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Rapid, but limited, zooplankton adaptation to simultaneous warming and acidification

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Predicting the response of marine animals to climate change is hampered by a lack of multigenerational studies on evolutionary adaptation, particularly to combined ocean warming and acidification (OWA). We provide evidence for rapid adaptation to OWA in the foundational copepod species, *Acartia tonsa*, by assessing changes in population fitness on the basis of a comprehensive suite of life-history traits, using an orthogonal experimental design of nominal temperature (18 °C, 22 °C) and p_{CO_2} (400, 2,000 μatm) for 25 generations (~1 year). Egg production and hatching success initially decreased under OWA, resulting in a 56% reduction in fitness. However, both traits recovered by the third generation, and average fitness was reduced thereafter by only 9%. Antagonistic interactions between warming and acidification in later generations decreased survival, thereby limiting full fitness recovery. Our results suggest that such interactions constrain evolutionary rescue and add complexity to predictions of the responses of animal populations to climate change.

T he current rate of increase in atmospheric CO_2 is unparalleled in the past 300 million years[1] and is a cause of rapid ocean warming (OW)[2,3] and ocean acidification (OA)[4.] Warming and acidification in coastal regions are projected to be more extreme than global averages[5,6]. Predicting how organisms and populations respond to this rapid global change[7] is a crucial, yet formidable, scientific challenge. Organisms can respond to changing environments through phenotypic plasticity (ability of a genotype to produce different phenotypes in response to distinct environmental conditions[8]) or genetic change, both of which can mitigate the deleterious effects of climate change[7,9–12]. In particular, long-term experimental evolution studies are a powerful tool to examine the role of adaptation in mitigating the effects of climate change on biota and to explore whether populations can evolve fast enough to keep pace[13].

Organismal performance typically decreases sharply when temperatures increase beyond the optimum[14], leading to disproportional deleterious effects on performance. In addition, under high CO_2 conditions, marine metazoans require the mobilization of energy-demanding acid–base regulatory processes to counteract decreases in internal pH to maintain homeostasis. This may result in increased metabolic costs at the expense of growth and reproduction, even for non-calcifying metazoans[15–17]. Although factorial assessments of species sensitivities to warming and acidification have increased rapidly over the past years, multigenerational studies on animal populations responding to future simultaneous warming and acidification (OWA) are rare[18–20]. Moreover, lack of population fitness measures in existing studies preclude considerations of evolutionary rescue—evolution occurring sufficiently fast to allow population recovery before extirpation[21–23].

We used an experimental evolution approach to test whether a marine zooplankton, the copepod *Acartia tonsa* (Dana, 1849), can adapt to environments created by OW, OA and OWA conditions, to identify the functional traits under selection and to assess evolutionary rescue. As the most abundant metazoans on the planet[24,25],

copepods link primary producers and other microbes to upper trophic levels, thereby influencing fisheries productivity[26,27] and mediating marine biogeochemical cycles[28]. Specifically, *Acartia tonsa* is a dominant copepod in estuarine systems from tropical to temperate regions[29] and a main prey item of forage fish[30], which makes this species an important zooplankton model. Using both improvements in trait performance and population fitness across generations, we show rapid, yet limited, copepod adaptation to OWA, which is probably driven by an antagonistic interaction between OW and OA.

We measured five fitness-relevant life-history traits (survival, egg production rate (EPR), egg hatching success (HS), development time and sex ratio) across 25 generations in an orthogonal design with two levels of CO_2 and temperature. A population of *Acartia tonsa* was collected from Long Island Sound (41.3° N, 72.0° W) and kept under standard laboratory conditions (Methods) for at least three generations before the experiment. Four lines of the population were established with four replicates of each condition. The target (actual ± standard deviation) conditions were as follows: ambient (AM) temperature = 18 °C (18 ± 0.34, N = 330), AM p_{CO_2} = 400 μatm (379 ± 36, N = 18; pH = 8.26 ± 0.1, N = 330); high temperature = 22 °C (22 ± 0.81, N = 336); and high p_{CO_2} = 2,000 μatm (2,301 ± 215, N = 18; pH = 7.55 ± 0.08, N = 330). AM target levels represented extant conditions for this species in northeast Atlantic estuaries (see Methods for choice of temperature), and high levels corresponded to future conditions based on global projections for the years 2100–2300[1–4], although *A. tonsa* already periodically experiences high temperature and CO_2 levels in its growth season in northeast Atlantic estuaries[31,32]. Summaries of the temperature, pH and CO_2 data are shown in Supplementary Tables 1–3. Details of statistical tests and their significance for the transgenerational experiment are in Methods.

Adaptation during the transgenerational experiment

During the first experimental generation (generation zero), EPR and HS declined in all three future (OW, OA and OWA) conditions

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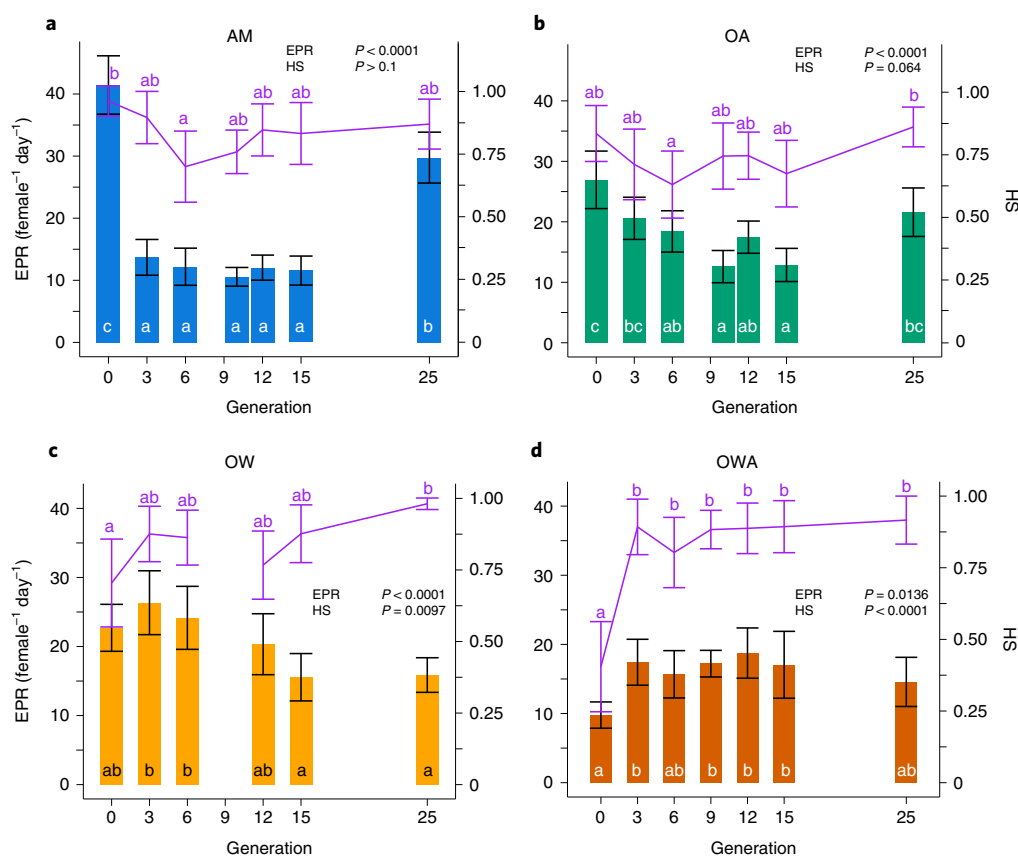
Fig. 1 | Changes in EPR and HS during the transgenerational experiment. EPR (histograms, left *y* axis) and HS (lines, right *y* axis) during the transgenerational experiment. **a**, AM conditions: blue (top left), AM temperature (18 °C) and CO_2 (400 µatm). **b**, OA conditions: green (top right), AM temperature and high CO_2 (2,000 µatm). **c**, OW conditions: orange (bottom left), high temperature (22 °C) and AM CO_2. **d**, OWA conditions: brown (bottom right), high temperature and high CO_2. Shown are mean values and 95% confidence intervals around the mean. Probability (*P*) values for EPR and HS for each panel are derived from the effect of generation on each trait taken from GAMs. Letters represent statistically similar groupings for post hoc *t*-test comparison. Box-and-whisker plots of the same graphs are displayed in Supplementary Fig. 6.

relative to AM conditions—OW (EPR: $P < 0.0001$; HS: $P < 0.01$; *t*-test); OA (EPR: $P < 0.0001$; HS: $P = 0.19$; *t*-test); OWA (EPR: $P < 0.0001$; HS: $p < 0.0001$; *t*-test; Fig. 1)—illustrating the ecological effects of these climate change variables. The decrease was strongest under OWA, particularly for HS, indicating synergistic deleterious effects of temperature and CO_2.

Significant interactions of temperature and CO_2 on HS were evident across all generations ($P < 0.0001$; three-way analysis of variance (ANOVA)). After the first generation, EPR decreased under AM ($P < 0.0001$; generalized additive model (GAM) ANOVA) and OA conditions ($P < 0.0001$; GAM ANOVA) but partially recovered in later generations. Meanwhile, HS remained stable (AM GAM ANOVA: $P = 0.14$; OA GAM ANOVA: $P = 0.064$; Fig. 1). Under OW, EPR decreased with generation ($P < 0.0001$; GAM ANOVA) but did not recover. Meanwhile, HS increased for OW ($P < 0.0001$; GAM ANOVA; Fig. 1c), suggesting some degree of adaptation. By contrast, under OWA, EPR increased by 50% ($P < 0.02$; GAM ANOVA), and HS doubled ($P < 0.0001$; GAM ANOVA; Fig. 1d) by generation 3; the improvements were maintained until generation 25. These changes yielded significant effects on population fitness (Table 1) and are consistent with rapid adaptation.

Survival from nauplii (first larval stages) to reproductively mature adults was independent of treatment ($P > 0.05$; two-way ANOVA) for the first 12 generations (Fig. 2). By generation 15, however, survival decreased by 30% under OW ($P = 0.02$; *t*-test) and OWA ($P = 0.01$; *t*-test) relative to AM conditions. Acidification alone elicited no

decrease in survival ($P > 0.1$; one-way ANOVA). Although survival recovered in the OW treatment by generation 25, survival decreased under OWA by an additional 50% relative to generation 15 for the same treatment ($P < 0.001$; *t*-test), demonstrating an antagonistic interaction[33] between OW and OA in later generations. Traits under selection are expected to increase, with generations, towards optimal values that maximize fitness[34–36]. This did not happen with the survival trait. Thus, survival does not appear to be a trait under selection for adaptation to OW, OA or OWA conditions. High temperature resulted in faster development in both the OW and the OWA treatments (Extended Data Fig. 1) with 22–24% shorter development times than at AM temperature ($P < 0.0001$; two-way ANOVA). High CO_2, by contrast, resulted in 5–6% slower development times than AM CO_2 across all generations ($P < 0.0001$; two-way ANOVA). Thus, warming and acidification acted antagonistically on development time. Finally, an ~1/1 sex ratio remained unchanged for three of the four treatments (Extended Data Fig. 2). Under OWA, however, the proportion of females decreased across generations, with a significantly lower proportion in generation 25 than in generation 0 ($P < 0.01$; Tukey honestly significant difference (HSD)).

To understand adaptation not just in terms of individual traits, we integrated all measured traits (survival, EPR, HS, development time and sex ratio) to estimate population fitness—the net reproductive rate[37], lambda (λ), which is the fraction of the population replaced in a generation (Fig. 3). In generation 0, OWA conditions resulted in a 56% reduction in λ relative to AM conditions, while OW

Table 1 | Model results of generation and treatment on fitness with replicates as random effects

Predictors	Estimates	CI	P
Count model			
AM	1.19	1.16 to 1.23	<0.001
OA	0.01	−0.04 to 0.06	0.817
OW	0.04	−0.01 to 0.09	0.115
OWA	0.13	0.07 to 0.18	<0.001
Generation × AM	0.00	0.00 to 0.00	0.001
Generation × OA	0.00	0.00 to 0.00	0.951
Generation × OW	0.00	0.00 to 0.00	0.009
Generation × OWA	−0.01	−0.01 to −0.01	<0.001
Zero-inflated model			
AM	−1.97	−2.62 to −1.32	<0.001
OA	0.55	−0.36 to 1.46	0.233
OW	1.27	0.39 to 2.16	0.005
OWA	1.54	0.65 to 2.44	0.001
Generation × AM	−0.01	−0.04 to 0.03	0.681
Generation × OA	−0.03	−0.07 to 0.02	0.212
Generation × OW	−0.04	−0.09 to 0.00	0.038
Generation × OWA	−0.19	−0.25 to −0.13	<0.001
Random effects			
σ^2	0.01		
$\tau_{00\ \text{replicate}}$	0.00		
Intra-correlation coefficient	0.08		
N replicate	16		
Observations	2,867		
Marginal R^2/conditional R^2	0.230/0.293		

Results represent combined effects of generation and treatment on fitness when $\lambda = 0$ values are omitted (count model) and when $\lambda = 0$ values are included (zero-inflation model). The AM treatment is the reference intercept for the model. CI, 95% confidence interval.

and OA resulted in 23% and 13% reductions relative to AM conditions, respectively ($P < 0.01$; t-test; Fig. 3). However, by generation 3, λ in OWA conditions had improved by 120% relative to generation 0 ($P < 0.0001$; Tukey HSD; Fig. 3) and recovered to levels equal to AM conditions ($P = 1.0$; Tukey HSD; Fig. 3), driven mainly by improved HS (Fig. 1). The λ-frequency distribution shows an inflation of zero values (Extended Data Fig. 3) due to the high abundance of mate pairs with low HS at generation 0, particularly under OWA conditions. In later generations, as HS increased so did the probability of non-zero λ, and this was especially evident under OWA conditions (Extended

Data Fig. 4). This suggests that, as generations progressed, selection under OWA conditions culled off low-fitness individuals in the population. Lambda remained high in OWA conditions until generation 15, after which there was a 19% reduction ($P < 0.05$; t-test; Fig. 3). This decrease was driven by reduced survival (Fig. 2), which suggests an inability of *Acartia tonsa* to maintain multiple optimal phenotypes (high HS and high survivorship) under OWA conditions. While λ was 22% lower under OWA than under AM conditions by generation 25, it was significantly higher than in generation 0 ($p < 0.0001$; t-test; Fig. 3), still consistent with adaptation over time. In accord, significant effects of generation on λ were evident under OW ($P < 0.04$; ANOVA) and OWA ($P < 0.001$; ANOVA) conditions, but not under AM ($P = 0.681$; ANOVA) or OA ($P = 0.212$; ANOVA) conditions (Table 1). This suggests that OA alone was not a strong selective force in our study.

Adaptation is evident when performance increases towards optimal phenotypes that increase fitness over time[34–36]. Here, we observed improved performance and fitness under OW and OWA conditions, but with important differences between the treatments: fitness was fully recovered under OW, but not under OWA conditions. *Acartia tonsa* exhibits strong tolerance to high temperatures, consistent with its seasonal dominance in the summer and wide-ranging latitudinal distribution[29,38]. In addition, estuarine regions where *A. tonsa* exists can experience high CO_2 conditions[5,6,31,32]. However, the combination of elevated temperature and CO_2 is known to affect ectotherm resource partitioning and energy distribution[15], which might account for the limited recovery under OWA conditions. During adaptation, multiple phenotypes are expected to reach optimal levels of performance concurrently to yield the maximum possible population fitness for a particular environment. We hypothesize that under OWA conditions, copepods could not sustain multiple optimized phenotypes, as evidenced by the observed reduction in survival following HS and EPR recovery to levels equal to or greater than those of AM conditions. Under OW, copepods improved both EPR and HS (Fig. 1) and maintained high survival (Fig. 2) across generations to yield the highest λ level relative to all conditions by generation 25 (Fig. 3). By contrast, under OWA conditions, the improvements in EPR and HS across generations yielded the highest λ values between generations 3 and 12, but decreases in survival reduced λ afterwards. These results are consistent with adaptation via selection, while simultaneously allowing maladaptive traits to persist[35] under OWA conditions.

Genetic drift affects global, genome-wide patterns of genetic diversity, while selective processes affect specific regions of the genome[39,40]. We tested for signatures of genetic drift caused by potential bottlenecks by exploring patterns of nucleotide diversity (π) among treatments using single nucleotide polymorphisms identified with pooled sequencing of genomic DNA from each replicate of each treatment at generation 25. We found that global levels of nucleotide diversity were equivalent across treatment groups (Extended Data Fig. 5; Wilcoxon rank sum test, $P > 0.05$; average π across treatments: AM: 0.0135 ± 0.006; OA: 0.0130 ± 0.006; OW: 0.0134 ± 0.006; OWA: 0.0138 ± 0.006), revealing no evidence of genetic bottlenecks or drift after 25 generations. In the same experiment, we previously reported divergence in both allele frequencies and gene expression at generation 20 between the AM and OWA lines[41]. Moreover, among the four lineages at generation 25, allele frequency estimates indicate differentiation between the four lineages despite the similar levels of nucleotide diversity (our own unpublished observations). Altogether, the improvement in traits and population fitness across generations coupled with the genetic differentiation between the OWA and the AM treatments are consistent with evolutionary adaptation in the OWA treatment.

Contribution of traits to adaptation

To assess the contribution of each life-history trait to adaptation, we quantified the strength of selection via the standardized linear
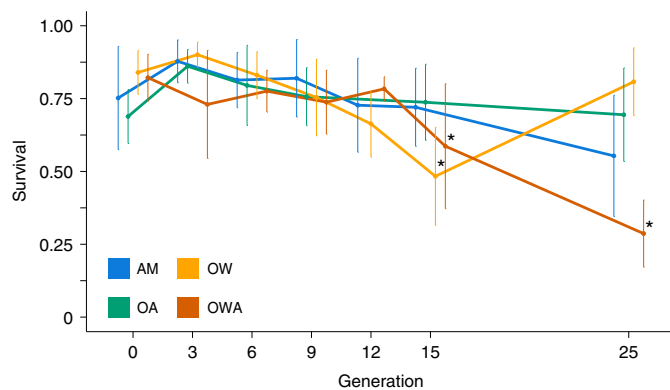
Fig. 2 | Changes in survival during the transgenerational study. Shown are mean survival values for nauplius stage 1 to adult stage. Error bars represent 95% confidence intervals around the mean. Asterisks indicate significant decrease relative to AM condition ($P < 0.001$; t-test). Treatment curves are offset for clarity. Box-and-whisker plots of the same graphs are displayed on Supplementary Fig. 7.

Fig. 3 | Mean fitness values, λ, calculated for the transgenerational study. Error bars represent 95% confidence intervals. Treatment curves are offset for clarity. Box-and-whisker plots of the same graphs are displayed on Supplementary Fig. 8.

selection coefficient of the fitness landscape[42]. High linear coefficients of selection for a given fitness landscape suggest that a particular trait is under selection, though not necessarily determining fitness. The coefficients are calculated from multiple regression models of relative λ against all changing life-history traits (Methods). We found that HS, but not EPR or survival, was under selection and had a positive impact on fitness. Specifically, relative λ increased as a function of HS with standardized linear selection coefficients (β) of 0.91 (AM), 0.94 (OA), 0.96 (OW) and 0.78 (OWA) at generation 0 ($P < 0.0001$; ANOVA; Fig. 4a). In addition, the linear selection coefficient for HS (effect of HS on relative fitness) was significantly different for all treatments between generations 0 and 25 ($P < 0.0001$; ANOVA), indicating changing degrees of selection on HS for all treatments between the first and last generations. By contrast, neither EPR nor survival was significant at generation 0 (Fig. 4b,c; $P > 0.1$; ANOVA) suggesting that selection acted in favour of higher HS, but not towards higher survival or EPR for the OW and OWA treatments. By generation 25, the impact of HS on relative fitness decreased relative to generation 0 by 88% and 10% for the OW and OWA treatments, respectively. Such decreases corresponded to a shift in phenotype distribution towards higher HS and suggested that selection had relaxed by generation 25 as HS within the population reached the phenotypic optimum (Fig. 4a). Finally, despite the significant decrease in sex ratio over the 25 generations in the OWA treatment (Extended Data Fig. 2), sex ratio had no effect on relative fitness between generations 0 and 25 ($P = 1.0$; ANOVA), suggesting this trait was not under selection.

We also employed path analysis on structural equation models (SEMs) to identify the hierarchical interactions of all life-history traits and the traits' effects on fitness[43,44]. High correlation coefficients of SEMs suggest causality of a given trait's effect towards fitness. The path analysis revealed that HS had the largest effect on fitness of all life-history traits across treatments, with the exception of the OW treatment at generation 25, and was significant at both generations 0 and 25 (Table 2). Taken together, we conclude that selection on HS both under OW and OWA conditions was the critical factor in adaptation to those environments.

Implications

Our study highlights the need for global change studies to consider population fitness, in addition to individual traits, as an integrative tool for measuring adaptation[27,45]. For example, in this experiment, using survival alone would have led to the erroneous conclusion of
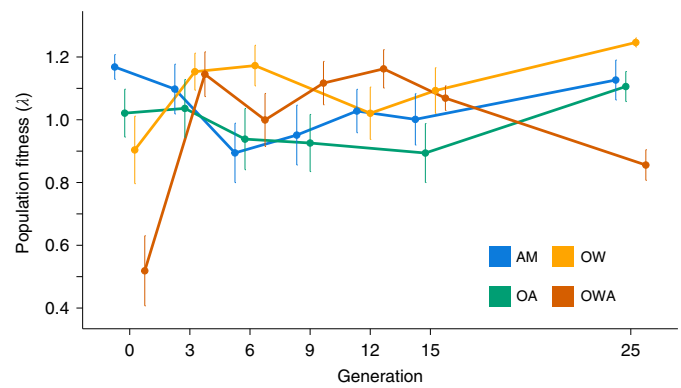
no adaptation under any treatment. Similarly, estimating fitness on the basis of EPR alone would have led to the erroneous conclusion that fitness decreased between generations 3 and 15 for AM. Despite this decrease in EPR, fitness remained unchanged across generations for AM (Fig. 3). Likewise, using EPR and HS alone would have missed the fitness decrease after generation 12 under OWA conditions. At the same time, the trade-offs of EPR and HS versus survival after generation 12 (Figs. 1 and 2) partly explain the limited evolutionary rescue under OWA conditions (Fig. 3).

The observed shifting interactive effects of OWA with generations highlight the need to evaluate long-term evolutionary studies with multiple stressors and underline the complexity that accompanies predicting organism responses to climate change[33,46]. Previous work has shown that *Acartia tonsa* can adapt to lower CO_2 levels (800 µatm) than those in our study[47]. Thus, it is conceivable that full evolutionary rescue to OWA can be achieved at lower CO_2 levels, suggesting a possible threshold. Furthermore, previous research has shown that exposure to OA over multiple generations improves EPR and selects for genes involved in RNA processing and regulation of metabolism in copepods[11,48]. Future work should investigate whether similar genes are also under selection for OW and OWA. Neither deleterious effects of OA nor adaptation to OA alone was observed across generations in our study (Fig. 3). Thus, our results highlight the evolutionary rescue and accompanying limitations that arise from multi-stressor adaptation. Synergistic deleterious effects of OW and OA on fitness were evident in generation 0 (Fig. 3). Later, full evolutionary rescue under OWA could have been achieved by generation 25 (OWA $\lambda \geq$ control λ) if OA and OW had additive effects, which was not observed (Fig. 3). Instead, OW and OA showed antagonistic effects on generation 25 (Supplementary Table 4). We suggest that the limited evolutionary rescue following rapid adaptation under OWA conditions in our study must have arisen from an antagonistic interaction between warming and acidification, as has been hypothesized earlier in polychaetes[19,20,49] or for other co-occurring environmental stressors in bivalves[50]. Thus, since OW and OA are occurring simultaneously, even crude predictions of population performance under climate change should consider non-additive effects of temperature and CO_2 interactions on population fitness.

Our study, showing both improved trait performance and population fitness across generations, constitutes a demonstration of rapid adaptation to global change conditions for a metazoan. We present evidence for adaptation to both warming and combined warming and acidification, but not acidification alone, based on standing genetic variation. Previous work has been limited to phytoplankton[39] or documenting marine metazoan traits for limited
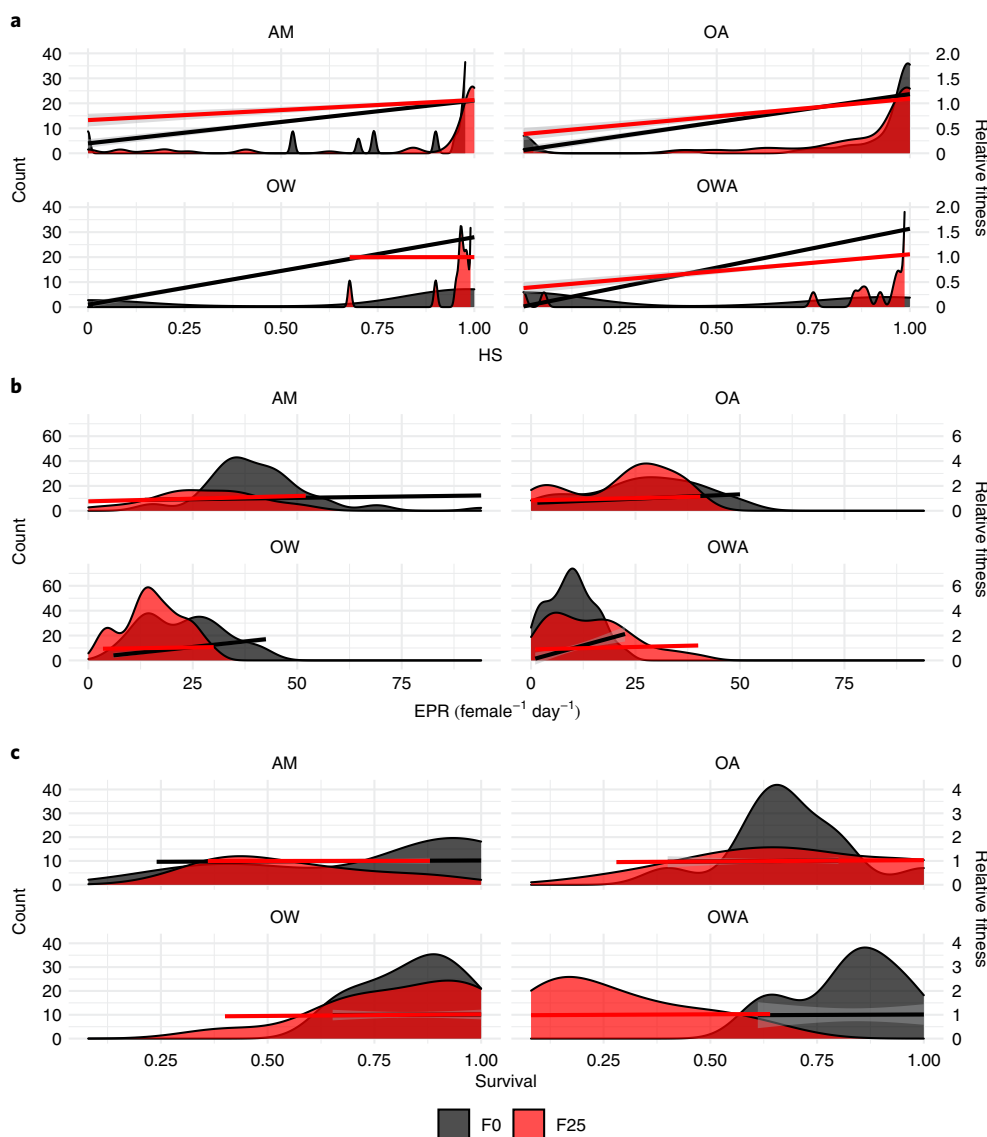
Fig. 4 | Fitness landscapes showing trait contribution to adaptation during the transgenerational experiment. a–c, Frequency distributions (left axes) and relative fitness landscape linear models (right axes) for HS (**a**), EPR (**b**) and survival (**c**) at F0 (generation 0; black) and F25 (red). Fitness landscapes illustrate the correlations between traits and relative fitness. Shading around lines represents standard error of the regression coefficients.

Table 2 | Trait effects on population fitness (λ) during the transgenerational experiment derived from path analysis

	Generation	AM	OA	OW	OWA
Survival	F0	0.12	−0.09	0.03	0.08
	F25	−0.09	0.13	0.72	0.33
EPR	F0	−0.07	0.04	0.03	0.10
	F25	0.34	0.25	0.86*	0.23
HS	F0	0.91*	0.94*	0.96*	0.78*
	F25	0.53*	0.65*	0.11	0.70*
Sex ratio	F0	0.12	−0.15	0.09	0.21
	F25	−0.39	0.37	0.37	−0.27

Shown are standardized parameter estimates from structural equation models for each trait's effect on λ at F0 and F25. *Parameter estimate is the largest estimate for a treatment at both F0 and F25.

generations without population fitness estimates[18–20]. Failing to account for adaptation potential may overestimate future population vulnerability[45,51]. For example, using the observed deleterious effects at generation 0 to predict future vulnerability would have estimated a fitness reduction for the OWA treatment relative to AM treatment of 56% (Fig. 3). However, accounting for evolutionary rescue (generations 3–25) resulted in an average fitness reduction of only 9%. At the same time, our results suggest that full evolutionary rescue under OWA conditions was not achieved (Fig. 3). This result is consistent with the hypothesis that adaptation to stress comes at a cost and that full recovery of populations to future climate conditions on the basis of extant genetic variation is limited[52], even though *A. tonsa* periodically experiences the warming and acidification conditions examined here[5,6,31,32]. The limited evolutionary rescue observed here also has consequences for future oceanic systems under OWA conditions. Because adaptation to OWA conditions appears to be costly even under the food-replete conditions

of our study, it is unclear to what extent evolutionary rescue would occur under scenarios that predict lower resource availability for zooplankton under climate change[51]. Limited evolutionary rescue, in turn, would reduce prey availability for fish, thereby negatively affecting fish production[51]. Finally, since copepods are a major vector of carbon transfer from the surface waters to the seafloor[28], limited evolutionary rescue would also reduce the efficiency of the biological carbon pump, with a concomitant lower drawdown of CO_2 from near-surface waters.

Conclusions

We show evidence for metazoan evolutionary adaptation to combined warming and acidification by explicitly assessing changes in population fitness on the basis of a comprehensive suite of life-history traits. Adaptation was evident in the fitness improvements for animals after a few generations, and confirmed separately by evidence of genetic differentiation[41]. The strength of selection coefficients and the path analysis suggested the key trait under selection affecting fitness in our study was HS. Evolutionary rescue was limited by the interaction of warming and acidification, which switched from synergistic to antagonistic from the beginning to the end of the experiment, adding complexity to predictions of population adaptation to climate change.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41558-021-01131-5.

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Methods

Copepod culturing and maintenance. Copepods were collected in June of 2016 from Esker Point Beach in Groton, Connecticut, USA (41.320725° N, 72.001643° W) and raised for at least three generations as stock cultures before the start of transgenerational experiments to limit maternal effects[53]. Stock cultures were split evenly into eight groups of 160 females and 80 males. Four of these eight groups were acclimatized to high temperature at 1 °C per day and used to seed the two high-temperature treatments (OW and OWA). The other four groups remained at AM temperature and were used to seed the AM and acidification treatments. After temperature acclimatization, groups of stock cultures seeded the parental (F0) individuals for two days. Stock culture groups yielded an average of 7,173 eggs per group to produce approximately 57,000 parental (F0) eggs. Resulting parental eggs and N1 nauplii were acclimated to one of four experimental treatments over the entire F0 generation: (1) AM (control; AM temperature = 18 °C, AM CO_2 ~400 µatm; pH ~8.2); (2) OA (AM temperature, high CO_2 ~2,000 µatm, pH ~7.5); (3) OW (high temperature = 22 °C, AM CO_2); (4) OWA (high temperature, high CO_2). Treatment replicates were derived from the stock culture groups (stock culture group 1 after high-temperature acclimatization seeded the F0 for OW replicate 1 and OWA replicate 1). The AM temperature was chosen from a Gaussian fit model[54] for optimal recruitment (EPR × HS) versus temperature derived from *Acartia tonsa* populations from Casco Bay (Gulf of

Maine), Long Island Sound and Chesapeake Bay (USA), $R = 11.1e^{-0.5\left(\frac{|T-17.7|}{6.4}\right)^2}$ where R is recruitment, T is temperature (°C), 17.7 is the optimal temperature and 6.4 is the standard deviation around the optimal temperature ($N = 54$, $r^2 = 0.42$; our own unpublished data). Each treatment was kept in a separate temperature-controlled incubator (Thermo FisherScientific Isotemp) and split into four replicate 10 l culture containers (Cambro). Copepods were fed every 48–72 h at food-replete concentrations (≥800 µg l^{-1} carbon) consisting of equal proportions of the phytoplankters *Tetraselmis* sp., *Rhodomonas* sp. and *Thalassiosira weissflogii* following long-standing copepod culture protocols in our lab[55]. The phytoplankton fed to copepods was deliberately raised under AM conditions for the entire length of the experiment to avoid confounding effects of possible changes in food quality due to the different temperature and CO_2 among treatments. We minimized the chance of selecting for early developers in the cultures. On the basis of development time and adult longevity, we allowed copepods to contribute progeny to the next generation for 7–10 days once we observed the first nauplii of a new generation.

Elevated CO_2 levels were achieved with gas proportioners (Cole-Parmer), mixing laboratory air with 100% bone-dry CO_2 that was delivered continuously to the bottom of each replicate culture. For small-volume life-history trait experiments (sections b–d), CO_2-mixed air was fed into custom Plexiglas enclosures within each temperature-controlled incubator to allow for passive diffusion of CO_2 into seawater. Target pH values were monitored using a handheld pH probe (Orion Ross Ultra pH/ATC Triode with Orion Star A121 pH Portable Meter; Thermo FisherScientific)). The pH probe was calibrated monthly using commercially available National Bureau of Standards (NBS) pH standards in a three-point calibration (pH 4.01, 7, 10.01; Thermo FisherScientific). Temperature and pH were monitored to ensure that those of small-volume experiments in the Plexiglas enclosures matched those of bulk cultures. To counteract metabolic CO_2 accumulation, control CO_2 conditions were achieved by forcing compressed AM air through a series of CO_2-stripping units containing granular soda lime (AirGas) and a particle filter (1 µm) and then to each culture container via airstone. Continuous bubbling maintained dissolved oxygen levels at >8 mg l^{-1}. Temperature, pH and actual p_{CO_2} were monitored throughout the experiment (Supplementary Table 1). Actual p_{CO_2} conditions were calculated in CO_2SYS[56] on the basis of measurements of salinity, temperature, pH and total alkalinity (A_T; Supplementary Tables 2 and 3) with k1/k2 from Lueker et al.[57], KHSO$_4$ from Dickson[58], total boron from Uppstrom[59] and pH based on NBS scale. Total alkalinity was measured in triplicates three times over the course of the experiment using endpoint titration (G20 Potentiometric Titrator; Mettler)[60].

Because treatments were housed in separate incubators, incubator-specific effects are theoretically possible but unlikely[61,62] given that incubators were held under identical AM conditions except for the constant temperature and CO_2 conditions that were meticulously monitored and verified by independent measures throughout the experiment (Supplementary Table 5). Because of logistical and personnel constraints, EPR, HS, survival and development time (see the following) could not be measured at every generation. Instead, measurements were taken at generations 0, 3, 6, 9, 12, 15 and 25.

EPR and HS. For each replicate culture within a treatment, ten pairs of newly developed males and females were placed into 20 ml petri dishes for 48 h ($n = 280$ per treatment). The dishes were housed in custom-made, airtight, Plexiglas enclosures whose atmosphere was controlled to the appropriate CO_2 concentration (see Copepod culturing and maintenance section). There was one enclosure per temperature-controlled incubator. After the 48 h egg-laying period, adults were checked for survival and removed from the petri dishes. Eggs were left in the dishes for an additional 72 h to allow for egg hatching, and their contents were preserved with non-acid Lugol's solution. Dishes with dead males were used for EPR, but not HS, since fertilization could not be assumed. Dishes with dead females were

discarded. EPR was calculated as $\frac{E_u + E_h}{t}$ where E_u represents unhatched eggs, E_h represents hatched eggs (nauplii) and t represents egg-laying time. HS was calculated as $\frac{E_h}{E_u + E_h}$.

Survival. Survival was measured from nauplius 1 (N1) stage to copepodid 6 (adult) stage[29]. For a given generation, all adults from the previous generation were removed from the culture and allowed to lay eggs in food-replete media for 48 h. Resulting nauplii were chosen for tracking survival. Unhatched eggs and any nauplii not chosen for survival analysis were returned to their respective replicate cultures for continued population maintenance. To measure survival for all generations where life-history traits were evaluated, three 250 ml beakers for each replicate culture were supplied with 25 randomly chosen N1 nauplii each and housed in the Plexiglas enclosure described ($n = 21$ per treatment). Copepods were checked every 48–72 h. The numbers of dead, live and missing copepods were logged for each beaker along with general stage (nauplius, copepodite, adult female or adult male). The fraction of survived individuals (l_x) was calculated as n_x/n_i where n_x represents the number of live individuals on day x, and n_i represents initial individuals. Nauplii were grown with media at levels of 500 µg l^{-1} C to prevent overgrowth of phytoplankton and allow for adequate nauplii grazing. Following the naupliar stages, copepods were grown with food-replete (800 µg l^{-1} C) media as described earlier. Food was replaced with fresh media on monitoring days. Average survival was calculated per each replicate culture at each generation measured. Differences in day-specific survival between replicates and treatments were assessed using the 'survival' package in R[63].

Development time. To calculate development time (time from N1 to adulthood), we recorded the number of days at which individuals reached the copepod VI stage (adult) during the survival experiments and averaged the observations.

Sex ratio. Sex ratio was calculated on the basis of the number of surviving adult females relative to surviving adult males in survival experiments.

Population fitness. The population net reproductive rate, λ, was calculated as the dominant eigenvalue of an assembled projected age-structured Leslie matrix constructed from survival and fecundity data[37]. Briefly, day-specific probabilities of survival are calculated from day-specific survival as $P_x = \frac{l_x}{l_{x-1}}$ where l_x represents the proportion of individuals on day x and l_{x-1} represents the proportion of individuals on day $x-1$. Probabilities of survival on day 1 are assumed to be 100%, or a value of 1.0. Per capita EPR and HS are calculated as described in the preceding, with fecundity rates equalling the product of EPR and HS. Because only females produce offspring, total fecundity rates must be scaled to the sex ratio (proportion of females to males). To account for differences in individual development time for each treatment, fecundity rates are assigned to all days after the first matured adult is observed. We assume that surviving individuals represented by the survival experiments are equally as likely to experience any of the fecundity values observed in EPR experiments. Therefore, each mate-pair fecundity rate was paired with each survival beaker to construct a matrix. This yields a maximum of 120 matrices per treatment per generation (3 survival beakers × 4 replicate cultures × 10 mate pairs).

Selection coefficients and path analysis. Linear selection coefficients (β) were evaluated by creating multiple linear regression models of relative fitness ($\lambda/\bar{\lambda}$) against survival, EPR, HS and sex ratio at both F0 and F25 for each treatment and calculating the partial regression coefficient estimate for each trait[64]. The path analysis was completed by creating SEMs[43,44] of relative fitness against survival, EPR, HS and sex ratio at both F0 and F25 for each treatment using the 'lavaan' and 'semPlot' packages in R[65,66]. Development time was omitted from the models because the lack of variance within a replicate violated assumptions of the model.

Genetic diversity. To quantify genetic diversity across the genome, capture probes were designed to target both coding and regulatory regions across the genome. For coding regions, we chose the highest-quality probe falling within the region. Regulatory probes were set within 1,000 bp upstream of the transcription start site. Genomic DNA was shipped to Rapid Genomics for library preparation and was captured with the 32,413 probes (21,311 coding, 11,102 regulatory). Enriched libraries were sequenced on a HiSeq 4000 with 150 bp paired end reads. Raw data were trimmed for quality and adaptor contamination with Trimmomatic v0.36[67]. Trimmed reads were mapped to the *A. tonsa* reference genome[68] with BWA-MEM[69]. SAMTOOLs[70] was used to generate a pileup for each sample, from which genetic diversity (π) was estimated with Popoolation[71]. We identified 1,450 100 bp windows in 704 unique scaffolds across the genome that were present across all samples. We estimated π in 100 bp sliding windows with a 100 bp step size. Each position required a minimum coverage of ×30, max coverage of ×1,000 (to avoid mapping errors) and at least 0.5 of the window meeting these thresholds. Resulting windows were required to be sequenced across all samples. To take into account the independent replicates within each treatment, we used pairwise Wilcoxon rank sum tests with a Holm correction for multiple testing. Genomic data are deposited in GenBank: BioProject number PRJNA590963.

Statistical analyses. All statistical analyses were done using the software package R (v4.0.2)[72]. To examine effects of generation on changing life-history traits, we created trait-specific GAMs smoothed across generations for each treatment[73,74]. To evaluate differences between life-history traits, we created separate linear mixed models with replicates as random effects and used post hoc t-tests and Tukey HSD tests to compare life-history trait values that were significantly different from other treatments at each generation ($\alpha < 0.05$). Analysis of fitness (λ) calculations also included estimations with a zero-inflated generalized linear mixed effects model with generation and treatment as fixed effects and replicates as random effects. Analysing the data with linear mixed models also allowed us to evaluate the effects of treatment replicates on life-history traits. A low intra-class correlation coefficient suggests no predictive effect of random variables. For the model constructed for fitness over generations, the variance due to replicates within a treatment is very low (intra-correlation coefficient (ICC) = 0.08; Table 1) and does not affect the model results. To estimate the predicted probabilities of $\lambda = 0$ across generations in Supplementary Fig. 4, we converted λ values to either 0 or 1 representing λ values of 0 and >0, respectively. We used the binomial-converted λ data to fit a linear mixed effects model against generation and treatment with replicates included as random effects. To evaluate individual effects of temperature, pH or generation on life-history traits, we constructed a third linear model that was tested with a three-way ANOVA. All scripts for the statistical analysis are provided[75].

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The phenotypic and physical data referred to in the text are deposited in *Zenodo* (https://doi.org/10.5281/zenodo.5119920)[75]. The genetic diversity data are deposited in GenBank: BioProject number PRJNA590963. Source data are provided with this paper.

Code availability

The scripts for analysis of the physical, phenotypic and genetic diversity data are deposited in *Zenodo* (https://doi.org/10.5281/zenodo.5119920).

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Author contributions

H.G.D. conceived the project, designed research, aided in data analysis and wrote the manuscript. J.A.deM. conducted experiments, analysed data, created figures and wrote the manuscript with H.G.D. G.P., L.N. and X.H. conducted experiments. M.B.F. conceived the project and designed research. H.B. conceived the project, designed research and designed the CO₂ delivery system. R.S.B. performed genomic diversity analysis. M.H.P. conceived the project, designed research and performed genomic analysis. All authors edited and approved the paper.

Competing interests

The authors declare no competing interests.

Additional information

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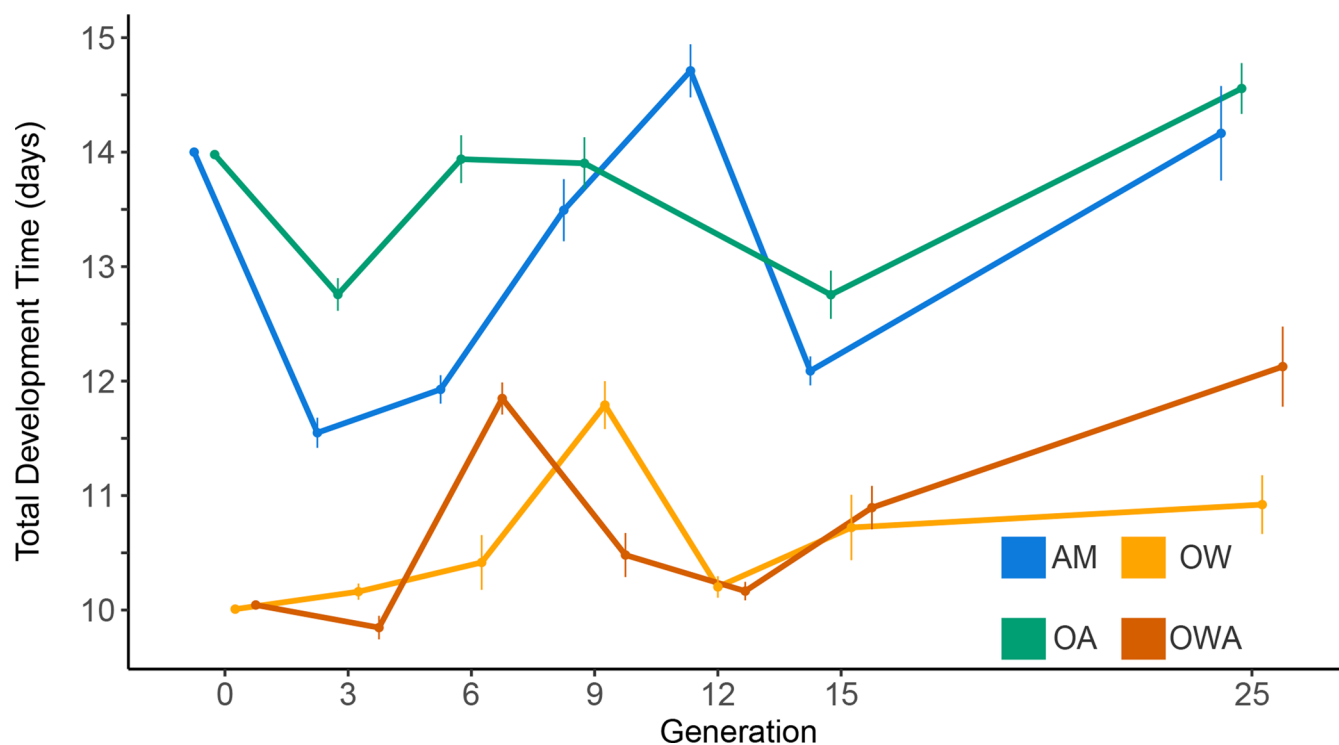
Extended Data Fig. 1 | Development time vs generation for transgenerational study. Shown are the mean calculated development times (naupliar stage 1 to adult) for each treatment at each generation where life-history traits are measured. Curves for treatments are offset for clarity. Treatment colors: blue: AM; green: OA; orange: OW; brown: OWA. Box and whisker plots for these data are available in Supplemental Fig. 4.
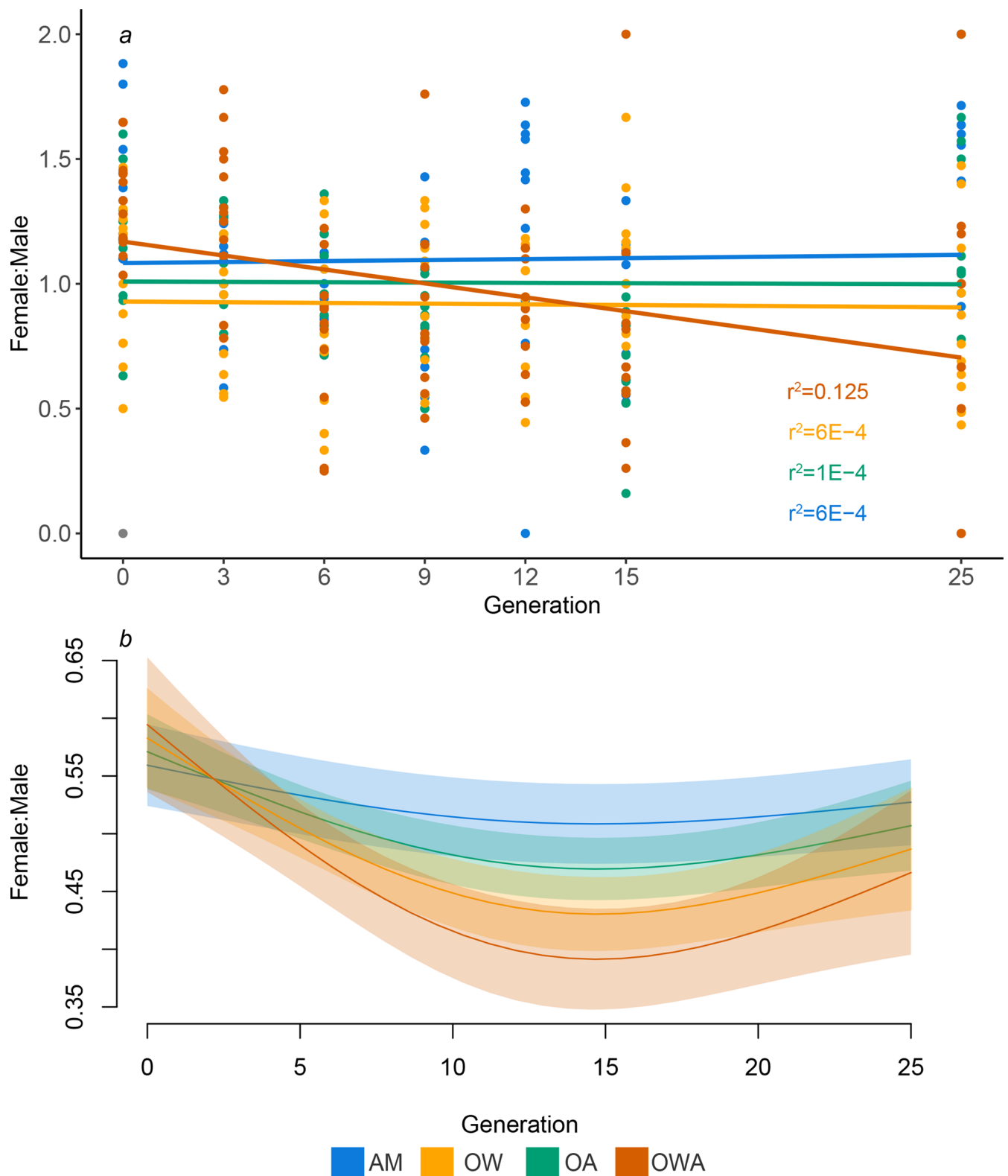
Extended Data Fig. 2 | Sex ratio vs generation for transgenerational study. Results for sex ratio across generations modeled as A) linear model and B) Generalized Additive Model. Treatment colors: blue: AM; green: OA; orange: OW; brown: OWA.
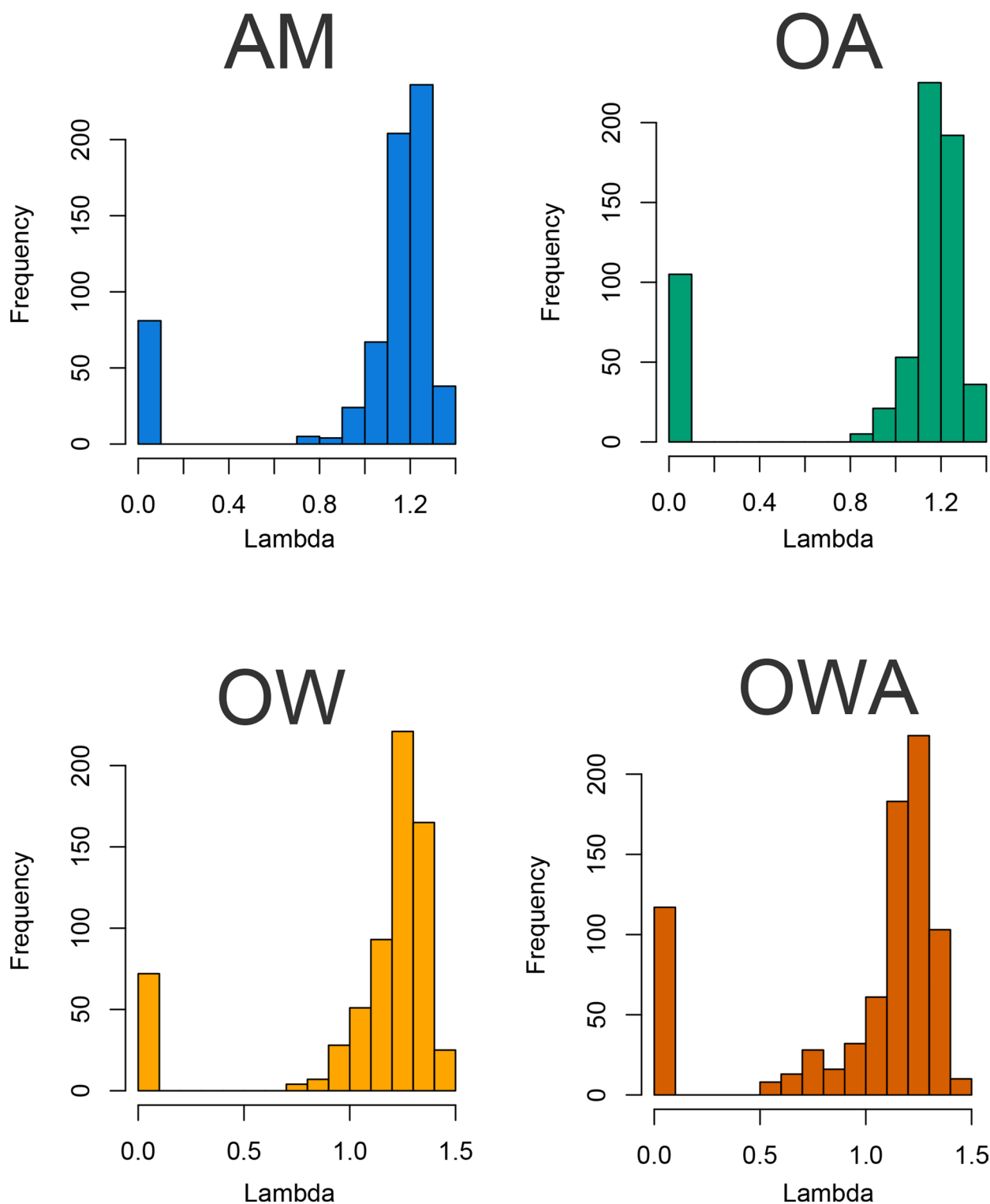
Extended Data Fig. 3 | Frequency distribution of population fitness values (λ) for the four treatments in the transgenerational experiment. *Treatment colors: blue: AM; green: OA; orange: OW; brown: OWA.*
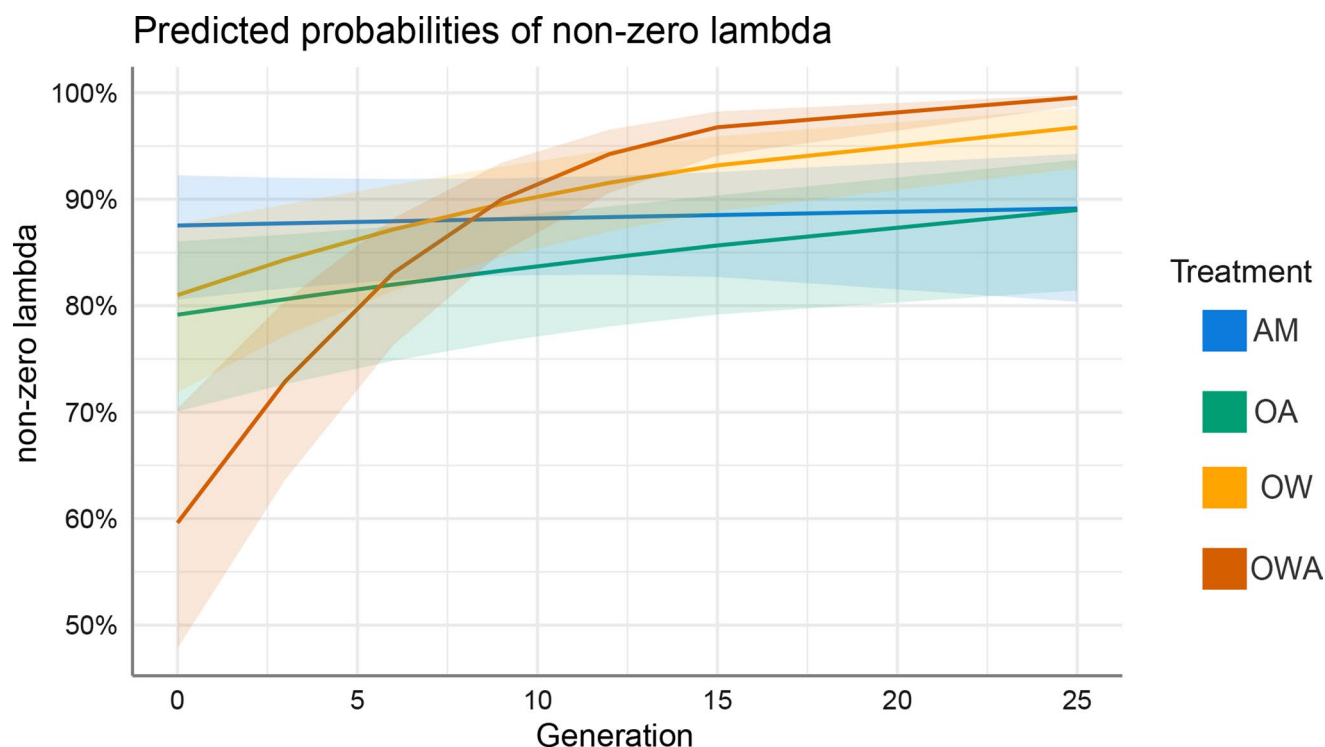
Extended Data Fig. 4 | Predicted probabilities of non-zero fitness (lambda) values vs generations across treatments in the transgenerational experiment. Shown are predicted mean non-zero lambda probabilities. Probabilities for ambient (AM), ocean acidification (OA), and ocean warming (OW) treatments are statistically independent of generations. Probabilities for the simultaneous ocean warming and acidification (OWA) significantly increase with generation. Shading represents 95% confidence intervals around the mean. Treatment colors: blue: AM; green: OA; orange: OW; brown: OWA.
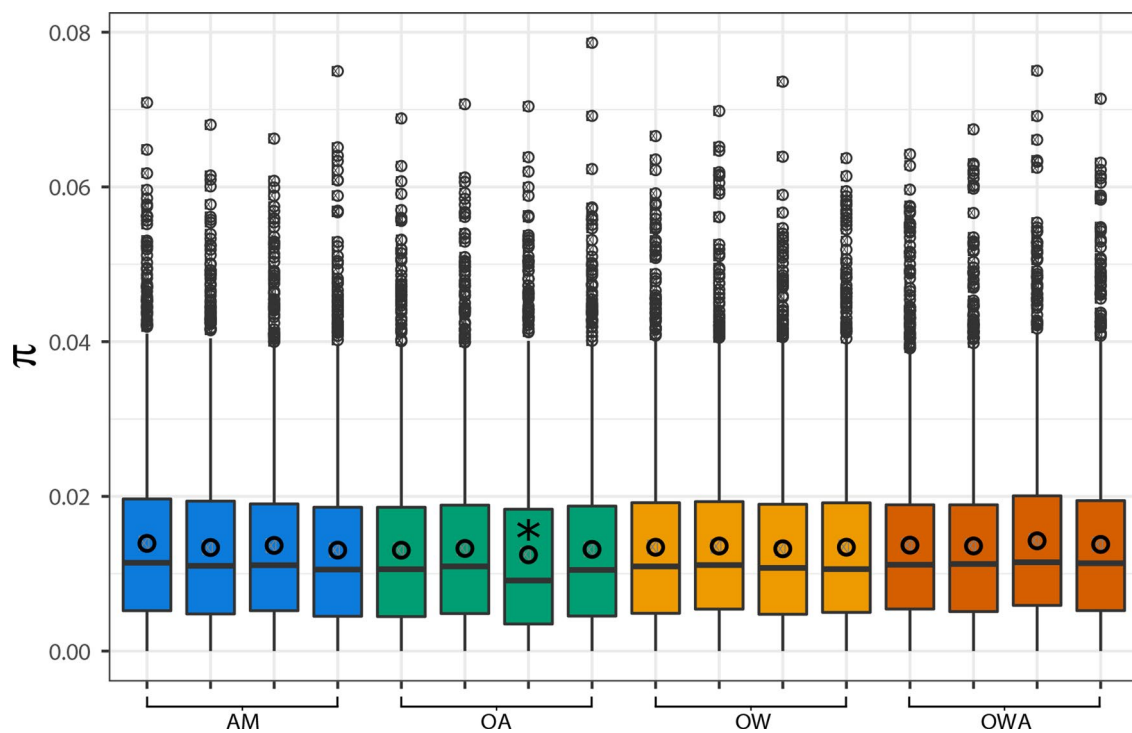
Extended Data Fig. 5 | Estimates of genetic diversity (π) at generation 25 vs treatments of the transgenerational experiment. Estimates were calculated in 100 bp non-overlapping sliding windows. Windows were included when at least 50% of sites had coverage between 30x and 1000x per sample and the window was covered across all samples. The asterisk indicates the sample in the OA treatment with reduced genetic diversity relative to other samples (Wilcoxon Rank Sum test with Holm correction for multiple testing; $p < 0.05$); all other samples were not significantly different ($p > 0.05$). In the boxes, the centre black line represents the median, the circles represent means, upper box edge represents the 75% quartile, lower box edge represents 25% quartile, whiskers represent 1.5x interquartile range, and points represent outliers. Treatment colors: blue: AM; green: OA; orange: OW; brown: OWA.

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Software and code

Policy information about availability of computer code

Data collection	No software was needed to collect data.
Data analysis	Custom data analysis code was generated using R v 4.0.2 and available at https://github.com/dam-lab/Transgenerational_manuscript.git

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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The datasets generated during and/or analysed during the current study are available in the "Transgenerational_manuscript" github repository, https://github.com/dam-lab/Transgenerational_manuscript.git with genomic data deposited in GenBank, BioProject number: PRJNA590963.

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Ecological, evolutionary & environmental sciences study design

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Study description	This study entails a multi-generational 2x2 factorial selection experiment on the copepod Acartia tonsa using two levels of temperature (18 C and 22 C) and CO2 (400 ppmv and 2000 ppmv) as experimentally manipulated environmental variables to yield four separate treatments: Control (18C 400 pppmv CO2), Warming (22C, 400 ppmv CO2), Acidification (18C, 2000 ppmv C02), and greenhouse condition (22C, 2000 ppmV CO2). Life-history traits (egg production rate, egg hatching frequency, survival from nauplius 1 to adulthood), development time, and sex ratio) and population fitness (the net reproductive rate per generation, lambda) were measured every third generation for 25 generations among four separate replicates per treatment, with >1000 individual copepods per replicate.
Research sample	Groups of Acartia tonsa were collected from field plankton tows in Long Island Sound, and >200 adult females collected during each field trip in June 2016. Females were supplemented with ≥100 males to ensure adequate fertilization for rearing cultures. Cultures were raised in the lab prior to exposure to each treatment.
Sampling strategy	Sample size upon collection was chosen based on previous work to ensure adequate genetic variation in generations leading to the initiation of the experiment.
Data collection	Data for traits was collected in lab notebooks by J. deMayo, G. Park, and L. Norton and then transferred to electronic data files and analyzed by J. deMayo. Genomic data was collected and analyzed by R. Brennan and M. Pespeni.
Timing and spatial scale	Data was collected every 3rd generation for each treatment with the initial start date beginning in August 2017. Because development is controlled by environment, generation times varied. Data for treatments at 22C were collected every ~36 days for the duration of the generation (~10-12 days). Data for treatments at 18C were collected every ~48 days for the duration of the generation (~14-16 days). The data for the last (25th) generation was collected in June of 2018.
Data exclusions	No data were excluded from analyses.
Reproducibility	Experiments were replicated among treatments with appropriate statistical tests used to verify that trait values were independent of replicate across generations. All attempts to complete experiments were successful for each trait except for F9 egg production in the high temperature, low CO2 (22 C, 400 ppmv) treatment and F12 survival in the low temperature, high CO2 (18 C, 2000 ppmv) treatment.
Randomization	For culture rearing within a treatment, copepods were split evenly into four replicates to acclimate to appropriate temperatures. Then, each replicate was split evenly in half again to acclimate to the appropriate CO2 level. For experiments, copepods were selected randomly from the entire population within a treatment replicate for each trait.
Blinding	Blinding was not necessary as all quantitative trait values were evaluated objectively across three different researchers.

Did the study involve field work? ☐ Yes ☒ No

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Animals and other organisms

Laboratory animals	Acartia tonsa
Wild animals	This study did not involve wild animals.
Field-collected samples	Acartia tonsa (male and female) were collected from the field using a 200 micrometer plankton net with a solid cod end and transported to the lab for selection and trait experiments. Copepods were transported in field collected water and transferred to temperature-controlled environmental chambers upon arrival to the lab. Copepods were raised in five-gallon buckets filled with 0.2-micrometer filtered seawater in the aforementioned environmental chambers with a 12:12 hour light cycle at a temperature of 18 C. For experiments, animals were moved from buckets in environmental chambers to 10 L clear polycarbonate containers in temperature controlled incubators set at 18 C or 22 C, with the appropriate level of CO_2-infused air (400 ppmv or 2000 ppmv).
Ethics oversight	No ethical oversight was necessary because no higher order invertebrates or vertebrates were involved in this study. No sampling permit was required.

Note that full information on the approval of the study protocol must also be provided in the manuscript.