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Fitness effects of spontaneous mutations in a warming world

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Spontaneous mutations fuel evolutionary processes and differ in consequence, but the consequences depend on the environment. Biophysical considerations of protein thermostability predict that warm temperatures may systematically increase the deleteriousness of mutation. We sought to test whether mutation reduced fitness more when measured in an environment that reflected climate change projections for temperature. We investigated the effects of spontaneous mutations on life history, size, and fitness in 21 mutation accumulation lines and 12 control lines of *Daphnia pulex* at standard and elevated (+4°C) temperatures. Warmer temperature accelerated life history and reduced body length and clutch sizes. Mutation led to reduced mean clutch sizes and fitness estimates at both temperatures. We found no evidence of a systematic temperature–mutation interaction on trait means, although some lines showed evidence of beneficial mutation at one temperature and deleterious mutation at the other. However, trait variances are also influenced by mutation, and we observed increased variances due to mutation for most traits. For variance of the intrinsic rate of increase and some reproductive traits, we found significant temperature–mutation interactions, with a larger increase due to mutation in the warmer environment. This suggests that selection on new mutations will be more efficient at elevated temperatures.

KEY WORDS: **beneficial mutation, *Daphnia pulex*, fitness, life history, mutation accumulation, temperature–size rule.**

Spontaneous mutations fuel evolutionary processes by introducing new genetic variation to populations. Their fitness effects range from highly advantageous to neutral to highly deleterious, altering the ecological and evolutionary dynamics that determine the fate of populations. In general, spontaneous mutations tend to be neutral or deleterious, rather than beneficial (Drake et al. 1998; Lynch et al. 1999; Halligan and Keightley 2009). However, in some cases, effects of mutations can also be condition dependent, where fitness effects change with the environment (e.g., Chang and Shaw 2003; Baer et al. 2006; Roles and Conner 2008; Rutter et al. 2012; Latta et al. 2015; Rutter et al. 2018). This may simply mean that the magnitude of deleteriousness depends on the environment in which fitness effects are manifest, but condition-dependent mutations can also switch from deleterious to beneficial or vice versa.

Researchers have used experimental mutation accumulation (MA) to study the phenotypic and fitness effects of spontaneous mutations for several decades (reviewed in Lynch et al. 1999; Baer et al. 2007; Halligan and Keightley 2009). In these experiments, replicate lineages are established from a common ancestor and maintained through random single-pair or single-individual descent to shield them from selection. Spontaneous mutations accumulate generation after generation, and eventually phenotypes of the lineages are assayed in a common garden. Variation among lines is due to genetic differences caused by the accumulated mutations. This approach has been applied to a variety of eukaryotes, including *Drosophila* (Houle et al. 1992; Fry 2001; Houle and Fierst 2012; Latimer et al. 2014), *Daphnia* (Lynch 1985; Lynch et al. 1998; Omilian et al. 2006; Schaack et al. 2013; Eberle et al. 2018; Bull et al. 2019), *Arabidopsis* (Chang and Shaw 2003; Ossowski et al. 2010; Rutter et al. 2010; Roles et al. 2016; Rutter et al. 2018; Weng et al. 2019), nematode worms (Baer et al. 2005, Baer et al. 2006; Davies et al. 2016), and others (Hall et al. 2008; Pannebakker, et al. 2008; Schultz and Scofield 2009; Ness

et al. 2012; Hall et al. 2013; Zhu et al. 2014; Krasovec, et al. 2017; Lovell et al. 2017). Phenotypes and fitness are usually assayed in a single environment, and hence we have comparatively little experimental information on condition dependence of the fitness effects of spontaneous mutation. Given that real environments are not static, evaluating fitness effects in multiple controlled environments provides a more robust understanding of the consequences of mutational processes. It also provides a bridge to studies where fitness is assayed in uncontrolled field environments (Roles and Conner 2008; Rutter et al. 2012).

The few published studies on spontaneous mutation that assayed phenotypes in multiple environments found evidence for condition dependence of fitness. Rutter et al. (2012) assayed five MA lines of *Arabidopsis thaliana* in the field during different seasons and at different locations. They found that comparisons between MA lines and ancestral controls differed qualitatively and quantitatively across environments for individual lines. Similar results were found in an analysis of a greater number of MA lines from the same experiment in two locations (Roles et al. 2016). Latta et al. (2015) worked with *Daphnia pulex*, comparing MA lines assayed in a baseline environment to treatments with mild copper stress, low food, and slightly elevated temperature. For a variety of fitness components and body size, they found that the effects of mutation shifted across environments. Other MA studies in multiple environments include Baer et al. (2006), which examined *Caenorhabditis elegans* MA lines at two temperatures, and Chang and Shaw (2003), which examined the effects of nutrient availability on *Arabidopsis* MA lines. Although these studies have shown that condition-dependent mutations can occur, they were not organized around testing underlying mechanisms that may drive widespread condition dependence.

Temperature could broadly influence the consequences of mutations through its effect on protein stability and integrity (Dandage et al. 2018; Agozzino and Dill 2018; Berger et al. 2020). Elevated temperature alone can disrupt protein folding (Varadarajan et al. 1996; Agozzino and Dill 2018), leading to diminished stability and a protein that is less able to tolerate the effects of an amino acid substitution. Berger et al. (2020) used enzyme kinetics and biophysical modeling to argue that global warming will increase the effects of mutation on protein stability, intensifying selection throughout the genome. Spontaneous mutations, particularly within a sequence coding for a protein's hydrophobic region, can destabilize the tertiary structure, making the protein more vulnerable to thermal degradation of biochemical function. These effects are sensitive to the magnitude of temperature change (Varadarajan et al. 1996). Dandage et al. (2018) used a large number of single-site mutants in *Escherichia coli* to show that mutations at central and typically protected locations are often linked to a greater decrease in fitness than those mutations affecting peripheral protein parts. The deleterious effects of these mutations were particularly pronounced at high temperature due to protein misfolding and binding problems. Taken together, there is clear reason to predict that elevated temperature will exacerbate the deleteriousness of spontaneous mutations.

We sought to test the predictability of condition-dependent effects of spontaneous mutations at a scale of temperature variation relevant to current climate change projections. Despite extensive research on the adaptability of populations to climate change, we are unaware of work aimed directly at evaluating how it could alter the mutation dynamics at the foundation of evolutionary change. In particular, we ask whether warmer temperatures amplify the deleteriousness of spontaneous mutations on life history traits and an aggregate estimate of fitness, the intrinsic rate of increase (r). To accomplish this, we used an MA experiment in *D. pulex*, conducting common garden life history assays for MA and ancestral control lines at two temperatures. We expected to see reduced fitness in MA lines compared to the ancestor, and to observe a more substantial reduction at the higher temperature.

Methods

ESTABLISHMENT AND MAINTENANCE OF THE MA EXPERIMENT

We initiated MA lines of *D. pulex* on May 15, 2016 with a single female of the LIS3 clone, which had been isolated several years prior from a pond near Listowel, Ontario, Canada. The LIS clone is "obligately asexual," that is, a clone that produces dormant eggs (ephippia) asexually (Hebert 1981), unlike the typical *Daphnia* life cycle in which dormant eggs are produced sexually.

We collected 30 daughters from a single individual and placed them in separate beakers under standard culture conditions (described below) to initiate the MA lines (Fig. 1). To propagate a line, a single, haphazardly chosen offspring of the current generation was transferred to a new beaker approximately every two weeks to be the next generation. At the same time, two additional offspring were kept separate to serve as back-ups in case the focal individual turned out to be male (sex identification as neonates can be challenging), reproduced only via ephippia, or died without reproducing. After establishing a new generation, we also kept the previous two generations at 10°C to serve as an additional reserve. Every 10 generations we collected dormant eggs, which can be used to re-establish a line following complete extinction, and stored them in microfuge tubes at −80°C. This procedure maintains the effective population size for each line at nearly 1.0 and shields the population from most effects of selection. Occasional generations based on more individuals occur when we use the back-ups; this raises effective population size only slightly because it is determined by the harmonic mean of the actual population size. Although this procedure precludes

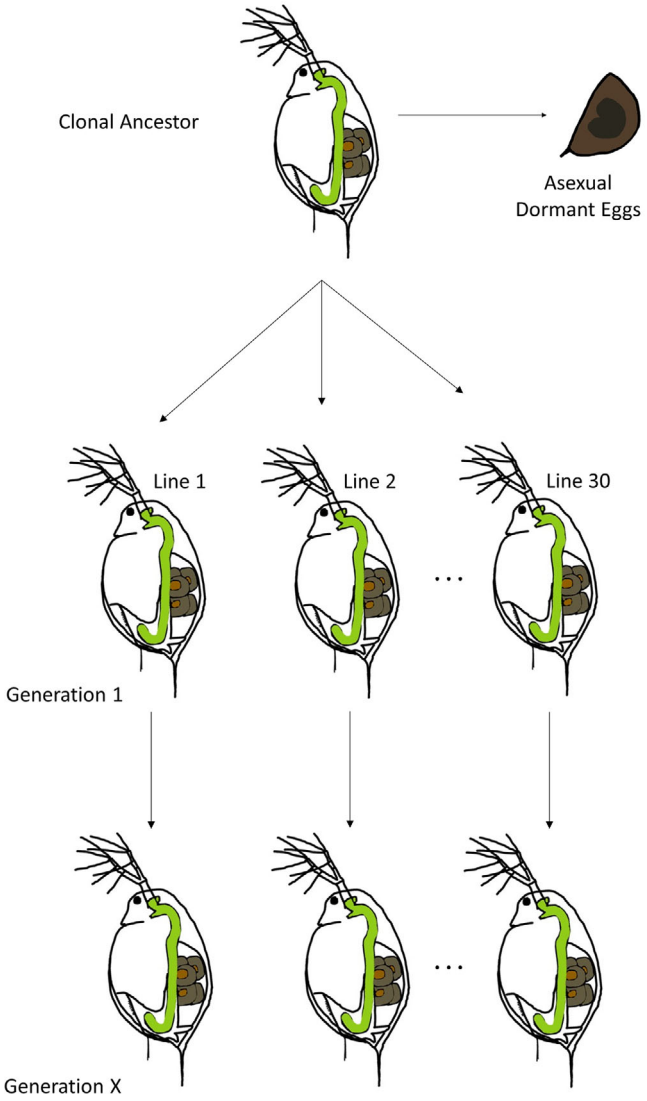

Figure 1. Mutation accumulation experimental set-up, starting with a clonal ancestor. Maternal lines are established with the female offspring of the ancestor and maintained separately for an indefinite number of generations via asexual reproduction. Over time, spontaneous mutations occur and accumulate in each line, causing lines to diverge from the ancestor and from one another. During the initial set-up, asexually produced dormant eggs (ephippia) were collected from the original ancestral population and frozen for later hatching.

mutations that have major effects on fitness in the heterozygous state (e.g., dominant lethals), it effectively accumulates the mutations thought to be responsible for most standing genetic variation.

ANCESTRAL CONTROLS

Because the LIS clone is obligately asexual, we were able to generate ephippia from the ancestor at the start of the experiment to use as controls in subsequent phenotypic assays. We used additional daughters from the ancestral individual to grow a large population and produce >1000 ephippia that we stored at −80°C. We estimate that the ephippia for the controls are three to four generations from the original ancestor.

To hatch controls for our phenotypic assay, we incrementally increased temperature and light exposure to mimic environmental conditions that *Daphnia* experience in the wild after removing them from storage at −80°C. First, ∼50 ancestral LIS ephippia were thawed and transferred to a tissue culture plate containing a small amount of filtered lakewater. The plate was moved to 4°C for two weeks in the dark before being transferred to a 20°C incubator set for high light intensity on a 12:12 (light:dark) photoperiod. After approximately two weeks under these conditions, neonates hatched and were transferred to individual beakers and maintained under standard culture conditions (described below). We collected 33 hatchlings this way, though we do not know whether ephippia that did not hatch actually had embryos in them and could have hatched if we had waited longer. All hatchlings reached reproductive maturity, and 12 of them were randomly chosen to be the replicate control lines for our phenotypic assay.

STANDARD CULTURE CONDITIONS

MA lines are propagated in filtered (to 1 μm) lakewater collected from a single location at the hypolimnetic outflow just below the Lake Murray Dam (Lexington County, SC, USA). Each individual is placed in 100 mL lakewater in a 150-mL Pyrex beaker. Beakers are kept in controlled-environment chambers at 20°C with a 12:12 (light: dark) photoperiod. Animals are provided vitamin-fortified *Ankistrodesmus falcatus* (Goulden and Hornig 1980) daily at a quantity that effectively allows ad libitum filter-feeding. To prevent surface film entrapment, a small amount of cetyl alcohol was dusted on the lakewater (Desmarais 1997).

PREPARATION FOR PHENOTYPIC ASSAY

MA lines had descended an average of 37 generations from the ancestor (range: 27–42) at the time we started preparation for our assay, and some lines were extinct due to production of only dormant eggs at some point (five lines that had not been "resurrected" at the time of the assay) or failure to reproduce (two lines), leaving 23 MA lines to potentially be used in the experiment. In an acclimation period before the phenotypic assay, three generations of both control and MA lines were reared under controlled conditions: 20°C, 12:12 L:D, and daily quantitative feeding at 20,000 cells/mL *A. falcatus*. Individuals were transferred to a new beaker and fresh lakewater on alternate days. This minimizes variation attributed to maternal effects. To start, two females of approximately the same size were haphazardly taken from each line and placed in separate beakers, marking the beginning of the great-grandmaternal generation. Twenty-one of our 23 MA lines and all 12 control lines of LIS produced

female offspring at this stage. Two MA lines produced only ephippia or male offspring. Waiting for these lines to generate female offspring was not possible given operational constraints, so they were excluded from the remainder of the experiment. We established each generation during the acclimation period from offspring of the third clutch or later, expanding the number of individuals in the grandmaternal and maternal generations to boost the likelihood that a sufficient number of offspring would be born within the desired timeframe for the assay.

PHENOTYPIC ASSAY

We conducted a phenotypic assay of fitness-related traits at two temperatures (20°C and 24°C) with $n = 10$ female individuals per line per temperature in each of two temporal blocks. We followed the previously described protocol for daily quantitative feeding and transfers every other day during the assay. We pipetted neonates out of beakers during daily counts of reproduction to prevent double-counting offspring. Survival was scored daily.

We discarded individuals once they had produced their fourth clutch because reproduction after that point has little influence on estimates of the intrinsic rate of increase. If a female did not produce offspring, she was observed for five to seven additional days, and if no eggs were deposited in her brood chamber by the end of this period, she was discarded. Daily procedures were identical for the 20°C and 24°C treatments.

The assay was divided into two temporal blocks, with the second block initiated about three weeks after the conclusion of the first. For each block, 20 females born within a 24-h period were collected from each line and placed into individual beakers. Ten of these 20 individuals were randomly assigned to the 24°C treatment and 10 to the 20°C treatment. Two MA lines (Lines 5 and 23) did not produce enough female offspring during the specified set-up period for block 1, so they were excluded from the first block but included in the second. In block 2, one line (MA Line 23) only produced 10 females to be used in the assay (five at each temperature).

We documented the clutch size of the first four clutches, survivorship, clutch frequency, and day of maturation for each individual. Each female in block 1 was photographed at maturation, and body length (measured from the crown of the head to the base of the tailspine) was recorded using a stage micrometer for calibration. Photographs were analyzed with ImageJ.

LIFE HISTORY CALCULATIONS

We tracked survival daily until production of the fourth clutch. We calculated age-specific survivorship (l_x) for each MA and control line after censoring data for the few individuals that were lost during transfers. Although most individuals within a line reproduced synchronously, some produced a fourth clutch several days after all other individuals. This was the case for 15 individuals (12 at 20°C and 3 at 24°C), so observations were terminated when they had not produced a clutch 4–7 days after other individuals of that line had. For the purpose of estimating r, survivorship was calculated for each day until the majority of experimental individuals produced a fourth clutch.

We calculated age-specific fecundity (m_x) for each female as a three-day moving average until she produced her fourth clutch. We then used Newton–Raphson approximation to combine the schedules of age-specific fecundity and survivorship and estimate the intrinsic rate of increase (r) from the Euler–Lotka equation. We pooled data from both blocks for these estimates.

STATISTICAL ANALYSIS

Our basic experimental design was an unbalanced two-way analysis of variance, with mutational history and temperature as main effects and lines as the primary unit of replication. We tested for effects of mutational history, temperature, and their interaction. Statistical models for reproductive traits included a block effect and block interactions to account for the two temporal blocks. We used type III sums-of-squares for our ANOVAs because we had an unbalanced experimental design with different numbers of MA and control lines. Although analyses using Levene's test showed deviations from homoscedasticity, we did not turn to nonparametric alternatives because our main hypothesis focused on interactions that can be masked by the transformations involved in nonparametric analyses. Additionally, ANOVA is robust to deviations of this nature. Although our statistical inferences should therefore be treated cautiously, none of our conclusions depend on comparisons that turned out to be marginally significant. We further used Levene's tests to test directly whether mutation increased the variance of traits, and to test whether trait variance was influenced by an interaction between mutation and temperature. We conducted all statistical analyses in R version 3.6.2 (R Core Team 2014) and figures were produced using the package ggplot2 (Wickham 2009).

Results

We recorded life-history traits from a total of 1,274 *D. pulex* from 21 MA and 12 control lines. Control Line 2 was an outlier with respect to most traits, so we excluded it from all analyses. Effects on fitness components were generally in line with expectations: elevated temperature accelerated life histories, while mutation reduced fecundity. Body size at maturation was reduced by both elevated temperature and mutation. Juvenile and adult survivorship was very high (>93%) throughout our experiment (Supporting Information Fig. S1), so we did not analyze it further. We did not find evidence of significant interactions between spontaneous mutations and temperature influencing trait means

Table 1. ANOVA tests of factors influencing the intrinsic rate of increase (*r*) and body size.

Trait	Effect	*F*	*p*
r	Mutation	5.2697	**0.0252**
	Temperature	99.8702	**2.191×10^{-14}**
	Mu × Temp	0.0116	0.9146
Relative fitness	Mutation	5.5605	**0.0217**
	Temperature	0.0351	0.8521
	Mu × Temp	0.0351	0.8521
Body length	Mutation	4.4461	**0.0395**
	Temperature	5.1767	**0.0267**
	Mu × Temp	0.1363	0.7134

Tests used Type III sums-of-squares and had *df* = 1, 60 (*r* and relative fitness) or *df* = 1, 56 (size). No block effects were tested because data were pooled across block to estimate *r*, and size was measured in only block 1. Bold values indicate $p < 0.05$. The identical values for temperature and Mu × temp effects on relative fitness are not a typographical error.

Table 2. ANOVA tests of factors influencing reproductive traits.

		Clutch Size		Timing	
	Effect	*F*	*p*	*F*	*p*
Clutch #1	Mutation	1.425	0.2350	5.3105	**0.0231**
	Temperature	0.0935	0.7603	298.7014	**2.0×10^{-16}**
	Mu × Temp	0.0487	0.8370	0.9775	0.3249
	Block	0.1767	0.6750	6.1668	**0.0145**
Clutch #2	Mutation	5.9866	**0.0159**	1.2868	0.2590
	Temperature	8.9728	**0.0033**	439.0989	**2.0×10^{-16}**
	Mu × Temp	1.5805	0.2112	0.1053	0.7461
	Block	7.6678	**0.0065**	5.4894	**0.0208**
Clutch #3	Mutation	23.8904	**3.3×10^{-6}**	0.4429	0.5070
	Temperature	7.2489	**0.0081**	610.6733	**2.2×10^{-16}**
	Mu × Temp	0.4364	0.5102	0.0715	0.7890
	Block	6.1261	**0.0147**	8.1272	**0.0052**
Clutch #4	Mutation	13.1205	**0.0004**	0.0481	0.8269
	Temperature	35.0501	**3.4×10^{-8}**	856.2458	**2.2×10^{-16}**
	Mu × Temp	0.9836	0.3234	0.5180	0.4730
	Block	6.7847	**0.0104**	9.9148	**0.0021**
Sum C1-C4	Mutation	17.5025	**5.6×10^{-5}**		
	Temperature	17.1569	**6.5×10^{-5}**		
	Mu × Temp	0.0715	0.7896		
	Block	6.5292	**0.0119**		

All tests used Type III sums-of-squares and had *df* = 1, 116 except Clutch 4, which had *df* = 1, 115. Bold values indicate $p < 0.05$. No interactions with block were significant (see Supporting Information Materials).

(Tables 1 and 2), but we did find evidence of interaction effects on some trait variances.

INTRINSIC RATE OF INCREASE

We estimated the intrinsic rate of increase (*r*) using fecundity and survivorship data. MA lines experienced a 6% decline in *r* at 20°C and a 5% decline in *r* at 24°C in comparison to controls (Table 1). At 20°C, mutation reduced the mean estimated *r* from 0.3258 ± 0.0097 SD in the controls to 0.3055 ± 0.0351 SD in the MA lines; at 24°C, the reduction was from 0.4196 ± 0.0152 SD to 0.3973 ± 0.0484 SD (Fig. 2). There was a significant increase in mean *r* for both MA and control lines in response to the increase in temperature (Fig. 2), driven by the accelerated reproductive schedule.

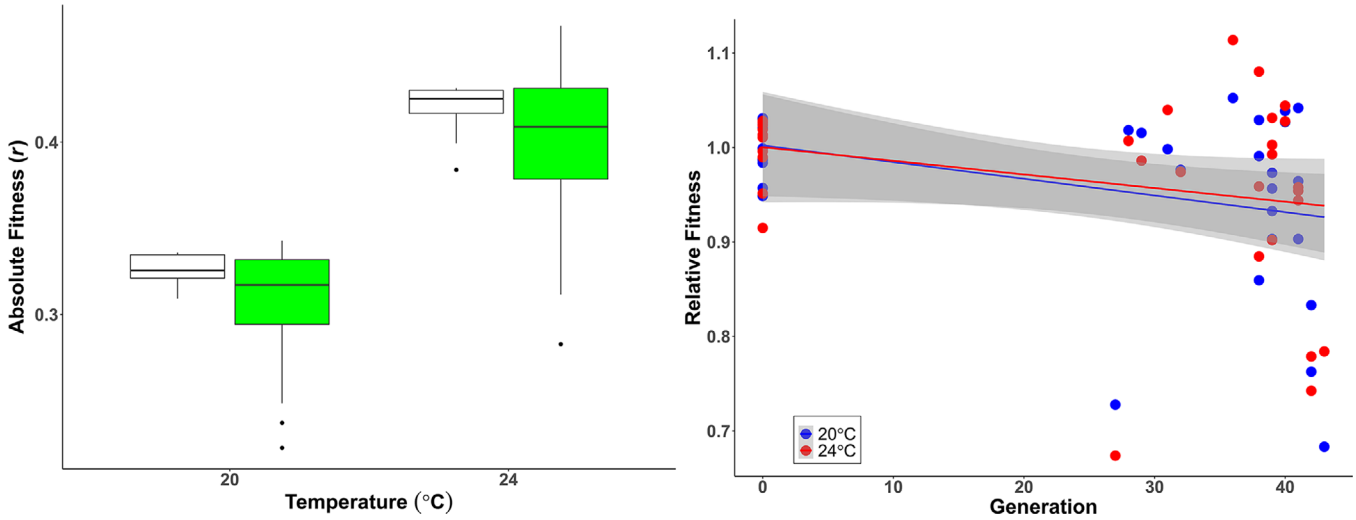

Figure 2. Intrinsic rate of increase (*r*) as a measure of absolute fitness (left panel) and scaled to the control mean in each temperature as a measure of relative fitness (right panel). Boxplots show distributions of *r* for control (black) and MA (green) lines. Relative fitness is illustrated as a function of generation; each point illustrates a replicate line, with control lines plotted at Generation 0. Blue points and regression line show data for 20°C, and red shows 24°C.

Contrary to our main hypothesis, there was no evidence for an interaction between spontaneous mutation and temperature with respect to mean *r* or relative fitness (Table 1).

The variance of estimated *r* was 55.2% greater for MA lines than control lines at 20°C and 65.8% greater for MA lines than control lines at 24°C. A Levene's test on the variance of estimated *r* revealed a significant interaction between mutational history and temperature (mutational history x temperature: $F = 3.7912$; $df = 3, 60$; $p = 0.0148$). Levene's tests also showed significant increases of *r* variance due to mutation at 20° ($F = 4.9699$; $df = 1, 30$; $p = 0.0334$) and 24° ($F = 5.52521$; $df = 1, 30$; $p = 0.0291$).

For both absolute and relative fitness, per-generation rates of change were similar at the two temperatures (Table 3). The intrinsic rate of increase declined by 0.00054 per generation at 20° and by 0.00062 at 24°; for relative fitness, the declines were 0.00169 and 0.00144 per generation, respectively. The estimated per-generation change in the coefficient of variation (ΔCV) was the same at both temperatures, 0.0023, and did not differ between absolute and relative measures of fitness. Estimates of mutational variance (V_m, the difference between V_{MA} and V_C) and mutational CV (CV_m, the difference between CV_{MA} and CV_C) were similar between the two temperatures (Table 3). Mutational heritabilities (the proportion of variance among MA lines attributable to mutation) on a per-generation basis were ~0.025 at 20° and ~0.024 at 24° (Table 3). These estimates reflect heritability in the broad sense because the *Daphnia* in our experiment were reproducing asexually.

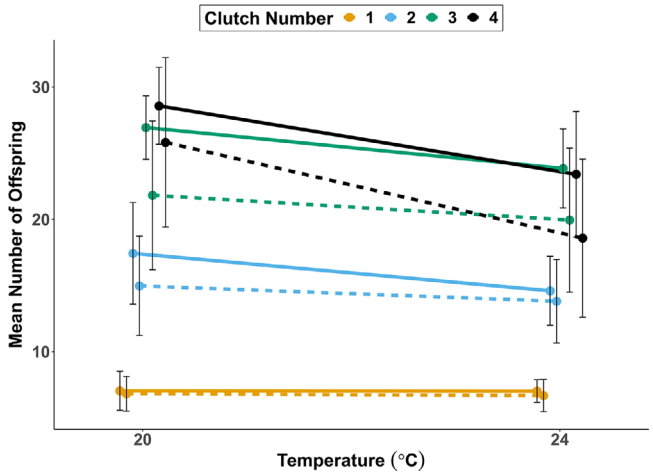

Figure 3. Number of offspring produced in each clutch at 20°C and 24°C. Solid lines show means of control lines, and dashed lines show MA lines. Error bars illustrate ±1 SD. Data are pooled across blocks.

CLUTCH SIZE

Neither temperature nor mutation, nor their interaction, affected mean size of the first clutch (MA lines: 20°C: 6.82 ± 0.25 SD; 24°C: 6.69 ± 0.014 SD; control lines: 20°C: 7.05 ± 0.04 SD; 24°C: 7.03 ± 0.06 SD) (Fig. 3; Table 2). There were no block effects or interactions with block (Supporting Information Materials).

We observed significant decreases in mean clutch size due to MA and elevated temperature at later clutches, with moderate

Table 3. Means and variances of absolute and relative fitness assayed at 20°C and 24°C, based on 11 control lines and 21 mutation accumulation lines.

	20°C		24°C	
	Absolute fitness (r)	Relative fitness	Absolute fitness (r)	Relative fitness
Control lines				
Mean	0.3259	1	0.4197	1
Variance	9.3×10^{-5}	0.000876	0.000230	0.00131
CV	0.0282	0.0282	0.0345	0.0345
MA Lines				
Mean	0.3055	0.9376	0.3973	0.9468
Variance	0.00123	0.0116	0.00234	0.0133
CV	0.1122	0.1122	0.1189	0.1189
Per-generation change				
Mean (ΔM)	−0.00054	−0.00169	−0.00062	−0.00144
Variance (ΔV)	3.07×10^{-5}	0.00029	5.70×10^{-5}	0.00032
CV (ΔCV)	0.0023	0.0023	0.0023	0.0023
Composite Parameters				
$V_m = V_{MA} - V_C$	0.001137	0.0107	0.00211	0.0120
$CV_m = CV_{MA} - CV_C$	0.0839	0.0839	0.0844	0.0844
Mutational heritability V_m/V_{MA}	0.9244	0.9224	0.9017	0.9023
Mutational heritability per-generation	0.0250	0.0249	0.0244	0.0243

differences in clutch 2, and greater differences in clutches 3 and 4 (Fig. 3). Mean size of clutch 2 was 14% smaller in MA lines (14.98 ± 1.55 SD) than in controls (17.44 ± 0.56 SD) at 20°C and 5% smaller (MA: 13.82 ± 0.86 SD; control: 14.61 ± 2.24 SD) at 24°C, although the interaction was not significant (Table 2). There was a block effect with clutches in block 1 ∼5% smaller than block 2 (Table 2) but no interaction between block and other factors (Supporting Information Materials).

In the third clutch, females from MA lines had clutch sizes that were 19% and 16% lower than control lines at 20°C (MA: 21.82 ± 0.11 SD; control: 26.94 ± 0.84 SD) and 24°C (MA: 19.95 ± 1.95 SD; control: 23.85 ± 3.58 SD), respectively (Fig. 3; Table 2). Again, there was a small block effect with block 1 ∼5% smaller than block 2 (Table 2). There was no interaction between main factors (Table 2) or block (Supporting Information Materials) for mean clutch size. However, the variance of third clutch size increased at 24°C for MA lines due to an interaction between temperature and mutational history (Fig. 4; mutational history x temperature: $F = 4.2151$; $df = 3, 120$; $p = 0.0071$).

For the fourth clutch, we observed a larger decline for MA lines in mean clutch size at 24°C than at 20°C (Fig. 3), but the interaction between temperature and mutation was not statistically significant. Clutch 4 in MA lines was 10% smaller than in control lines at 20°C (MA: 25.82 ± 1.38 SD; control: 28.58 ± 0.22 SD) and 20% smaller at 24°C (MA: 18.58 ± 3.15 SD; control: 23.41 ± 3.41 SD) (Fig. 3; Table 2). One MA line (Line 23) died out before producing a fourth clutch at 20°C and was treated as a missing value. A few individuals from this line produced a fourth clutch at the warmer temperature. We tested for interactions between block and other factors, and none were significant for mean clutch size (Supporting Information Materials). The variance of the fourth clutch increased at 24°C (Fig. 4), and MA lines experienced a more substantial change than the control lines because of a significant interaction between temperature and mutational history (mutational history x temperature: $F = 3.1675$; $df = 3, 119$; $p = 0.0269$).

Total reproduction summed across the first four clutches was 14% lower in MA lines than in controls at both 20°C (MA: 68.80 ± 1.88 SD; control: 80.01 ± 1.22 SD) and 24°C (MA: 59.03 ± 5.94 SD; control: 68.90 ± 9.17 SD), with substantially lower fecundity at the warmer temperature (Table 2). There were no significant interactions between factors for total number of offspring (Table 2, Supporting Information Materials). Variance of the summed reproduction did not differ due to temperature, but it increased due to mutation ($F = 7.2209$; $df = 1, 122$; $p = 0.0082$). There was no interaction effect on variance between temperature and mutation.

REPRODUCTIVE TIMING

MA lines produced their first clutches slightly later (20°C: by 0.31 days; 24°C: by 0.15 days) on average than individuals from control lines. However, the age of clutch production was not

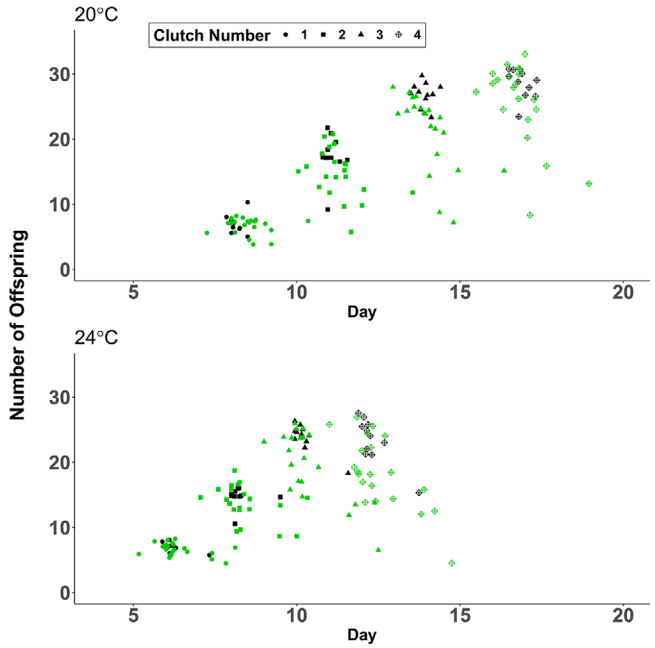

Figure 4. Timing and variation of reproduction in MA (green) and control (black) lines. Each point represents means of a replicate line. Top panel is 20°C, and bottom panel is 24°C. Data are pooled across blocks.

significantly different between MA lines and control lines for the second clutch, third clutch, or fourth clutch (Fig. 4; Table 2).

Reproduction was accelerated by the warmer temperature (Fig. 4). For both MA and control lines, elevated temperature led to significantly earlier production of the first four clutches (Table 2). We also observed significant block effects related to the timing of all four clutches with animals in block 1 reproducing later than those in block 2 (Table 2). There were no significant interactions between factors for the timing of any clutch (Table 2, Supporting Information Materials).

With the exception of the first clutch, Levene's tests showed no significant effect of mutation or mutation-by-temperature interaction on the variance of reproductive timing. For the first clutch, there was a significant interaction ($F = 3.8648$; $df = 3, 120$; $p = 0.0111$) that may have been caused by the small block effect on the timing of clutch 1.

BODY LENGTH AT MATURATION

Individuals from MA lines were significantly smaller than individuals from control lines (Table 1). The mean body length of MA lines was 34 μm less than controls at 20°C and 48 μm less at 24°C. Individuals were smaller at 24°C than at 20°C (Table 1), with a 51 μm difference in MA lines and a 37 μm difference in controls. Although the size difference between MA and control lines increased in the warmer temperature, the interaction was not significant (Table 1). There were no significant differences of variance due to temperature, mutation, or their interaction.

Discussion

Mutation provides the ultimate source of genetic variation, allowing for adaptation and other forms of evolutionary change. The effects of replication errors at the molecular scale may, however, depend on the environment in which they are manifest, and thus it is important to evaluate the phenotypic consequences of mutation in multiple environments. We experimentally accumulated spontaneous mutations in *D. pulex* for ~37 generations and then assayed life history, body size, and the intrinsic rate of increase (r) at standard and elevated temperatures. As expected, within each temperature, mutation was on average deleterious, leading to reduced reproduction (Fig. 3) and consequently lower absolute fitness as indicated by r (Fig. 2). Mutation also produced substantially higher variance of r and fitness components (Figs. 2–4). Similarly, effects of temperature matched general expectations: life history schedules were accelerated at the warmer temperature (Fig. 4), leading to higher estimates of r, even though body size and reproduction were reduced in accordance with the temperature–size rule.

We were particularly interested in the potential for an interaction between accumulated mutations and temperature. Protein structures are thought to function best at an optimal temperature. At a biochemical scale, the stability of proteins can be reduced at higher temperatures such that their functional effectiveness is compromised at temperatures that exceed their thermotolerance (Dandage et al. 2018; Berger et al. 2020). Mutations may limit the window of thermotolerance, causing protein function to be compromised at temperatures that otherwise would have been tolerated. We therefore predicted that MA and temperature would interact, such that the fitness degradation due to spontaneous mutation would increase at a warmer temperature. Our data did not support this prediction. Indeed, the magnitude of trait differences between ancestral lines and mutation lines was approximately the same at both temperatures for clutch size, reproductive timing, body size, and fitness as estimated by r.

Even though we did not see evidence for a widespread, directional influence of temperature on the fitness effects of mutation, we did find evidence suggesting condition-dependent effects in particular MA lines (illustrated as reaction norms in Fig. 5). In five MA lines, the fitness effects of mutation were qualitatively different at the two temperatures. In MA Lines 8 and 13, r was higher than controls at 20°, but lower than controls at 24°. The reverse occurred in MA Line 17. MA Line 25 had similar r to controls at 20°, and higher r at 24°. And MA Line 2 had lower r at 20°, but similar r at 24°. We do not have replicate estimates of r for the MA lines, so these observations should be viewed

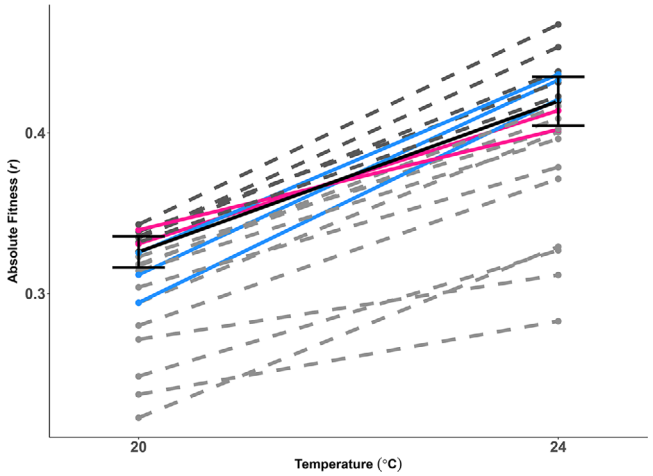

Figure 5. **Fitness reaction norms across the two temperatures. Solid black line shows the mean of the control lines, with error bars indicating ±1 SD. Colored lines indicate MA lines with qualitatively different fitness estimates at the two temperatures in comparison to controls. Blue lines represent MA lines with low estimated *r* at 20° and/or high estimated *r* at 24°; pink lines represent lines with high estimated *r* at 20° and/or low estimated *r* at 24°. Dashed lines represent MA lines with either higher (dark gray) or lower (light gray) estimates of *r* at both temperatures in comparison to controls.**

cautiously. However, all of these involve differences that are more than one, and sometimes more than two, standard deviations of the fitness mean for control lines, indicating that the differences are likely due to biological variation.

MA experiments do not usually uncover evidence for beneficial mutations, and the reasons some do despite the rarity of beneficial mutation are varied (Lynch et al. 1999; Bataillon 2000; Shaw et al. 2002; Eyre-Walker and Keightley 2007; Hall and Joseph 2010). One likely possibility is that any beneficial mutations get overwhelmed by the combined effects of larger numbers of slightly deleterious mutations (or any with large deleterious effects), and thus shorter periods of MA may be more likely to detect beneficial mutation. Another is that fitness effects of mutation depend on the environment in which phenotypes are assayed, so experiments may be more likely to detect beneficial mutation if they include multiple environments. A third possibility is that beneficial mutation can be overestimated if only some components of fitness are assayed; this could fail to account for tradeoffs arising from correlated deleterious effects in unobserved components of fitness. Here, we used estimates of *r*, one of the most complete representations of absolute fitness available, after a moderate duration of MA in two environments. In addition to the qualitatively condition-dependent mutations discussed above, estimated *r* for five MA lines was higher than controls at both temperatures. Of these, three had higher fitness by >2 SD of the mean control fitness. Thus, although our fitness estimates for individual MA lines are not replicated, our data indicate that in up to ∼14% of the lines the net effect of mutation could have been unconditionally beneficial. This suggests substantial scope for mutational improvement of fitness in the medium term.

Mutation does not simply influence the mean trait; mutation is also expected to lead to greater variance among lines, and our MA lines show this effect at both temperatures for body size, reproductive output and timing, and *r*. Temperature is not expected to have any inherent effect on variance. However, for some traits, we observed an interaction between mutation history and temperature with respect to variance. For reproduction in later clutches, total reproduction, and *r*, the increase in variance due to mutation was greater at 24° than at 20°. This suggests that in natural populations, the efficiency of selection on new mutations would be greater at 24° than at 20°.

In addition to effects on life history, we observed significant changes of body size in response to elevated temperature and accumulated mutations. Body size links reproduction in *Daphnia* to the temperature–size rule, and as predicted, our lines reached maturity earlier and at a smaller size in the warm temperature at a cost of smaller clutch sizes. Body size was reduced by mutation; this contrasts with prior MA studies in *Daphnia* that found no appreciable effect of mutation on mean adult body size (Latta et al. 2013; Bull et al. 2019). As with *r* and reproductive characteristics, we did not see an interaction between mutation and temperature.

Our experiments were begun with an ancestor that is "obligately" asexual, meaning that females (but not males) are restricted to asexual reproduction in both subitaneous and dormant eggs. One might expect that implies our clone started with a higher genetic load of deleterious mutations than a "typical" *Daphnia* clone, and consequently may be unusually sensitive to additional spontaneous mutation triggering temperature-dependent performance degradation. If that were the case, using a loaded clone would have biased the experiment against obtaining the results we found. However, we believe that our experiment is actually representative of a "typical" *Daphnia* for several reasons. First, the geographic origin of our clone suggests that it only recently acquired obligate asexuality (Paland et al. 2005). Second, our estimate of absolute fitness of the ancestor, as measured by *r*, is typical of *D. pulex* raised under the conditions we used. Estimates of *r* for *Daphnia pulex* under high food and temperatures near 20°C range from ∼0.1 to ∼0.45 (e.g., Scheiner and Berrigan 1998; Dudycha and Tessier 1999; Ferrão-Filho et al. 2000; Riessen 2012; Liu and Steiner 2017). Our ancestor appears to be on the high side of that. Third, because published data on *r* in *D. pulex* rarely are accompanied by definitive data on whether clones are sexual or obligately asexual, we reanalyzed life history data used in Dudycha and Hassel (2013), which reported full life histories for two obligately asexual and three confirmed sexual

clones (Heier and Dudycha 2009) of *D. pulex* under conditions similar to the present article. Data therein yield r estimates of 0.167 and 0.182 for the obligately asexual clones LIN and OL3, respectively, and 0.221, 0.468, and 0.236 for the sexual clones WEST, RW, and BW16. Those data show that the ancestral fitness of the MA experiment reported here (mean $r = 0.326$ at 20°) was higher than at least some sexual *D. pulex* clones. Therefore, our clone of *D. pulex* has an intrinsic rate of increase well within those that are typically expected and does not appear to have carried an unusually high genetic load into the experiment.

We are aware of three other reports comparing fitness assays of MA lines at two temperatures. Working with 10 MA lines of *D. pulex* after ∼93 generations of mutation, Latta et al. (2015) measured a variety of life history and morphological traits at 20° and ∼22–23°. Their experiment did not have controls, so they could not test for changes in mean traits. However, averaged across traits, they reported that mutational heritability was greater at the elevated temperature. Baer et al. (2006) compared ancestral controls and MA lines of four strains (two species) of rhabditid worms after >200 generations of mutation in 100 MA lines per strain. Comparisons between assays at 20° and 25° of the per-generation decline of fitness produced inconsistent results. For two strains, one each of *Caenorhabditis briggsae* and *C. elegans*, they found a greater fitness decrement at 20°, while the reverse was true for the other two strains. However, Baer et al. (2006) did not run the assays at the two temperatures contemporaneously. Assays at 20° were performed after 100 generations and again after 200 generations; assays at 25° were performed after 220 generations. This precluded them from directly testing for an interaction between temperature and mutation. Although they did not directly address the question, neither of these studies support the contention that elevated temperature will systematically exacerbate the deleteriousness of spontaneous mutation.

In a related study, Matsuba et al. (2013) also assayed fitness of *C. briggsae* and *C. elegans* MA lines at two temperatures, but this time with a greater temperature range of 18° and 26°, describing 26° as a stressful temperature for *C. elegans* (only), because it reduces fitness to one-third of the fitness at 18° in unmutated controls. Though not the focus of their study, their estimates of the per-generation reduction of fitness in *C. elegans* at 26° were roughly twice what they estimated at 18°. However, they provided no formal test comparing assay temperatures, and confidence intervals of parameter estimates overlapped substantially. In *C. briggsae*, differences were much smaller, though in the same direction. Thus, the data from this report are at least trending in the direction predicted by the biophysical properties at the molecular scale (Dandage et al. 2018; Agozzino and Dill 2018; Berger et al. 2020). Given that this study involved a temperature comparison well beyond most-probable estimates for climate change and found the strongest hint of an effect in a species brought to the thermal brink of fitness collapse, it provides guidance as to how extreme temperature would need to be to trigger a broad interaction with spontaneous mutations.

Although our data also did not support the interaction between mutation and temperature predicted by considering the thermostability of proteins at a molecular scale, it may still be premature to dismiss that hypothesis entirely. Either a warmer temperature or a longer period of MA may have revealed such an interaction, as the trend in Matsuba et al. (2013) suggests. Earlier work has shown that genetically diverse experimental populations of *D. pulex* suffer substantial performance degradation at 26° (Dudycha 2003), which would make it difficult to ascertain the compounded effects of mutation. Furthermore, our interest in relating results to projected climate change argued against evaluating a temperature differential substantially greater than +4°. After an average of 37 generations of mutation, our MA lines had ∼5% lower r than controls. This is a substantial selection differential in the context of evolutionary change, particularly considering that this differential is driven by mutations accumulating primarily in the heterozygous state (but see Omilian et al. 2006; Keith et al. 2016; Ho et al. 2020). Further accumulation of mutations may indeed result in some degree of general temperature-dependent exacerbation of the r reduction, though the effect would necessarily be small compared to the general effect of mutation. It is unlikely that such a small-magnitude effect would be important in populations experiencing climate change at the tempo and scales that are currently projected.

Climate change raises important questions about the capacity for populations to adapt in response to changes in their abiotic environment. Evolutionary responses to climate change depend on many attributes of populations, including the shape of environmental tolerance curves, variation among locally adapted populations, dispersal patterns, and standing genetic variation. Mutation may contribute additional variation on which selection may act. Our data indicate that mutation is unlikely to interact with rising temperatures in a manner that leads to broad thermal instability in proteins. Instead, our data suggest that mutational variance may increase with rising temperatures, increasing the efficiency of selection and consequently raising the rate of adaptation. Coupled with our observations that mutations beneficial in warm temperatures occur at least occasionally, spontaneous mutation may contribute to populations' adaptation to climate change. Our experiment was based on a total of 777 reproductive events that could produce heritable mutation (21 lines x 37 generations). It is easy to imagine that in a natural population of *D. pulex*, where populations size might be 10,000 to 1,000,000 with several asexual generations per year, there is ample opportunity for such mutations to occur.

AUTHOR CONTRIBUTIONS

ESD and JLD conceived the project. TCA managed the experimental MA. ESD, TCA, KBH, and JLD conducted research and collected data. ESD and TCA analyzed data. ESD and JLD wrote the manuscript.

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DATA ARCHIVING

Phenotypic data and relevant metadata are available in Dryad at https://doi.org/10.5061/dryad.np5hqbzsg.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1: Survivorship remained high (>93%) for both MA lines (dotted lines) and control lines (solid lines) through the production of four clutches. D. pulex in the warmer environment (shown in red) produced their first four clutches by day 12 and had slightly lower survivorship than D. pulex in the cooler environment (shown in blue).

Table S1: We calculated block effects for clutch size using averages for blocks 1 and 2. (Block effect = Block 1 Average – Block 2 Average). Significant block effects are bolded.

Table S2: We calculated block effects for clutch timing using averages for blocks 1 and 2. (Block effect = Block 1 Average – Block 2 Average). Significant block effects were observed for all clutches, and block 1 produced offspring later than block 2 in every case.