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RESEARCH ARTICLE

Can physiological engineering/programming increase multi-generational thermal tolerance to extreme temperature events?

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ABSTRACT

Organisms increasingly encounter higher frequencies of extreme weather events as a consequence of global climate change. Currently, few strategies are available to mitigate climate change effects on animals arising from acute extreme high-temperature events. We tested the capacity of physiological engineering to influence the intra- and multi-generational upper thermal tolerance capacity of a model organism, *Artemia*, subjected to extreme high temperatures. Enhancement of specific physiological regulators during development could affect thermal tolerance or life-history attributes affecting subsequent fitness. Using experimental *Artemia* populations, we exposed F0 individuals to one of four treatments: heat hardening (28°C to 36°C, 1°C per 10 min), heat hardening plus serotonin (0.056 μg ml^{-1}), heat hardening plus methionine (0.79 mg ml^{-1}) and a control treatment. Regulator concentrations were based on previous literature. Serotonin may promote thermal tolerance, acting upon metabolism and life history. Methionine acts as a methylation agent across generations. For all groups, measurements were collected for three performance traits of individual thermal tolerance (upper sublethal thermal limit, lethal limit and dysregulation range) over two generations. The results showed that no treatment increased the upper thermal limit during acute thermal stress, although serotonin-treated and methionine-treated individuals outperformed controls across multiple thermal performance traits. Additionally, some effects were evident across generations. Together, these results suggest that phenotypic engineering provides complex outcomes, and if implemented with heat hardening can further influence performance in multiple thermal tolerance traits, within and across generations. Potentially, such techniques could be up-scaled to provide resilience and stability in populations susceptible to extreme temperature events.

KEY WORDS: Phenotypic engineering, Thermal tolerance, Extreme heat events, Climate change, *Artemia*, Invertebrates

INTRODUCTION

The severe impact of short-term stochastic weather processes has long been recognised in ecology, human health and agriculture, especially with respect to causing high mortality that can even bring about rapid population crashes or local extinctions (McKechnie and Wolf, 2010; Fey et al., 2015). Heat waves have resulted in mass mortalities of domestic and wild animals and can potentially lead to large-scale catastrophic population declines. For example, successive heat waves have resulted in over 300,000 deaths in flying foxes (*Pteropus* sp.) (Welbergen et al., 2008), while entire intertidal coastal marine communities have collapsed along thousands of kilometres in the Northwest Mediterranean region during extreme summer temperatures (Garrabou et al., 2009).

Extreme temperature events clearly challenge the ability of animals to survive. Multiple factors including the frequency, duration and magnitude of short- to mid-term extreme climatic events are likely to explain the impact of pervasive heat waves on animals (Cerrano et al., 2000; Bailey and van de Pol, 2016). However, from an animal's perspective, the ability to mitigate the risks of heat events will also depend on its capacity for adaptive phenotypic responses (Hendry et al., 2011; Chevin and Hoffmann, 2017). Consequently, if animals cannot respond by either shifting their location (e.g. irruptive movement or seeking microhabitat refugia) or utilising physiological responses (e.g. phenotypic plasticity), then they remain at great risk from heat extremes. Importantly, there appears to be relatively limited use of strategies to mitigate the impact of heat waves on animals other than humans. For example, movement or translocation of animals is increasingly being considered as a way to deal with sustained environmental problems, but is not feasible for mitigating rapid climate change impacts on biodiversity (Lavergne et al., 2010; Hoffmann and Sgró, 2011). For species susceptible to extreme weather events, enhancement of phenotypic capacities provides an important potential option to improve survival.

Phenotypic engineering represents an experimental non-genetic approach that could lead to better fitness outcomes for animals exposed to weather extremes. Phenotypic engineering induces increased trait variation within an individual's phenotype to test the performance implications and ultimately the evolutionary implications of fitness consequences arising from manipulated traits (Ketterson et al., 1996). In evolutionary biology, there is extensive literature on the use of manipulation of phenotypic traits to examine the ensuing fitness consequences across a range of taxa and traits (Andersson, 1982; Sinervo and Licht, 1991; Hunt et al., 2004).

To date, the best example of physiological engineering is heat hardening (Dahlgaard et al., 1998; Loeschcke and Hoffmann, 2007; Sørensen et al., 2008). Heat hardening is a physiological process that exposes organisms to sublethal temperatures to induce short-term increased thermal tolerance to subsequent heat stressors (Sørensen et al., 2008). Additionally, heat hardening may be inherited across generations (Norouzitallab et al., 2014). However,

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this process can also have negative consequences (Hercus et al., 2003), indicating that adaptive consequences are highly dependent on environmental conditions (Loeschcke and Hoffmann, 2007), and suggesting that there are limitations to its use.

Natural thermal tolerance in *Artemia*, and the capacity for its induction, varies across life stages and populations (Frankenberg et al., 2000; Clegg and Trotman, 2002); for example, populations from Vietnam display greater tolerance of high temperatures than those from San Francisco. *Artemia* thermal tolerance across populations is probably regulated in part by heat shock protein 70 (HSP70) (Iryani et al., 2017). *Artemia* have been used in several previous heat-hardening experiments (Miller and McLennan, 1988; Frankenberg et al., 2000; Norouzitallab et al., 2014); induced thermal tolerance in these examples is achieved via repeated exposure to sublethal heat shocks varying from 40 to 50°C. Additionally, some hardening studies have also applied physiological engineering agents (e.g. Tex-OE®, a chaperone-stimulating factor) to enhance production of HSP70 (Baruah et al., 2012). Here, we extended these previous heat-hardening experiments by evaluating the application of phenotypic engineering regulators (serotonin and methionine) to influence thermal tolerance within and across generations. We were particularly interested in understanding whether dietary methionine could produce multi-generational effects on thermal tolerance in *Artemia*.

Serotonin (5-HT) plays major roles in invertebrates that may allow it to regulate an individual's thermal tolerance, particularly via effects on metabolism (Chaouloff et al., 1999). Serotonin is well known for its capacity to regulate broad-scale developmental and hormonal processes in both vertebrates and invertebrates (Buznikov et al., 2001; Tecott, 2007), is strongly linked to the stress response across multiple organisms (Chaouloff et al., 1999), and has previously been linked to induced thermal tolerance (Sharma and Hoopes, 2003). One major hormonal pathway that serotonin influences is the release of crustacean hyperglycaemic hormone (CHH) (Escamilla-Chimal et al., 2002; Lorenzon et al., 2005). In part, this may allow serotonin to regulate thermal tolerance via stimulation of the actions of this crustacean 'stress hormone' (Chang, 2005; Elwood et al., 2009). Additionally, serotonin is involved in protecting against protein misfolding (Tatum et al., 2015). Exogenous serotonin application early in ontogeny may therefore programme the animal for thermal tolerance throughout life through a combination of serotonergic effects and stimulated release of CHH and HSPs.

Dietary agents such as dietary methionine act as methylating agents through the provision of free methyl groups (Niculescu and Zeisel, 2002; Waterland, 2006). Although actual patterns of methylation vary across eukaryotes (Suzuki and Bird, 2008), the effects remain the same; DNA methylation in the promoter region of genes may repress transcription, effectively silencing those genes, as may the binding of repressor proteins to those methylated regions of DNA (Tate and Bird, 1993). Within *Artemia*, increased thermal tolerance is associated with altered global methylation patterns inherited across generations in the absence of the initial stressor (Norouzitallab et al., 2014). Methylation is a key process for epigenetic programming and is not easily reversed (Cedar and Bergman, 2009). Thus, methylation provides a putative process for regulating inheritance and conferring trans-generational responses to heat stress. Methionine has been used to alter physiology, especially within production animals. For example, dietary methionine can improve bovine cellular thermal tolerance via increased HSP70 production and reduced heat-induced morphological damage (Han et al., 2015). The addition of exogenous methionine into the diet of *Artemia* was used here to provide an opportunity for increased global methylation to further aid the effects of heat hardening.

This study applied a phenotypic engineering and programming methodology to enhance *Artemia* thermal tolerance, aiming to improve the survival of individuals during extreme acute heat events within and across generations. Specifically, we hypothesised that phenotypic engineering via the use of hormonal and dietary regulators (serotonin and methionine, respectively) would enhance heat-hardening responses to increase survival during extreme heat events. These regulators were applied to examine their effects on heat hardening over two generations of an invertebrate model species, the brine shrimp *Artemia franciscana*. *Artemia* species are widely recognised as valuable animal models to understand how organisms mount biochemical and physiological stress responses (Clegg and Trotman, 2002), and for toxicity studies (Neu et al., 2014), due in part to their short generation times and simplicity to culture.

MATERIALS AND METHODS

Experimental populations

Eight experimental populations of *Artemia franciscana* Kellogg 1906 were established from decapsulated cysts sourced from a local commercial supplier (Upmarket Pets Aquarium, Melbourne, VIC, Australia). Cysts (0.5 g) were placed into eight inverted 2 l plastic bottles (replicates). Bottles were immersed within 1220 mm×330 mm×410 mm glass tanks filled with water held at a constant temperature (28±1°C); tanks were fitted with water pumps to circulate water to prevent the formation of a thermocline. Temperature within the bottles was checked once daily to ensure similar water temperature exposure among populations during experiments. *Artemia franciscana* were maintained in saline water [0.1 ml of Seachem Prime (Madison, GA, USA), mixed with Kirby's Premium SPS aquarium salt (Melbourne, VIC, Australia)] to a final gravity of 1.042 to 1.044. These conditions reduce oviparity in other populations of *A. franciscana* (Velasco et al., 2016), ensuring ovoviviparity for production of an F1 generation, and maximise survival during maturation (Pinto et al., 2013). Water pH was maintained at 8.2–8.4, with constant aeration provided from air pumps (Sera Air 550 Plus, North Rhine-Westphalia, Germany). Populations were maintained on a constant 12 h light:12 h dark photoperiod, and fed 4 ml of feed daily. Regulators were added within 15 min of initiation of the light period (see below), with heat-hardening events (HHE) and thermal fitness tests occurring within 2 h of light period initiation. Feed comprised ground inactive baker's yeast (Lowan Instant Dried Yeast, Glendenning, NSW, Australia) mixed to 0.05 g ml^{-1} in tap water. Conditions were maintained throughout the study for both F0 and F1 populations, with full water changes occurring every 2 days to ensure water quality.

Experimental setup

Four treatment groups were randomly allocated two replicate populations each, stocked at *Artemia* densities of no greater than 2 ml^{-1}. Three groups comprised populations exposed to heat hardening; two of these groups were then additionally exposed to either serotonin or methionine, and the fourth group served as a control. These treatments were maintained for two generations (Fig. 1) and descriptions of their respective experimental protocols are given below.

Treatment 1: control

Control replicate populations were left untreated, aside from being exposed to similar movement among bottles on day 3 and day 5 to

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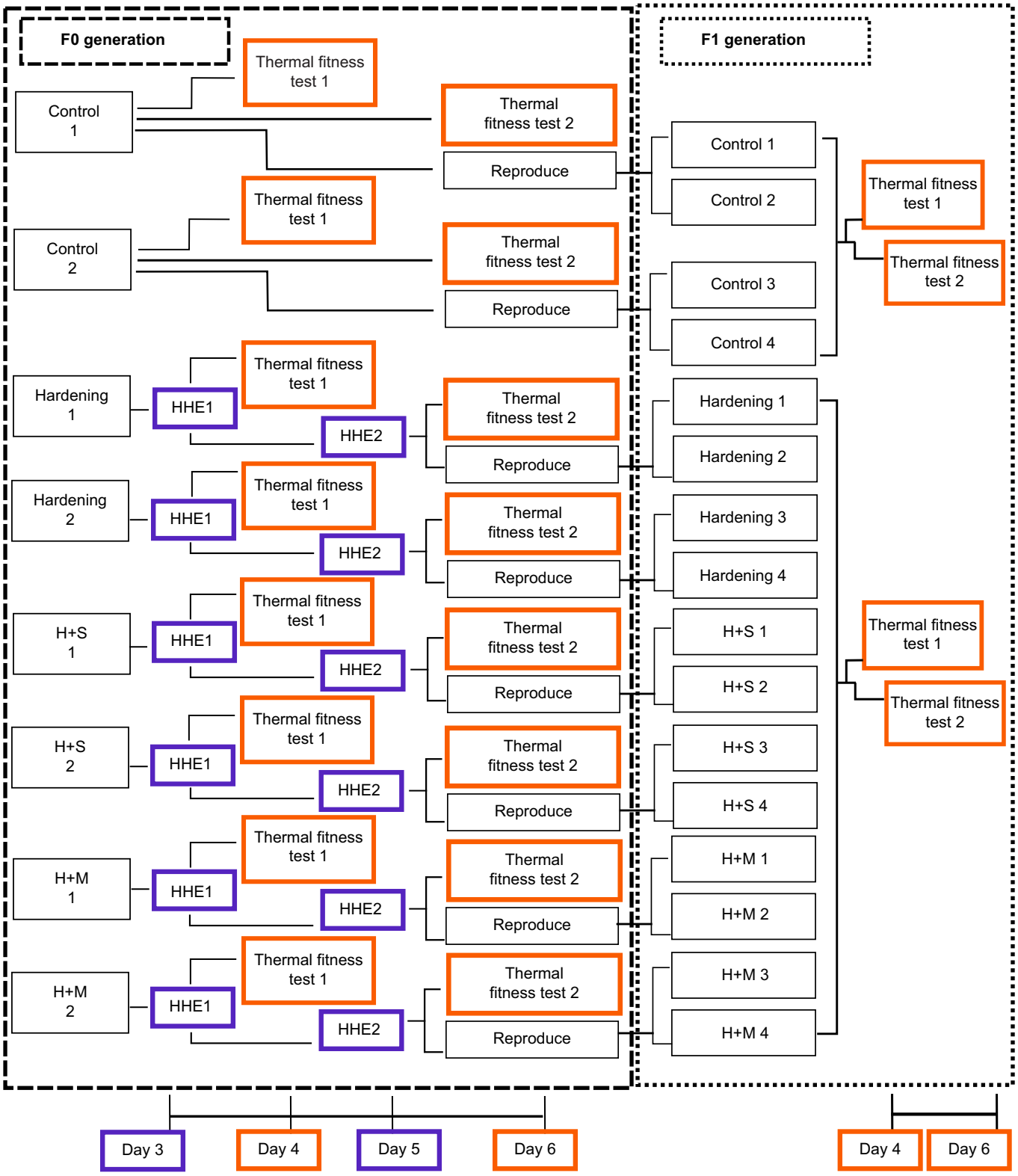

Fig. 1. Schematic diagram of the experimental design. Experiments were designed to test the efficacy of different hormonal or dietary regulators in combination with heat hardening to evaluate their effects on acute thermal-related fitness within and between generations of the brine shrimp (*Artemia franciscana*). Boxes on the far left of the F0 generation indicate treatment groups and replicates: Hardening: heat hardening; H+S: hardening+serotonin; H+M: hardening+methionine. HHE (heat-hardening events) are highlighted in purple, while thermal fitness tests are highlighted in orange. These colours are reflected in the timeline below the *x*-axis. Water changes occurred every 2 days (days 2, 4, 6, etc.).

mimic handling procedures that the other three treatments experienced during heat hardening.

Treatment 2: heat hardening

We induced heat hardening in *A. franciscana* to test its capacity for promoting increased thermal tolerance within and between generations of *Artemia* exposed to lethal temperatures. We exposed *A. franciscana* larvae (nauplii) to heat-hardening pre-treatment twice during development, on days 3 and 5 post-hatching. Replicate bottles were removed from glass immersion tanks, and populations were filtered with a 53 nm sieve from replicate bottles into 400 ml of housing water in 500 ml Duran Schott bottles (Duran Group, Wertheim, Germany). Populations were allowed to acclimate for 5 min, then Schott bottles were moved individually to a water bath and the temperature was raised 1°C every 10 min from 28°C to 36°C, a rate and time previously determined during protocol validation (Figs S1 and S2). Bottles were then removed and the water allowed to cool naturally to 28°C. Populations were then gently returned to replicate bottles and immersion tanks. Samples of nauplii were randomly removed from replicate bottles for physiological assays on days 4 and 6 post-hatching.

Treatment 3: hardening plus serotonin

We exposed two replicate populations of *A. franciscana* nauplii to extrinsic serotonin to test the ability of this neurotransmitter to influence multi-generational thermal tolerance effects alongside those due to heat hardening. Replicate bottles were dosed with 100 µg of serotonin (H9523, Sigma-Aldrich, Castle Hill, NSW, Australia) on hatching day (day 0), and on day 2 and day 4 (prior to thermal performance trials). Previous work dosing crustaceans with serotonin injected 50 µg g^{-1} body mass (Vaca and Alfaro, 2000; Wongprasert et al., 2006); here, we approximated total nauplii mass per bottle to not exceed 2 g by day 4, given an estimated 0.269 g of nauplii after hatching (Van Stappen, 1996). To ensure a near-constant exposure, serotonin was re-added during full water changes every 2 days; serotonin has an estimated half-life in water of 360 h (https://www.epa.gov/tsca-screening-tools/download-epi-suitetm-estimation-program-interface-v411), so concentrations were not expected to reduce significantly during these 48 h. Other than addition of serotonin, experimental conditions and times were equivalent to those undertaken for the heat-hardening group.

Treatment 4: hardening plus methionine

We exposed two replicate populations of *A. franciscana* nauplii to extrinsic L-methionine to test the ability of this dietary supplement to further influence multi-generational thermal tolerance. Replicate bottles were dosed with 1.423 g (5.3 mmol) of L-methionine (M9625, Sigma-Aldrich), a concentration previously used successfully for *Artemia* enrichment (Tonheim et al., 2000) on hatching day (day 0), day 2 and day 4. Addition of L-methionine alters water pH; to counter this effect and return pH to between 8.2 and 8.4, 0.1 ml of eight.four (Aquavitro, Madison, GA, USA) was also added to replicate bottles. As with serotonin treatments, L-methionine was re-added to the water every 2 days with water changes, to ensure constant exposure. L-Methionine was not expected to be absorbed into suspended solids within the water of treatment bottles (https://www.epa.gov/tsca-screening-tools/download-epi-suitetm-estimation-program-interface-v411). Additionally, L-methionine shows a half-life in water of 200 h, when the water is naturally lit by the sun and has sufficient oxygen (Zepp et al., 1977; Haag and Hoigne, 1986); these experiments were run under artificial lighting conditions, and so no significant degradation was expected within 48 h between doses. Treatments and timing otherwise were identical to those for the heat-hardening protocols of the heat-hardening group.

Second generation (F1) treatments

To evaluate whether any of the experimental treatments conferred multi-generational effects, we also tested the thermal tolerance of their progeny to heat exposure. Populations were maintained to allow remaining individuals (F0) to reach sexual maturity and reproduce to produce F1 progeny. All F0 adults were removed from bottles and their progeny (instar I) continued to develop. All F1 individuals were raised under conditions identical to F0 controls, without additional stressors; F1 physiological assays were performed for each replicate on day 4 and day 6.

Population survival

To ensure survival was not affected by treatments, we created additional populations (n=16) external to experiments as described above. These populations were maintained for 10 days under treatment conditions (n=4 per treatment), and the population was counted and averaged within each bottle every 2 days from day 0 to 10 (Fig. 2). Population density was initially recorded between one individual per 1.5 ml and one individual per 1.3 ml. No significant mortality was detected within any treatment during this time.

Thermal performance traits

Artemia franciscana (n=76, 38 per replicate, total across all treatments and generations n=1216) were randomly selected in cohorts of 19 from each treatment, placed individually into 2.0 ml (10.6 mm×40 mm) microcentrifuge tubes (Eppendorf, Hamburg, Germany) containing treatment water, and allowed to acclimate for 5 min. Micro-centrifuge tubes were heated within a Thermolyne dry block heater (75 mm×100 mm×50 mm; Barnstead International, Dubuque, IA, USA), beginning at 28°C and increasing in temperature by 1°C min^{-1}. After each 1°C increase, each individual was briefly examined and its condition scored as 1 (normal movement; smooth, directed motion which was slow but not sluggish), 2 (sublethal effects; disoriented movement and/or convulsions) or 3 (death). Temperature increases were stopped for each cohort of 19 when all individuals had reached a score of 3. This scoring system allowed us to measure upper sublethal thermal limit (USTL, score 2) and dynamic critical thermal maximum for upper thermal limit (UTL, score 3; Lutterschmidt and Hutchison, 1997), with the temperature difference between the two indicating the upper thermal dysregulation range (UTDR). These values are physiologically valuable to measure; UTL determines the geographical distribution of organisms (Pörtner, 2002), USTL determines the temperature at which organisms experience an increased 'cost of living' in their geographical range which may reduce overall fitness (Somero, 2002), and UTDR indicates the thermal range in which organisms, while surviving, may begin experiencing additional deleterious effects. Physiological assays were conducted 24 h post-heat hardening to ensure maximum hardening responses. To our knowledge there are no *Artemia*-specific studies on the duration of heat-hardening effects; however this time frame is consistent with previous studies utilising other species (Hoffmann et al., 2003).

Statistical analyses

We used a full-factorial multivariate and univariate linear mixed-effects model via a MANOVA to analyse the effects of phenotypic treatment, HHE and generation, and all second- and third-order interactions, on the three thermal traits (combined and independently) of *Artemia* exposed to an acute thermal exposure. Housing bottle was

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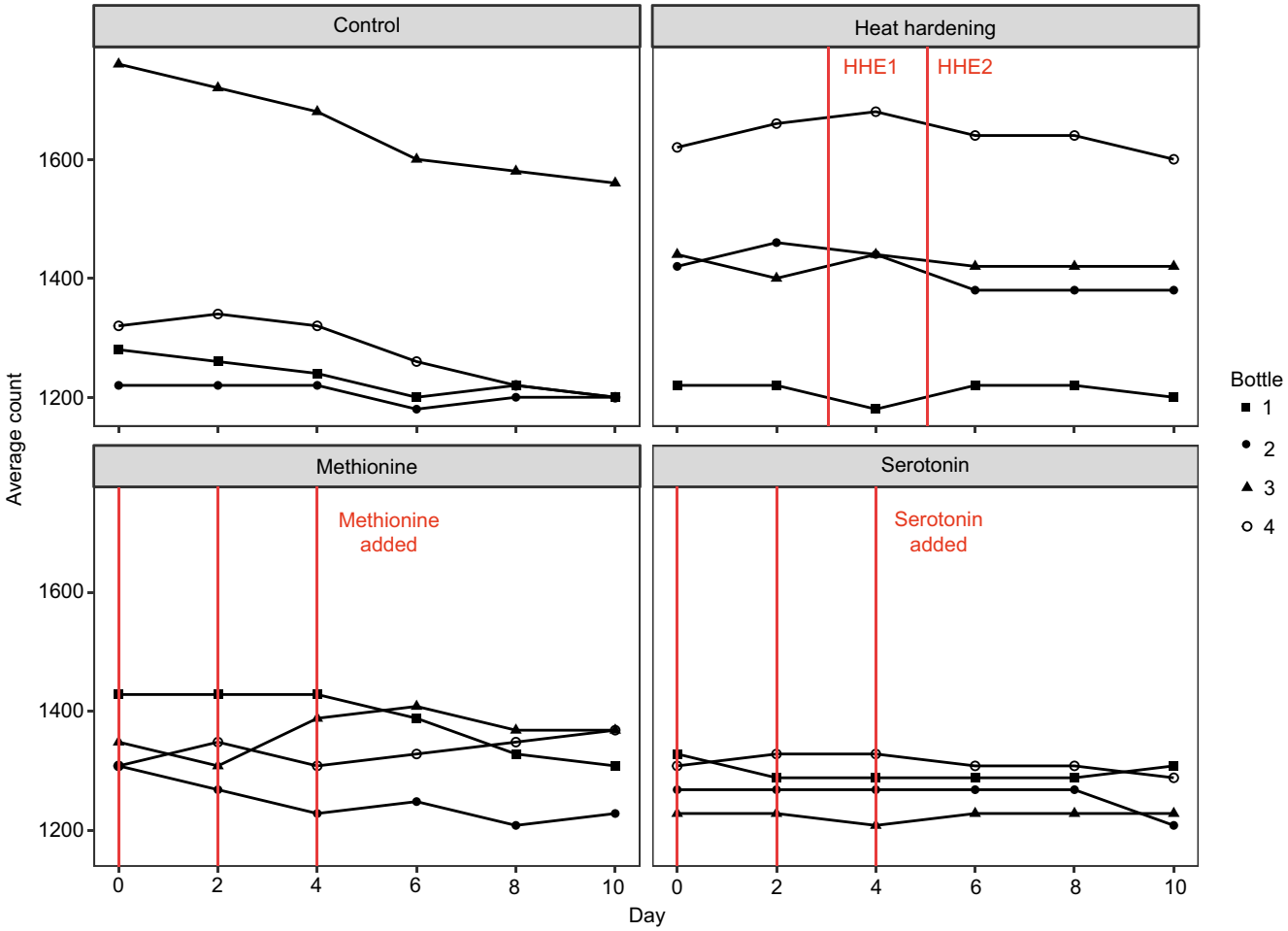

Fig. 2. Average *A. franciscana* population counts across treatments and bottles. Symbols indicate bottle number within treatments. Red lines and labels indicate the point at which interventions (HHE, serotonin/methionine addition) occurred. Bottle number was a significant random effect (P<0.01), although population counts did not differ significantly across treatments (heat hardening: P=0.42; methionine: P=0.52, serotonin: P=0.13) or across day (P=0.13). There was no interaction effect between day and treatment (P=0.73).

included as a random effect in each model. HHE reflected the day of acute thermal exposure. Additionally, HHE were included as a factor in order to examine potential cumulative or detrimental effects of repeated hardening events on *Artemia* thermal tolerance. For population survival, we performed an additional full-factorial linear mixed-effect model via an ANOVA to analyse the effect of treatment and day on *Artemia* survival, with bottle included as a random factor. We conducted our analyses in program R v3.4.1. (http://www.R-project.org/) using the packages LME4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017), Car (Fox and Weisberg, 2011) and ggplot2 (Wickham, 2009) to analyse and visualise the data. To ensure sufficient statistical power, we performed a *post hoc* power analysis using G*Power v3.1.9. (Faul et al., 2009); at a small effect size [$f^2(V)$=0.15], our total sample size (n=1216) using our full-factorial model returned a power of 1−β=1. Data are available from the Dryad Digital Repository (Sorby et al., 2018): https://doi.org/10.5061/dryad.05s45.

RESULTS

Population survival

To validate that physiological mediators did not impact survival and potentially confound estimates of thermal tolerance, we tested the effect of methionine and serotonin on *Artemia* survival relative to controls. Population count (an index of survival) did not significantly vary within treatments (heat hardening: P=0.42; methionine: P=0.52; serotonin: P=0.13) or across day (P=0.13). There was no interaction effect between day and treatment (P=0.73). There was, however, an effect of bottle as a significant random factor (P<0.01), indicating survival could vary among replicate groups used in the study.

Thermal performance traits

All main effects traits including phenotypic treatment (P<0.01), HHE (P<0.01) and generation (P<0.01) had significant effects on multivariate *Artemia* performance (Table 1A). Control populations showed a mean (±s.e.m.) USTL of 39.39±0.04°C, UTL of 46.03±0.03°C and UTDR of 6.65±0.04°C. Phenotypic treatment, generation and number of HHE each individually significantly affected the univariate traits USTL, UTL and UTDR (Table 1B; P<0.01 for all traits).

All second-order interactions had significant effects on multivariate *Artemia* thermal traits, including phenotypic treatment×generation (P<0.01), phenotypic treatment×HHE (P<0.01) and generation×HHE (P=0.02). Phenotypic treatment×generation and phenotypic treatment×HHE significantly affected all three univariate traits (P<0.01 for all traits). However, generation×HHE only significantly affected USTL (P=0.04).

Finally, the third-order interaction also had significant effects on multivariate *Artemia* thermal traits (P=0.02). This interaction

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Table 1. Effect of heat-hardening events (HHE), phenotypic treatment and generation and their interaction on *Artemia* performance traits

A. Multivariate performance traits

	d.f.	Test statistic	Approx. *F*-value	$d.f._{num}$	$d.f._{den}$	Pr (>*F*)
Treatment	3	0.137	54.955	3	1200	**<0.01**
Generation	1	0.063	25.119	3	1198	**<0.01**
HHE	1	0.031	12.318	3	1198	**<0.01**
Treatment×generation	3	0.037	14.640	3	1200	**<0.01**
Treatment×HHE	3	0.054	21.534	3	1200	**<0.01**
Generation×HHE	1	0.008	3.230	3	1198	**0.02**
Treatment×generation×HHE	3	0.021	8.470	3	1200	**<0.01**

B. Univariate performance traits

	Sum sq	d.f.	*F*-value	Pr (>*F*)
USTL				
Treatment	229.19	3	51.518	**<0.01**
Generation	80.57	1	54.331	**<0.01**
HHE	10.13	1	6.833	**<0.01**
Treatment×generation	47.54	3	10.687	**<0.01**
Treatment×HHE	94.31	3	21.199	**<0.01**
Generation×HHE	6.51	1	4.393	**0.04**
Treatment×generation×HHE	6.75	3	1.518	0.21
Residuals	1779.46	1200		
UTL				
Treatment	38.22	3	21.918	**<0.01**
Generation	9.59	1	16.504	**<0.01**
HHE	15.21	1	26.171	**<0.01**
Treatment×generation	20.97	3	12.025	**<0.01**
Treatment×HHE	6.86	3	3.935	**<0.01**
Generation×HHE	1.32	1	2.264	0.13
Treatment×generation×HHE	9.49	3	5.445	**<0.01**
Residuals	697.45	1200		
UTDR				
Treatment	286.08	3	52.600	**<0.01**
Generation	29.69	1	16.375	**<0.01**
HHE	48.96	1	27.006	**<0.01**
Treatment×generation	63.44	3	11.664	**<0.01**
Treatment×HHE	94.39	3	17.355	**<0.01**
Generation×HHE	2.96	1	1.633	0.20
Treatment×generation×HHE	16.27	3	2.991	**0.03**
Residuals	2175.53	1200		

Details of MANOVA test reporting the Roy test statistic, assessing the effects of the measured variables and their interactions on multivariate (A) and univariate (B) performance traits. USTL, upper sublethal thermal limit; UTL, upper thermal limit; UTDR, upper thermal dysregulation range. Pr (>*F*) indicates the *P*-value associated with the given *F*-value. Bold values indicate significance ($P<0.05$).

significantly affected the univariate traits UTL ($P<0.01$) and UTDR ($P=0.03$).

Effect of treatment 2: heat hardening

Populations treated with heat hardening alone showed a mean (±s.e.m.) USTL of 39.89±0.04°C, a UTL of 46.46±0.02°C and a UTDR of 6.57±0.05°C. Heat-hardening treatment alone did not significantly affect these three univariate traits compared with controls (USTL, $P=0.29$; UTL, $P=0.52$; UTDR, $P=0.21$; Table 2, Fig. 3). Hardened individuals exposed to two HHE showed a significantly increased USTL (+1.74±0.28°C, $P<0.01$) and significantly decreased UTDR (−1.62±0.3°C, $P<0.01$) compared with those exposed to only one HHE. Additionally, offspring (F1 generation) of heat-hardened populations showed a significantly increased UTL versus F1 controls (+0.50±0.33°C, $P<0.01$).

Effect of treatment 3: hardening plus serotonin

Populations treated with heat hardening plus serotonin showed a mean (±s.e.m.) USTL of 38.88±0.03°C, UTL of 46.42±0.02°C and UTDR of 7.54±0.04°C. Treatment with serotonin significantly decreased USTL (−1.13±0.27°C, $P<0.01$; Table 2), and significantly increased UTDR (+0.89±0.37°C, $P<0.01$; Table 2). Serotonin treatment also showed a tendency towards increased UTL, although this was not significant ($P=0.06$; Table 2). Serotonin treatment combined with two HHE significantly increased both USTL (+0.64±0.28°C, $P=0.02$) and UTL (+0.63±0.17°C, $P<0.01$), although there was no significant change to UTDR ($P=0.87$). Similarly, the F1 generation offspring of serotonin-treated individuals also showed significantly increased USTL (+0.84±0.39°C, $P<0.01$) and UTL (+0.93±0.33, $P<0.01$).

Effect of treatment 4: hardening plus methionine

Populations treated with heat hardening plus methionine showed a mean (±s.e.m.) USTL of 38.82±0.03°C, UTL of 46.43±0.02°C and UTDR of 7.61±0.04°C. Methionine treatment significantly decreased USTL (−1.00±0.27°C, $P<0.05$; Table 2) and significantly increased UTDR (+0.93±0.37°C, $P<0.01$; Table 2), although it did not significantly affect UTL ($P=0.60$; Table 2). Exposure to two HHE significantly increased UTL (+0.54±0.17°C, $P<0.01$), although it did not significantly affect USTL ($P=0.37$) or UTDR ($P=0.30$). F1 offspring of methionine-treated individuals showed a significantly

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Table 2. Effect of heat hardening, methionine and serotonin treatment, generation and their interaction on *Artemia* univariate performance traits

	USTL		UTL		UTDR	
	Estimate	s.e.	Estimate	s.e.	Estimate	s.e.
Intercept	39.41	0.19	46.38	0.17	6.97	0.26
Hardening	−0.21	0.27	0.08	0.23	0.28	0.37
M	−1.00	0.27	−0.07	0.23	0.93	0.37
S	−1.13	0.27	−0.24	0.23	0.89	0.37
F1	0.33	0.27	−0.25	0.23	−0.58	0.37
HHE2	−0.33	0.20	−0.46	0.12	−0.20	0.22
Hardening×F1	−0.05	0.39	0.50	0.33	0.58	0.53
M×F1	0.54	0.39	0.54	0.33	0.00	0.53
S×F1	0.84	0.39	0.93	0.33	0.09	0.53
Hardening×HHE2	1.74	0.28	0.04	0.17	−1.62	0.30
M×HHE2	0.25	0.28	0.54	0.17	0.36	0.30
S×HHE2	0.64	0.28	0.63	0.17	0.05	0.30
F1×HHE2	−0.08	0.28	0.01	0.17	0.26	0.30
Hardening×F1×HHE2	−0.51	0.39	0.33	0.24	0.64	0.43
M×F1×HHE2	0.16	0.39	−0.28	0.24	−0.61	0.43
S×F1×HHE2	−0.50	0.39	−0.63	0.24	−0.30	0.43

Details of univariate coefficients (estimate) and standard error assessing effects of variables on upper sublethal thermal limit (USTL), upper thermal limit (UTL) and upper thermal dysregulation range (UTDR). HHE, heat-hardening event; Hardening, heat hardening; M, methionine; S, serotonin; F1, F1 generation. These coefficients are represented graphically in Fig. 3.

increased UTL (+0.54±0.33, P<0.01) compared with F1 controls. Similarly, methionine-treated F1 individuals showed a tendency towards an increased USTL, although this was not significant (P=0.05).

DISCUSSION

This study examined the effects of heat hardening and physiological engineering on three thermal tolerance-related traits: UTL, USTL and UTDR. These traits are especially important as they influence an organism's phenotypic performance and fitness during exposure to thermal stress. Our results offer three valuable insights: first, that heat hardening may induce variable responses in different thermal performance traits; second, that effects of heat hardening can be enhanced by the addition of physiological regulators; and third, that phenotypic engineering can influence multi-generational thermal tolerance.

Our heat-hardening results add to substantive literature showing that heat hardening increases invertebrate thermal tolerance (Dahlgaard et al., 1998; Cypser and Johnson, 2002; Hopkin et al., 2006; Loeschcke and Hoffmann, 2007; Sørensen et al., 2008; Bahrndorff et al., 2009), and support previous examples of the multi-generational effects (Norouzitallab et al., 2014; Zizzari and Ellers, 2014). However, our results differ from previous literature regarding the effects of repeated exposure to HHE (Cypser and Johnson, 2002; Hercus et al., 2003). Here, we saw deleterious effects of a second HHE (though still more beneficial than no HHE), possibly explained by the cumulative negative effects of repeated treatments within a rapid timeframe (Terblanche et al., 2011). Additionally, this study differs in its methods from previous *Artemia*-specific heat-hardening studies (Miller and McLennan, 1988; Frankenberg et al., 2000), which either focused solely on adult treatment and responses or used higher hardening and testing

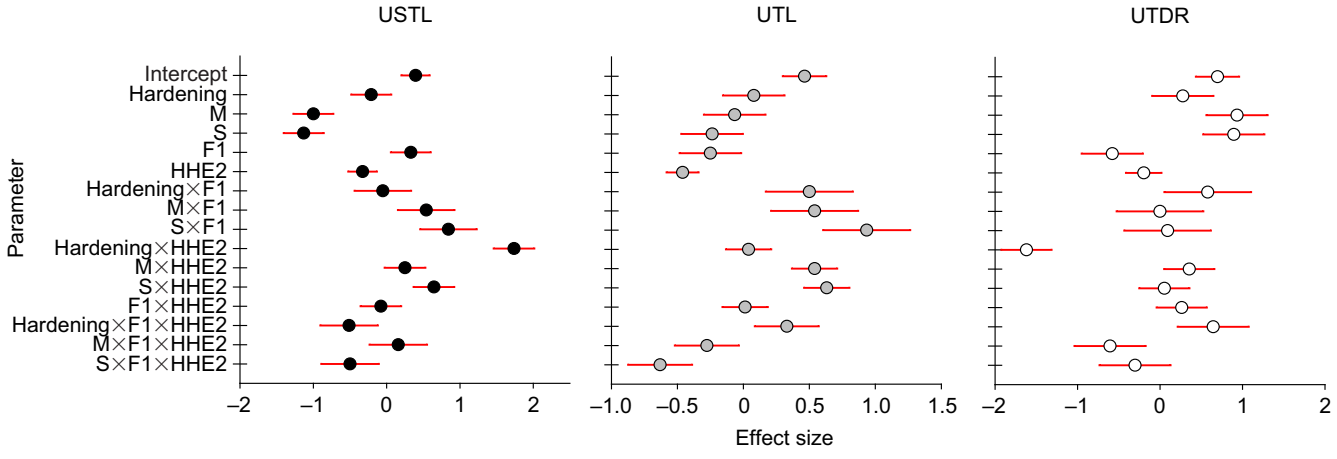

Fig. 3. Coefficients of measured variables and interactions, and their effects on performance traits across generations in *A. franciscana* (*n*=1216). Circles and lines indicate coefficients±s.e.m. from a full factorial linear mixed-effects model. These coefficients are tabulated in Table 2. Black circles represent the upper sublethal thermal limit (USTL), grey circles represent the upper thermal limit (UTL) and white circles represent the upper thermal dysregulation range (UTDR). Positive coefficient values indicate a thermal delay in the onset of the performance trait for UTL and USTL, and an increase in this thermal range for UTDR. Negative coefficient values indicate a thermal advance in trait onset for UTL and USTL, and a decrease in thermal range for UTDR. HHE, heat hardening event; Hardening, heat hardening; M, methionine; S, serotonin; F1, F1 generation.

temperatures. These methodological differences may explain the subsequent differences in results; where previous studies have shown increased thermal tolerance post-heat hardening, our results showed heat hardening alone did not significantly affect the three measured thermal tolerance-related traits. Finally, our experiment takes a novel approach through extension of the heat-hardening concepts within the literature, examining multiple thermal performance traits and the addition of physiological engineering. For example, two HHE appeared to have less effect on sublethal limits than on upper limits in our study, ultimately also affecting the dysregulation range. Although some previous heat-hardening work has examined thermal tolerance in conjunction with additional traits (Hercus et al., 2003), investigation into multiple thermal tolerance-related traits, as used here, is notably lacking.

We also found evidence of successful application of serotonin and methionine as physiological regulators to augment basic heat hardening. Both regulators, but particularly serotonin, further improved the beneficial effects of both one and two HHE (compared with no HHE), and overall increased upper thermal limits. Although these regulators decreased sublethal thermal limits, we note that rapid hardening improves an organism's ability to maintain or improve key behaviours and performance traits during exposure to otherwise sub-optimal or sublethal temperatures (Shreve et al., 2004; Nyamukondiwa and Terblanche, 2010), and so this may not indicate a negative effect. We have previously noted serotonin may act to improve thermal tolerance (Sharma and Hoopes, 2003), possibly through its heavy regulation of CHH and HSPs (Chang, 2005; Elwood et al., 2009; Tatum et al., 2015), and methionine via methylation (Norouzitallab et al., 2014), although we note additional mechanisms may be acting through these regulators. For example, both serotonin and methionine may have also acted as amino acid food sources, buffering negative effects and preventing protein degradation caused by acute thermal stress in a manner similar to HSPs. Examining these specific mechanisms was beyond the scope of this study, though we strongly recommend further investigation into the release of CHH and HSPs and changes to epigenetic profiles as a consequence of serotonin and methionine regulation, respectively.

Our experiments also indicated that heat hardening enhanced by phenotypic engineering resulted in individuals with enhanced thermal tolerance across generations, with effects greater than those resulting from heat hardening alone. Most interestingly, heat hardening regulated with serotonin decreased sublethal thermal limits within the F0 generation, but improved prevention of further sublethal limit reduction in the F1 generation compared with other treatments. This suggests that serotonin may work to buffer individuals against negative generational effects. Exogenous serotonin may act to increase the antioxidant capacity of treated individuals through increased synthesis of the antioxidant melatonin. In *Artemia*, as in other crustaceans, melatonin could act to reduce oxidation stress (Geihs et al., 2010) to protect gametes from the first generation more than those from other treatments. Further studies should examine circulating levels of serotonin and melatonin within treated individuals to confirm this systemic effect. We note that not all thermal-related performance traits were affected across generations by these regulators; dysregulation range was not significantly impacted by either serotonin or methionine in the F1 generation. Additionally, we recognise that further examination beyond the F1 generation is required to conclusively establish a trans-generational, rather than multi-generational effect. However, we argue our experiments provide exciting novel evidence of the ability of phenotypic engineering with specific engineering agents to act multi-generationally on particular thermal performance traits.

The present study was conducted under laboratory conditions, which presents inherent limitations associated with extending our findings to broader scale applications or extrapolation to natural populations. In particular, we acknowledge that the phenotypic regulators used here may not be appropriate for altering thermal tolerance of all species; there is evidence that excess methionine consumption leads to a range of pathological conditions (Benevenga and Steele, 1984), while serotonin may cause pathological behaviour (Fossat et al., 2014). However, we suggest our results offer evidence towards the application of phenotypic engineering to enhance heat-hardening responses that could increase the survival of individuals or populations exposed to extreme weather events.

For example, we note that rapid environmental change, including extreme heat events, is an ongoing challenge to successful ecological rewilding that attempts to reduce ongoing human intervention (Corlett, 2016), despite historical calls for the development of techniques and strategies that are proactive rather than strictly reactive (Hobbs and Cramer, 2008). Recent examples and reviews have focused heavily on the use of captive populations in reintroduction and rewilding attempts (Fernandez et al., 2017; Zamboni et al., 2017), and we suggest these captive populations may be an ideal focus for future conservation applications of this study. Through the application of organismal priming (e.g. heat hardening), and enhancing these natural capacities through use of developmental phenotypic programming and applied regulators (e.g. serotonin, methionine), conservationists may ultimately be able to augment lasting phenotypic organisation in select captive populations for use in these rewilding attempts. Specifically, this study provides preliminary evidence for a proactive strategy that not only prepares organisms for release into environments vastly different from their ancestral state but also programmes additional resistance and adaptive capacity in organisms and their offspring at risk of future unexpected extreme heat events.

This study revealed previously undescribed combinations of thermal performance traits in *Artemia* after heat hardening. Importantly, we have shown that multiple performance traits can be affected by heat hardening, and validated these as sound measures of phenotypic engineering in these types of experimental studies. We have also shown that these effects can be enhanced by the addition of specific physiological regulators, and that these benefits can be applied within and across generations. Together, this indicates complex outcomes of phenotypic engineering that can be applied to improve the capacity of organisms to respond to a rapidly changing environment and extreme heat events.

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Author contributions
Conceptualization: K.L.S., M.P.G., T.D.D., T.S.J.; Methodology: K.L.S., M.P.G., T.D.D., T.S.J.; Validation: K.L.S.; Formal analysis: K.L.S., T.S.J.; Investigation: K.L.S.; Resources: M.P.G., T.D.D., T.S.J.; Data curation: T.S.J.; Writing - original draft: K.L.S., T.S.J.; Writing - review & editing: K.L.S., M.P.G., T.D.D., T.S.J.; Visualization: K.L.S.; Supervision: M.P.G., T.D.D., T.S.J.; Project administration: T.S.J.

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Data availability

Data are available from the Dryad Digital Repository (Sorby et al., 2018): https://doi.org/10.5061/dryad.05s45

Supplementary information

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