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Trait variation in extreme thermal environments under constant and fluctuating temperatures

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Climate change is increasingly exposing populations to rare and novel environmental conditions. Theory suggests that extreme conditions will expose cryptic phenotypes, with a concomitant increase in trait variation. Although some empirical support for this exists, it is also well established that physiological mechanisms (e.g. heat shock protein expression) change when organisms are exposed to constant versus fluctuating temperatures. To determine the effect of common, rare and novel temperatures on the release of hidden variation, we exposed fathead minnows, *Pimephales promelas*, to five fluctuating and four constant temperature regimes (constant treatments: 23.5, 25, 28.5 and 31°C; all fluctuating treatments shared a minimum temperature of 22°C at 00.00 and a maximum of 25, 28, 31, 34 or 37°C at 12.00). We measured each individual's length weekly over 60 days, critical thermal maximum (CT_{max}), five morphometric traits (eye anterior–posterior distance, pelvic fin length, pectoral fin length, pelvic fin ray count and pectoral fin ray count) and fluctuating asymmetry (FA, absolute difference between left and right morphometric measurements; FA is typically associated with stress). Length-at-age in both constant and fluctuating conditions decreased with temperature, and this trait's variance decreased with temperature under fluctuating conditions but increased and then decreased in constant temperatures. CT_{max} in both treatments increased with increasing water temperature, while its variance decreased in warmer waters. No consistent pattern in mean or variance was found across morphometric traits or FA. Our results suggest that, for fathead minnows, variance can decrease in important traits (e.g. length-at-age and CT_{max}) as the environment becomes more stressful, so it may be difficult to establish comprehensive rules for the effects of rarer or stressful environments on trait variation.

This article is part of the theme issue 'The role of plasticity in phenotypic adaptation to rapid environmental change'.

1. Introduction

Earth is experiencing vast changes in both spatial and temporal patterns of climate variables such as temperature, wind and precipitation. The mean combined land and ocean surface temperature has increased 0.85°C between 1880 and 2012 [1]. In addition, precipitation [2], sea level [3], ice cover [4] and frequency of severe weather [5] are all changing at an unprecedented rate.

These changes are, in turn, having widespread effects in ecosystems, mediated by the effect of climatic variables on the physiology of organisms [6]. Geographical range [7] and migration pattern shifts [8], increased variability in population abundance through time [9] and modification of species interactions [10] are only some of the observed consequences. For these reasons, the ability to cope with future environmental changes will be crucial to the survival of many species [11,12]. In particular, how species respond to changes

in temperature, one of the most consequential abiotic variables (especially for ectotherms), will, in large part, determine their fate [13].

Although climate change is often discussed in terms of averages (e.g. increase in projected mean temperature), the increasing prevalence of extreme temperatures is also quite apparent. Increases in variance have already been observed and are predicted to become further exacerbated [1]. In fact, a complete departure from current temperature regimes is projected to have happened by 2047 (assuming a stay-the-course scenario; [14]). Periods of extreme temperature can have a disproportionate impact on the mortality and morbidity of plants and animals, yet are not necessarily well accounted for with means [15–17]. Thus, many populations will be increasingly challenged by rare and novel environmental conditions [18–20].

Some theory suggests that natural selection acts to canalize phenotypes in commonly faced environments [21,22], but rare and novel conditions could expose hidden phenotypes, with a concomitant increase in trait variation [22–24]. Variation is, of course, a critical component of the adaptive capacity of a population, as the rate of evolution is proportional to the additive genetic variance [25]. Increased trait variation can lead to significant ecological [26–28] and evolutionary changes [29–33], and the release of this variation in extreme environments has been suggested to be an important evolutionary process [34–36]. In yellow dung flies, for instance, exposure to heat stress led to an increase in variance in the number of sperm storage organs in females [37]. The expression of cryptic variation has also been shown to increase in experimental brown trout populations exposed to novel pH levels [38], and previously unencountered diet conditions are likely to have released variation and driven the evolutionary transition from omnivory to carnivory in spadefoot toad tadpoles [30]. More examples are summarized in [35,39].

However, it is unclear whether the theoretical prediction of increased variance in novel environments occurs consistently (i.e. whether reaction norms really 'fan out' as predicted; [40–42]). For instance, if optimal conditions are required to produce a fully expressed phenotype, then stressful environments will only induce a subset of potential phenotypes, thereby decreasing variation [40]. Studies on *Drosophila* revealed increases in the variance of some traits and decreases in others when exposed to stressful environments [43]. In a survey of 247 studies, Hollander & Bourdeau [44] found that organisms exposed to native predators (thus experiencing a 'common' environment) showed significantly more plasticity than those exposed to introduced (novel) ones. Yet another recent meta-analysis arrived at a similar conclusion, finding mixed evidence for the release of variation in rare or novel environments (increased coefficients of variation for life-history traits but not for morphological traits under highly stressful environments; [45]).

Additionally, almost all work on phenotypic variation in novel conditions is conducted under constant environments (but see [46]). Although simpler from an experimental standpoint, important differences exist when individuals are subjected to fluctuating conditions (reviewed in [47,48]). Growth, development and thermal tolerance in many ectotherms can differ significantly when exposed to daily thermal fluctuations versus a constant environment with the same mean temperature (e.g. [49,50]). For example, daily fluctuating temperatures can delay embryonic development in a

longhorned beetle [51] and can increase the upper thermal tolerance limit in zebrafish [52] relative to that of constant temperatures. Not surprisingly, failing to incorporate thermal variability can seriously bias predictions of species' responses to climate change [47,53].

Here, we use the fathead minnow, *Pimephales promelas*, to explore the interaction of these two phenomena in thermal biology: the effects of novel temperatures and of daily thermal variation on life-history and morphological traits. We reared these fish under constant (23.5, 25, 28.5 and 31°C) and fluctuating (22–25, 22–28, 22–31, 22–34 and 22–37°C) temperature environments that ranged from common to exceptionally hot. We then quantified variation in length-at-age, critical thermal maximum (CT_{max}), meristic and morphometric characters, and fluctuating asymmetry (FA). We hypothesized that fluctuating temperatures will result in improved performance at more stressful temperatures (when compared with fish in constant conditions), and that fluctuating thermal regimes will result in higher levels of phenotypic variation, as the underlying physiology is likely to be different.

2. Material and methods

(a) Model system

The fathead minnow is a cyprinid endemic to large parts of North America. Its range stretches from the Northwest Territories of Canada to northern Mexico, as well as from New York to Nevada and into parts of California in the continental USA [54]. Fathead minnows can tolerate a wide range of temperatures; when acclimated to 22°C, they exhibit a critical thermal maximum of 36.4 ± 0.75 (mean ± s.d.) [55]. Fathead minnows are used for a variety of purposes, including mosquito population control [56], bait [54,56] and toxicology studies [57].

(b) Temperature set-up

One-day-old *P. promelas* were shipped to our laboratory on 1 July 2017 from the US Environmental Protection Agency's Mid-Continent Ecology Division Laboratory. We allowed them to acclimate for 24 h at 25°C (temperature at which they were spawned) and then moved them to one of the temperature treatments. For the first 11 days, larvae were kept in groups of approximately 10 fish. Thereafter, fish were reared in individual chambers inside 225 l tanks. Individual growth chambers consisted of plastic Petri dish bottoms surrounded by cylinders of 762 μm mesh.

In all treatments, temperatures were controlled by tank-specific APEX Jr controllers (Neptune Systems, Morgan Hill, CA, USA) on an hourly basis and actual temperature was within ±0.2°C of the nominal treatment temperature. In all fluctuating treatments, the lowest temperature was 22°C at 00.00. Daily temperatures increased linearly to one of five maximum temperatures: 25 (mean daily temperature: 23.5°C), 28 (mean 25.2), 31 (mean 26.5), 34 (mean 28.4) or 37°C (mean 29.5) (figure 1). Twenty fish were grown at each of the fluctuating treatments. Controllers were checked daily for consistency/accuracy. For comparison, there were four constant temperature treatments: 23.5, 25, 28.5 and 31°C (±0.2°C), with 14 individuals per treatment (figure 1). Fish were kept under these conditions for the duration of the experiment (60 days). We replicated each temperature treatment in two 225 l tanks. For reference, water temperature at the Lake Superior National Estuarine Research Reserve (Superior, WI, USA) can reach 27–28°C during summer days, and water can remain greater than 27°C for 10 h these days (summer mean temperature: 20.6°C; electronic supplementary material, figure S1). Our temperature treatments were selected based on this water temperature data.
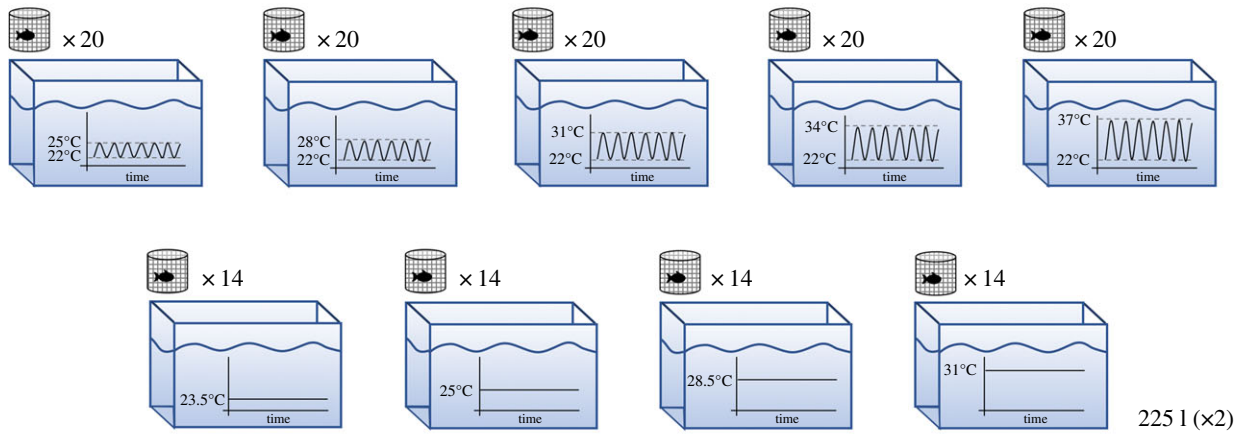
Figure 1. Schematic of the temperature treatments. (Online version in colour.)

(c) Husbandry

Water was filtered and checked weekly to ensure appropriate quality. We fed fish 1-day-old *Artemia* nauplii (San Francisco Bay Brand, Newark, CA, USA) for the first 11 days and then switched to TetraMin flake food (Tetra Spectrum Brands, Blacksburg, VA, USA). All fish were fed ad libitum three times daily, and the light cycle was maintained at a 16 L : 8 D cycle for the duration of the experiment. This rearing protocol is similar to the standard for these fish [58].

(d) Measurements

(i) Length-at-age

Starting with 13-day-old larvae, we measured length every week for a total of eight measurements per fish. We photographed each fish from above at a standard height of 65 cm, while the fish remained in 1 cm of water (within its chamber, to minimize stress). A Nikon D7200 camera with an AF-S Micro Nikkor 105 mm macro lens was used. Photos were measured using IMAGEJ 1.50i (NIH, Bethesda, MD, USA).

(ii) Critical thermal maximum

CT_{max}, defined as the temperature at which locomotion becomes disorganized [59], was measured at age 54–56 days. Fish were transferred from their experimental table to the test chamber and allowed to acclimate for 10 min. Water temperature was raised 1°C every 2 min until visual inspection indicated that the fish were unable to maintain equilibrium (uncontrolled swimming) for approximately 2 s. The CT_{max} assay was performed on all fish at their peak daily temperature.

(iii) Meristic/morphometric traits and fluctuating asymmetry

We measured five traits on the left and right side of individuals: eye anterior–posterior distance, pelvic fin length, pectoral fin length, pelvic fin ray count and pectoral fin ray count. Fish were sacrificed and then photographed; measurements were obtained electronically on IMAGEJ. These measurements were also used to calculate FA (i.e. the absolute difference between left and right measurements; a common approach to evaluate stress in organisms; [60]). The characters measured are all commonly used in studies of asymmetry [60].

(e) Statistical analysis

To assess trends with temperature in the mean and variance of different traits, we fitted a series of general linear and additive models. The goals of these analyses are to elucidate the main patterns, rather than test specific hypotheses. Specifically, we wished to know whether there was a trend with temperature and whether or not the trend was linear. Thus, to allow full flexibility for the trends, we considered linear models, piecewise linear models with two segments and penalized B-spline models. Model selection was done using Akaike's information criterion (AIC). However, the B-spline model was never the 'best' model for any trait and we did not consider it further. Thus, our model comparison was restricted to continuous models with one or two linear segments. These two-segment models are occasionally referred to in the literature as 'breakpoint regressions', and the value of the independent variable at which the slope changes is referred to as the 'breakpoint'.

For the mean, we fitted the raw data for each individual using a Gaussian likelihood. To determine a trend with temperature in the variance for each trait, we used squared deviations from the mean for each temperature, i.e. $z = [y - m(x)]^2$, as the input data [61]. To see why this works, note that if the raw data are normally distributed conditional on the independent variable, i.e. $y|x \sim N(m(x), v(x))$, then the likelihood for z is Gamma(3/2, 1/[2v(x)]) for which $E(z|x) = v(x)$. Since regression minimizes the distance between the model estimate and $E(z|x)$, it should provide an adequate description of the trend in variance.

In addition, because the collection of traits measured on a given individual are unlikely to be independent, we also conducted MANOVA analyses testing for differences between constant and fluctuating treatments, using mean temperature in each treatment as a covariate. This analysis was performed on (i) all of the traits combined, and (ii) traits grouped according to their correlation structure. Specifically, group A consisted of all of the length-at-age traits, group B included all of the morphometric and meristic traits and group C the asymmetry traits. Analysing the independent blocks separately allows somewhat greater interpretability without loss of information. We adopted a maximum-likelihood approach to performing the MANOVA using likelihood ratio tests to evaluate the effect of fluctuations, temperature and their interaction. All analyses were carried out in MATLAB 2017a.

3. Results

Mean length-at-age for both constant and fluctuating conditions tended to decrease with temperature in all ages measured (figure 2). Sixty-day-old fathead minnows were 10.6 mm shorter in fluctuating 22–37°C than in fluctuating 22–25°C water, and 7.1 mm shorter at constant 31°C than at constant 23.5°C. Under fluctuating conditions, the
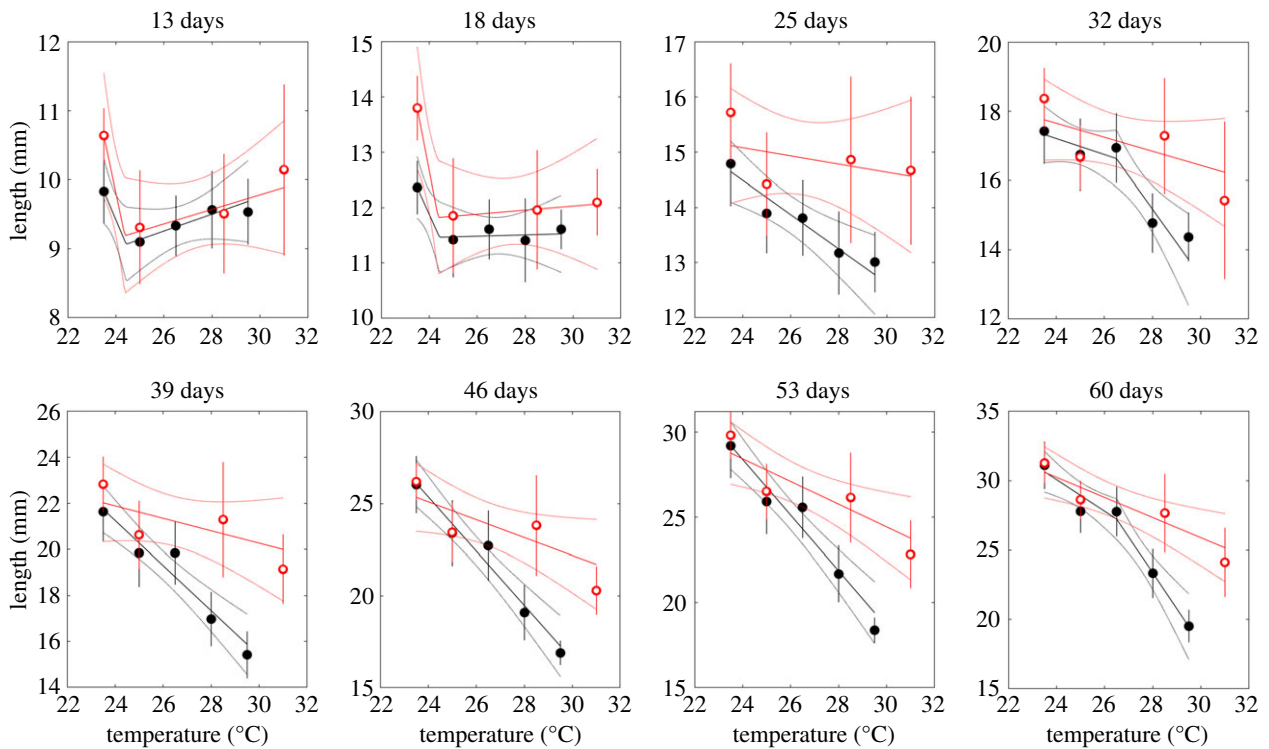
Figure 2. Mean length-at-age versus temperature under constant (open red circles) and fluctuating (closed black circles) temperature treatments. Measurements were taken when fish were 13, 18, 25, 32, 39, 46, 53 and 60 days old. Depending on model selection results, regressions are shown with either one or two segments. Means and regression lines shown with 2 standard error confidence bounds.

relationship between length and temperature was linear over the temperatures measured at early ages, yet the oldest ages were better modelled by a two-segment regression (electronic supplementary material, table S1). The break in the regression occurred between the fluctuating 22–31°C (mean 26.5°C) and fluctuating 22–34°C (mean 28°C) treatments. It is important to note that the exact break point in the regression is approximate, and indicates only that the response changes direction somewhere in the vicinity of the temperature indicated by the break.

Variance in length, on the other hand, exhibited a more complicated pattern (figure 3). Interestingly, it decreased consistently with temperature under fluctuating conditions, especially at older ages (figure 3). Under constant thermal environments, over the last three ages measured (46, 53 and 60 days old), variance increases as water warms up to a point (between 25 and 28.5°C) and then decreases at very high temperatures (as evidenced by AIC preferring the linear model with two segments; electronic supplementary material, table S2).

As temperature increased, so did mean CT_{max} for fish in both constant and fluctuating conditions (figure 4). Differences in CT_{max} between fish at the lowest and highest temperature treatments were 4.6°C (fluctuating) and 4.5°C (constant). Two-segment models outperformed their one-segment counterparts (electronic supplementary material, table S1), though the break was at a higher temperature in the constant environments. Variance in CT_{max} decreased as a function of temperature in both thermal treatments (figure 4).

FA in pelvic and pectoral fin length was generally smaller under constant environments, but this was not the case for eye diameter or pelvic and pectoral fin ray counts (figure 5). Variance in pelvic fin length and pelvic fin ray count was high at cooler and warmer temperatures in the constant treatment; under fluctuating conditions, there was no trend with temperature (figure 6).

Integrating all traits into a single MANOVA indicated a significant effect of temperature, temperature treatment (constant versus fluctuating) and their interaction (electronic supplementary material, table S3).

Survival in both treatments was depressed at higher temperatures: fluctuating 22–25°C = 0.95, fluctuating 22–28°C = 0.95, fluctuating 22–31°C = 0.95, fluctuating 22–34°C = 0.80, fluctuating 22–37°C = 0.55, constant 23.5°C = 0.57, constant 25°C = 1.00, constant 28.5°C = 1.00 and constant 31°C = 0.36.

4. Discussion

The importance of hidden phenotypic variation is underscored by recent calls for a mechanistic (i.e. physiological) understanding of climate change impacts [13,62]. We must understand how populations react to rare/novel environments if we are to accurately predict their fates over short and long time scales [63]. Consistent with earlier results on the temperature-size rule [64], we found that mean length-at-age in older fish decreases as temperatures increase. This was true in constant and fluctuating conditions, suggesting that our treatments were in the decreasing part of their thermal performance curve. In addition, the decrease with temperature was more extreme when temperatures fluctuated than when they were constant.

Although we were unable to fit nonlinear reaction norms to our data, this result is consistent with the idea that the reaction norm for the highest temperatures is convex (i.e. the reaction norm must accelerate downward in those temperatures that the fish in the fluctuating treatments experienced for which we have no constant temperature data). Specifically, because the growth trajectories are nearly linear, we can imagine that growth in length is approximately given by $dL/dt = g(T) + \sigma(T)\varepsilon$, where $g(T)$ is the mean growth rate
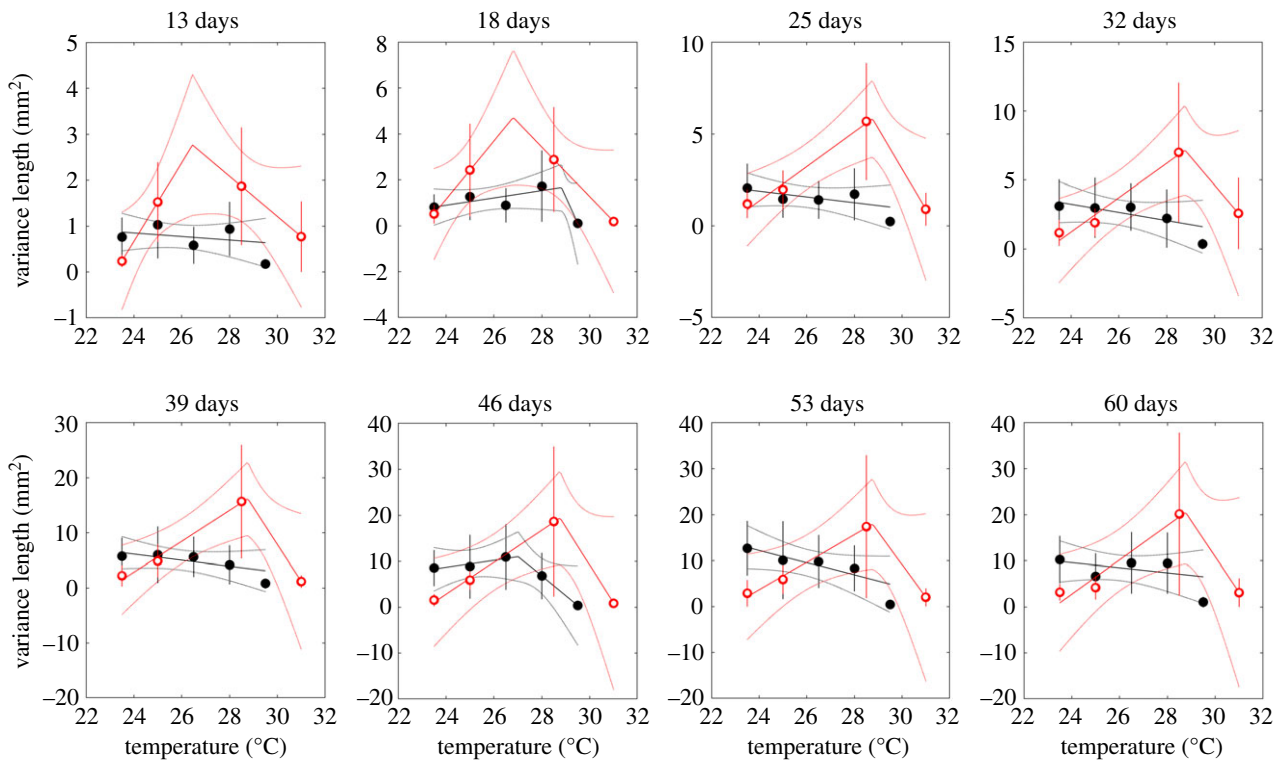
Figure 3. Variance in length-at-age versus temperature under constant (open red circles) and fluctuating (closed black circles, graphed on the *x*-axis at the corresponding mean temperature) temperature treatments. Depending on model selection results, regressions are shown with either one or two segments. Means and regression lines shown with 2 standard error confidence bounds.

and $\sigma(T)\varepsilon$ is white noise with zero mean and temperature-dependent variance and $\sigma^2(T)$. The mean size at age t is given by the integral $E[L(t)] = \int_0^t g(T)$. To see the effect of convexity in g, we can approximate this integral around the mean temperature as $\int_0^t g(T) \sim \int_0^t g(\bar{T}) + g'(\bar{T})(T-\bar{T}) + 1/2g''(\bar{T})(T-\bar{T})^2$ and hence $\int_0^t g(T) \leq tg(\bar{T})$ provided that $g''(\bar{T}) < 0$, which is Jensen's inequality [65,66]. Note also that this simple model predicts that the effect of varying temperature on the mean size of fish should increase with age as it does in our data.

Variance in length-at-age (in older fish) showed an analogous pattern with temperature in the two treatments: fish in waters whose temperatures fluctuated daily were less variable at the more extreme thermal conditions, while fish exposed to constant temperatures exhibited higher variability at intermediate temperatures (25 and 28.5°C). Under the simple model above, the variance in length is given by $V[L(t)] = \int_0^t \sigma^2(T)$, so in the constant temperature treatments, $V[L(t)] = \sigma^2(\bar{T})t$. Based on figure 3, this suggests that there is a unimodal relationship between the noise variance and temperature. Again, we expect the variation across temperature treatments to be more extreme in the older fish. In addition, the variance in the fluctuating temperature treatment will be less than in the constant temperature treatment when the noise magnitude is a convex function of temperature, i.e. $\sigma^{2''}(\bar{T}) < 0$, which is—at least broadly—consistent with what was observed in the constant temperature treatments. This is analogous to Jensen's inequality for variance (see also [67]).

Mean critical thermal maxima (upper thermal tolerance limits) for fish in both treatments increased with increasing water temperature, as is repeatedly observed [68]. CT_{max} was higher in fish reared under fluctuating conditions (comparison of black and red data points in figure 4 under similar mean temperatures). Hormesis (i.e. mild exposure to a
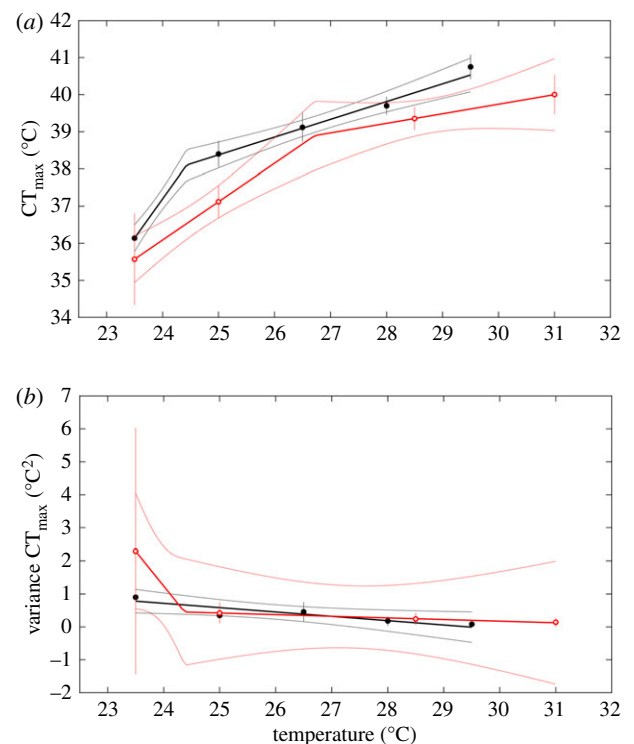

Figure 4. Mean (*a*) and variance (*b*) critical thermal maximum (CTmax) versus temperature under constant (open red circles) and fluctuating (closed black circles) temperature treatments. Depending on model selection results, regressions are shown with either one or two segments. Means and regression lines shown with 2 standard error confidence bounds.

stressful temperature having positive effects on an organism; [69]) appears to be at work here. Mechanistically, extreme temperatures influence the kinetic induction of heat shock proteins such as Hsp70, Hsp90, Hsp27 and Hsp22, which have differing transcriptional responses when exposed to
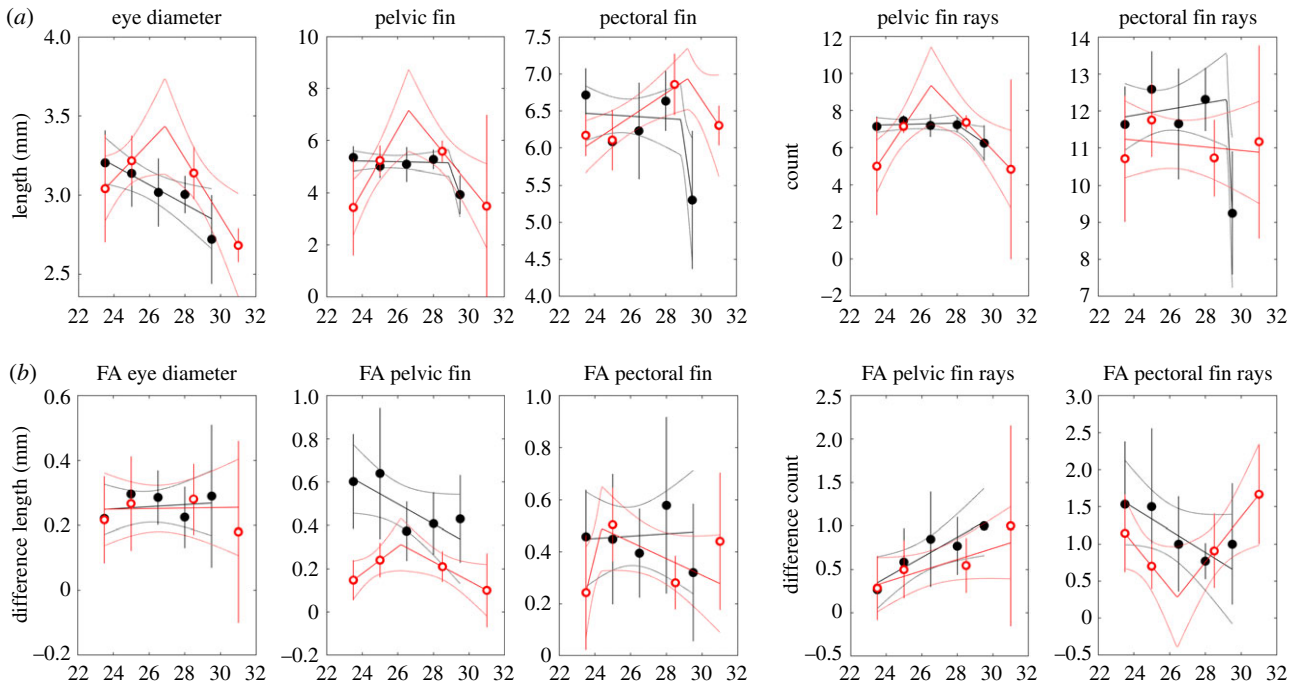
Figure 5. Mean morphometric trait values (*a*) and mean FA values (*b*) versus temperature under constant (open red circles) and fluctuating (closed black circles) temperature treatments. Depending on model selection results, regressions are shown with either one or two segments. Means and regression lines shown with 2 standard error confidence bounds.

Figure 6. Variance in morphometric trait value (*a*) and variance in FA values (*b*) versus temperature under constant (open red circles) and fluctuating (closed black circles) temperature treatments. Depending on model selection results, regressions are shown with either one or two segments. Means and regression lines shown with 2 standard error confidence bounds.
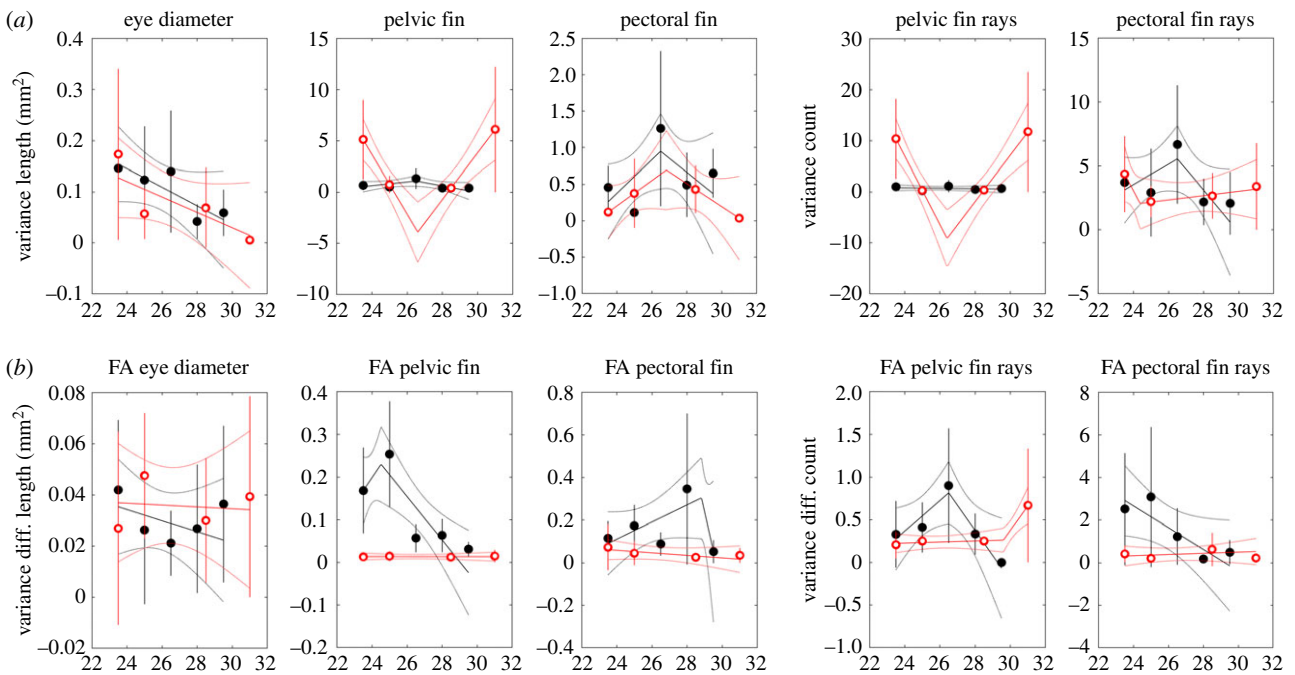
fluctuating versus constant temperature regimes [70]. Fathead minnows are also able to ramp up heat shock protein production in the spring as water temperatures increase [71]. Owing to the different magnitude and timing of induction, these proteins could play a role in mediating thermal tolerance, although the frequency of the exposure (i.e. daily versus seasonal versus episodic events like heat waves) could also be important [70,72]. It is possible, then, that exposing fathead minnows to higher temperatures daily could trigger upregulation of these chaperone genes. Interestingly, in the most extreme fluctuating treatments, the daily excursions to high temperatures provided a beneficial mild exposure for CT_{max} but a detrimental one when it came to growth, as was reported for zebrafish [52] and three Australian frogs [73]. This suggests that a trade-off may exist between growth and thermal tolerance [74], an important consideration when making predictions related to climate change. Variance in CT_{max}—like in length-at-age—decreased in warmer waters.

No consistent pattern in mean or variance was found across morphometric traits or FA, although variance in FA tended to be lower in constant temperatures than in fluctuating temperatures for pelvic and pectoral fin lengths.

Our results add to a growing body of evidence, suggesting that variance can decrease as the environment becomes more stressful [41,43] (but see [75,76]). This could be a result of strong genetic correlations between benign and rarer/more stressful environments, so that trait values at the extreme are determined in large part by the shape of the reaction norm in common environments [77,78]. We note, however, that sometimes different genes are upregulated based on the severity of the thermal stress [79], and it is unclear how the expression of these genes is correlated across environments. Furthermore, an organism's response to constant stress, as opposed to stress under fluctuating conditions, can be quite different. Fluctuating temperatures have been shown to cause differential gene expression [70] and to possibly call on different responses: constant temperature regimes appear to involve modification of a large part of the transcriptome while (non-stressful) fluctuations induce smaller changes in gene expression, but ones that are different and independent [80].

If stress responses are somewhat similar regardless of stressor [81], then we would expect less variation when exposing organisms to any number of stressful environments. Hoover *et al.* [82] reared fathead minnows under four salinity treatments and found more variation in reproductive traits (e.g. egg number, egg fertilization rate and time spent caring for nests), not less as we found for length-at-age and CT_{max}. Different stressors have been shown to have disparate effects on a given trait of wild mustard [83], and even a single stressor can have different effects on variance in multiple traits (e.g. low nutrient conditions reduced phenotypic variance in leaf length and number, among others, but increased it in seedling height [83]). Given the existence of several other examples (e.g. [43,83–85], establishing comprehensive rules for the effects of rarer environments on trait variation will be a challenge.

Most work to date has been focused on identifying the mean thermal limit of a population (see [86] for a historical perspective), and evolutionary forecasts have been attempted based on these population-average values [9,87–89]. Yet, many aspects of a population's thermal biology can be traced not to mean temperature measures, but to fluctuations, extremes and episodic events (e.g. [90–92]). Despite the difficulties, it is clear that we need to incorporate these into predictions of species responses to climate change.

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