

Thermal exposure and transgenerational plasticity influence embryonic success in a bivoltine estuarine sea hare

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ABSTRACT: Phenotypic plasticity has the potential to influence environmental adaptation on extremely short evolutionary timescales. Transgenerational plasticity (TGP) allows parents to provision their offspring for rapid environmental shifts in as little as one generation. We hypothesized that organisms that produce multiple generations of offspring each year use TGP to maximize their fitness under predictable fluctuations in seasonal environments. Using the direct-developing bivoltine eelgrass sea hare *Phyllaplysia taylori* as a test case, we examined the impacts of seasonal thermal variation (i.e. average temperature and acute heat stress) on physiological tolerance, maternal provisioning, and developmental plasticity across multiple generations. In the laboratory, we acclimated seasonally acclimatized adults from successive generations at 13, 17, and 21°C in order to assess plasticity of thermal tolerance limits. We also examined the effects of thermal acclimation and heat stress on reproductive output within a single generation in order to characterize TGP. Physiological plasticity, including but not limited to TGP, successfully maintained the viability of offspring under 2 seasonal conditions regularly experienced in the wild, despite differences in the density of offspring per clutch and large individual differences in offspring numbers under these conditions. These results indicate that warmer conditions (21°C) disrupt current patterns of plasticity and warrant further investigation of the long-term effects of chronic stress on TGP under climate change.

KEY WORDS: *Phyllaplysia taylori* · Physiological plasticity · Seasonal variation · Estuarine invertebrate · Thermal tolerance

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1. INTRODUCTION

Global change continues to amplify fluctuations in environmental conditions (e.g. temperature) that have physiological consequences for most organisms on earth. For example, average temperatures are rising (IPCC 2014), as are the frequency and intensity of heat waves (Min et al. 2013, Kharin et al. 2018). Organisms are thus required to respond to multiple

aspects of changes in temperature, often through shifting distribution to track their niche (Sunday et al. 2012) or through local adaptation of populations (Valdadares et al. 2014). Adaptation to chronic warming and increased acute thermal stress is expected to occur through selection on standing genetic diversity and, hence, to be dependent on population size, generation time, and existing genetic diversity (Chevin et al. 2010, Somero 2010, Franks & Hoffmann 2012).

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The interactive effects of standing genetic diversity and physiological plasticity, or the ability to change phenotype without an accompanying shift in genotype, can play a major role in buffering the effects of environmental variability on populations (Dewitt et al. 1998, Chevin et al. 2010, Del Giudice 2015).

Physiological plasticity can occur within a life-stage (e.g. among adults living in variable environments; Seebacher et al. 2015), within a generation characterized by life-stage-specific phenotypes (e.g. variation between embryos and adults; Del Giudice 2015), and transgenerationally via parental effects on the next generation (Galloway & Etterson 2007). The expression of physiological plasticity may be highly variable among individuals living at a small spatial scale (Dowd et al. 2015). Transgenerational plasticity (TGP) is defined broadly as the non-genetic effects observed in the offspring due to environmental exposure of the previous generations, and defined more specifically as the interaction between the current and previous generation's environmental conditions transduced by parental effects on offspring success (Salinas et al. 2013, Donelson et al. 2018). These non-genetic effects can be due to epigenetic inheritance from mother or father (i.e. histone modification, DNA methylation) or by maternal provisioning to the egg (i.e. different amounts and types of lipids, proteins, and RNAs) (Munday et al. 2013, Guillaume et al. 2016, Donelson et al. 2018). Since TGP is highly dependent on parental environment, individual variation in how organisms experience their environment (Dowd et al. 2015) can increase the expression of differences in TGP between individuals within a population. TGP can also modulate developmental trajectories of offspring (i.e. developmental plasticity). For example, whether annual killifish embryos enter diapause or develop without doing so depends both on the temperature conditions experienced by the mother and on the mother's age (Podrabsky et al. 2010). TGP is often adaptive (e.g. phenological shifts with environmental change in annual plants, Galloway & Etterson 2007; reproductive strategy [cf. r vs. K selection]; response to competition in bryozoans, Allen et al. 2008). However, TGP can also be non-adaptive (e.g. frost-acclimated trees, Cooper et al. 2019), and the effects of TGP are often subtle (e.g. viviparous lizards, Uller et al. 2011; also see Ghilambor et al. 2007 and Uller et al. 2013 for a broader discussion). Despite the importance of TGP in response to predictable environmental change, we know relatively little about the potential for TGP to influence long-term inheritance of favorable traits in response to climate change.

In estuarine ecosystems, environmental conditions of temperature, salinity, dissolved oxygen, and pH/pCO₂ are highly variable (Kimmerer 2004). Predictable fluctuations in these abiotic conditions occur seasonally. Other, less predictable, fluctuations occur due to precipitation and heat extremes, both of which are expected to increase with climate change (Min et al. 2013, IPCC 2014). In environments with predictable seasonal fluctuations, multivoltine (i.e. producing multiple generations per year) species experience positive selection for deterministic maternal effects as a consequence of the mother's ability to provision the offspring for a seasonal environmental shift (e.g. lower salinity during winter conditions in an estuary; Proulx & Teotonio 2017). During their early life stages, species in estuarine habitats have limited dispersal potential and therefore lack the ability to mitigate stress behaviorally via migration within or across generations, suggesting that TGP plays a role in the success of these species despite the resulting restricted gene flow (Galloway & Etterson 2007). In such situations, we may expect a high degree of physiological variation across individuals to benefit the inheritance of favorable TGP, as genetic diversity may be low. Although increased seasonal unpredictability may weaken the efficacy of TGP (Dey et al. 2016), it is possible that the high within-generation physiological plasticity promoted by TGP, where adult plasticity is maximized in offspring, is still an adaptive response under climate change.

Using the bivoltine direct-developing eelgrass sea hare *Phyllaplysia taylori* (Dall, 1900), we investigated the role of TGP in producing offspring that are physiologically matched to seasonal environmental fluctuations. We examined how exposure to acute heat stress, or an increase in unpredictable temperature extremes (Kharin et al. 2018), influence the efficacy of TGP. In estuaries of central California (USA), *P. taylori* has 2 non-overlapping generations per year, with reproduction occurring in late spring (summer generation) and early fall (winter generation) (Beeman 1963) (Fig. 1). The 2 generations have non-symmetrical durations, with the winter generation living significantly longer. These time periods characteristically differ in the average temperature and frequency of extreme heat days (Fig. 1). We hypothesized that mothers produce offspring of different quantities and sizes based on the temperatures they experience, and thus that the summer and winter generations will have different responses to temperature. We further hypothesized that when parental exposure temperatures deviate from seasonal averages (i.e. shifted to seasonal maxima), this seasonally

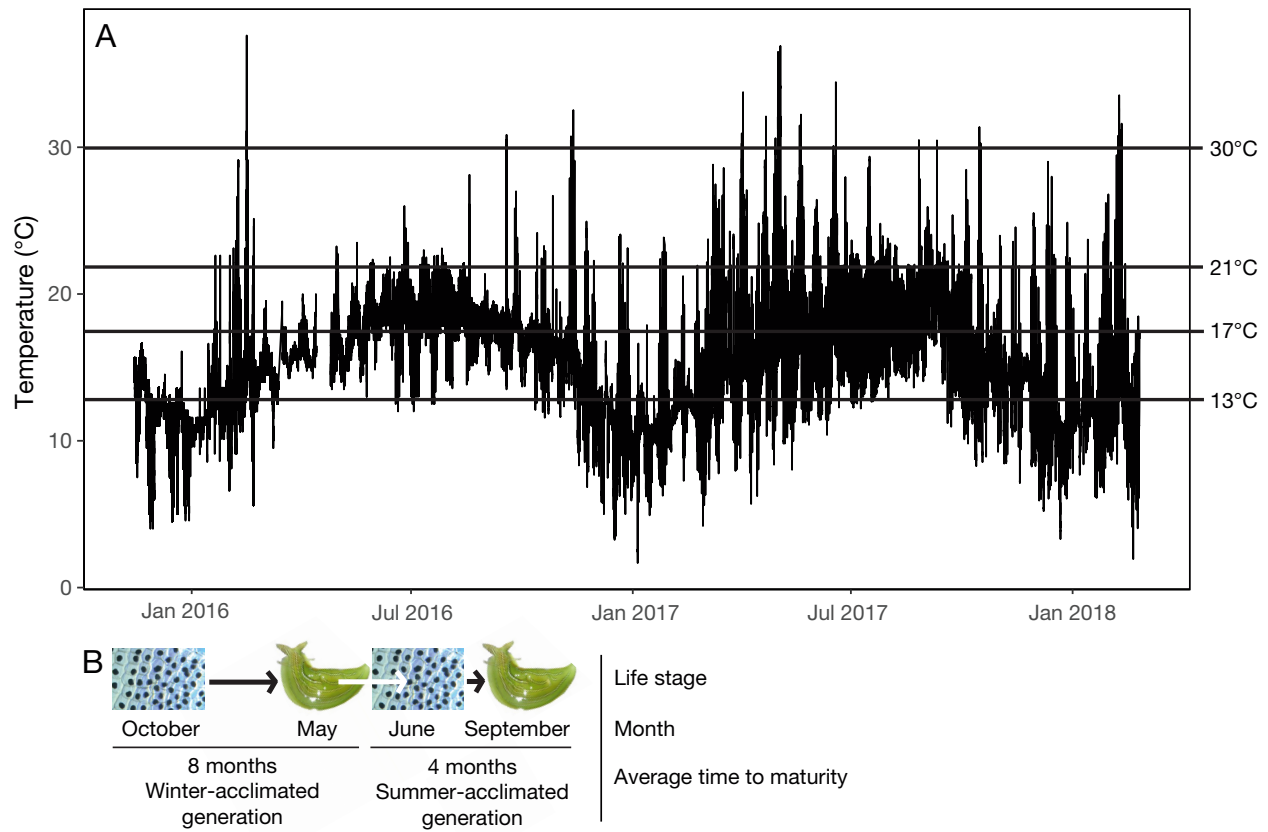


Fig. 1. (A) Temperature records (November 2016 to January 2018) from the *Phyllaplysia taylori* collection site at Point Molate, San Francisco Bay, CA, USA. Horizontal lines denote temperature treatments used in this study, reflecting summer (17°C) and winter (13°C) averages and summer maxima (21°C) in 2016. (B) Approximate timing of egg presence and development to adults in winter (October through May) and summer (June through September) generations

matched TGP will incompletely compensate for the effects of increases in average temperature and in heat extremes. To test the above hypotheses, we raised sea hare mating pairs through maturity in 3 temperature treatments that reflected the average of current winter, current summer, and future summer conditions. We then fully crossed these temperature treatments in a split-brood design for offspring developmental exposure. By crossing parental and developmental treatment conditions, we were able to investigate both temperature-induced TGP and individual-based variations in maternal egg provisioning along with the impact of the resultant developmental plasticity on enhancing brood success across temperatures. From the resulting egg masses, we predicted that embryos laid in future summer conditions would have reduced survival and decreased developmental plasticity across all development temperatures due to TGP. Additionally, we examined the effects of a brief intense heat shock within each generation in order to assess the relative effects of heat extremes in the context of thermal acclimation and TGP.

2. MATERIALS AND METHODS

2.1. Collection

Adult *Phyllaplysia taylori* from 2 successive generations within a 50 m² collection area were hand-collected on 24 May and 6 September 2016 at Point Molate, San Francisco Bay, California, USA (37.9453°N, 122.4143°W). Habitat temperatures at the times of collection were 17.5°C (May) and 18.5°C (September), and had averaged 16.1 and 18.6°C, respectively, over the prior 3 mo period. Data were collected every 30 min using a HOBO TidbiT v2 temperature data logger. Mean yearly temperature in 2016 increased from 14.23 to 15.40°C in 2017. The occurrence of temperatures above 30°C increased from 9 d in 2016 to 57 d in 2017. Across the entire eelgrass bed, the average sea hare length was 3.61 cm at a density of 0.11 sea hares m⁻² in May and 2.75 cm at a density of 0.99 sea hares m⁻² in September (Tanner 2018).

We maintained these sea hares in the laboratory (26 winter-acclimated and 21 summer-acclimated

specimens). After adjusting to laboratory conditions reflecting field temperature at time of collection for 3 d, specimens were separated randomly into 3 groups and acclimated at one of the following 3 conditions: temperatures of 13, 17, and 21°C, for a period of 10–17 d, at a constant salinity of 31 ± 1 psu, in flow-through aquaria with constant aeration and a 3 d feeding interval (for details of the feeding procedure, see Tanner et al. 2019). We selected this period based on existing evidence that 2 wk is adequate for acclimation to steady state in the majority of physiological phenotypes (Somero 2015, Khlebovich 2017).

2.2. Determination of critical thermal maxima in seasonally acclimated sea hares

After the 10–17 d acclimation period, we determined the critical thermal maxima (CT_{max}) of foot muscle function as follows. We placed 24 h-starved specimens individually on the inner wall of a 500 ml glass beaker filled with seawater at a starting temperature equivalent to the specimen's acclimation temperature and then warmed the beaker of seawater at a rate of 4°C h^{-1} . We recorded the CT_{max} as the temperature at which specimens fell off the beaker wall (see Armstrong et al. 2019 for details).

2.3. Genotyping

To confirm that successive generations were from the same population, we conducted a RADseq analysis. Using the Omega Biotek E.Z.N.A.[®] Tissue DNA kit, we extracted DNA from the frozen tissue of all individuals collected. We then quantified the dsDNA on a Qubit 2.0 fluorometer and conducted a downstream analysis on individual samples that met a 300 ng threshold (summer-acclimated $n = 21$; winter-acclimated $n = 25$). Following a modified ddRADseq protocol (Peterson et al. 2012), equal amounts of each individual sample were double digested with the restriction enzymes *Sph1-HF* (p1) and *MluCI* (p2) (New England Biolabs) (Saarman & Pogson 2015), adapter-ligated on both ends using Nextera DNA Flex adapters (New England Biolabs), pooled randomly into 12 libraries, and size-selected to 500–600 bp using a Pippin Prep at the Functional Genomics Laboratory at UC Berkeley. Libraries were sequenced on an Illumina HiSeq4000 (SR100) and analyzed for population relatedness between generations using the Stacks v2.2 pipeline with all presets for single-end reads without a reference genome (Paris et al. 2017).

Reported here are F_{ST} statistics, observed heterozygosity, and haplotype diversity across 265 365 loci comprised of 25 690 235 sites. Quality control was performed using FastQC v.0.11.7.

2.4. Transgenerational plasticity experiment: specimen collection and husbandry

Sea hare individuals ($n = 144$) were collected in August 2017 during a low tide at Point Molate, San Francisco Bay, in the 50 m² collection area used for our adult CT_{max} experiment. In this case, we collected individuals 1–1.5 cm in length before they had reached reproductive maturity and transported them to the laboratory in an aerated cooler within 45 min of collection. These specimens were held in individual enclosures at $17 \pm 1^\circ\text{C}$ and 31 ± 1 psu for 1 wk and monitored for egg laying. No individuals laid eggs during this period. After the first week, individuals were weighed, photographed, and paired for mating in acrylic flow-through cylinders (7 cm diameter \times 15 cm height) suspended in insulated and lidded acrylic tanks (Aqua Logic) in a 5 \times 8 cylinder grid supplied with a continuous flow of recirculated seawater with replacement (Paganini et al. 2014). Cylinders were randomly assigned to 1 of 3 temperature treatments (13, 17, and 21°C) at 31 ± 1 psu ($n = 24$ cylinders per treatment) and held for up to 3 mo. Sea hare pairs were fed every 3 d (for details of the feeding procedure, see Tanner et al. 2019), and cylinders were checked daily for egg masses.

Out of the 72 total mated pairs (24 pairs per temperature; 144 individuals total), only 16 pairs produced egg masses (see Table 1 for treatment sample sizes). Mating was not induced, only passively encouraged with single pairing in enclosures. Since this taxonomic group of sea hares is thought to be simultaneous hermaphrodites, each individual may act as both male and female and therefore egg masses may be from either individual. Anecdotally, sea hares in this experiment did not act as simultaneous hermaphrodites, instead only laying 1 egg mass at a time. The frequency and timing of egg laying varied among individuals, averaging ~1 wk between the first and second egg masses prior to parental heat shock, and ~3 d between the heat shock and the third and fourth egg masses; the only generalizable pattern to emerge was that eggs in the 21°C treatment were laid earlier than those in the other 2 treatments. To account for potential non-viable first clutches, which we had observed anecdotally during pilot testing, we noted but did not remove the first egg mass in

each cylinder until the second egg mass was laid; we then removed the second egg mass from each mating pair for experiments (see Section 2.5). In order to assess the effect of adult heat stress on egg masses, adults were acutely heat shocked for 1 h at 30°C, which reflects a currently environmentally relevant upper temperature (see Fig. 1, and Armstrong et al. 2019), and were then returned to their assigned temperature treatments (13, 17, or 21°C). The third egg mass was noted, and the fourth egg mass was also collected for experiments (see Section 2.5) in the manner just described. In an earlier experiment, we tested the interactive effects of parental heat shock and parental age at egg laying and found no significant correlation between embryo hatching success and egg mass number after controlling for parentage (linear mixed effects model, LME $t_{56} = -1.799$, $p = 0.08$; see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m634p199_supp.pdf).

2.5. Egg mass analysis

Within 24 h of deposition, we divided each of the second (pre heat shock) and fourth (post heat shock) egg masses into 6 sections, and we photographed 3 random selections from each section under the microscope in order to obtain initial measurements of egg quality and quantity. These 6 sections of the egg mass were crossed among the 3 temperature treatments (13, 17, and 21°C), with 3 masses assigned to no further manipulation (controls) and another 3 assigned to undergo a 1 h heat shock of 30°C less than 24 h post deposition (embryonic heat shock; experimental condition). Egg mass sections were placed in plastic containers (5 cm diameter \times 2 cm height) with 40 ml artificial seawater (32 ppt, Instant Ocean Aquarium Sea Salt) and enough remaining air headspace maintained to minimize oxygen limitation between changes of water. The water was changed every 3 d. Egg mass sections were maintained at temperature treatments of 13, 17, and 21°C, such that there were 2 sections of each egg mass in each temperature condition, one that had been heat shocked and one that had not (see graphical representation of experimental design in Fig. S2).

We examined the egg mass photos of both pre heat shock egg masses less than 12 h post deposition, in order to evaluate embryo density (number of embryos per picture), embryo diameter (3 random embryos per picture), and egg case diameter (3 random embryos per picture) using cloud-based counting software (source code from Chang 2018, hosted by

the Open Computing Facility at UC Berkeley). Size standard photos were taken during every day of sampling, and photographs of egg masses were scaled accordingly (see Fig. S3). We then assessed hatching success starting at ~25 d after egg deposition and every ~5 d thereafter until all live embryos had hatched. In order to determine the quantity of embryos that failed to hatch, we photographed 3 random areas that filled the microscope frame of the egg mass section after all live embryos had hatched, and we scaled these photos to the whole egg mass using the size standard photos and the total egg case size.

2.6. Statistical analyses

Analyses were conducted in R (version 3.5.1) with the packages 'ggplot2' and 'nlme' (Wickham 2009, R Team Core 2017, Pinheiro et al. 2019). Non-parametric tests were used when random effects were necessary, i.e. to account for parental ID. For the CT_{max} experiment, we used ANOVA to evaluate the differences between winter- and summer-acclimated individuals' critical limits. In this parametric test, normality was evaluated using a Shapiro-Wilk test ($p = 0.10$) and heterogeneity of variance was evaluated using Levene's Test (acclimation temperature: $p = 0.91$, season: $p = 0.29$). In the TGP experiment, we generated full ANCOVA models incorporating parent pair ID as a fixed effect (Table S1), along with corresponding LME models that incorporated parent pair ID as a random effect (Table S2). We used these models to evaluate all response variables in the TGP experiment against predictors of treatment temperature and the presence of heat shock (embryonic or adult). This paired approach was taken to ensure phenotypic variation as a signal, not noise, was accounted for as a possible physiological response to warming (Dowd et al. 2015, Tanner & Dowd 2019). Below, all results are reported from the LME models incorporating parent pair ID as a random effect to focus on the main effects of temperature exposure.

For the CT_{max} experiment, the acclimation response ratio (ARR) was calculated as the slope of the line between average critical limits at each of the 3 acclimation temperatures (Armstrong et al. 2019). In the TGP experiment, the total number of eggs per clutch, or clutch size, was calculated as the product of mean embryo density per mm and total egg mass area (mm²). Hatching success was calculated as the total number of unhatched embryos subtracted from the total number of eggs per clutch. Energy alloca-

tion per egg was calculated as the ratio between total number of eggs and the average embryo diameter per clutch. Total maternal investment at egg laying, or the total amount of egg material, was calculated as the average egg area (mm^2) multiplied by the number of eggs (se Fig. S3).

3. RESULTS

3.1. Seasonal acclimation of heat tolerance

Winter and summer generations of field-acclimated sea hares differed significantly in CT_{max} (ANOVA, $F_{4,37} = 7.743$, $p < 0.01$) and in the plasticity of CT_{max} inferred from the ARR (Fig. 2). The winter-acclimated individuals had a positive ARR between 13 and 21°C (slope = 0.45), while summer-acclimated individuals had a negative ARR between 13 and 17°C (slope = -0.95; Fig. 2). Summer-acclimated individuals did not survive the 21°C acclimation treatment.

3.2. Genetics

We determined that all individuals belonged genetically to the same population (mean $F_{\text{ST}} = 0.12$, mean $\phi_{\text{ST}} = 0.05$, mean $F_{\text{ST}'} = 0.05$). Mean nucleotide diversity was 0.63 in the summer-acclimated individuals and 0.59 in the winter-acclimated individuals. Observed heterozygosity was 0.00073 in both sets of individuals across all single nucleotide polymorphisms (SNPs), while observed heterozygosity among the variant SNPs only was 0.29 (winter-acclimated) and 0.30 (summer-acclimated).

3.3. Parental acclimation effects

Reproduction occurred successfully after heat shock in 4 of the 5 pairs in the 13°C treatment, in 4 of 5 pairs in the 17°C treatment, and in 1 of 5 pairs in the 21°C treatment. Due to low sample size, we found no significant difference among treatments for the portion of the population laying egg masses after heat shock (Pearson's r^2 test, $p = 0.713$).

All individuals in all treatments began at a similar size (328.4 ± 14.8 mg). Mass during initial egg laying differed significantly among treatments (Kruskal-Wallis $\chi^2 = 10.72$,

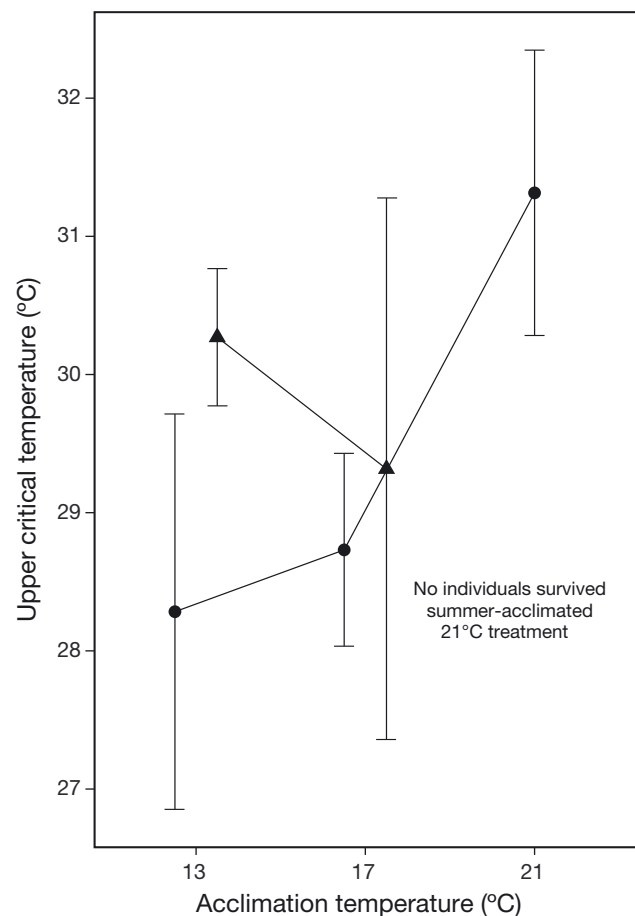


Fig. 2. Upper critical temperature and acclimation response (plasticity) of foot muscle function differed between 2 generations (triangles: summer-acclimated generation; circles: winter-acclimated generation) of the sea hare *Phyllaplysia taylori* collected from one population at Point Molate, San Francisco Bay, CA. Error bars are 95% CI

$df = 2$, $p < 0.005$), with the 21°C treatment parents being the lightest, followed by the 13°C and then 17°C parents (Table 1). Our sample size was too small to detect an effect of mass at last egg laying; the average mass gained over the captive period was 779.3 ± 125.8 mg.

Table 1. Mean (\pm SE) mass (mg) of *Phyllaplysia taylori* parental treatment groups, evaluated directly after egg mass laying for the experimental egg masses. Starting sample size for each of 3 treatments was $n = 48$ individuals (24 mated pairs), and not all individuals that mated survived (this is due to sperm storage; mortality before egg laying was $n = 5$, 4, and 19 at 13, 17, and 21°C, respectively). Therefore, some mated pairs are represented by only 1 individual here. HS: heat shock

Parental HS	13°C	n	17°C	n	21°C	n
No HS	931.0 \pm 72.7	12	1221.4 \pm 85.7	8	706.8 \pm 54.3	6
HS	1142.8 \pm 89.2	9	1274.0 \pm 141.8	6	892.6	1

Heavier parents laid eggs earlier (slope = -0.002 d g^{-1} , Kruskal-Wallis $\chi^2 = 112$, $\text{df} = 10$, $p < 0.0001$), had larger clutches (slope = $-22.15 \text{ embryos g}^{-1}$, Kruskal-Wallis $\chi^2 = 22.15$, $\text{df} = 9$, $p < 0.0001$), allocated less energy per egg (slope = $0.000004 \text{ energy units g}^{-1}$, Kruskal-Wallis $\chi^2 = 77.16$, $\text{df} = 9$, $p < 0.0001$), and invested more heavily in reproduction across treatments (slope = $-1.24 \text{ maternal investment units g}^{-1}$, Kruskal-Wallis $\chi^2 = 77.61$, $\text{df} = 9$, $p < 0.0001$), but not in an acclimation-specific pattern. While the parents' average weights were significantly different at first egg laying among treatments, the timing and allocation of egg laying were not significantly different due to treatment effects, and we therefore did not incorporate body size into the main models. Effects not due to acclimation were attributed to individual variation among parents.

3.4. Maternal investment

3.4.1. Acclimation effects

Regardless of parental origin, clutches in the 13°C treatment had 2.31 ± 0.27 (mean \pm SE) fewer eggs

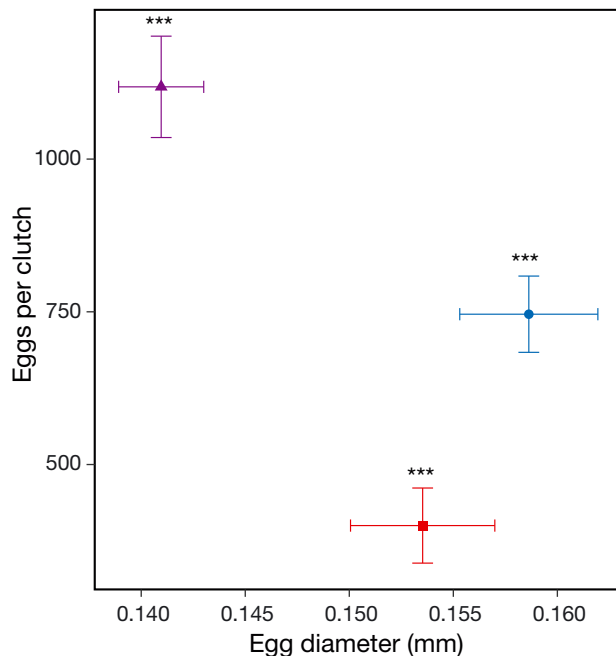


Fig. 3. *Phyllaplysia taylori* egg diameter and number of eggs per clutch were inversely related across all clutches per Thorson's rule at current seasonal variation in temperatures (13 and 17°C), but not at even warmer temperatures predicted with climate change. Parental acclimations were 13°C (blue circle), 17°C (purple triangle), and 21°C (red square). Error bars are 95% CI; asterisks denote significance at $p < 0.001$

per mm^2 and 11% larger eggs than those in the 17°C treatment. The 21°C treatment featured eggs of intermediate size, but the number of eggs was not significantly different from the 13°C treatment (Fig. 3, Tables S2 & S3). Independently from each other, embryo diameter and clutch size were significantly different by treatment temperature (embryo diameter: Kruskal-Wallis $\chi^2 = 64.516$, $\text{df} = 2$, $p < 0.0001$; eggs per clutch: Kruskal-Wallis $\chi^2 = 103.51$, $\text{df} = 2$, $p < 0.0001$). Earlier clutches had larger eggs (Kruskal-Wallis $\chi^2 = 72.23$, $\text{df} = 17$, $p < 0.0001$), and this relationship remained consistent within treatments (Table S3). Acute heat stress in parents did not influence embryo diameter (Kruskal-Wallis $\chi^2 = 0.33$, $\text{df} = 1$, $p = 0.57$). Parental acclimation temperature had no significant effect on total maternal investment, but individual pairs varied significantly in their total maternal investment (Tables S1 & S2). Clutches with lower maternal investment overall had higher investment per individual egg, but this rela-

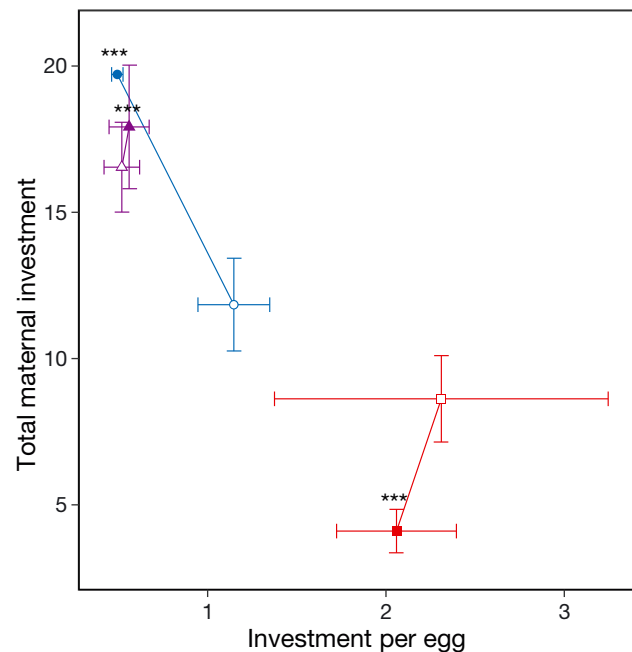


Fig. 4. Investment per egg and total maternal investment in *Phyllaplysia taylori* are dependent on parental temperature. Parental acclimations were 13°C (blue circles), 17°C (purple triangles), and 21°C (red squares). Open symbols indicate pre heat shock (HS) treatment, filled symbols indicate post HS treatment. Each point is the average \pm 95% CI. Total maternal investment was estimated from average embryo size per clutch across the total number of embryos in an egg mass. Investment per egg (total maternal investment divided by total number of embryos) is linearly scaled so that a value of 1 represents the average investment per egg across all measurements, negative values represent below-average measurements, and positive values represent above-average measurements

tionship was also independent of parental acclimation temperature (Fig. 4, Table S2). The highest maternal investment per egg was in the 21°C group, which had 3.2 times more investment per egg than the other 2 groups. We found higher maternal investment in parent pairs that laid eggs later in the season (Kruskal-Wallis $\chi^2 = 97.48$, $df = 16$, $p < 0.0001$) and at colder temperatures (Table S4). Clutch size and embryo diameter were strongly influenced by parental acclimation temperature (clutch size: Kruskal-Wallis $\chi^2 = 283.05$, $df = 12$, $p < 0.0001$; embryo diameter: Kruskal-Wallis $\chi^2 = 150.85$, $df = 13$, $p < 0.0001$; Fig. S3).

3.4.2. Heat stress effects

By comparing egg masses laid pre and post heat shock, we found that parental heat shock increased maternal egg provisioning in parents raised at 13 and 17°C, but decreased maternal egg provisioning in parents raised at 21°C (Fig. 4, Tables S2 & S3). At 13 and 17°C, parental heat shock increased total maternal investment by 25 %, while at 21°C, parental heat shock decreased total maternal investment by 52 %. Maternal investment was positively correlated with parental heat shock across treatments (slope = 2.04 maternal investment units per °C, Kruskal-Wallis $\chi^2 = 4.14$, $df = 1$, $p = 0.04$), but not within treatments (Table S4).

3.5. Hatching success of non-heat-shocked embryos

Total offspring per clutch was significantly lower in the 21°C parental and developmental temperature treatments, although the effect was not additive (ANOVA $F_{4,124} = 6.764$, $p < 0.0001$). Plasticity successfully maintained a constant offspring number in parental and developmental 13 and 17°C treatments, where there were no significant differences among any treatment combinations (Fig. 5B). Irrespective of treatment conditions, some parents produced significantly more successful offspring than others (2 parent pairs from 17°C and 1 pair from 13°C; see Fig. S4).

Individual effects of parental origin were stronger than treatment effects in determining hatching success (Fig. 5A, Table S1). When controlling for clutch effects, hatching success was negatively impacted by acclimation of parents and embryos to both chronic and acute stressful temperatures

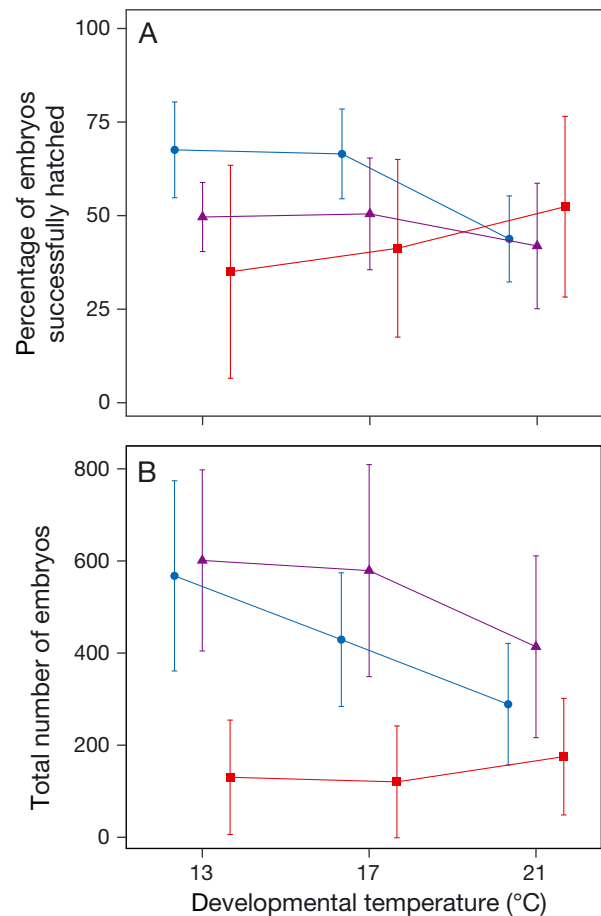


Fig. 5. (A) Total successful offspring and (B) offspring hatching success of *Phyllaplysia taylori* at 3 acclimation temperatures. Parental generation exposure temperatures were 13°C (blue circles), 17°C (purple triangles), and 21°C (red squares). Symbols are mean \pm 95% CI

(Table S5). Average hatching success at 21°C was 18% lower than the average hatching success at 13 and 17°C (Fig. 5A). Average hatching success at 13°C was higher than at 17°C; however, embryo density was inherently lower in offspring egg masses at 13°C. Total offspring per clutch was therefore not significantly different between these 2 treatments. Across parental treatments, development of offspring at 21°C significantly decreased the total number of embryos hatched by an average of 32% (Fig. 5A, Table S5). Embryonic hatching success ranged from 0–100% of the clutch hatched. There was more variation in embryonic hatching success in the parents exposed to 21°C with fewer parent pairs than the other 2 parental treatments. Irrespective of treatment, parental effects were strong in determining hatching success (Kruskal-Wallis $\chi^2 = 33.896$, $df = 13$, $p = 0.001$).

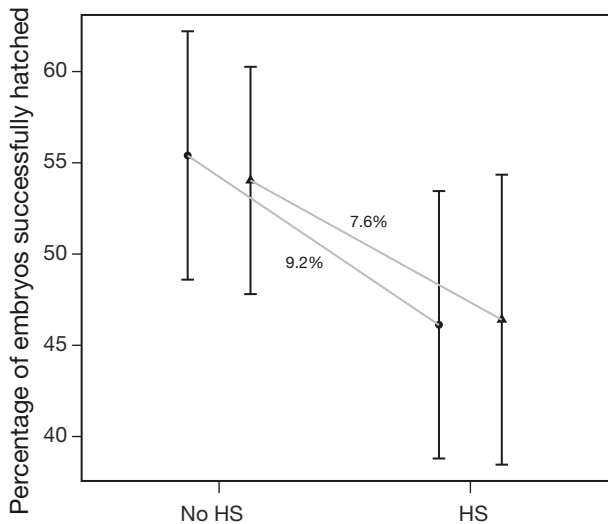


Fig. 6. Hatching success in *Phyllaplysia taylori* egg masses across all acclimation conditions was 7.6% lower under parental heat shock (HS) conditions when compared with control, or no HS (triangles) and 9.2% lower under embryo developmental HS conditions when compared with control (circles). Error bars are 95% CI

3.6. Hatching success of heat-shocked embryos

Parental heat shock and embryonic heat shock negatively impacted the percentage of embryos hatched by an average of 7.6 and 9.3%, respectively (Fig. 6, Table S5). There were no significant additive effects of experiencing both heat shocks. Parental and developmental treatments did not play a role in determining offspring hatching success after heat shock, although 4 parent pairs had significantly different hatching success after heat shock irrespective of acclimation treatment (see Table S1).

4. DISCUSSION

This study investigated the importance of TGP across seasonal variation in temperature for the bivoltine estuarine sea hare *Phyllaplysia taylori*. We documented the genetic structure of 2 consecutive field generations of *P. taylori*, confirming that differences between parents and offspring are attributable to physiological plasticity and negating the 'seasonally dominant genotype' hypothesis (Carvalho & Crisp 1987, Beaumont & Nichols 1996). We found evidence for TGP as well as individual-based variation in maternal egg provisioning and the resulting developmental plasticity that influence

brood success across temperature treatments. Plasticity, though not limited to TGP, was able to compensate for currently experienced seasonal shifts in average temperatures. However, plasticity was inadequate to maintain reproductive traits under temperatures that reflect likely future summer conditions, although further work is needed to parse the long-term effects of acclimation to these conditions. Additionally, some parental pairs were more fecund than average, suggesting that individual variation plays an important role in population persistence.

4.1. Plasticity maintains offspring numbers under predictable seasonal regimes

TGP often occurs in predictably fluctuating environments due to cue reliability (Stearns 1976, Reed et al. 2010). While there are many definitions of TGP in relation to its deterministic function, we broadly consider the effect of the parental condition on offspring quality and quantity here, as noted earlier. We found that the range of plasticity in embryo characteristics for adults exposed to 21°C temperature was not sufficient to maintain the same degree of offspring success under high temperatures as under current conditions (Fig. 5B). Plasticity resulted in similar numbers of total successful offspring in parents at 13 and 17°C, suggesting that a trade-off between egg quality and number, as predicted by Thorson's rule, results in similar numbers of successful offspring at different temperatures (Thorson 1950). At the average winter temperature (13°C), we observed increased individual provisioning and decreased numbers of total offspring, indicating that anticipatory or deterministic maternal provisioning maximized the percentage of successful offspring (Figs. 3 & 4). While compelling correlations, these may in fact be non-adaptive responses. At the average summer temperature (17°C), we observed decreased individual provisioning and increased numbers of offspring, indicating that randomized maternal provisioning maximized the number of successful offspring (Figs. 3 & 4). The winter and summer reproductive strategies resulted in the same number of successful offspring at both temperatures, including that the best adaptive TGP strategy—randomizing or deterministic—is dependent on the season. Computational models have shown that the optimal bet-hedging strategy is a combination of types of bet-hedging, meaning that egg size varies within

clutches and between seasons/years (Olofsson et al. 2009). Additionally, these models show that what is typically seen as non-adaptive variation within a population can be reflective of this mixed strategy (Fig. S4). Under conditions of climate change (21°C), a reduction in the potential available energy (Applebaum et al. 2014) due to increased unpredictable fluctuations and an increased mean temperature (Min et al. 2013) could disrupt the effectiveness of this mixed strategy.

A parental environment of 21°C, a temperature typifying the current maximum and average future temperatures, resulted in an 18% decrease in overall survival of offspring in the next generation, despite 3.2 times higher maternal provisioning per offspring, when compared with the 13 and 17°C acclimated parents. A low survival rate despite high provisioning per individual offspring suggests a breakdown of the relationship between temperature and maternal provisioning during exposure to maximal temperatures. While offspring egg size may have been larger at the stressful temperature of 21°C, resulting in increased energy stores for the next generation, the mechanisms underlying plastic responses during development may nevertheless have been disrupted. The only condition in which the 21°C acclimated parents had an advantage was at a developmental temperature of 21°C (Fig. 5A). These warmer parents did partially increase offspring success at their same developmental temperature, but in a seasonal context, this is unlikely to be adaptive. At that developmental temperature, we also saw that the offspring of parents raised in other thermal environments were similarly unsuccessful, suggesting that developmental plasticity at 21°C is reduced regardless of parental condition. Since the highest summer temperatures are followed by a sharp decrease to winter conditions, offspring born at 21°C are unlikely to develop at that same temperature. However, in the future with continued climate warming, offspring may experience a longer seasonal duration of 21°C developmental temperatures and therefore having parental exposure to that temperature could be advantageous, though not in a manner mediated by TGP. When parent and offspring environments, or maternally predicted offspring environments, do not match, TGP is not adaptive (Marshall & Uller 2007). In the 21°C parental treatment, offspring success was greatly reduced across all temperatures, showing that even increased provisioning per egg was not sufficient to ameliorate the effects of maternal exposure to high temperatures.

4.2. Temperature-dependent clutch allocation

Parental acclimation to 21°C resulted in greater investment per egg, but curtailed investment. Due to the bivoltine nature of *P. taylori*, life history trade-offs may be characterized as a 'fast-living' strategy, whereby reproduction is promoted at the expense of survival under acute stress (Zera & Harshman 2001). Egg size and total investment, or reproductive effort, are often considered part of a 2-step process and to be independently evolved (Winkler & Wallin 1987). However, these traits are linked across a wide clade of invertebrates, and allocation towards total offspring and individual partitioning happens simultaneously (Caley et al. 2001). Our study found that mothers at 13 and 17°C did not have limitations on their total maternal investment, and therefore the relationship between individual egg allocation and total reproductive effort was predictable. Thus, our results support the proposed linkage of allocation in clutches and individuals (Caley et al. 2001), but only under temperatures that reflect seasonal averages, not maxima or future conditions.

We also saw relatively low reproductive effort across all parent pairs in comparison with observations in the wild, which could be attributed to a number of things. One compelling argument is that this delayed reproduction was an adaptive TGP strategy to increase allocation to fewer offspring. While there was no significant within-mother variation in offspring quality, it is possible that the clutches were close enough in time to negate effects predicted to account for variation in clutch quality (Kindsvater et al. 2010). We did not see this delay in the 21°C parental treatment, which might account for the lower maternal investment overall but higher investment in each individual embryo, as the strategy of maximizing investment to individual offspring would stand.

4.3. Lasting effects of extreme heat days on offspring survival

Extreme heat days are expected to increase in frequency and duration with climate change (Min et al. 2013, IPCC 2014). An increase in the frequency and duration of heat waves may be outside of the current window of environmental heterogeneity encompassed in an estuarine organism's plastic response, therefore making extreme temperature a greater concern than increases in average temperature (Vasseur et al. 2014). Reduced hatching success

across parental and developmental heat shock treatments indicated that, overall, extreme heat days are detrimental to population persistence (Fig. 6). Since *P. taylori* are able to store sperm for up to 42 d (R. L. Tanner pers. obs.; previously 35 d in Chambers 1934) and since all parents laid eggs post heat shock within that time window, the parental gametes stored within the mother experienced the same heat shock and therefore showed similar, non-additive effects.

This study focused on maternal provisioning by measuring egg size, but paternal effects have been found to match or even exceed maternal effects with temperature stress (Guillaume et al. 2016). Therefore, heat shocking both parents may have had significant effects on offspring quality. Guillaume et al. (2016) found that thermal stress during gamete production prior to egg fertilization negatively impacted offspring success, with paternal gametes more susceptible to thermal stress than maternal gametes, which increased unviability with increased temperature (Guillaume et al. 2016). Reduced fertilization and reduced offspring success are reported with parental gamete heat shock at temperatures within the range used in this study, suggesting that addressing damage to all gametes with extreme heat days could shed light on reduced fecundity and offspring viability (Binet & Doyle 2013, Guillaume et al. 2016).

4.4. Individual variation in offspring size, number, and success

We observed high inter-individual variation in offspring number and quality regardless of temperature exposure. Three parent pairs (2 at 17°C and 1 at 13°C) had significantly more offspring than all other parent pairs (Fig. S5), but those parents were not the parent pairs that produced large embryos (Fig. S4). Inter-individual variation in offspring success can result in some parents contributing disproportionately more offspring to the next generation. What is usually considered non-adaptive variation could potentially reflect a mixed maternal strategy with regard to egg quality versus quantity (Olofsson et al. 2009). Inter-individual variation in adult physiology plays an important role in population-level mean responses (Denny et al. 2011, Dowd et al. 2015), and incorporating further considerations of variation in other life stages is the next step in understanding climate change effects across multiple generations in a single population.

4.5. Conclusions

Incomplete compensation by plasticity for chronic exposure to high temperatures suggests that seasonally dependent phenotypes in multivoltine species like *P. taylori* are likely to be disrupted by habitat warming, although the nature and degree of the disruption may depend on how many generations experience this chronic stress. Adverse responses to increased temperature and temperature variability in other estuarine organisms have been documented on the scale of a single generation; however, there is both a need and value in expanding this body of work to include the transgenerational effects of temperature in estuarine invertebrate responses (Incze et al. 1980, Smart et al. 2012). Limitations of developmental plasticity stem from chronic stress in mothers and acute stress across all life stages, but the magnitude of effects has much to do with individual variation regardless of treatment condition. These limitations have the potential to decrease population persistence in a warming ocean and reflect the importance of TGP in rapid adaptation to climate change, along with considerations of inter-individual variation in all plastic responses.

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