

Research



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Simultaneous warming and acidification limit population fitness and reveal phenotype costs for a marine copepod

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Phenotypic plasticity and evolutionary adaptation allow populations to cope with global change, but limits and costs to adaptation under multiple stressors are insufficiently understood. We reared a foundational copepod species, *Acartia hudsonica*, under ambient (AM), ocean warming (OW), ocean acidification (OA), and combined ocean warming and acidification (OWA) conditions for 11 generations (approx. 1 year) and measured population fitness (net reproductive rate) derived from six life-history traits (egg production, hatching success, survival, development time, body size and sex ratio). Copepods under OW and OWA exhibited an initial approximately 40% fitness decline relative to AM, but fully recovered within four generations, consistent with an adaptive response and demonstrating synergy between stressors. At generation 11, however, fitness was approximately 24% lower for OWA compared with the AM lineage, consistent with the cost of producing OWA-adapted phenotypes. Fitness of the OWA lineage was not affected by reversal to AM or low food environments, indicating sustained phenotypic plasticity. These results mimic those of a congener, *Acartia tonsa*, while additionally suggesting that synergistic effects of simultaneous stressors exert costs that limit fitness recovery but can sustain plasticity. Thus, even when closely related species experience similar stressors, species-specific costs shape their unique adaptive responses.

1. Introduction

A central problem facing biologists is predicting the long-term response of biota to global climate change. Empirical data on how organisms cope with multiple stressor environments across generations are still limited, rendering our understanding insufficient. In marine systems, two of the most important climate-driven changes are ocean warming (OW) and ocean acidification (OA). Most research to date has considered OW and OA separately, but this ignores the potential for interactive effects of concurrent stressors (i.e. combined warming and acidification; OWA) on performance and fitness of populations [1–5]. Non-additive effects (i.e. synergistic or antagonistic interactions) of multiple stressors can lead to fundamentally different selection regimes and therefore to novel phenotypes [6]. Both phenotypic plasticity (change in phenotype due to environment without a change in the genotype [7–9]) and evolutionary adaptation (improvements in fitness driven by natural selection in a given environment [10]) allow marine populations to cope with climate change [11–14]. How these mechanisms and their limitations shape the resilience of metazoan populations to climate change remains insufficiently understood.

Most empirical studies on the impact of climate change on marine organisms have been restricted to intra-generational experiments, which can only address phenotypic plasticity. For organisms with short generation times, multi-generation experimental evolution studies are useful to explore phenotypic plasticity and adaptation and to explore whether marine populations can evolve fast enough to keep pace with rapid climate change [15]. Experimental evolution studies in phytoplankton have been used to identify how adaptive phenotypes can persist in response to acidification and warming, the influence of phenotypic plasticity and how rates of environmental change influence adaptation [15–19]. Multigenerational experiments with marine copepods under ocean acidification have also identified effects of selection and the capacity to recover from reduced trait performance via phenotypic plasticity [13,20–22]. But long-term evolutionary studies in metazoans are still limited, as are studies that integrate multiple traits to estimate environmental effects on population fitness. Our recent work demonstrated rapid, but limited adaptation in a copepod (*Acartia tonsa*: *Dana*) to OWA by estimating fitness from multiple traits across 25 generations. This study was able to identify key traits, egg production rate and egg hatching success, contributing to improved fitness and those under selection [23]. In a related study, we showed that this adaptation carries costs: a loss of transcriptional plasticity and a loss of fitness when returned to the ancestral environments [24]. A follow-up study with the same species demonstrated that adaptation to OWA reduced thermal tolerance plasticity as well [25]. Overall, the results of multi-stressor studies suggest non-additive and costly OWA effects on phenotypic and physiological responses. Thus, multi-stressor environments select for different optimal traits [17,23,24,26,27], making generalizations challenging. Therefore, further tests are required to examine whether the observed adaptation patterns, costs and trait performance are consistent across species, and how they affect adaptive responses.

In theory, adaptation produces phenotypes that maximize either the net reproductive rate (i.e. the population fitness) for a given environment when growth is density independent [28–32] or increased carrying capacity otherwise [33]. But there may be constraints to the degree of adaptation. Consider a hypothetical copepod population once adapted to polar environments that eventually adapts to the tropics. The locally adapted tropical genotype should outcompete foreign invaders in its tropical environment, as should the polar genotype [31,34,35]. But maladaptive mechanisms may persist [28,31], resulting in costs like we previously observed [24,25] that keep populations from achieving maximum fitness. Adaptation costs arise when genotypes experience reduced fitness in non-native environments [31,34,35] or when the selection pressure leading to adaptation is relaxed [36]. An adaptation cost for the new tropical genotype is evident if fitness in the ancestral polar environment is lower than at the tropical one (electronic supplementary material, figure S1). Next, for the sake of argument assume that the polar genotype is more plastic than the tropical one. A plasticity cost for the polar genotype would occur if fitness was less than the tropical genotype when both populations are compared in their respective home environments [37–39] (electronic supplementary material, figure S1). Lastly, a phenotype cost exists when two genotypes (with correspondingly different phenotypes)

differ in fitness in the same environment (e.g. tropical or polar; electronic supplementary material, figure S1) [37–39]. While determining plasticity and phenotype costs involve comparisons between genotypes, adaptation costs are measured by comparing one genotype across environments. Other studies, however, have used interspecific comparisons to identify adaptation costs by comparing how a species performs with its own resources relative to the resources of another species [40]. All three types of costs may constrain adaptive responses [41], although they are not necessarily inherent to adaptation [31,34,35,38,42] or mutually exclusive.

Here, we used the copepod *Acartia hudsonica*, a foundational zooplankton species of the Northeast Atlantic [43] and a main prey item for larval fish [44], to study its multi-generational response to ocean warming and acidification. This species, and copepods generally, are useful models to test mechanisms and costs of adaptation because of their short generation time, large extant genetic variation and established culturing methods [45]. As arguably the ocean's most abundant metazoans [46,47], copepods dominate the zooplankton, comprise the most important link between primary producers and upper trophic level consumers [48], and are major modulators of biogeochemical cycles [49,50]. *Acartia hudsonica*, which is present in estuaries during winter and spring, experiences cooler temperatures and higher pH levels relative to its warm season congener, *A. tonsa* [51]. We exposed *A. hudsonica* over multiple generations to future ocean conditions to test how non-additive interactions of ocean warming and acidification may result in costs of adaptation, plasticity and phenotype. Importantly, our study contrasts with recent work [23–25,52] in patterns of adaptive responses to OWA, the traits responding to selection and the types of costs between even closely related species.

2. Experimental methods

Design: the orthogonal experimental design and methodology of this study are almost identical in design and execution to our previous studies on *A. tonsa* [23,24] except for the species and levels of temperature and CO₂. We tested for adaptation, plasticity, and phenotype costs using reciprocal transplant experiments under both replete and limited food conditions. The latter represents an indirect cost for vulnerability to reduced food concentration, a secondary potential consequence of climate change [53–55].

(a) Copepod culturing and maintenance

Three hundred copepods were collected in April 2018 from eastern Long Island Sound, CT, USA (41.3°N, 72.0°W) and raised for 1 year (approx. 12 generations) (14°C, 400 µatm CO₂, 30 ‰ salinity, 12:12 h light:dark) as stock cultures to limit maternal effects [56]. Three resulting stock cultures with greater than 2000 individuals each were combined and then split evenly into three groups for each of the four treatments. Groups were acclimatized within a generation to temperature (15°C or 13°C, 1°C per day) and pCO₂ (1000 µatm, 100 µatm per day, OA treatments only). Groups seeded the F0 individuals for 7–10 days yielding approximately 15 000 eggs per treatment. Resulting F0 eggs and nauplii were combined for each treatment, redistributed among three replicate cultures and returned to their respective experimental conditions. The experimental environmental

conditions (electronic supplementary material, tables S1–S3) were nominally: 1) ambient control (AM): 13°C, 400 μatm CO_2 , pH = 8.2; 2) ocean acidification (OA): 13°C, 1000 μatm CO_2 , pH = 7.85; 3) ocean warming (OW): 15°C, 400 μatm CO_2 , pH = 8.2; 4) combined warming and acidification (OWA): 15°C, 1000 μatm CO_2 , pH = 7.85. The high temperature and CO_2 conditions were chosen based on RCP8.5 projections for the year 2100 [57,58]. Each treatment was kept in a separate temperature-controlled incubator (Thermo Fisher Scientific Isotemp; Waltham, MA, USA) and split into three replicate 10 l culture containers (Cambro; Huntington Beach, CA, USA). Copepods were fed equal proportions of the live phytoplankters *Tetraselmis* sp., *Rhodomonas* sp. and *Thalassiosira weissflogii* every 48–72 h to achieve food-replete conditions ($\geq 600 \mu\text{g}$ carbon l^{-1} ; C l^{-1}) [59] deliberately raised under ambient conditions to avoid confounding effects of possible food quality changes. Adults were removed for a minimum of one week, and a maximum of two weeks, after we observed the first nauplii of a new generation to create non-overlapping generations.

(b) Life-history traits

Traits and fitness were assessed at generations F0, F2 and F4 for AM, OA, OW and OWA, and at F11 for AM, OWA and transplant treatments. Concentrations of phytoplankton food during food limitation experiments were: $> 600 \mu\text{g}$ C l^{-1} for food replete, $250 \mu\text{g}$ C l^{-1} for food limited and $0 \mu\text{g}$ C l^{-1} for starved. Life-history trait experiments were conducted in small-volume containers (e.g. beakers, Petri-dishes) housed within custom, airtight plexiglass enclosures where CO_2 -mixed air was fed into the surrounding atmosphere. Each enclosure was housed within an incubator where temperature and pH were monitored to ensure that small-volume experiments in the plexiglass enclosures matched those of bulk cultures.

(i) Population fitness

For population fitness, we calculated the net reproductive rate per generation (λ) as the dominant eigenvalue of a projected, age-structured Leslie Matrix assembled from survival and offspring production data as previously described [23,24,60]. Because one male can fertilize multiple females, the total number of offspring depends on the availability of females rather than males. Therefore, total offspring production rates were scaled to the proportion of females: males. To account for differences in development time for each treatment, and because these copepods are iteroparous, offspring production rates were assigned to all days after the first matured adult was observed. We assumed that surviving individuals represented by the survival experiments were equally likely to experience any of the offspring production values observed in EPR experiments. Therefore, each mate-pair offspring production rate was paired with each survival probability to yield a maximum of 90 matrices per treatment per generation (3 survival probabilities \times 3 replicate cultures \times 10 mate pairs).

(ii) Egg production rate (EPR) and hatching success (HS)

For each replicate culture within a treatment, 12 pairs of newly developed adult males and females were placed into 25 ml Petri dishes housed in the plexiglass enclosure described above for 96 h ($N = 108$ per treatment for

F0 to F4). Adults were transferred to a new dish after 48 h. For food limitation experiments (section C) in F11, the 12 pairs were split equally among the three food concentrations. Adults in food limitation experiments laid eggs for 72 h and were transferred to a new dish daily to maintain food concentration during the experiment. After the 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 hatch and then 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. We independently evaluated survival in additional assays, thus the measurements for this assay were only used to estimate the number of offspring produced for live copepods. EPR was calculated as the number of eggs produced per female per day and HS was calculated as the proportion of live nauplii from produced eggs as described previously [23,24].

(iii) Survival

Survival was measured from nauplius 1 (N1) to copepodid 6 (C6; adult). 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 cultures for continued population maintenance. To measure survival, three 250 ml beakers for each replicate culture were supplied with 25 randomly chosen N1 nauplii each and housed in the plexiglass enclosure ($n = 9$ per treatment). Copepods were checked every 48–72 h. The number of dead, live and missing copepods were recorded for each beaker along with developmental stage (nauplius, copepodite, adult female or adult male). Nauplii were grown with media at levels of $250 \mu\text{g}$ C l^{-1} for the first four days to prevent overgrowth of phytoplankton and allow for adequate nauplii grazing. Then, copepods were grown with food-replete media. For food limitation experiments in F11, the three beakers for each replicate culture were split evenly between the three food concentrations. Food media was replaced on monitoring days. Average survival probabilities were calculated for each replicate culture at each generation as the proportion of surviving individuals on monitoring days as described previously [23,24].

(iv) Development time

Development time was recorded as the number of days it took individuals to progress from N1 to C6 stage during the survival experiments. Individual development time values ($N = 34$ –187) were averaged across each treatment for each generation from F0 to F4.

(v) Sex ratio

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

(vi) Body size and somatic growth rates

Body size was measured as prosome length (mm) at C1 and C6 stages using Image-J (<https://imagej.nih.gov/ij/>) for individuals grown in 250 ml beakers alongside survivorship experiments. Ten individuals per replicate and treatment

(i.e. 10 C1, 10 males and 10 females) were preserved in non-acid Lugol's solution each generation for life-history trait measurements. Individuals were isolated in a drop of filtered seawater and photographed using a Lumenera Infinity5–5 camera (Teledyne Lumenera, Ottawa, ON, CAN) attached to an inverted microscope (Olympus IX70, Olympus, Waltham, MA, USA) after the water droplet had been removed.

To estimate somatic mass growth rates, we first converted prosome lengths at C1 and C6 to dry mass using the relationship:

Mass = $13.185 \times \text{Length}^{3.1858}$ for C1 individuals, and the relationship:

Mass = $12.37 \times \text{Length}^{3.6276}$ for C6 individuals [61] at each generation for each treatment. Somatic mass growth was then estimated for each treatment and generation as:

$$\frac{M_{C6} - M_{C1}}{T_{C6} - T_{C1}},$$

where M_{C6} = mass at C6 (μg), M_{C1} = mass at C1 (μg), T_{C6} = development time at C6 (days) and T_{C1} = development time at C1 (days).

(c) Reciprocal transplants

Unfortunately, prolonged periods of experimental interruption due to the COVID-19 pandemic precluded performing experiments between F4 and F11. Thus, F11 was the next possible generation assessed after F4 for transplant experiments. At F11, we reciprocally transplanted copepods from AM and OWA. Each replicate from each treatment was split to yield three additional replicates for each of two new transplant treatments: AM→OWA and OWA→AM (as well as control transfers: AM→AM and OWA→OWA) leading to 12 total cultures (2 treatments \times 2 transplant/non-transplant \times 3 replicates). Copepods were raised for an additional generation and maintained as described above.

(d) Statistical analyses

Statistical analyses were done using R (v 4.0.2) [62]. All lineages were evaluated for changes from F0 to F4, whereas changes to F11 were only followed for the AM and OWA lineages. To examine the effects of generation on life-history traits, we used trait-specific generalized additive models (GAMs) smoothed across generations for each treatment [63]. To evaluate differences between life-history traits, we used separate linear mixed models (LMMs) with temperature, pH, and generation as fixed effects and replicates as random effects. *Post hoc* *t*-tests were corrected with Tukey honest significant difference (HSD) to compare trait values that were significantly different from other treatments at each generation ($\alpha < 0.05$). If both GAMs and LMMs found significant treatment effects on a trait, we chose the model with the lowest Akaike Information Criterion (AIC). Differences in day-specific survival were assessed using the 'survival' package in R [64]. Analysis of fitness (λ) also included estimates with a zero-inflated generalized linear mixed-effects model with generation and treatment as main effects and replicates as random effects. Lastly, we also included a three-way ANOVA with generation, temperature and pH as fixed main effects, which allowed testing for significant synergistic and antagonistic stressor interactions [6].

To evaluate traits under selection, we constructed generalized linear models of relative fitness ($\lambda/\bar{\lambda}$) against EPR, HS and survival. Standardized linear regression coefficients (β)

for each main effect indicate the strength of selection for that particular trait [28]. We also evaluated the contribution of individual life-history traits to relative fitness with path analysis by creating structural equation models [65] of relative fitness against EPR, HS and survival at F0 and F4 for each treatment using the 'lavaan' and 'semPlot' packages [66,67]. Sex ratio and development time were omitted because of lack of variance for some treatment replicates, which violates assumptions of the models.

To evaluate effects of temperature, pH or generation on life-history traits, we constructed another linear model that was tested with a three-way ANOVA. We constructed linear mixed-effects models for data collected during the reciprocal transplant experiments with lineage and environment as fixed effects, and treatment replicates as random effects. A significant environment effect on performance indicates maintained phenotypic plasticity after adaptation. Conditions of similar temperature and CO₂ within the incubators were tested with a two-way ANOVA to ensure that incubators intended to be at the same temperature or CO₂ level were similar (electronic supplementary material, table S4).

(e) Determining additive, synergistic and antagonistic effects

We identified traits for which there was a significant interaction for temperature, pH and generation [6]. After an interaction was identified, we compared the mean difference (MD) for a life-history trait between a single stressor (OA or OW) and AM (e.g. MD_{AM-OA} and MD_{AM-OW}, respectively) [6,68]. Then, we compared this value to the difference between OWA and AM (MD_{AM-OWA}). The effect was *additive* if MD_{AM-OWA} was equal to the sum of the mean differences for each individual treatment (i.e. MD_{AM-OWA} = MD_{AM-OA} + MD_{AM-OW}). The effect was *antagonistic* if MD_{AM-OWA} was less than the sum of the mean differences for each individual treatment (i.e. MD_{AM-OWA} < MD_{AM-OA} + MD_{AM-OW}). The effect was *synergistic* if MD_{AM-OWA} was greater than the sum of the mean differences for each individual treatment (i.e. MD_{AM-OWA} > MD_{AM-OA} + MD_{AM-OW}).

(f) Genetic separation between treatments and generations

To quantify the degree of genetic separation across treatments and across generations, we compared allele frequency shifts using single nucleotide polymorphisms (SNPs) called from RNAseq data [69] in the same manner as previously reported [24]. Extractions were performed using TRIzol (Invitrogen, Carlsbad, CA, USA) and with Qiagen RNeasy spin columns (Qiagen, Germantown, MD, USA). Library preparation and sequencing was conducted by Novogene (Sacramento, CA, USA) and sequenced with 150 bp paired end reads with an Illumina NovaSeq6000. Reads were quality- and adapter-trimmed with Trimmomatic v0.36 with a leading and trailing quality of 2, sliding window length of 4 with a quality of 2 and minimum length of 31. Full details on transcriptome assembly and SNP variant calling can be found in the electronic supplementary material. Pairwise F_{ST} was calculated using the R package *poolfstat* [70] and differences in mean genome-wide values were compared across treatments and generations using a two-way ANOVA with an interaction.

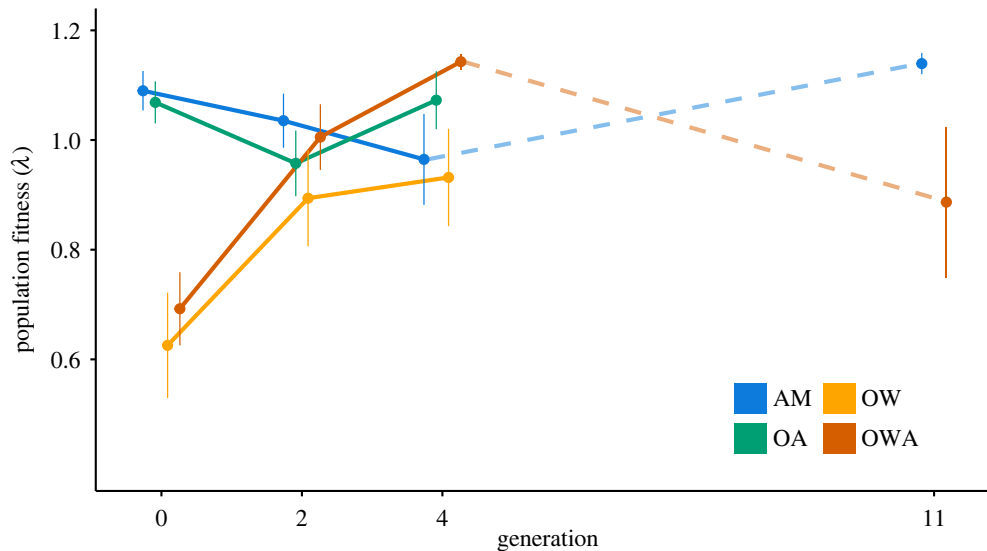


Figure 1. Population fitness of *Acartia hudsonica* during the transgenerational experiment. Fitness represents the net reproductive rate per generation (see methods). Values calculated from food-replete F11 are connected by a dashed line to F4 values. Treatment lines are offset for clarity. Colours represent treatments. Error bars represent 95% confidence intervals. Values for OW and OA were not evaluated at F11.

3. Results

(a) Multigenerational population fitness patterns

A rapid adaptive response was evident in the OW and OWA treatments by the increases in population fitness (λ) after an initial decline at F0 relative to the AM treatment (figure 1). In F0, λ decreased by 37% for the OWA and 44% for OW treatments relative to the AM treatment ($p < 0.001$ for OW and $p < 0.001$ for OWA; figure 1). The decrease in λ under OWA conditions represents an additive effect because the OW \times OA interaction was not significant ($p > 0.2$, two-way ANOVA). However, by F2, λ under OW and OWA conditions recovered to levels equal to those of AM conditions. By F4, λ under OWA was greater than either OW (LMM ANOVA, $p_{\text{Tukey}} < 0.001$) or AM (LMM ANOVA, $p_{\text{Tukey}} < 0.001$), and greater than the sum of OW λ and OA λ , consistent with a synergistic effect of OW and OA (LMM three-way ANOVA, $p < 0.001$, electronic supplementary material, table S7). The increase in λ from F0 to F4 was underlain by sustained increased survival to adulthood (figure 2a). This is also supported by probability estimates of observing λ values of zero and by predicted non-zero values of λ (electronic supplementary material, figure S5); that is, the probability of λ values that equal 0 decreased over time for both OW and OWA through F4. Thus, with increasing generations, low-fitness individuals decreased in frequency, driving an increase in overall population fitness. At F4, there was no significant difference in λ values for either the OW or OA treatments relative to AM. However, fitness in the OWA treatment was 19% higher relative to AM at this generation (LMM ANOVA, $p_{\text{Tukey}} < 0.001$). Lastly, λ declined in F11 relative to F4, but was still 29% higher than F0 (LMM ANOVA, $p_{\text{Tukey}} < 0.01$, figure 1).

(b) Multigenerational trait patterns

The OWA treatment showed a 73% reduction in survival relative to AM at F0 (figure 2a; LMM ANOVA, $p_{\text{Tukey}} < 0.001$) in contrast to the OA and OW treatments (t -test, $p > 0.3$ for OA, $p > 0.1$ for OW). However, a threefold increase in survivorship in the OWA treatment was evident from the F0 to

the F4 (GAM ANOVA $p < 0.001$). Likewise, survivorship in the OW treatment increased significantly by 56% from the F0 generation to the F4 generation (GAM ANOVA, $p < 0.02$), but neither OA nor AM survivorship changed across generations (GAM ANOVA, $p > 0.3$ for OA, $p > 0.06$ for AM).

Egg production rate (EPR) also increased across generations in OW and OWA treatments (LMM ANOVA, $p < 0.01$). Relative to AM, OW EPR decreased by 50% in the first generation (figure 2b; LMM ANOVA, $p_{\text{Tukey}} < 0.01$), but then increased across generations until F4 (LMM ANOVA, $p < 0.01$). The OWA treatment showed a similar pattern where EPR was 32% lower than the AM at F0 (LMM ANOVA, $p_{\text{Tukey}} = 0.05$), but then increased across generations until F4 (LMM ANOVA, $p < 0.01$). By contrast to the OW and OWA treatment, OA EPR did not decrease relative to AM at F0 ($p > 0.8$), but did decrease across generations (GAM ANOVA, $p < 0.01$). However, EPR decreased by 53% at F11 for OWA (LMM ANOVA, $p_{\text{Tukey}} < 0.01$). Hatching success (HS) remained unchanged for most treatments across generations (figure 2c). The exception was the OWA treatment, where HS increased from F0 to F4 by 8% (LMM ANOVA, $p = 0.01$; GAM ANOVA, $p < 0.04$).

OW and OWA treatments experienced 20% shorter development times than the AM treatment (three-way ANOVA, $p_{\text{Tukey}} < 0.001$; figure 2d). Similarly, OA reduced development time relative to AM, but only by 3–7% at F2 (LMM ANOVA, $p_{\text{Tukey}} < 0.04$). Across generations, OA did not affect development time (GAM ANOVA, $p > 0.1$), but OW and OWA did (GAM ANOVA, $p < 0.001$ for OW and $p < 0.0001$ for OWA). Moreover, for OWA, by F4, development time was shortest relative to the other three treatments (figure 2d; LMM ANOVA, $p_{\text{Tukey}} < 0.001$ compared with AM, $p_{\text{Tukey}} < 0.001$ compared with OA, $p_{\text{Tukey}} < 0.03$ compared with OW), illustrating the synergistic effects of warming and acidification ($p < 0.001$, three-way ANOVA).

The sex ratio in this study was independent of treatment and generation (GAM ANOVA, $p > 0.6$ for AM, $p > 0.1$ for OA, $p > 0.05$ for OW, $p > 0.08$ for OWA; electronic supplementary material, figure S2).

Copepod body size in the OWA treatment increased in both males and females across generations (GAM ANOVA, males: $p < 0.001$, females: $p < 0.001$, electronic supplementary material, figure S3) as development time shortened, leading

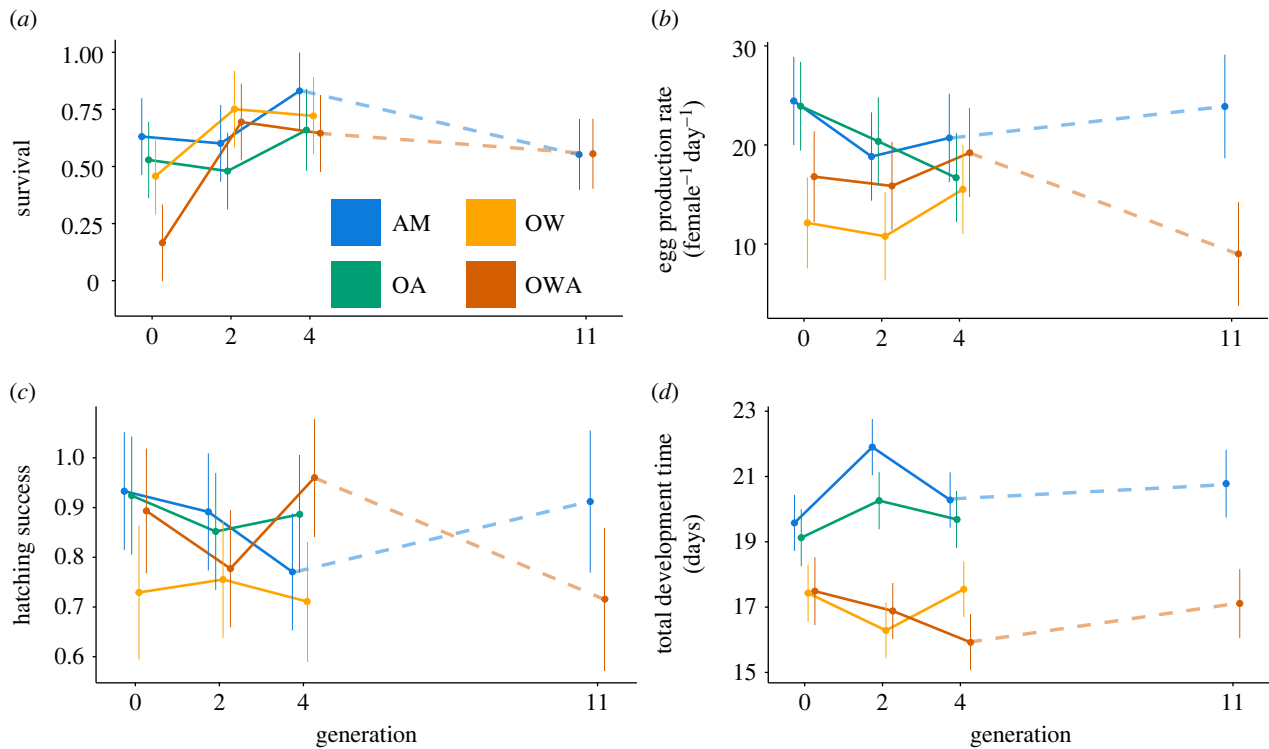


Figure 2. Multigenerational trait patterns for *Acartia hudsonica*. Mean values of (a) survival, (b) egg production rate, (c) hatching success and (d) total development time. Values at F11 from the food-replete treatment are plotted and connected to the F4 values with a dashed line. Treatment lines are offset for clarity. Legend colours in panel (a) represent treatments in all panels. Error bars represent 95% confidence intervals. Values for OW and OA were not evaluated at F11.

to increases in somatic growth rate between F0 and F11 (electronic supplementary material, figure S4). Body size decreased in the OW treatment (GAM ANOVA, $p_{\text{Tukey}} < 0.001$ for males, $p_{\text{Tukey}} < 0.001$ for females, electronic supplementary material, figure S3) and the OA treatment (LMM ANOVA, $p_{\text{Tukey}} < 0.001$ for males, $p_{\text{Tukey}} < 0.001$ for females). Somatic growth rate for both sexes decreased across generations (GAM ANOVA, $p < 0.001$ for OA, $p < 0.001$ for OW, electronic supplementary material, figure S4).

(c) Costs of producing adaptive phenotypes

To evaluate potential costs, we compared performance and fitness of the OWA and the AM lineages in reciprocal transplant experiments after 11 generations. We observed a significant effect of developmental environment on EPR in the OWA lineage (two-way ANOVA, $p < 0.001$, figure 3a), with a 2.5-fold increase in EPR after transplant to AM conditions ($p_{\text{Tukey}} = 0.02$, figure 3a). By contrast, survival was independent of environment in the OWA lineage but decreased significantly when the AM lineage was transplanted to OWA conditions (figure 3b). This result resembled the effect on survival observed at F0 (figure 2a). Overall, there was no significant decrease in fitness for the OWA lineage when it was transplanted to AM conditions (t -test, $p = 0.1$, figure 3c). However, fitness from the OWA lineage was significantly lower than the AM lineage at F11 regardless of environment (two-way ANOVA, $p < 0.001$).

We explored additional costs to OWA adaptation by exposing both the AM and OWA lineages to food-limited conditions. Under food limitation, both lineages show a significant decline in EPR (t -test, AM lineage $p_{\text{Tukey}} < 0.04$, OWA lineage $p_{\text{Tukey}} < 0.05$, figure 3a) but no decrease in fitness (t -test, both lineages $p > 0.1$, figure 3c), suggesting that

food limitation does not represent an additional cost of adapting to OWA.

(d) Shifting traits under selection

Egg-hatching success and survival showed the strongest evidence of selection. Relative λ increased as a function of HS with standardized linear selection coefficients (β – see methods) of 0.85 (AM), 0.56 (OA), 0.92 (OW) and 0.50 (OWA) at generation 0 (electronic supplementary material, table S5, ANOVA, $p < 0.001$). Notably, for the OWA treatment the linear selection coefficients for EPR and HS were nearly equal (EPR: 0.49, HS: 0.50) indicating that these traits experienced equal strengths of selection at F0 for OWA. In addition, between F0 and F4, the linear selection coefficients of the OWA treatment decreased for EPR and HS by 45% and 62%, respectively, but increased sevenfold for survival leading to the highest coefficient of selection for the OWA treatment. Path analysis (electronic supplementary material, table S6) revealed that HS had the largest effect on fitness of all life-history traits across treatments, except for the OWA treatment at generation 4 where survival exhibited the largest effect on fitness.

(e) Genetic separation between treatments and generations

Allele frequency estimates identified 286 139 single nucleotide polymorphisms within and between lineages (electronic supplementary material, figure S6). Principle components analysis (PCA) showed that samples clustered by treatment across all generations and there was generally separation between treatments, indicating consistent genetic differentiation. F_{ST} estimates further revealed increasing genetic differentiation from AM control lines across generations for all treatment

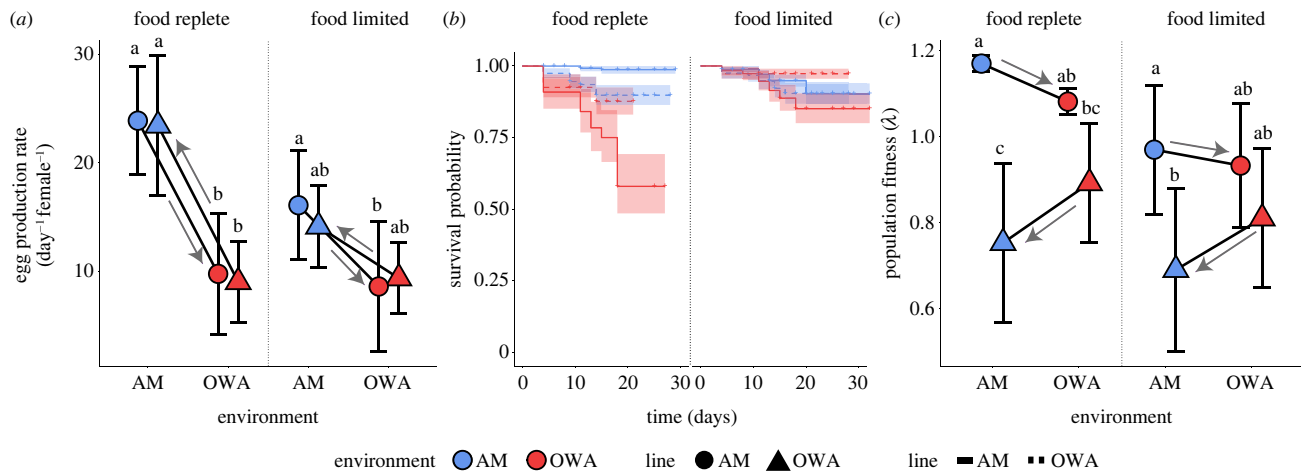


Figure 3. Transplant experiments. For all panels, shape indicates selection lineage, colour indicates environmental condition at that generation and error bars represent 95% confidence intervals. (a) Egg production. (b) Survivorship. (c) Population fitness (λ). Letters in (a) and (c) represent statistically similar groups for each food treatment. Arrows in (a) and (c) indicate direction of transplant.

groups ($p = 0.001$) as well as higher F_{ST} of OWA from AM at F11 than all other groups and generations ($F_{ST} = 0.062 \pm 0.005$, electronic supplementary material, figure S7).

4. Discussion

We present evidence for a rapid recovery of fitness across multiple generations after initial declines in response to combined warming and acidification for a foundational zooplankton species, *A. hudsonica*. This rapid phenotypic response was consistent across replicates within a treatment and did not reverse upon transplantation to the ancestral environment after 11 generations. Allele frequency segregation by treatment also suggests genetic differentiation. Altogether, these observations are consistent with evolutionary adaptation to OWA, although plastic mechanisms cannot be completely ruled out and we discuss caveats below. The phenotypic shifts in the OWA lines also appear to carry costs and limit maximum fitness, which highlights the challenges of achieving full fitness recovery under OWA. Lastly, continued synergy of warming and acidification through F4 seems to lead to persistent plasticity. Together, our study highlights the strikingly different patterns and costs related to adaptation that can exist between congeners.

The fitness changes for *A. hudsonica* were caused by different mechanisms that varied across the generations. In early generations, HS was the trait under selection. However, survival to adulthood improved across generations due to increasing somatic growth rates, becoming the primary driver for recovering fitness and the trait under selection by F4. This selective shift could be due to the persistent synergistic effects of OW and OA, either through epistasis or other mechanisms [71]. Moreover, the continuing synergy of OW and OA across generations led to a maintenance of phenotypic plasticity for OWA lineages but a lower overall fitness maximum relative to AM control lineages caused by decreasing EPR. These results show the complexity of adaptive responses in a multiple stressor environment where selection can act on numerous traits and where the effects of multiple stressors may interact. Because interactive effects of additional levels of warming and acidification have not been reported for this species, we cannot definitively say that this interaction holds across other temperature or pH

levels. However, the non-additive effects of OW and OA at various levels are well documented in other taxa [72]. In addition, as the number of traits under selection and stressors increase, the probability of adaptation decreases due to the challenges of multi-dimensionality [73]. Our results resemble recent work on rotifers, which suggests that multi-dimensional stress produces lineages that are initially specialized to their adaptive environment and in later generations become more generalist [74]. In the case of *A. hudsonica*, fitness was recovered, but at a submaximal level and appeared to carry costs over the longer term.

The trait shifts observed for *A. hudsonica* were markedly different compared with its congener *A. tonsa*. Superficially, both species display rapid but limited adaptation to OWA where OA did not appear to be a selective agent. However, while *A. hudsonica* had a shift in the traits under selection across generations, HS was under selection for the duration of the experiment in *A. tonsa* [23]. Additionally, the non-additive effects of OA and OW switch from synergistic to antagonistic for *A. tonsa* [23], but remain synergistic for *A. hudsonica*. Finally, the lack of performance/fitness decrease under food limitation suggests that *A. hudsonica* tolerates this additional stress well, which contrasts with *A. tonsa* which performs poorly under limited food conditions after OWA adaptation [24].

As populations adapt to new environments, selection may drive genotypic specialization to optimize phenotypes in new environments [75] and maximize fitness [28,31]. However, maladaptive mechanisms like the costs observed in *A. tonsa* [24,25] may limit the extent to which fitness can be sustained at high levels [31] if selection acts on genes or alleles that divert resources away from population growth in favour of maintaining homeostasis [24,52]. Selection may also promote adaptive plasticity [7,8,76–78]. In the present study, EPR decreased when the AM lineage was transplanted at F11, but fitness did not. This is opposite to the pattern observed at F0 when the AM lineage was first exposed to OWA to begin the transgenerational experiment. Thus, plasticity seems to have increased from F0 to F11 for the AM lineage. Genetic drift is one possible mechanism for producing a new, more plastic phenotype by F11 that does not involve selection driving changes in performance [8,38,41,79]. However, the parallel responses of the replicates within treatments are not consistent with drift. It is also

possible that plasticity within the AM lineage could be selected for, along with increased mutational variation (i.e. genetic accommodation [80,81]). Because selection acts on relative fitness within the population [28], individuals with higher degrees of phenotypic plasticity could be selected if the selection coefficient for plasticity was sufficiently strong. This could lead to a saturation of individuals with high levels of phenotypic plasticity over time. Consequently, it is conceivable that populations could maintain absolute fitness while increasing the proportion of the population with higher relative fitness. This could explain the lack of change in absolute fitness and the increased genomic variation we observed across generations in the AM lineage. Thus, it is possible that we selected for a population where plasticity positively affected the relative fitness of the population. However, a clear distinction of the selective pressure acting on these lineages is, seemingly, absent given that the environment was maintained at a stable, benign temperature and CO₂ concentration for this species. Studies that explicitly intend to address impacts of changing plasticity on relative fitness are essential to understanding the degree to which organisms may experience selection for increased plasticity.

The OWA lineage shows phenotypic plasticity at F11 in two ways: fitness is maintained when the environment changes from food replete to food limited, and when the environment changes from OWA to AM. Results for the former suggest that *A. hudsonica* requires few resources to maintain homeostasis and population fitness is less susceptible to food limitation than *A. tonsa* [24]. Indeed, *A. hudsonica* is smaller in size than *A. tonsa* [48], which could lead to lower nutritional requirements. Thus, adaptation to OWA is not resource dependent for this species, at least for the temperature, CO₂ and food conditions evaluated here. This suggests that adaptation to combined warming and acidification can be achieved at suboptimal resource conditions – a benefit of sustaining phenotypic plasticity.

Animals from the OWA lineage that were transplanted to ambient conditions at F11 showed similar levels of phenotypic plasticity (change in EPR with environment) as the AM lineage. This result is similar to previous research where transgenerational rearing in low pH environments improved copepod egg production relative to populations with no prior exposure to OA and was reversible, indicating transgenerational plasticity (i.e. multigenerational plasticity) [13,82]. Those studies, however, did not explore the relationship of copepod fecundity to population fitness. In the present study, fitness declined in later generations and was not reversible at F11 like EPR was. This suggests that multigenerational plasticity could have contributed to our observed fitness and trait patterns. Indeed, there has been empirical support for multigenerational plasticity acting as a mechanism for tolerating climate change in marine systems [14,83,84]. However, a recent review on cross- and multigenerational plasticity in closely related marine invertebrates experiencing climate-based stressors highlights that parental conditioning offers no long-term benefits to performance or fitness [82]. Multigenerational plasticity is also expected to decline after two generations [85]. Given that our experiment lasted 11 generations, we expect multigenerational plasticity to contribute minimally to the transgenerational trait and fitness patterns, though potentially not altogether absent.

Testing for cost of plasticity requires differential plasticity between genotypes. Both the OWA and AM lineages showed

similar degrees of plasticity at F11, leaving us unable to unequivocally test for this cost. However, the maximal fitness in the OWA lineage at F11 was lower than the AM lineage, consistent with a cost of the OWA phenotype [37–39]. Although this cost may constrain adaptive responses [41], it does not completely prevent further adaptation to environmental change, nor restrict phenotypic plasticity, and is not inherent to adaptation [31,34,35,38,42]. Rather, this cost reduces fitness under OWA conditions relative to AM conditions presumably due to physiological limitations resulting from simultaneous warming and acidification that prevent high performance across all traits and result in trade-offs. Additionally, this cost can reflect the variation across an individual fitness landscape whereby the overall fitness is reduced as the environment or trait becomes suboptimal. Variation across an individual fitness landscape should yield peaks and valleys that reflect changes due to environment or individual phenotype [10,28,31]. Thus, the physiological limitations imposed by OWA conditions that prevented maximum performance across all traits probably drove a key trait (EPR) to suboptimal performance at F11 in the OWA environment, which sent fitness into a landscape valley or an alternative lower fitness peak. Likewise, the change in environment from OWA to AM conditions reflects an introduction to a suboptimal environment. While the combination of these two phenomena indicates sustained plasticity for *A. hudsonica*, the overall fitness is still below the maximum attainable level. Thus, the shift in selection from offspring production towards survival seems to lead to prolonged, but reduced population growth.

Our observed patterns for *A. hudsonica* contrast with *A. tonsa*, which shows a loss of plasticity following adaptation to OWA [24,25] and consistent selection on offspring production [23]. The differences in plasticity maintenance could result from the contrasting responses to selection driven by the complex effects of OW and OA on either copepod species in later generations (antagonistic for *A. tonsa* and synergistic for *A. hudsonica*). Another possibility is that cold-adapted species, like *A. hudsonica*, show more developmental plasticity than warm-adapted species, like *A. tonsa* [86]. The differences between these two copepod species are consistent with those observed on transgenerational studies of congeneric annelids exposed to OWA conditions [26,84,87,88] in that a ubiquitously distributed generalist, like *A. tonsa*, is more tolerant to OWA than an endemic specialist, like *A. hudsonica*, with both annelids exhibiting some degree of transgenerational plasticity [84]. Studies like these, in addition to our own, support the notion that closely related phylogenetic groups respond differently to combined stressors depending on their native environments. Additionally, increased environmental variability across seasons and across days can lead to differences in plasticity and its persistence [7,8]. As such, natural populations of *A. tonsa* and *A. hudsonica* are poised to exhibit differing responses given their respective seasons and differences in daily thermal/pH fluctuations within those seasons relative to laboratory environments. During the year-long acclimation period, static conditions could alter plasticity of *A. hudsonica* relative to natural populations. However, we observed no reduction in plasticity for *A. tonsa* when exposed to ambient, static laboratory conditions for more generations over the same time period [24]. Thus, we do not expect the acclimation period to have altered plasticity with respect to natural populations

in this study. Future studies should aim to incorporate effects of natural environmental variability for multiple stressors on plasticity and adaptation.

(a) Broader implications

The rapid responses of *A. hudsonica* (this study) and *A. tonsa* [23,24,52] to OWA conditions suggest that coastal zooplankton species harbour sufficient standing genetic variation to cope with rapid climate change. The limited adaptative response and the costs, however, also suggest that evolutionary rescue may not be complete for the projected levels of OWA even in the somewhat benign environment of unlimited food conditions of our study, thereby restricting future population resilience. Importantly, the different selection mechanisms between the two species determine their respective adaptative and plastic responses. Thus, phylogenetically close species can respond to the same stressor, and combination of stressors, differently. Other stressors such as hypoxia [89], toxic algal blooms [90] and heat waves [91] that may co-occur with warming and acidification, may further limit fitness recovery. An open question is whether populations that experience a high degree of local warming and acidification can be rescued from maladaptation and costs by gene flow to allow population persistence. Continuing to explore the balance between adaptation and plasticity will provide novel insights into adaptive responses during rapid global change.

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

Data accessibility. All data and code for analysing and visualizing the data are deposited into a Zenodo repository located at: <https://zenodo.org/badge/latestdoi/505896789> [92]. Transcriptomic data are deposited in Genbank with BioProject Accession number PRJNA966098.

Supplementary material is provided online [93].

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

Authors' contributions. J.A.d.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, software, supervision, validation, visualization, writing—original draft, writing—review and editing; R.B.: methodology, writing—review and editing; M.H.P.: conceptualization, funding acquisition, methodology, writing—review and editing; M.B.F.: conceptualization, funding acquisition, methodology, writing—original draft, writing—review and editing; G.P.: conceptualization, investigation, writing—review and editing; L.N.: investigation, methodology, writing—review and editing; H.B.: conceptualization, funding acquisition, writing—review and editing; H.G.D.: conceptualization, funding acquisition, investigation, methodology, writing—original draft, writing—review and editing.

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

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

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