Published in final edited form as:

Exp Gerontol. 2016 December 15; 86: 62–72. doi:10.1016/j.exger.2016.06.010.

Effects of fluctuating temperature and food availability on reproduction and lifespan

Tonia S. Schwartz^{a,b,c}, Phillip Pearson^{c,d}, John Dawson^{a,e}, David B. Allison^{a,f}, and Julia M. Gohlke^{d,g}

^aOffice of Energetics, School of Public Health, University of Alabama at Birmingham

^fNutrition Obesity Research Center, University of Alabama at Birmingham

^dDepartment of Environmental Health Sciences, School of Public Health, University of Alabama at Birmingham

Abstract

Experimental studies on energetics and aging often remove two major factors that in part regulate the energy budget in a normal healthy individual: reproduction and fluctuating environmental conditions that challenge homeostasis. Here we use the cyclical parthenogenetic *Daphnia pulex* to evaluate the role of a fluctuating thermal environment on both reproduction and lifespan across six food concentrations. We test the hypotheses that (1) caloric restriction extends lifespan; (2) maximal reproduction will come with a cost of shortened lifespan; and (3) at a given food concentration, relative to a metabolically equivalent constant temperature environment a diel fluctuating thermal environment will alter the allocation of energy to reproduction and lifespan to maintain homeostasis. We did not identify a level of food concentration that extended lifespan in response to caloric restriction, and we found no cost of reproduction in terms of lifespan. Rather, the individuals at the highest food levels generally had the highest reproductive output and the longest lifespans, the individuals at the intermediate food level decreased reproduction and maintained lifespan, and the individuals at the three lower food concentrations had a decrease in reproduction and lifespan as would be predicted with increasing levels of starvation. Fluctuating temperature had no effect on lifespan at any food concentration, but delayed time to reproductive maturity and decreased early reproductive output at all food concentrations. This suggests that a fluctuating temperature regimen activates molecular pathways that alter energy allocation. The costs of fluctuating temperature on reproduction were not consistent across the lifespan. Statistical interactions for age of peak reproduction and lifetime fecundity suggest that senescence of the reproductive system may vary between temperature regimens at the different food concentrations.

Keywords

thermal fluctuations; caloric restriction; dietary restriction; life span; adaptive plasticity; intergenerational effects; fecundity

^bCorresponding Author: Tonia S. Schwartz, Auburn University, 101 Rouse Life Sciences Building, Auburn, AL 36849 USA, Phone: (334) 844-1555, tschwartz@auburn.edu.

^cPresent addresses: Department of Biological Sciences, Auburn University

^ePresent addresses: Department of Nutritional Sciences, Texas Tech University

^gPresent addresses: Department of Population Health Sciences, Virginia Polytechnic Institute and State University

1. Introduction

The thermal environment is a determinant of energy utilization. In endotherms this is driven by the need to maintain a constant body temperature for thermal homeostasis (Bicego and others 2007). While ectotherms do not maintain a constant body temperature, they make behavioral, physiological and cellular adjustments to maintain thermal homeostasis. Further, metabolic rate in ectotherms is largely defined by temperature following the Boltzmann-Arrhenius equation for chemical reaction kinetics, with higher temperatures (within the range over which organisms commonly operate) resulting in higher metabolic rates and typically faster growth to reproduction (Dell and others 2011; Hochachka and Somero 2002). The interaction between energetics and life history traits is evident from studies showing different diet regimens producing a range of outcomes in traits such as reproduction and lifespan. For example, dietary restriction can extend lifespan in many species (Swindell 2012), whereas increased food availability can result in more and bigger offspring (Warner and others 2015). This response to energy availability can be described as a form of phenotypic plasticity (Wada and Sewall 2014). Understanding the relationships between the metabolic demands of the thermal environment and their interaction with diet can provide further insight on the determinants of plasticity in reproductive lifespan and total lifespan.

Lifespan studies typically maintain animals in a constant laboratory environment despite most animals living in (and having evolved to live in) fluctuating thermal environments, from diel fluctuations over a 24-hour period to seasonal fluctuations across a year (Dell and others 2011; Steinberg 2012). Organisms must regulate their cellular function to adjust to variable environments, as demonstrated by gene expression studies that show altered gene expression in fluctuating versus constant temperature environments (Podrabsky and Somero 2004). Studies that specifically test for the effect of fluctuating temperature environments on life history traits have demonstrated that growth rates and development often do not meet expectations predicted from constant mean temperatures (Kern and others 2015; Kingsolver and others 2015; Niehaus and others 2012). In terms of lifespan, fluctuating temperatures have been reported to either increase, decrease, have no effect, or have sex-specific effects depending the amplitude of the fluctuations and their frequency (Colinet and others 2015; Economos and Lints 1986; Mironidis and Savopoulou-Soultani 2008). Additionally, in response to thermal fluctuations some animals can increase their thermal tolerance and reduce their metabolic plasticity (Chen and Stillman 2012; Williams and others 2012). Animals can alter their metabolic molecular networks, and more specifically their responsiveness (plasticity) to the environmental conditions, in order to optimize the animal's physiology under a fluctuating thermal regimen (e.g. acclimation) (Angilletta 2009; Podrabsky and Somero 2004; Seebacher and others 2009). Altering these networks may have pleiotropic effects across the life history, including the allocation of energy to growth, reproduction, and longevity (Colinet and others 2015; Kern and others 2015).

Life history theory and resource allocation models predict trade-offs between early life traits (e.g. growth and early reproduction) and later-life traits such that limiting energy (caloric restriction) will delay reproduction, and prolong reproductive and total lifespan (Shanley and Kirkwood 2000; Stearns 1992). The disposable soma theory of aging predicts trade-offs

between early life and late-life traits, such that fast growth and early reproduction would limit investment in cell maintenance resulting in a shortened lifespan (Kirkwood 1993). Using this framework we can make predictions as to the effects of a fluctuating thermal environment on reproduction and lifespan. Here we are testing for an interaction between temperature regimen (fluctuating versus constant) and food concentration on key determinants of fitness: reproduction across the lifespan, and lifespan itself using *Daphnia pulex*.

1.2. *Daphnia* and specific questions

Daphnia pulex is a cyclical parthenogenic microcrustacean that has long been studied as a freshwater ecology model organism and has more recently been recognized by the NIH as a model organism for biomedical research (NIH). They provide an ideal system for studying the effects of temperature regimens on lifespan and reproduction as they: are short lived (< 2 months); have direct development after hatching from the egg within the mother's brood pouch; reach reproductive maturity in 1–2 weeks; have discrete reproductive events that can be quantified throughout the lifespan; and have more genes in common with humans than do *Drosophila* or nematodes (Colbourne and others 2011). Several seminal studies have described physiological effects in *Daphnia* due to temperature, food levels, diet composition, and population of origin, (Chen and Stillman 2012; Dudycha and Lynch 2005; Heugens and others 2006; Lynch and Ennis 1983; Lynch and others 1999; MacArthur and Baillie 1929). Most studies have been conducted under constant temperatures and these demonstrate that higher food concentration shortens time to maturation, increases size at maturation, increases proportion of resources allocated to reproduction, and increases fecundity (Dudycha and Lynch 2005; Heugens and others 2006; Lynch 1989). At higher (non-stress) temperatures, *Daphnia* grow faster to maturity, but are smaller, and have small early clutches, yet overall fecundity is not reduced (Heugens and others 2006). Further higher temperatures are more metabolically demanding (Heugens and others 2006). Under fluctuating thermal conditions *Daphnia* have increased thermal tolerance, and a depressed metabolic rate when measured at the lowest temperature (Chen and Stillman 2012) and a faster rate of population increase (van As and others 1980).

More recently *Daphnia* has been recognized as a model for aging (Kim and others 2014; Murthy and Ram 2015; Schumpert and others 2014). Previous studies on the plasticity of lifespan have shown conflicting results as to whether *D. pulex* demonstrates an extension of lifespan in response to dietary restriction (Dudycha 2003; Kim and others 2014; Latta and others 2011; Lynch 1989; Lynch and Ennis 1983). Here we build on these studies to assess the effect of a daily fluctuating temperature, compared to a metabolically equivalent constant temperature, on lifespan and reproduction across six food concentrations. First, we ask whether decreasing energy availability, across six food concentrations, results in a reallocation of resources from growth and reproduction to somatic maintenance as determined by an extension of lifespan and a reduction in reproduction (prediction in Fig. 1A). Further, we hypothesize that at maximal food concentration there will be a cost of reproduction in terms of lifespan. Second, we ask whether a fluctuating thermal environment is energetically costly such that the extension of lifespan and reduction in reproduction will occur at a higher food concentration relative to a metabolically equivalent constant

temperature (Fig. 1B). In other words, at the same intermediate food intake level, living in a fluctuating environment, even if at the same metabolic mean over a 24-hour period, would alter molecular pathways to increase the energy allocated to maintaining homeostasis and less energy to reproduction and lifespan relative to a constant environment. Third, we ask whether the cost of a fluctuating thermal regimen is consistent as a function of food availability (Fig. 1C), indicating the fluctuations alter metabolic pathways to reallocate energy away from reproduction at all food concentrations; alternatively, if the cost of thermal fluctuations is evident at lower food concentrations but virtually eliminated at the higher (*ad lib*) food concentrations (Fig. 1D) this would indicate that with unlimited food all energy demands can be met and there is no need to reallocate energy away from reproduction.

2. Materials and Methods

We used a strain of *Daphnia pulex* received from Dr. J. Shaw's laboratory at Indiana University in June of 2011. Since then, it has been maintained in Dr. J. Gohlke's laboratory at University of Alabama at Birmingham using previously established protocols (Asselman and others 2013; Shaw and others 2007). Media consisted of COMBO media (final concentrations: 388.16 μM Boric Acid, 330.87 μM Calcium Chloride Dihydrate, 307.24 μM Magnesium Sulfate Heptahydrate, 99.93 μM Potassium Chloride, 149.98 μM Sodium Bicarbonate, 232.84 μM Sodium Metasilicate Nihydrate). Selenium (final concentration 0.0126 μM) and Animate solution (final concentrations: 3656.52 μM Lithium Chloride, 19.88 μM Potassium Iodine, 155.51 μM Sodium Bromide, 578.90 μM Rubidium Chloride, 562.60 μM Strontium Chloride Hexahydrate) were added fresh during media changes every other day.

2.1. Treatments

A total of 366 individual *Daphnia* were used in this study. We exposed animals to one of 12 treatments (six food concentrations by two temperature regimens) and tracked individuals for reproductive output over their lifespan and measured total lifespan.

2.1.1. Food Treatments—To provide consistency across the experiment, and the potential for repeatability across experiments and labs, we used RGcomplete as the food source (Reed Mariculture Inc.). RGcomplete is a blend of four microalgae (size 1.5 – 15 μm ; *Nannochloropsis oculata*, *Tetraselmis* sp., *Chlorella vulgaris*, and *Schizochytrium* sp.) that has been nutritionally formulated as a food source for rotifers grown for aquaculture. The algae are dead so there are no effects of the temperature regimens on the algal growth that would alter the energy available in each treatment. Since there is no literature on the use of RGcomplete in *Daphnia*, we use this experiment as a means of validating it as a reliable food source for *Daphnia* and for identifying a concentration that would be ideal for future experiments. Upon purchase of a single batch of RGcomplete, 1 ml aliquots were frozen and stored for use throughout the experiment (3 mo.). Aliquots were thawed once and used for that single day and discarded. We used six food concentrations, A (lowest) to F (highest) based on a dilution factor of 0.6 (Fig. S1). Food treatments were defined by the following

amount of RGcomplete in 48 ml of COMBO provided every two days: A=1.318 µl, B=2.396 µl, C=4.356 µl, D=7.920 µl, E=14.400 µl, F=26.182 µl.

2.1.2 Temperature Regimens—Because metabolic rate does not linearly increase with temperature we estimated two temperature regimens that are metabolically equivalent by using a range of previously calculated temperature coefficient values (Q10, the factor of reaction changes over a 10°C increase in temperature) for *Daphnia* (MacArthur and Baillie 1929; Richman 1958). Using a Q10 of 2.5 based on multiple species (Dell and others 2011) or 1.6 based on heart rate (MacArthur and Baillie 1929) we calculated the expected change in metabolic rate for a fluctuating temperature from 19°C to 27°C back to 19°C over a 24 hour period with 1 hour time-steps of 0.58°C change per hour. We used the expected change in metabolic rate to calculate an equivalent constant temperature of 23°C. Thereby, the two temperature regimens estimated to be metabolically equivalent over a 24-hour day were: constant 23°C and fluctuating 19°C to 27°C.

Data loggers were placed in tubes of media in each chamber to record temperature every minute (Fig. 2). Based on the average over five days, the constant temperature treatment averaged 23.42°C with an average range from 23.03°C to 23.83°C in an environmental chamber (Percival Intellus Ultra Controller Model I36NLXC8). The fluctuating thermal temperature treatment had an average of 23.05°C and an average range from 19.24°C to 26.95°C over a 24 hour cycle with the temperature peaking ~ 7PM and at a low ~ 7AM in an environmental chamber (LH-6 Laboratory Humidity Chamber, Associated Environmental Systems, Inc., Ayer, MA). Thus the actual temperature in the constant chamber was slightly warmer than our target. This equated to the area under the curve of calculated increase in metabolic rate for a 24-hour period of 33.95 for fluctuating vs. 35.06 for constant based on a Q10 of 2.5, or an area under the curve of 28.49 for fluctuating versus 28.76 for constant if using the Q10 of 1.6.

2.2. Experimental Design

For five generations prior to this experiment *Daphnia* populations were maintained in liter jars on 300 µl of RGcomplete/L of media with one daphnid per 50 mls of media (same concentration as the E treatment) (Fig. S1). Offspring produced from the second generation on RGcomplete were randomly assigned to either the constant or the fluctuating thermal environment (see details below) with a 12-hour light/dark cycle (Fig. 2). To minimize the maternal effects of the temperature regimen, we maintained the populations at these two temperature regimens for an additional two generations. Thus, the animals in the constant treatment were the 5th generation on the 300 µl/L RGcomplete and 5th generation in the constant temperature regimen, the animals in the fluctuating temperature treatment were the 5th generation on 300 µl/L RGcomplete and the 3rd generation at a fluctuating temperature regimen.

In two day-blocks (27 March and 28 March 2014), offspring 0–24 hours old (moms 14–17 days old) were collected from each temperature regimen and randomly assigned to food treatments that varied in the amount of RGcomplete (A – F, Fig S1) with balance enforced among the different food treatments (this may be thought of as a blocked design with ‘one

big block'). While we attempted to have all food groups balanced within day-block, perfect balance was not achieved because the number of offspring obtained was not always a multiple of 6 within each regimen; so day-by-food-group totals sometimes differed by one. One week later a third block of animals (moms 21–24 days old) were randomly assigned to the food treatments within the fluctuating thermal treatment to replace early deaths and increase sample size since the fluctuating chamber was limited in space compared to the constant chamber. Final sample size at each of the 6 food treatments in the fluctuating temperature regimen was $n=28$ and in the constant temperature regimen was $n=33$ individuals.

Animals were housed singly in 50 ml plastic conical tubes in 48 ml of media. Within each temperature chamber, food treatments were placed non-randomly in a space-filling design, so that the tubes for each food treatment were not clustered together, and the racks were rotated throughout the chamber every other day. This was done to minimize the chance of an adverse event, such as getting fried by a heater malfunction, disproportionately affecting the tubes in any one food treatment.

2.3 Data Collection and Traits Measured

Media was changed every other day for all tubes, during which the individual was moved to a new container of media using a transfer pipet. Changes were scheduled between 10AM–3PM when the fluctuating chamber was near room temperature to minimize thermal shock. The spent medium was poured through a 100 μm mesh cell strainer (Fisher Scientific, cat: 22363549) to count the number of offspring (*brood size*). For the first 16 days, each tube was checked daily for the first reproduction event to determine *time to reproduction*. *Lifetime fecundity* indicates the total number of live offspring produced by an individual throughout its life. *Peak day of reproduction* was the first day the animal reached its max brood size. *Reproductive lifespan* is the number of days from the first brood produced until the last brood was produced (often this was the day of death). *Lifespan* beyond day 16 was determined in two-day intervals. Deaths in the tubes were recorded on the day the daphnid was found unresponsive, and accidental deaths were censored. We checked for a visual heartbeat using a microscope in the event it was unclear if an individual was dead.

2.4 Statistical Analyses

All analyses were performed in R with statistical significance set at $\alpha = 0.05$ (2-tailed). Reported P-values are uncorrected for multiple comparisons. *Reproductive profile over the lifespan* was evaluated using a Poisson regression model where *brood size* could vary by food concentration or temperature regimen as a function of time.

Time to reproduction was analyzed as time to event data using Kaplan-Meier curves with animals that died prior to reaching reproductive maturity being right-censored on their day of death. We used the Cox proportional hazards model to test for the effect of temperature regimen (either constant or fluctuating) and food concentration (six levels: A through F) and their interaction.

For *age of peak reproduction* and *percent reproductive lifespan* we removed individuals that did not reproduce due to death before reaching reproductive maturity ($n=39, 32, 12, 3, 7$, and

6 across A, B, C, D, E and F treatments respectively). For *day of peak reproduction* we fit a quasi-Poisson regression to test for the effect of temperature regimen (Temp: either constant or fluctuating) and food concentration (Food: six levels) and their interaction. For *percent reproductive lifespan* we fit a linear model to the natural log of the percent reproductive lifespan. For *reproductive lifespan* and *lifetime fecundity* we fit an overdispersed quasi-Poisson regression model, since reproductive lifespan is a non-negative count from the day of first reproduction to the last day of reproduction that may (and frequently does) include 0 in the lower food concentrations.

To specifically test whether fluctuating temperature altered reproductive output at different ages we calculated average reproductive output within four age classes: Early (Day 0 – Day 15), Mid (Day 16 – Day 25), Late (Day 26 – Day 35) and Old (over Day 35). Only individuals that lived through an age class were included in the count for that particular age class. Animals in the “Old-Reproduction” group had to live until at least 40 to be included in that group. We fit a linear model to test for the effects of temperature regimen (either constant or fluctuating) and food concentration (six levels) and their interaction.

Lifespan (survival) was compared across the twelve groups using Cox Proportional Hazard model via the survival package in R. In both the GLM and Cox Proportional Hazard models, p-values are based on likelihood ratio tests. These tests make some assumptions (e.g., proportionality of hazards) that, while not observed to be egregiously violated in this experiment, are unlikely to perfectly hold in practice. For this reason, and because we have some very stark differences among the outcomes in the most extreme food treatment groups, presenting exact p-values with a level of precision such as $p=3 \times 10^{-264}$ would not be meaningful in this context, other than indicating that we have ‘really small p-values’. Thus we will report $p < 10^{-10}$ in these cases.

3. Results

In this experiment we measured reproduction and lifespan across two temperature regimes, constant versus thermal fluctuations and six food concentrations ranging from starvation to *ad libitum* (Fig. S1), the results are summarized in Fig. S2.

3.1. Food concentration but not fluctuating temperature affects lifespan

Survival was driven by food concentration with the lower food concentration (A, B, C) causing a reduction in lifespan compared to the higher food groups D, E, and F ($p < 10^{-10}$) (Fig. S2 and Fig. 3). In contrast to our predictions in Fig. 1A and B, we found no empirical evidence of extension of lifespan at mid-level food concentrations in either the constant or the fluctuation thermal environments as would be predicted with caloric restriction (e.g. Fig. 1A and 1B). Although the D food treatment shows the trend of extension of lifespan (Fig. 3, Fig. 6D), it is not significantly different from E and F ($p=0.62$) when ignoring temperature regimen. We found a nearly statistically significant interaction with food concentration and temperature treatment ($p=0.072$). Testing specifically for differences between D, E and F, the temperature difference is largely due to the shortened lifespan of the fluctuating E group ($p=0.04$). We interpret these results to indicate A, B, and C were decreasing levels of starvation (respectively), E and F were over abundance (supported by the lawn of algae

remaining on the bottom of the tube at the time of media changes), and D to be an intermediate level of food (left-over algae not obvious at media change when adults).

3.2. Fluctuating temperature delays reproduction

Reproductive profiles over the lifespan had very strong “food concentration by time” ($p < 10^{-10}$) and “temperature regimen by time” ($p < 10^{-10}$) effects (Fig. 4). To explore this further, we examined the timing of reproductive events. In evaluating *time to first reproduction*, we found that fluctuating temperature ($p < 10^{-10}$) and extreme low food treatments (A, B, and C treatments) delay time to reproductive maturity ($p < 10^{-10}$) (Fig. 5, Fig. 6A). In terms of our predictions in Fig. 1C, this supports the hypothesis that fluctuating temperatures are energetically costly to growth and reproduction in early life. The interaction was not statistically significant ($p=0.08$), but was suggestive that fluctuating temperature had a stronger effect on early reproduction under low food treatments (Fig. 6 versus prediction in Fig. 1C and D).

For *age of peak reproduction*, we found an interaction between food concentration and temperature ($p=0.026$), where the thermal fluctuation groups in the two lower food treatments and the highest food treatment reached their reproductive maximum at a later age (Table 1, Fig. 6B).

We found little evidence that *Daphnia* have a post-reproductive life since they often reproduced up until the day of or day before death (Fig. S2). In *reproductive lifespan*, we did not find an interaction between temperature regimen and food concentration ($p=0.072$), but both food concentration ($p < 10^{-10}$, lower food concentrations had a shorter reproductive lifespan) and temperature ($p=0.04$, fluctuating temperature had a shorter reproductive lifespan, strongly driven by E treatment) have significant additive effects. This pattern of reproductive lifespan closely mimics total lifespan (i.e. Fig. 6D); thereby, in the *percent reproductive lifespan* there was no evidence for an interaction ($p=0.14$) or an effect of temperature regimen ($p=0.88$). But there was a strong effect of food concentration ($p < 10^{-10}$).

For *lifetime fecundity* there was a strong food concentration by temperature regimen interaction ($p=5 \times 10^{-5}$), with the E food treatment fluctuating group performing worse than their constant temperature counterparts (Fig. 6C).

Reproductive output peaked in mid- and late-life age groups (Fig. 6B, Fig. 7A–D). When testing specifically for differences in age-class specific reproductive output, in *Early reproduction* food concentration ($p < 10^{-10}$) and temperature regimen had additive effect ($p < 10^{-10}$) with low food and fluctuating environments causing reduced reproductive output (Fig. 7A, Fig. 7B). *Mid-life reproduction* and *Late-life reproduction* showed interactions between food and temperature treatments ($p=0.0046$ and $p=0.0023$ respectively). Relative to the constant temperature, fluctuating temperatures had higher reproductive output at the lower food concentrations, and lower reproductive output at the higher food concentrations (Fig. 7B and C). Neither food concentration or temperature treatment had an effect on *Old-life reproduction* (Fig. 7D). In terms of our predictions in Fig. 1C and D, the effect of fluctuating temperature regimen on reproduction was not constant across food treatments.

Further the costs were not constant across the different life stages. These results suggest a difference among the thermal treatments in how energy is allocated across the lifespan and a potential role for the acclimation of metabolic networks.

4. Discussion

If the translation of laboratory experiments to natural populations is a goal of biomedical research then it is essential to understand the physiological effect of experiencing natural environmental fluctuations and how thermal fluctuations may alter the response to other environmental variables. Here we use *Daphnia pulex* to test the energetic effects of fluctuating temperature across six food concentrations on the life history traits reproduction and lifespan. In this experiment, the fluctuating temperature treatment had a nearly equivalent metabolic mean as the constant temperature over 24 hours; thereby the effects of the temperature regimens on reproduction and survival would not simply be due to differences in the metabolic rate but rather how fluctuating temperature may alter how energy is allocated to maintain homeostasis versus allocated to life history traits.

Cost of caloric restriction in reproduction or lifespan?

Our first aim was to determine whether decreasing energy availability, across six food concentrations, results in a reallocation of resources from growth and reproduction to somatic maintenance as determined by an extension of lifespan and a reduction in reproduction (prediction in Fig. 1A). Multiple studies have demonstrated an increase in lifespan under caloric restriction with the corresponding trade-off in reproduction in *Daphnia pulex* (Dudycha 2003; Lynch 1989; McCauley and others 1990) or *Daphnia magna* (Pietrzak 2011). In contrast to these studies and the predictions for the disposable soma hypothesis, at the food levels tested we found no evidence of increased somatic maintenance to extend lifespan in response to food restriction or thermal fluctuations. Rather, we see with the transition from high to intermediate levels of food (*ad lib* to caloric restriction), lifespan is maintained and reproduction is reduced (see transition from F to D). This is consistent with (Kirkwood and Shanley 2005) in that investment in maintaining lifespan is prioritized over investment in reproduction (see intermediate food level D). As expected, with insufficient resources (starvation) both reproduction and lifespan decrease accordingly (C, B, A). The second highest food concentration (E) had a decrease in both lifespan and fecundity in the fluctuating treatment relative to the highest food treatment (F) and the next lowest treatment (D); a result for which we have no clear explanation. Further, we did not see a trade-off in reproduction versus lifespan as the maximum food levels had the maximum lifespan, the fastest time to reproduction, highest early reproduction, and maximum fecundity. Our results are similar to those of Kim and others (2014) who also found the trend of higher food, higher fecundity, and longer lifespan in *D. pulex*; which is in contrast to Latta and others (2011) that showed both lifespan and fecundity decreased with increasing food levels.

The variation among these experiments could be due to variation among genetic strains as has been previously documented in *D. pulex* (Dudycha 2003; Pietrzak 2011), and in mice (Liao and others 2010). Variation in food source, macronutrient composition, and quality

may also alter trade-offs in reproduction and longevity responses (Solon-Biet and others 2015). Standardizing diets and experimental conditions to compare across *Daphnia* genotypes and laboratories will improve our ability to compare and interpret these differences. Additionally, it is possible that we skipped over the window of “food scarcity” where the switch to *increasing* investment in somatic maintenance would be predicted (Kirkwood and others 2000) and where we would see the characteristic extension of lifespan. Indeed Lynch (1989) demonstrated a sharp drop from lifespan extension to starvation. If lifespan extension in response to dietary restriction is possible for this strain of *D. pulex*, we would expect to see it between food concentrations C and D, and we have ongoing experiments to address this question.

It is important to note the contrasts in dietary restriction between this experiment in *Daphnia* and those conducted in rodents. Most rodent studies initiate dietary restriction after the animals reach adulthood. Thereby, treatments C-F would be most relevant for comparing to rodent studies, where treatments C and D were likely *ad lib* for most of development and then had increasingly severe caloric restriction starting early in adult life (treatment C), and later in adult life (treatment D), and treatments E and F were unlimited food throughout the lifespan.

Energetic Cost of Thermal Fluctuations

Our second goal was to determine if a fluctuating thermal environment was energetically costly to life history traits. We predicted the response to caloric restriction, with the extension of lifespan and reduction in reproduction, to occur at a higher food concentration in the fluctuating environment relative to a metabolically equivalent constant temperature. As discussed above we did not see an extension of lifespan with caloric restriction, and this was consistent across both thermal regimen treatments. We did see an effect of daily thermal fluctuations within the normal non-stressful range having an energetic cost on reproduction, particularly early in life, although the effects were small relative to the range of effects due to food availability. Thermal fluctuations delayed time to reproduction (especially in low food treatments), reduced early reproductive output (all food treatments) and delayed age of peak reproduction. These early life reproduction results demonstrate that living in a fluctuating thermal regimen has an energetic cost in early life.

Alterations in Energy Allocation

Our third goal was to determine if the effects of thermal fluctuations were purely energetic (Fig. 1D) or if they may also alter molecular networks underlying resource allocation (Fig. 1C). Our results were intermediate between these predictions. Fitting with our prediction in Fig. 1C, we found that the energetic costs of the fluctuating temperatures were highly evident in early reproduction since time to first reproduction was consistently longer in the fluctuating temperature environment across all food treatments. Delays in reproduction are typical of a reduced energy budget as demonstrated with caloric restriction in this and other *Daphnia* experiments (Dudycha 2003; Kim and others 2014) as well as in rodents (Nelson and others 1985). Comparisons among the constant versus fluctuating temperatures at the same food treatment assumes the same amount of energy is available (same total energy budget), thereby these differences suggest alterations early in life in the molecular pathways

to regulate how much energy is allocated to maintain homeostasis and decrease energy allocated to early life reproduction.

The effect of the thermal fluctuations reducing reproduction was not consistent across the lifespan. Particularly at the later age stages (Mid and Late-life) reproduction under the fluctuating regimen were just as high or higher than in the constant temperature regime (more similar to prediction in Fig. 1D). In other terms, there was an interaction between food concentration and temperature regimen on overall fecundity, age of peak reproduction, and Mid- and Late-life reproductive output; thus, fluctuating temperatures could be described as slowing the reproductive schedule but not reducing maximal fecundity. Together these interactions suggest that senescence of the reproductive system may vary between temperature regimens as they move from low to high food availability (Fig. 4 and 7), and that molecular pathways being activated or altered by the thermal fluctuations may have an effect on energy utilization translating to life history traits across the lifespan.

This concept of fluctuating environments altering metabolic pathways is supported by Chen and Stillman (2012) who demonstrated that after *Daphnia* acclimate to a fluctuating environment both respiration rate and time to reproduction are still reduced when the organisms are put in a constant environment.

What are some of the molecular pathways that may be altered by acclimation to the fluctuating thermal regimens? The thermal fluctuation regimen we used mimics a natural diel circadian rhythm. Podrasky and Somero (2004) tested the effect of a diel thermal cycle relative to constant temperature on liver gene expression in fish and found temperature regime to affect expression of hundreds of genes including those involved in energy metabolism. The compound resveratrol is known to extend lifespan in some rodent models (Barger and others 2008), and is also known to regulate circadian rhythms at the cellular level (Sun and others 2015). Intriguingly, Kim and others (2014) found that resveratrol does not affect lifespan in *D. pulex*, but it did delay time to reproduction and reduced early life reproduction in a similar pattern to fluctuating temperatures in our study. Based on gene expression studies in mammals, genes and molecular pathways that link resveratrol's effect on circadian rhythm includes CLOCK genes, Sirt1, and Ppara (Kulkarni and Canto 2015; Park and others 2014). We suggest these would be the candidate pathways to test for the effects of fluctuating temperatures and a potential mechanistic basis for how fluctuating temperature regimens may alter the reproductive schedule in *Daphnia*.

Limitations of this study include the lack of measurements of actual food intake and body size, both of which can affect animal energetics and reproduction. While differences in body size were obvious among the range of food treatments (from starvation to *ad lib*), they were not obvious among the constant and fluctuating treatments. Future studies would be needed to test for effects of thermal fluctuations on both these variables.

4.2. Conclusions

We found that in *D. pulex*, thermal fluctuations did not alter lifespan but slowed the reproductive life history, even when the thermal fluctuations are centered on the metabolic mean of the constant environment and are not “stress” temperatures. Further these effects

were inconsistent across the food treatments suggesting they alter molecular pathways underlying energy allocation. Understanding how these pathways are altered by environmental fluctuations will assist our ability to translate findings from laboratory studies to natural populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported the James S. McDonnell Foundation 21st Century Science Initiative – Postdoctoral Program in Complexity Science/Complex Systems-Fellowship Award Grant number 220020353, and NIH grants P30DK056336, T32HL072757, and R01AG043972. The opinions expressed are those of the authors and do not necessarily represent those of the NIH or any other organization. We thank S. Hudson and D. Doke for assistance and expertise in maintaining the *Daphnia* cultures. We appreciate the thoughtful and constructive comments from A. Niehaus and an anonymous reviewer that have improved this manuscript.

References

- Angilletta, MJ. Thermal adaptation: A theoretical and empirical synthesis. Oxford: Oxford University Press; 2009.
- Asselman J, Shaw JR, Glaholt SP, Colbourne JK, De Schampelaere KAC. Transcription patterns of genes encoding four metallothionein homologs in *Daphnia pulex* exposed to copper and cadmium are time- and homolog-dependent. *Aquatic Toxicology*. 2013;142–143. 422–430.
- Barger JL, Kayo T, Vann JM, Arias EB, Wang J, Hacker TA, Wang Y, Raederstorff D, Morrow JD, Leeuwenburgh C, Allison DB, Saupe KW, Cartee GD, Weindruch R, Prolla TA. A low dose of dietary resveratrol partially mimics caloric restriction and retards aging parameters in mice. *PLoS ONE*. 2008; 3:e2264. [PubMed: 18523577]
- Bicego KC, Barros RCH, Branco LGS. Physiology of temperature regulation: Comparative aspects. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*. 2007; 147:616–639.
- Chen X, Stillman JH. Multigenerational analysis of temperature and salinity variability affects on metabolic rate, generation time, and acute thermal and salinity tolerance. *Daphnia pulex*. 2012; 37:185–194.
- Colbourne JK, Pfrender ME, Gilbert D, Thomas WK, Tucker A, Oakley TH, Tokishita S, Aerts A, Arnold GJ, Basu MK, Bauer DJ, Caceres CE, Carmel L, Casola C, Choi JH, Dettler JC, Dong Q, Dusheyko S, Eads BD, Frohlich T, Geiler-Samerotte KA, Gerlach D, Hatcher P, Jogdeo S, Krijgsvelde J, Kriventseva EV, Kultz D, Laforsch C, Lindquist E, Lopez J, Manak JR, Muller J, Pangilinan J, Patwardhan RP, Pitluck S, Pritham EJ, Rechtsteiner A, Rho M, Rogozin IB, Sakarya O, Salamov A, Schaack S, Shapiro H, Shiga Y, Skalitzyk C, Smith Z, Souvorov A, Sung W, Tang Z, Tsuchiya D, Tu H, Vos H, Wang M, Wolf YI, Yamagata H, Yamada T, Ye Y, Shaw JR, Andrews J, Crease TJ, Tang H, Lucas SM, Robertson HM, Bork P, Koonin EV, Zdobnov EM, Grigoriev IV, Lynch M, Boore JL. The ecoresponsive genome of *Daphnia pulex*. *Science*. 2011; 331:555–561. [PubMed: 21292972]
- Colinet H, Sinclair BJ, Vernon P, Renault D. Insects in fluctuating thermal environments. *Annual Review of Entomology*. 2015; 60:123–140.
- Dell AI, Pawar S, Savage VM. Systematic variation in the temperature dependence of physiological and ecological traits. *Proceedings of the National Academy of Sciences*. 2011; 108:10591–10596.
- Dudycha JL. A multi-environment comparison of senescence between sister species of *Daphnia*. *Oecologia*. 2003; 135:555–563. [PubMed: 16228255]
- Dudycha JL, Lynch M. Conserved ontogeny and allometric scaling of resource acquisition and allocation in *Daphniidae*. *Evolution*. 2005; 59:565–576. [PubMed: 15856699]

- Economos AC, Lints FA. Developmental temperature and life span in *Drosophila melanogaster*. II. Oscillating temperature. *Gerontology*. 1986; 32:28–36. [PubMed: 3081406]
- Heugens EH, Tokkie LT, Kraak MH, Hendriks AJ, Van Straalen NM, Admiraal W. Population growth of *Daphnia magna* under multiple stress conditions: joint effects of temperature, food, and cadmium. *Environ Toxicol Chem*. 2006; 25:1399–1407. [PubMed: 16704075]
- Hochachka, P.; Somero, G. *Physiological Evolution*. Oxford-New York: Oxford University Press; 2002. *Biochemical Adaptation: Mechanism and Process*.
- Kern P, Cramp RL, Franklin CE. Physiological responses of ectotherms to daily temperature variation. *J Exp Biol*. 2015
- Kim E, Ansell CM, Dudycha JL. Resveratrol and food effects on lifespan and reproduction in the model crustacean *Daphnia*. *Journal of experimental zoology Part A, Ecological genetics and physiology*. 2014; 321:48–56.
- Kingsolver JG, Higgins JK, Augustine KE. Fluctuating temperatures and ectotherm growth: distinguishing non-linear and time-dependent effects. *The Journal of Experimental Biology*. 2015; 218:2218–2225. [PubMed: 25987738]
- Kirkwood TBL. The disposable soma theory - evidence and implications. *Netherlands Journal of Zoology*. 1993; 43:359–363.
- Kirkwood TBL, Kapahi P, Shanley DP. Evolution, stress, and longevity. *Journal of Anatomy*. 2000; 197:587–590. [PubMed: 11197532]
- Kirkwood TBL, Shanley DP. Food restriction, evolution and ageing. *Mechanisms of Ageing and Development*. 2005; 126:1011–1016. [PubMed: 15893805]
- Kulkarni SS, Canto C. The molecular targets of resveratrol. *Biochim Biophys Acta*. 2015; 1852:1114–1123. [PubMed: 25315298]
- Latta LC, Frederick S, Pfreder ME. Diet restriction and life history trade-offs in short- and long-lived species of *Daphnia*. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*. 2011; 315A:610–617.
- Liao CY, Rikke BA, Johnson TE, Diaz V, Nelson JF. Genetic variation in the murine lifespan response to dietary restriction: from life extension to life shortening. *Aging Cell*. 2010; 9:92–95. [PubMed: 19878144]
- Lynch M. The life history consequences of resource depression in *Daphnia pulex*. *Ecology*. 1989; 70:246–256.
- Lynch M, Ennis R. Resource availability, maternal effects, and longevity. *Exp Gerontol*. 1983; 18:147–165. [PubMed: 6884438]
- Lynch M, Pfreder M, Spitze K, Lehman N, Hicks J, Allen D, Latta L, Ottene M, Bogue F, Colbourne J. The quantitative and molecular genetic architecture of a subdivided species. *Evolution*. 1999; 53:100–110.
- MacArthur JW, Baillie WHT. Metabolic activity and duration of life. I. Influence of temperature on longevity in *Daphnia magna*. *Journal of Experimental Zoology*. 1929; 53:221–242.
- McCauley E, Murdoch WW, Nisbet RM. Growth Reproduction Mortality of *Daphnia pulex* Leydig: Life at Low Food. *Functional Ecology*. 1990; 4:505–514.
- Mironidis GK, Savopoulou-Soultani M. Development, survivorship, and reproduction of *Helicoverpa armigera* (Lepidoptera: Noctuidae) under constant and alternating temperatures. *Environ Entomol*. 2008; 37:16–28. [PubMed: 18348792]
- Murthy M, Ram JL. Invertebrates as model organisms for research on aging biology. *Invertebr Reprod Dev*. 2015; 59:1–4. [PubMed: 26241448]
- Nelson JF, Gosden RG, Felicio LS. Effect of dietary restriction on estrous cyclicity and follicular reserves in aging C57BL/6J mice. *Biology of Reproduction*. 1985; 32:515–522. [PubMed: 4039610]
- Niehaus AC, Angilletta MJ Jr, Sears MW, Franklin CE, Wilson RS. Predicting the physiological performance of ectotherms in fluctuating thermal environments. *J Exp Biol*. 2012; 215:694–701. [PubMed: 22279077]
- NIH. Genetic & Genomic Resources for Model Organisms. <https://www.nigms.nih.gov/Research/models/Pages/default.aspx> in: <http://www.nih.gov/science/models/daphnia/>, ed

- Park I, Lee Y, Kim HD, Kim K. Effect of Resveratrol, a SIRT1 Activator, on the Interactions of the CLOCK/BMAL1 Complex. *Endocrinol Metab* (Seoul). 2014; 29:379–387. [PubMed: 25309798]
- Pietrzak B. Interclonal differences in age-specific performance in *Daphnia magna*. *Journal of Limnology*. 2011:1–8.
- Podrabsky JE, Somero GN. Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish. *Austrofundulus limnaeus Journal of Experimental Biology*. 2004; 207:2237–2254. [PubMed: 15159429]
- Richman S. The transformation of energy by *Daphia pulex*. *Ecological Monographs*. 1958; 28:273–291.
- Schumpert C, Handy I, Dudycha JL, Patel RC. Relationship between heat shock protein 70 expression and life span in *Daphnia*. *Mechanisms of Ageing and Development*. 2014; 139:1–10. [PubMed: 24814302]
- Seebacher F, Murray SA, Else PL. Thermal Acclimation and Regulation of Metabolism in a Reptile (*Crocodylus porosus*): The Importance of Transcriptional Mechanisms and Membrane Composition. *Physiological & Biochemical Zoology*. 2009; 82:766–775. [PubMed: 19732017]
- Shanley DP, Kirkwood TBL. Calorie restriction and aging: A life-history analysis. *Evolution*. 2000; 54:740–750. [PubMed: 10937249]
- Shaw J, Colbourne J, Davey J, Glaholt S, Hampton T, Chen C, Folt C, Hamilton J. Gene response profiles for *Daphnia pulex* exposed to the environmental stressor cadmium reveals novel crustacean metallothioneins. *BMC Genomics*. 2007; 8(477)
- Solon-Biet SM, Walters KA, Simanainen UK, McMahon AC, Ruohonen K, Ballard JWO, Raubenheimer D, Handelsman DJ, Le Couteur DG, Simpson SJ. Macronutrient balance, reproductive function, and lifespan in aging mice. *Proceedings of the National Academy of Sciences*. 2015; 112:3481–3486.
- Stearns, SC. *The evolution of life histories*. Oxford, UK: Oxford University Press; 1992.
- Steinberg, CEW. *Player in Evolution*. Dordrecht: Springer, Netherlands; 2012. *Stress Ecology: Environmental Stress as Ecological Driving Force and Key*.
- Sun L, Wang Y, Song Y, Cheng XR, Xia S, Rahman MR, Shi Y, Le G. Resveratrol restores the circadian rhythmic disorder of lipid metabolism induced by high-fat diet in mice. *Biochem Biophys Res Commun*. 2015; 458:86–91. [PubMed: 25640840]
- Swindell WR. Dietary restriction in rats and mice: a meta-analysis and review of the evidence for genotype-dependent effects on lifespan. *Ageing Res Rev*. 2012; 11:254–270. [PubMed: 22210149]
- van As JG, Combrinck C, Reinecke AJ. An experimental evaluation of the influence of temperature on the natural rate of increase of *Daphnia pulex* De Geer. *Journal of the Limnological Society of South Africa*. 1980; 6:1–4.
- Wada H, Sewall KB. Introduction to the symposium-uniting evolutionary and physiological approaches to understanding phenotypic plasticity. *Integr Comp Biol*. 2014; 54:774–782. [PubMed: 24973363]
- Warner DA, Buckelew AM, Pearson PR, Dhwan A. The effect of prey availability on offspring survival depends on maternal food resources. *Biological Journal of the Linnean Society*. 2015; 115:437–447.
- Williams CM, Marshall KE, MacMillan HA, Dzurisin JD, Hellmann JJ, Sinclair BJ. Thermal variability increases the impact of autumnal warming and drives metabolic depression in an overwintering butterfly. *PLoS One*. 2012; 7:e34470. [PubMed: 22479634]

Highlights

1. A daily fluctuating temperature regimen delays reproduction in *Daphnia pulex* compared to an equivalent constant temperature regimen.
2. Additional energy input does not eliminate the cost of fluctuating temperature on early life reproduction in daphnia.
3. Fluctuating temperature had minimal effect on lifespan.
4. Caloric restriction at the chosen food levels did not extend lifespan.
5. No cost of reproduction was realized in terms of lifespan at maximal energy input.

Fig. 1. Predictions. (A) Predictions for life history trade-offs of lifespan (left axis with black line) and reproduction (right axis with grey line) with varying food concentrations. At the highest food concentrations, we predict to see highest reproduction with a trade-off in lifespan. With caloric restriction we expect to see a reallocation of energy to extend of lifespan with a decrease in reproduction as indicated by the arrows on the Y axes. (B) Prediction for the effect of fluctuating temperature (dashed line) on extension of lifespan relative to constant temperature (solid line). If the fluctuations are energetically costly we would expect to see the effects of caloric restriction with the extension of lifespan to occur at a higher food concentration relative to the constant temperature. Stars indicate the maximum lifespans. C and D are prediction for the fluctuating temperature (dashed lines) relative to the constant temperature (solid lines) on reproductive output across the lifespan at two food concentrations (grey versus black lines). (C) Predicts the energetic cost of the fluctuating environment is consistent in decreasing reproduction across food treatments, (D) predicts high food conditions can alleviate the cost of fluctuations.

Fig. 2.

Daily temperature profiles for the constant (black) and fluctuating (red) temperature chambers. Plotted are averages and standard errors for every 5 minutes over a week.

Fig. 3. Survival curves for the twelve populations. Food concentration is color-coded: A red, B orange, C green, D cyan, E dark blue, F purple. Solid lines represent constant temperature treatments, dashed lines represent fluctuating temperature treatments. A. Both temperature regimes combined. B. Temperature regimes separated. (ABC) have significantly shorter survival than higher food concentrations.

Fig. 4. Reproductive profiles over the lifespan. Food concentration treatments are rainbow color-coded to indicate increasing food: A red, B orange, C green, D cyan, E dark blue, F purple. The left column shows the raw data and right column shows the smoothed curves. Panels A and B have temperature regimes are combined. Panels C and D have food treatments combined with constant temperature as the solid line and fluctuating temperature the dashed line. Panels E and F have each treatment separated.

Fig. 5. Time to first reproduction. Food concentration treatments are rainbow color-coded to indicate increasing food: A red, B orange, C green, D cyan, E dark blue, F purple. Extreme low food ratio delays reproduction (A and B treatment). (A) Both temperature regimes combined. (B) Temperature regimes separated; Constant solid lines, fluctuating dashed lines.

Fig. 6.

Reaction norms for age related traits across increasing levels of food. Solid lines represent Constant temperature regimens, and dashed lines are Fluctuating temperature regimens. We provide these reactions norms as an alternative way to visualize and summarize the data for comparison to other studies, but they do not represent the primary analytical strategy. Points represent averages with standard errors.

Fig. 7.

Reaction norms for average reproductive output over that age range for individuals that reached each of the respective age categories. Solid lines represent Constant temperature regimen, and dashed lines are Fluctuating temperature regimen. We provide these reactions norms as an alternative way to visualize and summarize the data, but they do not represent the primary analytical strategy. Points represent averages with standard errors for the number of offspring produced in (A) early-life, first 15 days; (B) mid-life, between days 16–25; and (C) late-life, between days 26–35, and (D) old-age, 36 days and older.

Table 1

Summary of statistical results, where Temp refers to the two temperature regimens of either constant or fluctuating temperature. Food refers to the six food concentration treatments, and Time refers to the day of the life.

Variable	Temp	Food	Temp × Food	Temp × Time; Food × Time	Pattern
Reproduction over the lifespan	N/A ^(‡)	N/A	p = 0.25	p < 10 ⁻¹⁰ for both interactions	Broods are smaller and (a bit) later with fluctuating temp; lower food means smaller broods
Time to Reproduction	p < 10 ⁻¹⁰ ([†])	p < 10 ⁻¹⁰	p = 0.08		Fluctuating temp delays onset; lower food delays onset
Day of Peak Reproduction	N/A	N/A	p = 0.026		Fluctuating temp delays peak day in the lowest and highest food treatments (A, B, F)
Lifetime Fecundity	N/A	N/A	p = 5 × 10 ⁻⁵		Fluctuating temp results in lower fecundity at intermediate food concentrations (C, D, E)
Reproductive Lifespan	0.04	p < 10 ⁻¹⁰	p = 0.072		Fluctuating temp means shorter reproductive lifespans (due to starting later); same for lower food
Reproductive Lifespan (if we remove those with 0 spans, as in the % repro and day peak)	0.53	p < 10 ⁻¹⁰	p = 0.21		Lower food means shorter reproductive lifespans
% Repro Lifespan	p = 0.88	p < 10 ⁻¹⁰	p = 0.14		Lower food means less of the lifespan is reproductive
Lifespan	p = 0.94	p < 10 ⁻¹⁰	p = 0.072		Lower food concentrations (A, B, C) reduce lifespan

([†]) As mentioned in the main text, the tests that we are using make some assumptions (e.g., proportionality of hazards) that, while not observed to be egregiously violated in this experiment, are unlikely to perfectly hold in practice. For this reason, and because we have some very stark differences among the outcomes in the most extreme food treatment groups, presenting exact p-values with a level of precision such as p = 3 × 10⁻²⁶⁴ would not be meaningful in this context, other than indicating that we have 'really small p-values'. Thus we will report p < 10⁻¹⁰ in these cases.

([‡]) If an interaction involving a measurement is significant, it is not meaningful to test for the significance of the main effect by itself. We denote such cases via N/A