period (hours) | A | | B | |
|---|---|---|---|---|
| | Survival (%) | | Survival (%) | |
| | CTR | HS | CTR | HS |
| 2 h | 33 ± 3[a] | 65 ± 5[a,*] | 37 ± 2[a] | 60 ± 2[a,*] |
| 6 h | 29 ± 5[a] | 63 ± 6[a,*] | 30 ± 6[a] | 65 ± 6[a,*] |
| 12 h | 24 ± 4[a] | 45 ± 5[b,*] | 25 ± 5[a] | 43 ± 2[b,*] |
| 24 h | 9 ± 4[b] | 30 ± 2[c,*] | 13 ± 1[b] | 18 ± 7[c] |

For each average, the respective standard deviation is added (mean ± S.D.). Each experiment was repeated twice: A and B. CTR − control without heat treatment (28 °C); HS − heat shock. Values in the same column (for each experiment) showing the same superscript letter are not significantly different ($P > 0.05$). * indicates significance different between HS and control (non-HS) treatments ($P < 0.05$).

high larval mortality (data not shown) and therefore was not considered to be suitable. High mortality was also observed in the challenge test when nauplii were given a NLHS followed by a 24 h recovery period most probably due to starvation. Based on these results, it was also concluded that a HS from 28 °C to 32 °C for 30 min would be sufficient to boost protective effects of *Artemia* nauplii towards resistance against pathogenic bacteria, but HS37 (with the highest survival rates) was subsequently chosen for use in experiments 2 and 3.

## 3.2. Recovery periods

*Artemia* nauplii that were given a recovery period of 2 h and 6 h after heat treatment showed significantly higher survival rates ($P < 0.05$) in the challenge test as compared to the non-HS controls (Table 2). Larval survival was observed to decline after prolonged recovery periods in both HS and the controls treatments (CTR). A 6 h recovery period (with the largest difference in larval survival between HS and CTR) was chosen for usage in experiment 3.

## 3.3. Duration of heat shock

Significant differences ($P < 0.05$) in larval survival were observed in the challenge test between HS nauplii and the control (Table 3). However, data revealed that the duration of the HS does not have a big impact on the enhancement of the larval survival although a 15 min NLHS might be the minimum. A 30 min NLHS (resulting in the highest survival) was chosen for the following experiments.

## 3.4. Challenge test at optimal NLHS condition

HS treated *Artemia* nauplii were always better protected in the challenge test. Survival more or less doubled by applying a NLHS (37 °C, 30 min, and 6 h recovery). Significant differences in survival rates ($P < 0.05$) were

Table 3
Average survival (%) of *Artemia* nauplii after 24 h challenge test using $10^7$ cells ml$^{-1}$ of *Vibrio campbellii* in function of different heat shock duration with non-lethal heat shock temperature of 37 °C and 6 h recovery period (Experiment 3)

| HS duration (minutes) | A | B |
|---|---|---|
| | Survival (%) | Survival (%) |
| CTR | 36 ± 4[b] | 33 ± 3[b] |
| 15 min | 67 ± 10[a] | 64 ± 4[a] |
| 30 min | 77 ± 6[a] | 74 ± 5[a] |
| 45 min | 76 ± 4[a] | 71 ± 8[a] |
| 60 min | 74 ± 9[a] | 65 ± 5[a] |

For each average, the respective standard deviation is added (mean ± S.D.). Each experiment was repeated twice: A and B. CTR − control without heat treatment (28 °C); HS − heat shock. Values in the same column (for each experiment) showing the same superscript letter are not significantly different ($P > 0.05$).
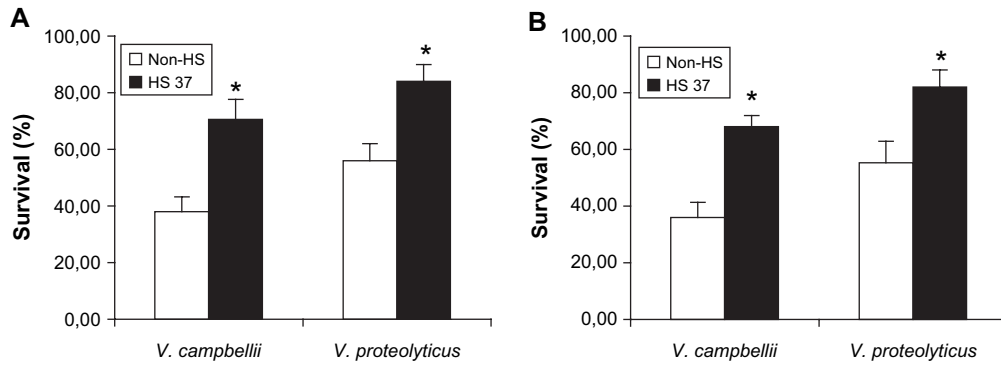
Fig. 1. Survival (%) after 24 h challenge test using $10^7$ cells ml$^{-1}$ of *Vibrio campbellii* and *Vibrio proteolyticus* at optimal HS condition (NLHS at 37 °C for 30 min and 6 h recovery period). Each experiment was repeated twice: A and B. * indicates significant difference ($P < 0.05$) between HS treatment and control (Non-HS).

observed between controls and HS treated *Artemia* in both challenge tests (*V. campbellii* and *V. proteolyticus*) (Fig. 1A,B). Data obtained showed that the overall survival of both the controls and HS treated nauplii were slightly higher when challenged with *V. proteolyticus* as compared to *V. campbellii*, suggesting that *V. proteolyticus* is less virulent. Both the negative control treatments, which consisted of unchallenged HS and non-HS animals recorded no larval mortalities and insignificant differences ($P > 0.05$) in the larval survival (data not shown), thus verifying that the beneficial increase of larval survival in the challenge test is due to cross-protection.

### 3.5. HSP expression

Western blot detection of HSP-70 in the controls (non-HS) and heat shocked *Artemia* showed a cross-reaction with one protein band of approximately 70-kDa. There was a significant increase in the expression of HSP-70 in comparison to the control using equal amount of proteins (Fig. 2A,B) or equal amount of aliquots (result not shown).

## 4. Discussion

The NLHS conditions were optimised as a way to protect *Artemia* nauplii against pathogenic bacteria. In this particular study on the effects of a non-lethal heat shock, gnotobiotically-grown *Artemia* nauplii were used throughout the experiment. The established gnotobiotic system was previously used in the evaluation of different yeast cell-wall mutants and microalgal strains as feed in *Artemia* [25]. With the same culture system, the immunostimulatory nature
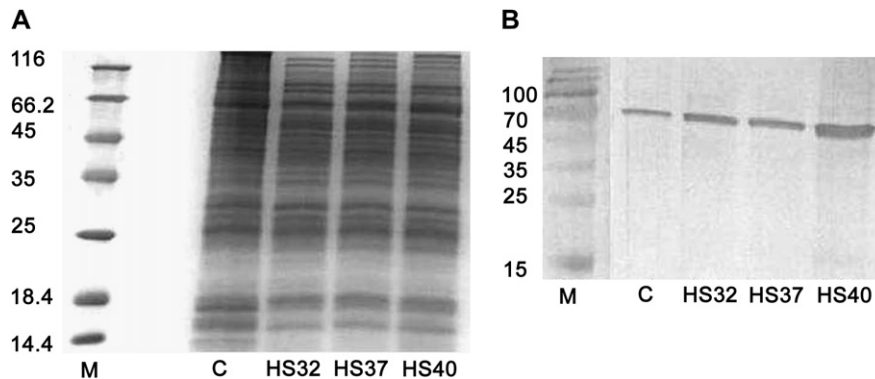


Fig. 2. Protein profiles of control (C) and heat-shocked (HS) treated *Artemia franciscana* larvae. (A) Approximately (50 µg µl$^{-1}$) of larval protein was loaded in each lane of the gel and stained with Coomassie brilliant blue. (B) Alternatively, the gel was blotted and Western blot analysis was performed to detect members of the HSP-70 family. Protein standards (M) in kilodaltons are shown on the left of the figures.

of β-glucans and baker's yeast in a challenge test of *Artemia* was verified [28,31]. This gnotobiotic system provides an excellent and fully controllable host-pathogen environment. Furthermore it facilitates the investigation and verification of the effects of a NLHS towards a bacterial pathogen in *Artemia* nauplii. The experimental system avoids interference of factors such as modified physiology of host-associated microorganisms or even shift in microbial composition as the NLHS was applied in axenically hatched *Artemia* nauplii.

As often discussed, most physiological and environmental stressors may lead to impaired survival of cells or animals [32−35]. In addition, long-term stress increases the susceptibility to infectious diseases, for example in fish [36] and shrimp [37]. However, a remarkable increase in the larval survival was observed when a short-term stress was imposed on the challenged animals. HS-treated *Artemia* nauplii were shown to perform better in the *Vibrio* challenge test (*V. campbelli* and *V. proteolyticus*), while the controls (non-HS treated) were generally more susceptible to pathogenic *Vibrios*. The optimal NLHS conditions consisted of temperature increases from 28 °C to 37 °C for 30 min, with subsequent recovery for 6 h at 28 °C before exposure to the pathogen. Survival could be doubled in the *Vibrio* challenge test. An extended duration of the NLHS does not further increase the effects, while prolonged recovery periods showed a relative increase in mortality both in controls and treatments, which may be due to starvation as animals were not fed throughout the experiments. In agreement with the observation made by Marques et al. [28], it was also noted that *V. campbelli* is slightly more virulent than *V. proteolyticus* as the mortality is higher in the challenge test.

Using Western blot analysis, it was documented [38] that the application of a NLHS at optimal conditions increased the expression of the 70-kDa families of stress proteins (Fig. 2B). As previously documented, a 5 min HS at 40 °C in 24 h larvae is sufficient to enhance the subsequent expression of p68 and p89 in nauplii. Two-dimensional IEF/SDS-PAGE analysis coupled to immunoblotting showed the p68 to consist of inducible HSP-68 and HSP-70 and constitutively synthesized HSC-70 forms [38]. By one dimensional/SDS-PAGE and immunoblotting, it was possible to detect the up-regulation of the HSP-70 family. In adult *Artemia franciscana*, the low level of constitutive HSC-70 and HSP-67 was still strongly up-regulated by a sub-lethal HS at 37 °C for 30 min [14]. Recent studies documented that isolated erythrocytes from the eurythermal species, *Fundulus heteroclitus* reached a maximum induction rate in the first 6 h of recovery [39] while maximal induction of HSP-70 were observed during a 6−9 h recovery in an endothermic cell line of the Chinese hamster ovary (CHO) cells culture [40], similar to the recovery period described by us, which in our case results in maximum protection against the pathogens.

Many studies continue to disclose the remarkable diversity and functions of HSPs in *Artemia*. Cross protection against a secondary insult were frequently observed after an initial stress. Concomitant with this stress, the expression of HSPs was observed. For instance, besides verifying that up-regulation of HSP-70 was associated with the protection of *Artemia* (nauplii and adult) against extreme thermal stresses [41−45], other HSPs such as p26 were also postulated to protect mammalian cells (Cos-1) against oxidative damage [23] and further acts synergistically with trehalose to confer desiccation tolerance in mammalian cells [24]. In this study, we verified if a primary stress by an application of a NLHS could protect *Artemia* nauplii against a subsequent pathogenic bacteria attack. Also in this case of gnotobiotic conditions, an up-regulation of the HSP-70 protein family by the applied NLHS was observed. Based on the current observations, the beneficial effects on survival can possibly be explained by an activation of the innate immune system of *Artemia* nauplii in the short term, while we anticipate, based on the many reports on this subject, that long-term stress for many animals might increase the vulnerability to a pathogenic attack.

Piles of information indicate that stress-regulated HSPs are capable of inducing strong immune responses in vertebrates [11,45]. Acting as a ''red flag'', extracellular HSPs, as a result of necrosis, are postulated to alarm the immune system on the existence of a foreign invader and thereby activate a prompt and potent immune response (both innate and adaptive immunity) enabling the host to combat the disease [46]. The lack of data and research on short-term stress and the immune response in invertebrates, particularly in crustaceans, may have hampered the establishment of a clear association between HSPs production and activation of the innate immune response. Nevertheless, de la Vega et al. [47] have recently demonstrated that a short-term hyperthermic treatment and the associated up-regulation of the HSP-70 protein family might play a substantial role in the beneficial reduction in the replication of gill-associated virus (GAV) in *Penaeus monodon*, postulating that HSPs play a vital role in the immune response of the whole organism.

The data reported in this paper do not provide evidence that the innate immune system in *Artemia* was triggered by HSP-70 expression. They merely illustrate that a NLHS, associated with a proven up-regulation

of HSP-70 and probably other HSPs results in the protection against two pathogens. In view of the mounting evidence that HSPs can stimulate the innate immune response in vertebrates, we would like to put forward the hypothesis that HSPs are directly involved in triggering the innate immune response in the invertebrate *Artemia*. Unequivocal evidence for this hypothesis awaits the development of the proper genetic tools such as knock-out *Artemia* or the development of RNA interference (RNAi) technology [48] to add to the currently available gnotobiotic system.

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