UNIVERSITY OF CALIFORNIA SAN DIEGO

The roles of adaptation and plasticity in populations persistence in the face of multiple stressors

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by

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The Dissertation of Hamanda Badona Cavalheri is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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ABSTRACT OF THE DISSERTATION

The roles of adaptation and plasticity in populations persistence in the face of multiple stressors

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Global environmental changes are rapidly transforming ecosystems. As a result, populations will face changes in environmental conditions over short timescales that can threaten their persistence. When dispersal to more suitable habitats is not possible, the threat of extinction can be ameliorated through distinct, but non-exclusive biological mechanisms, including phenotypic plasticity, which refers to changes in traits mostly common due to shifts in gene expression, and genetic adaptation by rapid evolutionary shifts in gene frequencies. In my dissertation research, I studied the phenotypic and molecular population responses of a freshwater zooplankton (*Daphnia*) from Sierra

Nevada mountain lakes of California to anthropogenic environmental variation caused by climate change and introduced predators. Hence, populations with different environmental histories may diverge in response to changing selective pressures, such as those caused by such as warming and introduced predators, because they might differ on the level of plasticity and adaptation they show to current environmental conditions. In Chapter 1, I show that different *Daphnia* populations were equally able to evolve plasticity in response to selection by different temperature regimes for two years in field experiment. This results highlights the importance of evolution of plasticity in response to temperature regime, thus in Chapter 2, I explore whether or not a population of *Daphnia* exhibits genetic variation that confers shifting plasticity in response to temperature throughout the annual cycle, since there is strong seasonal variability in temperature in most lakes located in temperate climates Studying *Daphnia* population from Blue Lake I found a marked distinction in plastic responses between *Daphnia* collected in the beginning and end of the growing season, indicating that seasonal temperature variability has distinct effects on plasticity for different traits. In Chapter 3 I studied the possible molecular mechanisms behind phenotypic response to temperature and how it is affected by predation. I studied two *Daphnia* genotypes collected from Blue and Gardisky Lakes. Overall our results suggest that both genotypes can reach similar fitness through distinct underlying molecular mechanisms, but also that they have distinct environmental sensitivities to stressors. Together these findings illustrate the variation and the importance of plasticity in addition to the potential for its evolution in maintaining *Daphnia* populations as key species in freshwater ecosystems, helping to mitigate the effects of climate change on food webs and ecosystem processes.

INTRODUCTION

Global environmental changes are rapidly transforming ecosystems, challenging the survival and persistence of organisms. The duration, frequency and extent of extreme events (e.g. droughts, heat waves) are expected to change over the next century such that daily and seasonal minimum and maximum temperatures are projected to increase globally, and the amplitude of climate cycles may also shift (Easterling et al. 2000, Meehl and Tebaldi 2004, Vasseur et al. 2014). As a result, populations will face changes in environmental conditions over short timescales that can threaten their persistence. The threat of extinction can be ameliorated through distinct, but non exclusive biological mechanisms, including 1) phenotypic plasticity, which refers to changes in traits mostly common due to shifts in gene expression (Bradshaw 2006, Lande 2009, Chevin et al. 2010); 2) range shifting, by dispersing to more suitable habitats (Parmesan 2006, Chen et al. 2011); 3) and genetic adaptation by rapid evolutionary shifts in gene frequencies (Bell and Collins 2008).

Recent studies demonstrate that genetic adaptation can rescue populations from extinction in response to changing climatic conditions (Nussey et al. 2005, Bradshaw 2006, Bradshaw and Holzapfel 2008), and several studies have found genetic variation among populations in relevant traits, indicating a potential for genetic adaptation over ecological time-scales (Jump and Penuelas 2005, Millien 2006). Adaptive responses can prevent population decline if there is sufficient genetic variation and selection on heritable traits that increases fitness (Gomulkiewicz and Holt 1995). However, there is no consensus as to how effective microevolution will be in mitigating consequences of ongoing environmental

changes. In fact, if insufficient standing genetic variation is present in a population, or if migration is too slow to introduce new adaptive genotypes, a population may decline to extinction before evolutionary rescue can occur (Carlson et al. 2014). Hence, if dispersal to more suitable habitats is not possible, plasticity may play a vital role in rescuing populations from rapid environmental change because plasticity can enable organisms to rapidly adjust to novel conditions (Charmantier et al. 2008, Gienapp et al. 2008, Merilä 2012, Munday et al. 2013).

Phenotypic plasticity, or plastic response, is the ability of an organism's genotype to adjust its phenotype in response to an environmental cue (DeWitt et al. 1998, Pigliucci 2001). Most traits are plastic to some degree, but the level of plasticity may vary in different populations in response to the same stressor. The degree to which plasticity varies can be adaptive, depending on whether the induced phenotype is closer to the optimum phenotype for a particular environmental condition (e.g. Ghalambor et al. 2007). Nevertheless, evolution of adaptive plasticity may be limited by a lack of genetic variation, by developmental constraints on the capacity of the genotype to produce alternative phenotypes, or by trade-offs where the potential increase in fitness due to plasticity is offset by a decrease in fitness due to negative effects on other traits. Therefore, populations that have evolved under different environmental conditions may exhibit different levels of plasticity in response to the same environmental cue, which can have a significant impact on an organisms' fitness in response to environmental change.

Plasticity is expected to be favored and maintained by natural selection in variable environments. Theory predicts that plasticity will evolve in more heterogeneous environments with reliable cues, where its benefits outweigh its costs and genetic basis for

plasticity exists in the population (Berrigan and Scheiner 2004, Chevin and Hoffmann 2017). A powerful way to evaluate the relationship between environmental heterogeneity and plasticity is to either compare populations arrayed along natural gradients, such as latitudinal or elevational gradients, or compare turnover of individuals in seasonal environments (Parmesan and Yohe 2003, Ghalambor et al. 2007, Deutsch et al. 2008, Hoffmann and Sgrò 2011). For instance, the degree of thermal plasticity has been shown to be proportional to the magnitude of temperature variation experienced in the local environment (Addo-Bediako et al. 2000, Khaliq et al. 2014). Thus, populations with different environmental histories may differ in the level of plasticity they present, which in turn may cause divergence in their response to novel selective pressures (Adams and Collyer 2009).

The most important mechanism of plasticity is changes in gene expression due to up- or down-regulation of genes (Ancel 2000, West-Eberhard 2003). Many studies emphasize that plasticity at the level of gene expression is one of the most important mechanisms of coping with thermal stress (Townsend et al. 2003, Ranz and Machado 2006; Hoffmann and Willi 2008, Yampolski et al. 2014a, Chowdhury et al. 2015). Investigating the adaptive value of regulatory plasticity is important for elucidating whether or not this mechanism may rescue populations from extinction in a warming climate, and whether populations respond with enough plasticity to environmental changes to dampen their effects.

For my dissertation research, I studied the phenotypic and molecular population responses of a freshwater zooplankton (*Daphnia*) to anthropogenic environmental variation caused by climate change and introduced predators. My study system was Sierra

Nevada mountain lakes of California, which are clearly defined habitats situated along independent gradients of temperature and fish stocking, with distinct populations in each lake. *Daphnia* exhibit small body sizes, asexual reproduction and short life span, making them an ideal model system for studying the effects of plasticity in mitigating stress caused by anthropogenic environmental changes (Miner et al. 2012).

Freshwater ecosystems and environmental change

Freshwater ecosystems are among the most vulnerable to anthropogenic stressors. Physical processes in lakes are changing as a result of climate warming, including longer durations of ice-free periods and reduced vertical mixing (McCormick 1990, Schindler et al. 1990, Adrian et al. 1995). Additionally, lake chemistry can be indirectly affected by warming, due to changes in the amount of precipitation and snowmelt that supply water, dissolved nutrients and organic matter to lakes. Despite these changes, trophic structure of lake food webs, among other biotic factors, may mediate the ecosystem response to warming. In high elevation mountain lakes worldwide, naturally fishless lakes have been stocked with fish to create recreational fishing opportunities, with marked ecological effects (Sarnelle and Knapp 2005, Woodward et al. 2010) due to fish predation that causes cascading trophic interactions (Carpenter et al. 1985). Therefore, the presence of an introduced predator may interact with temperature to determine the ecosystem response to warming. The impact of both stressors on the composition and traits of species may lead to cascading effects on trophic interactions, impacting freshwater ecosystem functioning (e.g. Poff et al. 2002, Jeppesen et al. 2010, Symons and Shurin 2015).

Study system

Sierra Nevada alpine lakes (CA) vary along an elevational gradient in temperature and in history of fish stocking. These lakes are located in subalpine and alpine zones and are generally small (0.5-10 ha of surface area), shallow (less than 15 m in depth) and oligotrophic (Harper-Smith et al. 2005). There is little variation in physical and chemical characteristics since the majority of lakes are located on substrates of granite and granodiorite rock (Melack et al. 1985). Moreover, the ice-free period lasts approximately 4 months per year, resulting in low biodiversity (Melack et al. 1985).

In the Sierra Nevada, fish stocking began in the mid-1800s for recreational purposes (Knapp 1996); prior to this period, lakes were fishless because physical barriers prevented the movement of fish upstream (Knapp 1996). Although the fish stocking program has ended, currently more than 80% of lakes contain fish, and the most common species are rainbow trout (*Oncorhynchus mykiss*), golden trout (*O. m. auguabonita*), and brook trout (*Salvelinus fontinalis*) (Knapp 1996, Knapp and Matthews 2000, Harper-Smith et al. 2005). While fish went extinct in some lakes after the termination of stocking, other lakes have self-sustaining populations (Harper-Smith et al. 2005).

The primary ecological consequences of fish introduction in these lakes were a decrease in zooplankton richness and shift in community composition with extinction of large crustacean zooplankton species, which were replaced by small crustacean species (Knapp and Matthews 2000, Knapp et al. 2001). Zooplankton species that have survived fish introduction rapidly evolved traits associated to predation, such as smaller body size or decreased expression of melanin (Latta et al. 2007, 2010, Scoville and Pfrender 2010). Because zooplankton species are smaller in lakes with fish, their per capita grazing rate has

decreased (Bradford et al. 1994, Knapp 1996). Lakes with fish have higher phytoplankton biomass than fishless lakes, where zooplankton species are larger (Sarnelle and Knapp 2005). Nevertheless, in lakes where fish were removed or became extinct, zooplankton communities were able to recover and are now similar to those of fishless lakes (Knapp and Sarnelle 2008). Lakes situated at different elevations also exhibit different zooplankton community composition and traits. Symons and Shurin (2016) found that fishless lakes arrayed along an elevation gradient in Sierra Nevada (CA) have marked differences in zooplankton species composition. However, lakes with fish converge to a similar composition as low elevation lakes, indicating that higher temperature and fish presence selects for similar species composition. Hence, organisms distributed across an elevation gradient in temperature, as seen in alpine lakes throughout the Sierra Nevada of California (USA), are ideally suited for investigation of the contribution of adaptive plasticity in response to heat stress as well as the effects of introduced predator on thermal response, and whether the degree of population differentiation influences the level of plasticity.

The large bodied zooplankter *Daphnia* occurs in permanent and intermittent bodies of water worldwide and is a key species in freshwater ecosystems due to its significant role in food-web dynamics (Miner et al. 2012). *Daphnia* is a pelagic filter feeder with the potential for high population growth rate. Its large influence on phytoplankton grazing and nutrient cycling, combined with its role as a preferred prey species for fish, place *Daphnia* in a central position in lake food webs (Brooks and Dodson 1965, Miner et al. 2012). In addition, *Daphnia* populations possess a range of genetically based morphological, physiological and behavioral adaptations that influence the strength of interactions with its predators and resources (Miner et al. 2012, Boersma et al. 1998, Havens et al. 2015).

Studies have repeatedly shown a decrease in body size of zooplankton in response to warming because high temperature increases the cost of development and respiration more than ingestion (Moore et al. 1997). Additionally, large-bodied zooplankton show declines in fecundity with warming, which is compensated by a decrease in developmental rates, resulting in smaller size at maturity, fewer eggs produced and shorter brood duration time (Orcutt and Porter 1983, Moore et al. 1997). Populations of *Daphnia* are capable of rapid evolution in response to warmer temperatures, but this response is context dependent, such that traits are affected differentially by temperature depending on other co-occuring environmental conditions, for instance presence of predators, parasites, or competitors (Van Doorslaer et al. 2009, De Meester et al. 2011).

Fish predation is also a strong selective force on zooplankton populations. A reduction in individual body size within and between species is a frequently observed response to size selective fish predation in zooplankton (Jeppesen et al. 2007, Hart and Bychek 2011, Havens et al. 2015). Cladocerans, such as *Daphnia*, can also respond by changing life history traits, for instance, producing many small eggs and juveniles that mature at a smaller size, as an adaptation to avoid reaching a size where they are visible to fish. Morphological traits like helmet and neck spine development, or behaviors, like vertical migration, phototactic swimming, and alertness (Boersma et al. 1998) have also been shown to respond to fish. The adaptive value of morphological and behavior defenses has been demonstrated, as *Daphnia* from populations that were previously exposed to predators had higher survival, due to change in phototactic behavior or developing neck spine or helmet in the presence of predator (Pijanowska et al. 1993, De Meester 1996, Laas and Spaak 2003 and references herein). Finally, Cousyn et al. (2001) studied adaptation in

D. magna that lived under predation for 30 years and found correlated genetic markers in predator-avoidance traits, which are in agreement with the hypothesis of genetic adaptation. This body of research demonstrates that predation is a strong selective pressure and causes phenotypic and genetic changes in zooplankton species. Furthermore, *Daphnia* show similar phenotypic responses to predation by fish and high temperature because both exert pressure on some of the same traits and in the same direction, including smaller size and earlier age at maturity, as well as fewer eggs and smaller neonates (Abrams and Rowe 1996, De Meester et al. 2011, Moore et al. 1997, Taylor and Gabriel 1993).

Daphnia show high responsiveness to stressors not only at the phenotypic level but also at the molecular level. Several genes related to oxidative stress metabolic pathways are important in ectotherms living under thermal stress (Yampolsky et al. 2014). Additionally, Colbourne et al. (2011) studied the *D. pulex* genome and found that variation in expression of specific genes was associated with several ecological and environmental factors. There is evidence that both temperature and predation change expression of stress proteins. (Kawaga and Mugiya 2002, Paulwels et al. 2005, 2007). However, a large number of genes are potentially differently regulated under predation (Tollrian and Leese 2010). Hence, responses to both stressors can involve different genetic pathways and their interaction might lead to additive effects (Chu et al. 2014). This makes *Daphnia* populations from Sierra Nevada lakes (CA) an ideal system in which to investigate the impact of different predation histories on plastic responses to anthropogenic warming and the possible molecular mechanism behind this response.

Summary of the chapters

Anthropogenic environmental challenges such as climate warming and interactions with non-native predators are strong selective pressures that constantly shape phenotypic traits of species (e.g. Darimont et al. 2009). These novel selective pressures can influence reaction norms of traits and are context-dependent because other environmental stressors might increase or buffer the vulnerability of populations to global warming (e.g. Parmesan 2006). Hence, populations with different environmental histories may diverge in response to changing selective pressures because they differ in the level of ancestral plasticity and/or adaptation to previous conditions (Adams and Collyer 2009). Thus, the nature of the response to both temperature and predation can vary among *Daphnia* populations from Sierra Nevada lakes due to local adaptation under divergent selection.

In Chapter 1, I show that *Daphnia* populations from high and low elevation lakes with and without histories of fish predation were equally able to evolve plasticity in response to selection by different temperature regimes for two years in field mesocosms. Some life history traits showed the imprint of the natal environment after two years of selection at different temperatures. However, phenotypic plasticity in response to temperature evolved over the short time frame of the experiment, indicating that genetic variation for plasticity provides broad phenotypic variation on which selection may act.

Since plasticity evolved in response to temperature regime (Chapter 1), in Chapter 2, I explore whether or not a population of *Daphnia* exhibits genetic variation that confers shifting plasticity in response to temperature throughout the annual cycle, when water temperature in lakes can vary from 4°C to greater than 20°C. Strong seasonal variability in temperature causes most lakes located in temperate climates to experience thermal

stratification, a phenomenon wherein lakes separate into distinct thermal layers. In addition, large zooplankton species that occur in temperate lakes often perform diel vertical migration throughout the water column. Hence, stratification has important effects on zooplankton as they regularly experience a large daily temperature range as they travel throughout the water column in stratified lakes (Stich and Lampert 1981), resulting in a high degree of phenotypic plasticity in response to temperature variability. Plasticity differed seasonally between *Daphnia* collected in mid-summer and early-fall. Some traits showed higher levels of plasticity in mid-summer when the lake experienced peak thermal stratification, compared to early-fall conditions, while for other traits the opposite happened, plasticity was lower in the mid-summer and increased in early-fall. These results suggest a marked distinction in plastic responses between organisms collected in the beginning and end of the growing season and indicate that seasonal temperature variability has distinct effects on plasticity for different traits.

Given the importance of plasticity for the *Daphnia* populations studied in the previous chapters, in Chapter 3 I studied the possible molecular mechanisms behind phenotypic response to temperature and how it is affected by other factors, such as predation. Plasticity is directly related to gene expression and has been shown to play a role in adaptive evolution (Miner et al. 2005), and populations' persistence or extinction in changing environments (Chevin et al. 2010). However, the mechanism by which a plastic response is triggered by environmental cues is not clear, especially the response to combined effects of stressors, such as introduced predators and climate. We studied response of two distinct genotypes of *Daphnia* collected from different lakes in Sierra Nevada (CA). Results show that phenotypic response was similar for the two clones we

tested. In both genotypes temperature showed more differentially expressed genes than predation. We found opposite regulation patterns for genes related to metabolism, despite the fact that genotypes showed similar phenotypic responses. We also found overrepresentation of genes related to reproduction and oxygen binding in high temperatures at the molecular, which matched phenotypic response of larger individuals at high temperature and more offspring. Both clones down-regulated anabolic activities, which indicates decreasing energy expenditure. Predation triggered up-regulation genes possibly related to meiosis. Overall our results suggest that organisms can reach similar fitness through distinct underlying molecular mechanisms, but also that they have distinct environmental sensitivities to stressors.

These studies elucidate how biotic and abiotic factors impact phenotypic and molecular responses in *Daphnia*. At a broader level, studying the adaptive potential that arises from genetic and phenotypic processes is important in order to understand the mechanism more likely to confer resilience to keystone species in response to a warming climate and also indicate whether other selective agents, such as introduced predators, amplifies or dampens the potential for adaptation or phenotypic plasticity to rescue *Daphnia* populations from extinction due to climate warming. Here, I corroborate previous findings that *Daphnia* populations are able to evolve in response to anthropogenic stressors at temporal and spatial scales (e.g. Yampolsky et al. 2014b, Geers et al. 2015, Brans et al. 2017) and showed that despite phenotypic response can be similar among populations, the molecular mechanism leading to similar response can vary considerably. These findings illustrate the importance of plasticity and the potential for its evolution in maintaining *Daphnia* populations as key species in freshwater ecosystems, helping to mitigate the

effects of climate change on food webs and ecosystem processes.

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CHAPTER 1

Rapid evolution of thermal plasticity in mountain lake *Daphnia* populations

Abstract

Populations at risk of extinction due to climate change may be rescued by adaptive evolution or plasticity. Selective agents, such as introduced predators, may enhance or constrain plastic or adaptive responses to temperature. We tested responses of *Daphnia* to temperature by collecting populations from lakes across an elevational gradient in the presence and absence of fish predators (long-term selection). We subsequently grew these populations at two elevations in field mesocosms over two years (short-term selection), followed by a common-garden experiment at two temperatures in the lab to measure life-history traits. Both long-term and short-term selection affected traits, suggesting that genetic variation of plasticity within populations enabled individuals to rapidly evolve plasticity in response to high temperature. We found that short-term selection by high temperature increased plasticity for growth rate in all populations. Fecundity was higher in populations from fishless lakes and body size showed greater plasticity in populations from warm lakes (long-term selection). Neither body size nor fecundity were affected by short-term thermal selection. These results demonstrate that plasticity is an important component of the life-history response of *Daphnia*, and that genetic variation within populations enabled rapid evolution of plasticity in response to selection by temperature.

Introduction

Rapid environmental change including changes in temperature can push species beyond their physiological boundaries, threatening their persistence (Parmesan 2006). When dispersal to suitable habitats is limited, populations can be rescued from extinction through two distinct but non-exclusive mechanisms: genetic adaptation, or phenotypic plasticity (Merilä and Hendry 2014). Genetic adaptation occurs by shifts in gene frequencies (Bell and Collins 2008), while phenotypic plasticity involves changes in traits without changes in DNA that can occur by mechanisms such as shifts in gene expression or DNA methylation (Lande 2009, Chevin et al. 2010). Understanding the relative importance of genetic adaptation and phenotypic plasticity as mechanisms to cope with environmental change is crucial for predicting population persistence.

Recent studies demonstrate that genetic adaptation can rescue populations from extinction in response to changing climatic conditions (Nussey et al. 2005, Bradshaw 2006, Bradshaw and Holzapfel 2008), including rapid adaptation of increasing thermal tolerance (e.g. Geerts et al. 2015, Padfield et al. 2016). Adaptive responses can prevent population decline if there is sufficient genetic variation and selection on heritable traits that increases fitness (Gomulkiewicz and Holt 1995). However, there is no consensus as to how effective microevolution will be in mitigating consequences of ongoing environmental changes. In fact, if insufficient standing genetic variation is present in a population, or if migration is too slow to introduce new adaptive genotypes, a population may decline to extinction before evolutionary rescue can occur (Carlson et al. 2014). Hence, plasticity may play an important role as a mechanism to help populations to cope with rapid environmental change

because plasticity can enable organisms to rapidly adjust to novel conditions (Charmantier et al. 2008, Gienapp et al. 2008, Merilä 2012, Munday et al. 2013).

Plasticity is expected to be favored and maintained by natural selection in variable environments. Theory predicts that plasticity will evolve in more heterogeneous environments with reliable cues, where its benefits outweigh its costs and genetic basis for plasticity exists in the population (Berrigan and Scheiner 2004, Chevin and Hoffmann 2017). A powerful way to evaluate the relationship between environmental heterogeneity and plasticity is to compare populations arrayed along natural temperature gradients in latitude or elevation (Parmesan and Yohe 2003, Ghalambor et al. 2007, Deutsch et al. 2008, Hoffmann and Sgrò 2011). The degree of thermal plasticity has been shown to be proportional to the magnitude of temperature variation experienced in the local environment (Addo-Bediako et al. 2000, Khaliq et al. 2014). Thus, populations with different environmental histories may differ in the level of plasticity they present, which in turn may cause divergence in their response to novel selective pressures (Adams and Collyer 2009).

Temperature is one of many aspects of the environment undergoing rapid change (Sala et al. 2000) and selection imposed by other environmental changes could influence population vulnerability to thermal stress (Parmesan 2006). For instance, water fleas (genus *Daphnia*), a dominant freshwater zooplankter, respond similarly to predation by fish and high temperature because both exert pressure on the same traits and in the same direction, including smaller size and earlier age at maturity (Taylor and Gabriel 1993, Moore et al. 1997, De Meester et al. 2011). Previous studies have shown that elevation and fish predation can have important consequences for populations and communities. For instance,

lake surveys in the Sierra Nevada (CA) showed that communities sympatric with fish at all elevations contain taxa characteristic of low elevation fishless lakes (Symons and Shurin 2016). Selection by fish predation and warmer temperatures tend to result in a decrease in body size, which reduces the ability of fish to detect *Daphnia*, and higher investment in reproduction (Riessen 1999). Therefore, both biotic and abiotic factors can drive population responses to warming (e.g. Tseng and O'Connor 2015).

Here we examine how biotic and abiotic selection interact to influence thermal plasticity in *Daphnia* populations originating from different long-term (lake conditions) and short-term (2-year mesocosms) environments. These *Daphnia* populations are native to alpine lakes in the Sierra Nevada (CA). The populations originate from lakes that vary in temperature due to elevation, and fish presence due to a century of salmonid stocking in these historically fishless lakes (hereafter long-term selection). We collected populations from high and mid-elevation lakes with and without fish predators, and then exposed them to cold and warm temperatures in a mesocosm experiment for two years (hereafter short-term selection). We then isolated clones from the field experiment and used a common-garden laboratory experiment to determine how selection on short- and long-time scales influenced the degree of phenotypic plasticity in *Daphnia* life-history in response to temperature.

We predicted that local adaptation would result in clones with the highest fitness in their home environment condition, i.e. (i) populations from cold lakes would exhibit higher fitness at low temperature, while populations from warm lakes would have higher fitness at higher temperature (crossing reaction norms). We also expected that (ii) after two years of selection populations would evolve greater levels of plasticity in response to increased

maximum temperature in the warm mesocosm treatment (i.e. steeper slopes of the reaction norms), since they would be experiencing higher temperatures.

Materials and Methods

Thermal field experiment

To determine how long- and short-term selection by temperature and fish predation influence life-history responses to temperature, we exposed populations of *Daphnia pulicaria* collected from four different categories of lakes (cold or warm, with fish present or absent), to high or low temperature (19°C and 13°C summer averages respectively) in field mesocosms that mimicked lake environments at two elevations (Symons 2017, Table 1.3 and Figure 1.4). In July 2013, plankton and sediment were collected in 12 lakes located in Sierra Nevada (CA) (Table 1.3). Lakes were categorized by elevation, high and low, and by presence or absence of fish, thus we had four lake categories: warm (low elevation) with fish, warm (low elevation) without fish, cold (high elevation) with fish, and cold (high elevation) without fish. Collections were made from three lakes within each category to inoculate the tanks in order to insure genetic and species diversity within each community type. Plankton were collected at the deepest point in the lake by drawing a 30 cm diameter and 1 m length zooplankton net through the water column, starting 1 m above the lake bottom. Live plankton from the same lake category were mixed together to establish experimental communities. In addition, we collected 6 L of sediment from the shore of each lake in order to include resting stages of invertebrates in the inoculum.

In order to initiate the thermal selection experiment we placed 1000 L mesocosms in UC Natural Reserves at two different elevations: 1200 m at the Sierra Nevada Research

Station in Wawona and 3093 m at White Mountain Research Center. At each elevation we established 5 replicate mesocosms per lake category for 20 mesocosms in total at each elevation. We divided sediment equally among replicates of the same lake category and inoculated zooplankton at the mean density found in the lakes from the same category. We found that the community composition in the mesocosm experiment reflects general patterns found in natural lakes (Symons 2017). The complete design of the thermal field experiment is given in Symons (2017), and includes 20 additional mesocosms per elevation for treatment with fish predators (rainbow trout) present. No *D. pulicaria* persisted in mesocosms with fish, therefore the fish treatment was excluded from this study.

After two years, *Daphnia pulicaria* adult females from each mesocosm were selected to establish maternal lineages. Two years of selection would correspond to about 30 to 50 generations during Spring and Summer in these populations based on what was found in the common-garden experiment (see below). Maternal lines that survived came from 12 different warm mesocosms and 7 different cold mesocosms (Table 1.4). Mortality of maternal lines occurred during transportation from the field to the lab and acclimation to lab conditions. *Daphnia* produced ephippia (dormant eggs resulting from sexual reproduction) in the mesocosms, indicating that genetic diversity was present within and between mesocosms, but we did not establish whether maternal lines were genetically distinct. We quantified life-history parameters of maternal lines isolated from the mesocosms at two incubation temperatures: 13°C and 17°C (± 1°C - hereafter referred to as "test temperatures") under standardized laboratory conditions. Each maternal line was grown at the two test temperatures in the lab, and we constructed life tables for each one of the total 89 *D. pulicaria* maternal lines that survived. Our approach allowed us to

partition the phenotypic response to selection by temperature over long (as determined by the lake of origin) and short, i.e., two years (as determined by the temperature in the mesocosm experiment) time scales.

Life-history experiment

This experiment consisted of 89 maternal lines total (replicates) obtained across all lake categories (four) and mesocosm temperatures (two). Each maternal line was grown at two test temperatures in the lab. To minimize maternal effects, each maternal line was cultured individually in a separate 50 mL tube filled with COMBO medium (Kilham et al. 1998) under standardized conditions (17±1°C and photoperiod 12:12 L:D) for two generations. All animals were fed live culture of the green alga *Nanochloropsis* sp. at a constant high rate of 24×10^6 cells per day. Neonates of the second clutch of the second generation were randomly assigned to each of the two test temperatures (13°C and 17°C). After culturing the animals for an additional two generations at their test temperature, juveniles from the second clutch were used as the experimental generation. Individuals were monitored daily and transferred every 48 hours to clean jars with fresh media until they produced their third clutch. We scored the following life-history variables: age at maturity (when individual released eggs in the brood pouch), size at maturity, and age and number of offspring from each clutch. Data for the first three clutches were used to calculate intrinsic population growth rate (including only those individuals that survived for the duration of the experiment) for each maternal line following the Lotka–Euler equation (Roff 1997).

Statistical Analysis

Correlations between life history traits were tested using Pearson's correlation tests (Figure 1.5). We analyzed the effects of the treatments using linear mixed-effects models for each trait using the *lmer* function from the *lmerTest* package in the statistical software R (Kuznetsova et al. 2017, R Core Team 2017) implemented with restricted maximum likelihood estimation. We also used a cross-validation approach (the *upSample* function from the *caret* package and *vfold_cv* function from the *rsample* package; R Core Team 2017; Kuhn 2018; Kuhn & Wickham 2017) to confirm that our results were not influenced by sample size differences (Figure 1.7). For each response variable, lake elevation, presence or absence of fish, mesocosm temperature and test temperature were modeled as fixed effects, and maternal line was nested within mesocosm as a random effect. We used the same procedure for number of offspring, but included age as an explanatory variable in the model as well. Any interaction that includes test temperature indicates an effect on the level of plasticity, i.e. effects on the slope of the reaction norms. We started with the most complex models for fixed effects (the random effect was kept in all models) and dropped higher order interactions if they did not significantly improve model fit (using log-ratio tests) until we arrived at a best-fit model. All variables, except intrinsic growth rate, were log transformed after visual inspection of data distribution.

Results

The linear mixed-effects model revealed a range of factors that influenced life history (Table 1.1). All *D. pulicaria* maternal lines showed life-history plasticity in response to test temperature. Age at maturity was only affected by test temperature (T, *P*

< 0.001) and decreased 4.5 ± 0.07 days on average when clones were grown at 17°C compared with clones tested at 13°C regardless of the previous conditions experienced by the maternal line (Figure 1.1A, B).

D. pulicaria were smaller at maturity at test temperature 13°C compared to 17°C by 0.1 ± 0.01 mm on average (Figure 1.1C, D). However, the impact of test temperature on body size depended on lake elevation (L * T, $P = 0.012$), indicating different slopes of the reaction norms between populations from warm and cold lakes. Maternal lines from warm lakes typically exhibited greater plasticity in size at maturity than those from cold lakes (Figure 1.2, long-term selection), with crossing reaction norms where warm lake populations matured at smaller size at 13°C but larger size at 17°C.

The number of offspring produced per clutch was influenced independently by fish presence in the ancestral environment. Individuals from lakes with fish had 0.6 ± 0.16 fewer offspring on average than clones from fishless lakes (F, $P = 0.030$, Table 1.2). Fecundity was also influenced by an interaction between age and test temperature (Age x T, $P = 0.019$, Table 1.2). As an individual ages, it produces more offspring per clutch, but the increase in number of offspring per clutch is greater when individuals were tested at 17°C compared to 13°C (Figure 1.3). The number of offspring produced at different test temperatures also varied with mesocosm temperature (M x T, $P = 0.004$, Table 1.2). At 13°C maternal lines from cold mesocosms showed a steeper slope than those from warm mesocosms, while the opposite is seen for maternal lines tested at 17°C (Figure 1.3).

Intrinsic growth rate was approximately two times greater at test temperature 17°C ($0.25*day^{-1}$) than at test temperature 13°C ($0.12*day^{-}1$; T, $P < 0.001$). An interaction between mesocosm temperature and test temperature revealed that the intrinsic growth rate

of maternal lines from warm mesocosms increased by 0.03*day^{-1} more than maternal lines from cold mesocosms when grown at 17°C compared to 13°C (M * T, P = 0.004; Figure 1.1E, F), i.e., populations that spent two years in warm mesocosms showed steeper slopes of the reaction norms than ones from cold mesocosms (Figure 1.1E, F). All individuals reared in the 13°C test temperature had the same growth rate, regardless of long- or short-term selection while differences among lake and mesocosm treatments were expressed at 17°C. Specifically, at 17°C*, Daphnia* from warm mesocosms had a higher intrinsic growth rate compared to maternal lines from cold mesocosms (Figure 1.2 short-term selection).

Table 1.1. Results of stepwise model selection between long- and short-term selection and their interactions for *Daphnia pulicaria* life-history traits.

	Estimate	Std. Error	df	T value	Pr(>|t|)
Age at maturity					
(Intercept)	2.335	0.027	175	85.099	**<0.001**
Lake temperature (L)	-0.018	0.022	175	-0.843	0.400
Fish in the lake (F)	-0.010	0.022	175	-0.462	0.644
Mesocosm temperature (M)	-0.015	0.022	175	-0.705	0.482
Test temperature (T)	-0.582	0.022	175	-26.153	**<0.001**
Size at maturity					
(Intercept)	0.339	0.020	32.50	16.932	**<0.001**
Lake temperature (L)	-0.038	0.022	15.87	-1.735	0.102
Fish in the lake (F)	0.011	0.021	21.24	0.520	0.608
Mesocosm temperature (M)	-0.013	0.021	24.32	-0.633	0.532
Test temperature (T)	0.054	0.021	14.67	2.491	**0.013**
L x F	-0.026	0.020	12.73	-1.288	0.220
L x M	0.037	0.020	12.38	1.833	0.091
L x T	0.050	0.019	14.75	2.531	**0.012**
F x M	0.060	0.020	11.15	0.293	0.774
F x T	-0.029	0.019	14.76	-1.499	0.136
M x T	0.047	0.020	14.73	0.002	0.998
Intrinsic growth rate					
(Intercept)	0.134	0.001	127	11.268	**<0.001**
Lake temperature (L)	-0.009	0.001	127	-0.791	0.430
Fish in the lake (F)	0.000	0.001	127	0.018	0.985
Mesocosm temperature (M)	0.000	0.001	127	-0.680	0.497
Test temperature (T)	0.116	0.001	127	9.678	**<0.001**
L x F	0.001	0.001	127	1.035	0.302
L x M	0.001	0.001	127	1.171	0.243
L x T	0.000	0.001	127	0.791	0.675
F x M	-0.002	0.001	127	-1.864	0.094
F x T	-0.001	0.001	127	-1.621	0.387
M x T	0.002	0.001	127	2.651	**0.004**

Table 1.2. Results of stepwise model selection between age, long- and short-term selection and their interactions describing number of offspring in *Daphnia pulicaria*. Age and number of offspring were log-transformed.

Number of offspring	Estimate	Std. Error	df	T value	Pr(>|t|)
(Intercept)	0.173	0.304	653.392	0.569	0.569
Age	0.514	0.091	695.776	5.614	**<0.001**
Mesocosm temperature (M)	-0.171	0.258	502.705	-0.665	0.506
Test temperature (T)	0.025	0.230	699.070	0.112	0.910
Fish in the lake (F)	-0.561	0.258	460.858	-2.170	**0.030**
Lake temperature (L)	0.234	0.255	373.650	0.919	0.358
Age x M	0.012	0.074	693.231	0.162	0.871
Age x T	0.172	0.073	700.952	2.348	**0.019**
M x T	0.193	0.067	698.363	2.883	**0.004**
Age x F	0.133	0.074	691.552	1.797	0.072
M x F	-0.069	0.089	9.435	-0.779	0.455
T x F	0.081	0.067	699.438	1.206	0.228
Age x L	-0.109	0.073	688.015	-1.501	0.133
M x L	0.123	0.088	9.481	1.390	0.196
T x L	-0.022	0.066	698.031	-0.335	0.737
F x L	0.111	0.087	9.882	1.266	0.234

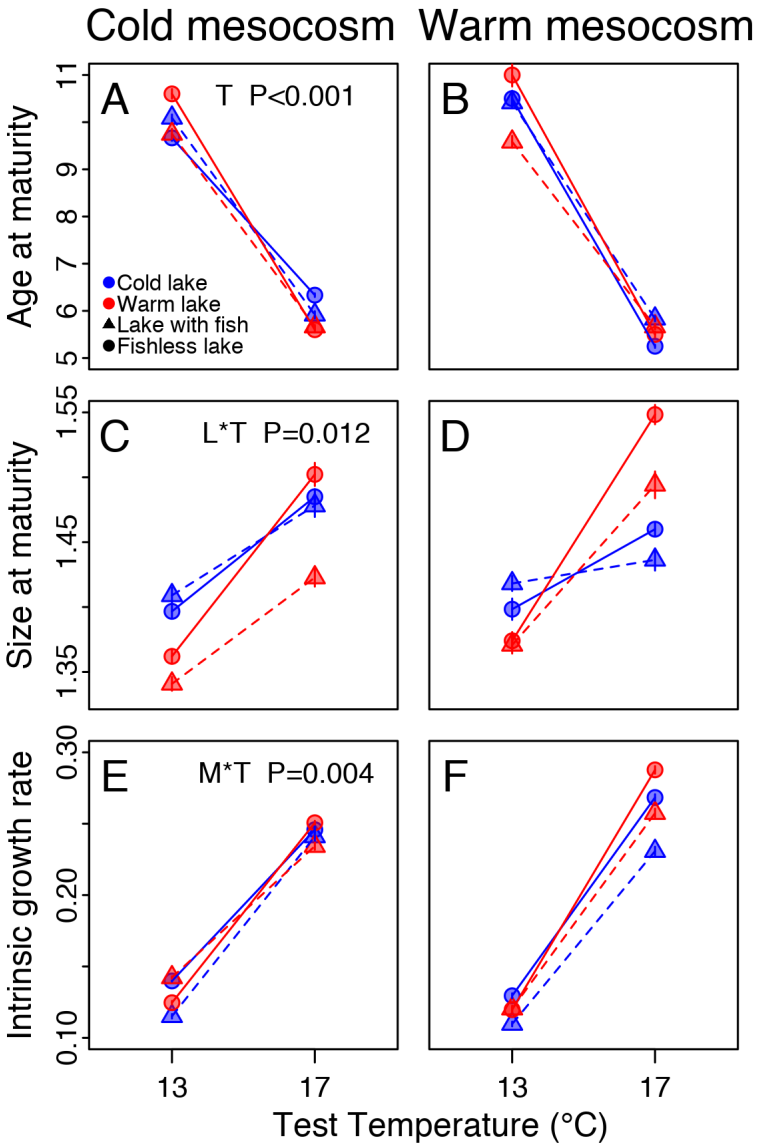

Figure 1.1. Means ± 1 S.E.M. of age at maturity in days (A, B), size at maturity in millimeters (C, D) and intrinsic growth rate (E, F) of the populations of *Daphnia pulicaria* as a function of test temperature. Populations that spent two years in cold mesocosms (elevation: 3093m) are shown on the left, and populations in warm mesocosms (elevation: 1200m) are on the right. Significant effects for each trait after model selection are shown in the left panel (for letter reference see Table 1.1). Red symbols indicate populations from warm, low elevation lakes (elevation: 2399-2801m), and blue symbols are clones from cold lakes (elevation: 3150-3337m). Circles indicate fishless lakes and triangles lakes with fish.

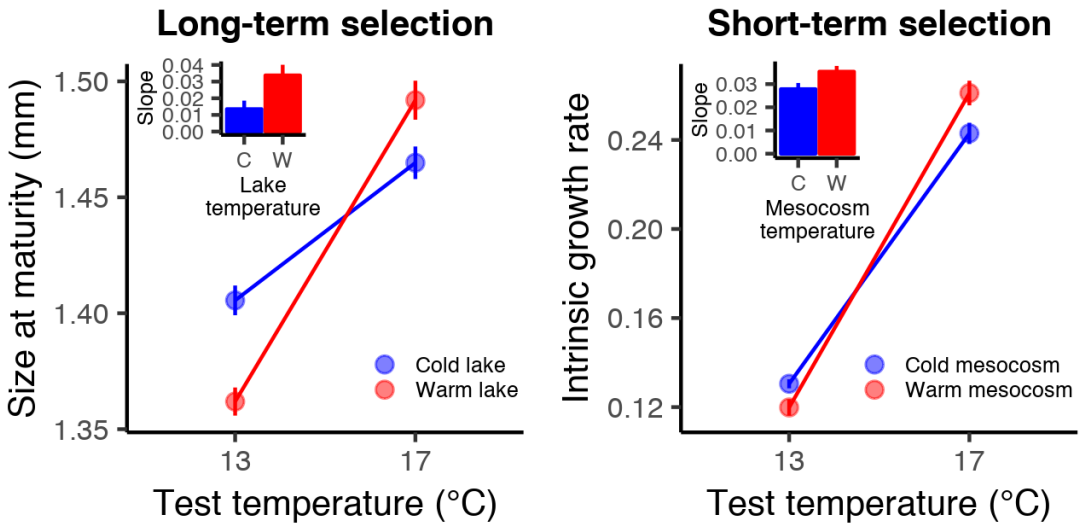

Figure 1.2. Effect of long- and short-term selection on level of plasticity in *Daphnia pulicaria* populations. Long-term selection: means ± 1 S.E.M. of size at maturity across all populations from cold (blue) and warm (red) lakes in response to test temperature (13°C and 17°C). Inset shows the difference in slope for the reaction norms for size at maturity from populations of cold (blue, elevation: 3150-3337m) and warm (red, elevation: 2399-2801m) lakes. Short-term selection: means ± 1 S.E.M. of intrinsic growth rate across populations from cold (blue, elevation: 3093m) and warm (red, elevation: 1200m) mesocosms in response to test temperature (13°C and 17°C). Inset shows the difference in slope for the reaction norms for intrinsic growth rate from populations of cold (C, blue) and warm (W, red) mesocosms.

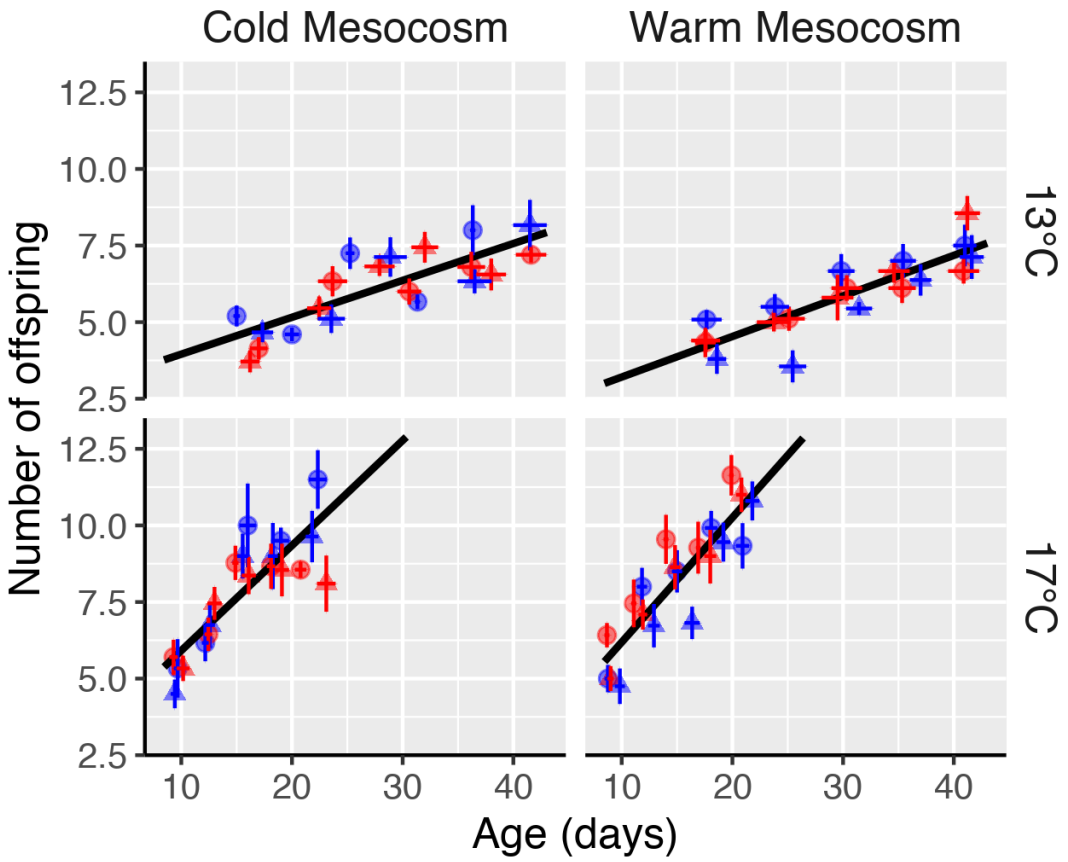

Figure 1.3. Average number of offspring against average age for each clutch measured for *Daphnia* reared at 17°C and 13°C test temperatures after spending two years in cold or warm mesocoms. Number of offspring ± 1 S.E.M. and age in days ± 1 S.E.M. are shown per population. Blue symbols represent populations from cold lakes and red, warm lakes. Triangles, populations from lakes with fish and circles, fishless lakes. Lines indicate relationship between age and number of offspring.

Discussion

We found evidence for the influence of both the short- and long-term selective environment on life-history traits and growth rates of *Daphnia pulicaria* populations from California mountain lakes. While the response of individual life-history traits to temperature showed the imprint of the ancestral lake environment, plasticity in population growth rate was most influenced by selection over the past two years. Together, these

results suggest that rapid evolution of life-history plasticity maintains population growth near the optimum for *Daphnia*, although different life-history traits varied in their response to short-term selection. Such rapid evolution of plasticity may have arisen from *de novo* mutations in the experimental populations or, perhaps more likely, selection among coexisting clones with variable responses to temperature within lakes found at the same elevation. Our results suggest that *Daphnia* populations display considerable adaptive potential to respond to changes in temperature within relatively short time scales.

Our data add to the growing body of evidence for evolutionary response to temperature-driven selection in *Daphnia* populations. In a study with *D. pulex*, Van Doorslaer et al. (2010) observed an effect of short-term selection treatments in size at maturity, as clones from heated mesocosms (24°C) reached maturity at a larger body size than those from non-heated mesocosms (20°C), indicating a microevolutionary response to temperature. Similarly, we found that *Daphnia* were larger on average at the warmer test temperature. This result contradicts the general expectation that warm temperature should result in decreases in size (Deutsch et al. 2008, Daufresne et al. 2009). Smaller size at high temperatures increases heat tolerance (Geerts et al. 2015, Brans et al. 2017) and enables more efficient oxygen transport. However, the larger size at higher temperature we observed could have arisen because 17°C is an intermediate temperature, hence, not warm enough to cause effects of thermal stress that produces smaller size.

In addition, the level of plasticity in size at maturity depended on the water temperature at the lake of origin as *Daphnia* populations from warm lakes had higher thermal plasticity than populations from cold lakes. However, size at maturity did not respond to short-term temperature selection, suggesting that this trait may evolve more

slowly, or that less genetic variation for plasticity may be maintained within populations for size at maturity than for other life-history traits.

Populations that originated from warm lakes showed greater thermal plasticity in size at maturity than populations from cold lakes by being both larger at higher temperatures and smaller at colder temperatures, consistent with our hypothesis that populations are locally adapted to the thermal conditions in their environment. This result agrees with studies of other *Daphnia* populations. For example, Chopelet et al. (2008) found a similar pattern for metabolic rates in a comparison of subarctic and temperate populations of *D. magna* tested at 15°C and 25°C. Metabolic cold adaptation, a counter gradient variation that occurs when genetic differences counteract temperature effects (Krogh 1916) suggests that populations of ectotherms that inhabit colder environments should have increased metabolic rates to compensate for the negative effect of low temperatures on growth rates and, possibly, reaching a larger size. However, cold adapted organisms may not be able to maintain their metabolism at warm temperatures, at the same level as organisms from warm environments (Conover and Schultz 1995, Gaitán-Espitia and Nespolo 2014), thus metabolism in warm conditions would be lower, affecting body size. Our results support the cold metabolism hypothesis if large size at maturity confers fitness benefits, such as larger individuals producing larger clutches.

Changes in temperature might directly affect zooplankton physiology or indirectly influence zooplankton through bottom-up effects. Often the effects of temperature cannot be disentangled from the effects of nutrients, as low elevation, warm, lakes in Sierra Nevada (CA) also have greater primary productivity as well as higher levels of detritus and dissolved organic carbon (Symons and Shurin 2016). Hence, the effect of lake temperature

on size at maturity could be confounded or be a synergistic response to high temperature and food availability, which can potentially increase growth and/or egg production of zooplankton (e.g. Kvile et al. 2016). In our common-garden experiment, food availability was controlled, leaving only the effect of temperature, thus indicating that differences in size at maturity are physiological adaptations in response to temperature.

We also found evidence of life-history evolution of *Daphnia* in response to the presence of fish in the ancestral environment, which was maintained over two years in mesocosms without fish. Long-term selection by fish did not show an interactive effect with test temperature, indicating that fish presence did not influence thermal plasticity. Populations from fishless lakes produced on average more offspring than populations sympatric with fish. Non-native fishes have been widely introduced into naturally fishless alpine lakes throughout the world with profound effects on native zooplankton species, including *Daphnia* (Knapp 1996). Previous work has documented contrasting life-history responses to fish predation where *Daphnia* populations from high predation environments show higher fecundity and faster development (Walsh and Post 2011, Stoks et al. 2016). Latta et al. (2007) compared life history of *D. melanica* among lakes throughout Sierra Nevada (CA) with different fish stocking histories and found that populations from lakes with fish showed reduction in number of eggs and size when compared to populations from fishless lakes. That study corroborates our findings that fishless populations had more offspring per clutch under all mesocosm and lab conditions, indicating that long-term selection by fish led to evolutionary divergence between fish and fishless populations. We did not have *Daphnia* populations from mesocosms with fish (i.e., short-term selection by fish) because *Daphnia* were extirpated from this treatment in the field experiment. It is

possible that release from fish predation caused populations sympatric with fish to reduce resource allocation to reproduction, but further work will be necessary to isolate how evolutionary response to fish shape life-history responses to temperature.

We found that the intrinsic population growth rate of *Daphnia* populations was more closely associated with the selective environment of the past two years than the lake of origin, suggesting a rapid response of growth rates to selection by temperature. Indeed, clones isolated from the warm mesocosms in our experiment showed higher intrinsic growth rate at 17°C, and therefore steeper slopes of the reaction norms, than populations collected from the cold mesocosm, indicating increased levels of plasticity, which can also be seem by the steeper slope between age and number of offspring on clones from warm mesocosms tested at 17°C. This result supports our hypothesis, in that after two years of selection, populations would adjust plasticity to cope with shifting thermal environments (Lande 2009, Crispo et al. 2010). The ancestral environment had no lasting impact on how population growth rate responded to temperature in our experiment. Similarly, Van Doorslaer et al. (2009) found that phenotypic plasticity increased the intrinsic growth rate for *D. magna* after only three months of thermal selection. By contrast, similar experiments with *D. magna*, *D. pulex* and *Simocephalus vetulus* revealed plastic growth rate responses to temperature, but no effect of short-term selection after 6 months of selection (Van Doorslaer et al. 2007, Van Doorslaer et al. 2010), giving no indication of a microevolutionary response for population growth rate.

Our experiment showed that regardless of environmental history, all studied populations of *D. pulicaria* had elevated intrinsic growth rates at higher temperatures in response to short-term selection in warm mesocosms. Intrinsic growth rate is often greater

at higher temperatures due to an increased metabolism (e.g. Mitchell and Lampert 2000, Weetman and Atkinson 2004), which permits rapid maturation and reduction in age at release of each clutch, resulting in larger populations in warmer temperatures in the absence of limiting conditions (Stich and Lampert 1981, Kingsolver and Huey 2008, Henning-Lucass et al. 2016). In fact, we found that number of offspring per clutch increased and the interval between clutches decreased in individuals that spent two years in warm mesocosms (Table 1.2 and Figure 1.3). Although populations in high elevation mesocosms probably underwent fewer generations in the time period of the experiment than populations in the low elevation treatment, populations at both elevations overwintered and produced ephippia (i.e. sexual reproduction occurred), which could have increased genetic variation, through recombination, increasing the scope for selection on plasticity.

Theory predicts that higher plasticity should evolve in more heterogeneous environments (Berrigan and Scheiner 2004). However, it is not always easy to identify the environmental factors that affect the development and maintenance of plastic responses. Analyses of temperature differences in our mesocosms experiment indicated higher temperature variation throughout the summer in cold mesocosms compared to warm mesocosms, but warm mesocosms had the greatest daily minimum and maximum temperatures (Figure A5). Studies with ectotherms show that temperature variation can have important impacts on life history traits (Tuck and Romanuk 2012, Hong and Shurin 2015). However, daily fluctuations in temperature can have qualitatively different effects at different mean temperatures (Vasseur et al. 2014, Kingsolver et al. 2015). Orcutt and Porter (1983) compared growth and reproductive responses of *D. parvula* in two

fluctuating temperature regimes, high (15°-25°C) and low (10°-20°C) with those at the average and maximum constant temperatures at in each range (15°C and 25°C). They found that *Daphnia* at the constant maximum (25°C) and fluctuating high treatments (15°-25°C) matured at the youngest age, and had the shortest time between clutches and the highest intrinsic growth rate, suggesting that fitness is higher at the warmest temperature. In addition, no differences were observed among *Daphnia* populations from different latitudes in temperature performance curves as measured in life-table analyses (Mitchell and Lampert 2000), whereas a positive relationship was observed between thermal tolerance and the average temperature of the warmest month (Yampolsky et al. 2014). This supports our result of an increase in plasticity in the warmer mesocosm, suggesting that experiencing high temperatures continuously, instead of temperature variability, could be the cause of increasing plasticity.

We found variable life-history responses to temperature among *Daphnia* populations originating from lakes that differ in thermal and predation history and that had experienced two years of selection by different temperatures in the field. Our results indicate that *Daphnia* populations have high adaptive capacity to respond to environmental change in short time scales possibly through changes in the genetic composition of local populations. In addition, plasticity may evolve rapidly as maternal lines that had undergone two years of selection at warm temperatures showed greater thermal plasticity. This finding suggests that considerable adaptive potential within and among *Daphnia* populations arises from genetic and variation in phenotypic plasticity and may indicate that those populations could be resilient to temperature changes.

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Appendix

Table 1.3. Lakes sampled for the thermal field experiment.

Lakes	Latitude	Longitude	Elevation (m)	Depth (m)	Fish
Lower Gaylor	37.9126°N	119.2688°W	3150	8	Present
Upper Gaylor	37.9226°N	119.2673°W	3205	5	Present
Helen	37.8306°N	119.2287°W	3337	10.7	Present
Lower Skelton	37.9376°N	119.2984°W	3322	6	Absent
Upper Skelton	37.9368°N	119.2972°W	3320	7.2	Absent
Secret	37.9985°N	119.3095°W	3317	3.5	Absent
Lukens	37.8598°N	119.6161°W	2506	5.1	Present
Harden	37.8962°N	119.6750°W	2399	10.5	Present
Lower Sunrise	37.8041°N	119.4521°W	2801	5.5	Present
Dog	37.8911°N	119.3396°W	2798	7	Absent
Polly Dome 1	37.8613°N	119.4558°W	2652	3.5	Absent
Polly Dome 2	37.8655°N	119.4493°W	2663	4	Absent

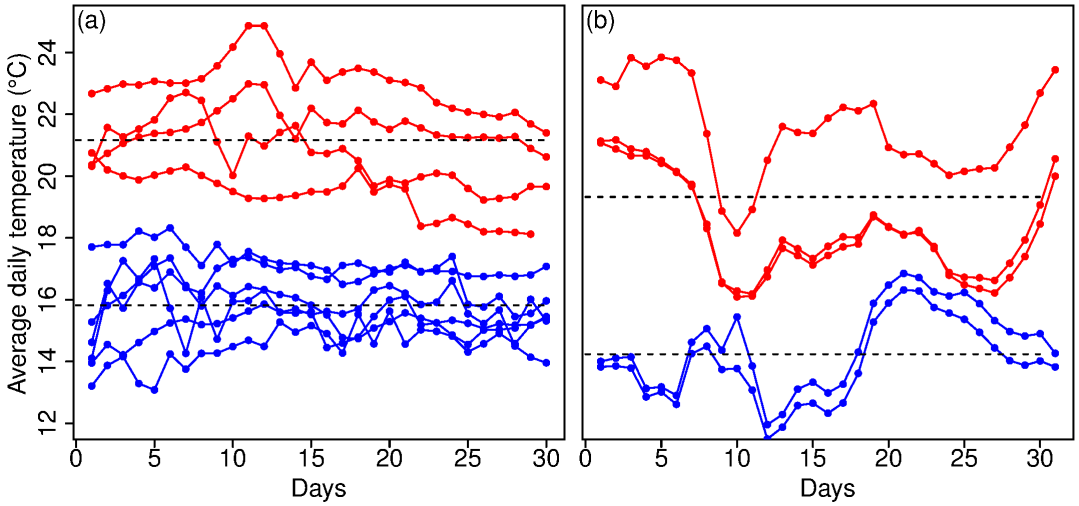

Figure 1.4. Average daily temperature of the lakes sampled during July 2014 (a). In (a) red lines represent temperature in Harden, Lukens, Polly Dome 1 and Polly Dome 2 lakes, while blue lines show the temperature in Gaylor 1 and 2, Helen, Secret, Skelton 1 and 2 lakes. In (b) the red lines indicate the average daily temperature of the mesocosms during July 2015. Red lines, warm mesocosms, and blue lines, cold mesocosms. In both graphs the dashed line indicates the average temperature across all warm or cold lakes in (a) and across warm and cold mesocosms in (b

Table 1.4. Number of mesocosms from where maternal lines were obtained for each lake category.

Lakes category	Mesocosm	Maternal lines
Warm without fish	Cold: 2	12
	Warm: 3	12
Warm with fish	Cold: 1	11
	Warm: 2	12
Cold without fish	Cold: 2	6
	Warm: 4	12
Cold with fish	Cold: 2	12
	Warm: 3	12
Total	Cold: 7 Warm: 12	89

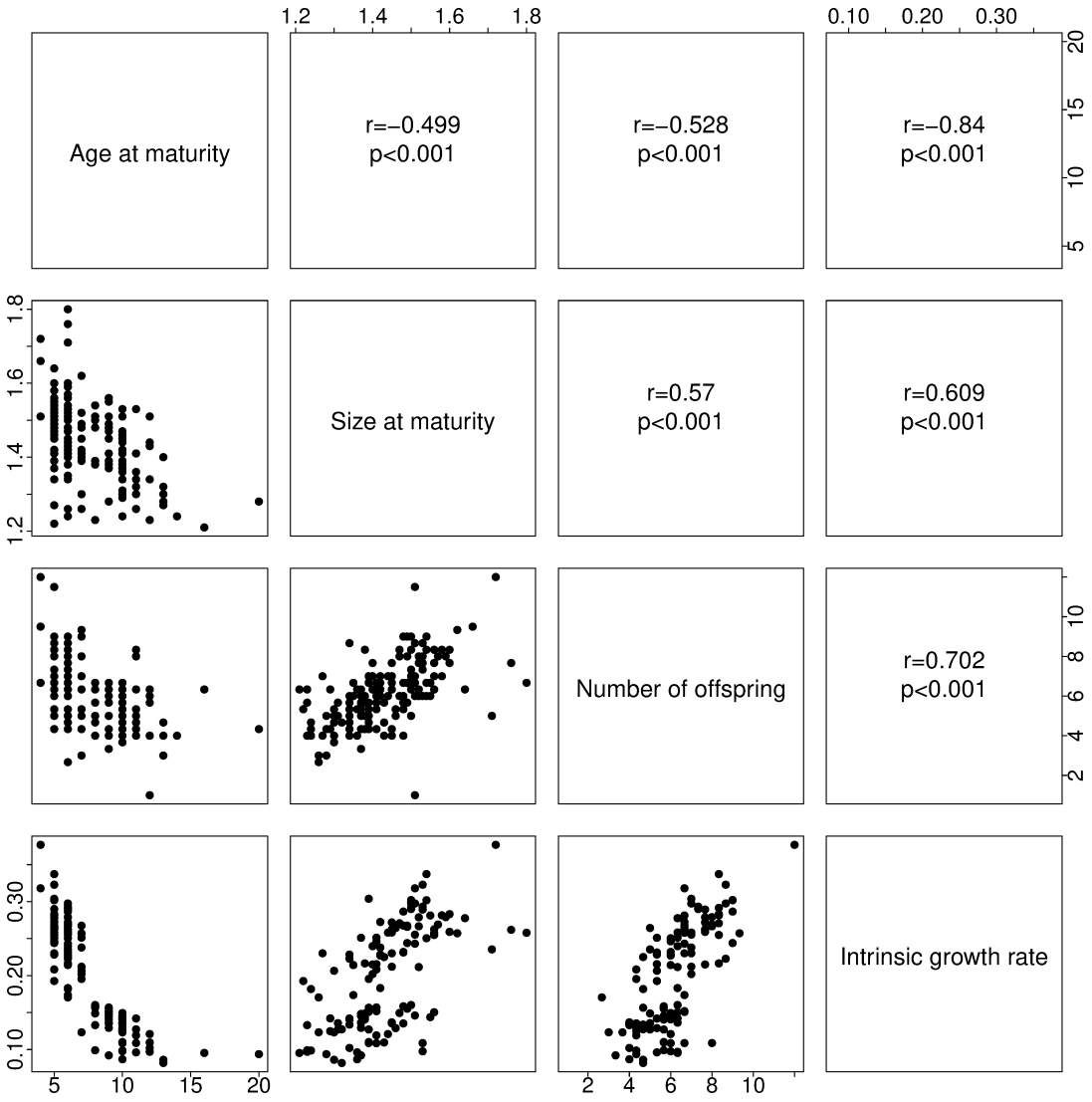

Figure 1.5. Correlation matrix of all traits measured in this study. The upper triangle of the matrix denotes Pearson's correlation coefficients among the variables.

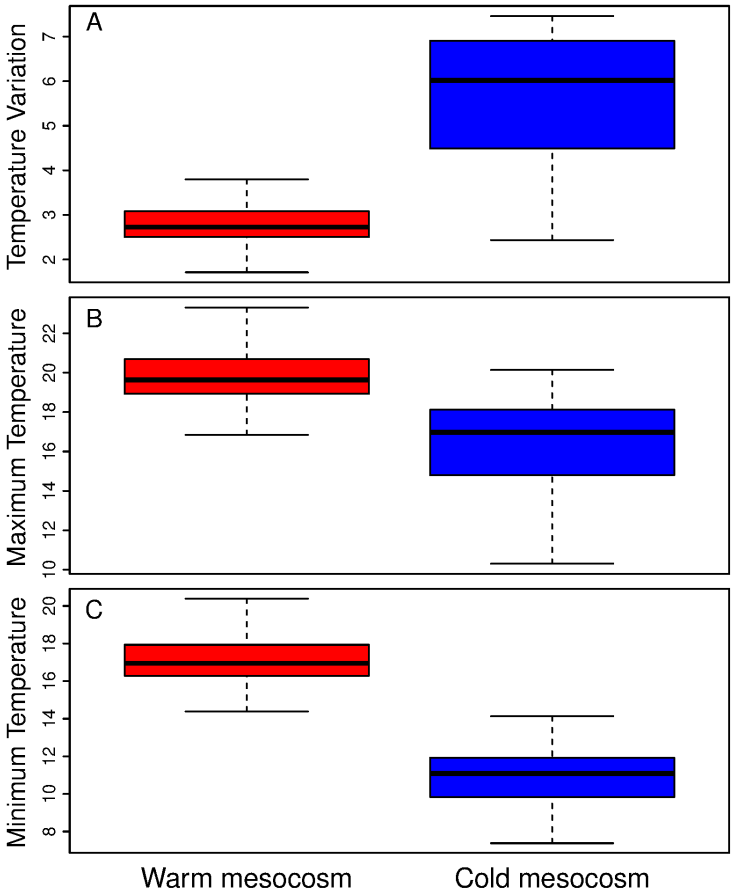

Figure 1.6. Differences in average daily variability (A, ANOVA; $F_{1,87}$=463.7, P<0.001), maximum (B, ANOVA; $F_{1,87}$=180.2, P<0.001) and minimum (C, ANOVA; $F_{1,87}$=1339, P<0.001) temperature (°C) during Summer 2015 of three mesocosms placed at Sierra Nevada Research Station in Wawona (red, elevation: 1200m) and two placed at White Mountain Research Center (blue, elevation: 3093m). Middle line shows the median, boxes show first and third quartiles and error bars show maximum and minimum values.

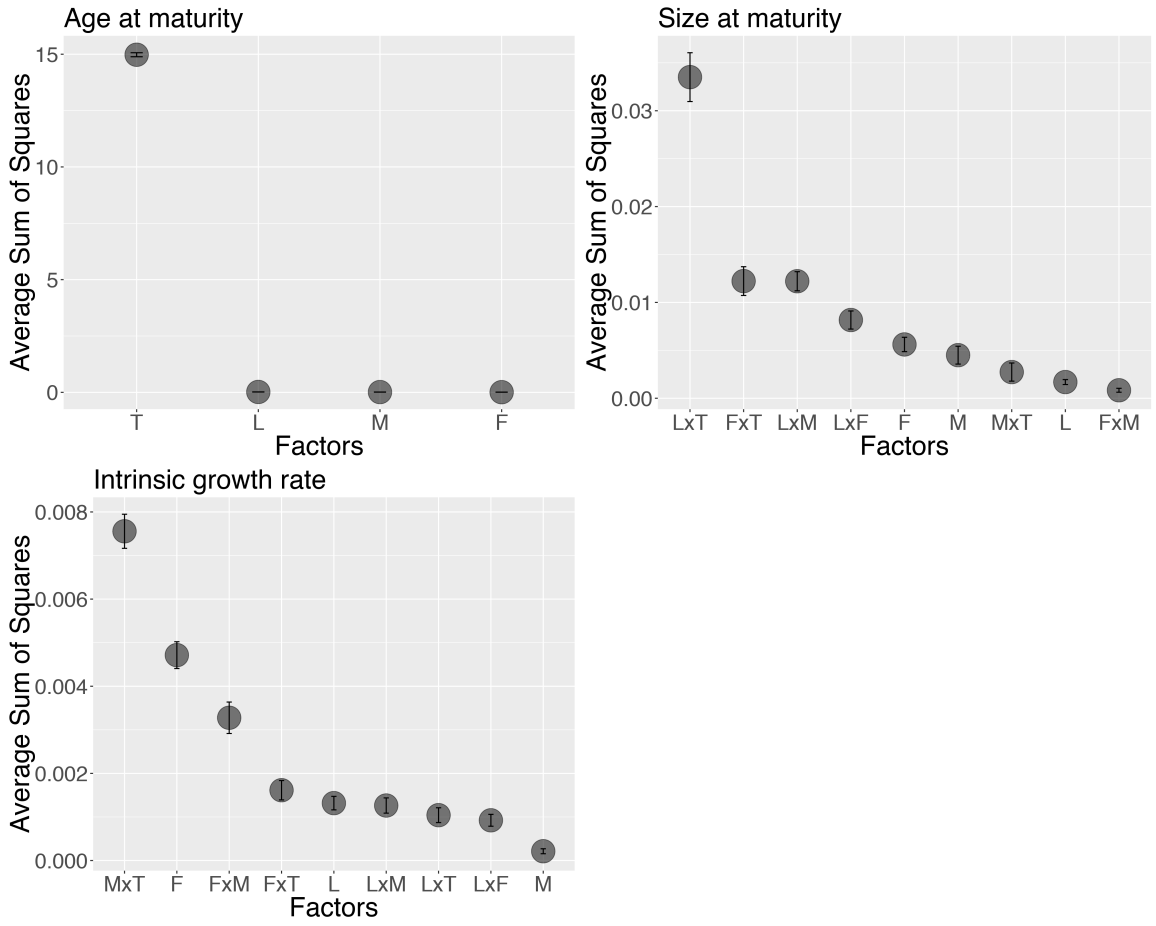

Figure 1.7. Average sum of squares ± twice standard error of linear mixed models produced by 10-fold cross-validation procedure with up sampling. We used the same final model selected after stepwise model selection, where we kept the simplest model that best described the data (see Materials and Methods). Factors are fixed effects, including interactions, kept in the final models. L = Lake temperature; M = Mesocosm temperature, F = Fish in the lake; and T = Test temperature. In size at maturity and intrinsic growth rate, average sum of squares for test temperature was not plotted to allow better visualization. For size at maturity, average sum of squares for T = 0.190 ± 0.003 and for intrinsic growth rate, average sum of squares for T = 0.451 ± 0.004.

CHAPTER 2

Seasonal variation in thermal plasticity of an alpine lake *Daphnia* population

Abstract

Populations are expected to maintain a greater degree of plasticity in more heterogeneous environments. Temperature changes dramatically throughout the growing season in temperate latitudes, and organisms experience different thermal regimes depending on their phenology. Seasonal changes in temperature are especially pronounced in lakes where water stratifies into distinct thermal layers during summer. We studied seasonal variation in plasticity of a population of *Daphnia*, a key grazer in alpine lakes. We isolated maternal lines of *Daphnia pulicaria* from Blue Lake (Sierra Nevada, CA, USA) at four different times throughout the growing season, then measured phenotypic traits and survivorship after individuals were reared at two temperatures (17°C and 24°C). Plasticity, measured by the steepness of the slopes of the reaction norms, was different for maternal lines collected in mid-summer (when the lake was stratified) and early-fall (when the lake was vertically mixed), while the two intermediate samples were not clearly differentiated from each other. Lower plasticity for the interval between *Daphnia* clutches occurred when the lake experienced peak thermal stratification in mid-summer compared to early-fall conditions, while plasticity for critical maximum temperature was higher in the mid-summer and decreased in early-fall. These results suggest that the degree of plasticity in response to temperature varies throughout the season in relation to thermal stratification, with different life history traits showing distinct seasonal patterns of plasticity.

Introduction

Temporal variation in environmental conditions is ubiquitous in nature. Populations living in intermediate and high latitude climates encounter tremendous seasonal variation in environmental conditions. For instance, the water temperature of temperate lakes can vary from 4°C during the winter to over 20°C during the summer months (O'Reilly et al. 2015). Thus, organisms in seasonal environments face selective pressure to maintain fitness under variable conditions. Phenotypic plasticity and genetic adaptation are two mechanisms that help organisms respond to environmental variation. Phenotypic plasticity allows a single individual to express the optimum phenotype in response to variation in conditions (Scheiner 1993, Sultan 2003). Genetic adaptation, on the other hand, results from environment-dependent selection among individuals based on heritable variation in traits (Debat and David 2001). Seasonal environments in general exhibit predicable changes in conditions and by sensing cues early in the season, individuals can adjust, thereby matching their phenotypes to the expected conditions (Reed et al. 2010).

Strong seasonal variability in temperature causes most lakes located in temperate climates to experience thermal stratification where the water column separates into distinct thermal layers. The most extreme thermal stratification occurs during the summer, when lakes have a warm epilimnion (upper layer) and a cold hypolimnion (lower layer) separated by a steep cline in temperature (thermocline). Stratification is an important phenomenon because it affects physical, chemical and biological processes in lakes, such as transport of nutrients and oxygen between the surface and deep water, the light environment of phytoplankton cells in the mixed layer, and zooplankton behavior (Boehrer and Schultze 2008).

Thermal stratification affects zooplankton in temperate lakes because they often exhibit a behavioral syndrome where they perform diel vertical migration (DVM) throughout the water column. DVM represents a trade-off between the functions of food gathering and predator avoidance (Kaartvedt et al. 1996). In classic DMV behavior individuals spend the daytime in the deeper darker layer (hypolimnion) of a lake to reduce the probability of an attack by optically orientated predators, then migrate upwards at night to warmer layers to either feed on phytoplankton or take advantage of higher temperatures to speed up metabolism in order to increase growth and reproductive rates (Lampert 1989, Loose et al. 1993, Dawidowicz and Loose, 1992). Hence, stratification affects zooplankton as they regularly experience a large daily temperature range as they travel throughout the water column (Stich and Lampert 1981, Ringelberg 1991). Ectotherms that occupy heterogeneous thermal environments are hypothesized to evolve physiological or behavioral capacity to optimize performance in variable thermal environments (Chown and Terblanche 2007, Angilletta 2009). Thus, organisms from variable environments are expected to display a high degree of phenotypic plasticity (Kingsolver et al. 2016).

Daphnia is a key species' in freshwater ecosystems due to its role as an effective phytoplankton grazer and preferred prey for fish. *Daphnia* also often exhibit DMV in alpine lakes. Previous studies have found genetic turnover throughout the growing season for *Daphnia*, with clones from different time periods having distinct responses (e.g. Carvalho 1987, Paul et al. 2012). For instance, winter clones have lower survivorship and thermal tolerance than summer clones (Carvalho 1987, Paul et al. 2012). In addition, *Daphnia* populations can evolve plasticity in response to temperature over short time scales. Cavalheri et al. (2018) found that *D. pulicaria* populations evolve higher plasticity for

intrinsic growth rate after two years of selection at warm temperatures. Similarly, Van Doorslaer et al. (2009) found that phenotypic plasticity increased the intrinsic growth rate for *D. magna* after only three months of selection at elevated temperatures. These studies show that natural populations of *Daphnia* contain standing genetic variation for plasticity. Since theory predicts that higher plasticity should evolve in more heterogeneous environments (Berrigan and Scheiner 2004) and the amount of temperature variation experienced by *Daphnia* changes during the growing season. Individuals that occur during periods of thermal stratification and migrate vertically may experience the greatest thermal variability over a spatial scale of meters. The ability of genotypes to induce phenotypic plasticity may therefore also have a temporal signal. However, to date we lack experiments that test how seasonal variation shapes the phenotypic plasticity of *Daphnia* populations in response to temperature.

Here, we collected *D. pulicaria* individuals from Blue Lake (Sierra Nevada, CA) four times throughout the growing season, then measured phenotypic traits and survivorship to determine how plasticity for tolerance at their thermal maximum changes over time. We conducted an experiment using constant rearing conditions of 17°C (benign temperature) or 24°C (high temperature) to determine whether individuals collected at different periods during the growing season show a distinct response to acclimation temperature. Hence, we tested whether individuals collected during periods of greater vertical temperature variation in the water column (stratification) exhibit higher levels of plasticity. The results of this work provide insights on the importance of seasonal variation in adaptive plasticity for underlying thermal adaptation across the growing season.

Materials and Methods

Lake sampling

Blue lake (latitude = 38.051164, longitude = -119. 270342, elevation 3013 m), located in Inyo National Forest (CA), was sampled six times during Summer 2017 (15-Jul-2017, 01-Aug-2017, 16-Aug-2017, 04-Sep-2017, 13-Sep-2017 and 05-Oct-2017). *Daphnia* tested in the common-garden experiment were collected in the last four sample days since *Daphnia* abundance was very low in the first two samples (Figure 2.1c). At every sampling date, we recorded water temperature at 1 m intervals throughout the water column using a field probe (YSI Incorporated, Yellow Springs, Ohio, USA, Figure 2.1a and 2.1b) and collected zooplankton from the deepest point of the lake using a 63 μm mesh conical net with a 30 cm diameter drawn through the water column, starting 1 m above the lake bottom. Zooplankton samples were preserved in 70% ethanol and the total number of *Daphnia* was counted under a stereo microscope (Figure 2.1c).

We also collected live zooplankton samples from the deepest point of the lake (11 meters) using the same approach. These samples were kept cold until we returned to the laboratory, where we searched for live *Daphnia*. When present, 30 *D. pulicaria* females carrying eggs in the brood pouch were separated in individual 50 ml falcon tubes filled with COMBO medium (Kilham et al. 1998). Each individual *Daphnia* was considered a maternal line and cultured for at least five generations in separate 50 mL falcon tubes filled with COMBO medium under standardized conditions (17±1°C and photoperiod 12:12 h light/dark). All animals were fed the green alga *Nanochloropsis* sp. at a constant high rate of 24×10^6 cells every two days.

We measured the chlorophyll-a concentration the first five samples by collecting lake water from 1 m below the surface. A known volume of water was filtered through a GF/F filter that was frozen until processing. We measured the concentration of chlorophyll-a using a Turner Trilogy fluorometer (Turner, USA) following a 24 hour ~ 4°C methanol extraction (Figure 2.1c).

Common-garden experiment

We performed a common-garden experiment with maternal lines that survived and reproduced until the end of the summer. In total, we had two maternal lines from 16-Aug-2017, one from 04-Sep-2017, one from 13-Sep-2017 and three from 05-Oct-2017. We separated all neonates born within a 24h period and kept up to three *Daphnia* individuals from the same maternal line in 100 ml jars under standardized conditions to minimize maternal effects (17 ± 1°C and photoperiod 12:12 h light/dark). Individuals were fed daily and transferred every two days to fresh media. We established three independent grandmother cultures of each maternal line. The second and third clutches of the grandmother generation were pooled together and used to establish twelve cultures of the mother generation per maternal line. This approach enabled us to establish twelve replicate cultures of each maternal line from the mother generation onwards.

Neonates of the second clutch of the daughter generation were pooled together and randomly assigned to one of the two acclimation treatments (17°C and 24°C). We chose the acclimation temperatures based on field sampling and a pilot experiment; 17°C is close to the maximum temperature *Daphnia* experience in Blue Lake (Figure 1) and is near the optimum temperature for most populations of *Daphnia* from Sierra Nevada (CA) lakes

including Blue Lake. 24°C was chosen as the "stress" acclimation temperature because it is high enough to impact life history traits, but low enough to not induce high mortality. After culturing the *Daphnia* for another two generations at their acclimatization temperatures (17°C or 24°C), all neonates born from the second and third clutches were separated in individual 50 ml falcon tubes and used as the experimental generation. Upon reaching maturity, 30 individuals per maternal line were randomly assigned to one out of six stress temperatures and scored for survivorship: control (staying at ambient temperature for 1h and return to acclimatization temperature), 28°C, 30°C, 32°C, 34°C and 36°C. In addition, five individuals per maternal line were assigned to i) a life history assay or ii) a critical maximum temperature assay. In total 560 individuals were tested.

Critical maximum temperature

We defined critical maximum temperature (CT_{max}) as the temperature at which individuals lose motor control (Angilletta 2009). To assess *Daphnia* critical maximum temperature we placed individuals in 0.5 ml Eppendorf tubes after a resting period of 30 minutes in ambient temperature (~ 20°C). The measurement was done in a thermal heater (4 x 6 Corning Digital Dry Bath Heater Dual Block). The tubes were randomly divided over the thermal heater. The starting temperature was 25°C, and the temperature was gradually increased from 25°C to 40°C in 1°C steps of 45 ± 5 seconds. Once *Daphnia* lost motor control and stopped moving they were transferred to ambient conditions to recover for 30 minutes. Because body size can also impact critical maximum temperature (Portner and Farrell 2008), after recovery we measured the body size of each individual from the top of the head to the base of the tail (Gliwicz 1990, Yurista and O'brien 2001).

Life history assay

For the life history assay, we scored age and size at maturity, and age and number of offspring from the first three clutches for individuals from the experimental generation. We used the average number of offspring of the first three clutches and the average interval between clutches as variables. The average interval was the mean difference between the first and second and second and third clutches. Age at maturity, age at each clutch and number of offspring in each clutch were used to calculate intrinsic population growth rate for each maternal line following the Lotka–Euler equation (Roff 1997).

Survivorship

We examined the effect of temperature stress on survivorship by placing individuals in 0.5 ml Eppendorf tubes placed in a thermal heater (4x6 thermal heater, Corning Digital Dry Bath Heater Dual Block). First, individuals had a 1h rest period at ambient temperature (approximately 20°C). Next, individuals were exposed to a stress temperature for 1h. Following the exposure to temperature stress, individuals were moved back to 50 ml falcon tubes and rested for 30 minutes at ambient temperature before being transferred back to their respective acclimatization temperature (17°C or 24°). We assessed survivorship at two-degree intervals for temperatures ranging from 28°C to 36°C. A pilot experiment found that individuals can reproduce at 27°C, thus we elected to begin the thermal trials at 28°C and increased temperatures up to 36°C, which was the minimum temperature at which all individuals died. In all trials we included a control, which consisted of placing the individual in 0.5 ml Eppendorf tube for 1h at ambient temperature

and transferring it back to 50 ml tube and its acclimatization temperature. Survivorship was scored after 72 hours.

Statistical analysis

We tested for seasonal variation in phenotypic plasticity by including sample day (time) in our analyses, and plasticity by analyzing the effect of the temperature acclimation treatments. The effect of collection date might indicate either genetic adaptation through seasonal turnover in *Daphnia* genotypes, or long-term, transgenerational effects of lake temperature. Since all maternal lines were maintained in the lab for several generations and acclimated to the experimental temperatures for two generations before the experiment, it is likely that differences among sampling dates reflect genetic variation. We tested effects of time, acclimation treatment and target temperature (28°C – 36°C) and their interactions on survivorship using generalized linear models. We also tested whether survivorship at each stress temperature differed with acclimation temperatures (17°C and 24°C) across all maternal lines using a non-parametric Wilcoxon test on survivorship data at each stress temperature.

We log-transformed all continuous variables after visually assessing the probability distribution that best fit the data. We analyzed the effects of the acclimation treatments (17°C and 24°) and time using linear mixed-effects models for each trait using the *lmerTest* package in the statistical software R (Kuznetsova et al. 2017, R Core Team 2018). Acclimation treatment and time were modeled as fixed effects and replicate was nested within maternal line as random effects for age and size at maturity, number of offspring, average interval between clutches and intrinsic growth rate. Any interaction that includes

acclimation treatment would indicate an effect on the level of plasticity, i.e. effects on the slope of the reaction norms.

We also used linear mixed-effects model to analyze the effects of acclimation treatment and time on critical maximum temperature with the same fixed and random parameters, but with the addition of size at maturity as fixed effect because CT_{max} can be negatively correlated with body size. Larger individuals can have lower thermal tolerance than smaller individuals (Geers et al. 2014; 2015). In this case we used a backward selection model where we started with the most complex model for fixed effects (the random effect was kept in all models) and dropped higher order interactions if they did not significantly improve model fit (using log-ratio tests) until we arrived at a best-fit model.

To evaluate differences in the magnitude of plasticity through time, we calculated the pairwise slopes of each replicate tested at 17°C to all tested at 24°C within a maternal line. Then, we tested for differences in slopes among traits and sample time using generalized linear model with an interaction between trait and sample time as a fixed effects. We also computed pairwise differences between sample days for each trait using the least square mean values based on the generalized linear model using *lsmeans* function in the *lmerTest* package in R statistical language (Kuznetsova et al. 2017, R Core Team 2018). Significant difference test for multiple contrasts alpha level was set to 0.001 following Bonferroni correction. All analyses were performed using R statistical language (R Core Team 2018).

Results

Environment

The temperature of Blue Lake changed considerably throughout the summer (Figure 2.1a). Surface temperature ranged from 8.6°C - 14.6°C, while bottom temperature ranged from 7.8°C - 11.6°C. The highest temperatures were reached during late August and early September. The largest difference between minimum and maximum temperatures due to thermal stratification occurred in mid-summer, from August to September (Figure 2.1a). The lake had stratified by 01-Aug-2017, with the thermocline occurring at approximately 7.5 m in depth (Figure 2.1b). Following the first observation of thermal stratification, the thermocline became deeper until disappearing in 13-Sep-2017. The water column was vertically mixed in the final two samples, in 13-Sep-2017 and 04-Oct-2017, the temperature decreased from 12°C to 8°C during that period, as fall began in the eastern Sierra mountain range (Figure 2.1b). Zooplankton abundance and chlorophyll-a concentration changed coincidentally with temperature throughout the growing season. The highest chlorophyll concentration occurred in mid-August and was followed in early-September by a peak in zooplankton density (Figure 2.1c), the same period when the water reached the highest temperatures. *Daphnia* reached the highest densities in early-September followed by an overall decrease in abundance as water temperature declined (Figure 2.1c).

Traits

The linear mixed-effects model revealed that traits responded differently to acclimation temperature (Table 2.1). Age at maturity was affected by acclimation

temperature (Acclim: p-value < 0.001, Table 2.1) and decreased 2.33 ± 0.04 days on average when clones were grown at 24°C compared with clones tested at 17°C (Figure 2.2a). There was also an interactive effect of acclimation temperature and time (Acclim x Time: p-value < 0.001, Table 2.1). Similarly, size at maturity also showed an interactive effect of acclimation temperature and time (Acclim x Time: p = 0.021, Table 2.1), and we also observed a significant effect of maternal line (random effect, p < 0.001, Table 2.1), indicating that for the sampling dates where we tested multiple maternal lines (the first and last sample) had different responses to acclimation temperature (Figure 2.2b). In contrast, the average number of offspring was unaffected by acclimation temperature or time (Table 2.1, Figure 2.2c). The interval between clutches showed a significant impact of acclimation temperature (Acclim: p < 0.001, Table 2.1), with a 0.60 day shorter interval at 24°C compared to 17°C conditions (Figure 2.2d). Similarly, intrinsic growth rate is 7% higher when maternal lines were raised at 24°C than in 17°C (Figure 2.2e; Acclim: p < 0.001, Table 2.1).

Across all sample dates, acclimation at 24°C resulted in directional shift that increased the critical maximum temperature. The 7°C difference between acclimation treatments increased critical maximum temperature by an average of 1.2 °C (Figure 2.2f). Although critical maximum temperature did not vary with body size, it did differ among sample dates (Time: p-value = 0.014 and Size x Time: p-value = 0.037, Table 2.1), mostly because different maternal lines show distinct responses to acclimation temperature for size at maturity. There was a significant interaction between acclimation temperature and time, showing differences among maternal lines in response to acclimation temperature (Acclim x Time: p-value = 0.022, Table 2.1).

Levels of plasticity are affected by both the trait and the sample time (Trait: $F_{5, 1401}$ = 510.735, p-value < 0.001; Sample: $F_{3, 1406}$ = 20.107, p-value < 0.001; Trait x Sample: $F_{15, 1386}$ = 13.648, p-value < 0.001). Pairwise comparisons of least square means shows that samples have different slopes for age at maturity, number of offspring, clutch interval and CT_{max}. The *Daphnia* sampled on 13-Sep-2017, after the lake was no longer stratified, had the greatest plasticity for age at maturity, i.e. steeper slopes for the reaction norms, compared to all the other maternal lines (Figure 3a). In contrast, the *Daphnia* sampled on 4-Sep-2017, during the stratified period, was the only maternal line that had more offspring when reared at 24°C (Figure 2.3b). For interval between clutches the first (16-Aug-2017) and last samples (5-Oct-2017) had the lowest and highest levels of plasticity, respectively (Figure 2.3c). For CT_{max} the earliest sample, taken on 16-Aug-2017, during stratification, had the highest level of plasticity, followed by 13-Sep-2017 and 5-Oct-2017, while the sample collected on 4-Sep-2017 had the lowest (Figure 2.3d). All samples were grouped together for size at maturity and intrinsic growth rate, indicating that slopes were not affected through time.

Survivorship

The two temperature acclimation treatments affected *Daphnia* survivorship, but those effects were not influenced by time (Table 2.2, Acclim: p-value = 0.030, Time: p-value = 0.118). Survivorship curves approximated a sigmoidal shape, starting with a plateau of high survivorship at low temperatures, followed by a steep decrease in survivorship as temperatures increased between 30°C and 32°C (Figure 2.4). Stress temperature (28°C – 36°C) also significantly affected thermal performance curves (Table

2.2, Stress: p-value < 0.001). However, there was no significant effect of time in our model, indicating that *Daphnia* collected at different time periods throughout the growing season i) showed similar survivorship responses to stress temperature, and ii) acclimatization equally affected survivorship. Wilcoxon tests show that, across all maternal lines, acclimation only increased survivorship at 32°C, but not at any of the other stress temperatures (W = 525, p-value = 0.039).

Table 2.1. Results of the linear mixed-effects model analysis of the acclimation temperature (Acclim) and time on phenotypic traits of *Daphnia pulicaria* collected during 2017 growing season in Blue Lake. Likelihood ratio test statistic (LRT) and p-value of random effect of replicate nested in maternal line is shown when significant.

Factor	df	Sum sq	F - value	P - value
Age at maturity				
Acclim	1, 563	19.141	1230.362	**< 0.001**
Time	3, 563	0.048	1.039	0.374
Acclim x Time	3, 563	0.327	7.012	**< 0.001**
Size at maturity				
Acclim	1, 133.02	0.012	2.561	0.111
Time	3, 7.001	0.032	2.208	0.174
Acclim x Time	3, 133.03	0.048	3.325	**0.021**
Random effect	**LRT**		**Pr(>Chisq)**	
Maternal line	13.043		**< 0.001**	
Average number of offspring				
Acclim	1, 20.579	0.013	0.406	0.530
Time	3, 40.782	0.029	0.302	0.823
Acclim x Time	3, 20.903	0.197	2.040	0.139
Interval between clutches				
Acclim	1, 37.754	0.54234	13.925	**< 0.001**
Time	3, 41.253	0.04757	0.407	0.748
Acclim x Time	3, 38.480	0.15007	1.284	0.293
Intrinsic growth rate				
Acclim	1, 70	0.087	77.516	**< 0.001**
Time	3, 70	0.006	1.898	0.137
Acclim x Time	3, 70	0.004	1.221	0.308
Critical maximum temperature				
Size at maturity	1, 71	0.001	0.272	0.603
Acclim	1, 71	0.001	0.065	0.799
Time	3, 71	0.003	3.747	**0.014**
Size x Acclim	1, 71	0.001	3.106	0.082
Size x Time	3, 71	0.002	2.971	**0.037**
Acclim x Time	3, 71	0.003	3.410	**0.022**

Table 2.2. Results of the generalized linear model analysis of the survivorship probability at each stress temperature (Stress) of *Daphnia pulicaria* collected throughout growing season 2017 (Time) and reared under different acclimatization treatments (Acclim, 17°C and 24°C). Error distribution: binomial, link function: logit for survivorship analysis.

Factor	df	Deviance	Residuals deviance	Pr (>Chi)
Survivorship				
Stress	1, 350	361.35	120.06	**< 0.001**
Acclim	1, 349	4.67	115.38	**0.030**
Time	3, 346	5.86	109.52	0.118
Stress x Acclim	1, 345	1.43	108.09	0.230
Stress x Time	3, 342	5.99	102.09	0.111
Acclim x Time	3, 339	0.19	101.90	0.978
Stress x Acclim x Time	3, 336	7.10	94.80	0.069

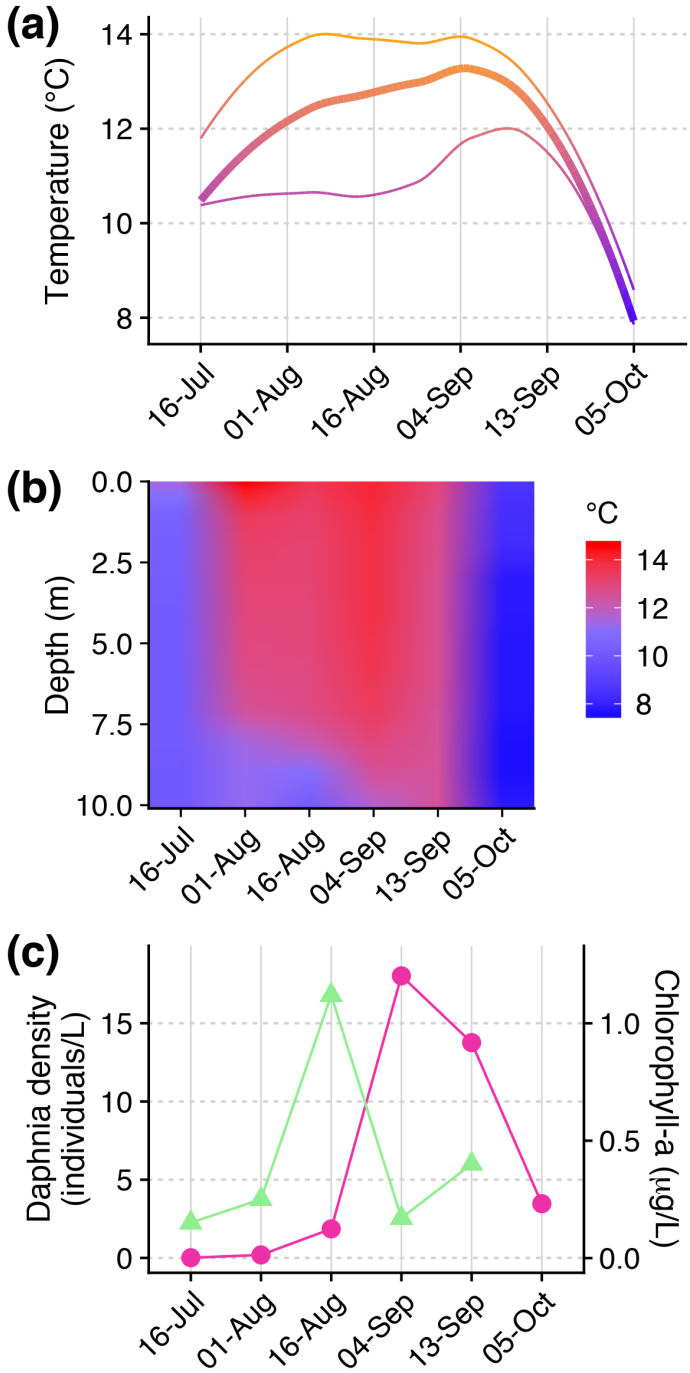

Figure 2.1. (a) Maximum (top line), average (middle line) and minimum (bottom line) temperature of the water column measured throughout the 2017 summer in Blue Lake (Sierra Nevada, CA). (b) Temperature variation of water column of Blue Lake during summer 2017. (c) Variation in *Daphnia* density (circles) and chlorophyll–a concentration (triangles) in Blue Lake during summer 2017.

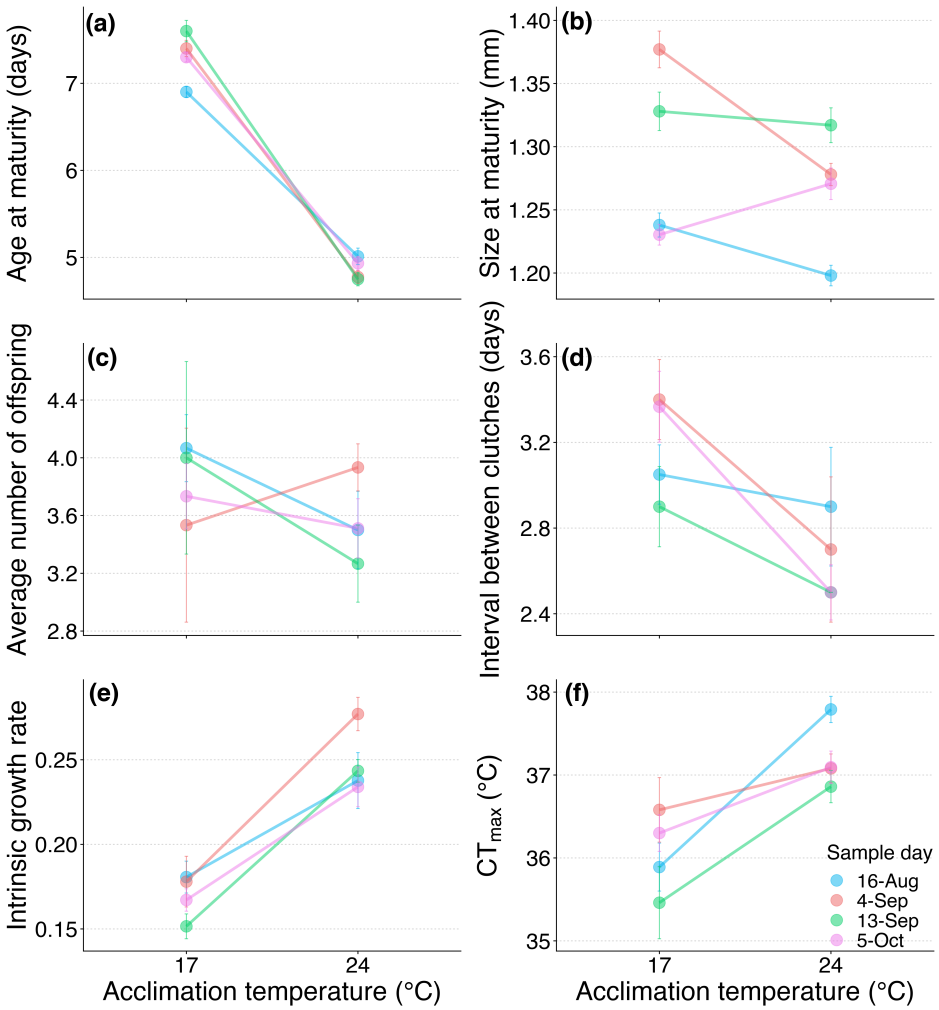

Figure 2.2. Means ± 1 S.E.M. of age at maturity (a), size at maturity (b), average number of offspring of the first three clutches (c), interval between clutches (d), intrinsic growth rate (e), and critical maximum temperature (f) of *Daphnia pulicaria* collected at different time periods during summer 2017 at Blue Lake as a function of acclimation temperature.

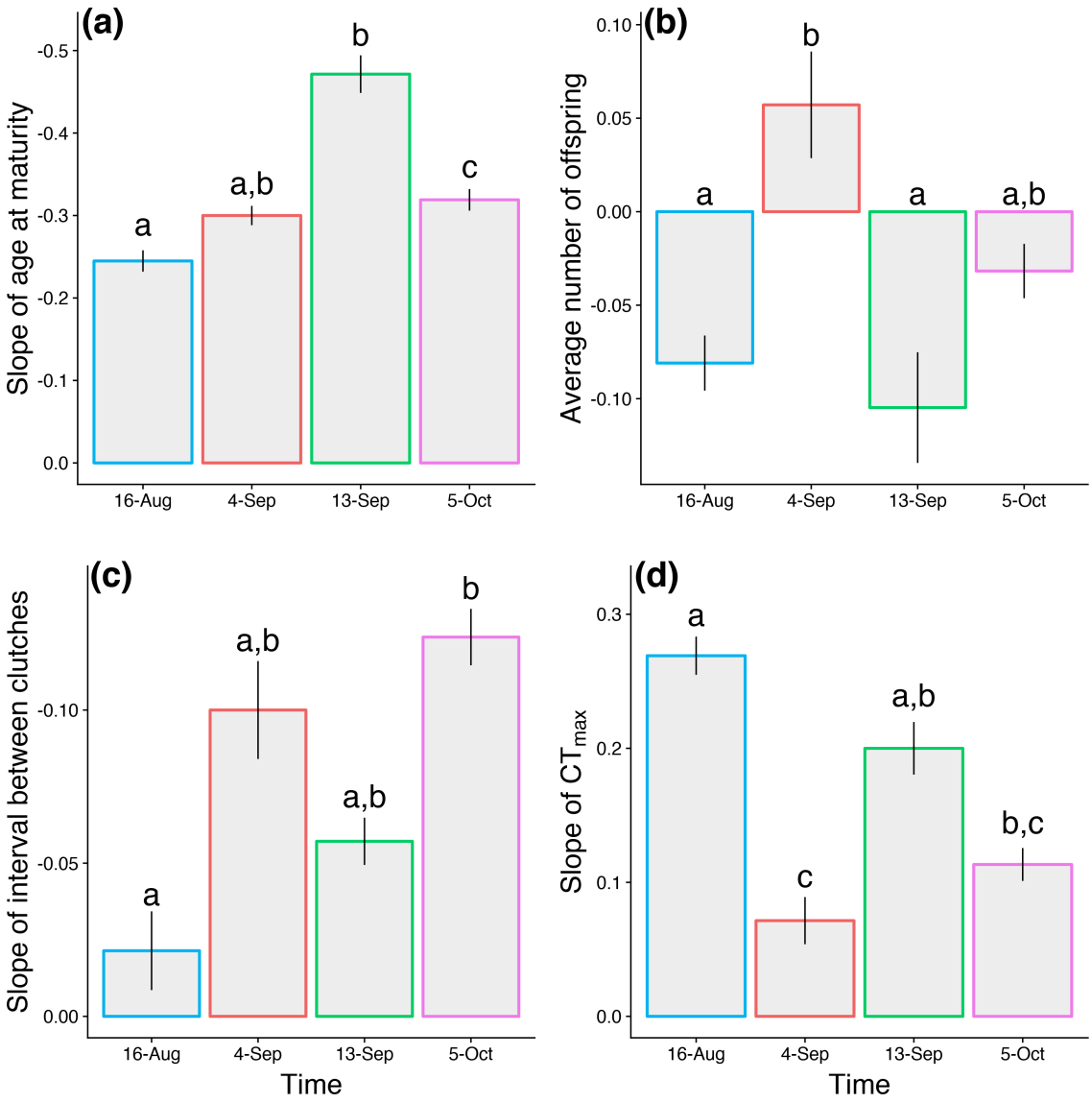

Figure 2.3. Means ± 1 S.E.M. of the slopes for the reaction norms of age at maturity (a), average number of offspring (b), interval between clutches (c) and critical maximum temperature (d) of *Daphnia* in response to two acclimation temperatures (17°C and 24°C) collected throughout the growing season 2017 in Blue Lake. Letters indicate significant different values based on least square means pairwise differences. Colors represent the sampling date as in Figure 2.2.

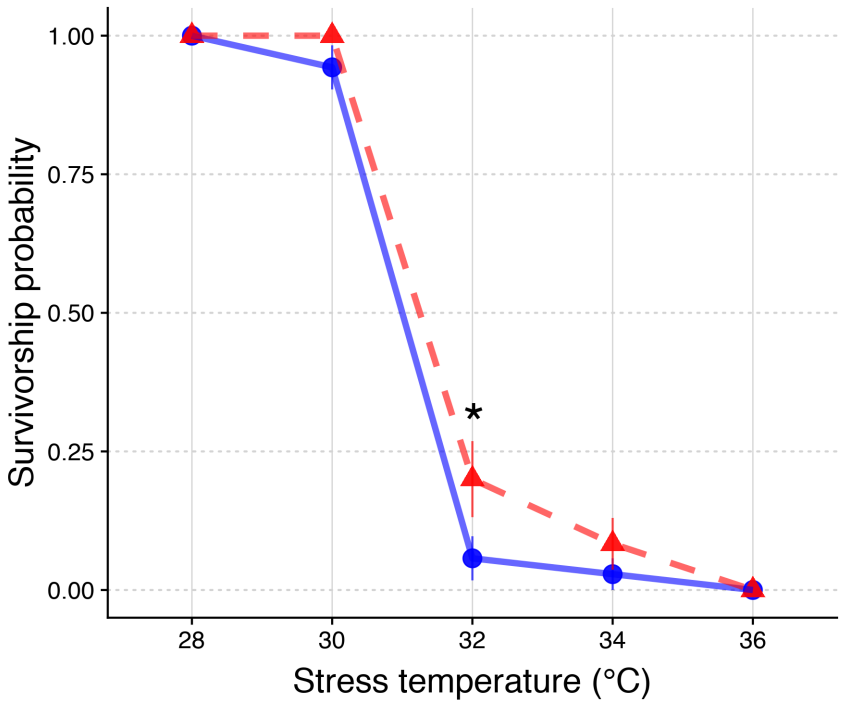

Figure 2.4. Probability of survival of *Daphnia* from Blue Lake sampled during summer 2017 to exposure to stress temperatures for 1h across all maternal lines. Survivorship to stress temperature was tested in individuals from different samples reared at 17°C (blue) and 24°C (red) acclimation temperatures. Asterisk indicates significant differences in survivorship between acclimatization treatments.

Discussion

Our study found variation in phenotypic plasticity in response to temperature among *Daphnia* clones isolated at different times of the growing season in an alpine lake. Phenotypic traits showed distinct responses to time and acclimation. Thermal survivorship curves were similar over time indicating that only acclimation temperature affected survivorship. Similarly, the effect of acclimation temperature on growth rate and age at maturity did not change throughout the growing season. By contrast, plasticity in size at maturity, interval between clutches and CT_{max} varied among maternal lines isolated on different dates, indicating potential seasonal genetic variation in plasticity in response to

temperature. Different traits showed distinct seasonal variation in plasticity, indicating that plasticity in general does not show clear seasonal patterns. Rather, different traits tend to be most plastic at different times of year. Our results indicate that genetic diversity and seasonal variation in plasticity may maintain fitness and allow *Daphnia* to persist over a broad phenological temperature range encountered in alpine lakes.

Our data support other studies of the role of genetic adaptation and plasticity in *Daphnia's* response to temperature and seasonal cycles. Carvalho (1987) compared clones of *Daphnia magna* before and after seasonal shifts in temperature and found that the transition from spring to summer was associated with selection for clones that exhibit increased survivorship and fecundity at high temperatures. Other studies also have shown that *D. magna*, *D. pulex, D. pulicaria,* and *Simocephalus vetulus* (another freshwater zooplankton) have plastic growth rate and age at maturity responses to acclimation temperature (e.g. Van Doorslaer et al. 2007, Van Doorslaer et al. 2010, Cavalheri et al. 2018). Intrinsic growth rate is often greater at higher temperatures due to an increased metabolism (e.g. Mitchell and Lampert 2000, Weetman and Atkinson 2004), which permits rapid maturation and reduction in age at release of each clutch, resulting in larger populations in warmer temperatures (Stich and Lampert 1981, Kingsolver and Huey 2008, Henning-Lucass et al. 2016). We also found that the maternal line collected in 13-Sep-2017 exhibit higher level of plasticity for age at maturity than the other maternal lines. This is also consistent with the result for the *Daphnia* collected in 04-Sep-2017, which showed an increase in offspring number at the higher acclimation temperature. Studies have shown that *Daphnia* can show genetic variation in habitat preference, including DMV behavior (Weider 1984, De Meester 1993, Tessier and Leibold 1997, Stirling and Roff 2000). Hence,

this supports a growing body of evidence that both genetic and plastic variation in response to temperature is important for *Daphnia* population dynamics.

Inter-clutch intervals can be shortened by a decrease in time for egg development caused by higher metabolic rates in response to temperature (Gulbrandsen and Johnsen 1990), but also by timing of egg production. Reichwaldt et al. (2005) studied *D. magna* and *D. hyalina* responses to constant cold (12°C), constant warm (19°C) and fluctuating (12h in warm and 12h in cold) temperature regimes. They found that egg development time was significantly different between treatments. *Daphnia* reared at a cold temperature had the longest time to develop eggs, followed by the fluctuating and warm treatments, showing that temperature fluctuations experienced by organisms during diel vertical migration may have direct effects on egg development time. Our results are consistent with that work, as the interval between clutches was the longest when maternal lines were reared at 17°C and the shortest when reared at 24°C.

The amount of plasticity in the interval between clutches changed throughout the growing season. *Daphnia* from mid-summer (16-Aug-2017) had the lowest level of plasticity while individuals from early-fall (05-Oct-2017) had the highest. This result contradicts our hypothesis that spatial environmental heterogeneity in the form of vertical stratification should select for greater plasticity. Plasticity for interval between clutches could be related to timing for egg production that could respond to food availability (Bradley et al. 1991). Phytoplankton are abundant in mid-summer (Figure 1c), but declined in the early-fall. When food is scarce, *Daphnia* decrease total energy investment in reproduction, but optimize the investment per offspring (Goulden et al. 1987), since larger egg size is positively correlated with offspring fitness. Thus, individuals produce fewer,

but larger eggs. Even though different life history traits showed seasonally variable plasticity, plasticity in intrinsic growth rate was consistent among sampling periods. This result supports Lynch (1989) who concluded that despite life history changes in *D. pulex* in response to food availability, reproductive tradeoff can lead to the same population growth rate with many different life history strategies.

Previous studies have shown that *Daphnia* reared in higher temperatures had increased thermal tolerance, indicating that this trait is plastic and the response is adaptive (Paul et al. 2004, Yampolsky et al. 2014). CT_{max} is a measurement of the upper thermal limits, i.e. tolerance to extreme temperatures, thus, adaptive changes in the mean CT_{max} response is most likely to occur due to maximum temperatures or heat waves (Ward et al. 2016, Yampolsky et al. 2014, Geers et al. 2015, Brans et al. 2017). Furthermore, thermal tolerance is related to aerobic capacity, which reflects the relationship between oxygen supply and demand of organisms' tissues (Portner and Knust 2007). Thus, adaptive changes in slopes of the reaction norms indicate that organisms have greater aerobic capacity to cope with a wider range of temperatures. Our results show that maternal lines from the beginning of the growing season (16-Aug-2018) have an increase of up to 1.9°C in CT_{max} after acclimation in higher temperature (24°C), followed by increases of 1.4°C (13-Sep-2018) and 0.8°C (5-Oct-2018). In general, CT_{max} decreased during fall compared to summer, except for the *Daphnia* collected on 04-Sep-2017. Paul et al. (2012) found that thermal tolerance of summer clones was greater than spring or fall clones, followed by winter clones. This corroborates the general pattern found in this study that CT_{max} seems to decrease throughout the growing season, suggesting that thermal variability might play an important role in selecting plasticity for CT_{max}.

The inferences we draw from our study are constrained by a number of limitations. Most importantly, we only had one maternal line from the two intermediate samples due to high mortality, thus our maternal line might not represent the average plasticity for that time. Low survival in laboratory conditions is often observed in alpine and high latitude zooplankton. Additionally, establishment and maintenance of maternal lines in the laboratory might cause artificial selection that may have prevented us from recording more prominent differences in life-history traits between maternal lines. A more comprehensive assessment of the genetic diversity present in Blue Lake is needed to assess the generality of our results. Nevertheless, we observed clear evidence for phenotypic plasticity and variation among clones isolated at different times of growing season.

Genetic variation reflects an evolutionary potential to respond to future disturbances or gradual changes, such as warming, either through adaptation (Brans et al. 2017, Geers et al. 2015) or adaptive plasticity (Cavalheri et al. 2018). Furthermore, our previous work (Cavalheri et al. 2018) showed that different populations of *D. pulicaria* from Sierra Nevada (CA) evolved plasticity in response to temperature, indicating the presence of genetic variation for plasticity among populations. Our results build on our prior work by documenting variation in plasticity through time, possibly as a result of tracking changes in temperature of the water column. Lakes are already affected by climate warming by longer duration of ice-free periods, stratification, and reduced vertical mixing (McCormick 1990, Schindler et al. 1990, Adrian et al. 1995, O'Reilly et al. 2015). Thus, it will be expected changes in genetic structure of *Daphnia* populations favoring individuals capable of coping with a wider range of environmental conditions, i.e. higher levels of plasticity. Our results show that *Daphnia* populations exhibit genetic variation for plasticity

in response to temperature, suggesting that these keystone species are likely to be resistant to the direct effects of global warming and may have the capacity to persist in freshwater ecosystems in a warmer world.

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CHAPTER 3

Phenotypic and transcriptional response of *Daphnia* to the combined effects of temperature and predation

Abstract

Plasticity at the level of gene expression is one of the most important mechanisms for coping with biotic and abiotic stress. *Daphnia*, an ecologically important zooplankton species in lakes, shows both genetic adaptation and phenotypic plasticity in response to temperature and fish predation, but little is known about the molecular basis of this response and the potential for these responses to interact. We performed a factorial experiment exposing two *Daphnia* genotypes from two lakes in the Sierra Nevada mountains (CA) to high or optimal temperature (25°C or 15°C) in the presence or absence of fish cues (kairomones), then measured changes gene expression and phenotype. At the molecular level, both clones had more differently expressed genes in response to temperature than predation. Differently expressed genes in response to temperature involved down-regulation of genetic pathways related to protein biosynthesis, indicating decreasing energy expenditure in both clones, while metabolic pathways were up regulated in one clone and down regulated in another, suggesting possible compensation in response to temperature. Both genotypes matured at a younger age in response to higher temperature and fish cues, while size at maturity, fecundity and population intrinsic growth were only affected by temperature. In contrast, exposure to fish cues increased upper thermal tolerance limits of both clones. Temperature also triggered expression of genes related to

oxygen binding and reproduction. Predation triggered up-regulation of genes possibly related to meiosis. Our results show that despite similar phenotypic responses in both clones, overall regulation pattern for energy intake was clone-specific, while energy expenditure was reduced in both clones. Our results suggest that phenotypic plasticity in response to temperature interacts synergistically as exposure to fish predators increases *Daphnia's* tolerance of stressful temperatures and revealed that the similar phenotypic responses to temperature and predators arise from divergent patterns of gene regulation.

Introduction

Many species are at risk of extinction as the environment changes at an unprecedented pace. A fundamental objective of ecological and evolutionary research is to predict species' responses to anthropogenic environmental change, such as global warming and introduction of predators (Parmesan 2006). In order to cope with such stressors, species can either migrate to more suitable places or adapt to new conditions. To predict persistence of populations and changes in biodiversity under environmental change, it is necessary to understand the potential and limits of genetic adaptation and phenotypic plasticity to maintain fitness (Urban et al. 2016). Understanding the mechanisms by which organisms are adapted to present day conditions throughout their ranges is therefore paramount to predicting extinction, persistence, and changes in biodiversity in a rapidly changing environment (Gienapp et al. 2008, Merilä and Hendry, 2014).

Plasticity and genetic adaptation impact organismal fitness in response to environmental change. Plasticity allows a genotype to express multiple phenotypes in different environments whereas genetic adaptation arises from environment-dependent

variation in fitness of different genotypes. Evidence for rapid evolution in natural systems, particularly in response to human-induced environmental change, indicates that most of the observed changes are not genetically based, but rather a consequence of plasticity (Hendry et al., 2008). Plasticity at level of gene expression is one of the most important mechanisms for coping with stress (Hoffmann et al. 2005, Yampolsky et al. 2014b), yet the magnitude of this plasticity remains largely unknown.

Plasticity arises from differential gene expression patterns in response to environmental cues (Whitehead and Crawford 2006, 2007). Variable levels of plasticity may evolve in a population if reaction norms differ across genotypes and the slope of the reaction norm is correlated with fitness (Aubin-Horth and Renn 2009). Either decreases or increases in phenotypic plasticity could contribute to adaptation to variable environments depending on the rate and predictability of environmental change (DeWitt et al. 1998, Via and Lande 1985).

Several factors could constrain the evolution of plasticity at the transcriptome level. First, genetic variation for plasticity could be limited or absent. Alternatively, even when genetic variation for plasticity is present, its evolution could be constrained by costs of the mechanisms underlying plastic responses (Sørensen et al. 2003). A trade-off is expected where enhanced plasticity would be beneficial in more spatially or temporally variable environments, but detrimental in a stable environment (Huang and Agrawal 2016, Kenkel and Matz 2016). In addition, if environmental variation is unpredictable, then plasticity may fail to match phenotypes to the environment to produce higher fitness.

Zooplankton, such as *Daphnia*, an ecologically important genus in lakes that transfers energy from phytoplankton to fish and exert top-down control on primary

production by grazing (Miner et al. 2012), show both genetic adaptation and plasticity at phenotypic level in tolerance to two important determinants of fitness: temperature and predation. Previous studies have documented that the response of *Daphnia* populations to thermal stress is strongly correlated with environmental conditions and can be either genetic, plastic or both (Williams et al. 2012, Geerts et al. 2015, Yampolsky et al. 2014a). Although these studies show plastic and evolutionary changes, the co-occurrence of other environmental stressors, such as predation, with temperature might interact in natural populations. Fish predation and temperature impose selection on many of the same traits, and in the same direction (Williams et al. 2012, Stoks et al. 2016). For instance, *Daphnia* often mature at smaller sizes and younger ages in warmer water and when fish are present, yet little is known about the molecular basis of this response among populations or their potential interactive effects.

We collected *Daphnia* from two lakes in the Sierra Nevada Mountains (CA) that differ in thermal conditions then conducted an experiment to evaluate the degree to which life history, thermal tolerance and gene expression are influenced by temperature and predation. We predicted that warm temperatures and exposure to predators should produce similar responses life history traits, then used transcriptomics to ask whether the same genes underlie the plastic response to fish and warming, or if the similar phenotypic effects arise from pleiotropic effects where altered gene regulation affects multiple phenotypic traits. Our goal was to ask whether *Daphnia's* plastic response to one selective agent (fish or warming) magnified or dampened the response to the other in terms of both phenotype and gene expression.

Materials and methods

Lake sampling

Gardisky (37.955774, -119.251198) and Blue lakes (38.051164, -119.270342) are located in Inyo National Forest (CA). Lakes were sampled in late August or early September 2017. In both lakes we measured temperature throughout the water column at each meter (Figure 3.6) using field probe (YSI Incorporated, Yellow Springs, Ohio, USA). We also collected live zooplankton from the deepest point of the lake using a 63 μm mesh conical net with a 30 cm diameter and 1 m length through the water column, starting 1 m above the lake bottom. These samples were kept cold until returning to the laboratory, where we searched for the presence of *Daphnia pulicaria*. When present, 30 *D. pulicaria* females carrying eggs in the brood pouch were separated in 50 ml falcon tubes filled with COMBO medium (Kilham et al. 1998). Each *Daphnia* was, then, considered a maternal line. Each maternal line was cultured for at least twelve generations in separate 50 mL Falcon tubes filled with COMBO medium (Kilham et al. 1998) under ambient conditions (around 22°C and natural light). All animals were fed the green alga *Nanochloropsis* sp. at a constant high rate of 24×10^6 cells every two days.

Experimental design

We randomly picked one individual for each lake to propagate. *Daphnia* reproduces asexually under benign conditions. Thus, all *D. pulicaria* were propagated asexually in the experiment. We started by randomly picking one mature female from populations of each lake clone. Upon the release of the second clutch we isolated three neonates that were separated and moved to new 100 mL containers containing the same media and algae. All

individuals were transferred to fresh media and algae three times a week and were reared at 15 ± 1°C and photoperiod 12:12 h light/dark. This allowed us to establish three independent grandmother cultures of each maternal line. The second and third clutches of the grandmother generation were pooled together and used to start up twelve cultures of the mother generation per maternal line. We established twelve independent cultures of each maternal line from the mother generation onwards. Upon release the second and third clutches of the mother generation, 3 - 4 neonates (daughter generation) of each mother generation were distributed in individual 50 mL falcon tubes, totalizing 50 individuals per maternal line. Finally, two neonates of the second clutch of each daughter were separated in individual tubes, totaling 100 individuals per maternal line. We collected all neonates from the second and third clutches that were born over the previous 12 h from each of the parental jars. All neonates were placed into 100 ml jars containing COMBO medium at a density of 3 *Daphnia*/jar (Kilham et al. 1998). Each jar was randomly allocated to one of four treatments: (1) optimum temperature (15°C, which is close to the maximum temperature measured in the lakes and considered a benign temperature, Figure 3.6) without kairomones (fish cues), (2) high temperature (25°C) without fish cues, (3) optimum temperature with fish cues, and (4) high temperature with fish cues. All *Daphnia* were transferred to fresh medium, with algae and kairomones (in the fish cue treatment) daily. We monitored jars daily for maturation (i.e., release of first clutch into the brood chamber). Upon reaching maturity, individuals were preserved in RNAlater (Qiagen, USA) and kept in -20°C for subsequent RNAseq analyses assigned to phenotypic assay (see below). Since RNA was extracted from whole individuals, no food was added during the last 12 h before sampling in order to minimize algal RNA contamination (most of which will be digested

and hence degraded after 12 h). The period without food was kept relatively short to minimize starvation-dependent gene regulation.

Kairomones

COMBO medium conditioned by the presence of planktivorous fish was collected daily from a tank containing 5 juvenile rainbow trout (*Oncorhynchus mykiss*; ~ 5 cm in total length) in 72 L of COMBO. Each day, media containing fish chemical cues was filtered through 0.7 μm mesh membrane filters and added at a concentration of 0.007 fish/L to the predator treatments.

Phenotype assays

Individuals from the experimental generation assigned to the phenotypic assay were scored for the following life-history variables: age and size at maturity, and age and number of offspring from the first three clutches. These data were used to calculate intrinsic population growth rate for each maternal line following the Lotka–Euler equation (Roff 1997). We also measured critical maximum temperature (CT_{max}) using a heat ramping assay. After a 30-minute resting period at ambient temperature, *Daphnia* from the four treatments were transferred to 0.5ml Eppendorf tubes and placed in a thermal heater (4x6 thermal heater, Corning Digital Dry Bath Heater Dual Block). The water temperature increased 0.1°C every 20 seconds. We continuously monitored the state of individuals, and recorded the temperature when each individual *Daphnia* lost swimming ability and sank to the bottom of the tube. The temperature when *Daphnia* became immobilized was used as a proxy for CT_{max} (e.g. Geers et al. 2016). We measured phenotypic traits of 11 to 13

individuals per treatment per lake and CT_{max} of 10 to 12 individuals per treatment per lake. In total, we scored phenotypes for 186 individuals.

Statistical analysis for phenotype

We log-transformed all continuous variables, except intrinsic growth rate, after visually assessing the probability distribution that best fit the data. We analyzed the effects of the treatments using generalized linear models for each trait using the statistical software R (R Core Team 2018). Temperature treatment, fish cue treatment, lake and their interactions were modeled as fixed effects for age and size at maturity, average number of offspring, intrinsic growth rate and CT_{max}. Since we expected that CT_{max} would be influenced by body size we included an additive effect of size.

Additionally, in order to test the effects of slopes of the reaction norms, we calculated the pairwise differences for each dependent variable between each treatment combination within a lake. We computed pairwise differences using the least square mean values based on the generalized linear models for each trait using *lsmeans* function in the *lmerTest* package in R statistical language (Kuznetsova et al. 2017, R Core Team 2018). Pairwise difference test were corrected by Tukey-adjusted for multiple comparison. All analyses were performed using R statistical language (R Core Team 2018).

RNA isolation, library preparation and sequencing

We extracted RNA using the TRI Reagent Protocol (Sigma-Aldrich, USA) from samples preserved in RNAlater (Qiagen, USA). Any remaining genomic DNA was removed using the TURBO DNA-free Kit (Invitrogen, USA). Each treatment included five

biological replicates, with each replicate comprised of 35 clonal *Daphnia* individuals. Extracted RNA was stored in -80°C until sequencing. Quality of the isolated RNA was assessed using RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA) and mRNA-seq libraries were constructed using NEBNext UltraTM RNA Library Prep Kit for Ilumina (NEB, USA) following manufacturer's recommendations. A total amount of 1 μg RNA per replicate was used as input material for RNA-seq library preparations. Index codes were added to attribute sequences to each sample and all 40 libraries were clustered on a cBot System using the PE Cluster kit cBot-HS (Illumina). After generating the clusters, libraries were sequenced using the Illumina HiSeq 2000 platform and 100 bp paired-end reads were generated.

Transcriptome assembly and functional annotation

The raw reads were preprocessed through a custom Perl scripts from Novogene Bioinformatics Technology Co., Ltd. (Sacramento, CA) to discard low-quality reads. Reads were filtered to remove those with adaptors, PHRED quality scores < 20, reads that were < 20 bp in length and reads with > 5% unknown nucleotides. Sequence duplication, Q20, Q30 and the GC content of the clean reads were calculated (Table 3.2). All downstream analyses were based on the cleaned, high-quality data. *De novo* assembly of the transcriptome was performed for each genotype separately. We used 18 replicates from Blue Lake and 17 replicates from Gardisky Lake, samples with < 5G output and assembly was realized using Trinity (Grabherr et al. 2011), with min_kmer_cov set to 2, and the other parameters left on default settings.

For each genotype the assembly was annotated through BLAST searches against the following seven databases: nr (nonredundant NCBI protein sequences, e-value = 1e-5), nt (nonredundant NCBI nucleotide sequences, e-value = 1e-5), Pfam (Protein family, e-value = 0.01), KOG/COG (Clusters of Orthologous Groups of proteins, e-value = 1e-5), Swiss-Prot (manually annotated and reviewed protein sequence database, e-value = 1e-5), KO (KEGG Orthology database, e-value = 1e-10) and GO (e-value = 1e-6). Gene names were assigned based on the annotation of the closest UniProt match, with uninformative descriptions excluded. A BLASTn search was also performed against the NCBI nt (nucleotide sequence) database using a protein query. The Blast2GO Program was used to assign GO (Gene Ontology) terms with an e-value <= 1e-5. After the unigenes were successfully aligned and the CDSs were translated into amino acid sequences, transcript open reading frames were extracted. The software ESTScan (Iseli et al. 1999) was used to decide the sequence direction of unigenes that did not align to any of the above databases.

Differential expression analysis

In order to estimate the relative expression levels of the unigenes, we mapped the RNA-Seq data back to the transcriptome assemblies for each sample using RSEM (RNA-Seq by expectation-maximization; Li and Dewey 2011) and BOWTIE (mismatch 2; parameters set default; Langmead et al. 2009). RSEM/BOWTIE mapping was implemented using scripts packaged with the Trinity pipeline. The significance of difference in gene expression of each treatment for each genotype was determined using the R package DESeq (Wang et al. 2010, R Core Team 2018). DESeq provides statistical routines for determining differential expression in gene expression data using a model

based on the negative binomial distribution. Statistical significance (p-value) was adjusted using the q-value obtained from the false discovery rate (Storey and Tibshirani 2003), with a q-value < 0.005 and |log2(foldchange)| > 1 set as the threshold for significantly differential expression. Pearson correlation analysis and hierarchical clustering analysis was performed with log2-transformed median-centred FPKM (fragments per kilobase of transcript per million mapped reads) of expression values to visualized patterns of expression for significantly differentially expressed genes (DEGs) in R statistical software (R Core Team 2018).

Enrichment analysis was performed to illustrate the biological functions of the identified DEGs. We used up- and down-regulated DEGs to perform enrichment analysis. We performed GO and KEGG enrichment analyses to determine if significantly differentially expressed gene sets were enriched for particular functional categories of genes. GO database classifies unigenes in three distinct domains: molecular functions, biological processes, and cellular components, while KEGG database categorizes the pathways; the KEGG pathway database is a widely accepted source for molecular pathway maps. Mapping of the DEGs to the KEGG pathways can provide insights into the functional relevance of the gene lists corresponding to the high-throughput expression data (Manyam et al. 2015). The interactions of multiple genes may be involved in certain biological functions, thus pathway enrichment analysis identifies significantly enriched pathways associated with DEGs compared with the whole genome background. To compare these results with the transcriptome background, all DEGs were subjected to GO enrichment analysis using the GOseq R package (Young et al. 2010, R Core Team 2018), which is based on the hyper-geometric distribution, and KEGG pathway enrichment

analysis using the KOBAS (Mao et al. 2005) software to test the statistical enrichment of DEGs in KEGG pathways. We considered categories as significantly enriched if the ratio test resulted in a Bonferroni-corrected p-value ≤ 0.05.

Results

Phenotype

The generalized linear model revealed that traits and the two clones responded differently to temperature and fish cues (Table 3.1). The clone form Gardisky Lake matured at a younger age than Blue Lake (Lake: $p < 0.001$, Table 3.1). At 15°C, Gardisky clones matured at 0.70 ± 0.09 day younger than the Blue Lake clone. Age at maturity was also earlier in the presence of fish cues at high temperature (Temp x Fish: p-value = 0.006, Table 3.1, Figure 3.1). The post-hoc test (least square means, see methods) showed that fish had an effect only in Blue reared at 25°C (Table 3.3). At 25°C, Blue Lake replicates reared with fish cues matured 0.55 ± 0.16 days earlier compared to replicates without fish cues.

Temperature affected size at maturity differently in the two populations (Temp x Lake: $p < 0.001$, Table 3.1) but fish cues had no effect (Fish: $p = 0.093$, Table 3.1, Figure 1). Post hoc tests revealed that the Blue Lake clone matured at 1.26 ± 0.006 mm regardless of temperature, while Gardisky matured at 15°C with 1.31 ± 0.01 mm compared to 1.41 ± 0.01 mm at 25°C.

The average number of offspring for the first three clutches showed a significant three-way interaction (Temp x Fish x Lake: $p = 0.029$, Table 3.1, Figure 3.1). For the Blue Lake clone, offspring number only varied with temperature (Table 3.3); on average Blue

had 1.1 ± 0.11 more offspring at 25°C compared to 15°C. In contrast, the effect of temperature on fecundity was greater for the Gardisky Lake clone when fish cues were present. Individuals had 1.67 ± 0.30 more offspring at 25°C than 15°C in the presence of fish cues, while without fish cues the difference between 25°C and 15°C response was 0.98 ± 0.23 offspring.

The intrinsic growth rate was significantly affected by the main effects of temperature and lake (Temp: $p < 0.001$ and Lake: $p < 0.001$, Table 3.1, Figure 3.1). At each temperature, the Gardisky Lake clone had higher intrinsic growth rates compared to the Blue Lake clone. For instance, the Gardisky clone had an $r = 0.15 \pm 0.001 * day^{-1}$ at 15°C, while Blue had $r = 0.11 \pm 0.004 * day^{-1}$. At 25°C, the intrinsic growth rate was $0.32 \pm 0.006 * day^{-1}$ for the Gardisky Lake clone, while the growth rate for the Blue Lake clone was $0.28 \pm 0.007 * day^{-1}$.

CT_{max} increased significantly when *Daphnia* were reared at 25°C compared to 15°C. CT_{max} was significantly affected by the interaction between temperature and fish cues (Temp x Fish: $p = 0.001$, Table 3.1) and showed a marginally significant interaction between temperature and lake (Temp x Lake: $p = 0.052$, Table 3.1, Figure 3.1), but no main effect of lake. Post hoc tests showed that at 15°C, fish cues had no effect on CT_{max} (Table 3.3). The thermal tolerance of the Gardisky clone increased by 0.8 ± 0.12°C in the presence of fish cues, while the Blue Lake clone increased by 0.3 ± 0.14°C at 25°C.

Gene expression

For the Blue Lake clone, sequencing produced on average 42,306,315 raw pair-ended reads for each sample. In total, for the Blue Lake samples, 746,514,988 clean reads

(after removing adaptor sequences, low-quality and ambiguous sequences) were used to assemble the transcriptome data using the Trinity method (Table 3.3). Using the Trinity program, clean reads were assembled into 108,410 transcripts with a mean size of 1510 bp. The transcripts were subjected to cluster and assembly analyses, resulting in a total of 108,325 unigenes, having an average size of 1511 bp and including 25,259 unigenes (23.32%) with lengths greater than 2k bp. The Gardisky clone produced on average 44,025,859 raw pair-ended reads for each sample and 732,462,246 clean reads (Table 3.2). Moreover, the Gardisky clone assembly resulted in 95,691 transcripts with mean size 1656 bp and after cluster analysis 95,632 unigenes were identified with average length of 1656 bp, including 25,140 (26.29%) unigenes with length greater than 2k bp.

Temperature treatments (25N vs. 15N and 25Y vs. 15Y) produced the greatest number of differentially expressed genes (DEGs). A total of 152 genes were differentially expressed between treatments for the Blue Lake clone. Exposure to different temperatures (15°C and 25°C) without predator cues was associated with 52 significantly differentially expressed genes (25N vs. 15N) specific to this treatment comparison, while exposure to different temperatures with predator cues showed 65 differentially expressed genes (25Y vs. 15Y, Figure 3.2a). Thirteen genes were differentially expressed at high vs. low temperature regardless of the presence of fish (Figure 3.2a). Only eight unique genes responded to fish in the 15°C temperature treatment (15Y vs. 15N) and 14 in the 25°C temperature treatment (25Y vs. 25N, Figure 3.2a).

For both Blue and Gardisky clones, temperature affected expression of a larger set of genes than predator cues. For the Gardisky Lake clone, we identified 700 DEGs and similarly to the Blue clone, most DEGs responded to temperature treatments. In total, 532

genes were specific to 25N vs. 15N (Figure 3.2b) and 49 were specific to 25Y vs. 15Y. These two treatment comparisons shared 48 genes (Figure 3.2b). Predator cues had a lower impact on gene expression. At 15°C ,13 genes were differentially expressed (15Y vs. 15N), while at 25°C 58 DEGs were identified (25Y vs. 25N, Figure 3.2b).

Gene expression patterns tended to be consistent across replicates in the same treatment (Figure 3.3). Cluster analysis separated samples by temperature treatment (15°C or 25°C, Figure 3.3) for both Blue and Gardisky. It also separated by predation treatment (N or Y, Figure 3.3), with the exception of two Gardisky samples reared at 15°C (Figure 3.3b).

For the Blue clones, we were able to successfully annotate 76.77% of unigenes and 70.7% were matched with *Daphnia pulex*. In total, 51.17% of the unigenes were annotated with at least one term by the Gene Ontology (GO) database and 16.55% were successfully annotated with the KO database (see Table 3.4 for annotation from other databases). For Gardisky clones, we successfully annotated 75.86% of the unigenes in at least one database and from those 78.5% matched with *Daphnia pulex*. 51% were annotated using GO database and 23.54% using KO database. GO terms classify genes according to one of three categories: molecular function, biological process and cellular components. Comparisons of significant GO terms for differentially expressed genes showed that only molecular function was significantly enriched for the Blue Lake clones, while molecular function and biological process were enriched for Gardisky Lake clones.

GO helps to clarify the functionality of a gene and GO enrichment analysis determines which term appears more often than would be expected by chance when comparing treatment conditions. In this study, the most enriched GO terms were related to

molecular function: catalytic activity, binding and structural molecule activity, as well as the biological process, proteolysis (Figure 3.4). Significant enriched GO terms were only found for treatment comparisons between temperatures. In the Blue clone, differences in temperature without predator cues (25N vs. 15N) involved changes in peptidase (serine-type endopeptidase, serine-type peptidase and endopeptidase) and hydrolase activity (serine hydrolase, Figure 3.4). A different set of genes responded to temperature with predator cues (25Y vs. 15Y), but those were still involved in catalytic activity: transferase (sulfotransferase and sulfate adenylyltransferase) and carboxy-lyase (phosphoenolpyruvate carboxykinase and sulfate adenylyltransferase, Figure 3.4a). Ten pathways were significantly enriched (adjusted p-value ≤ 0.05) in the Blue clone when DEGs were mapped to the terms in the KEGG database. Response to temperature without predator cues (25N vs. 15N) was only significantly enriched for the Blue clone, exposure to high temperature down-regulated energy metabolism (nitrogen metabolism), carbohydrate metabolism (amino sugar and nucleotide sugar metabolism), folding, sorting and degradation (ubiquitin mediated proteolysis) and translation (ribosome biogenesis, Figure 3.5a). High temperature combined with predator cues (25Y vs. 15Y) showed down regulation of carbohydrate metabolism (starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism), digestive system (carbohydrate digestion and absorption) and metabolism of cofactors and vitamins (retinol metabolism), while the metabolism of other amino acids (glutathione metabolism) was up-regulated (Figure 3.5a). Comparison between predator cues treatments at high temperature (25Y vs. 25N) showed up-regulation of replication and repair in the Blue clone (Fanconi anemia pathway, Figure 3.5a). The predator cue treatment

at 15°C (15Y vs. 15N) showed down-regulation of translation (mRNA surveillance pathway, Figure 3.5a).

The Gardisky clone also showed enrichment for peptidase activity (endopeptidase, serine-type endopeptidase, and serine-type peptidase) and hydrolase activity (serine hydrolase) (Figure 3.4b). Reaction to high temperature was similar with or without fish cues, but other molecular functions besides catalytic activity were enriched for Gardisky when experiencing high temperature without fish cues. Specifically, those related to protein degradation (proteolysis), oxygen transport and exoskeleton (Figure 3.4b). Twelve pathways were significantly enriched in Gardisky clone in the KEGG database. Significant up-regulated pathways for 25Y vs. 15Y were: glycan biosynthesis and metabolism (glycosaminoglycan biosynthesis), carbohydrate metabolism (pentose and glucuronate interconversions, fructose and mannose metabolism and galactose metabolism), lipid metabolism (glycerolipid metabolism), folding, sorting and degradation (ubiquitin mediated proteolysis) and transport and catabolism (lysosome); down-regulated: replication and repair (mismatch repair), metabolism of cofactors and vitamins (porphyrin and chlorophyll metabolism), glycan biosynthesis and metabolism (other types of O-glycan biosynthesis), amino acid metabolism (glycine, serine and threonine metabolism) and translation (aminoacyl-tRNA biosynthesis, Figure 3.5b). Treatment with predator cues at 15°C (15Y vs. 15N) showed up-regulation of glycan biosynthesis and metabolism (glycosphingolipid biosynthesis), replication and repair (Fanconi anemia pathway) and cell growth and death (cell cycle and p53 signaling pathway) (Figure 3.5b).

Table 3.1. Results of the generalized linear model analysis of the life-history traits of *Daphnia pulicaria* collected in Blue and Gardisky Lakes and reared under different temperature and fish cues treatments (Temp: 15°C and 25°C; Fish cues: N – without fish cues – and Y – with fish cues).

Factor	df	Deviance	Residuals deviance	Pr (>Chi)
Age at maturity				
Temperature	1, 184	16.0918	3.1609	**< 0.001**
Fish cues	1, 183	0.1340	3.0270	**0.002**
Lake	1, 182	0.3961	2.6309	**< 0.001**
Temp x Fish	1, 181	0.1036	2.5273	**0.006**
Temp x Lake	1, 180	0.0020	2.5253	0.7098
Fish x Lake	1, 179	0.0147	2.5106	0.3074
Temp x Fish x Lake	1, 178	0.0011	2.5096	0.7800
Size at maturity				
Temperature	1, 182	0.1078	0.9068	**< 0.001**
Fish cues	1, 181	0.00002	0.9068	0.9349
Lake	1, 180	0.2726	0.6341	**< 0.001**
Temp x Fish	1, 179	0.0054	0.6286	0.1989
Temp x Lake	1, 178	0.0382	0.5904	**< 0.001**
Fish x Lake	1, 177	0.0046	0.5858	0.2368
Temp x Fish x Lake	1, 176	0.0024	0.5833	0.3917
Average number of offspring				
Temperature	1, 87	2.7408	9.7072	**< 0.001**
Fish cues	1, 86	0.0403	9.6669	0.3436
Lake	1, 85	5.7215	3.9454	**< 0.001**
Temp x Fish	1, 84	0.0105	3.9349	0.6290
Temp x Lake	1, 83	0.0826	3.8524	0.1751
Fish x Lake	1, 82	0.0030	3.8494	0.7963
Temp x Fish x Lake	1, 81	0.2120	3.6374	**0.0297**
Intrinsic growth rate				
Temperature	1, 87	0.6194	0.0962	**< 0.001**
Fish cues	1, 86	0.0007	0.0954	0.3087
Lake	1, 85	0.0327	0.0627	**< 0.001**
Temp x Fish	1, 84	0.0000	0.0627	0.9713
Temp x Lake	1, 83	0.0003	0.0624	0.5032
Fish x Lake	1, 82	0.0003	0.0620	0.4853
Temp x Fish x Lake	1, 81	0.0014	0.0606	0.1665
Critical maximum temperature (CT_{max})				
Size at maturity	1, 91	0.0003	0.0256	0.1655
Temperature	1, 90	0.0082	0.0173	**< 0.001**
Fish cues	1, 89	0.0009	0.0163	**0.0125**
Lake	1, 88	0.0004	0.0159	0.1112
Temp x Fish	1, 87	0.0015	0.0144	**0.0019**
Temp x Lake	1, 86	0.0006	0.0138	0.0523
Fish x Lake	1, 85	0.0003	0.0134	0.1356
Temp x Fish x Lake	1, 84	0.0000	0.0134	0.5433

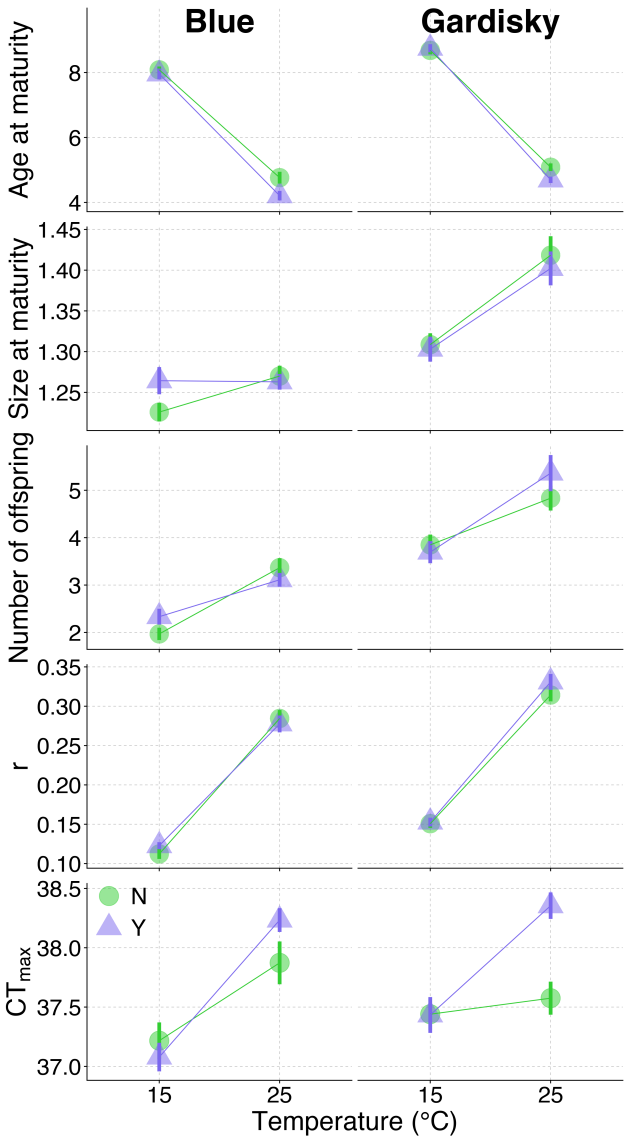

Figure 3.1. Means ± 1 S.E.M. of age at maturity (days), size at maturity (mm), average number of offspring of the first three clutches, intrinsic growth rate (r), and critical maximum temperature (CT_{max}, °C) of *Daphnia pulicaria* collected at Blue and Gardisky Lakes during summer 2017 in response to temperature (15°C and 25°C, x-axis) and predation cues treatments. Circles show response without fish cues and triangles response to fish cues.

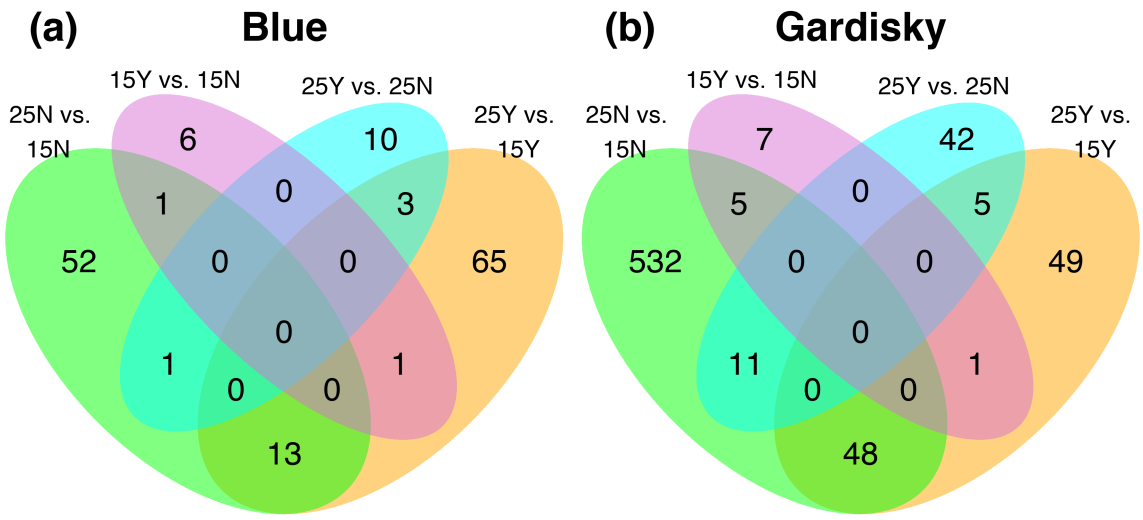

Figure 3.2. Venn diagram illustrating the number of differentially expressed genes (DEGs) genes for *Daphnia* from Blue (a) and Gardisky (b) in response to temperature (15°C and 25°C) and predation cues (N = without cues and Y = with cues) treatments.

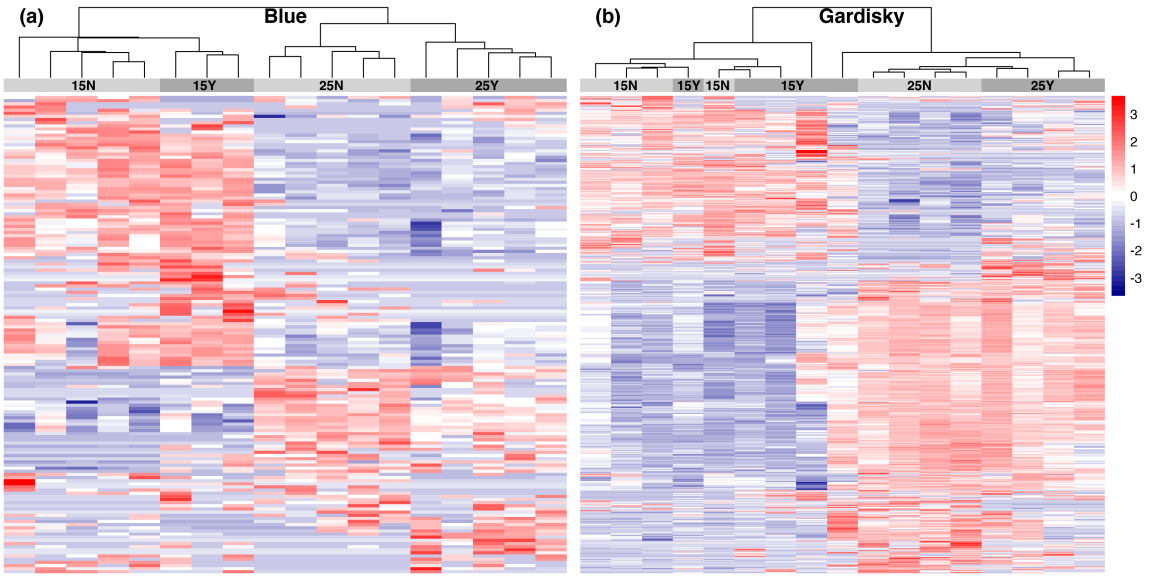

Figure 3.3. Heat map showing RNA-Seq expression levels of differentially expressed genes (DEGs) for *Daphnia* clones from Blue (a, 152 DEGs) and Gardisky (b, 700 DEGs). Expression values are log2-transformed median-centred FPKM. Red and blue colour intensity indicates upregulation and downregulation, respectively. Dendrogram clustering on the x-axis indicates sample similarity. In the gray bars, "15" and "25" refer to temperature treatments (15°C or 25°C) and "N" and "Y" refer to predator cues (N = without predator cues; Y = with predator cues).

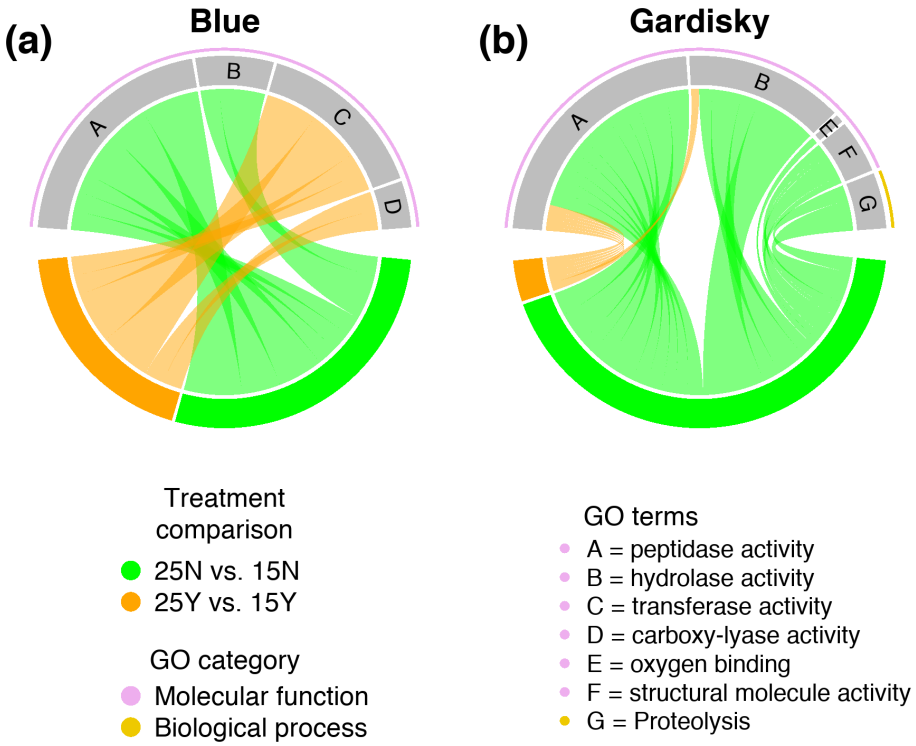

Figure 3.4. Enrichment of significant GO terms for *Daphnia* from Blue (a) and Gardisky (b). Green and orange ribbons represent enrichment of significantly differentially expressed genes between treatments (25N vs. 15N and 25Y vs. 15Y, respectively). Gray peripheral rings represent GO terms within molecular function and biological process categories (dark yellow and purple, respectively). The larger the ribbon connecting to a gray ring (or GO term), the more genes described by that GO term.

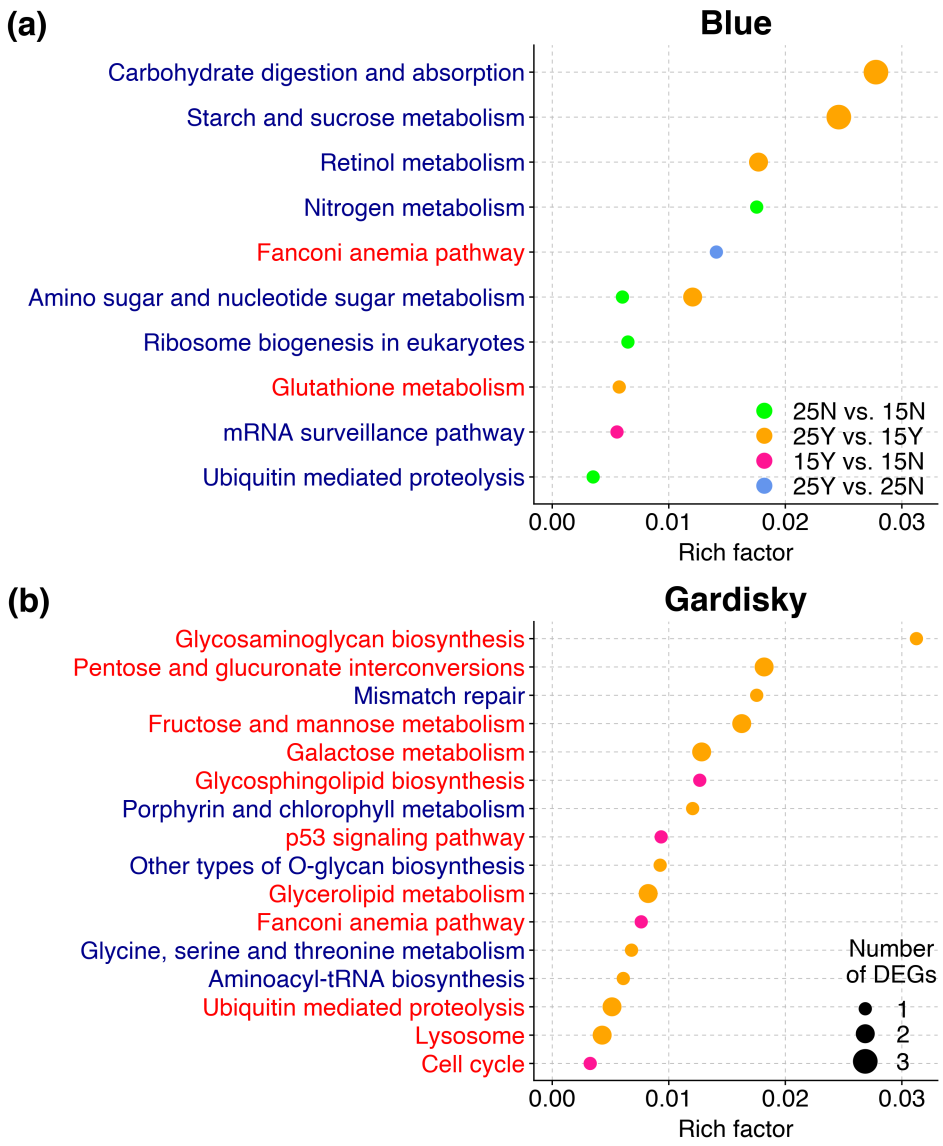

Figure 3.5. Enriched KEGG of up-regulated (red) and down-regulated (blue) DEGs with Bonferroni-corrected p-value ≤ 0.05 for Blue (a) and Gardisky (b). The x-axis indicates the rich factor, which represents the number of DEGs in the related pathway (point size) divided by the number of all the annotated genes in this pathway. Each color indicates distinct treatment comparisons.

Discussion

We found similar phenotypic response between two *Daphnia* clonal lines to the effects of temperature and predator cues. Fish cues magnified the effect of 10°C of

warming on age of maturity, and increased the critical maximum temperature at which *Daphnia* lose motor control when individuals were reared at high temperatures, but not at optimum temperature. In our experiment, we found that temperature is the most important factor driving changes in genes expression, most of the DEGs in both clones responded to changes in temperature (Figure 3.2). We also found different regulation of genetic pathways that underlies the plastic response to predators and temperature (Figure 3.5). The results suggest that the phenotypic response to introduced predators increases thermal tolerance and affects life history response to warming. Coexisting with novel predators may therefore increase *Daphnia's* fitness in a warming environment. Additionally, though clones show similar phenotypic response to temperature and predation, the genetic regulation leading to similar phenotypes is different, suggesting distinct environmental sensitivities to stress.

Phenotypic responses

Both clones generally matured at a younger age when reared in high temperature and also produced more offspring and thus a higher intrinsic growth rate (Figure 3.1). Indeed, high temperature increases metabolic rate, often leading to a larger reproductive investment and faster development (Stibor 1992, Riessen 1999). In addition, we found an interactive effect of temperature and predation on age at maturity and critical maximum temperature. These results agree with others studies that also have found synergistic effect of these two stressors in *Daphnia* and other organisms (e.g. Miler et al. 2014, Janssens et al. 2015, Riessen 2015, Tseng and O'Conner 2015, Luhring and DeLong 2016, Zhang et al. 2016, Luhring et al. 2018). We found that individuals reared with predator cues had

higher thermal tolerance and matured younger at 25°C than those reared without predator cues. In *D. pulex*, after seven generations, Tseng and O'Connor (2015) observed increased thermal plasticity in individuals reared in higher temperatures but only when they were also reared with predators. Zhang et al. (2016) tested resurrected *D. magna* population from pre-fish period and high-density fish period in response to temperature and predation. They found that *Daphnia* that coexisted with fish exhibited earlier maturation in high temperatures compared to *Daphnia* from the pre-fish period. Earlier maturation under predation risk is common in size-dependent predation in *Daphnia* where predators, such as fish, prefer larger prey items, inducing a smaller size at maturity and earlier age of first reproduction (Riessen 1999). Our results agree with these earlier studies as fish cues magnify the life history response to temperature, and also show that they can increase the tolerance of stressfully high temperatures.

Temperature response in fishless condition

We observed up-regulation of serine peptidases and hydrolases in high temperature treatment for both clones (Figure 3.4). These enzymes are the most important digestive proteases in *D. magna* (Von Elert et al. 2004). They are involved in proteolysis in the digestive system, i.e. the process by which peptide bonds in proteins are broken generating free amino acids. This up-regulation may indicate a necessity to accommodate higher feeding rates caused by increased metabolism in high temperatures (Stibor 1992, Riessen 1999). Moreover, the *D. pulex* genome contains many peptidase genes, which might indicate adaptation to high variation in food availability in aquatic environment (Colbourne et al. 2007).

The responsiveness of metabolic genes suggests an important correlation between temperature and metabolism (Tewksbury et al. 2008). A recent study with *D. magna* reveled plasticity in the expression of several genes in response to extreme temperature events (Jansen et al. 2017). Moreover, Jansen et al. (2017) documented evolutionary changes in gene expression due to shifts in temperature occurring both over short time scales (2 years in populations from mesocosms) and long time scales (40 years in populations resurrected from different layers of the lake sediment). Populations from the mesocosm responded to high temperature by regulating genes that belong to central metabolic functions, immunoregulation and oxidative stress response.

In our study, although we found in general most up-regulation of peptidases and hydrolases in both clones, the enriched genetic pathways from KEGG database showed the opposite regulation pattern for the Blue clone (Figure 3.5). Individuals from Blue Lake, showed a down-regulation of carbohydrate and energy metabolisms. This discrepancy could be because, in addition to acting as digestive enzymes, serine proteases are also involved in several other physiological functions such as degradation, blood clotting, immunity, and development (Krem et al. 2000) and we could not identify the exact enzymes in the peptidase and hydrolase families because Gene Ontology only enriched broad categories. Moreover, down-regulation of metabolic pathways might occur if ventilation and perfusion rates approach their maximum levels (Paul et al. 2004), where *Daphnia* need to adjust demands for energy supply by changing gene expression patterns. Yampolsky et al. (2014b) evaluated gene expression response of two heat-tolerant and two heat-sensitive populations of *D. pulex* after acclimatization at 18°C and 28°C. They observed differential expression in numerous metabolic and regulatory pathways, but more

genes were down-regulated than up-regulated and this effect was stronger in higher plasticity, i.e. in heat-tolerant genotypes, than in heat-sensitive genotypes, suggesting metabolic compensation as a possible acclimation mechanism. Thus, down-regulation of metabolic pathways could indicate a compensatory response to high temperature.

Up-regulation of genes related to protein sorting, folding and degradation is a general, common response to stressors. For instance, heat-shocked *D. magna* have been observed to enhance production of heat shock proteins (HSPs, Mikulski et al. 2009). Although we did not find enriched genetic pathways related to HSPs, we observed down-regulation of ubiquitin mediated proteolysis pathway and translation in Blue in response to high temperature, which might also indicate a molecular adjustment to improve energy supply (e.g. by adjusting feeding rate) and/or reducing energy expenditures, such as down-regulating expression of genes for anabolic processes (e.g. protein biosynthesis) (Becker et al. 2018).

In the Gardisky clone, we also observed up-regulation of oxygen binding and structural molecule activity that may be related to hemoglobin production and moulting, respectively. In higher temperature body tissues impose an increased demand for oxygen demand (Pörtner and Farrell 2008). Moreover, genes linked to components of the exoskeleton, such as those related to structural molecule activity, suggests more frequent moulting in higher temperature. Those genes regulate the ability of the organism to destroy and build chitin structures that are important for the formation of a new exoskeleton for the ecdysis (Merzendorfer and Zimoch 2003).

Temperature response under predation risk

Similar to the response to temperature without fish cues, the Blue clone showed a down-regulation of carbohydrate metabolism (Figure 3.5) and also down-regulation sulfotransferases and carboxy-lyases (Figure 3.4). Genes coding for sulfotransferases, including sulfate adenylyltransferases, catalyze the addition of sulfate in several compounds (Josephy 1997). There is evidence that addition of sulfate to some molecules in *D. magna* may represent a detoxification process (Ikenaka et al. 2006), while sulfate can also be involved in processes related to purine and selenium amino acid metabolisms, which are important for DNA and RNA processes. On the other hand, carboxy-lyases convert oxaloacetate into phosphoenolpyruvate, playing an essential role in glucose metabolism. Again, GO terms were broad categories and we could not determine the exact function of the genes assigned for the categories mentioned above.

The Blue clone up-regulated stress genes, such as those part of the glutathione metabolism. The up-regulation of glutathione proteins may reflect a higher production of stress damaged cell components and molecules, which can be exported by membrane transporters (Ballatori et al. 2009). Up-regulation of glutathione proteins is a common response under temperature stress in *Daphnia* (e.g. Heckmann et al. 2008, Roy Chowdhury et al. 2015, Becker et al. 2018).

In contrast, the Gardisky clone up-regulated genes related to carbohydrate metabolism as well as lysosome genetic pathways. Lysosome pathways are related to intracellular digestion. Lysosomes contain several hydrolases that help in the degradation of macromolecules, such as proteins. This result matches the expectation of an increased generation of energy from feeding to supply the higher rate of metabolic processes in

higher temperatures. In higher temperatures, up-regulation of gene expression is expected for the digestion of proteins and carbohydrates to match the higher metabolic demands at higher temperatures (Dölling et al. 2016). When organisms experience stressful conditions, proteins can denature or be misfolded. These proteins are often labeled by ubiquitins for degradation (Hershko and Ciechanover 1998). Hence, our results indicate that the up-regulation of ubiquitins in Gardisky might be associated to stressful conditions, such as high temperature.

The Gardisky clone also up-regulated lipid metabolism, particularly glycerolipids (Figure 3.5). Reproductive *D. magna* females accumulate large quantities of fatty acids as glycerolipids, which are stored in lipid droplets and allocated to the moult and egg formation (Tessier and Goulden, 1982, Goulden and Place 1990). Evidence has shown that *Daphnia* species bioaccumulate highly polyunsaturated fatty acids from their diet and allocate them to the eggs (Sengupta et al. 2016). Jansen et al. (2017) found that resurrected core populations of *D. magna* that experienced distinct temperatures showed down-regulation for genes associated with egg formation (DamVTG1), which encodes the egg yolk precursor protein vitellogenin, which has been shown to be up-regulated in asexual females of *D. pulex* as compared to sexual, resting-egg bearing females (Raborn et al. 2016).

Predation at optimum and high temperatures

Gene expression in response to only fish predation at low temperature (15Y vs. 15N) showed only one enriched pathway in Blue Lake and five in Gardisky Lake (Figure 3.5). Blue clone down-regulated genes associated with translation, which could be related in reducing energy expenditures, by down-regulating expression of genes involved in

anabolic processes (e.g. protein biosynthesis) (Becker et al. 2018). On the other hand, Gardisky up-regulated pathways related to replication and repair, cell cycle, such as p53 signaling pathway, and glycosphingolipid biosynthesis. Cell cycle pathways, including expression of p53 transcription factor, were found up-regulated in *D. pulex* asexual females compared to sexual females and genes associated with this pathway were annotated as meiotic genes in *D. pulex*, suggesting a role in reproduction (Raborn et al. 2016). Hales et al. (2017) observed up-regulation of genes related to egg formation and embryo development, as well as larger clutches after exposure to predator cues in *D. ambigua* (Walsh et al. 2015). Genes involved in the glycosphingolipid pathway are important membrane building blocks and may play an important role in the composition of the membrane. The effect of predation at 25°C (25Y vs. 25N) showed only enrichment for genetic pathways in Blue clone, which up-regulated replication and repair pathways (which were up-regulated in Gardisky but only at low temperature).

Our results differ from other studies that have found several genes coding in protein metabolism with differential expression to predator exposure, such as translation genes (40S and 60S ribosomal proteins, eukaryotic translation initiation factor; Jansen et al., 2013), synthesis (18S and 28S; Schwarzenberger et al. 2009), folding (cyclophilin; Schwarzenberger et al. 2009), and degradation (ubiquitin specific protease; Schwarzenberg et al. 2009, Jansen et al. 2013). Degradation and reactivation of damaged proteins in known to be involved in stressful conditions, for instance induction of HSPs have been previously reported in response to predators, which facilitate the synthesis and folding of proteins (Pauwels et al. 2005, Pauwels et al. 2007, Pijanowska and Kloc 2004). However, we did not find any of these genetic pathways enriched when comparing predation treatments.

Furthermore, Jansen et al. (2013) used microarray analysis to document differential expression of allergen and globin genes in *D. magna* after fish cues exposure. High levels of allergens could be caused by a response to the predator, serving as a defense mechanism during predation. Globins, on the other hand, are related to oxygen binding hemoglobin and myoglobin and may be a compensating mechanism related to behavioral response to predation. Several studies have reported diel vertical migration as a defense to fish predation in several *Daphnia* species (Boersma et al. 1998, De Meester and Cousyn 1997, De Meester et al. 1999, Cousyn et al. 2001, Jansen et al. 2013). Diel vertical migration occurs when *Daphnia* migrates to the deeper layers during the day, which often has less dissolved oxygen, in order to avoid encounter with fish that occupies upper layers (Salonen and Lehtovaara 1992). Contrasting with our results, *Daphnia* usually respond to predation with a smaller body size at maturity, which could be a result of either earlier allocation of resources into vitellogenin, triggering asexual reproduction at an early age (Stibor 2002) or a decrease in actin and tubulin concentrations (Pijanowska and Kloc 2004). However, we only find up-regulation of oxygen binding genes in response to temperature and both clones in our study did not change body size in response to predator cues.

We recognize that characterizing the gene expression levels of one clone per lake, the consequence of difficulties rearing natural populations of *Daphnia*, is a limitation of our study, However, we did not aim to characterize genetic variation within populations in the response to temperature and predation. We included clones from two lakes only to ensure genetic variation between the two clonal lines. We also used separated *de-novo* RNA transcriptome assembly due to possible genetic divergence between clones, thus we are not able to quantitatively compare levels of expression between clones. Ultimately, we

see this work as providing a first overview of general genetic pathways involved in response to temperature and predation in two *Daphnia* clones and characterize possible variation in enriched genetic pathways between clones.

Conclusion

Our study highlights that expression patterns of genes differed between *Daphnia* clones while phenotypic responses and interactions were qualitatively similar, suggesting that different transcriptomic responses can result in similar phenotypes. Other studies have found distinct gene expression patterns among individuals from different zooplankton species (e.g. Roy Chowdhury et al. 2015, Lima and Willett 2017). These results suggest that diverse genetic pathways can give rise to similar phenotypic plastic responses to environmental stress. We also showed synergistic interactions between temperature and predation for some traits. Overall, our results show that *Daphnia* can have similar phenotypes through distinct molecular mechanisms and the synergistic effects of temperature and predation may represent an important mechanism for organisms to adapt to a rapidly changing environment.

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Appendix

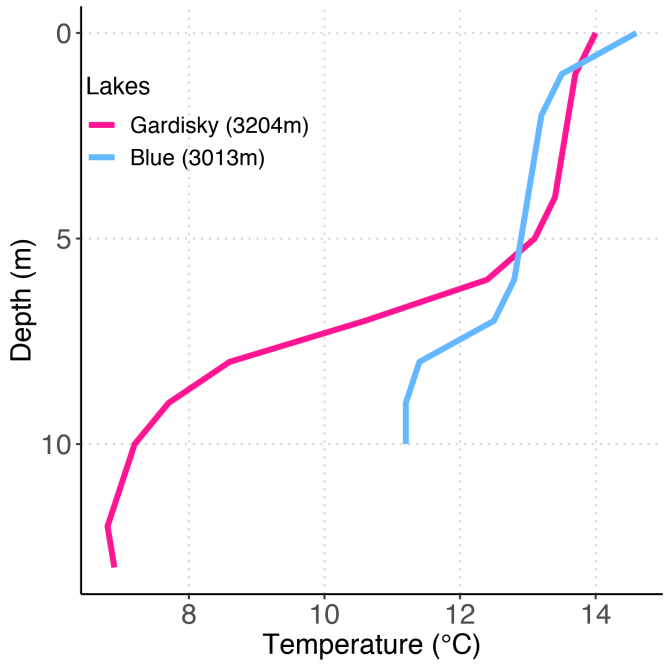

Figure 3.6. Water column temperature profile of Blue and Gardisky Lakes measured in late August or early September during summer 2017. In parenthesis: elevation.

Table 3.2. Data quality control summary for RNA-Seq.

Sample (replicate)	Raw reads	Clean reads	Clean Bases	Error (%)	Q20 (%)	Q30 (%)	GC Content (%)
Blue Lake							
s22	43973956	43219328	6.5G	0.02	98.63	95.65	45.08
s33	43217124	42199480	6.3G	0.02	98.75	95.88	46.39
s34	39155128	38615788	5.8G	0.02	98.64	95.65	45.31
s23	34860246	34291946	5.1G	0.02	98.70	95.87	46.32
s15	40745166	39360958	5.9G	0.02	98.43	95.28	44.89
s35	50074920	49450854	7.4G	0.02	98.75	95.83	44.52
Comb_9	36537648	35875020	5.4G	0.02	98.40	94.85	44.64
Comb_36	48713866	47666366	7.1G	0.02	98.56	95.30	45.60
Comb_1	48068206	47162022	7.1G	0.02	98.34	94.94	46.34
Comb_32	39143324	38525164	5.8G	0.02	98.54	95.24	45.18
s31	45687954	44428186	6.7G	0.02	98.70	95.80	47.49
s14	39788536	38877516	5.8G	0.02	98.72	95.86	46.44
s18	50328200	49198090	7.4G	0.02	98.68	95.83	45.59
s25	40936186	40104412	6G	0.02	98.65	95.72	46.90
s8	41031090	40099214	6G	0.02	98.62	95.58	45.56
s13	36127218	35386006	5.3G	0.02	98.62	95.68	46.09
s24	43593710	43016746	6.5G	0.02	98.79	96.03	45.86
s30	39531198	39037892	5.9G	0.02	98.73	95.88	45.74
Gardisky Lake							
S19	48588148	47570624	7.1G	0.02	98.71	95.84	45.60
S28	42594982	41426256	6.2G	0.02	98.71	95.85	47.06
S5	44356400	43057718	6.5G	0.02	98.59	95.68	44.87
S10	40416570	39510194	5.9G	0.02	98.65	95.66	46.83
S38	47004660	46347016	7G	0.02	98.73	95.86	44.87
S11	42528098	41524206	6.2G	0.02	98.83	96.14	46.50
S20	36927282	36232366	5.4G	0.02	98.66	95.71	44.59
S26	41943264	41438036	6.2G	0.02	98.66	95.73	46.37
S37	44916548	44264526	6.6G	0.02	98.70	95.75	45.39
S29	44656226	43374154	6.5G	0.02	98.78	96.00	46.93
S39	45913042	44947618	6.7G	0.02	98.66	95.69	47.20
S16	48798556	47866930	7.2G	0.02	98.63	95.61	46.24
S7	46574406	45524106	6.8G	0.02	98.76	95.93	45.19
S21	44226560	43253904	6.5G	0.02	98.72	95.87	46.18
27	45227736	44304004	6.6G	0.02	98.80	96.06	46.99
S40	39884796	38962992	5.8G	0.02	98.67	95.75	46.70
S12	43882324	42857596	6.4G	0.03	98.13	94.00	46.03

Table 3.3. Least square mean (LS mean) values calculated using the generalized linear model analysis of the life-history traits of *Daphnia pulicaria* collected in Blue and Gardisky Lakes and reared under different temperature and fish cues treatments (Temp: 15°C and 25°C; Fish cues: N – without fish cues – and Y – with fish cues). Different Group letters indicate significantly different LS mean value after Bonferroni correction for multiple comparisons.

Lake	Temperature	Predator cues	LS mean	SE	Group
Age at maturity					
Blue	15	N	2.087	0.024	a
Blue	15	Y	2.069	0.024	a
Blue	25	N	1.545	0.025	b
Blue	25	Y	1.422	0.024	c
Gardisky	15	N	2.159	0.025	a
Gardisky	15	Y	2.166	0.024	a
Gardisky	25	N	1.619	0.024	b
Gardisky	25	Y	1.541	0.024	b
Size at maturity					
Blue	15	N	0.202	0.011	a
Blue	15	Y	0.232	0.012	a, b
Blue	25	N	0.238	0.012	a, b
Blue	25	Y	0.232	0.011	a, b
Gardisky	15	N	0.267	0.012	b
Gardisky	15	Y	0.263	0.012	b
Gardisky	25	N	0.346	0.011	c
Gardisky	25	Y	0.335	0.012	c
Average number of offspring					
Blue	15	N	0.652	0.063	a
Blue	15	Y	0.829	0.070	a
Blue	25	N	1.197	0.067	b
Blue	25	Y	1.123	0.061	b
Gardisky	15	N	1.332	0.063	b, c
Gardisky	15	Y	1.282	0.061	b
Gardisky	25	N	1.558	0.061	c, d
Gardisky	25	Y	1.650	0.061	d
Intrinsic growth rate (r)					
Blue	15	N	0.112	0.008	a
Blue	15	Y	0.122	0.009	a, b
Blue	25	N	0.284	0.008	c, d
Blue	25	Y	0.277	0.007	c
Gardisky	15	N	0.150	0.008	b
Gardisky	15	Y	0.152	0.007	b
Gardisky	25	N	0.314	0.007	d, e
Gardisky	25	Y	0.330	0.007	e
Critical maximum temperature (CT_{max})					
Blue	15	N	3.616	0.003	a
Blue	15	Y	3.613	0.003	a
Blue	25	N	3.634	0.003	b, c, d
Blue	25	Y	3.643	0.003	c, d
Gardisky	15	N	3.622	0.004	a, b
Gardisky	15	Y	3.623	0.003	a, b
Gardisky	25	N	3.625	0.004	a, b, c
Gardisky	25	Y	3.646	0.004	d

Table 3.4. Number of successfully unigenes annotated for Blue and Gardisky clones using individual *de novo* RNA-Seq assembly.

Database	Number of unigenes	Percentage (%)
Blue		
NR	70512	65.09
NT	33365	30.80
KO	17932	16.55
SwissProt	51707	47.73
PFAM	54762	50.55
GO	55440	51.17
KOG	33347	30.78
Annotated in all databases	6459	05.96
Annotated in at least one database	83169	76.77
Total unigenes	108325	100.00
Gardisky		
NR	65304	68.28
NT	23022	24.07
KO	22518	23.54
SwissProt	43986	45.99
PFAM	48374	50.58
GO	48780	51.00
KOG	30044	31.34
Annotated in all databases	7955	08.31
Annotated in at least one database	72550	75.86
Total unigenes	95632	100.00

CONCLUSIONS

Plasticity and genetic adaptation impact organismal fitness in response to environmental change. Plasticity allows a genotype to express multiple phenotypes in different environments whereas genetic adaptation increases fitness only in its local environment. Although there is evidence for rapid evolution in natural systems in response to human-induced environmental change, most of the observed changes are, in fact, not genetically based, but rather a consequence of plasticity (Hendry et al. 2008).

In Chapter 1 I explored how environmental history affected *Daphnia pulicaria* population response to temperature by exposing Sierra Nevada (CA) populations from high and low elevation lakes that had fish present or absent to two distinct temperature regimes for two years. Most traits responded to temperature treatment they were exposed in the lab, but most importantly I found evidence for the influence of both the short- and long-term selective environment on life-history traits and growth rates of *Daphnia* populations. Although size at maturity showed imprint of the ancestral lake environment, high elevation lakes had higher levels of plasticity in response to temperature than low elevation lakes, plasticity in population growth rate was most influenced by selection over the past two years. This result supports our hypothesis, in that after two years of selection, populations would adjust plasticity to cope with shifting thermal environments (Lande 2009, Crispo et al. 2010). Another study found similar results phenotypic plasticity increased the intrinsic growth rate for *D. magna* after only three months of thermal selection (Van Doorslaer et al. 2009). Analyses of temperature differences in the mesocosms experiment indicated higher temperature variation throughout the summer in cold mesocosms compared to warm

mesocosms, but warm mesocosms had the greatest daily minimum and maximum temperatures. Study with *Daphnia* has shown that maximum temperatures either on constant or fluctuating regime caused individuals to mature at the youngest age, and have the shortest time between clutches and the highest intrinsic growth rate, suggesting that fitness is higher at the warmest temperature (Orcutt and Porter 1983). In addition, across latitude populations of *D. magna* show a positive relationship between thermal tolerance and average temperature of the warmest month (Yampolsky et al. 2014a). This supports our result of an increase in plasticity in the warmer mesocosm, suggesting that experiencing high temperatures continuously, instead of temperature variability, could be the cause of increasing plasticity. Together, these results suggest that rapid evolution of life-history plasticity maintains population growth for *Daphnia.* Such rapid evolution of plasticity may have arisen from selection among coexisting clones with variable responses to temperature within lakes found at the same elevation. The results suggest that *Daphnia* populations display considerable adaptive potential to respond to changes in temperature within relatively short time scales.

In Chapter 2 I looked at variation in plasticity through time in a *D. pulicaria* population in Sierra Nevada (CA). Freshwater environments have extensive fluctuations temperature and all these factors can affect plastic levels. Hence, temperature fluctuations experienced by *Daphnia* may have direct influence on life history parameters. Here we tested the adaptive response to thermal stratification by collecting maternal lines throughout the growing season and measuring plastic levels of life-history traits in response to two distinct acclimation temperatures. We found that an increase in 7°C in the acclimation temperature resulted in a significant increase in survivorship, thermal tolerance,

age at maturity, and reduction in the interval between clutches, consequently, increasing intrinsic growth rate. Carvalho (1987) compared clones of *Daphnia magna* before and after seasonal shifts in temperature and found that the transition from spring to summer was associated with selection for clones that exhibit increased survivorship and fecundity at high temperatures. Other studies also have shown that *D. magna*, *D. pulex, D. pulicaria,* and *Simocephalus vetulus* (another freshwater zooplankton) have plastic growth rate and age at maturity responses to acclimation temperature (e.g. Van Doorslaer et al. 2007, Van Doorslaer et al. 2010, Cavalheri et al. 2018). These results reveal that phenotypic plasticity occurs in response to acclimation temperature in all maternal lines, but more importantly, significant differences in thermal tolerance and interval between clutches were found in the level of plasticity exhibited by maternal lines collected at the extremes of the growing season, i.e. from Mid-Summer and Early-Fall, reflecting that plasticity varies throughout the growing season and it can vary among traits. Paul et al. (2012) found seasonal variation in thermal tolerance in clones of *D. longispina* species complex from a reservoir in Germany. Those authors found that thermal tolerance of summer clones were higher than spring or fall clones, followed by winter clones. This corroborates the general pattern found in this study that thermal tolerance seems to decrease throughout the growing season. Our results show that *Daphnia* populations exhibit genetic variation for plasticity in response to temperature, suggesting that this keystone species is likely to be resistant to the effects of climate change and should be able to persist in freshwater ecosystems in a warmer world.

In Chapter 3 I investigate the potential molecular mechanisms behind plastic response in *Daphnia*. I performed a factorial experiment exposing two *Daphnia* genotypes from two lakes in the Sierra Nevada mountains (CA) to high or optimal temperature (25°C

or 15°C) in the presence or absence of fish cues (kairomones) and measured changes in phenotype (life history and thermal tolerance) and gene expression. Plasticity at level of gene expression is one of the most important mechanisms for coping with stress. *Daphnia*, an ecologically important zooplankton species in lakes, shows both genetic adaptation and phenotypic plasticity in response to temperature and fish predation, but little is known about the molecular basis of this response their potential interactive effects. I found similar phenotypic response between two *Daphnia* clonal lines to the effects of temperature and predator cues. Fish cues magnified the effect of high temperature on age of maturity, and increased the critical maximum temperature, but not at optimum temperature. High temperature increases metabolic rate, which often leads to a larger reproductive investment and faster development (Stibor 1992, Riessen 1999). In addition, earlier maturation under predation risk is common in size-dependent predation in *Daphnia* where predators, such as fish, prefer larger prey items, inducing a smaller size at maturity and earlier age of first reproduction (Riessen 1999). Increases thermal tolerance at high temperature when at risk of predation suggests introduced predators might affect life history response to warming. I also found that temperature is the most important factor driving changes in genes expression. The responsiveness of metabolic genes suggests an important correlation between temperature and metabolism (Tewksbury et al. 2008). Clones showed different regulation of genetic pathways that underlies the plastic response to predators and temperature. For instance, one clone up regulated metabolic pathways in response to temperature, while other down regulated. In higher temperatures, up-regulation of gene expression is expected for the digestion of proteins and carbohydrates to match the higher metabolic demands at higher temperatures (Dölling et al. 2016). However, down-

regulation of metabolic pathways could indicate a compensatory response to high temperature (e.g. Yampolsky et al. 2014b). In addition, genetic pathways related to stress, translation and reproduction were different between clones and between treatments. Hence, the results indicate that coexisting with novel predators may increase *Daphnia's* fitness in a warming environment and that *Daphnia* can have similar phenotypes through distinct molecular mechanisms.

A fundamental objective of ecological and evolutionary research is to predict species' responses to anthropogenic environmental change, such as global warming and introduction of predators (Parmesan 2006). To predict persistence of populations and changes in biodiversity under environmental change, it is necessary to understand the potential and limits of genetic adaptation and phenotypic plasticity to maintain fitness (Urban et al. 2016). My work shows that *Daphnia* populations are able to evolve plasticity in response to temperature over short-time scales (Chapter 1) and that a natural population has genetic variation for plasticity (Chapter 2). The molecular mechanism behind the plastic response triggered response to distinct genetic pathways, regulation is variable and this response is much stronger for temperature stress than predation (Chapter 3). These results collectively indicate that *Daphnia* differ in the mechanisms behind plasticity but populations have genetic variation that allows selection of distinct levels of plasticity in response to rapid selective pressures.

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