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Cite this article: Neylan IP, Vaidya RV, Crable EL, Faircloth BC, Dassanayake M, Kelly MW. 2026 Genetic assimilation and accommodation shape adaptation to heat stress in a splash pool copepod. *Proc. R. Soc. B* **293**: 20253265. https://doi.org/10.1098/rspb.2025.3265

Received: 18 December 2025
Accepted: 13 April 2026

Subject Category:
Evolution

Subject Areas:
evolution, ecology, physiology

Keywords:
thermal stress tolerance, plasticity-led evolution, acclimation, transcriptomics, developmental plasticity

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.8430439.

THE ROYAL SOCIETY PUBLISHING

Genetic assimilation and accommodation shape adaptation to heat stress in a splash pool copepod

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Understanding organismal responses to dynamic environments is becoming increasingly important in our rapidly changing world. Beyond genetic adaptation, plastic responses to the environment can alter phenotypes and fitness, ultimately driving evolution. However, the interaction between plasticity and adaptation during environmental change is complex and hard to measure in natural systems. Here, we used two populations of *Tigriopus californicus* copepods, a thermally tolerant southern population and a thermally sensitive northern population, to conduct a fully factorial, split-brood experiment where we exposed animals as larvae and adults to either a sublethal heat stress or control (no heat treatment) before measuring phenotypic plasticity of heat tolerance and gene expression plasticity patterns. We found that increased thermal tolerance across populations came at the expense of physiological plasticity and evolved through both higher baseline expression and increased gene expression plasticity of heat stress-related genes. Importantly, we found evidence for plasticity-led evolution in this system, with the same set of genes contributing to long-term evolutionary changes across populations and to short-term physiological adjustments within populations, providing insight into how these populations will adapt and respond to future environmental change.

1. Introduction

How species adapt to changing environments is a key problem in evolutionary biology and is becoming increasingly critical to understand in our rapidly changing world [1,2]. Evolutionary responses occur via genetic adaptation, but a single genome can produce multiple phenotypes in response to the environment, meaning that phenotypic plasticity can also play an important role in determining which individuals successfully adapt and persist [3,4]. However, the interplay between plasticity and adaptation during environmental change is complex [5,6]. Plasticity may mask underlying genetic variation, slowing responses to selection [7], or plasticity may facilitate adaptation by allowing populations to persist long enough to allow evolutionary rescue [8,9]. But most intriguingly, the evolution of plasticity itself can pave the way for subsequent adaptation by facilitating the production of genetic variation in environmentally responsive traits [10–12]. To predict future evolutionary outcomes in changing environments, it is critical to understand the role plasticity plays in genetic adaptation over time as well as the underlying mechanisms that promote or suppress plasticity in a population [13].

Gene networks that underlie plastic traits are built by many regulatory and protein coding changes over long evolutionary time scales [14,15]. But once these networks exist, changes in gene regulation can create variation in the environmental responsiveness of a trait [16], which is, in turn, the raw material necessary for further evolutionary changes in plasticity [17,18]. In variable or changing environments, selection for better environmental tracking should favour the evolution of increased plasticity, a process known as genetic accommodation [5,19]. Over time, if the environment becomes stable or if plasticity becomes maladaptive, selection will favour the loss of environmental responsiveness via the process of genetic assimilation [20,21]. Traits with greater plasticity and environmental sensitivity tend to harbour greater genetic variation and thus a higher capacity for evolutionary change [22,23]. Therefore, evolutionary changes that lead to reduced plasticity, such as genetic assimilation, can increase vulnerability to changing environments by limiting genetic variation.

The magnitude of plasticity can be modulated by the timing of exposure to a given cue. Many of the most well studied plastic traits are irreversible and triggered by cues encountered during development [24–26]. But reversible acclimation throughout adulthood also plays a role in adaptive evolution, especially in response to rapid environmental shifts, and both forms of plasticity interact to shape adult phenotypes and fitness [20,27]. Developmental priming can heighten or dampen the environmental sensitivity of a trait during adulthood, an interaction ultimately shaped by changes in gene expression [28]. Early exposure to a cue may result in upregulation of environmentally responsive genes during development that are maintained at a higher baseline into adulthood, resulting in carryover effects of early exposure and the loss of adult plasticity. On the other hand, early exposure may alter transcriptional machinery to produce higher plasticity in adult gene expression and increase the environmental sensitivity of a trait [29]. Over time, selection can act on these same gene regulatory modifications to drive the process of genetic accommodation or assimilation [30].

Evaluating the contribution of genetic accommodation and assimilation to adaptation is notoriously difficult in natural systems where it is only possible to measure end points of evolution [31]. One approach is to use a space-for-time substitution by measuring the means and plasticities of environmentally responsive traits across populations that are locally adapted to a known environmental gradient [32]. In these cases, phenotypic measurements can be combined with transcriptomic data to investigate whether loci that are environmentally responsive within populations are also disproportionately involved in population divergence [33,34]. In environmental change studies, this involves comparing a more 'tolerant' population with a more 'sensitive' population and exposing each to control and stress conditions to compare phenotypic and molecular responses [29,35]. If stress tolerance has evolved through genetic assimilation, we expect the tolerant population to have a higher baseline expression of beneficial genes in control conditions and to have less gene expression plasticity when exposed to the stressor [36]. If stress tolerance has evolved through genetic accommodation, we expect the tolerant population to show greater gene expression plasticity in environmentally responsive genes after exposure to the stressor than the sensitive population. We also expect that the identity of these accommodated genes in the tolerant population should significantly overlap with those involved in the plastic responses to heat shock in the sensitive population [22] (figure 1).

Tigriopus californicus copepods are a tractable, well established system in molecular ecology and physiology and well suited for environmental change studies. *Tigriopus californicus* occupies high intertidal splash pools along the west coast of North America from Baja California to Alaska, where they experience substantial latitudinal, seasonal and diurnal variation in temperature [37]. These copepods have short generation times (about three weeks) and limited gene flow, resulting in genetically diverged and locally adapted populations [38]. Importantly, *T. californicus* populations vary in their ability to thermally acclimate as adults after exposure to a sublethal heat shock [39,40] as well as how exposure to stressful temperatures during larval development impacts subsequent heat tolerance in adulthood [41,42]. Here, we used two populations of *T. californicus* from along the coast of California, USA—a thermally sensitive northern population and a thermally tolerant southern population. We measured phenotypic (thermal tolerance and plasticity) and transcriptomic (differential gene expression) responses to temperature stress experienced during early life and adulthood across the two populations using a fully factorial design. Our goals were to address: (i) how exposure to heat stress across life stages impacts thermal tolerance and plasticity, and (ii) whether the heat-tolerant southern population gained an increased tolerance through evolution of plasticity via increased trait mean and loss of plasticity (genetic assimilation) or through the evolution of increased acclimation potential (genetic accommodation) by comparing patterns of differential gene expression, and to determine (iii) which genes are involved in phenotypic plasticity and evaluate how the environmental responsiveness of these genes was modified in the tolerant southern population.

2. Material and methods

(a) Animal collection and maintenance

During the summer of 2022, we collected *T. californicus* copepods from two field sites in California (CA), USA: Salt Point State Park near Jenner, CA (northern population; 38°33′58.34″ N, −123°19′55.32″ W) and Sunset Cliffs in San Diego, CA (southern population; 32°43′56.83″ N, −117°15′24.04″ W), and used these to establish lab cultures following procedures described in [37,40,43]. We chose these two populations because they have well documented differences in mean thermal tolerance and thermal plasticity, and both have a replicate, geographically paired site that shows similar patterns of thermal tolerance and plasticity [37,40] as well as gene expression patterns in response to heat stress [44] (electronic supplementary material, figure S1). Our northern population experiences mean monthly air temperatures of about 14.9°C, with a monthly minimum of 7.8°C and a monthly maximum of 22.7°C, while our southern population experiences a mean of 16.6°C, with a minimum of 9.0°C and a maximum of 22.4°C, according to data extracted from WorldClim [45]. Because *Tigriopus* inhabits shallow splash pools that are not regularly filled by tides, air temperatures are a better proxy than ocean temperatures for the conditions experienced by this species [37].

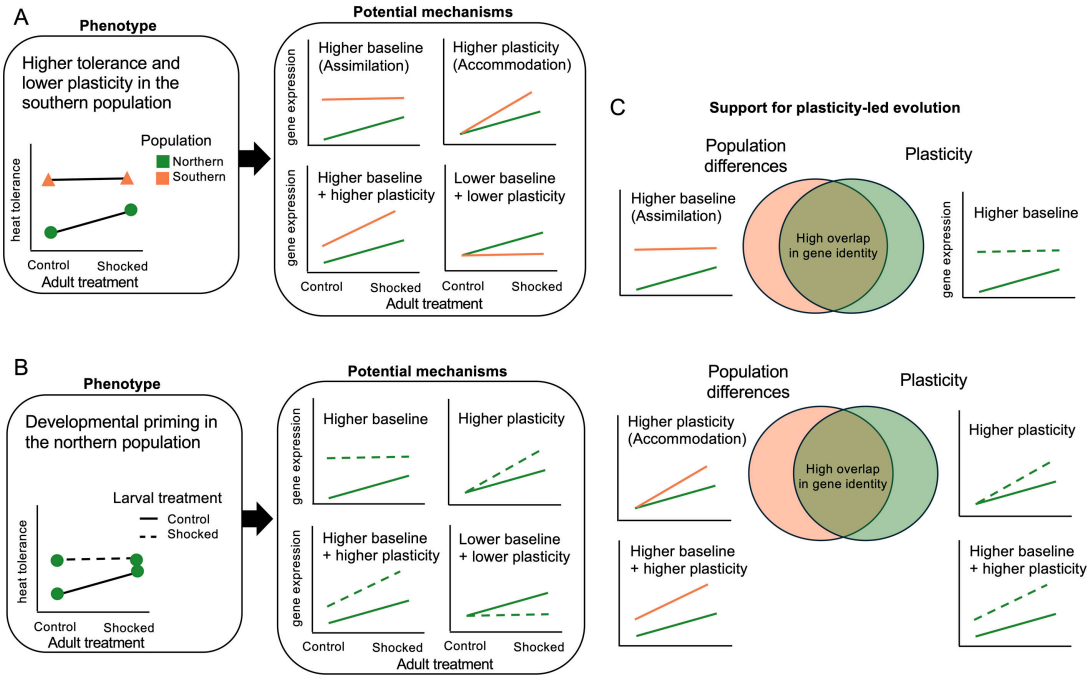

Figure 1. A conceptual overview schematic illustrating (A) on the left, phenotypic differences in heat tolerance and plasticity (e.g. adults experience sublethal heat shock or control (no heat treatment)) between the sensitive northern (in green) and tolerant southern (in orange) populations. On the right, four hypothetical patterns of gene expression across heat-responsive genes that may explain the phenotypic patterns. (B) On the left, phenotypic differences in heat tolerance and plasticity within the northern population in animals that experience sublethal heat shock or control treatments as adults (*x*-axis) and as larvae (line type). On the right, four hypothetical patterns of gene expression across heat-responsive genes that may explain the phenotypic patterns. (C) Our expectations if plasticity-led evolution is acting in this system, which includes high overlap in gene identity across our population comparison (northern versus southern, see (A)) and the developmental treatment comparison (within the northern population, see (B)) for genes that fall in the higher baseline/assimilation or higher plasticity/accommodation categories.

We maintained our lab cultures in an incubator (Percival 136VL) at 19°C under a 12 h light/12 h dark cycle and using artificial seawater (Instant Ocean®) at a salinity of 35 ppt for at least 15 generations before the start of these experiments. We chose these conditions because they are benign and non-stressful across both populations. All animals were fed ground-up *Spirulina* fish flakes ad libitum. Given that both populations experienced identical conditions in the lab for many generations, any observed differences between populations are due to evolved differences because any lab adaptation will make responses more similar. To reach adequate sample sizes for our thermal tolerance assays, we repeated our experimental procedure five times to create five temporal replicates spaced about a week apart, with the collection of the first six larval stages (nauplii) running from September to October of 2023. We repeated the same procedure in May of 2024 to create the F1 animals used in our transcriptomic experiments.

(b) F1 larval exposure

We created the F1 generation by collecting 50 gravid females from our stock containers (F0) and placing each individual female into one well of a six-well plate and allowing her to hatch multiple broods of nauplius larvae over the course of two weeks. Using a dissecting scope and glass pipette, we carefully transferred each brood equally to four 200 ml, lidded plastic cups filled with 150 ml of seawater (representing each of our four larval–adult treatment combinations) to ensure that every mother contributed to the treatments equally (split-brood design). Each cup housed 200–350 nauplii (except for replicate 1, which only had 150), and mothers typically produced between 0 and 50 nauplii.

After the nauplii were collected, we placed two of the four cups into a water bath (VWR WBE20 General Purpose Water Bath) set at 34°C for 1 h to serve as our sublethal larval heat shock, while we maintained the other two as room temperature (approximately 22°C) controls. Previous work has demonstrated that 1 h at 34°C serves as a stressful but non-lethal heat shock for both populations [37,40]. After the hour, we removed the cups from the bath and maintained all treatment cups in the same conditions as our lab cultures (in an incubator at 19°C, 12 h light/dark cycle, 35 ppt) until the copepods reached adulthood.

(c) F1 adult exposure

Once the majority of animals had reached adulthood (past final moult stage, about two weeks later), we transferred six animals into 0.6 ml microtubes filled with 200 µl of seawater at 35 ppt to create as many replicates as we could for the thermal tolerance assay (n = 6–27 tubes per temporal replicate per population and larval–adult treatment combination). We used males and females and included mate-guarding pairs but excluded gravid females and any copepods that had not matured to adulthood at the time of the assay.

We placed half of the tubes into the water bath for 1 h at a temperature of 34°C while the other half remained at room temperature to create our four treatment combinations spanning our larval and adult treatments (control–control (control),

shock–control (larval shock), control–shock (adult shock) and shock–shock (larval + adult shock) (figure 2A)). While there was some background mortality during the maturation of the copepods, these rates were low and did not differ across our treatments, confirming that our larval shock was sublethal [46]. We did not see any mortality during the period between the adult shock and the thermal tolerance assay, as found in previous studies [37,40,44].

(d) Thermal tolerance and physiological plasticity assays

Within 24–48 h after the adult heat shock, we subjected tubes from all four treatment combinations to our thermal tolerance assay designed to measure lethal temperatures and calculate an LT50 value (the temperature at which 50% of animals die) [37,47]. We exposed three replicate tubes with six animals each (18 total) to each target temperature. We began our assays at the estimated LT50 found in previous studies from our lab that included these two populations (35.8°C for northern control, 37.0°C for northern shock, 37.2°C for southern control, and 37.6°C for southern shock; [37]) and then progressed by 0.2°C up and down from that point until we found temperatures with 100% mortality and 0% mortality (13–16 temperatures tested per population and treatment combination). We ran additional replicates for temperatures that produced around 50% mortality to increase accuracy for these critical temperatures (n = 3–16 tubes or 18–96 animals per temperature across populations and treatments). The assay consisted of a 1 h acclimation period at 30°C before ramping up to the target temperature for an additional hour within a thermocycler (Techne 3PRIMEX/02, Bibby Scientific) to precisely control temperatures [37,40]. We allowed animals to recover for 24 h before we counted the number of survivors in each tube.

(e) Thermal tolerance and plasticity statistical analyses

We performed all analyses in R v. 4.4.0 [48]. We calculated the LT50 for each treatment and population combination using the *drc* package [45]. For all treatments across both populations and across all five temporal replicates, we fitted a logistic regression model (LL.2) that allowed us to estimate an LT50 value as well as the 95% confidence interval and standard error associated with this estimate. We corrected for simultaneous inferences using the *glht* (for standard error) and *confit* (for confidence intervals) functions from the *multcomp* package [49]. We considered treatments to have significantly different LT50 values when their 95% confidence intervals did not overlap [47,50].

(f) Tissue collection and RNA extraction

We used a different subset of copepods for our RNA extractions, but followed the same protocols for F1 nauplii collection and early-life larval exposure as the thermal tolerance assay animals (see above). We maintained the animals at control conditions in the incubator until maturation before we began gene expression experiments on the adult animals. We placed copepods from the larval control and larval shock treatment into 50 ml conical tubes filled with seawater and exposed these to either: (i) control, animals did not experience any adult heat shock, or (ii) shock, animals were exposed to a sublethal adult shock at 34°C for 1 h and immediately frozen. We subdivided each 50 ml conical tube into four replicate 1.5 ml Eppendorf tubes with 50–60 animals in each (n = 32 tubes total, four replicates for each population and treatment combination). We flash-froze all animals using liquid nitrogen and stored them in 1.5 ml tubes in a −80°C freezer until extraction.

(g) RNA extraction and sequencing

We extracted total RNA from flash-frozen copepods using a combination of TRIzol (Invitrogen; catalogue no. 15596026) and the Qiagen RNAeasy Plus kit (Qiagen, catalogue no. 74134). We homogenized copepods using a TissueRuptor II (Qiagen, catalogue no. 9002755) before we followed steps 1–7 of the TRIzol protocol, followed by steps 4–11 of the RNAeasy Plus kit protocol [51]. We extracted total RNA from 24 samples and sent extracts to Novogene Corporation (Sacramento, California), where RNA quality was confirmed using a 2100 Agilent Bioanalyzer on a eukaryote total RNA nano chip, followed by the preparation of non-directional RNASeq libraries using poly-A tail selection. The resulting libraries were sequenced on NovaSeq X Plus, with 150 bp paired-end reads.

Sequencing quality was checked using the program FASTQC [52]. We removed adapter sequences and low-quality reads using Trimmomatic [53]. We then mapped the reads to the *T. californicus* reference genome ([54]; NCBI GCF_007210705.1]) using the STAR RNA-seq aligner (v. 2.6.0a; [55]). Reads were mapped to gene features with the options (--quantMode GeneCounts --outFilterScoreMinOverLread 0.50 --outFilterMatchNminOverLread 0.50) to adjust for poly-A tail contamination [56], which generates a count matrix using ReadsPerGene.out.tab output. We generated transcripts per million (TPM) values used for visualizing our RNASeq dataset using the *RSEM* package [57].

(h) Differential gene expression analysis

We removed genes that were expressed at a low level, which we defined as genes that did not have at least 10 counts in 25% of samples from our dataset [58]. This retained 16 821 genes that we used in subsequent analyses. We conducted differential gene expression analyses using DESeq2 (v. 1.24.0) and normalized gene counts [59]. For each pairwise comparison, we obtained a list of differentially expressed genes (DEGs) and calculated false discovery rates (FDRs) using the Benjamini–Hochberg method

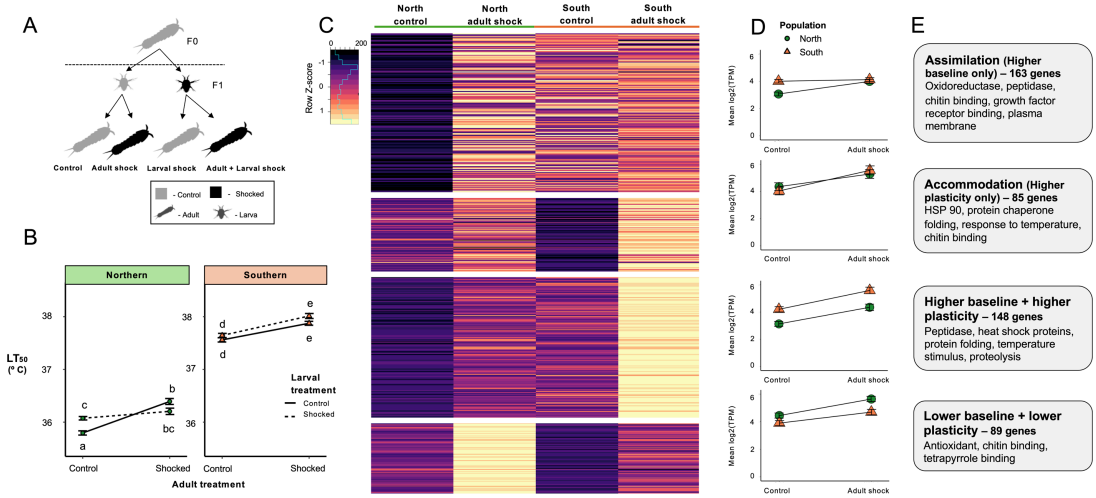

Figure 2. Comparison of thermal tolerance and gene expression patterns in response to heat stress across a thermally sensitive northern and a thermally tolerant southern population of *Tigriopus californicus*. (A) A schematic illustrating the experimental design for both populations. Larvae were collected from parents maintained in control conditions, and half were exposed to a sublethal but stressful heat shock (shocked, black) while the other half were maintained in control conditions (control, grey). These larvae were allowed to grow into adulthood where again half of the adult animals were exposed to a sublethal heat stress while the other half remained in control conditions for a fully factorial design. (B) The results of the LT50 (temperature at which 50% of animals die) heat tolerance assays for both the northern population (left panel) and southern population (right panel). Values along the *y*-axis represent the temperature (°C) at which 50% of the animals in each treatment group died. Adult treatment is indicated across the *x*-axis (control or shocked) and larval treatment is indicated by the line type (solid indicates control, dashed indicates shocked). Different letters represent significantly different values between treatment groups (non-overlapping 95% confidence intervals around the LT50 estimate), and error bars represent standard error. (C) Expression heatmap of the 485 genes that were significantly upregulated in response to adult heat stress across northern and southern copepods. The mean TPM (no. transcripts per million) value was used for each population and treatment combination (calculated using three biological replicates) to calculate a relative expression value (*Z*-score). The categories were determined by comparing (i) the expression level of each gene in the southern control with the northern control (i.e. whether the southern population had higher baseline expression in controls, evidence for assimilation) and (ii) the difference in expression between southern control and adult shock and northern control and adult shock treatments (i.e. the difference in slope, indicating higher plasticity or accommodation). (D) Reaction norm plots illustrating the mean expression level (measured in $\log_2$(TPM)) within each category and across the four populations and treatment combinations. Control and adult shock along the *x*-axis indicate treatment, and colour and shape indicate population (green circles indicate northern population, orange triangles indicate southern population). Error bars indicate standard error. (E) A summary of the category, the number of genes within the category and the gene ontology enrichment analysis indicating terms that were significantly enriched within that category.

[60]. We considered genes with an adjusted *p*-value cutoff of <0.05 and a $\log_2$foldchange of >1 (for upregulated genes) or <−1 (for downregulated genes) to be significantly differentially expressed (see electronic supplementary material, table S1 for all counts of up- and downregulated genes per pairwise comparison). We performed gene ontology enrichment by means of GO_MWU [61], using Fisher's exact test ($p < 0.05$). We used TPM values to create heatmaps using the *heatmap.2* function in the *gplots* package [52], and the reaction norm graphs using the *ggplot2* package [62]. We constructed proportional Venn diagrams using the DEG counts determined through our various pairwise comparisons and visualized these using the *eulerr* package [63].

(i) Analysis of northern versus southern population evolved gene expression responses

We assessed gene expression responses in the northern and southern populations by comparing adults exposed to heat shock with their respective control groups. Our goals were to identify gene expression changes associated with heat stress acclimation observed in both populations and to explore the mechanisms driving higher tolerance in the southern population. To obtain a candidate list of heat responsive genes, we conducted four differential gene expression analyses: (i) between northern control and northern adult shock treatments, (ii) between northern control and northern larval + adult shock treatments, (iii) between southern control and southern adult shock treatments, and (iv) between southern control and southern larval + adult treatments. Including both adult shock treatments was important because it allowed us to examine the full suite of genes potentially involved in the heat stress response across contexts. Through these analyses, we found 485 genes significantly upregulated in response to heat stress across our two populations (p-adjusted < 0.05).

To place these genes within each of our hypothetical categories (assimilation, accommodation, higher baseline + higher plasticity, lower baseline + lower plasticity), we created two true–false binaries and compared the averaged TPM within a treatment (i.e. across the three replicates). The first binary asked whether the baseline expression level of these DEGs in southern controls was greater than that in northern controls, which was considered potential evidence of assimilation. The second binary asked whether the difference between control and adult shock in the southern population was greater than the difference in the northern population, evidence of increased plasticity or accommodation. Genes that showed evidence for being true for both were considered to support a 'higher baseline + higher plasticity' hypothesis (figure 1A). Genes that were true for the first and false for the second were considered evidence for 'assimilation'. Genes that were false and true were considered evidence for 'accommodation'. Finally, genes that were false for both did not fit into any of those categories ('lower baseline + lower plasticity').

(j) Analysis of northern developmental priming through gene expression

We used the results of the differential expression analysis between control versus adult shock animals and control versus larval + adult shock animals in the northern population to create a list of 419 northern heat-responsive genes on which to conduct our analysis of the larval priming mechanism in the northern population. We constructed two true–false binaries and compared TPM values, but this time across the four treatments in the northern population—control, adult shock, larval shock, and adult + larval shock. The first binary asked whether the expression level of a given gene in the larval shock treatment was higher than in controls (no adult shock for either), evidence of higher baseline expression. The second binary asked whether the difference between control and adult shock was higher in copepods that had a larval shock (larval shock versus larval + adult shock) than in those that lacked one (control versus adult shock), evidence for increased plasticity after an adult shock. This allowed us to assign each gene to one of our four categories (higher baseline only, higher plasticity only, higher baseline + higher plasticity, and lower baseline + lower plasticity; figure 1B). We also used this list of DEGs to better understand the mechanistic differences induced by larval priming by constructing the Venn diagram in figure 3D and comparing common and unique genes across the northern treatments.

(k) Analysis of southern developmental priming through gene expression

Finally, we found no evidence for larval priming effects in the southern population (i.e. no difference in thermal tolerance (figure 2B), zero DEGs between southern control copepods and southern larval shock copepods and little difference in the number of DEGs between the pairwise comparisons of control versus adult shock (227) and control versus larval + adult shock (258; electronic supplementary material, table S1)). However, we wanted to ensure our conclusions were complete, so we performed a similar developmental priming analysis to that for the northern population (described above). Using the results from the differential expression analyses comparing control versus adult and control versus larval + adult shock treatments in the southern population, we created a list of 186 significantly upregulated genes responsive to heat shock. We used the same binary framework as with the northern population to determine which genes belonged in each of our four categories (higher baseline only, higher plasticity only, higher baseline + higher plasticity, and lower baseline + lower plasticity; electronic supplementary material, figure S2 and tables S13–S15). Ultimately, genes were evenly distributed across our four categories without a clear signature of higher plasticity or higher baseline expression.

3. Results

(a) Thermal tolerance assays reveal higher tolerance but lower physiological plasticity in the southern population

As expected, the southern population had a higher thermal tolerance (37.6 ± 0.04°C for southern controls compared with 35.8 ± 0.04°C for northern controls (mean LT50 ± s.e.; figure 2B). We also found that the northern population had a stronger adult acclimation effect than the southern population (an increase of 0.6 versus 0.3°C) although both populations demonstrated increases in tolerance after a sublethal adult shock (figure 2B; solid lines). We did not find an effect of larval shock in the southern population, but we observed a significant effect of larval heat shock in the northern population (figure 2B; dashed lines). Specifically, a larval shock in the northern population increased tolerance almost as much as an adult shock alone, but there was no additional benefit to being shocked as a larva and again as an adult.

(b) Gene expression comparisons suggest higher tolerance in the southern population evolved via assimilation and accommodation

Out of the 485 genes significantly upregulated in response to heat stress, we found that 163 genes supported a pattern of genetic assimilation, meaning that they had a higher baseline expression in southern control than northern control animals and did not show increased expression after adult shock in the southern population (figure 2C–E). Using a gene ontology enrichment analysis, we determined that these genes were associated with peptidases (17 genes, $p \leq 0.06$), oxidoreductases (21 genes, $p < 0.001$) and chitin binding (9 genes, $p < 0.001$; figure 2E; electronic supplementary material, table S2). We found 85 genes that supported a pattern of accommodation, meaning that they were not constitutively upregulated in southern controls, but had a greater increase in expression after adult heat stress in the southern as compared with the northern population. These genes were enriched for heat shock proteins (HSPs) and protein folding processes (10 genes, $p < 0.001$; figure 2E; electronic supplementary material, table S3). A total of 148 genes had higher baseline expression in southern than northern controls but were also more plastic in the southern population after heat shock, meaning that these genes retained their plasticity and better support a pattern of accommodation rather than assimilation. These genes were significantly enriched for HSPs and protein folding processes (13 genes, $p < 0.05$) as well as peptidases and proteolysis (26 genes, $p \leq 0.07$; figure 2E; electronic supplementary material, table S4). Finally, 89 genes did not support a pattern of either genetic assimilation or genetic accommodation because they showed lower baseline expression and lower plasticity in the southern population. We found this category to be enriched

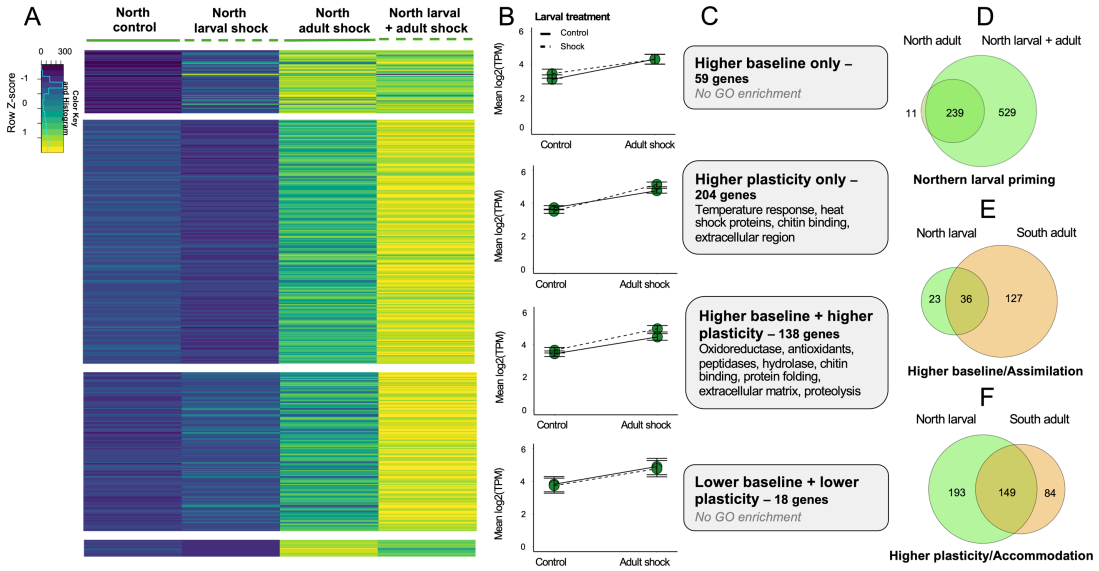

Figure 3. Comparison of the differentially expressed genes in the northern *Tigriopus californicus* population across four treatments (control, larval shock, adult shock, and larval + adult shock). (A) Expression heatmap of the 419 genes that are significantly upregulated in copepods exposed to adult heat shock as compared to controls. The mean transcripts per million (TPM) value was used for each population and treatment combination (calculated using three biological replicates) to calculate a relative expression value (*Z*-score). The categories were determined by (i) comparing the expression level of each gene in the larval shock treatment with the control treatment (i.e. if the larval shock treatment had a higher baseline expression) and (ii) the difference in expression between control and adult shock and larval shock and larval + adult shock treatments (i.e. the difference in slope, indicating higher plasticity). (B) Reaction norm plots illustrating the mean expression level (measured in $\log_2$(TPM)) within each category and across the four treatment combinations. The *x*-axis indicates adult treatment, and the line type indicates larval treatment (solid line indicates control, dashed line indicates larval shock). Error bars indicate standard error. (C) A summary of the category, the number of genes within the category, and the gene ontology enrichment analysis indicating terms were significantly enriched within that category. (D) Proportional Venn diagram displaying the significantly upregulated genes in copepods from the northern population that experienced adult shock alone or an adult + larval shock as compared with controls. (E) Proportional Venn diagram displaying the genes that were considered assimilated in the southern population (figure 2) and genes with higher baseline expression in northern larval priming. (F) Proportional Venn diagram displaying the genes that were considered accommodated or those with higher plasticity + higher baseline expression in the southern population (figure 2) and genes with higher plasticity (with and without higher baseline expression) in northern larval priming.

for genes related to antioxidants and oxidoreductases (12 genes, $p \leq 0.05$) and chitin binding (6 genes, $p < 0.001$; figure 2E; electronic supplementary material, table S5).

(c) Developmental priming in the northern population leads to increased transcriptional plasticity in adulthood

Out of the 419 heat-responsive genes in our northern population, we found 59 genes that demonstrated a pattern of higher baseline expression without an increase in plasticity, but we did not find significant gene enrichment within this category (figure 3A–C). A total of 204 genes showed higher plasticity but not higher baseline expression in animals receiving a larval shock, and this category was significantly enriched for genes related to the heat shock protein response and protein folding processes (14 genes, $p < 0.001$; figure 3C; electronic supplementary material, table S6). One hundred and thirty-eight genes demonstrated higher baseline expression and higher plasticity and were enriched for categories related to peptidases and proteolysis (22 genes, $p < 0.001$) as well as oxidoreductases and antioxidants (19 genes, $p \leq 0.05$; figure 3C; electronic supplementary material, table S7). Finally, the remaining 18 genes showed similar responses to adult heat shock between larval treatments, and we were unable to identify any enriched genes in this category.

Using the results from our differential gene expression analyses between control and adult shock as well as control and larval + adult shock treatments in the northern population, we determined that while most genes upregulated in the adult shock treatment overlapped with those upregulated in the larval + adult shock treatment (96%), there were many additional genes unique to the larval + adult shock category, with over three times as many significantly upregulated genes overall (figure 3D). Gene ontology enrichment revealed that the genes shared across both treatments were related to HSPs and protein folding processes (17 genes, $p < 0.05$) while the genes unique to the larval + adult shock treatment involved additional stress responses, such as genes related to peptidases and proteolysis (42 genes, $p < 0.04$) as well as antioxidants and oxidoreductases (35 genes, $p < 0.001$; electronic supplementary material, tables S8 and S9).

(d) Genes responsible for evolved tolerance in the southern population largely overlap with those responsible for plasticity in the northern population

Finally, we wanted to explore whether the genes responsible for increased thermal tolerance due to evolved differences in the southern population were shared or distinct from those conferring increased tolerance due to larval priming in the northern population (figure 1C). Of the genes associated with constitutive responses lacking plasticity—assimilation in the southern

population (163) and higher baseline expression in the northern population (59)—the northern population shared many of the individual genes with the southern population (61%) while the southern population expressed a much higher proportion of unique genes (78%; figure 3E). These uniquely expressed southern genes were enriched for growth factors and receptors, chitin binding, metabolism, autophosphorylation and cell population proliferation (electronic supplementary material, table S10). For genes associated with evolved genetic accommodation in the south (233) and higher plasticity due to developmental priming in the northern population (342), we found that the majority of genes in the southern population overlapped with those in the northern priming response (88%) and these common genes were enriched for HSPs (18 genes, $p < 0.05$) as well as peptidases and proteolysis (24 genes, $p < 0.01$; figure 3F). Conversely, 66% of these genes were uniquely differentially expressed in the northern population and were enriched for multiple peptidases and hydrolases (22 genes, $p < 0.06$) as well as antioxidants and oxidoreductase (27 genes, $p \leq 0.05$; electronic supplementary material, tables S11 and S12). Importantly, we found that the individual genes associated with assimilation and accommodation in the southern population were significantly enriched for the northern plastic response ($p < 0.001$; Fisher's exact test), indicating a larger overlap than we would expect by chance and suggesting that the same genes are largely responsible for evolved responses in the south and the developmentally primed heat shock responses in the north.

4. Discussion

We were interested in understanding the mechanistic overlap between plastic responses to heat stress within populations and evolved differences in heat tolerance between populations of *T. californicus*. If the evolution of increased heat tolerance occurred through accommodation or assimilation of plastic responses, we expected the evolved responses to heat stress in the thermally tolerant southern population to resemble the plastic responses to heat stress in the thermally sensitive northern population. Consistent with these expectations, we observed similarities between evolved and plastic responses, but our inference of evolution through genetic accommodation versus assimilation depended on whether we considered the response to heat stress at a physiological or molecular level. At the physiological level, our LT50 results indicated that increased thermal tolerance in the southern population evolved through an increase in constitutive mean tolerance across environmental contexts and diminished plasticity of heat tolerance, a pattern consistent with genetic assimilation that is widespread across metazoans [40,64,65]. Developmental priming caused the northern population to become more like the southern population, as northern copepods that experienced a larval shock had higher baseline thermal tolerance and lower adult heat hardening capacity (figure 2B).

At the molecular level, we found evidence that the increase in mean heat tolerance in the southern population was produced by genetic assimilation and accommodation because we found transcripts showing higher baseline regulation but lower plasticity in response to heat stress (genetic assimilation) as well as transcripts showing greater plasticity in response to heat stress after an adult shock (genetic accommodation; figure 2C–E). It is worth noting that we found evidence for all four of our hypothetical categories, not just assimilation or accommodation, suggesting that the nature of the heat shock response is complex and multifaceted. Additional compelling evidence for genetic accommodation came from the similarities in gene expression patterns between evolved responses in the southern population and developmental plasticity in the northern population (figure 3E,F). Of the 233 transcripts with greater upregulation after heat shock in the south compared with the north, 149 also showed greater heat shock response after larval priming in the northern population, a highly significant enrichment of the *evolved* accommodation response in the southern population for genes involved in the *developmentally plastic* responses to heat shock in the northern population. A similar pattern of canalized plasticity has been observed in other systems, such as tolerance to organophosphates in mosquitoes, where changes within a generation occurred through epigenetic modifications while evolved differences occurred through nuclear genome modifications [66].

Notably, gene expression patterns differed across functional categories in the northern and southern populations, with peptidases showing signatures of higher baseline expression and assimilation while HSPs showed signatures of higher plasticity and accommodation. During heat stress, peptidases can break up protein aggregations caused by misfolded or denatured proteins and help improve cellular function during stress [67,68]. Peptidases can be plastically induced in response to a stressor but can also be produced ahead of time and stored in lytic vacuoles within cells until needed [69]. Consistent production of peptidases through higher baseline expression would alleviate the need for acclimation in this subset of genes. Ultimately, assimilation of peptidase-related genes may buffer against environmental perturbation to maintain homeostasis [11]. A similar pattern of transcriptional front loading has been found between resilient and sensitive populations in their response to heat stress in several other marine species [36,70–72].

Genes related to HSPs were consistently associated with accommodation and higher plasticity categories in both the northern and southern responses. HSPs are conserved across the tree of life and are known to be highly inducible in response to heat stress [73,74]. However, HSPs can also be a part of a front-loaded response, with tolerant populations displaying higher constitutive HSP levels at baseline [36,75]. In our southern population, we found some evidence for higher baseline expression of HSPs, but this higher baseline was always accompanied by upregulation in response to heat stress, indicating that plasticity is maintained. One potential explanation for this retention of plasticity is that these genes have not had enough time or selection pressure to canalize [76]. The alternative explanation is that the southern population experienced enough variability in their evolutionary history to maintain sensitivity. Temperature data from the field support this explanation. While temperature in the northern population is more variable across seasons, the southern population has higher daily fluctuations as the tidepools with copepods tend to be shallower and experience more direct sunlight [37]. The preservation of plasticity may also be due to the nature of the proteins themselves. Retaining HSPs when not needed can be a maladaptive response detrimental to cellular

function, and thus plasticity may be maintained regardless of the selection regime [73]. Additionally, HSPs can also be used in response to stressors other than temperature, such as salinity, making retained plasticity beneficial even if temperature regimes remain stable [43].

Another overarching pattern across our results was a saturation of response with increased exposure to heat stress. In the northern population, we found that the level of adult plasticity was modulated by an individual's early-life experience, with no additional benefit of being shocked as larvae and as adults. This saturation may arise because the increase in physiological plasticity is driven by an increase in gene expression plasticity that is sensitive to thermal stress experienced during early life and in adulthood. While we found a substantial set of DEGs that were upregulated in response to adult shock when an individual had also received a larval shock (529; figure 3D), the transcriptional plastic response of an adult shock, alone, was sufficient to confer similar benefits in tolerance. This result suggests that the 239 shared genes associated with HSPs and other plastically induced responses were sufficient to confer increased tolerance and may have already reached maximum transcriptional plasticity, such that two shocks (larval + adult) provided no additional increase in transcriptional plasticity above and beyond that generated by a single shock. The retention of plasticity and lack of canalization in multiple genes in the northern and southern populations suggests that there may be room for further adjustment as temperatures increase or continue to vary, but there may be a hard limit to the amount of heat tolerance these plastic responses can confer owing to this pattern of saturation.

Our results provide evidence for two types of trade-offs incurred during thermal adaptation: metabolic costs and loss of plasticity. HSPs are metabolically costly, and we consistently saw greater upregulation in the more thermally tolerant southern population [73,74]. The metabolic cost of the heat shock response is one possible explanation for lower fecundity previously observed in lines of *Tigriopus* bred for higher heat tolerance [39]. We also found decreased acclimation plasticity in the southern population compared with the northern population in response to adult shock, supporting a potential plasticity–tolerance trade-off [40,77,78]. These observations are consistent with prior work showing that *Tigriopus* lines bred for higher heat tolerance had lower phenotypic plasticity [39]. Indeed, a broad taxonomic diversity of ectotherms consistently shows diminished plasticity of heat tolerance in the most heat-tolerant population [79]. Taken together, these observed trade-offs suggest that, while *Tigriopus* populations have some capacity to adapt to climate warming, this adaptation comes with a cost.

Overall, the mechanisms underlying the adaptive responses in the southern population were largely shared with the larval acclimation effect in the northern population, linking current physiological plasticity with long-term evolutionary responses and providing evidence for plasticity-led evolution. Our empirical data support the theories that physiological responses to the environment can respond rapidly to selection because environmental stressors are heterogeneous and frequently vary over generational time [80] and that selection should act on genes with environmentally responsive expression because these genes underlie phenotypes relevant to different fitness optima across environments [22,23]. In this system, adaptation to environmental change appears to be occurring through modification of shared regulatory networks, as evidenced by the differences in expression of environmentally responsive transcripts between the northern and southern populations underpinned by shared genetic pathways [27,34]. The ability to adjust the environmental responsiveness of gene expression and ultimately alter phenotype in response to environmental variation appears to be a driver of adaptive evolution in this system. Our results suggest that the northern population with its larger reservoir of plasticity should have a greater potential to evolve as the climate continues to warm, but that there is a limit to this adaptive capacity, which the southern population appears to be approaching. Further understanding about how trait innovation and stress tolerance evolve across a diversity of taxa will improve our ability to predict species and population persistence and evolutionary potential into the future.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. All code and data are available on Zenodo [81]. Raw sequencing reads are available at NCBI BioProject ID: PRJNA1327809. Supplementary material is available online [82].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. I.P.N.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, writing—original draft, writing—review and editing; R.V.V.: formal analysis, investigation, methodology, writing—review and editing; E.L.C.: investigation, methodology; B.C.F.: funding acquisition, project administration, resources, supervision, writing—review and editing; M.D.: funding acquisition, methodology, project administration, writing—review and editing; M.W.K.: conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed herein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. Funding was provided by the National Science Foundation through a Postdoctoral Research Fellowship in Biology 2305966 (to I.P.N.) and by grant IOS 2154283 (to M.W.K., B.C.F. and M.D.).

Acknowledgements. We thank the multiple undergraduate students who contributed many hours of microscope work and assisted in copepod care, especially Xochitl Gonzalez and Ria Salway. Portions of this research were conducted with high-performance computing resources provided by Louisiana State University (http://www.hpc.lsu.edu).

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