Environmental heat stress induces epigenetic transgenerational inheritance of robustness in parthenogenetic *Artemia* model

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ABSTRACT **The notion that phenotypic traits emerging from environmental experiences are heritable remains under debate. However, the recent report of nonmendelian transgenerational epigenetic inheritance, *i.e.*, the inheritance of traits not determined by the DNA sequence, might make such a phenomenon plausible. In our study, by carrying out common garden experiments, we could provide clear evidences that, on exposure to nonlethal heat shocks, a parental population of parthenogenetic (all female) *Artemia* (originating from one single female) experiences an increase in levels of Hsp70 production, tolerance toward lethal heat stress, and resistance against pathogenic *Vibrio campbellii*. Interestingly, these acquired phenotypic traits were transmitted to three successive generations, none of which were exposed to the parental stressor. This transgenerational inheritance of the acquired traits was associated with altered levels of global DNA methylation and acetylated histones H3 and H4 in the heat-shocked group compared to the control group, where both the parental and successive generations were reared at standard temperature. These results indicated that epigenetic mechanisms, such as global DNA methylation and histones H3 and H4 acetylation, have particular dynamics that are crucial in the heritability of the acquired adaptive phenotypic traits across generations.—Norouzitallab, P., Baruah, K., Vandegehuchte, M., Van Stappen, G., Catania, F., Vanden Bussche, J., Vanhaecke, L., Sorgeloos, P., Bossier, P. Environmental heat stress induces epigenetic transgenerational inheritance of robustness in parthenogenetic *Artemia* model. *FASEB J.* 28, 3552–3563 (2014). www.fasebj.org**

Key Words: heat shock protein 70 · DNA methylation · histone modification · stress resistance · heritability

LIVING ORGANISMS, BOTH terrestrial and aquatic, are constantly influenced by a multitude of environmental (abiotic and biotic) stressors to which they react with a battery of responses (1). The plastic responses of a population to environmental insults are typically thought to be associated with its genetic diversity, with higher levels of genetic diversity providing increased adaptive potentials (2, 3). This idea originated from the general consensus that most phenotypes determining stress tolerance have a genetic basis and are subject to darwinian natural selection and mendelian inheritance (4). While there is no doubt about the validity of these principles, epigenetic modification has recently been suggested as an additional source of potentially adaptive response (5, 6). Epigenetic inheritance refers to mechanisms that permit the development and stable transmission of potentially heritable traits to a subsequent generation or generations without any alteration in the DNA sequence (7). This nongenetic transgenerational transfer of phenotypes is regulated by numerous mechanisms, such as DNA methylation, histone modifications and post-transcriptional gene regulation by noncoding RNA (8, 9). Epigenetic modifications can be influenced directly by environmental cues, potentially allowing an organism to respond and adapt to environmental fluctuation over a short timescale (4, 10–12). The notion that acquired traits induced by environmental cues could become heritable dates back to Lamarck and has been controversial ever since (13, 14). Over the past century, various researchers using different experimental models have attempted to demonstrate the direct influence of the parental environment on the phenotypes and fitness of the offspring (15–17). However, other authors demonstrated that stress-induced epigenetic modifications are not always transmitted to subsequent nonexposed generations (18, 19). At present, little evidence appears in the

Abbreviations: dG, deoxyguanosine; Hsp, heat-shock protein; mdC, 5-methyl-2′-deoxycytidine; PVDF, polyvinylidene fluoride; UHPLC, ultra-high performance liquid chromatography

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literature that unequivocally demonstrates transgenerational epigenetic inheritance of environmentally induced phenotypes (4, 20). Indeed, many studies failed to exclude parental effects, could not or did not demonstrate the persistence of traits across generations, were not sufficiently replicated to distinguish stochastic effects from treatment, or did not integrate phenotypic characterization with more detailed molecular and genetic analysis (4, 21). Studies systematically examining such a large range of generations under stress and control conditions are still scarce but are crucial to understand if and how environmental conditions can induce heritable phenotypic changes, potentially through epigenetic inheritance.

Selection of an appropriate animal model that allows to better delineate the contribution of epigenetics to the inheritance of acquired traits is of high importance. The apomictic parthenogenetic *Artemia*, an aquatic invertebrate, represents an exceptional model for studying transgenerational epigenetic inheritance. It has a short generation time and can parthenogenetically generate clonal offspring, a feature that minimizes genetic variability in the experiments. Moreover, this invertebrate possesses two independent reproductive pathways that allow it to produce either dormant eggs (cysts) by oviparous reproduction under unfavorable environmental conditions or swimming larvae ovoviviparously under favorable conditions (22, 23). The produced cysts can be stored for several weeks without losing viability and can be hatched on demand. The cysts are also a powerful gene bank that allows preserving the genetic memory of the parental population. Moreover, the storability of cysts allows for "common garden experiments," *i.e.*, the simultaneous testing of animals from subsequent generations (24), a convenient property if one seeks to minimize environmental interference in experiments.

Among the environmental stressors animals can encounter, pathogen attack and heat stress are quite common. In addition, heat stress is expected to increase in frequency in the coming decades as a consequence of climate change (25). On exposure to heat stress, animal's cells express a family of proteins termed heat-shock proteins (Hsps), whose function is to protect the cells from stress-induced damages by maintaining protein biogenesis and homeostasis (26, 27). In our previous study, we have demonstrated that exposure of *Artemia* to a nonlethal heat stress at 37°C for 30 min followed by 6 h recovery induced Hsp70 production within the animals and conferred protection against subsequent lethal stress (41°C for 20 min) or pathogenic vibrios (*Vibrio campbellii, Vibrio proteolyticus*; refs. 27, 28). In addition, in another study, it was shown that heat shock induces a series of epigenetic modifications, providing evidence for the existence of a heat stress-related histone code (29).

Here, using a population of parthenogenetic *Artemia* obtained for a single female, we investigated the effects that environmental stressors can have on the emergence and transgenerational inheritance of phenotypic traits in *Artemia*. After daily exposure of a parental population of parthenogenetic *Artemia* to nonlethal heat shocks at early life stages, we assessed the phenotypic and epigenetic changes in the treated population and in 3 subsequent (untreated) generations. We found that, on exposure to nonlethal heat shocks, parental *Artemia* experience an increase in levels of Hsp70 production, tolerance toward lethal heat stress, and resistance against pathogenic *V. campbellii*. These acquired traits were transmitted to 3 successive generations, none of which was exposed to the parental stressor. The transgenerational inheritance of the acquired phenotypes was associated with significantly altered levels of global DNA methylation and acetylated histones H3 and H4 in the treatment group compared to a control group, where both the parental and its successive generations were reared at standard temperature.

MATERIALS AND METHODS

Culture of single female offspring

A population of apomictic parthenogenetic *Artemia* was obtained ovoviviparously from a single tetraploid female of Megalon Embolon saltworks in Greece (30). The animals were grown till adult in 35 g/L artificial seawater under controlled laboratory conditions (constant temperature 28°C, light intensity 27 $\mu mol/m^2/s$ for 24 h/d, adequate aeration). Throughout the culture period, the animals were fed *ad libitum* everyday with live green microalgae *Tetraselmis suecica* obtained from the Culture Collection of Algae and Protozoa (CCAP) Department (Dunstaffnage Marine Laboratory, Oban, UK). Dead animals were removed daily, and complete water exchange was performed 2×/wk.

Artemia cyst production and hatching

Under nonoptimal environmental conditions, such as high salinity or low oxygen, *Artemia* switches from an ovoviviparous to an oviparous mode of reproduction. To induce the *Artemia* to produce cysts, the salinity of the culture water was gradually increased from 35 to 80 g/L over a period of 10 d. Deposited cysts enter in a state of diapause and are not ready for hatching (31, 32). To terminate cyst diapause, the collected cysts were dehydrated in a saturated NaCl brine solution and exposed to a hibernation of 3 mo at −20°C and then stored as activated cysts at 4°C until use. For hatching, the activated cysts were washed with sterile distilled water to remove the brine, rehydrated in sterile distilled water for 1 h, and then transferred to a 1 L glass bottle containing 35 g/L sterile artificial seawater. Following incubation at 28°C under constant illumination for 48 h, the emerged instar II larvae (stage at which mouth is open for ingestion of food) were used for experimental treatments.

Daily nonlethal heat shock treatment of the parental (F0) generation

A group of 1800 instar II larvae was distributed in 2 groups (treatment and control), each with 3 replicates. Each group was maintained in a 2 L glass bottle containing sterile artificial seawater (35 g/L), maintained at 28°C under constant illumination (~27 $\mu mol/m^2/s$) and aeration. For daily hyperthermic treatments, *Artemia* larvae were exposed to 2 nonlethal heat shocks starting from d 2 posthatching and continued for 14 d ahead of the reproductive period (under standard laboratory conditions, *Artemia's* uterus develops on d

16 posthatching). Stress exposure at early life stages only was applied to ensure that the uterus carrying the cysts/embryos was not directly exposed to the experimental stress conditions. The first heat shock was given at 35°C for 30 min, followed by a recovery period of 75 min at 28°C. The second heat shock was applied at 38°C for another 30 min. For this purpose, the nauplii from the 2 L glass bottles were collected over a sieve (250 μm), rinsed several times with sterile seawater to reduce bacterial load, and then resuspended in 500 ml glass bottles that contained sterile artificial sea water (stocking density, ~4 animals/ml) maintained at 28°C. The animals were exposed to a nonlethal heat shock at a Δt rate of 7°C/min in a preheated and controlled water bath system with thermostat heaters accurate at ±0.01°C. Heat-shocked *Artemia* were slowly acclimated back to 28°C (Δt=3°C/min). After the second heat shock, animals were collected again over sieves, rinsed with sterile seawater, and then resuspended in the 2 L bottles containing 1.8 L of sterile seawater (28°C). The control group, cultured isothermally at 28°C, went through the same handling process. This regime of nonlethal heat shocks was established based on preliminary studies (results not shown).

Production and collection of the progenies as nauplii and cysts

Approximately 23 d posthatching, adult females started producing larvae (F1 generation). The F1 larvae from 3 replicates were isolated, pooled, and further cultured isothermally at 28°C to maturity, after which the F2 larvae were collected. The experiment was continued until, and including, the F3 generation. The adult parental (F0), F1, and F2 animals (from d 28 posthatching) in both control and treatment groups were induced to produce F1, F2, and F3 cysts, respectively, by gradually increasing the salinity from 35 to 80 g/L over a 10 d period. An aliquot of the larvae collected during each generation was subjected to thermal and *V. campbellii* challenge tests as described below. This entire procedure of nonlethal heat-shock treatment of the F0 generation followed by offspring production and challenge assays was repeated once to confirm the reproducibility of the results. The detailed experimental design is shown schematically in **Fig. 1**.

Thermotolerance assay

Artemia larvae (instar II stage) collected from each generation (F1 to F3) were rinsed several times with sterile (artificial) seawater and then resuspended in 35 g/L sterile seawater (28°C). Groups of 20 larvae were transferred in 7 replicates into separate sterile 40 ml glass tubes that contained 30 ml of 35 g/L sterile seawater (28°C). The larvae were challenged with a lethal heat shock at 42°C for 15 min (Δt=7°C/min) and then transferred to 28°C. Resistance to this thermal shock was determined by counting the live nauplii at every indicated time interval (33).

V. campbellii challenge assay

V. campbellii strain LMG21363, stored in 40% glycerol at −80°C, was incubated at 28°C for 24 h on Marine Agar 2216 (Difco Laboratories, Detroit, MI, USA) and then grown to log phase in Marine Broth 2216 (Difco Laboratories) by incubation at 28°C with shaking. The bacteria were transferred to a sterile tube, centrifuged at 2200 *g* for 15 min at 28°C, suspended in filtered (0.2 μm) sterile seawater, and immediately used in challenge experiments. Bacteria cell numbers were determined spectrophotometrically at 550 nm according to McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells/ml. Groups of 20 *Artemia* nauplii (from each generation) were transferred in 7 replicates into separate 40 ml glass tubes containing 30 ml of 3.5 g/L sterile seawater. They were then challenged with *V. campbellii* at 10^7 cells/ml (34). The survival of *Artemia* was scored at every indicated time interval.

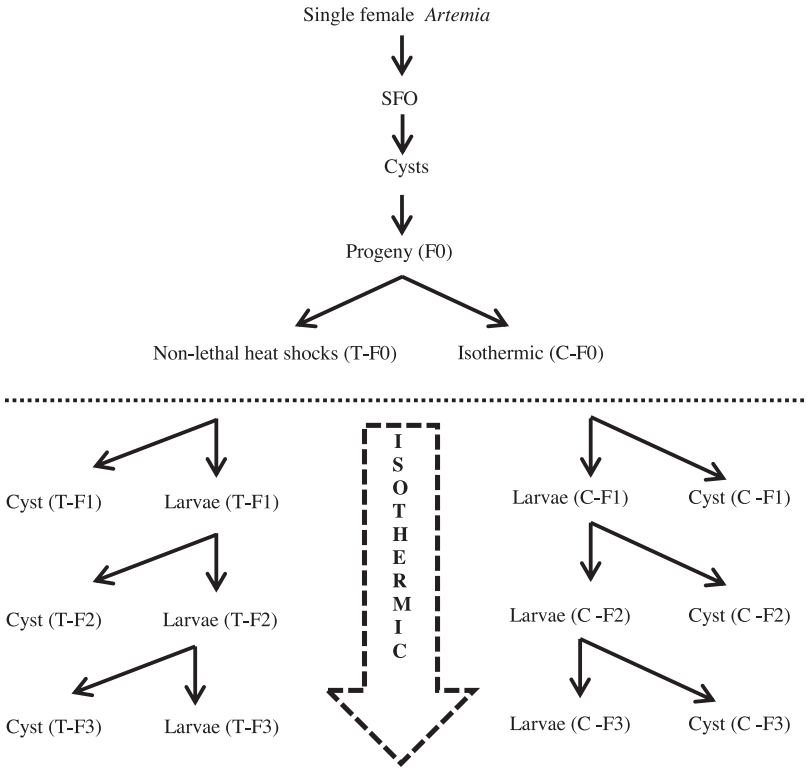

Figure 1. Scheme of the experiment. A single female parthenogenetic *Artemia* was propagated to the next generation [*i.e.*, single female offspring (SFO)] under normal growth conditions. The SFO (all females) were reared normally until adulthood, and were then induced to produce cysts (F0). On hatching, the F0 progeny were divided into 2 groups. One group was exposed daily to 2 nonlethal heat shocks (the first at 35°C for 30 min, followed by 75 min recovery period at 28°C, and the second at 38°C for another 30 min) starting from d 2 posthatching until d 15 posthatching; *i.e.*, ahead of the reproductive period (T-F0). The other group was grown isothermally at 28°C (C-F0). Approximately 23 d posthatching, the parental (F0) females from the treatment (T-F0) and control (C-F0) groups produced their next-generation larvae; *i.e.*, T-F1 and C-F1, respectively. The F1 larvae from both groups were further cultured isothermally at 28°C to maturity, after which the F2 larvae were collected. The experiment was continued until, and including, the F3 generation. The adult parental (F0), F1 and F2 *Artemia* in both the control and treatment were induced to produce F1, F2 and F3 cysts, respectively, by gradually increasing the salinity from 35 to 80 g/L.

Common garden test

For the experiment, age- and size-synchronized larvae were used, which were obtained by hatching the oviparously produced cysts as described previously (35). Briefly, *Artemia* cysts from the control and treatment groups collected from all the generations during the experiment were hatched simultaneously by incubating in 35 g/L sterile (artificial) seawater at 28°C for 48 h. The required number of emerged larvae was used for thermal and *Vibrio* challenge assays as described above. The remaining numbers were further reared isothermally (in triplicates) till juvenile stage. Once at juvenile stage, animals were sampled, pooled, rinsed in sterile distilled water, immediately frozen in liquid nitrogen, and stored at −80°C for analysis of epigenetic marks.

Protein extraction

Artemia juveniles (16 d old, 18 individuals) were homogenized by rapid agitation with a required amount of 0.5 mm diameter glass beads in cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, and 40 mM HEPES, pH 7.4; ref. 34) containing 10% protease inhibitor cocktail (Sigma-Aldrich, Diegem, Belgium). Subsequent to centrifugation at 4000 *g* for 5 min at 4°C, supernatant was collected and protein concentration was determined following the Bradford method (36) using bovine serum albumin as standard.

Total histone extraction

Histone extraction of *Artemia* samples (16-d-old juveniles, 18 individuals) was carried out using the EpiSeeker histone extraction kit (ab113476; Abcam, Cambridge, UK) according to the manufacture's instruction. Histone concentration was determined following the Bradford method using bovine serum albumin as standard (36).

Western blot analysis of Hsp70 and histone H4 total acetylation

Histone or protein samples were combined with loading buffer, vortexed, heated at 95°C for 5 min, and then electrophoresed in 10% SDS-PAGE gel, with each lane receiving equivalent amounts of protein (10 μg). HeLa (heat-shocked) cells (6 μg; Enzo Life Sciences, Farmingdale, NY, USA) were loaded in one well to serve as a positive control and for further calculation of the amount of Hsp70 and histone H4 acetylation in the samples. Gels were then transferred to polyvinylidene fluoride (PVDF) membranes (Immun-Blot; Bio-Rad, Nazareth Eke, Belgium) for antibody probing. Membranes were incubated with blocking buffer [50 ml of 1× phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin] for 60 min at room temperature. For Hsp70 analysis, the membrane was incubated with mouse monoclonal Hsp70 antibody, clone 3A3 (Affinity BioReagents Inc., Golden, CO, USA), which recognizes both constitutive and inducible Hsp70 (27), at the recommended dilution of 1:5000. Horseradish peroxidase-conjugated donkey anti-mouse IgG was used as secondary antibody at the recommended dilution of 1:2500 (Affinity BioReagents). For histone H4 acetylation analysis, the membrane was incubated with rabbit polyclonal anti-acetylated protein primary antibody (ab193; Abcam), which is sensitive to histone H4 acetylation at the recommended dilution of 1:1000. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Gentaur, Kampenhout, Belgium) was used as secondary antibody at the recommended dilution of 1:2500. The membranes were then treated with enhanced chemiluminescence (ECL) reagent (GE Healthcare, Little Chalfont, UK), with subsequent signal detection by means of a ChemiDoc MP Imaging System (Bio-Rad). The relative signal intensity was quantified by densitometry with Bio-Rad Image Lab 4.1 software.

Analysis of histone H3 total acetylation

Histone (5 μg) samples from all the generations (F0 to F3) of the control and treatment groups were analyzed for histone H3 total acetylation using the EpiSeeker histone H3 total acetylation detection fast kit, fluorometric (ab131561; Abcam) according to the manufacturer's instructions.

Global DNA methylation

DNA was extracted from 16-d-old *Artemia* juveniles (10 individuals) with a ReliaPrep kit (Promega, Leiden, The Netherlands) following the manufacturer's protocol. DNA (1 μg) was enzymatically digested to nucleosides [including 5-methyl-2′-deoxycytidine (mdC)] with benzonase (Merck, Darmstadt, Germany), phosphodiesterase I (Sigma-Aldrich) and alkaline phosphatase (Sigma-Aldrich) according to Quinlivan and Gregory (37). Finally, nucleosides were separated, detected, and quantified on an Accela ultra-high performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, San Jose, CA, USA) with a Waters Acquity high-strength silica (HSS) T3 column (2.1×100 mm, 1.8 μm; Waters Corp., Milford, MA, USA) coupled to a TSQ Vantage triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific) by means of a heated electrospray ionization interface (HESI-II). The mobile phases consisted of 0.08% aqueous formic acid (solution A) and 0.08% formic acid in acetonitrile (solution B). A stepwise linear gradient was used, starting from 99% solution A and going up to 90% solution B. Mass/charge (*m/z*) ratios of detected ions for mdC were 242.076 (precursor ion) > 126.032 (product ion); for deoxyguanosine (dG) these were 268.058 (precursor ion) > 134.983, 152.021 (product ions). These product ions were used for quantifying the levels of mdC and dG in the digests, based on the analysis of external standard series of mdC in a nucleoside mixture containing dG, dA, and T, and mixtures of dG, dA, dC, and T. The concentration ratio [mdC]/[dG] was used to calculate the fraction of methylated cytosines in *Artemia* DNA, as described by Vandegehuchte *et al.* (38).

Statistical analysis

At each generation, significant differences in the epigenetic marks (DNA methylation and histone H3 acetylation) between the control and the treatment were determined by Student's *t* test analysis using the Statistical Package for the Social Sciences (SPSS) 19.0 (IBM, Armonk, NY, USA). Survival data were analyzed for statistical differences by subjecting the data to logistic regression analysis using GenStat 16 (VSN International, Hemel Hempstead, UK). Significance level was set at $P < 0.05$.

RESULTS

Progeny of daily nonlethal heat-shocked ancestors have increased levels of Hsp70 in the 3 subsequent unstressed generations

We have shown previously that exposing *Artemia* to a nonlethal heat shock induces Hsp70 production within

the animal (27). Here, we tested whether the phenomenon of nonlethal heat-shock-mediated Hsp70 induction in parental generation of *Artemia* could be transmitted to its progeny across 3 subsequent generations. To this end, we carried out Western blot analysis on protein extracts from the (heat-shocked) parental *Artemia* and their isothermally grown F1, F2, and F3 progeny. We found that exposure of the parental *Artemia* to daily nonlethal heat shocks (T-F0) markedly increased Hsp70 production relative to the non-heat-shocked control groups (C-F0; **Fig. 2**). Remarkably, this increased Hsp70 level was also observed in the isothermically reared progeny for 3 subsequent generations. In these individuals, the level of Hsp70 remains consistently higher across generations and appears to fluctuate, initially decreasing (generation F1 and F2) and then spiking (generation F3). More specifically, T-F0 animals that were daily exposed to nonlethal heat shocks exhibited a 7.3-fold increase in Hsp70 production level compared to the respective C-F0 control. This relative increase in the Hsp70 level reached 4.6-fold at the T-F1 generation and 2.4-fold at the T-F2 generation, whereas at the T-F3 generation, the level of Hsp70 increased once more compared to the control C-F3 progeny by a factor of 17-fold.

Progeny of daily nonlethal heat-shocked ancestors exhibit increased resistance against *V. campbellii* in the 3 subsequent unstressed generations

Since elevated Hsp70 (or nonlethal heat shock) has been reported to induce resistance against biotic and abiotic stressors in *Artemia* (28, 39, 40), we next investigated whether the parental T-F0 population also could transmit the acquired phenotype of resistance toward pathogenic *V. campbellii* to its (isothermally reared) subsequent generations. To address this, *Artemia* larvae that were produced ovoviviparously at every generation were challenged with pathogenic *V. campbellii* as described in Materials and Methods. As shown in **Fig. 3*A***, the nonexposed T-F1 progeny of F0 *Artemia* that were exposed daily to nonlethal heat shocks exhibited a significantly higher survival when challenged to *V. campbellii* than did the respective progeny of control C-F1 *Artemia*. This relatively higher survival extended to *Vibrio*-challenged T-F2 and T-F3 progeny, whose grandparents and great grandparents, respectively, were exposed to nonlethal heat shocks (Fig. 3*B*, *C*). In the following experiment, we validated the results of the *Vibrio*-challenge assay by carrying out a common garden experiment, where *Artemia* cysts from all 3 (F1 to F3) generations were hatched, and the obtained larvae were reared under similar laboratory conditions. Such experiments are useful as they can provide a more mechanistic understanding for the causes of phenotypic variation in the 3 successive generations in response to heat treatment of the F0 generation. The results showed a similar survival trend as observed for the animals from ovoviviparous reproduction (Fig. 3*D*–*F*). These data from the common garden experiment reported here, together with the data from the above (generation-specific) challenge tests, suggested that nonlethal heat-shock treatment of parental *Artemia* also leads to transgenerationally inherited increase in *Artemia* resistance against the *Vibrio* pathogen.

Progeny of daily nonlethal heat-shocked ancestors exhibit increased resistance to lethal heat shock in the 3 subsequent unstressed generations

To substantiate the results of the above challenge studies, we tested how the progeny from the F1 to F3 generations of *Artemia* that were exposed to nonlethal heat shocks, and the respective progeny of control *Artemia*, respond to a heat shock that is lethal for *Artemia* grown at standard temperature. We found that, on exposure to a typically lethal heat shock (42°C for 15 min), the survival of the T-F1 progeny of nonlethal heat-shocked F0 parents was significantly higher than

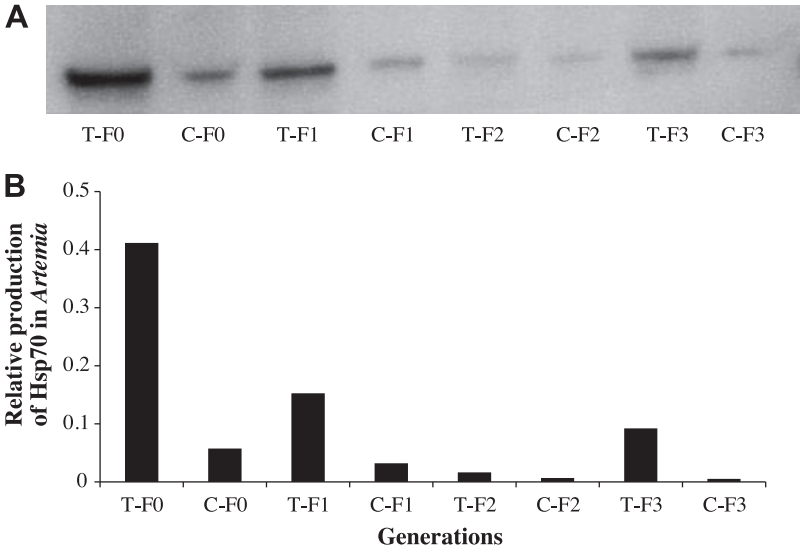

Figure 2. Immunoblot analysis showing Hsp70 production level in *Artemia* from the F0 parental generation and 3 successive generations of progeny (common garden test). *Artemia* F0 larvae were exposed to either daily nonlethal heat shocks prior to uterus development (T, treatment) or were not treated (C, control). Thereafter, *Artemia* from each group were grown isothermally to produce F1 cysts and larvae. The F1 larvae were further grown isothermally to produce F2 cysts and nauplii, from which F3 cysts were produced. Cysts from F1 to F3 were hatched simultaneously; the larvae produced were grown isothermally till juveniles and sampled. *A*) Protein extracted from F0 to F3 juveniles was resolved by SDS-PAGE and then transferred to a PVDF membrane and probed with an antibody specific to *Artemia* Hsp70. *Artemia* protein (10 μg) was loaded in each lane. HeLa (heat shocked) cells (6 μg) were loaded onto one well to serve as a positive control and for calculating the amount of HSP70 in the sample. *B*) Quantitative analysis of Hsp70 in *Artemia*, expressed as nanograms per microgram of protein.

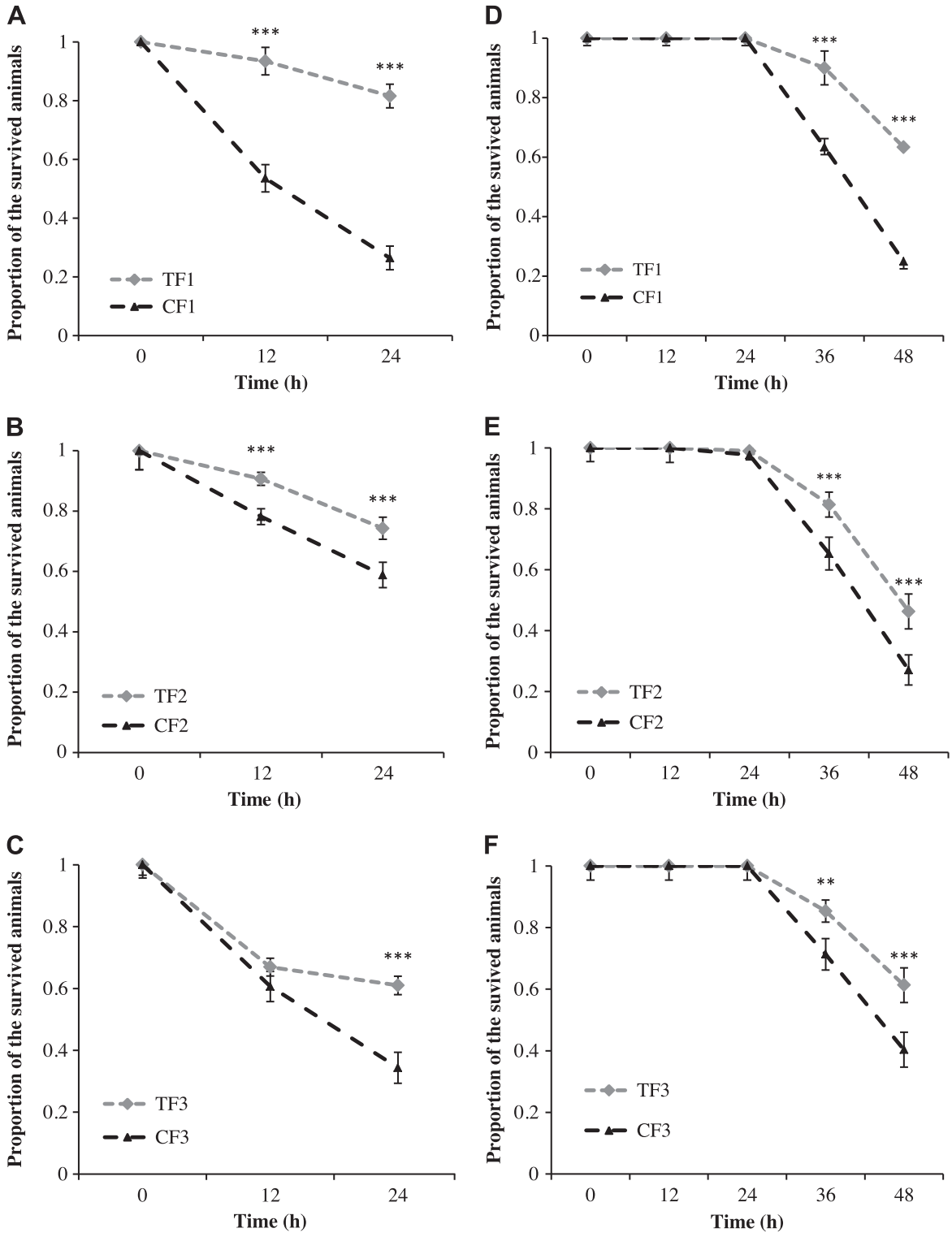

Figure 3. Proportion of the surviving *Artemia* larvae challenged with *V. campbellii*. See Fig. 2 for explanation of the treatment (T) and control (C) groups. *A–C*) At every generation [F1 (*A*), F2 (*B*), and F3 (*C*)], the ovoviviparously produced larvae (at instar II stage) were challenged with *V. campbellii* at 10^7 cells/ml and survival was scored at 12 h intervals. *D–F*) Cysts from generations F1 (*D*), F2 (*E*), and F3 (*F*), were hatched simultaneously, after which the larvae were challenged with *V. campbellii*, and survival was scored (common garden test). Values are presented as means ± SE (n=3). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ *vs.* corresponding control group.

that of the C-F1 progeny of control parents. This result implies that the T-F1 progeny of the nonlethal heat-shocked parents have higher thermotolerance than their respective C-F1 controls (**Fig. 4*A***). Interestingly, the increase in thermotolerance (with respect to the control groups) was similarly exhibited by T-F2 and T-F3 progeny whose T-F0 ancestors were treated with nonlethal heat shocks (Fig. 4*B, C*). It is worth noting

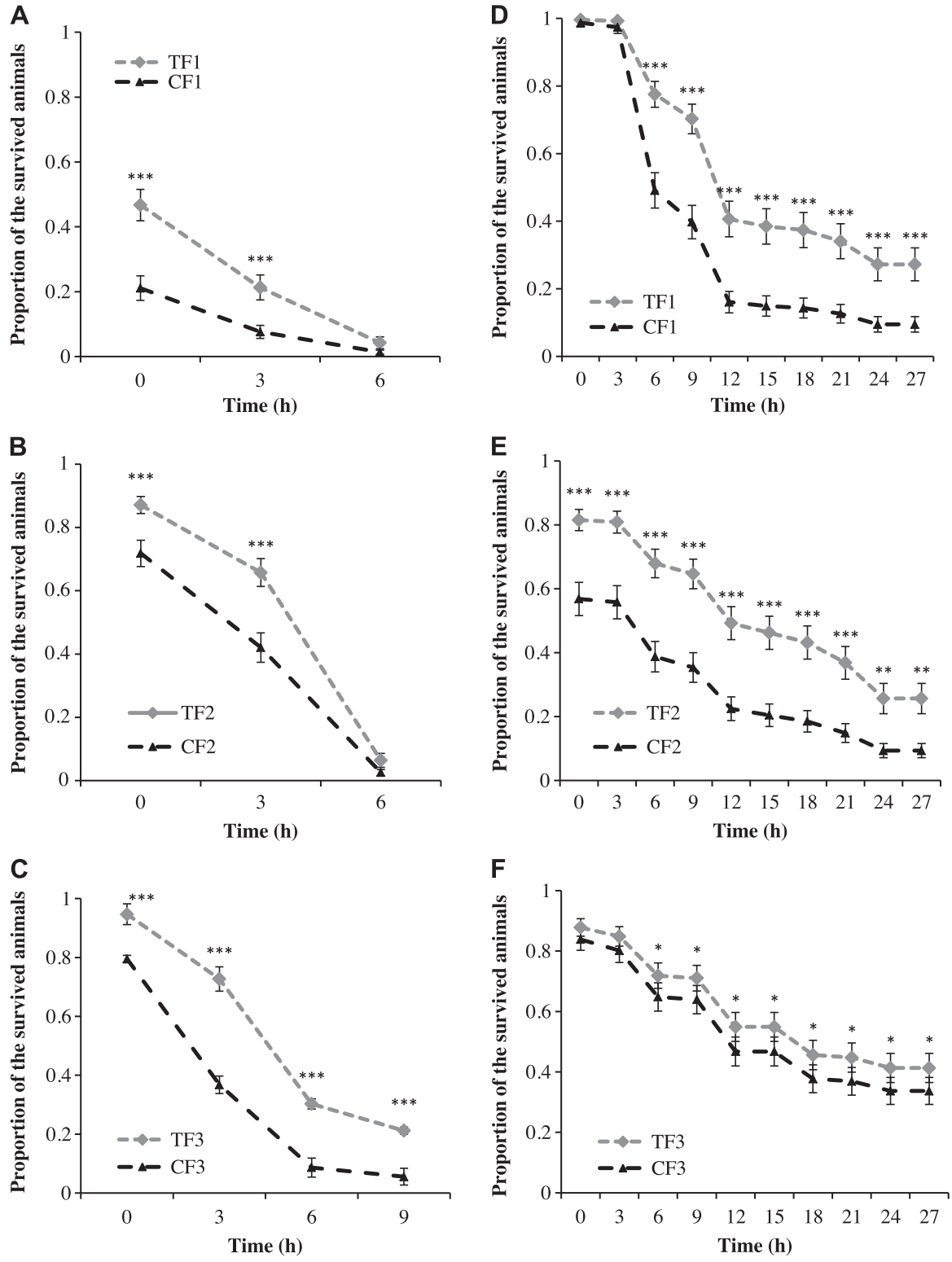

Figure 4. Proportion of the surviving *Artemia* larvae challenged with lethal heat shock. See Fig. 2 for explanation of the treatment (T) and control (C) groups. *A–C*) At every generation [F1 (*A*), F2 (*B*), and F3 (*C*)], the ovoviviparously produced larvae (at instar II stage) were challenged with lethal heat shock at 42°C for 15 min, and survival was scored at 3 h intervals. *D–F*) Cysts from generations F1 (*D*), F2 (*E*), and F3 (*F*), were hatched simultaneously, after which the larvae were challenged with *V. campbelii*, and survival was scored (common garden test). Values are presented as means ± SE (n=3). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ *vs.* corresponding control group.

that the same phenotype was displayed by *Artemia* larvae obtained from ovoviviparous reproduction and by the larvae obtained from cysts in a common garden test (Fig. 4*D–F*). These results indicate that, much like the increased Hsp70 production and improved resistance toward pathogenic *V. campbellii*, the nonlethal heat-

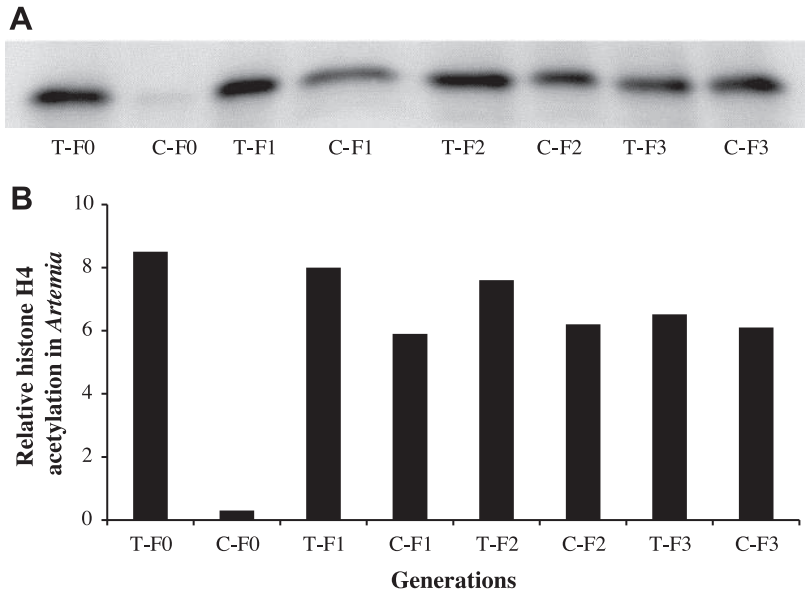

Figure 5. Immunoblot analysis showing acetylation level of histone H4 in the parental *Artemia* and 3 successive generations of progeny (common garden test). See Fig. 2 for explanation of the treatment (T) and control (C) groups. *A*) Total histone extracted from the generation F0 to F3 juveniles was resolved by SDS-PAGE and then either stained with Coomassie Biosafe or transferred to PVDF membrane and probed with antibody that recognizes acetylated lysines at histone H4. *Artemia* histone protein (10 μg) was loaded in each lane. HeLa cells (6 μg) were loaded onto one well to serve as a positive control and for calculating the amount of histone H4 acetylation in the sample. *B*) Quantitative analysis of histone H4 in *Artemia*, expressed as nanograms per microgram of histone.

shock-induced stress tolerance has persisted in 3 successive generations.

Progeny of daily nonlethal heat-shocked ancestors exhibit acetylation of histone H4 and H3 under isothermic conditions

Because previous studies have implicated the critical involvement of histone acetylation in the transcriptional regulation of *hsp70* gene in animals (29, 41–43), we determined whether this process is also involved in the observed transgenerationally inherited up-regulation of *Artemia* Hsp70. We addressed this by analyzing the acetylation level of histone H4 and H3 (details in Materials and Methods). As shown in **Figs. 5** and **6**, the level of histone (H4 and H3) acetylation was markedly higher in the nonlethal heat-shocked T-F0 parental *Artemia* than in the isothermally grown C-F0 parental group, which suggests that nonlethal heat shocks exert direct or indirect controls on the global level of histone acetylation. Hyperacetylation of histone H4 and H3 was also detected in the T-F1 to T-F3 progeny, whose ancestors were exposed to nonlethal heat shocks. The level of H4 and H3 acetylation appeared to decrease steadily over the generations, a pattern that is reminiscent of the level of Hsp70 production described above. Indeed, in the F3 generation, no significant difference was found in the levels of histone H3 acetylation between the C-F3 control and T-F3 treatment groups (Fig. 6). However, the acetylation of H3 in the animals with treated ancestors remained relatively higher than the control. An almost similar trend was observed for histone H4 (Fig. 5). These data constitute a powerful argument that acetylation of both H3 and H4 histones contributes, at least to some extent, to the up-regulation of Hsp70 production in nonlethal heat-shocked parental *Artemia* and to the inheritance of the increased Hsp70 production in successive unstressed generations.

Progeny of daily nonlethal heat-shocked ancestors exhibit changes in global DNA methylation under unstressed conditions

Transgenerational effects in both plants and animals are often associated with alterations in methylation of genomic DNA (44–46). This finding prompted us to compare the 5-methylcytosine (5-MeC) content of genomic DNA isolated from the T-F0 and C-F0 generation, as well as from the first 3 generations of progeny, by UHPLC-MS/MS analysis. This analysis revealed that

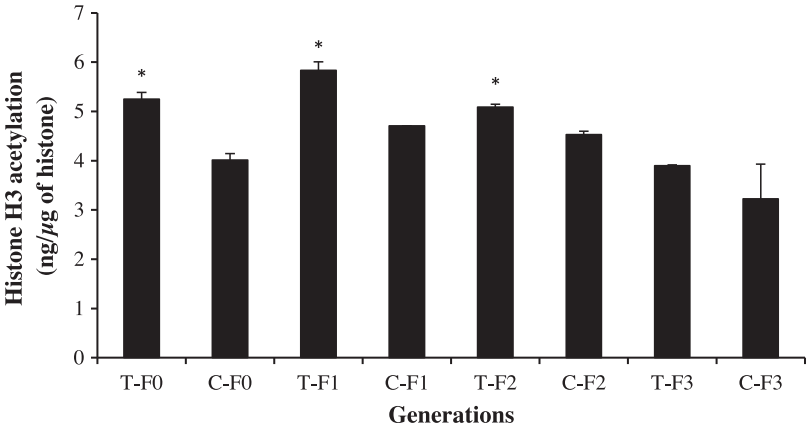

Figure 6. Acetylation level of histone H3 *Artemia* in the parental *Artemia* and 3 successive generations of progeny (common garden test). See Fig. 2 for explanation of the treatment (T) and control (C) groups. Total histone (5 μg) extracted from F0 to F3 juveniles was analyzed for total histone H3 acetylation using the Epi-Seeker histone H3 total acetylation detection flurometric fast kit. Values are presented as means ± SE (n=3). *$P < 0.05$ *vs.* corresponding control group.

the percentage of methylated cytosine in the T-F0 *Artemia* was ~3-fold lower than in the C-F0 group ($P<0.05$; **Fig. 7**). Interestingly, this response was reversed in the F1 generation, where the T-F1 progeny, reared isothermally, exhibited a significant 3-fold increase in the percentage of cytosine methylation compared to the same-generation progeny of control parents (C-F1 progeny). In the F2 and F3 generations, the percentage of DNA methylation in the T-F2 and T-F3 groups dropped by ~2.5-fold compared to the T-F1 progeny of heat-shocked parents and was statistically indistinguishable from that of the respective C-F2 and C-F3 progeny of the control parental group.

DISCUSSION

The epigenetic inheritance of phenotypic traits across multiple generations is a matter of great interest and of continuing debate (47–50). In this study, by using a population produced by apomictic parthenogenetic *Artemia* (*i.e.*, lineages derived from a single mother), we provided the first experimental evidence that environmentally induced phenotypic traits (increased production of Hsp70, tolerance toward lethal heat shock, and resistance against pathogenic *V. campbellii*) were transmitted to 3 successive, unexposed generations. Similar phenotypes were observed when this experiment was repeated, indicating the reproducibility of the results. Moreover, we also demonstrated explicitly that this transmission of the phenotypic traits across 3 successive generations was associated with changes in the molecular epigenetic marks, such as histone H3 and H4 modification and global DNA methylation.

Studies demonstrating the inheritance of the environmentally responsive phenotypes over multiple generations in (genetically identical) animal or plant models are limited. However a few studies focus on multigenerational transmission of acquired phenotypes. For instance, using a genetically identical *Arabidopsis thaliana* Heynh line plant model, Whittle *et al.* (51) demonstrated that plants exposed to a mild heat (30°C) treatment in the parental and F1 generations exhibited markedly improved fitness (5-fold increase in seed production per individual) in a later generation (F3). The heat-specific fitness improvements among F3 plants were preserved even after one generation (F2) of reproduction under normal temperature circumstances, which led to the conclusion of an environmentally induced epigenetic and heritable adaptive phenomenon. Another relevant example of multigenerational inheritance of acquired traits was reported using rats as a model (noninbred lines) by Benyshek *et al.* (52), who demonstrated that impaired glucose metabolism in F1 rats exposed to a nutritional stress during gestation persisted through maternal transmission to the F3 generation. In a further study on a noninbred line mice model, it was shown that chronic and unpredictable stress in early life of mice altered behavioral response not only in the stressed animals when adult but also in their successive, unstressed generations (up to generation F3; ref. 53). These examples illustrate that altered phenotypes induced by environmental stress may be transmitted across multiple generations. In our study, we observed the environmentally induced phenotypic traits in all 3 successive generations of the parthenogenetic *Artemia*, which are having apomictic breeding behavior. Apomixis implies that a parthenogenetic *Artemia* population clone has no other mechanism for genotypic change but mutation, which may induce genetic differentiation (54). At this point, we cannot exclude the possibility of a genetic (DNA sequence) mutational event (such as environmentally facilitated single-nucleotide polymorphisms) in the development of observed phenotypes. However, from the available information, it may be suggested that a nongenetic process is also involved in the emergence/inheritance of the observed phenotypic traits or regulation and persistence of epigenetic modifications.

Ample evidence indicates that expression/production of stress proteins, mainly Hsp70, plays important roles in defining the tolerance of organisms to several stressors. For instance, in the invertebrate animal model *Artemia*, we have shown that induction of Hsp70 on exposure to nonlethal heat shock significantly improved the resistance of the animal toward subsequent lethal heat shock or pathogenic *Vibrio* (27, 28). This improved resistance was due to the fact that Hsp70 plays a crucial function as molecular chaperone and is involved in protein biogenesis and protein homeostasis in the cells or it contributes to the generation of protective immune responses in the host (33, 34). Similar increased tolerance to extreme temperatures, both hot and cold, subsequent to the induction of Hsp70 by mild heat shock as the initial stressor, has also been demonstrated in other organisms from diverse

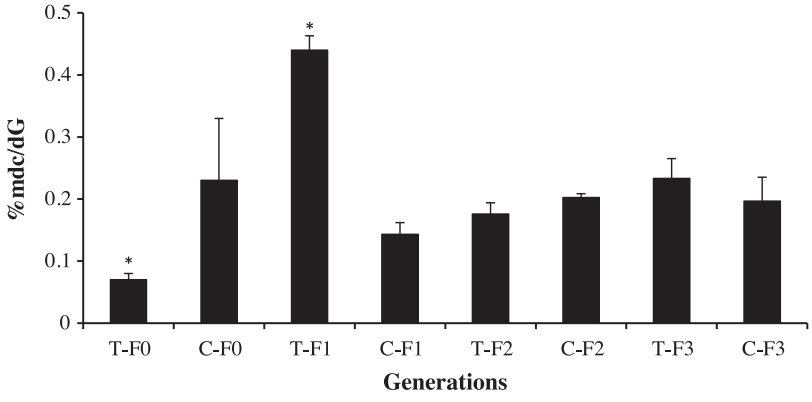

Figure 7. Percentage of methylated cytosine (%mdC/dG) in the parental *Artemia* and 3 successive generations of progeny (common garden test). See Fig. 2 for explanation of the treatment (T) and control (C) groups. DNA (1 μg) extracted from F0 to F3 juveniles was analyzed for global DNA methylation by UHPLC-MS/MS analysis. Values are presented as means ± SE ($n=3$). $P < 0.05$ *vs.* corresponding control group.

phyla, including bacteria, coelenterates, molluscs, fish, shrimp, echinoderms, and humans (34, 55–58). The positive correlation that we detected in our study between elevated Hsp70 levels (as induced by daily nonlethal heat shocks of the T-F0 generation) and increased survival in each generation (T-F1 to T-F3) is in accordance with the above-mentioned studies.

The underlying mechanisms for the inheritance of phenotypes, such as stress tolerance and elevated Hsp70 production as observed in our study, remain elusive. However, our analyses suggest that changes in histone acetylation, associated with chromatin relaxation and the initiation of transcription (59), and DNA methylation, mostly known for its gene repression activity (60), contribute to the emergence and/or multigenerational inheritance of stress tolerance and elevated Hsp70 production in *Artemia*. This suggestion is consistent with the known role that these two processes have in shaping epigenetic landscapes and is compatible with previous observations. As for histone acetylation, for example, a number of studies in animal and plant models have shown that histone acetylation modifications play a key role in epigenetic control and the organism's functional status under stress (58, 61) by controlling the functional state of chromatin and gene expression (62, 63). For instance, Chen *et al.* (41) demonstrated that histone acetylation modification significantly enhanced both the basal and the inducible expression of *hsp70* gene in *Drosophila melanogaster*. In another study, Tetievsky and Horowitz (64) provided evidence that acetylation of histone H4 (but not H3) in *hsp70* chromatin is associated with heat-shock-induced changes in the *hsp70* gene expression. Notably, these researchers also showed that histone H4 acetylation is responsible for heat acclimation-mediated cytoprotective memory. In our study, the acetylation states of histone H4 and H3 in the parental generation increased in response to daily mild heat shocks (Figs. 5 and 6). These modifications propagated up to the F3 generation, in parallel with elevated Hsp70 levels and increased stress tolerance in the T-F1 to T-F3 progenies. It has indeed been shown that chromatin modifications can be transmitted to subsequent unstressed generations (65). These findings are compatible with a scenario in which acetylated histone H3 and H4 in the T-F1 to T-F3 progenies of treated F0 animals are related in a causal way to Hsp70 production. Under this scenario, histone modifications favor prompt binding of transcription factors to heat-shock regulatory elements of *hsp70* (or other *hsp* genes) and subsequent rapid activation of the cytoprotective arsenal (43, 61, 64).

As for the role that DNA methylation may have in transgenerational epigenetic inheritance, a number of previous studies in animal and plant models have detected an association between alterations in DNA methylation in response to environmental cues and transmission of gene expression patterns and/or adaptation to stress (18, 19, 66). In our study, the methylation patterns of specific genes were not investigated, but global DNA methylation was analyzed as an overall measure of potential stress-induced modifications. Global DNA methylation across four successive generations in response to mild heat shocks of the first generation was highly variable. In fact, at T-F0, we observed a decrease in the global DNA methylation level due to mild heat shocks. This finding suggests that hypomethylation is needed for up-regulation/production of Hsps as observed by a marked increase in the Hsp70 level in this T-F0 group. This is in accordance with the results of Gan *et al.* (67), who demonstrated that DNA methylation of the Hsp70 promoter was negatively associated with the mRNA expression level of *hsp70* in the muscle tissue of chicken. However, the reduced DNA methylation might be a prerequisite for the observed increase histone acetylation (68, 69). This increased histone acetylation was subsequently transferred to the nonexposed progeny, whereas the DNA methylation was not. By contrast, the T-F1 progenies of heat-shocked parents exhibited a markedly increased global DNA methylation. A similar shift in global DNA methylation patterns of nonexposed offspring has been observed before in the invertebrate *Daphnia* (38). This methylation increase can possibly be a compensation effect for the general hypomethylation effect of the heat-shock exposure in the T-F0 generations, which might already have been manifested in the F1 germ cells during the F0 exposure. In the T-F2 and T-F3 progeny, the methylation level remained unaffected compared to the C-F2 and C-F3 groups. These generations have never been directly exposed to the heat shocks, even as germ cells.

Our results also showed that there was a decrease in the Hsp70 production over the generations. This decrease, however, did not correspond with the phenotypic traits related to increased tolerance against lethal heat shock or against pathogenic *V. campbellii*. In fact, the stress-resistant phenotypes appeared to increase over the generations, with T-F3 progeny being the most robust. This could be explained by the fact that F0-imposed nonlethal heat shocks not only induced Hsp70 within the exposed animals but also induced a constellation of stress proteins like Hsp27, Hsp40, Hsp90 (27, 70) and other stress-resistant genes (71). It is possible that the transgenerational inheritance of stress-resistant traits across generations observed here is mediated by transgenerational inheritance of elevated stress protein expression, including Hsp70 and/or other heat-inducible genes potentially involved in stress tolerance. These possibilities need to be addressed by identifying the stress-resistant genes and their corresponding proteins.

In essence, this study provides strong evidence for the phenomenon that nonlethal heat shocks can induce epigenetic inheritance of phenotypes across three successive generations concomitant with sustained modification of histones H3 and H4. No apparent mechanistic link between global DNA methylation and the acquired phenotypes could be established here. It remains to be determined whether the inheritance of acquired phenotypic traits across generations is linked to DNA methylation, histone acetylation, or interplay between them at specific loci, particularly Hsp70. Such analysis will provide further mechanistic insight into the observed phenomenon. Overall, these observations add some intriguing insights to augment

our current understanding of transgenerational epigenetic inheritance in animals. FJ

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