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Functional Ecology

Measuring phenotypes in fluctuating environments

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keywords: thermal tolerance, heat tolerance, reversible plasticity, unpredictable environments, bet-hedging, insurance

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Author contributions

All authors participated in the design and execution of the study. TB and SE conceived the idea for the study, analysed the data and drafted the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability

Data deposited in the Dryad digital repository: <http://doi.org/10.5061/dryad.3xsj3txbg>, (Burton et al 2019)

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Abstract

1. Despite considerable theoretical interest in how the evolution of phenotypic plasticity should be shaped by environmental variability and stochasticity, how individuals actually respond to these aspects of the environment within their own lifetimes remains unclear.
2. We propose that this understanding has been hampered by experimental approaches that expose organisms to fluctuating environments (typically treatments where fluctuations in the environment are cyclical *versus* erratic) for a pre-determined duration, while ensuring that the mean environment over that the entire exposure period is invariable. This approach implicitly assumes that responses to the mean and variance/predictability in the environment occur over the same time scale. If this assumption is false, one potential outcome is that phenotypic differences among the treatment groups might arise in response to differences in the mean environment that are present over shorter time periods among those same treatment groups.
3. We illustrate an experimental design that (i) creates variation in the level of environmental predictability, (ii) allows for estimation of the time scale over which

the phenotypic response to the mean environment occurs, and (iii) permits statistical estimation of the effect of predictability in the environmental variable of interest while controlling for any effect of the mean environment over the relevant temporal scale.

4. Using the clonally reproducing zooplankton species *Daphnia magna*, we test for within-generation plasticity in the ability to tolerate high temperature following exposure to multiple temperature treatments with the same overall mean, but where the pattern of fluctuations differed among them. This approach revealed that heat tolerance in *Daphnia* was not influenced by variability in temperature *per se* nor the predictability of fluctuations in temperature but adjusted in response to the mean temperature they experienced 24 hours prior to measurement.
5. Our results suggest that conclusions arising from studies that employ a single manipulation of environmental predictability and which cannot consider such potentially confounding effects may be premature.

Introduction

Natural populations experience environmental variation over a broad range of temporal and spatial scales, and as a result the optimal phenotype at any given time and place can vary accordingly (Auld, Agrawal & Relyea 2010). To track these shifting optima, individuals must respond to environmental cues and adjust their phenotype through phenotypic plasticity (West-Eberhard 2003). Consequently, phenotypes can differ substantially across different mean environmental conditions, even in the absence of genetic differentiation (Shine 1999; Fischer, Brakefield & Zwaan 2003; Yampolsky, Schaer & Ebert 2014). It is also becoming increasingly apparent that organisms should, and do, respond to short-term environmental variability that they experience throughout their lives. A growing number of studies reveal that predictably variable environments can induce phenotypic change relative to a stable environment of the same mean value. For example, predictable fluctuations in temperature around a given mean have been shown to influence the growth, development and physiology of individuals (e.g. Niehaus, Wilson & Franklin 2006; Kern, Cramp & Franklin 2015; Sørensen *et al.* 2016; Kielland, Bech & Einum 2017; Verheyen & Stoks 2019). Furthermore, theoretical models assert that the optimal phenotype may also be influenced by the level of

environmental predictability (Donaldson-Matasci, Lachmann & Bergstrom 2008; Reed *et al.* 2010; Arnoldini *et al.* 2012). However, far less is known about how individuals assess, and respond through plasticity, to the level of fine temporal grain (i.e. within-generation) environmental predictability. Empirical data is scarce and afforded various interpretations. For example, observed increases in body size and heat tolerance in less predictable environments have been interpreted as risk-management/bet-hedging responses (Drake, Miller & Todgham 2017; Shama 2017). Whereas decreases in the ability to tolerate desiccation and poor food availability in response to decreased environmental predictability have been attributed to the stress that results from attempting to track an environment which provides little information as to how and when it might change (Manenti *et al.* 2014).

Experimental investigations of the relationship between trait expression and environmental predictability typically compare three treatments: one where the environment is stable, and two where the environment fluctuates either predictably or unpredictably but with the same overall variance (in the latter, all individuals tend to experience the same permutation of environmental fluctuations over the treatment period, e.g. Schaefer & Ryan 2006; Manenti *et al.* 2014; Shama 2017). Exposure occurs over a pre-determined period prior to trait measurements, over which the mean environment is ensured to be constant among the respective treatments. Thus, this design makes the implicit assumption that animals respond to the mean and variance over the same time period. If this assumption is invalid, the two fluctuating treatments might produce mean environments that are different over the time period when the trait(s) in question actually respond to the environmental variable under manipulation. Specifically, one may envision that whereas phenotypic responses to the mean or current environment can occur rapidly (Kelty & Lee 2001; Nord *et al.* 2009; Ratikainen & Wright 2013), responses to variability in the environment may be slower. A statistical argument for this is that environmental variables are often temporally autocorrelated, such that information about the mean environment in the future can be obtained from a single observation of the current environment, whereas a minimum of two observations are necessary to obtain information about the variance. If animals respond to the mean environment over a shorter time scale than they respond to variance, any pattern in phenotypes produced by the experimental design described above might result from differences in the mean environment experienced towards the end of the exposure period (which may differ between the respective fluctuating treatments) and not differences in predictability as intended (Fig 1). Thus, when investigating such phenomena, we propose that

it is more sensible to adopt a design where it is possible to quantify the level of predictability in the fluctuating environmental variable of interest and then statistically estimate its effect relative to that of any potentially confounding influence of the recent or current environment.

Temperature is one of the environmental factors that can vary dramatically over different temporal scales, and which has pervasive effects on biochemical and physiological processes, particularly in ectothermic organisms (Angilletta 2009). Indeed, the temperature-sensitivity of ectotherms is expected to make them particularly reliant on reversible plasticity (e.g. Gabriel 2005; Gabriel 2006). When considering physiological change, this is often referred to as acclimation, a term which describes the reversible changes that occur in an organism as it attempts to maintain an optimal phenotypic state following a shift in e.g. ambient temperature (Loeschcke & Sørensen 2005). In this context, one trait that has been of particular interest is the short-term ability to tolerate high temperature (Manenti *et al.* 2014; Yampolsky, Schaer & Ebert 2014; Phillips *et al.* 2016; Brans *et al.* 2017). Both static and dynamic measures of heat tolerance can show a clear acclimation response to mean temperature, whereby an increase in mean temperature triggers physiological responses that improve tolerance of acute high temperature events, with the effect of acclimation becoming more apparent at less acute temperature exposures (Gunderson & Stillman 2015; Cambroner, Zeis & Orsini 2017; Shah, Ghalambor & Funk 2017; Semsar-kazerouni & Verberk 2018). Moreover, a recent experiment revealed consistent differences in heat tolerance among three permutations of a stochastic thermal regime that had the same overall mean and variance but where the temporal predictability of fluctuations differed (Drake, Miller & Todgham 2017). In the current study we measured heat tolerance of the keystone freshwater zooplankter *Daphnia magna* (Crustacea: Cladocera). This species is ideally suited to studies of phenotypic plasticity because its facultatively parthenogenetic mode of reproduction means that ‘true’ norms of reaction to environmentally manipulated states can be measured. Moreover, an ecologically important role for heat tolerance or another correlated trait seems plausible in *D. magna* because (a) this species can experience fluctuations in temperature that can become acute in their shallow pond habitats, for instance, during summer heatwaves and (b) the increasing frequency of such events has been implicated as the likely driver behind an evolutionary increase in this trait that was recently reported in a natural population (Geerts *et al.* 2015).

Our measurements of heat tolerance followed exposure to manipulations of temperature (within-generation) that had the same overall mean but where the temporal pattern of fluctuations was either (i) non-existent/stable, (ii) completely predictable or (iii) unpredictable, the latter in a series of sub-groups, where the order and magnitude of fluctuations differed but with the same overall variance as (ii). Following Drake et al. (2017), we predicted that *Daphnia* would adjust their physiology to the information contained in the pattern of temperature fluctuations in their environment. Namely, that when this information is unpredictable rather than predictable or stable, individuals should be better prepared to cope with an unexpected increase in temperature, i.e. as a form of within-generation bet-hedging or insurance (Haaland *et al.* 2018), which would manifest as a greater tolerance of high temperature. We first demonstrate a statistical approach for identifying the time-period over which a given trait responds to the mean of an environmental variable that fluctuates in time. We then quantify the level of unpredictability in each of the fluctuating treatment groups (i.e. predictable group and unpredictable sub-groups) and estimate the effect of unpredictability relative to that of the mean environment (over the specific time period identified above) experienced by individuals in the same groups. Using the same data, we lastly illustrate how it is possible to draw false conclusions from experimental designs that do not enable separate estimation of such potentially confounding effects.

Methods

We obtained developmentally synchronized clonal animals by isolating neonates produced within a 48 hour period (2nd to 5th clutch) from stock cultures of an experimental clone (EF49, hatched from an ephippium collected at a pond on Værøy Island, northern Norway, 67.687°N, 12.672°E). The clone had been maintained at 17°C in 250 ml jars at a density of 5 - 7 individuals per jar for at least 3 asexual generations. A 24 hour photoperiod mimicked the normal summer day length experienced by the source population. Commercial shellfish diet (1800, Reed Mariculture Inc, USA) was provided to these cultures at *ad libitum* levels 3 times per week. Culture medium (ADaM, Klüttgen *et al.* 1994) in the stock cultures was changed 1-2 times per week.

Neonates from the stock cultures were allocated to four different temperature acclimation treatments: stable average (AVE), fluctuating predictable (PR), fluctuating unpredictable (UP) and stable maximum (MAX). Temperature in the AVE treatment was constant at 19.3°C. In the PR treatment, temperature cycled diurnally between 24.0°C and 17.0°C. The UP acclimation treatment was composed of 16 different sub-treatments: unique temperature *versus* time profiles where temperature could cycle randomly between the same minimum and maximum temperatures as the PR treatment but also the intermediate values of 19.3 and 21.6 °C. Temperature in the MAX treatment was set to the maximum that could be experienced by individuals in the both PR and UP treatments (i.e. 24°C, actual mean \pm SD, 24.12°C \pm 0.04). The MAX treatment was included as a positive control group. In the fluctuating temperature treatments, changes in water temperature were induced by manually shifting the experimental cultures (at 8:00 AM and 16:00 PM, \pm 15 min each day) between climate cabinets set to 17, 19.3, 21.6 and 24°C. Cultures in the AVE and MAX treatments were handled in the same way as the fluctuating treatments, i.e. simulated shifting/handling was performed at 8:00 am and 16:00 pm each day. Due to logistical constraints, all 16 UP sub-treatments could not be performed simultaneously, meaning that the experiment was divided into a series of 4 blocks, with the AVE, PR, MAX and 4 of the UP sub-treatments being performed in each block. With the exception of the MAX group, the duration of each treatment was 12 days. To ensure that MAX individuals were of a similar size/physiological age to the other treatments at the time of measurement, MAX cultures were established 2 d later (i.e. treatment duration 10 d) than the other treatments within each block. In all treatments, the neonates from 20 separate maternal cultures were pooled before a random subsample was allocated to 250 ml jars (starting temperature of culture medium 17 °C) at a density of 10 individuals per jar ($n = 8$ replicate jars per treatment per block). Each maternal culture was used to provide experimental neonates for a single experimental block only. All treatments were maintained under the same photoperiod as the stock cultures and were fed *ad libitum* amounts of shellfish diet daily. Culture medium was changed twice during the treatment period. Temperature *versus* time profiles for each of the sub-treatments were constructed by logging (every min, HOBO UX120-014M) the temperature of medium in 250 ml jars that were shifted among climate cabinets set to 17, 19.3, 21.6 and 24 °C in all the combinations possible in the experimental design. The logger data revealed that it took approximately 5.5 hours for the largest change in water temperature to occur (17 to 24 °C and *vice versa*). Over the 12 day treatment period, both average temperature and variation in temperature in the PR and UP sub-treatments was similar (range in mean \pm SD: 19.28°C \pm

2.57 to $19.51^{\circ}\text{C} \pm 2.78$, Figure 2) and closely approximated the temperature experienced by individuals in the AVE group (mean \pm SD, $19.35^{\circ}\text{C} \pm 0.05$).

We measured heat tolerance in this species as the ability to maintain bodily function at high temperature, recorded as the ‘time to immobilization’ (referred to hereafter as T_{imm}) at 37°C , which is lethal after up to several hours of exposure (Yampolsky, Schaer & Ebert 2014). T_{imm} is a thermal endpoint defined by the time taken for the loss of locomotory function to occur at constant, lethal temperature (Terblanche *et al.* 2011). While such acute measurements of thermal tolerance employ temperatures that do not directly reflect natural conditions, they are becoming a widely used relatively high-throughput tool for measuring among individual performance at high temperature (Manenti *et al.* 2014; Yampolsky, Schaer & Ebert 2014; Cambronero, Zeis & Orsini 2017) that have been shown to correlate with variables that describe distribution patterns (Clusella-Trullas, Blackburn & Chown 2011; Kellermann *et al.* 2012) and likely reflect the relative thermal tolerance of individuals experiencing chronic, suboptimal temperatures in the natural environment (Messmer *et al.* 2017). T_{imm} was estimated using a custom algorithm in the R computing environment that can objectively identify the loss of locomotory function from video-derived tracking data (Burton, Zeis & Einum 2018). Briefly, we exposed individual daphnids from each of the acclimation treatments to 37°C using a custom-built, aluminium and glass thermostatic well-plate (see Fig 1 in Burton, Zeis & Einum 2018). Forty-five individual glass wells, open on their upper surface (well diameter 16 mm, depth 21 mm) were inserted in a rectangular 5×9 array on an aluminium plate (length 265 mm, width 125 mm, thickness 3 mm). This plate was fitted (and sealed via a series of screws) on top of a rectangular aluminium frame (depth 25 mm, length 265 mm, width 125 mm, thickness 20 mm). A sheet of glass (thickness 3 mm) of the same dimensions as the aluminium frame was glued to its underside. Water, warmed to 37°C (Grant Instruments water bath, UK, capacity 15.0 L), was pumped into the water-jacket via 5 inlet points (Eheim Compact 600 pump, Deizisau, Germany) and then flowed back to the water bath via five outlet points located at the opposite end of the plate. Up to 45 min before video recording in the well-plate commenced, females from each acclimation treatment ($n = 1-2$ per jar in each run) were placed individually into plastic vials along with 3.5 ml of fresh ADaM (without food) equilibrated to the same temperature that they experienced during their final day of treatment (i.e. potentially 17, 19.3, 21.6 or 24°C). These vials were then returned to their respective climate cabinets until recording began.

All individuals within a given experimental block were measured in three runs during a single day between 8:30 am and 13:30 pm. Within a given run, the 45 chosen individuals were transferred into the wells (i.e. one individual per well), that had been pre-heated to the test temperature by circulating water from the water bath, noting down the well number and time (in seconds) elapsed from the moment that the first individual was placed in a well (on average, it took 3 - 4 min to introduce all 45 individuals to the well plate). The well-plate was filmed from above with digital video cameras (Basler aCA1300-60gm, fitted with 5 – 50 mm, F1.4, CS mount lenses). Video recording commenced after the last individual within a run was introduced to a well. The well-plate was backlit with an LED light board (Huion A4 LED light pad, set to maximum intensity), to provide contrast between the individual in each well and the background. Video recording ceased when visual inspection confirmed that all individuals were motionless. The resulting video files were processed in Ethovision (version XT 11.5, Noldus Information Technology, The Netherlands, settings: greyscale pixel range 0 - 120, pixel size range 2 – 130, sample rate 3 observations s⁻¹), to produce a time-series of velocity data (in mm s⁻¹, travelled by the center-point of each individual. Then, using a custom R-script based upon a moving median (med.filter function from robfilter package, Fried, Schettlinger & Borowski 2015), we calculated the time taken (in seconds) for an individual's swimming velocity to drop below an a priori specified threshold value (0.03 mm s⁻¹) that is indicative of a state of immobility for *D. magna*. A full description of the hardware and R algorithm is given in Burton et al. (2018). After filming, all animals were photographed digitally under a stereomicroscope and body size (carapace length, mm) was measured with ImageJ software (Rasband 1997-2016). In total, we obtained T_{imm} estimates for 76 - 79 individuals from each of the AVE, PR and MAX treatments and 304 individuals from the UP treatments (18 – 21 per treatment group, n = 537 total).

Data analysis

Preliminary analyses revealed differences in T_{imm} among the 17 fluctuating acclimation treatments (n = 383 individuals distributed across 1 × PR treatment & 16 × UP sub-treatments, data from AVE & MAX treatments excluded, see Fig S1 and supplementary material for further details). Thus, in the first part of our analysis we further evaluated the

influence of temperature predictability by using this subset of the data to test for an association between T_{imm} and the predictability of temperature fluctuations. For each of the 17 fluctuating treatments, we quantified predictability of fluctuations in temperature as sample entropy, a measure of system complexity or regularity that was developed for the analysis of clinical physiological time-series data (Pincus 1991; Richman & Moorman 2000). Briefly, given N data points, sample entropy (commonly referred to as SampEn) is the negative natural logarithm of the conditional probability that two sequences that are similar for m points remain similar, within a given tolerance r for the next sequence of m points (see Richman & Moorman 2000 for a discussion of the relative merits of SampEn versus related complexity measures). A relatively low SampEn value indicates greater regularity or predictability in the time series in question. We calculated SampEn from hourly mean temperatures for each of the 17 fluctuating temperature treatments using values of $m = 24$ (thus considering predictability of daily temperature fluctuations) and $r = 0.2$ (recommended default value).

However, one challenge with disentangling the effects of mean and variation in experiments like the current one is that trait values might be more strongly influenced by more recently experienced temperatures rather than temperatures experienced further in the past (several studies show organisms can closely 'track' changing temperature, e.g. Kelty 2007; Overgaard & Sørensen 2008). Accordingly, any effect of overall predictability in temperature fluctuations on T_{imm} might be outweighed or at least moderated by the influence of mean temperature, which on average was the same across the complete 12 day duration of the treatments, but over shorter intervals towards the end of the treatment varied substantially (Fig 2a). Thus, we developed an approach for controlling for the variation in T_{imm} introduced by such effects when investigating the influence of predictability in temperature. We posited that the influence I on T_{imm} of an experienced temperature at a given time t prior to trait measurement should decline (either linearly or non-linearly) with increasing t . Our calculation of time-specific 'influence' values of previously experienced temperature on T_{imm} was based on calculation of the parameter T_{wt} which represents the sum of the weighted temperatures experienced by an individual over a specific time interval. To estimate T_{wt} for the individuals grouped within each of the 17 fluctuating acclimation treatments, we first calculated mean temperature (of culture medium) over 6 hour time intervals ($Temp_t$) for each

treatment over the full duration of the 12 day experimental period. T_{wt} for each fluctuating treatment was then calculated according to:

$$T_{wt} = \sum_{t=0}^{47} I_t \times Temp_t \quad \text{where } I_t \text{ is the weighting value for the mean temperature during interval}$$

number t prior to the T_{imm} measurement ($t = 0$ is the final 6 hours of the treatment).

I_t was calculated to decline from 1 to 0 with increasing values of t linearly:

$$I_t = 1 - (t \times \beta) \quad , \quad t \times \beta \leq 1$$

$$I_t = 0 \quad , \quad t \times \beta > 1$$

or exponentially:

$$I_t = e^{-\lambda t}$$

For both types of decline we first calculated I_t for each interval t for a range of values of the respective parameters (β or λ , range 0.0 - 1.0 in increments of 0.01). These were then used to calculate the corresponding value of T_{wt} for each specific treatment, before fitting a linear mixed effect model of the form $\log(T_{imm}) \sim T_{wt} + \text{body size}$. Each model shared the same random intercept structure: treatment was nested within measuring ‘run’ which in turn was nested within ‘measuring block’. We then plotted the AIC values from each model against the corresponding values of β or λ . For both types of decline (linear and exponential), an intermediate rate of decline in influence with increasing time prior to measurement of T_{imm} minimized AIC (summarized in Fig S2), and the model where T_{wt} was calculated based on a linear declining I with a slope of $\beta = 0.25$ was the most parsimonious. This means that the time-scale over which T_{imm} responded to acclimation temperature in the current experiment was approximately 24 hours (see Fig S3). Thus, in the analysis used to test the prediction that T_{imm} might respond to the level of unpredictability in temperature fluctuations, we calculated mean temperature over the final 24 hours of treatment as a covariate to control for the effect of mean temperature on T_{imm} . We then employed an information theoretic approach to evaluate the relative influence of the unpredictability in temperature fluctuations (i.e. sample entropy) on the variation in T_{imm} observed among the fluctuating acclimation treatments versus that of the differences in mean temperature experienced over the final 24

hours of exposure in each of those same treatments. Thus, we evaluated the relative fit of four models (using AICc-based Akaike weights w_i , models fit with maximum likelihood), where individual T_{imm} observations (transformed to log-scale) were set as the response variable. Two of these models considered the influence of either sample entropy or average temperature in the final 24 hours of treatment as explanatory variables. The remaining two models considered the influence of these two variables either additively or interactively. Body size was excluded as a covariate from this analysis due to the low variation in this trait among the 17 fluctuating acclimation treatments (range in mean size, mm \pm SE, $2.55 \pm 0.02 - 2.72 \pm 0.03$, maximum difference in mean size among treatments: 6.5%). Each model shared the same nested random intercept structure: treatment was nested within measuring run which in turn was nested within block.

In the second part of our analysis, we illustrate the potential peril of failing to quantify the effect of predictability in the environmental driver of interest independently of any effect of variation in the recently experienced mean of the same environment. To do so, we compare the T_{imm} of individuals from the AVE, PR and *pooled* UP treatments, thus mimicking experimental designs that implement treatment as a categorical variable composed of three levels: a stable environment, an environment that fluctuates predictably and an environment that fluctuates unpredictably. Data from the MAX treatment group was included in this analysis to help illustrate the effect of acclimation to different mean temperatures. Thus, we fitted a linear mixed effect model with individual observations of T_{imm} as the response variable (transformed to log-scale), treatment type as a categorical predictor (i.e. 4 different levels), body size as a covariate (there were minor treatment specific differences in body size that might contribute to variation in T_{imm} , see Table S3, Fig S4) and a random intercept of measuring run nested within measuring block. In each part of our analyses, we confirmed the assumptions of linear modelling via visual inspection of residuals for heteroscedasticity and normality. All statistical analyses were conducted in R (R Development Core Team 2019): Mixed effect models and information theoretic analyses were implemented with the lme4 (Bates *et al.* 2019) and AICcmodavg (Mazerolle 2019) libraries respectively. Sample entropy was calculated using the TSEntropies library (Tomcala 2018). Data are deposited in the Dryad Digital repository (Burton, Lakka & Einum 2019).

Results

In the first part of our analysis, the model considering only mean temperature in the final 24 hours of treatment provided a better fit of the data than models that considered sample entropy or the influence of these variables together (see Table 1 for AIC_c model weightings, and Table 2 for the summary of the best fitting model). This model showed that the variation in T_{imm} among the 17 fluctuating acclimation treatments could be explained partly by the differences in mean temperature experienced over the final 24 hours of the exposure period, but not the predictability of temperature fluctuations experienced (Fig 3a,b). Although the effect size attributable to mean temperature was only moderate (Table 2), it nonetheless indicated that *daphnia* from the fluctuating treatments who experienced a relatively high mean temperature during the final 24 hours of treatment had a higher T_{imm} than those who experienced cooler temperature over the same period.

However, in the second part of our analysis, where we deliberately excluded the information that enabled us to separate the effect of predictability in temperature from the effect of recently experienced mean temperature (i.e. by grouping together the unpredictably fluctuating treatments and testing the influence of temperature predictability as a categorical variable), this relationship was no longer evident. Instead, we observed a pattern of variation in T_{imm} that was consistent with our prediction. T_{imm} was found to be higher among individuals who experienced temperature that fluctuated unpredictably in comparison to individuals who experienced constant temperature of the same average value overall (Table 3, Fig 4). In this part of the analysis, T_{imm} was also observed to be highest among individuals who experienced the highest average temperature (i.e. the maximum constant treatment, Table 3, Fig 4). This effect was pronounced, with the MAX (i.e. stable 24 °C) treatment having a 43% higher T_{imm} than the AVE (i.e. stable 19.3 °C) treatment (mean T_{imm} = 2115 sec and 1366 sec for MAX and AVE treatments, respectively). T_{imm} did not differ among individuals that experienced predictable fluctuating *versus* constant temperature of the same average value (Table 3, Fig 4). Body size, which was marginally smaller in individuals from both of the fluctuating acclimation treatments relative to the two constant acclimation

treatments (maximum difference in mean size 1.87%, Fig S4), had no apparent influence on T_{imm} (Table 3).

Discussion

The present study provides novel insight for the investigation of within-generation responses to fluctuating environments. By exposing *Daphnia magna* to constant, predictably fluctuating and multiple unpredictably fluctuating temperature regimes, we were able to experimentally disentangle the effects of the mean, variability, and predictability in temperature on the ability of this zooplankton species to withstand high temperature. Moreover, by adopting the framework proposed here, we were then able to test for the effect of predictability in the environmental driver of interest whilst controlling for the potentially confounding influence of the recently experienced mean environment. Our data indicates that plastic adjustments of heat tolerance respond primarily to changes in mean temperature, but not to variability or predictability in temperature. First, variation in T_{imm} among the fluctuating treatment groups (i.e. $1 \times$ predictably fluctuating and $16 \times$ unpredictably fluctuating) could not be attributed to the level of unpredictability in temperature fluctuations in those groups but instead was partly explained by variation in average temperature among those groups over the final 24 hours of the treatment period (Fig 3b, Table 1,2). Second, T_{imm} was highest in the treatment group which experienced the warmest temperature on average (MAX treatment group, Fig 4. And third, no difference in T_{imm} was observed among individuals who experienced stable or predictably variable temperature of the same mean value (Fig 4). As such, our results are consistent with the positive effect of mean acclimation temperature on physiological traits that is often reported for ectotherms (Gunderson & Stillman 2015; Cambroner, Zeis & Orsini 2017; Burton, Zeis & Einum 2018; Semsar-kazerouni & Verberk 2018) . However, these results also suggest that despite unreliable information regarding the magnitude and direction of temperature change, *Daphnia* were nonetheless able to partially acclimate heat tolerance (in response to short-term mean temperature), an observation slightly at odds with theoretical work regarding the expression of reversible plasticity (DeWitt, Sih & Wilson 1998; Gabriel 2005). Why did *Daphnia* not draw upon cues describing the predictability of temperature change, for example as an ‘insurance’ strategy (Haaland *et al.* 2018) of increasing heat tolerance given uncertainty over the direction of future temperature change

(Drake, Miller & Todgham 2017). Such a strategy, instead of tracking mean temperature, would likely evolve only if there is low temporal autocorrelation in the environment, such that recently experienced conditions are a poor predictor of the future. For the pond from which the experimental genotype originates, there is a high autocorrelation in mean temperature over short time scales (e.g. $r > 0.9$ with a one day lag, supplementary data in Kielland et al. 2017). Thus, the fine-scale tracking in temperature tolerance observed here (ca. 24 h) is likely an efficient means for adapting to near-future conditions. Furthermore, the fact that *D. magna* adjust to fluctuating temperatures in this manner suggests that the continuous physiological adjustments required can be achieved without significant cost. Alternatively, the fine-grained adjustments of heat tolerance observed here might simply occur as a correlated response to recent mean temperature that results when *Daphnia* attempt to shift their overall thermal performance curve so that the optimum or near optimum coincides with the current environmental temperature. One question that we did not address in the current study is whether tracking of the mean environment would have been observed if the amplitude of temperature fluctuations was also subject to manipulation? When temperature fluctuates predictably, the phenotypic response is known to depend on the amplitude of the fluctuations relative to the mean (Colinet *et al.* 2015). Furthermore, the adaptive value of plasticity in physiological responses to environmental change might only be revealed in relatively extreme environments (Wang & Althoff 2019). In the current study, treatment temperatures never approached such a level (e.g. somatic growth rates in the Værøy population increase monotonically up to at least 26°C, Fossen, Pélabon & Einum 2018). Thus, further experimental work is required to determine if this pattern remains consistent over a broader amplitude of temperature fluctuations that extend to the chronically tolerable limit of the species in question. The work presented here also serves as a cautionary note both for interpretation of previous studies on this topic and future investigations: the apparent relationship between temperature unpredictability and thermal tolerance (Fig 4) was not evident when the differences in temperature toward the end of the exposure period among the fluctuating treatments were accounted for statistically (Fig 3b). This suggests that conclusions drawn from studies that employ a single unpredictable environment, and thus cannot test for such potentially confounding effects, may be premature (Schaefer & Ryan 2006; Manenti *et al.* 2014; Drake, Miller & Todgham 2017).

In conclusion, we present an approach for investigating within-generation responses to environmental variability and stochasticity. By comparing multiple fluctuating unpredictable treatment groups, with treatment groups that fluctuated predictably or not at all, we were able to test for the effect of overall predictability in the environment whilst controlling for the potentially confounding influence of variation in the recently experienced environment. Whilst our data pertain to heat tolerance in *Daphnia* and indicate that plastic adjustment of this trait occurs in response to recently experienced mean temperature, not variability in temperature nor the predictability of fluctuations in temperature, our approach should be readily generalizable to other phenotypic characters and environmental parameters. Given that our results suggest that phenotypic responses to the mean and variance in the environment may not always occur over the same time-scale, we urge future studies of responses to variability/stochasticity in the environment to employ experimental designs that can estimate these effects separately.

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Figure Legends

Figure 1. In a fluctuating environment, the mean environmental state can differ depending on the time-span considered. Shown are environments that fluctuate predictably (upper) and unpredictably (lower). Over the complete time-span depicted, both environments have the same overall variance and mean (the latter represented by dashed lines encompassing the full range of the x-axis). However, over shorter periods of time, the mean of each environment can differ substantially (shorter dashed lines).

Figure 2. (a) Temperature (of daphnid culture medium) *versus* time profiles for each the fluctuating acclimation treatments (1 × predictable, PR and 16 × unpredictable, UP) used in the experiment. (b) Mean temperature (\pm SD) over the 12 day treatment period for each of the fluctuating treatments shown in (a).

Figure 3. (a) Relationship between mean T_{imm} of *Daphnia magna* (time until immobilization, plotted on log-scale, \pm 1 SE) and unpredictability (quantified as sample entropy) of temperature fluctuations in each of the fluctuating (i.e. UP and PR) acclimation treatments. The fluctuating predictable treatment is represented by the datum point closest to the y-axis. Further details on quantification of unpredictability is given in the text. (b) Relationship between mean T_{imm} (plotted on log-scale, \pm 1 SE) and mean temperature they experienced over the final 24 hours of treatment period in each of the fluctuating acclimation treatments. For a full description describing why this time interval was chosen, see the Methods. Dashed line represents the model estimate for the relationship between T_{imm} and mean temperature in the final 24 hours of the treatment period.

Figure 4. Mean T_{imm} (time until immobilization, plotted on log scale \pm 95% confidence intervals) of *Daphnia magna* in relation to acclimation treatment. To aid comparison of treatments with the same overall mean temperature (stable average, fluctuating predictable and pooled fluctuating unpredictable groups), the zoomed facet excludes the stable maximum (MAX) treatment group. Statistical differences among the treatment groups in the zoomed facet are indicated by different letters (a & b).

Tables

Table 1. Set of candidate models testing the relationship between heat tolerance (T_{imm} , time until immobilization) of *Daphnia magna* in relation to the overall unpredictability of temperature fluctuations ('SampEn') and mean temperature experienced over the final 24 hours of exposure ('MeanTemp') in each of the fluctuating acclimation treatments. k : number of terms in a given model, ΔAIC_c : difference in AIC_c values between a given model and the best fitting model of those considered and w_i : probability that a given model is the best model of those considered.

model	fixed effects	k	AIC_c	ΔAIC_c	w_i	acc w_i
1	MeanTemp	6	-136.79	0.00	0.63	0.63
3	MeanTemp + SampEn	7	-135.13	1.66	0.27	0.90
4	MeanTemp \times SampEn	8	-133.06	3.73	0.10	1.00
2	SampEn	6	-120.99	15.79	0.00	1.00

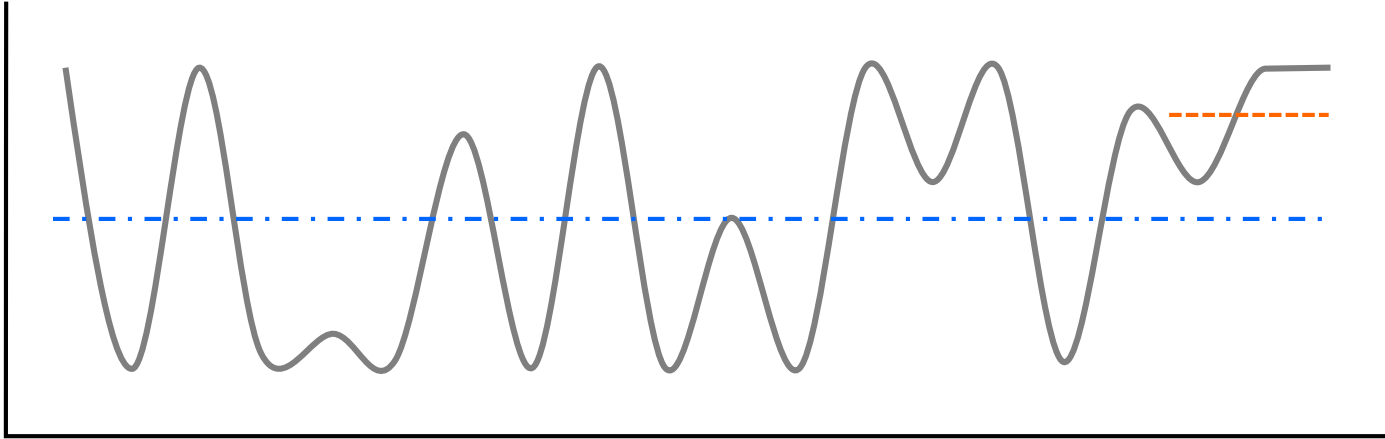
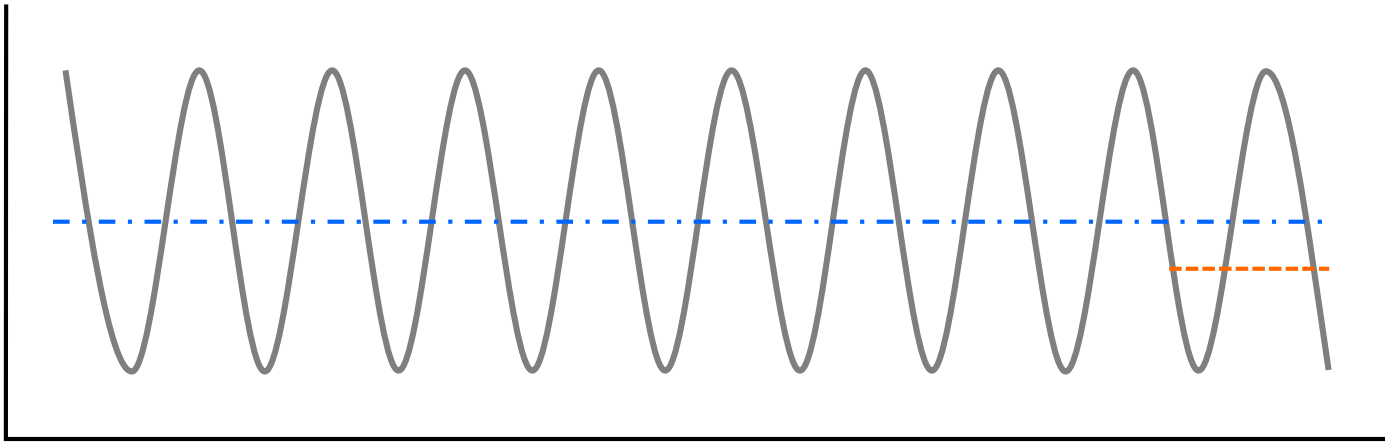
Table 2. Summary of Model 1, showing relative influence of the mean temperature experienced by individuals during the final 24 hours of exposure in each the 17 fluctuating acclimation treatments on heat tolerance of *Daphnia magna* (T_{imm} , time until immobilization).

	estimate	SE	t-value	p-value
intercept	6.48	0.18	36.42	< 0.0001
mean temperature	0.04	0.01	4.491	< 0.0001

Table 3. Summary of the mixed-effect model exploring the relative influence of body size and temperature treatment on heat tolerance of *Daphnia magna* (T_{imm} , time until immobilization). In this analysis, temperature treatment was fitted as a categorical variable composed of four levels: the stable average group, the fluctuating predictable group, the 16 pooled fluctuating unpredictable groups and the stable maximum group. Model estimates for temperature treatment are presented relative to the effect of the stable average group.

	estimate	SE	t-value	p-value
intercept (stable average)	7.21	0.19	37.83	< 0.0001
fluctuating predictable	0.04	0.03	1.24	0.21
pooled fluctuating unpredictable	0.07	0.03	2.85	< 0.01
stable maximum	0.43	0.03	13.57	< 0.0001
body size	0.00	0.07	-0.03	0.98

Environment



Time

Temperature (°C)

