LIFE HISTORY EVOLUTION IN SEASONAL ENVIRONMENTS: PHENOLOGICAL AND ENVIRONMENTAL DETERMINANTS OF THERMAL ADAPTATION IN *WYEOMYIA SMITHII*

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ABSTRACT

GREGORY RAGLAND: Life history evolution in seasonal environments: phenological and
environmental determinants of thermal adaptation in *Wyeomyia smithii*
(Under the direction of Joel Kingsolver)

Environmental variation poses a major evolutionary challenge to organisms. This is
particularly true for seasonal environments where environmental factors fluctuate radically
but predictably on an annual basis. Dormant life history stages often evolve to mitigate
exposure to harsh seasonal environments (*e.g.*, winter). In addition, norms of reaction, or the
relationship between phenotype and environment, often evolve as a response to local
environmental heterogeneity. My thesis explores how the seasonal timing of dormancy
affects selection on reaction norms of active, non-dormant life history stages in temperate
insects. Changing the dates of initiation and termination of winter dormancy changes the
thermal habitat experienced during active growth and reproduction. Thus, geographic
variation in the timing of dormancy complicates geographic patterns of thermal selection on
active life history stages. Using available inter- and intraspecific life history data in
conjunction with long-term weather data, I show that geographic clines in dormancy timing
cause populations along the cline to experience similar exposure to cold temperatures during
active growth. As a result, strong latitudinal trends in the timing of dormancy predict weaker
latitudinal trends in thermal adaptation of active stages. I further illustrate this concept by
examining geographic variation in the timing of winter dormancy, thermal sensitivity of
development, and tolerance to thermal stress in the pitcher plant mosquito, *Wyeomyia smithii*.
The results from *W. smithii* suggest that selection applied specifically by the thermal

environment of the growing season best explains geographic variation in the thermal sensitivity of development time. In contrast, geographic variation in the thermal environment of the entire year best explains geographic trends in thermal stress tolerance of active life history stages. These results suggest two major conclusions. First, thermal sensitivity and thermal tolerance can exhibit local adaptation in populations that also demonstrate local adaptation in diapause timing. Thus the evolution of one type of adaptation does not preclude the other. Second, dormancy timing unquestionably influences direct selection on active life history stages, but correlated selection on overwinter survival may strongly influence temperature tolerance of active life history stages.

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CHAPTER 1

INTRODUCTION

A major challenge for organisms in nearly every type of habitat is to maintain positive fitness when faced with environmental variation. All organisms are limited to a defined range of tolerable environmental conditions, and environmental fluctuations that approach or exceed the boundaries of that range will impose fitness costs. The challenge is to achieve a relatively constant fitness value across environments. A common solution to this problem is to employ flexibility, or plasticity of the phenotype to achieve inflexibility of fitness.

Plastic phenotypes that mitigate fitness costs in sub-optimal environments are only adaptive, however, if they are appropriately timed to coincide with the environment in which they maximize fitness (Pigliucci 2001). Adaptive plasticity is thus a common evolutionary strategy in seasonal environments, where biotic and abiotic environmental factors fluctuate radically but predictably on an annual scale. For example, in *Colias* butterflies from North America there are different seasonal morphs that vary in the degree of wing melanism (a developmentally determined, irreversible phenotype). The expression of increased melanism occurs in early spring broods, and this season-specific timing is determined by photoperiodic cues (Watt 1968; Watt 1969). Wing melanism in *Colias* butterflies also serves as an example of adaptive phenology: spring broods benefit from increased melanism because basking adults can attain threshold temperatures for flight more readily during a time of year in which

available heat is relatively minimal compared to summer conditions. Summer broods, on the other hand, express decreased melanism presumably because the higher temperatures experienced pose a greater risk for overheating. Thus, the adaptive value of the expression of wing melanism is highly dependent upon the seasonal timing of expression (Watt 1969).

Similarly, the fitness benefits of diapause, the physiologically buffered dormant state of insects, are highly dependent upon seasonal timing (Tauber et al. 1986). Diapause is induced in order to avoid stressful environmental conditions such as low precipitation (Denlinger 1986), lack of food availability (Istock et al. 1975), or stressful temperatures (Bradshaw et al. 2004) that fluctuate on an annual basis. Timed appropriately, diapause allows persistence through unfavorable conditions under which active growth and reproduction would result in population decline. Too long a diapause period and the diapausing organism loses out on valuable time and resources that could be exploited to increase reproductive success. Too short a diapause period and the organism will be exposed to deleterious or lethal conditions. Thus, the timing of diapause not only determines the adaptive value of the diapause stage itself, but also determines the conditions that an organism experiences during active growth and reproduction.

This dissertation addresses the dependence between seasonal timing and the selective environment experienced by active life history stages. I explore this relationship by examining geographic variation in the environment, the seasonal timing of diapause, and temperature-dependent life history traits in temperate insects. The logic behind the hypotheses that I test has two main components. The first is the recognition that environmentally-mediated selection on a life history stage, in this case non-diapause (active) stages, critically depends on the seasonal timing of the initiation and termination of

dormancy in relation to seasonal environmental fluctuations. The second is the observation that the seasonal timing of dormancy varies with geography. For example, winters become increasingly harsher and longer proceeding away from the equator, and the timing of dormancy in a broad range of taxa changes with geography such that populations further from the equator have shorter growing seasons. This geographic variation in dormancy timing will then affect geographic patterns of selection on active life history stages.

Herein I examine the consequences of geographic variation in diapause timing for selection on and evolution of life history traits that are sensitive to temperature. I begin with a broad overview of geographic patterns in temperature variation and life history evolution in temperate insects (Chapter 2). By reviewing both intra- and interspecific comparative data on geographic variation in diapause and on a temperature-sensitive trait, I show that seasonal timing is generally more correlated with latitude than is the thermal sensitivity of development. I also show that evolved differences in seasonal timing can minimize the relationship between cold temperature exposure and latitude. This relationship is often implicitly assumed in studies that infer adaptation from clinal trait variation, illustrating how knowledge of seasonal timing can better inform adaptive hypotheses. In the following three chapters I address more specific questions about seasonality and life history evolution in the pitcher plant mosquito, *Wyeomyia smithii*.

Several ecological and organismal features make *W. smithii* an exceptional model system for investigating the geography of life history adaptation in seasonal environments. Adult females obligately oviposit into the water filled leaves of the purple pitcher plant, *Sarracenia purpurea*, and thus track the broad geographic range of *S. purpurea* from northern Florida up to the Great Lakes region inland and Newfoundland along the coast of

North America (Armbruster et al. 1998). Further, since *S. purpurea* only grows in acidic, boggy substrates, the distribution of the plant is spatially patchy. Adult mosquitoes are weak flying, and the distances between patches of *S. purpurea* are such that gene flow between *W. smithii* populations is virtually absent (Istock and Weisburg 1989). Additionally, *W. smithii* has a well characterized larval diapause stage that is cued by photoperiod and varies with latitude such that the termination and initiation of diapause become progressively later and earlier, respectively, with increasing latitude (Bradshaw and Lounibos 1977). Each geographic population of *W. smithii* represents an independently evolving unit on which selection has been acting since the last glacial maximum (Armbruster et al. 1998) to optimize diapause timing and the environmental sensitivity of active life history stages.

Chapters 3 and 4 test the hypothesis that the timing of diapause has evolved to reproduce similar thermal habitats across disparate geographic populations in *W. smithii* and examine the consequences for thermal selection on development time, body mass, and thermal tolerance. Using long-term temperature data I show that geographical gradients in the thermal habitat experienced during active growth and reproduction is different than the gradient predicted by simple metrics such as annual maximum and minimum temperatures. Specifically, the thermal habitat of the growing season, or the annual time period occurring after diapause termination but before diapause initiation, is more geographically divergent at high compared to low temperatures. Considering temperatures experienced over the course of an entire year, winter minimum temperatures decline faster than summer maximum temperatures with increasing latitude. Thus, the observed geographic trend shows that evolved differences in diapause timing have created similar thermal habitats during the

growing season by similarly limiting exposure to cold temperatures in all the geographic populations examined.

Trends in life history traits partially conform to predictions based on the diapause-defined growing season, but patterns are somewhat complex. Development time is geographically more variable at high than at low rearing temperatures, consistent with predictions based on the greater geographic variability of frequencies of high compared to low temperatures during the growing season (Chapter 3). In contrast, tolerance of stressful low temperatures of active stages is more geographically variable than tolerance of stressful high temperatures (Chapter 4). I therefore suggest that geographic patterns in low temperature tolerance of active life history stages may often reflect correlated selection on overwintering survival rather than direct selection on survival, performance, and reproduction during active growth.

Chapter 5 ranges a bit further afield, examining the impact of temperature variation within a geographic site (rather than mean temperature) on geographic variation in life history traits. I show that differences in annual temperature variation among sites have not greatly influenced the evolution of life history traits in *W. smithii*. However, the results do suggest that population differences in sensitivity to thermal variation will be most pronounced when the shape (*e.g.*, slope or curvature) of the underlying function relating life history traits to mean temperature is the most variable among population.

I close with a brief concluding statement, synthesizing the collective results and exploring broader implications. These joint investigations of seasonality and environmental sensitivity raise many more questions than they answer. The selective mechanisms that lead to specific combinations of seasonal timing and thermal responses remain unclear, as do

many of the physiological and genetic underpinnings of inter- and intraspecific variation. However, the results do suggest testable hypotheses to explore these questions. With global climate change imminent, a working knowledge of how environmental physiology and seasonal timing evolve will greatly inform predictions of range expansions, range shifts, and extinctions.

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CHAPTER 2

THE EVOLUTION OF PHENOLOGY AND THERMAL SENSITIVITY IN SEASONAL ENVIRONMENTS

Abstract

Both the timing, or phenology of life history stages and the environmental sensitivity of those stages represent major adaptations to seasonally fluctuating environments. Substantial empirical evidence shows that these traits diverge between geographic populations and between species with different geographic ranges. However, it remains unclear how phenology and environmentally-dependent physiology evolve in tandem. In particular, selection on the seasonal timing of a life history stage will affect selection on that stage mediated by an environmental factor that fluctuates seasonally and on the timescale of a single generation. Herein, I examine latitudinal trends in phenology, thermal physiology, and temperature to illustrate how this relationship between timing and environmentally-mediated selection affects seasonal adaptation in temperate insects. The timing of photoperiodically-cued diapause (dormancy) varies predictably with latitude, while the thermal sensitivity of development is only marginally related to latitude. Comparative data on intraspecific variation show that this disparity in pattern is probably not caused by genetic constraint on thermal sensitivity. However, latitudinal variation in temperature conditions experienced at seasonal transitions suggests that thermal selection on diapause phenology is more closely related to latitude than is selection on the thermal sensitivity of development. These results illustrate the importance of accounting for the phenological dependence of

selection on thermal physiology when formulating adaptive hypotheses based on

geographical variation.

Introduction

      Temperate organisms typically experience environmental fluctuations that vary on both a short-term (*e.g.*, diurnal) and long-term, seasonal scale. Faced with this high degree of environmental variability, what are the major adaptations that allow persistence in seasonal environments? This question has been addressed in a broad range of taxa for a number of phenotypes by exploring associations between native seasonal environments and various morphological, physiological, and life-history traits (*e.g.*, Blanckenhorn and Fairbairn 1995; Bradshaw and Lounibos 1977; Burke et al. 2005; Hoffmann et al. 2003; Kimura 2004; Masaki 1972; Mousseau and Roff 1995; Schmidt et al. 2005; Sorensen et al. 2005). Conclusions from these studies point towards two distinct, if not mutually exclusive classes of adaptations to seasonal environments: 1) the timing, or phenology life history events, life history stages, or alternate phenotypes, and 2) the norm of reaction for short-term reversible physiological and behavioral traits.

      Both of these types of traits are important in adaptation to seasonal fluctuations in temperature. Ectotherm physiology in particular is highly temperature-dependent, and seasonal temperature variation exerts selection on the thermal sensitivity of survival, growth, and reproduction (Angilletta et al. 2002; Angilletta et al. 2006; Gilchrist 1995; Huey and Berrigan 2001; Huey and Kingsolver 1989; Kingsolver and Gomulkiewicz 2003). Further, alternate phenotypes induced by developmental plasticity may enhance survival of extreme, stressful conditions and exploitation of more favorable conditions (Adedokun and Denlinger 1984; Brakefield 1996; Fric and Konvicka 2002; Hazel 2002; Jacobs and Watt 1994; Kingsolver 1995; Nice and Fordyce 2006; Rinehart et al. 2006; Saunders and Hayward 1998; Tanaka 1997). The seasonal timing of these alternate phenotypes is often critical,

synchronizing life cycles to match phenotypes to environments (*e.g.*, Bradshaw et al. 2000).

Selection on timing, however, is not independent of selection on thermal sensitivity,

complicating predictions about how these traits should evolve in tandem. Latitudinal patterns

in temperate insects provide an exceptional example of how this link between seasonal

timing and temperature-mediated selection affects life history evolution in seasonal

environments.

Phenology, or seasonal timing, determines the environmental context that a particular

life history stage or alternate phenotype will experience. For example, the fitness benefits of

diapause, the physiologically buffered dormant state of insects, are highly dependent upon

seasonal timing. Diapause is induced in order to avoid stressful environmental conditions

such as low precipitation (Denlinger 1986), lack of food availability (Istock et al. 1975), or

stressful temperatures (Bradshaw et al. 2004). In the context of seasonal temperature

fluctuations, the timing of diapause appears to be an especially critical trait for several

reasons. First, diapause is an avoidance response to temperatures that would be highly

deleterious and often lethal were an insect actively growing (Bradshaw et al. 2004). Seasonal

temperature variation, though technically continuous, generates discreet environments from

an insect's-eye-view: conditions that are favorable for growth and reproduction, and

conditions that are not. Failure to induce diapause before the transition between these two

environments often results in a fitness value of zero (Tauber et al. 1986). Second, the costs of

inducing (or failing to terminate) diapause during favorable growth conditions can be high

because certain differences between the diapause and non-diapause phenotypes are also

discrete. Most importantly, diapausing individuals arrest development and do not reproduce,

trading off reproductive success for survival. Thus, fitness may be greatly reduced in individuals that maintain diapause while conditions are favorable for growth.

In contrast to traits such as diapause, many physiological responses to the thermal environment occur rapidly, are short-term reversible (*i.e.*, on the order of hours or days), and are induced directly by temperature. The relationship between temperature and physiology in ectotherms is often largely determined by biochemical reaction kinetics (Schoolfield et al. 1981; Sharpe and Demichele 1977). Thus, thermal sensitivity of many traits can be viewed simply as a biochemical constraint. The specific parameters (*e.g.*, shape) of the relationship between physiology and temperature (a thermal reaction norm), however, can vary both inter- and intraspecifically (David et al. 2004; Delpuech et al. 1995; Kingsolver et al. 2004; Scheiner and Lyman 1989) and often reflect native thermal conditions: that is, the parameters of thermal reaction norms can be adaptive (Angilletta et al. 2002; Huey and Kingsolver 1989).

Physiological traits related to development, growth, and reproduction are all aspects of organismal performance and contribute to overall fitness through reproductive success and generation time (Garland and Carter 1994). In organisms that undergo a diapause state, however, selection acts on performance traits mainly during the favorable season when active growth and reproduction takes place. Selection on survival through unfavorable conditions, on the other hand, will act on various aspects of diapause, including the timing of induction and termination (Bradshaw and Lounibos 1977; Tauber et al. 1986). Changes in diapause timing will shift the seasonal 'window' of temperatures experienced during active growth and reproduction. In this respect, selection on diapause phenology and thermal performance

is not independent because diapause timing affects the temperature distribution of the favorable season.

Comparative studies of geographic populations have shown that there is often pronounced geographic variation for both diapause phenology (*e.g.*, Bradshaw and Lounibos 1977; Gomi and Takeda 1996; Masaki 1972; Nechols et al. 1987; Schmidt et al. 2005; Tauber et al. 1988b) and thermal physiology (*e.g.*, Ayres and Scriber 1994; Baldwin and Dingle 1986; Barnes et al. 1989; Birkemoe and Leinaas 2001; Carriere and Boivin 1997; Delpuech et al. 1995; James and Partridge 1995; Robinson and Partridge 2001). Often, this variation is correlated with local thermal and seasonal conditions such that individuals from a population obtain maximum fitness (or maximum values of fitness components) in the thermal/seasonal environments most similar to that population's native habitat. The results of these types of studies generally indicate that selection imposed by the thermal/seasonal environment indeed modifies both diapause timing and thermal physiology.

On the intra-specific level, there is some disparity between latitudinal trends in diapause timing and thermal physiology. In insects, the thermal sensitivity of development rate is often either unrelated or only marginally related to latitude (Beck and Apple 1961; Calvin et al. 1991; Campbell et al. 1974; Campbell and Mackaur 1975; Goryshin et al. 1987; Khomyakova 1976; Tauber and Tauber 1982; Tauber and Tauber 1987). In contrast, the relationship between seasonal timing of dormancy and latitude is nearly always tight, and in the expected direction (Beck and Apple 1961; Calvin et al. 1991; Campbell et al. 1974; Campbell and Mackaur 1975; Goryshin et al. 1987; Khomyakova 1976; Tauber and Tauber 1982; Tauber and Tauber 1987). With increasing latitude, the calendar date of spring emergence or resumption of growth and the date of the onset of dormancy increase and

decrease, respectively. Quite reliably, higher latitude populations have shorter growing seasons, whereas those same populations may or may not differ in thermal responses.

Modifications to seasonal timing and thermal physiology are both viable adaptive responses to selection mediated by temperature, so why does seasonal timing exhibit a more consistent latitudinal trend? There are three possible explanations for this disparity in patterns: 1) thermal physiology is more evolutionarily constrained than seasonal timing, 2) natural selection acts more strongly on seasonal timing, or 3) latitude is a better predictor of gradients in selection on seasonal timing than of gradients in selection on thermal physiology. Without detailed studies correlating specific phenotypes to fitness, (2) is difficult to address. However, available data on phenology and thermal physiology allow hypothesis (1) and (3) to be evaluated for temperate insects.

To illustrate the relative roles of selection and constraint in shaping these contrasting latitudinal patterns, I examine geographic variation in photoperiodic diapause responses and temperature-development rate relationships in terrestrial insects. I begin by summarizing latitudinal variation inferred from previous meta-analyses to establish the differences in geographic patterns. To explore potential evolutionary constraint, I then use insect species for which geographic variation in both diapause phenology and development rate have been characterized to ask whether the two types of traits are equally divergent among populations. Finally, I examine latitudinal trends in several aspects of seasonal temperature variation to ask how well latitude predicts selection on seasonal timing and the thermal physiology of development.

Empirical data on latitudinal trends

Hibernal diapause timing and norms of reaction for development rate vs. temperature have clear fitness implications mediated by temperature. The timing of initiation and termination of hibernal diapause will determine exposure of actively growing individuals to stressful winter conditions, including lethal low temperatures (Bradshaw et al. 2004). When an insect is in non-diapause development, the relationship between development rate and temperature will partially dictate whether individuals reach the diapause stage before the onset of winter (Gotthard et al. 2000), and in multivoltine populations it will also determine the number of generations produced during the growing season. Seasonal variation in temperature will thus influence the optimal timing of diapause and the optimal shape of the developmental rate vs. temperature reaction norm. Variation in seasonality across geography will drive divergence among populations or among species with different geographic ranges.

Trends in Critical Photoperiod

Photoperiodic (*i.e.*, daylength) cues commonly initiate and terminate diapause in insects. Temperature, photoperiod, or a combination of both can influence diapause timing (Tauber et al. 1986), but studies of latitudinal variation often focus exclusively on photoperiodic responses. The most common quantitative description of diapause timing is critical photoperiod (CP), the photoperiod at which 50% of a sample induces or terminates diapause (Bradshaw and Lounibos 1977; Taylor and Spalding 1986). CP may be reported for diapause induction, termination, or both, and increasing CP leads to later and earlier termination and induction, respectively.

Insects that use photoperiodic cues to induce or terminate diapause generally exhibit a trend of increasing critical photoperiod with increasing latitude, where later termination and

earlier initiation at higher latitudes causes a reduction in the length of the growing season.

This trend makes sense from an adaptive standpoint, as in addition to intensity, the duration

of winter increases with increasing latitude. In a meta-analysis of geographic variation in CP,

Taylor and Spalding (1986) show consistent trends of increasing CP with increasing latitude

within and across species. Figure 2.1, reproduced from Taylor and Spalding (1986), plots CP

versus latitude for 12 species of insects and for one mite species. Considering interspecific

variation, there is a clear pattern of increasing CP with increasing latitude. This same trend is

consistently demonstrated intraspecifically as well (Figure 2.1; the majority of lines

connecting geographic populations have similar, positive slopes). Since the publication of

this study, positive relationships between latitude and CP measured for geographic

populations have also been reported for a number of species, including (but not limited to)

six species of Lepidoptera (Gomi 1997; Jia 1993; Kato 2005; Ujiye 1985; Yoshio and Ishii

1998), two species of Diptera (Moribayashi et al. 2001; Riihimaa et al. 1996), one species of

Heteroptera (Ito and Nakata 2000), and one species of spider mite (Suwa and Gotoh 2006).

Further, of the four species summarized in Taylor and Spalding (1986) with available

estimates of r^2 for the regression of CP on latitude, three are significant at the $p < 0.05$ level

and all have $r^2 > 0.73$. The near-universality of these trends strongly suggest that

photoperiodic timing of diapause is a major adaptation to seasonal environments that is

highly predictable based on latitude (Bradshaw and Lounibos 1977; Tauber et al. 1986;

Taylor and Spalding 1986). Two decades ago, Taylor and Spalding (1986) even went so far

as to suggest that latitudinal trends in CP are so well substantiated that further evolutionary

studies directed exclusively at examining this question would be redundant.

Figure 2.1. Plot of critical photoperiod vs. latitude redrawn from Taylor and Spalding (1986) to include only studies including two or more geographic populations. Lines connect geographic populations considered in the same study, and each line represents a separate species (species list in Taylor and Spalding (1986). Filled circles represent geographic populations within each study (line).
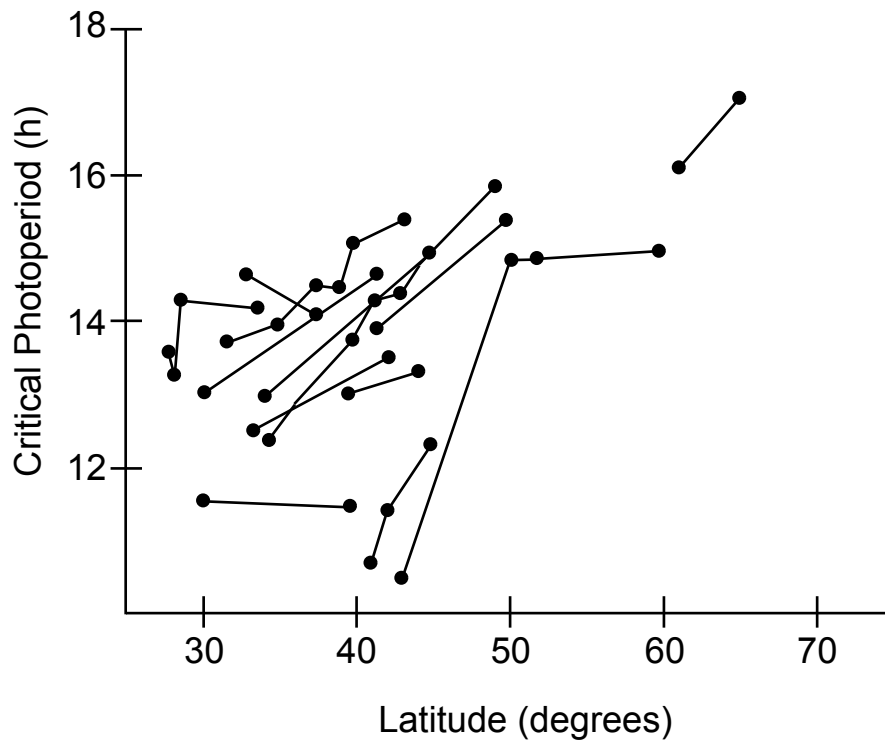
Trends in Degree-day parameters

In ectotherms, the functional relationship between development rate and temperature (a thermal reaction norm) typically assumes a curvilinear, concave-down shape such that there is a single temperature at which the rate is maximized and an asymmetrical decline (steeper at higher temperatures) in rate on either side of the maximum. Ideally, an average reaction norm for a genotype, population, species, etc., would be quantified by fitting an appropriate curvilinear function to development rate measures at a set of landmark temperatures (Fig. 2.2a). In practice, especially in the entomological literature, the relationship is simplified by measuring development rate only at temperatures at or below the optimum (estimated or inferred) and assuming a linear relationship across these temperatures (Fig. 2.2b). This is the "Degree-day" model familiar to entomologists, and since the relationship is linear it can be described by two parameters with straightforward biological meaning. The x- (temperature) intercept (t) is defined as the lower thermal threshold below which development does not occur, and the inverse of the slope (K) is defined as the thermal requirement (units are [degrees C*days], thus the "degree-day" model) for development above the lower thermal threshold (Fig. 2.2b). The parameters t and K define the lower thermal limits of development and the required accumulation of heat for the completion of development, respectively. Despite the model's simplicity, it often serves as an accurate predictor of insect development and is widely used to predict phenology in the field (Trudgill et al. 2005).

Figure 2.2. a) 4rth degree polynomial fit (solid line) to hypothetical measures of development rate (inverse of the numbers of days to eclosion) taken at landmark temperatures (diamond symbols). b) Linear fit to the same hypothetical data using only data points below 35° C (solid black diamonds) and excluding data at or above 35° C (open diamonds). This represents the degree-day model where t (the x-intercept) is the lower thermal threshold for development and K (the reciprocal of the slope), is the degree-day requirements above the lower thermal threshold.
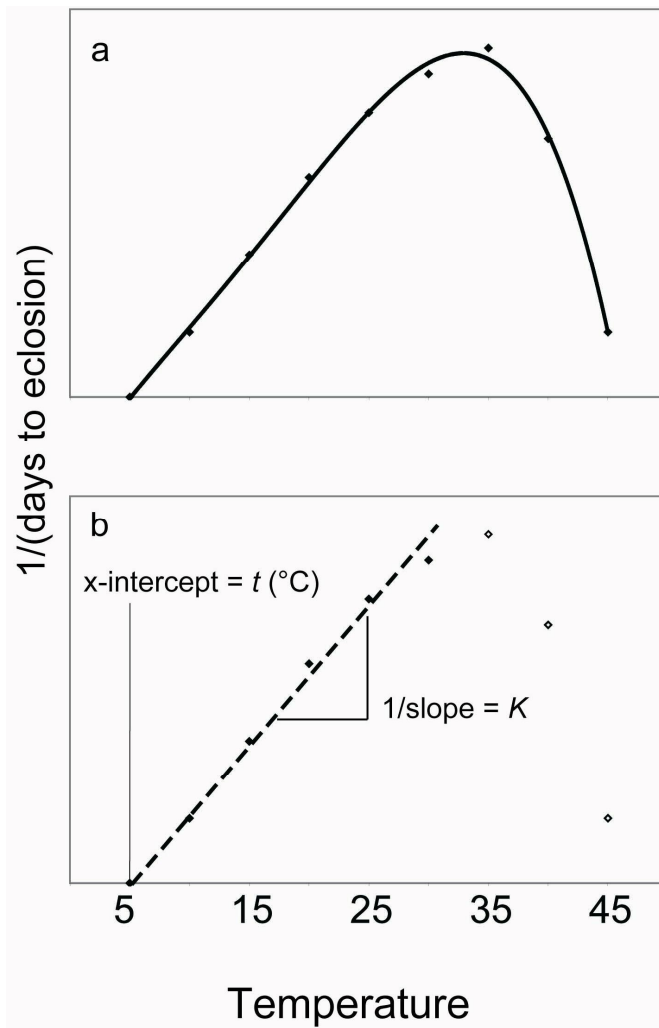
Degree-day parameter estimates appear to be less tightly related to latitude than critical photoperiod. The decrease in annual average temperatures with increasing latitude predicts that the lower thermal threshold t will decrease with increasing latitude (Trudgill et al. 2005). Somewhat less intuitively, the adaptive expectation is an increase in K with increasing latitude because K and t seem to often be negatively correlated, and multivoltinism at lower latitudes often favors decreased development time, *i.e.*, a lower value of K (Trudgill 1995; Trudgill and Perry 1994). A comparative study by Honek (1996) partially supports these predictions, but the inferred relationships with latitude are relatively weak. Using data from 335 insect species from 13 orders sampled from 24 - 60° (North or South) latitude, Honek (1996) estimated r^2 values for regressions of t and K on latitude. Regressions were estimated for total development and separately for several developmental stages, but results were qualitatively consistent across developmental stages. Despite a high degree of scatter in the data, t was significantly negatively correlated with latitude, but latitude explained only a small proportion of the variation ($r^2 = 0.12$ for total development). There was no discernable relationship between K for total development and latitude, although latitude did explain a small but significant proportion of the variation in K for the egg stage alone ($r^2 = 0.034$).

The data set analyzed by Honek (1996) represents interspecific variation, and total variation is almost certainly highly inflated by ecological differences among species (*e.g.*, variation in nutritional and microhabitat requirements). However, two lines of evidence suggest that the non-trends or weak trends are not simply an artifact of noisy data. First, a re-analysis of the data applying separate regressions of t on latitude for several taxonomic divisions did not substantially improve the estimates (average r^2 of 0.141; Honek 1996). Second, geographic populations of several insect species demonstrate either limited inter-

population variation in *K* and *t* or variation that is not related to latitude (Beck and Apple 1961; Calvin et al. 1991; Campbell et al. 1974; Campbell and Mackaur 1975; Goryshin et al. 1987; Khomyakova 1976; Tauber and Tauber 1982; Tauber and Tauber 1987). Thus, the weak trends inferred from interspecific variation are also demonstrated by intraspecific variation. Together, the available evidence suggests that compared to the highly supported, tight relationship between CP and latitude, thermal sensitivity of development rate is only weakly related to latitude.

Evolvability of CP, *t*, and *K*

Clearly there is ample variation in CP and degree-day parameters at the interspecific level, but does a lack of intraspecific variation constrain the evolution of *t* and *K* relative to CP? If this were true, geographically disparate populations of insects with photoperiodically-controlled diapause responses should differ markedly for CP and little for *t* and *K*. A test of this hypothesis requires data on CP, *t*, and *K* for multiple geographic populations of the same species. There are few species for which all of these estimates are available, a somewhat surprising result given the amount of attention devoted to phenological models in the insect literature. Nevertheless, what examples are available prove informative.

Geographic populations appear to diverge as much in degree-day parameters as they do in CP. Table 2.1 summarizes intraspecific variation in 5 insect species sampled from two or more geographic populations. I calculated maximum divergence (MD) between populations in a given study for a given trait as the greatest difference in trait value (from all possible pairwise comparisons) between two populations divided by the mean trait value of all populations. Generally, these mean-standardized estimates did not differ greatly between CP, *t*, and *K* within a given species: all estimates were well within an order of magnitude.

Table 2.1. Maximum divergence between geographic populations (MD) for degree day requirements for non-diapause (K_{non}) and post-diapause (K_{post}) development, lower thermal threshold for non-diapause (t_{non}) and post-diapause (t_{post}) development, and critical photoperiod (CP; in bold).

Species	Order	N (pops)	Latitudinal range (degrees)	Trait	MD
[1]*C. oculata*	Neuroptera	7	20.0	K_{post}	0.42
[2]*C. oculata*	Neuroptera	5	25.7	K_{non}	0.19
[1]*C. oculata*	Neuroptera	7	20.0	t_{post}	0.21
[2]*C. oculata*	Neuroptera	5	25.7	t_{non}	0.15
[1]**C. oculata**	**Neuroptera**	**10**	**25.0**	**CP**	**0.37**
[3]*C. carnea*	Neuroptera	9	35.0	K_{post}	0.61
[3]*C. carnea*	Neuroptera	6	35.0	K_{non}	0.23
[3]*C. carnea*	Neuroptera	9	35.0	t_{post}	0.59
[3]*C. carnea*	Neuroptera	6	35.0	t_{non}	0.14
[3]**C. carnea**	**Neuroptera**	**9**	**20**	**CP**	**0.19**
[4]*L. decemlineata*	Coleoptera	2	1.5	K_{post}	0.46
[5]*L. decemlineata***	Coleoptera	2	1.5	K_{non}	0.015
[4]*L. decemlineata*	Coleoptera	2	1.5	t_{post}	0.041
[5]*L. decemlineata*	Coleoptera	2	1.5	t_{non}	0.074
[6]*L. decemlineata***	Coleoptera	2	6.8	K_{non}	0.017
[4]**L. decemlineata**	**Coleoptera**	**2**	**1.5**	**CP**	**0.051**
[7]*H. cunea*	Lepidoptera	2	1.9	K_{non}	0.058
[7]*H. cunea*	Lepidoptera	2	1.9	t_{non}	0.028
[8]**H. cunea**	**Lepidoptera**	**13**	**5.5**	**CP**	**0.013**
[9]*O. nubilalis*	Lepidoptera	4	10.3	K_{non}	0
[9]*O. nubilalis*	Lepidoptera	4	10.3	t_{non}	0.13
[10]**O. nubilalis**†	**Lepidoptera**	**9**	**8.0**	**CP**	**0.10**

† CP estimated by linear extrapolation from reference (9)
[1-10] Index of references listed in Appendix

Table 2.2. Direction of divergence in degree-day parameters between the two latitudinally most divergent populations in each study. A "+", "-", or "0" indicate an increase, decrease, or no change in trait (*K* or *t*) value with increasing latitude.

species	K		T	
	Non-diapause	Post-diapause	Non-diapause	Post-diapause
C. oculata	-	+	+	-
C. carnea	-	-	-	+
O. nubilalis	0	*na*	-	*na*
H. cunea	-	*na*	+	*na*
L. decemlineata*	+	+	-	-
L. decemlineata*	-	*na*		*na*

*   two rows for L. decemlineata represent data from independent studies
na data not available

Moreover, maximum divergence was actually greater for either t or K than for CP within every species listed. These estimates of divergence are admittedly rough, and do not include any estimates of variation about the mean. However, they provide no evidence that CP is more divergent among populations than are degree-day parameters. Thus, there is no *a priori* expectation of constraint for degree-day parameters. Three of these species are recent colonizers (introductions within the last 100 to 300 years), showing that both CP and the thermal sensitivity of development may evolve relatively rapidly. Life history evolution on such short time scales would be unlikely were these traits highly constrained.

Comparisons of direction of divergence between CP and t and K also support the hypothesis that CP has a more predictable relationship with latitude. In all of the studies of CP listed in Table 2.1, CP increased from the population furthest south to the population furthest north. In contrast, degree-day parameters sometimes increased and sometimes decreased with increasing latitude (Table 2.2). This contrast in consistency between CP and t and K mirrors the discrepancy between the results of Taylor and Spalding (1986) and the results of Honek (1996): CP is tightly linked to latitude, while degree-day parameters are not.

Geographic patterns of selection

If both critical photoperiod and the thermal sensitivity of development are free to evolve, a selective explanation must account for the consistent latitudinal trend in CP and the inconsistent latitudinal trends in t and K. As previously mentioned, detailed fitness measures are necessary to quantify the relative strength of selection on these traits, and such data are not available in a sufficient number of species to inform any broad conclusions. However, if we make a few plausible assumptions about what selective forces shape seasonal adaptations,

we can use climatic data to infer how selection on development rate and seasonal timing varies with latitude.

The need to synchronize life cycles with seasonally fluctuating biotic factors such as resource availability undoubtedly contributes to the optimal timing of hibernal diapause (Wolda 1988). Climatic fluctuation in temperature and rainfall ultimately drive fluctuations in resource levels, however, and these factors will also directly constrain diapause timing by imposing physiological stress. For example, sub-zero temperatures are often lethal to non-dormant insects (and other ectotherms and plants, for that matter), and even freeze-tolerant insects cannot actively grow and reproduce in freezing temperatures. Thus, the seasonal boundaries of freezing temperatures (in spring and fall) ultimately dictate the length of the growing season in most temperate environments (Tauber et al. 1986). Latitudinal variation in the timing of spring and fall freezes, therefore, will often predict latitudinal variation in the timing of hibernal diapause (and thus, CP). Likewise, latitudinal trends in thermal conditions occurring after or before the spring and fall freezes (respectively) will depend on trends in freeze timing. Environmental temperature should select on the thermal sensitivity of development (Kingsolver and Gomulkiewicz 2003), so latitudinal trends in thermal conditions occurring after the last freeze should predict latitudinal variation in degree-day parameters.

Accepting these assumptions of thermal selection on diapause timing and development rate suggests straightforward predictions for geographic variation in CP, t, and K. The higher the correlation between latitude and date of last spring and first fall freeze, the higher the expected correlation between CP and latitude. Likewise, higher correlations between latitude and the thermal conditions experienced between first and last freeze (*i.e.*,

during the growing season) predict higher correlations between degree-day parameters and latitude. Temperatures experienced during the spring and fall are particularly important for temperature-mediated selection during the growing season. Evolutionary studies of geographic clines rarely consider the thermal conditions occurring at the seasonal transitions, and these conditions can significantly alter predictions of clinal variation in selection. Consider two geographic populations of a seasonal insect from two different latitudes. The northern population invariably experiences a colder, longer winter, but if this population is seasonally adapted it will emerge from diapause later in the year than the southern population. By emerging earlier in the year, the southern population could potentially experience similar, or even colder temperatures during development, or at least for the first generation if the insect is multivoltine. Thus, the thermal habitat of the growing season depends on the timing of diapause (or on the timing of seasonal freeze cycles if we assume that this determines diapause timing).

To explore these latitudinal trends, I calculated several metrics of the thermal/seasonal environment using data from 21 weather stations arrayed along a latitudinal cline in eastern North America (see Appendix). I calculated the average date of the final spring freeze (daily minimum below -1° C), the average minimum temperature for the first 50 days after the spring freeze, and the average degree-day accumulation of thermal units over those same 50 days. A span of 50 days provides enough time to accumulate more than the average degree-day requirement for total development reported in Honek (1996). Starting with 50 years of data, I excluded all years in which there was more than one week of missing data for any of the weather stations, leaving 30 years of data to include in the analysis. After calculating the means, I performed a simple linear regression of each measure on latitude to

estimate coefficients of determination (r^2). A term for altitude was also included in each

model, and altitude-corrected latitudes were calculated for use in Figure 2.3 (see Appendix).

The weather data provide a clear picture of geographic clines in seasonal and thermal

selection. Date of last spring freeze is tightly correlated with latitude, while the average

minimum temperature and cumulative degree-days for the first 50 days after the last freeze

are substantially less correlated with latitude. The latitudinal trend in the date of last freeze

predicts the consistent latitudinal trend in CP observed in most insects with a

photoperiodically-cued diapause response. Dates of last spring freeze increase predictably

with latitude (Fig. 2.3a; $r^2 = 0.87$, $p < 0.001$), as does CP (Fig. 2.1). Trends in degree-day

accumulation and average minimum temperature predict a relatively weaker relationship

between degree-day parameters and latitude. Average minimum temperature of the 50 days

after the last frost does decline with increasing latitude, but a smaller proportion of the

variation is explained by latitude (Fig. 2.3b; $r^2 = 0.56$, $p = 0.002$). Latitude accounts for an

even smaller, non-significant proportion of the geographic variation in cumulative degree

days (Fig. 2.3c; $r^2 = 0.21$, $p = 0.12$). Geographic trends in degree-day parameters are also

relatively weak: lower thermal threshold (t) decreases only marginally with latitude, while

degree-day requirements (K) show no detectable relationship with latitude. Year-to-year

variability of these measures could also influence the estimated correlations, but CP, t, and K

all had comparable coefficients of variation (CV) at all sites.

Using the above analyses to link variation in thermal selection to variation in latitude

ignores variation in peak summer temperatures, but this omission is unlikely to invalidate the

predicted pattern. Particularly in multivoltine populations, hot summer temperatures surely

contribute to selection on degree-day parameters. However, unless thermal sensitivity of

Figure 2.3. Mean (± SE) day of last freeze (last day of spring where minimum < -1° C; [a]) and mean annual average minimum temperature (b) and cumulative degree-days (sum of daily mean temperatures; [c])) for the first 50 days after the last freeze estimated from 30 years of weather data versus altitude-adjusted latitude (see Appendix for adjustment calculations).
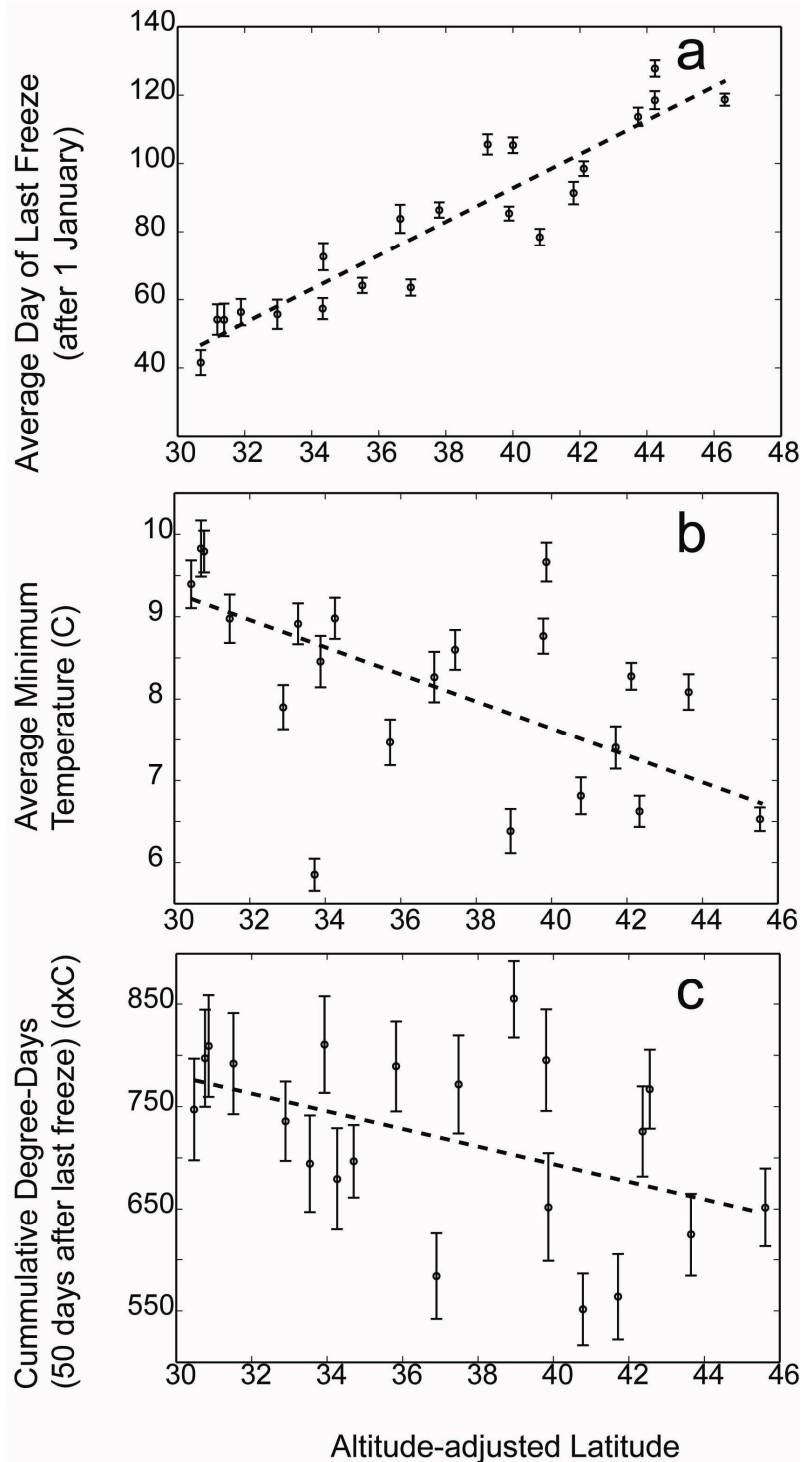
mid-summer generations is genetically uncorrelated with that of the post-diapause generation, degree-day parameters that maximize fitness will reflect a balance between performance at cooler and warmer temperatures. Thus, thermal conditions experienced after the last spring freeze will influence the evolution of both post-diapause and non-diapause development in a substantial proportion of insect species.

Concordance between climatic and phenotypic trends combined with an apparent lack of genetic constraint suggests that geographic variation in temperature-mediated selection drives geographic variation in CP, t, and K. These predictions for geographic variation in the phenotypes are only apparent when the timing of particular life history stages (*i.e.*, timing of the first spring generation) are accounted for, emphasizing the importance of phenology for selection on thermal physiology. Metrics such as latitude and altitude can provide valuable information about climatic differences among geographic regions. In this case, however, identifying the relevant seasonal context is a critical step in the process of forming and testing appropriate adaptive hypotheses.

Implications for life history evolution in fluctuating environments

The relationship between diapause timing and seasonal temperature conditions illustrates how selection on a particular life history stage is inextricably linked to selection on seasonal timing. Seasonal fluctuations in any environmental or biotic factor will generate this dependency. For example, seasonal abundance of predators partially controls the timing of dormancy in some populations of *Daphnia magna* (Slusarczyk 2001). Temperature conditions and resource levels occurring during non-dormant periods will thus be determined by cycles in predator abundance. Similarly, the intensity of predation on pond breeding pool

frog (*Rana lessonae*) larvae varies with the timing of egg hatch (Altwegg 2002), so the timing of reproduction will affect selection on anti-predator behavior. These examples share two critical features with the example of seasonal temperature variation and diapause timing: an environmental factor that fluctuates within a generation and a life history transition (*i.e.*, induction or termination of diapause or initiation of juvenile development) that occurs while the environment is fluctuating. These conditions are sufficient for selection on a defined life history stage to depend upon the timing of that stage. This general relationship is well supported, and serves as the basis for a number of models of the optimal timing of life history stages (Roff 2002).

Because life history timing affects selection on particular stages, geographic variation in timing may alter geographic patterns in selection. In the example presented here, geographic populations of insects arrayed along a latitudinal cline experience a gradient in temperature conditions, but these populations also differ in the seasonal timing of diapause. Differences in diapause timing alter the thermal environment of the growing season such that northern populations may not experience colder temperatures than southern populations during active growth and reproduction. Here this explanation is inferred from multiple comparative data sets, but it is also supported by a specific empirical example. Geographic populations of *Wyeomyia smithii* diverge in critical photoperiod inducing and terminating diapause, and CP is tightly correlated with latitude (Bradshaw and Lounibos 1977). Local temperature data from four populations ranging from 30.8 to 45.6º N latitude show that after correcting for population differences in seasonal timing, three of the four populations experience roughly equivalent frequencies of daily average temperatures below 10º C during the growing season (see Chapter 3). This pattern is reflected in geographic variation in

development rate: populations have indistinguishable development rates at low temperatures, while rates do vary among populations at higher temperatures. Variation in diapause timing homogenizes the thermal environment of the growing season across geographic clines, modifying geographic predictions of thermal adaptation.

The trends examined in this paper do not address whether there is a consistent difference between the relative strength of selection on phenology and selection on thermal physiology, and this issue is rarely, if ever directly addressed either empirically or theoretically. Recent studies of evolutionary response to climate change suggest that seasonal phenology of a number of species has evolved to accommodate warming trends (Bearhop et al. 2005; Bradshaw and Holzapfel 2001; Jonzen et al. 2007; Nussey et al. 2005; Parmesan 2006; Reale et al. 2003; Umina et al. 2005), whereas examples of the evolution of thermal responses are rare or non-existent (Bradshaw and Holzapfel 2006). Although these examples are far from conclusive, they suggest that selection to shift the timing of life history stages (*e.g.*, diapause) or events (*e.g.*, reproduction) may be stronger than selection to adjust the thermal sensitivity of those stages. Alternatively, evolutionary responses may be largely determined by genetic constraints. A major challenge in evolutionary studies of climate change will be to distinguish between these hypotheses, as the resolution of this issue will have important implications for local extinction and geographic range shifts in temperate organisms.

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CHAPTER 3

INFLUENCE OF SEASONAL TIMING ON THERMAL ECOLOGY AND THERMAL REACTION NORM EVOLUTION IN *WYEOMYIA SMITHII*

Abstract

Evolutionary changes in the seasonal timing of life history events can alter a population's exposure to seasonally variable environmental factors. I illustrate this principle in *Wyeomyia smithii* by showing that 1) geographic divergence in diapause timing reduces differences among populations in the thermal habitat experienced by non-diapause stages, and 2) the thermal habitat of the growing season is more divergent at high compared to low temperatures with respect to daily mean temperatures. Geographic variation in thermal reaction norms for development time was greater in a warm compared to a cool rearing treatment, mirroring the geographic trend in daily mean temperature. Geographic variation in body size was unrelated to geographic temperature variation, but was also unrelated to development time or fecundity. Our results suggest that proper interpretation of geographic trends may often require detailed knowledge of life history timing.

Introduction

In seasonal environments, selection on a particular life history stage or event depends strongly upon timing, or phenology. At a given geographic location, season-dependent selection applies to any life history stage or event that occurs in a predictable and defined annual time window when selective factors are seasonally fluctuating. For example, selection on floral traits mediated by pollinators is dependent on the timing of flowering in desert cacti (Fleming et al. 2001). Similarly, periods of dormancy in *Daphnia* often coincide with periods of high predation (Slusarczyk 2001) so that selection on anti-predator responses is dependent on the timing of initiation and termination of dormancy. Migratory events or stages also fit into this framework, as the timing of migration is often intimately tied to seasonal environmental fluctuation (Dingle and Drake 2007).

Just as selection is time-dependent within a particular site with seasonal fluctuations, variation in selection among geographic sites is dependent on the seasonal context. Traits such as adult body size, propagule size, growth rate, and development rate exhibit latitudinal and altitudinal trends in diverse animal and plant taxa (*e.g.*, Blanckenhorn and Fairbairn 1995; Galen et al. 1991; Gilchrist et al. 2004; Tracy and Walsberg 2001). Latitude and altitude serve as geographic proxies for environmental variation, suggesting that these clines are the result of local adaptation (Endler 1986). In seasonal environments, however, both environmental factors and the activity pattern of organisms vary throughout the year, and the timing of a particular life history stage can greatly affect the selective environment experienced by that life history stage. Thus, simple geographic proxies provide little insight into the actual selective factors driving such clines without knowledge of life history timing and patterns of annual environmental variation. In addition, evolved differences in life

history timing among geographic populations will further modify how selection on a particular life history stage varies with geography.

In response to geographic variation in seasonality, geographic populations often vary in the seasonal timing of life history events or stages, and this variation in timing affects geographic clines in selection. For example, geographic clines in the timing of hibernal diapause (dormancy) are widespread in temperate insects. Studies in a number of species show that that the timing of the onset and termination of diapause diverges between geographic populations such that the length of the active, or growing season decreases with increasing altitude or latitude (Tauber et al. 1985; Taylor and Spalding 1986). Geographic variation in diapause timing affects geographic clines in selection in two ways. First, shifts in diapause timing change the length of the growing season, causing geographic variation in developmental time constraints. The consequences of this effect have been well studied both theoretically (Roff 1980) and empirically (Burke et al. 2005; Fischer and Fiedler 2002; Laugen et al. 2003; Masaki 1972), particularly as they relate to geographic clines in development time. Second, changes in diapause timing can alter the environment experienced by a given life history stage. The most obvious adaptive value of diapause timing is to mitigate the exposure of actively growing individuals to harsh winter conditions (Tauber et al. 1985), but there is an additional consequence. The timing of diapause will also affect the environment experienced by actively growing (*i.e.*, non-diapause) individuals by changing the window of exposure to seasonally fluctuating environmental factors.

Temperature is an important selective factor that varies seasonally and geographically, and diapause timing will impact patterns of selection on and adaptation of thermal reaction norms. For traits that are temperature-sensitive such as body size and

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development time in insects, selection mediated by temperature acts on the relationship between trait value and temperature, termed a thermal norm of reaction or reaction norm. Theoretical models predict that the strength of selection on trait value at a particular temperature will be proportional to the frequency at which that temperature is experienced in the natural environment (Gilchrist 1995; Gilchrist 2000; Kingsolver and Gomulkiewicz 2003). Consequently, the frequency distribution of temperatures at a given site describes the relative strength of selection applied across temperatures of a thermal reaction norm (Kingsolver and Gomulkiewicz 2003). Changes in diapause timing effectively change the frequency distribution of temperatures experienced during active growth and reproduction, altering thermal selection on non-dormant life history stages. Similarly, geographic variation in diapause timing will influence geographic variation in the temperature frequency distribution of the growing season. In this way, geographic clines in thermal selection may depend on geographic clines in diapause timing. Despite these clear ecological and evolutionary implications, the influence of diapause timing on exposure to seasonally fluctuating environments is rarely considered (but see Bradshaw et al. 2004). To our knowledge, this phenomenon has not been the direct focus of any theoretical or empirical studies.

The pitcher plant mosquito, *Wyeomyia smithii*, exhibits a well characterized cline in diapause timing across both latitude and altitude (Bradshaw and Lounibos 1977). Here I examine how geographic variation for diapause timing affects geographic variation in the thermal environment of the growing season and geographic divergence in thermal reaction norms for life history traits. The length of the growing season decreases with increasing altitude and latitude in *W. smithii* (Bradshaw and Lounibos 1977), limiting exposure of

actively growing individuals to cold winter temperatures. Thus, I hypothesized that geographic variation in the timing of hibernal diapause reduces geographic variation in the thermal environment experienced during the growing season.

For several geographic populations I combined available diapause timing data with long-term weather records to determine the seasonal time window and temperature conditions associated with active growth and reproduction. In addition, I tested for geographic variation in thermal reaction norms for development time, body size, and fecundity, life history traits that often vary substantially across latitude and altitude in insects. Because of logistical issues I were only able to measure fecundity at a single temperature, but these data prove useful for assessing whether body size impacts fitness via fecundity. I compare geographic variation in the thermal environment with geographic variation for thermal reaction norms to explore the consequences of local adaptation of diapause timing.

Methods

*Study organism and sample site*s

W. smithii obligately oviposits into the water-filled leaves of the purple pitcher plant, *Sarracenia purpurea*, and the geographic distribution of *W. smithii* tracks that of *S. purpurea* from the Gulf Coast of Florida to Newfoundland (Armbruster et al. 1998). The initiation and termination of diapause in *W. smithii* is cued by photoperiod (Bradshaw and Lounibos 1972), and geographic populations demonstrate a cline of increasing critical photoperiod (CP, the photoperiod at which 50% of a sample initiates or terminates diapause) with increasing latitude and altitude (Bradshaw and Lounibos 1977). Diapause occurs in the larval stage and critical photoperiod for initiation and termination is symmetrical (Bradshaw and Lounibos 1977).

I collected approximately 1000 larvae from each of four geographic populations (Table 3.1) during the spring and fall of 2004, sampling a minimum of 50 pitcher plants per population. With the exception of the Massachusetts population (MAS), CP has previously been estimated for each population that I sampled (FL, NC Coast, and NC Mtn. are WI, GS, and DB, respectively, in Bradshaw and Lounibos (1977). Here I computed an estimate of CP for MAS using equation 1 of Bradshaw and Lounibos (1977). Field observations of phenology agree well with this estimate (Aaron Ellison, personal communication). Phylogeographic data suggest that these populations cluster into two distinct clades based on morphology (Bradshaw and Lounibos 1977), allozyme (Armbruster et al. 1998), and mtDNA variation (William Bradshaw, unpublished data). All geographic populations of *W. smithii* are considered a single species (Bradshaw and Lounibos 1977). FL and NC Coast fall into a southern clade, while NC Mtn. and MAS fall into a northern clade. Hereafter I refer to FL and NC Coast as southern clade populations and NC Mtn. and MAS as northern clade populations to reflect this phylogeographic clustering. I did not choose these populations to specifically test the effects of altitude or latitude. Rather, I chose populations that provided replication within clade (northern and southern) and exhibited substantial differences in environmental temperature (see results).

Temperature data

To characterize the thermal environment of each population I obtained weather data from 1950 to 2001 including daily maximum and minimum temperatures from nearby (< 5 km) weather stations. I calculated daily mean temperatures as the mean of the daily maximum and minimum; in comparison to actual mean temperatures this calculation is generally biased during warmer months particularly at lower latitudes, but generally by no

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Table 3.1: Geographic locations and critical photoperiods for the four *W. smithii* study populations.

Geographic Location	Lat./Lon. (deg)	Altitude (m)	Critical Photoperiod (h)
Florida gulf coast (FL)	30°N 85°W	10	12.25*
North Carolina coast (NC Coast)	34°N 78°W	20	12.75*
North Carolina mountains (NC Mtn.)	35°N 83°W	900	14.35*
Central Massachusetts (MAS)	42°N 72°W	265	14.50†

* from Bradshaw and Lounibos (1977)
† calculated from Eq. 1 in Bradshaw and Lounibos (1977): see methods

more than one degree Celsius (for August, 2000, temperature means calculated from daily maxima and minima were on average biased by 0.69 and -0.10° C in Wilmington, NC and Portland, ME, respectively). Data from temperature loggers placed in pitcher plant leaves at each site indicate that when there is no snow cover, thermal conditions at the actual field sites are highly correlated with nearby weather station data (G. Ragland, unpublished data; Bradshaw et al. 2000). Snow cover moderates exposure of diapausing larvae to temperatures below freezing (Bradshaw et al. 2004), but this does not affect temperatures experienced during the growing season.

Excluding years for which there were any missing data from any month of the year at any site (leaving 35 years of data), I estimated frequency distributions (binned into 1° C intervals) of daily mean temperatures from the long-term weather data for each population for 1) the entire year and 2) only the growing season predicted by CP for each population. CP-corrected frequency distributions were computed by including temperature data only for days of the year longer than the CP at each geographic location. A previous study suggests that *W. smithii* includes incoming light during twilight periods in its perception of daylength (Bradshaw and Phillips 1980), so I included civil twilight in our estimates. Excluding civil twilight did not qualitatively change the results. Choosing the last day longer than the CP as a fall cutoff for the growing season is somewhat arbitrary as *W. smithii* is photosensitive at least one instar before the diapausing instar and must develop through to diapause (Bradshaw and Lounibos 1972); however, extending this cutoff by 2.5 weeks did not qualitatively change the results.

Colony establishment and maintenance

Larvae sampled from each population were reared to adulthood under standard laboratory conditions (L:D 16h:8h, temperature oscillating as a sine curve from 13 to 29° C) as in Hard *et al.* (1992). Adults were allowed to oviposit into freshly cut pitcher plant leaves in 5 gallon mating cages and the resulting eggs were reared to the diapause stage at L:D 8:16 (shorter than the shortest photoperiod necessary to induce diapause in all populations) and 20° C. Individuals selected for the next generation represented a haphazard sample across the entire oviposition period of each mating cage. Once all populations were synchronized in diapause, I switched diapausing larvae back to standard, long-day conditions and reared them to adulthood to produce eggs for the next generation. I initiated experimental cohorts with hatching larvae from the F3 laboratory generation. Lab colonies were maintained with at least 500 mating individuals per population per generation.

Experimental conditions

Our experiment was designed to test for the influence of temperature, clade, and population on development time, pupal mass, and fecundity. Temperature treatments were applied as levels of a crossed factor using temperature-controlled growth chambers, and larvae from each population were reared in cohorts, introducing a random effect. Development time and pupal mass were measured on individuals, while fecundity was measured on cohorts.

If diapause timing evolves towards progressively shorter growing seasons with increasing altitude and latitude, the temperature frequency distribution of the growing season should become increasingly truncated (at low temperatures) at higher latitude/altitude. The coldest temperatures of the growing season occur near the transitions between diapause and

active growth. Therefore, such a geographic trend in diapause timing should reduce

geographic variation primarily in the thermal conditions associated with these transitional

periods. As the growing season generally includes the warmest temperatures of the year (*i.e.*,

the summer) in many insect species including *W. smithii*, transitions between diapause and

non-diapause stages usually occur in the spring or fall.

I chose two fluctuating temperature rearing conditions that reflected both warm

summer-like conditions and cool, spring-like conditions. The warm treatment simulates a hot

summer day in the south (T_{ave}=27° C, T_{min}=23° C, T_{max}=39° C) and the cool treatment

simulates a typical cool summer day in the north (T_{ave}=20° C, T_{min}=16° C, T_{max}=32° C).

Each treatment was designed to mimic diurnal temperature fluctuations measured by

temperature loggers in the field and each had identical diurnal profiles offset by 7° C. Figure

3.1 shows the relationship between the experimental treatments and actual diurnal

temperature variation measured in pitcher plant leaves in a southern (NC coast) and northern

(NC Mtn.) clade population.

Eggs were collected every 3 days from colony cages and checked for hatching daily.

Newly hatched larvae were haphazardly selected by pipetting from a well-mixed Petri dish

and placed in 170 ml of distilled water in 150 by 25mm culture dishes, 25 larvae per dish,

and assigned to one of two Percival 36-VL environmental chambers simulating the warm and

cool temperature treatments. Each chamber was set at 16:8 L:D to simulate long-day

conditions. Use of this light cycle is a standard practice when comparing direct development

of geographic populations of *W. smithii* (*e.g.,* Bradshaw et al. 2004) and other insect species

(*e.g.*, Tauber and Tauber 1987). Daylengths more than an hour longer than the critical

photoperiod have negligible effects on development rates in *W. smithii* (Bradshaw and

Figure 3.1. Diurnal temperature cycle for experimental 'warm' (black solid line) and 'cool' (grey solid line) rearing treatments and diurnal fluctuations of a typical early summer day at the NC coast (southern clade; black dashed line) and NC Mtn. (northern clade; grey dashed line) populations.
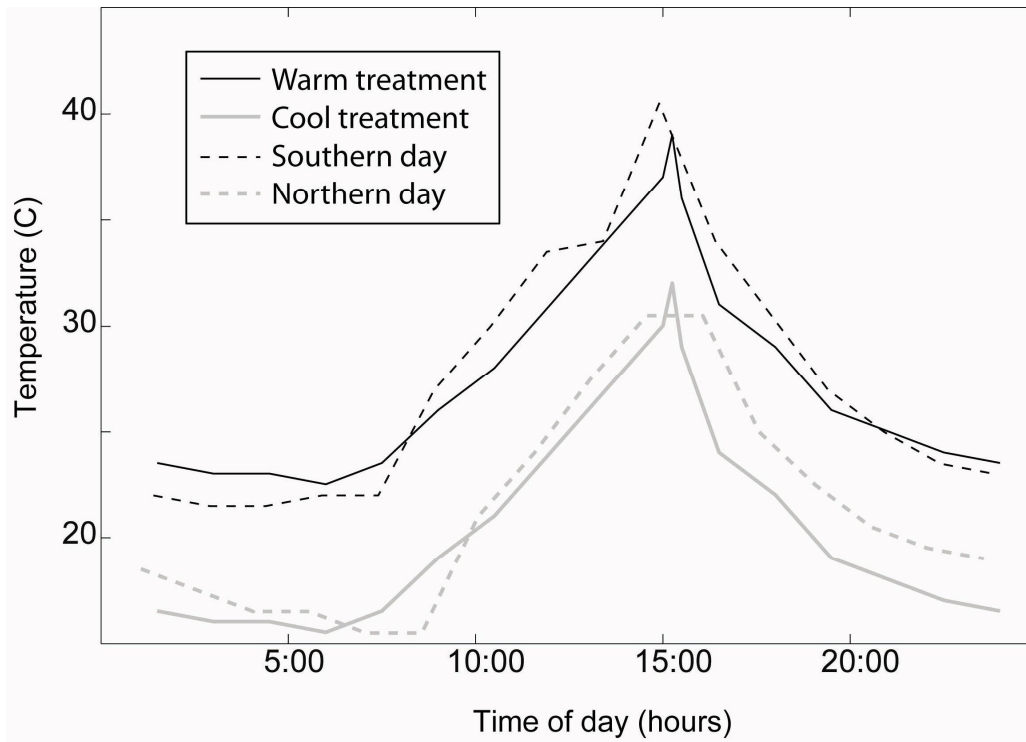
Lounibos 1972; Bradshaw and Lounibos 1977; Bradshaw, personal communication). In addition, a day length at least as long as the longest day of the year proportionally represents the same time point in the season for all populations because of the symmetry of critical photoperiod for diapause initiation and termination in *W. smithii*. A total of 12 dishes were assigned to each treatment per population and all dishes for a given population*treatment combination were initiated within a 10-day window.

Once per week larvae from each dish were transferred into a fresh dish of distilled water and fed with a 0.05g/ml suspension of standard diet for *W. smithii* (4:1 guinea pig chow to freeze-dried brine shrimp). Larvae were fed weekly, and for the first three weeks I progressively changed the amount of food to maintain *ad libitum* conditions without fouling the water. 1.0, 1.75, 2.5, and 3.0ml of food suspension were added in weeks one through four and 2.5ml weekly thereafter. Pilot studies suggested that adding additional food did not substantially change development time or final mass, and there were no statistically significant interactions among food treatments and rearing temperatures for either pupal mass or development time (based on mixed model ANOVA, $\alpha = 0.05$; G. Ragland, unpublished data). Additionally, data from Bradshaw and Holzapfel (1986) show that there is no interaction between larval density and population of origin for generation time and replacement rate, suggesting that effects of larval density do not vary consistently among geographic populations. Sex, time to pupation, and mass at pupation were recorded for all individuals that survived to pupation; survival in all treatment*population combinations was high (95% on average).

Fecundity was measured in a subset of the experimental cohorts. I measured cohort fecundity by allowing pupae from 4 dishes (closest to each other in hatching date; n=100

total) to eclose into 5.6L mating cages, yielding three replicate mating cohorts per population. The bottom of each mating cage was covered with moistened paper towels, and periodic measurements confirmed that this maintained 80 – 85% relative humidity. Every 6 days, cages were provided with a fresh sponge moistened with honey-water for adult nutrition and a freshly cut pitcher plant leaf for oviposition. Eggs were collected every three days until the last adult in a cage had died. I initiated mating cohorts in both the warm and cool treatments, but high mortality in the warm treatment mating cages due to logistical difficulties precluded measurements of fecundity. Here I report fecundity data only for the cool rearing treatment.

Statistical analyses

Pupal mass and development time data were analyzed in separate linear mixed model ANOVAs with dish effects as a random factor. In this design, dish effects serve as the error term in all F tests of fixed effects (Kuehl 1994). Both dependent variables were natural log-transformed to improve normality and homoscedasticity. An AIC score was calculated from the maximum likelihood value of models containing all possible combinations of the fixed factors clade (northern and southern), population nested within clade, sex, temperature, and all two- and three-way interactions. This experiment was designed to draw inferences about specific populations whose thermal environments were well characterized. Consequently, I specified population effects as fixed rather than random. I present the best model selected by (minimizing) the AIC and F statistics associated with each term in that model. To test whether the average linear relationship between temperature and each response variable differed between the northern clade (NC Mtn. and MAS) and southern clade (FL and NC Coast) populations in 1) average value across temperatures and 2) slope, I estimated linear

contrasts from the selected model. These tests assess parameters of the norm of reaction for trait value (development time or pupal mass) vs. temperature. Methods for testing the significance of slope parameters of reaction norms using orthogonal polynomial contrasts are described by Huey *et al.* (1999). The tests I apply here are similar, but rather than testing whether a given slope parameter is different from zero I generated contrast coefficients to test whether a difference between slope parameters is different from zero (standard methods in Kuehl 1994). I performed all analyses in SAS version 9.1 using Proc Mixed (SAS Institute 2004).

Cohort fecundities were calculated as the total number of eggs produced divided by the number of females for each mating cage. I performed ANOVA (SAS proc Mixed) with clade and population nested within clade as fixed factors to test for population differences in fecundity (untransformed data were reasonably homoscedastic and normal). To estimate the effect of body size on cohort fecundity I performed ANCOVA (SAS Proc Mixed) with clade and population nested within clade as fixed factors and average female body size as the covariate. No interaction effects including body size were significant, confirming parallelism.

Results

Thermal environment

Considering the entire year, frequencies of low temperatures were highly divergent among populations (Fig. 3.2a). Northern clade populations clearly experience higher frequencies of freezing temperatures than do southern clade populations. After accounting for geographic differences in diapause timing, however, frequencies of temperatures below 10° C were much more similar among populations during the growing season. Moreover,

Figure 3.2. Frequency distributions of daily mean temperatures for each geographic location as estimated from the 35-year weather data set for an entire year (a) and for the growing season alone (b), as defined by diapause timing.
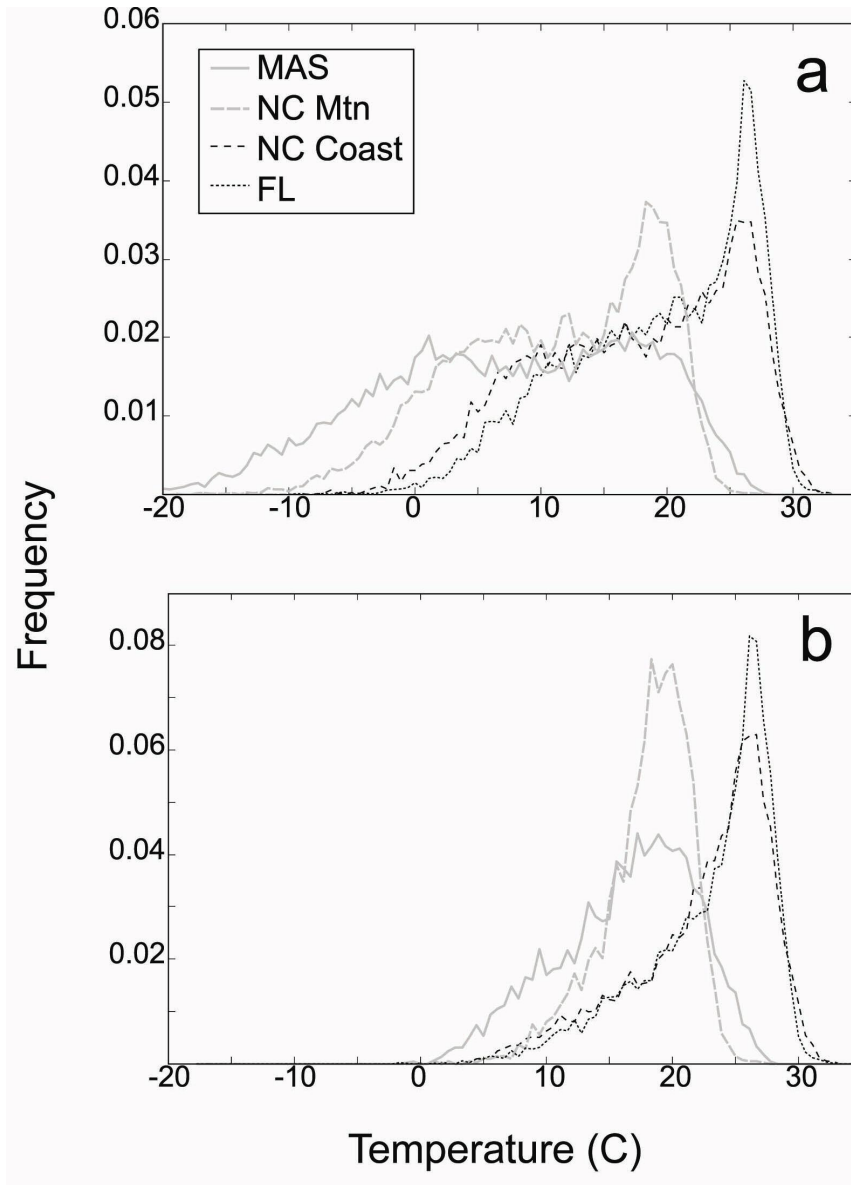
none of the populations experience daily mean temperature below 0° C during active growth and reproduction.

Accounting for diapause timing also suggests a pattern of high vs. low temperature variation during the growing season that is opposite of the pattern observed for the entire year. Growing season frequency distributions show a clear peak at about 20 and 27° C for northern and southern clade populations, respectively (Fig. 3.2b). Averaged across populations within clades, northern clade populations experience daily means of 20° C about 3 times more frequently than southern clade populations. However, daily means of 27° C occur more than an order of magnitude less frequently in northern compared to southern clade populations. These comparisons suggest that on a scale of daily mean temperature, the thermal environment of the growing season is more divergent among populations at high compared to low temperatures, whereas the reverse is true when considering the entire year (Fig. 3.2a). In addition, compared to distributions of mean temperatures for the growing season alone, clear separation between northern and southern clades in the modes (peaks) was markedly less pronounced for distributions for the entire year (Fig. 3.2a).

Development time and body mass

Temperature, sex, population of origin and clade all had significant effects on development time (Table 3.2). The clade*temperature interaction was also significant, indicating that the slope of the thermal reaction norm for development time varied between clades. Although there was a significant pop*sex*temperature three-way interaction, clade*sex*temperature interaction effects were not significant, indicating that the temperature-development time relationship is not sex-specific across clades. Males developed faster than females at all temperatures (Fig. 3.3a,c), a pattern typical of many

Table 3.2: ANOVA results from mixed model analyses of development time and pupal mass. Interaction terms excluded from the best model via AIC are excluded from the table. Parentheses reflect nesting relationships (e.g., 'Pop(Clade)' is population nested within clade).

Trait	Effect	Df.[*]	F value	P value
Development time	Clade	1	41.72	< 0.001
	Pop(Clade)	2	6.01	0.004
	Temp	1	275.88	< 0.001
	Sex	1	817.18	< 0.001
	Clade*Temp	1	11.78	< 0.001
	Pop*Temp*Sex (Clade)	9	2.83	0.006
Pupal mass	Clade	1	0.16	0.6887
	Pop(Clade)	2	11.88	< 0.001
	Temp	1	603.35	< 0.001
	Sex	1	5268.35	< 0.001
	Clade*sex	1	88.68	< 0.001
	Pop*temp (Clade)	2	5.60	0.005
	Pop*sex (Clade)	2	8.01	< 0.001
	Pop*Sex*temp (Clade)	4	5.63	< 0.001
	Clade*sex*temp	2	5.60	0.005

[*] denominator df are 86 and 85 for analyses of development time and body mass, respectively

insects, including *W. smithii* (Holzapfel and Bradshaw 2002). Northern clade populations developed more slowly than southern clade populations, averaged across temperatures and sexes ($F_{1,86} = 41.72$, $p < 0.001$; Fig. 3.3a,c). All populations exhibited the typical negative relationship between development time and temperature, but southern clade populations had on average a more negative slope than northern clade populations ($F_{1,86} = 11.78$, $p < 0.001$). Despite differences in slope, however, the rank order of population mean development time did not significantly change across temperatures.

Geographic patterns for pupal mass were more complex. Pupal mass was also significantly affected by temperature, sex, and population of origin, but the main effects of clade were non-significant (Table 3.2). The interaction effects population*temperature, population*sex, and population*sex*temperature were significant, indicating variable relationships between mass and temperature across sexes and across populations within clades. Although clade*sex and clade*sex*temperature interactions were significant, there were no detectable differences in the slope of the temperature-mass relationship between clades within females ($F_{1,85} = 2.07$, $p = 0.15$; Fig 3.3b) or males ($F_{1,85} = 2.93$, $p = 0.091$; Fig. 3.3d). Trends in average mass were sex-specific. Averaged across temperatures, southern clade females were slightly larger than northern clade females ($F_{1,85} = 18.43$, $p < 0.001$; Fig. 3.3b). In contrast, southern clade males were on average slightly smaller than northern clade males ($F_{1,85} = 25.18$, $p < 0.001$; Fig. 3.3d), although this difference was largely determined by the relatively small size of one southern population (FL). Overall there was no obvious relationship between latitude/altitude of origin and pupal mass in either males or females (Fig 3.3b,d).

Figure 3.3. Least-squared means ± SE for log-transformed development time from egg hatch to pupation (a,c) and pupal mass (b,d) vs. average rearing temperature in females (a,b) and males (c,d). Black and grey lines represent southern (FL, short dashed; NC coast, long dashed) and northern (MAS, solid; NC Mtn., dashed) clade populations, respectively.
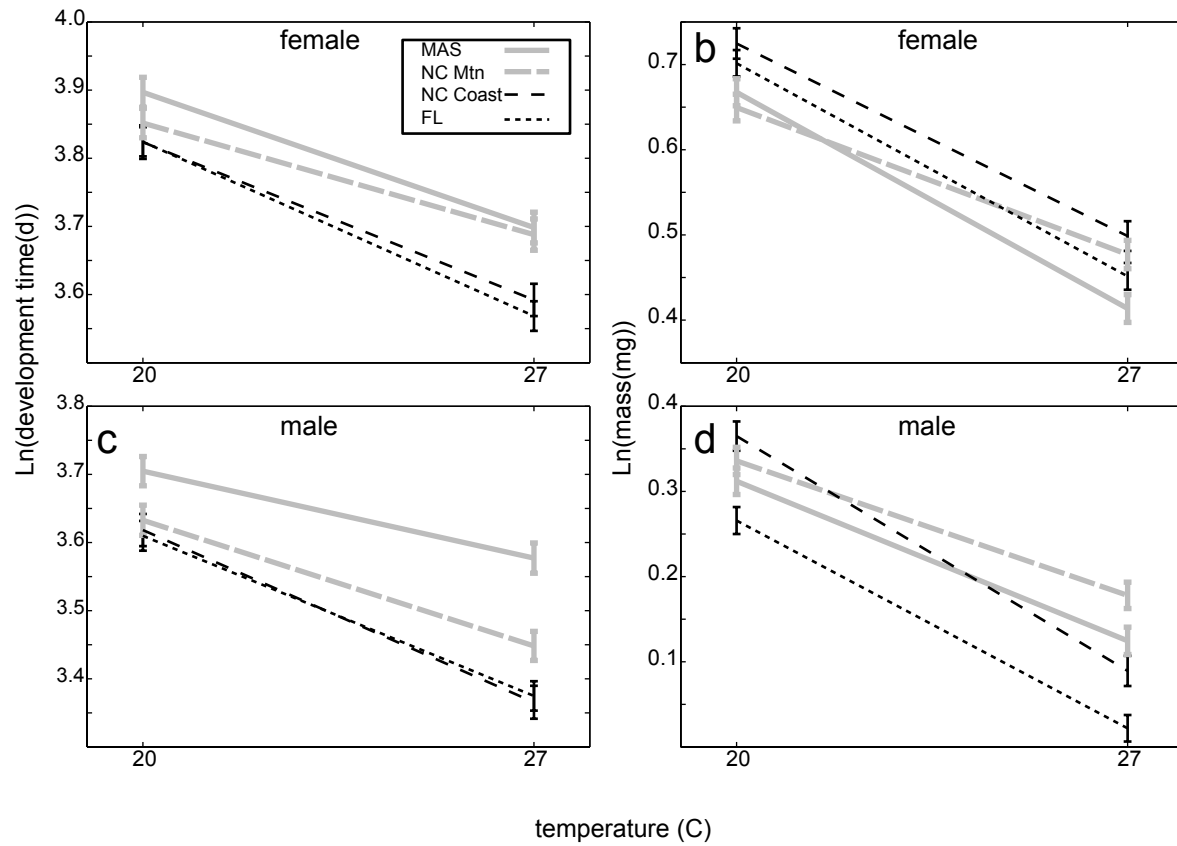
Development time and pupal mass were moderately correlated *within* populations and rearing temperatures (average $r^2 < 0.5$; data not shown), but development time was not strongly related to pupal mass in either sex *across* populations. Despite developing more rapidly (Fig. 3.3a), southern clade females were slightly larger than northern clade females (Fig. 3.3b). In addition, even though males from both southern clade populations developed at similar rates at each rearing temperature (Fig. 3.3c), NC Coast males were significantly larger than FL males averaged across rearing temperatures ($F_{1,85} = 9.83$, $p = 0.002$; Fig. 3.3d). These data suggest that mean pupal mass and mean development time have evolved independently across populations.

Fecundity

ANOVA revealed a significant effect of clade on cohort fecundity ($F_{1,8} = 25.89$, $p < 0.001$), while population-within-clade effects were non-significant ($F_{2,8} = 0.07$, $p = 0.93$). The results of the ANCOVA suggested that pupal mass had a marginally non-significant effect ($F_{1,7} = 4.73$, $p = 0.066$), accounting for 28% of the variance (compared to 65% explained by clade effects) in fecundity. Further, females from northern clade populations were slightly smaller than females from southern clade populations (Fig. 3.3b), yet northern clade populations achieved higher average cohort fecundity than southern clade populations (Fig. 3.4) in the cooler rearing treatment. These results suggest that female pupal mass may moderately affect fecundity within populations but fails to explain geographic trends in fecundity across populations.

Figure 3.4. Average fecundity ± SE of experimental cohorts (n~100 per cohort, 3 cohorts per population) from each population under the $\bar{T} = 20\,°C$ rearing treatment.
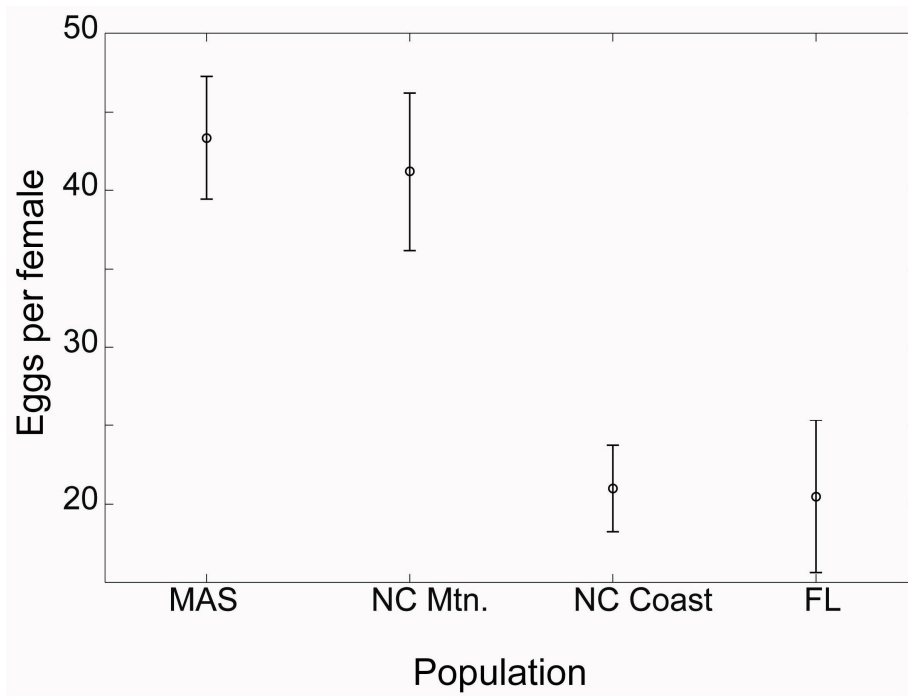
Discussion

In seasonal organisms with hibernal diapause, phenology evolves as a response to a seasonally fluctuating environment. Evolution may be driven by fluctuations in biotic resources such as food or oviposition sites, or fluctuations in abiotic factors such as temperature (Tauber et al. 1985). Adult *W. smithii* exhibit a strong oviposition preference for freshly opened pitcher plant leaves (Bradshaw and Holzappel 1986), and *S. purpurea* only produce new leaves during the warmer months of the year. Thus, diapause phenology in *W. smithii* could be evolving in response to host availability rather than in response to temperature-mediated selection. Regardless of the specific selective factors, however, the result is that non-dormant life history stages experience more similar thermal habitats across geographic populations.

Exposure to low daily mean temperatures during active growth and reproduction in *W. smithii* is relatively similar among populations because of evolved differences in diapause timing. As illustrated by the low ends of the temperature distributions in Figure 3.2a and 3.2b, northern clade populations experience much colder temperatures than southern clade populations during the winter, but not during the growing season. Considering the entire year, winter minimum temperatures generally decline faster than summer maxima with increasing latitude, suggesting that adaptations to cold temperature should be more geographically variable than adaptations to high temperature (Addo-Bediako et al. 2000). The evolution of diapause timing in *W. smithii* effectively counters this trend for non-diapause life history stages, homogenizing the low temperature environment of the growing season. Positive linear relationships between critical photoperiod and latitude have been documented in a number of insect species (Tauber et al. 1986; Taylor and Spalding 1986),

suggesting that the effects of diapause timing on the thermal environment of *W. smithii* may be common among insects with photoperiodically cued diapause.

With respect to daily mean temperature, variation among populations of *W. smithii* is actually more pronounced at the high compared to the low end of the temperature distribution for the growing season. Theory predicts that the strength of thermal selection at a particular temperature is proportional to the relative frequency at which that temperature is experienced (Gilchrist 1995; Gilchrist 2000; Kingsolver and Gomulkiewicz 2003). Thus, greater geographic variation in the relative frequency of a temperature or temperatures predicts greater geographic variation in thermal selection at that temperature or temperatures. Thermal reaction norms for life history traits that are primarily associated with active growth and exhibit consistent geographic trends should therefore demonstrate greater geographic variation at higher temperatures in *W. smithii*.

Development time varies consistently with respect to both geography and phylogeography. Averaged across temperatures, southern clade populations developed faster than northern clade populations. Observations of pulses in *W. smithii* pupa across the growing season at each field site indicate that the Massachusetts and North Carolina mountain populations have one to two generations per year (Aaron Ellison, personal communication; G. Ragland, unpublished data) whereas the Florida and North Carolina coast populations have 3 or more generations per year (Bradshaw and Holzapfel 1983; Bradshaw and Holzapfel 1986). Thus, populations with the greatest voltinism exhibit the most rapid development, results consistent with selection to fit in additional generations in the south.

These results contrast somewhat with previous results showing that generation time from egg hatch to mean date of oviposition actually declines with increasing latitude from 30

to 42° N (Bradshaw and Holzapfel 1983). However, Hard *et al.* (1993) found no consistent differences among geographic populations in development time at temperatures fluctuating around a mean of 21° C (similar to our results at 20° C), suggesting that differences in generation time may be driven by variation in oviposition schedules. Southern populations are more iteroparous than northern populations (Bradshaw 1986), thus extending the time period of oviposition. Faster development at higher temperatures may partially compensate for the effects iteropary on generation time, *i.e.*, prolonging the oviposition period increases mean generation time, while faster development decreases generation time. Selection is strongly density-dependent and generations are asynchronous in southern populations (Bradshaw and Holzapfel 1986), likely diluting the selective advantage of a particular temperature-development time relationship. However, the fitness payoff of successfully completing an additional generation is substantial, and irrespective of density-dependence there are a limited number of accumulated heat units (the physiological scale on which ectotherm development is measured) in a given growing season. Selection will thus favor those individuals that best exploit the thermal environment to maximize yearlong replacement rate.

Geographic variation in development rate reaction norms is more pronounced at higher temperature, consistent with the geographic variation in the thermal environment of the growing season. The rank order of population means did not change significantly across rearing temperatures, but development time reaction norms for southern clade populations had a significantly steeper negative slope on average than did those for northern clade populations (Fig. 3.3a,c). Differences between northern and southern clade populations were thus greater in magnitude in the warm compared to the cool rearing treatment. Moreover,

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data from an additional study indicate that northern and southern clade populations have statistically indistinguishable development times at a lower rearing temperature (16° C constant; see Chapter 5). Several studies present similar results in other insect species (Burke et al. 2005; Fischer and Fiedler 2001; Norry et al. 2001), suggesting that evolutionary divergence in development time among populations may often be greater at higher rearing temperatures in temperate insects. Development time is a trait typically associated exclusively with non-dormant life history stages, so the thermal environment of the growing season rather than that of the entire year should be a better predictor of direct selection on development time. Our data are consistent with this hypothesis, as geographic variation in the thermal habitat best explains geographic variation in the development time-temperature relationship when evolved differences in diapause timing are accounted for.

Pupal mass vs. temperature reaction norms also differed among geographic populations but did not co-vary with development time. If body size and development time are positively genetically correlated, life history models generally predict that selection will act most strongly on development time (because encountering a catastrophic event such as a hard frost while not in diapause results in massive mortality) and that body size will evolve as a correlated character, often resulting in converse Bergmann's clines (size decreases with increasing latitude) in body size (Blanckenhorn and Demont 2004; Mousseau 1997). In *W. smithii*, however, a previous study shows that artificial selection on development time produces no correlated response in pupal mass (Bradshaw and Holzapfel 1996), suggesting that these traits are not genetically correlated. Our results are consistent with those data, as body size and development time appear to evolve independently across populations.

Unconstrained by the life history tradeoff with development time, body size fails to show a consistent geographic trend, and geographic variation was roughly equivalent at cool and warm rearing temperatures. Significant differences in body size across populations in both sexes and at both rearing temperatures show that body size can evolve, but the lack of a geographic trend suggests that selection for body size does not vary consistently from south to north. Selection on female body size is often mediated through selection on fecundity (Kingsolver and Pfennig 2004; Roff 2002), and cohort fecundity in the cool rearing treatment varied significantly among populations. Within the single density level (25 larvae per dish) applied in our study, however, variation in body size did not strongly influence fecundity, nor does it explain fecundity differences among populations. Pupal size is negatively related to larval density (or resource availability) in *W. smithii*, and measured across a range of pupal sizes generated by a broad range of density treatments there is a positive relationship between female size and fecundity (Bradshaw and Holzapfel 1992). However, the relationship between density and fecundity does not consistently vary with geography (Bradshaw 1986), so the existence of these correlations does not alter our inferences about population differences based on a single density treatment. Our combined fecundity and body size measures suggest that fecundity selection is neither acting to maximize female body size in a given population nor to maintain an optimum female body size across populations. Given the lack of a consistent geographic trend, it is somewhat unsurprising that geographic variation in the thermal environment does not predict geographic variation in the thermal reaction norm for body size.

To summarize, our results illustrate how evolutionary differences in seasonal timing can impact geographic variation in selection on thermal reaction norms for life history traits.   In

W. smithii, this is reflected in geographic variation in the slopes of thermal reaction norms for some life history traits (development time) but not others (body size).   Our analyses emphasize that proper interpretation of geographic patterns in thermal reaction norms requires an understanding of the phenological context, a point largely neglected in the substantial literature about reaction norm evolution (*e.g.*, Angilletta et al. 2002; Huey and Kingsolver 1989; Kingsolver and Gomulkiewicz 2003).

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CHAPTER 4

GEOGRAPHY OF THERMAL ADAPTATION IN *WYEOMYIA SMITHII*: THERMOTOLERANCE, ACCLIMATION, AND SEASONAL TIMING

Abstract

Tolerance of extreme temperatures is a major adaptation to seasonal environments, often exhibiting intra- and interspecific geographic variation that is correlated with environmental temperatures. However, selection on thermal tolerance of organisms with complex life cycles may be stage-specific because particular life history stages are seasonally timed to coincide with stressful temperatures. Geographic variation in seasonal timing may therefore complicate geographic trends in thermal selection by changing the thermal environment experienced by a particular stage. Here I illustrate this concept by examining geographic variation in annual thermal environments, seasonal timing of dormancy, and cold and heat tolerance in pitcher plant mosquitoes. The results show that 1) evolved differences in diapause timing cause active (non-diapause) stages to experience similar levels of cold stress and more variable levels of heat stress among geographic populations, and 2) despite this pattern of temperature variation, adult mosquitoes from northern populations are more cold tolerant but not less heat tolerant than southern populations. I suggest that the observed geographic pattern in cold tolerance of active life history stages may be driven by correlated selection on overwintering survivorship rather than direct selection on growth, development, and reproduction. In addition, I discuss the joint geographic patterns of cold and heat stress tolerance in relation to broader, interspecific patterns.

Introduction

Tolerance of stressful temperatures is a major adaptation to thermally variable

environments. All organisms are physiologically limited to a defined temperature range

(Hoffmann et al. 2003; Huey and Kingsolver 1993), and particularly in seasonal

environments with high annual temperature variation, stressful (*i.e.*, deleterious)

temperatures at the limits of that range are inevitably encountered at some point during the

life cycle (Stevens 1989). Short term physiological responses to temperature stress are

common across a broad range of taxa (*e.g.*, the well-characterized heat shock response) and

often vary with geography in temperate organisms. The general expectation is that

populations from colder habitats will be more cold tolerant, while populations from warmer

habitats will be more heat tolerant. This expectation is often confirmed, demonstrating

geographic variation in temperature-mediated selection (Armbruster et al. 1999; Castaneda et

al. 2004; Castaneda et al. 2005; Collinge et al. 2006; David et al. 2003; Sorensen et al. 2005;

Van Berkum 1988; Winne and Keck 2005).

Geographic patterns of selection on thermotolerance may be complicated, however,

by the occurrence of alternate phenotypes. Alternate phenotypes resistant to environmental

stress are a common feature of complex life cycles (Caceres 1997). In seasonal environments

temperature variation is relatively predictable, so these alternate phenotypes often coincide

with the seasonal occurrence of stressful temperatures. For example, temperate insects often

have a dormant overwintering stage with reduced metabolic rate and enhanced cold tolerance

(Danilevsky 1965; Danks 2002; Tauber et al. 1986). Because of the seasonal timing, the

dormant stage is exposed to the coldest temperatures annually, while active life history stages

experience the hottest. Thus, selection on thermotolerance will depend on both seasonal

timing and life history context in organisms such as insects with seasonally variable life history stages. Geographic variation in seasonal timing can therefore affect geographic variation in selection on thermotolerance of a given life history stage. However, the geography of life cycle timing and thermal tolerance are rarely considered simultaneously (but see Bradshaw et al. 2004).

To examine the joint evolution of dormancy timing and thermotolerance I analyzed geographic patterns of annual temperature variation, diapause timing, and high and low temperature tolerance in the pitcher plant mosquito, *Wyeomyia smithii*. *W. smithii* overwinters in larval diapause (a dormant, metabolically depressed stage), and diapause in higher latitude populations is timed such that the growing season is relatively shorter than that of lower latitude populations (Bradshaw and Lounibos 1977). Bradshaw *et el.* (2004) have previously shown that higher latitude populations attain higher fitness than lower latitude populations when exposed to stressful winter conditions in the diapausing stage, whereas the reverse is true in stressful summer conditions experienced during active growth and reproduction. However, it is unclear from this study whether there is geographic variation in high and low temperature tolerance in non-diapause, active stages. Here I address two questions relating to selection on and evolution of thermotolerance in *W. smithii*: 1) Is there significant geographic variation in cold stress experienced during active growth after accounting for geographic variation in diapause timing, and 2) does geographic variation in cold and heat tolerance reflect geographic variation in the frequency of high and low temperatures experienced during the growing season, *i.e.*, the portion of the year between termination and initiation of dormancy?

Methods

Study organism and sampling

Adult female pitcher plant mosquitoes obligately oviposit into the water-filled leaves of the purple pitcher plant (*Sarracenia purpurea*), where the larvae develop until eclosion. Winter (larval) diapause is induced and terminated by photoperiodic cues during larval development; long-day conditions promote direct development, while short-day conditions induce diapause (Bradshaw and Lounibos 1972).

Geographic populations of *W. smithii* are found in eastern North America, ranging from northern Florida to the Great Lakes region inland and Newfoundland along the coast (Armbruster et al. 1998). Adults are weak flying and the host plants are patchily distributed, so gene flow between populations is minimal or absent (Istock and Weisburg 1989). Populations arrayed along a latitudinal cline are highly divergent in critical photoperiod (CP), the photoperiod at which 50% of a sample initiates or terminates diapause (Bradshaw and Lounibos 1977). CP increases (and the length of the growing season concomitantly decreases) with increasing altitude and latitude (Bradshaw and Lounibos 1977). Phylogeographic analyses suggest that geographic populations fall into two fairly distinct, intraspecific clades: a northern clade occurring north of North Carolina or at high elevations in North Carolina, and a southern clade from North Carolina to Florida (Armbruster et al. 1998; WE Bradshaw, unpublished).

From each of the four geographic populations listed in Table 4.1 I collected ~ 1000 larvae from 50 or more individual pitcher plants in the spring and fall of 2004. Two of these populations are from the southern clade, two from the northern, and all have diverged in

Table 4.1: Geographic locations and critical photoperiods for the four *W. smithii* study populations.

Geographic Location	Lat./Lon. (deg)	Altitude (m)	Critical Photoperiod (h)
Florida gulf coast (FL)	30°N 85°W	10	12.25*
North Carolina coast (NC Coast)	34°N 78°W	20	12.75*
North Carolina mountains (NC Mtn.)	35°N 83°W	900	14.35*
Central Massachusetts (MAS)	42°N 72°W	265	14.50†

* from Bradshaw and Lounibos (1977)
† calculated from Eq. 1 in Bradshaw and Lounibos (1977): see methods

diapause timing as determined by CP. Sampled larvae were returned to the lab and reared to adulthood as in Hard *et al.* (1992) under long day conditions (LD 16:8) and fluctuating temperatures (from 13 to 29° C as a sine curve). Adults were allowed to swarm in 5 gallon cages provided bi-weekly with a fresh pitcher leaf and a honey-water-soaked sponge for nutrition. Eggs were collected every three days until all adults in a cage had died. Freshly hatched larvae were then reared under diapause inducing conditions (LD 8:16, 19º C) to synchronize all individuals in the same developmental stage. Diapausing larvae were then returned to long-day conditions to initiate the next generation. I maintained laboratory colonies at > 500 individuals per population per generation to preserve genetic variation, and initiated new generations with a constant proportion of eggs from each egg collection date.

Weather data

Fifty years of daily minimum and maximum temperature data were retrieved from the US National Climatic Data Center (http://www.ncdc.noaa.gov/oa/ncdc.html) for weather stations less than 5km from each sampled site. Data from temperature loggers placed in pitcher plants in the field show that temperatures at the study sites are highly correlated with weather station data (Bradshaw et al. 2000; G. Ragland, unpublished). A cover of snow will insulate pitcher plants from sub-zero (°C) temperatures (Bradshaw et al. 2004). I present the results with this complication in mind, although snow is not a confounding factor during the growing season.

Years for which any of the stations reported missing values for any month of the year were excluded, and the resulting 35-year subset was analyzed. For each site I calculated the frequency distribution of maximum and minimum temperatures for a single, entire year binned into 1° C intervals. In addition, I calculated the frequency distribution of maximum

and minimum temperatures for only the growing season of each site, defined as the days of the year with daylength longer than the critical photoperiod of that population. I included civil twilight in my estimates of daylength because *W. smithii* likely includes twilight cues in photoperiod perception (Bradshaw and Phillips 1980), although excluding civil twilight did not qualitatively change the results.

Experimental design

Cold and heat tolerance assays were performed in two separate experiments: one examining acclimation and cold tolerance in two fluctuating temperature rearing environments (Experiment I), and one examining acclimation and heat tolerance in two different fluctuating temperature rearing environments (Experiment II).

Experiment I. Chill coma recovery

Chill coma recovery assays are commonly used to assess cold tolerance in arthropods (Castaneda et al. 2005; David et al. 2003; David et al. 1998; Gibert and Huey 2001; Gibert et al. 2001; Zeilstra and Fischer 2005). In the assay, individuals are exposed to a stressful cold temperature (usually 0° C) for an extended period of time, inducing a comatose state. After this exposure, individuals are returned to a more benign temperature and measured for recovery time, or the latency to regain locomotory capacity (David et al. 1998). This assay has been shown to correlate well with other measures of cold tolerance in *Drosophila* (Anderson et al. 2005). Further, tropical *Drosophila* species exhibit consistently longer chill coma recovery times than temperate species, suggesting that the chill coma assay is a reliable indicator of general resistance to cold stress (Gibert et al. 2001).

To assess geographic variation in both plastic responses to rearing temperature and average cold tolerance, I measured chill coma recovery of adults from each of the four

geographic populations (NC Coast, NC Mtn., and FL) reared from egg hatch to adulthood in two fluctuating environments designed to mimic natural diurnal temperatures measured in pitcher plants in the field during a relatively warm ('Warm' treatment, $\overline{T}$ = 22 º C) and relatively cool ('Cool' treatment, $\overline{T}$ = 18 º C) spring day (Fig. 4.1). Daily minima at or below 0° C are much more likely to occur during the spring than during the summer, so warmer rearing temperatures are less realistic. I avoided using colder temperatures to avoid potentially high mortality, as survival to pupation is relatively low (< 50%) when larvae are reared at 16º C constant (G Ragland, unpublished).

From the F_2 lab generation I initiated cohorts of newly hatched (within 24 hours) larvae by haphazardly selecting and transferring 25 individuals to a 150 x 25mm culture dish filled with 170ml distilled water. Eight cohorts per population were assigned to each temperature treatment. Each dish was initially provided with 1.00 ml standard food suspension (0.05 g/ml 4:1 guinea pig chow to freeze-dried brine shrimp), and larvae were transferred to fresh dishes once per week until all larvae had pupated. Fresh food suspension was provided at each transfer in a temporal pattern designed to mimic food levels in a pitcher plant leaf (Bradshaw and Holzapfel 1986): 1.75, 2.5, and 3.0ml were added for the first three weeks, and 2.5ml every week thereafter. Pilot studies suggested that this feeding regime maintained *ad libitum* conditions (G. Ragland, unpublished).

Commencing on the day that the first pupa appeared, pupae were removed from dishes, sexed, and transferred to individual wells in 24-well culture plates. From these pupae I selected a subset from each dish to obtain a uniform sample across the entire range of pupation dates in a single dish. Selected pupae were transferred to 50ml centrifuge tubes with 15ml distilled water for eclosion, and two days after eclosion each adult was assayed for chill

Figure 4.1. Diurnal temperature profiles for the rearing treatments in the chill coma recovery (black lines) and heat knockdown (grey lines) experiments.
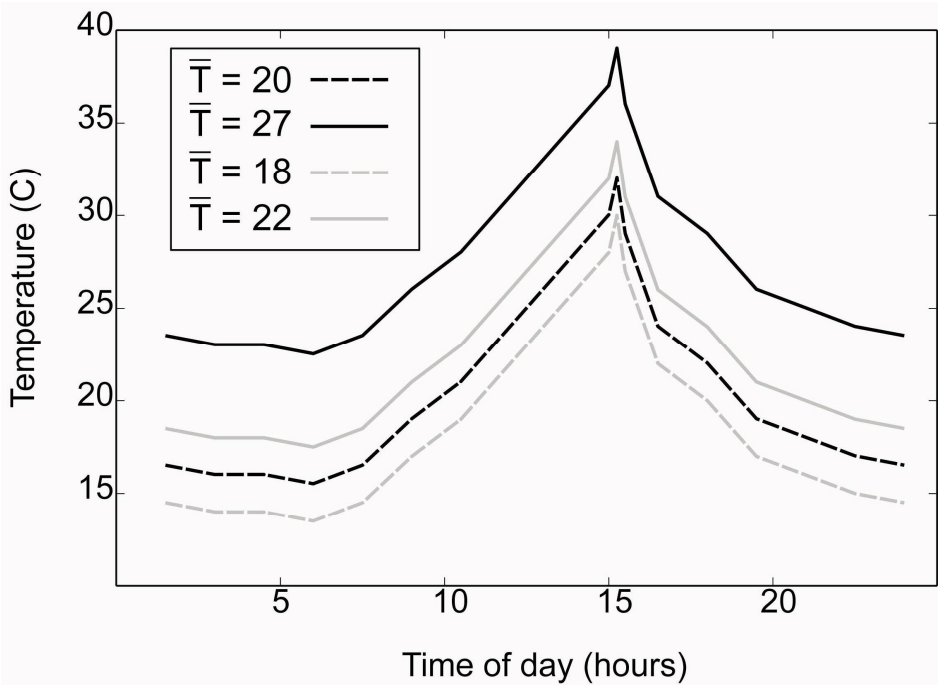
coma recovery. All assays were carried out from 1:00 to 2:00 PM to avoid any diurnal effects. The assay itself consisted of 1) a 20 minute exposure to 24° C, during which individuals were transferred to stoppered glass test tubes), 2) immersion in melting ice (0° C) for 1.5 hours, and 3) return to 24° C. Immediately after returning the comatose adults to 24° C, I recorded the time required for a righting response (comatose individuals lie on their backs). Mortality was low in both rearing environments (< 8%; similar for all populations). Sample sizes for the 18 and 22º C mean treatments were 83 and 77 (FL), 75 and 54 (NC Coast), 79 and 73 (NC Mtn.), and 67 and 51 (MAS).

Thermal tolerance in insects can be dependent on life history stage (Krebs and Loeschcke 1995), and in *W. smithii* the post-diapause generation (individuals undergoing larval diapause before developing to adulthood) that develops in the spring is more likely to experience stressful cold temperatures than non-diapause generations. Any carry-over physiological effects of diapause could potentially alter the physiology of post-diapause compared to non-diapause development. Since the above experiment was conducted during non-diapause development, I also measured cold tolerance of post-diapause individuals from two populations. From the F_1 lab generation I haphazardly selected diapausing larvae from the FL and MAS lab colonies to initiate 5 cohorts per population. These larvae were maintained for three months at 8:16 LD and 18° C constant, with 30 larvae per dish. After three months, the cohorts of diapausing larvae were transferred to the 'cool' treatment and reared concurrently with the non-diapausing larvae (long-day conditions caused the larvae to break diapause and develop to adulthood). Chill coma assays were performed as described above.

<u>Experiment II. Heat knockdown</u>

Just as chill coma assays are commonly used to measure cold tolerance in arthropods, knockdown temperature is often used to measure heat tolerance (Berrigan and Hoffmann 1998; Bubliy and Loeschcke 2005; Folk et al. 2006; Hoffmann et al. 2002; Hoffmann et al. 2005; Huey et al. 1992; Sorensen et al. 2005). This trait is typically measured by exposing individuals or groups to ramping temperatures and recording the temperature at which an individual becomes comatose (Huey et al. 1992). Heat knockdown temperature responds readily to selection in *Drosophila* and is also correlated with a suite of heat tolerance-related traits (Bubliy and Loeschcke 2005). I used a modification of this assay to measure heat tolerance in *W. smithii*. Rather than measuring knockdown temperature, I measured the proportion of a population sample that maintained locomotory ability after a fixed exposure to a fixed temperature. Since a low knockdown proportion indicates high heat tolerance, I report 1- knockdown proportion as the measure of heat tolerance.

To measure both acclimation and general tolerance as in the previous experiment, I reared 8 cohorts of larvae from each population under two fluctuating temperature regimes designed to mimic a typical cool ('Cool' treatment, $\bar{T} = 20°$ C) and warm ('Warm' treatment, $\bar{T} = 27°$ C) summer day in the field (Fig. 4.1). From the F_3 generation I selected newly hatched larvae, reared cohorts, and selected pupae as described above. Two days after eclosion, adults were transferred to 15 ml centrifuge tubes and allowed to equilibrate at 24° C for 15 minutes. The centrifuge tubes were then immersed in a temperature-controlled water bath held at $42.5 \pm 0.1°$ C for 5 minutes. This temperature and duration of exposure were pre-determined to induce heat coma in 20 – 60% of adults sampled from each population. After the 5 minute exposure, tubes were returned to 24° C and individuals were scored as either

able or unable to fly. Under benign environmental conditions *W. smithii* will fly upwards when disturbed. Gently rapping mosquito-containing centrifuge tubes on the lab bench elicited a very clear-cut response: the individual either remained at the bottom of the tube or flew towards the top. Sample sizes for the 20 and 27º C mean treatments were 84 and 104 (FL), 113 and 84 (NC Coast), 54 and 76 (NC Mtn.), and 90 and 94 (MAS).

Statistical analyses

For the analysis of non-diapause development, the fixed effects of population, temperature, and sex on chill coma recovery time were analyzed in a likelihood framework using a linear fixed effects model implemented in Proc Mixed, SAS version 9.1 (SAS Institute 2004). Prior to analysis, chill coma recovery times were natural log transformed to improve normality and homoscedasticity. Initially I analyzed the data in a mixed model including the random effect of cohort nested within temperature by population, but since this effect did not significantly improve the fit of the model (assessed by the Akaike Information Criterion, or AIC), it was excluded. I calculated AIC scores for all possible models containing all of the main effects and all possible combinations of interactions between population, temperature, and sex. The results I present include only the best model selected by the (lowest) AIC. From the linear model I also estimated linear contrasts to compare the average value across temperatures and the slope of the relationship between rearing temperature and chill coma among populations (see Chapter 3: Methods). Rather than calculating all pairwise contrasts, however, I compared the average slope of the two northern clade populations to the average slope of the two southern clade populations.

The effects of developmental mode (non- vs. post-diapause development) on chill coma recovery were also analyzed in a fixed effects linear model (SAS Proc Mixed)

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including terms for population, sex, temperature, and developmental mode. Cohort effects were again excluded from the model as they did not significantly lower the AIC. The best fitting model was selected as above, and only the best model is presented in the results.

Since heat knockdown data were binary, I applied a logistic fixed effects model including population, temperature, and sex as effects (SAS Proc Glimmix). Model selection and linear contrasts were carried out as above.

Results

Weather Data

The two southern clade populations (NC Coast and FL) clearly experience higher frequencies of high daily maximum temperatures than the two northern clade populations (NC Mtn. and MAS; Fig. 4.2c). Accounting for phenological differences does not drastically change the high-temperature end of these distributions (Fig. 4.2d). After correcting for the timing of diapause at each sight, however, none of the populations experience daily minimum air temperatures below about -7° C during the growing season (Fig. 4.2b). A cover of snow will minimize exposure of frozen pitcher leaves to temperatures below zero (Bradshaw et al. 2004), so actual temperatures in a pitcher plant are often much closer to zero than below-zero measurements of air temperature would suggest. This effect would truncate the low end of the minimum temperature distribution (primarily for northern clade populations) for the entire year. Accounting for the insulating effects of snow does not change the observation, however, that northern clade populations experience temperatures at or below zero much more frequently than southern clade populations during the entire year (Fig. 4.2a). Because of phenological differences all populations experience much more

Figure 4.2. Frequency distributions of daily minimum (a,b) and maximum (c,d) temperatures for each sampled geographic location as estimated from the 35-year weather data set for an entire year (a,c) and for the phenologically-defined growing season only (b,d).
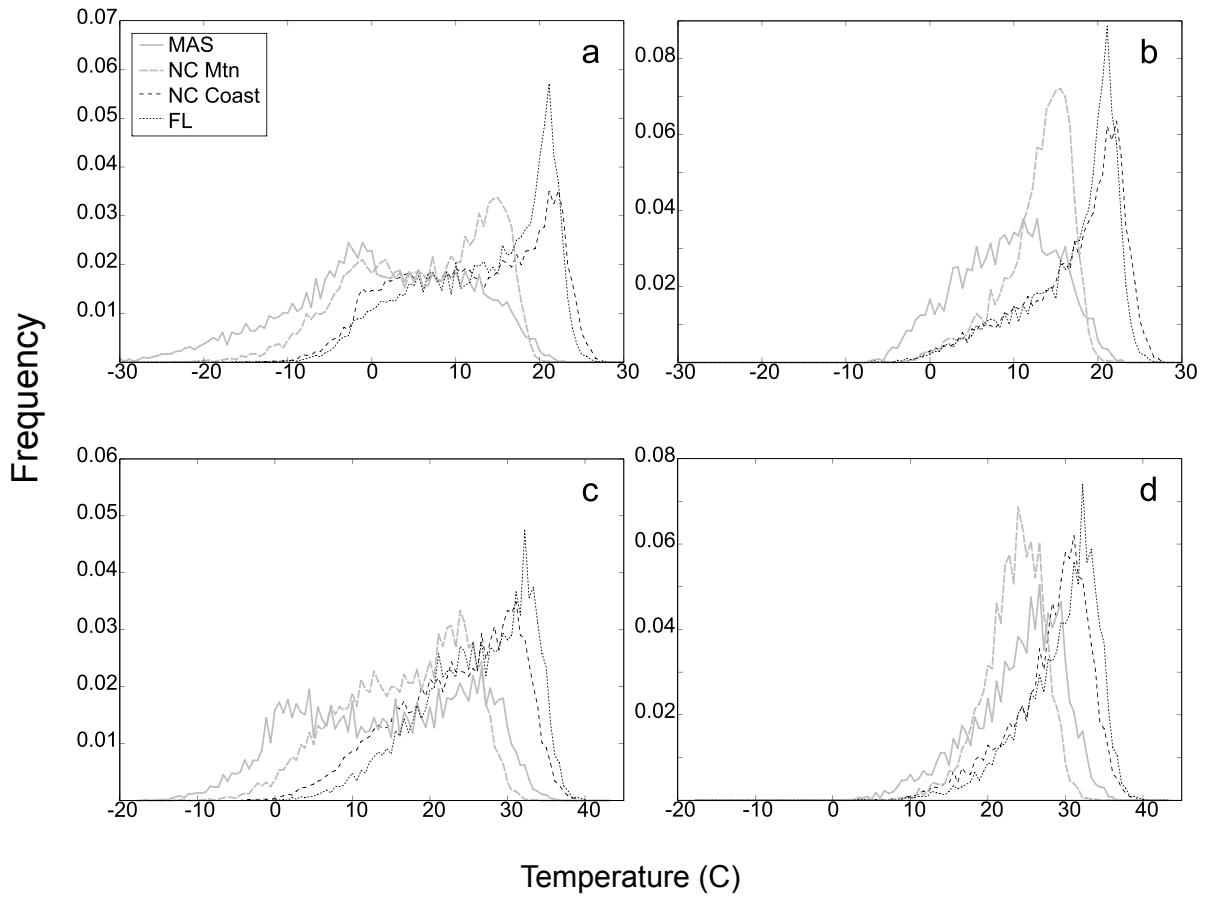
Figure 4.3. Least-squared means ± SE for log-transformed chill coma recovery time (a) and 1- heat knockdown proportion (heat tolerance; b). Black and grey lines represent southern (FL, short dashed; NC coast, long dashed) and northern (MAS, solid; NC Mtn., dashed) clade populations, respectively. In panel 'a', the lines connect means for non-diapause development, while the open square and open circle symbols depict the means of post-diapause development for FL and MAS, respectively.
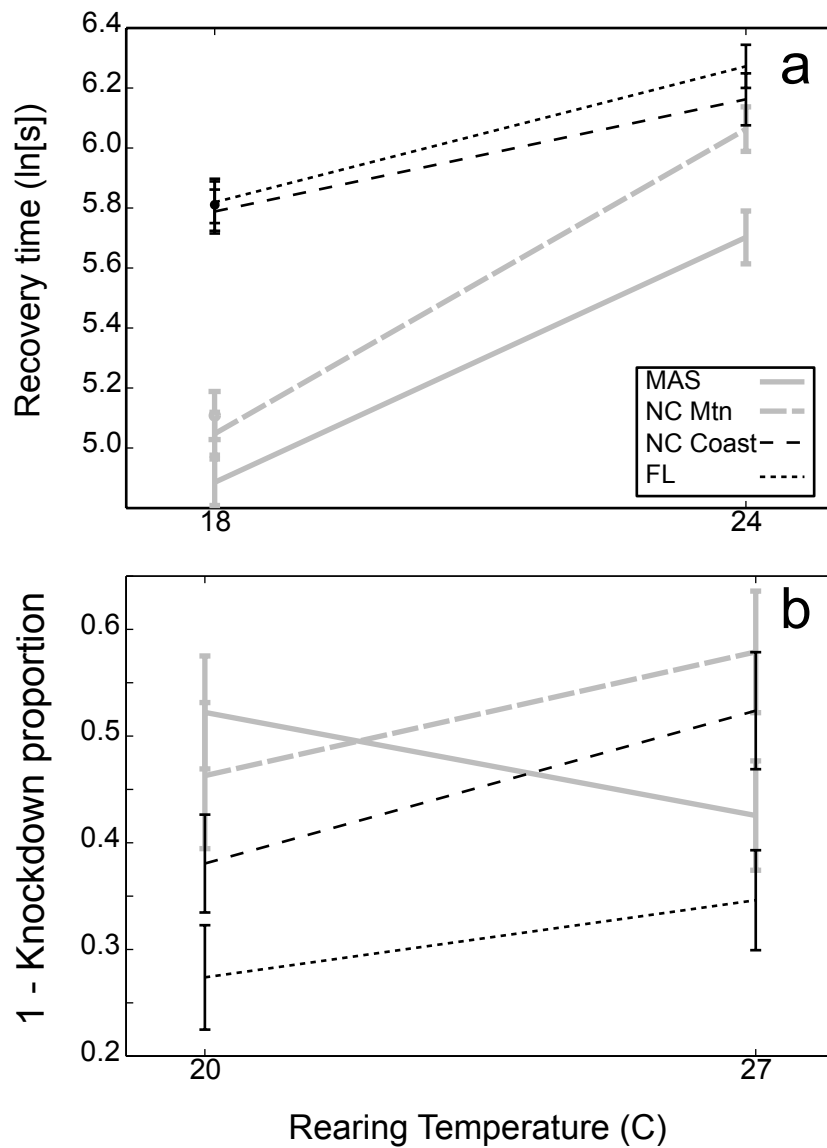
Table 4.2. Results of linear mixed model (a,b) and logistic model (c) ANOVA for chill coma recovery at constant (a) and fluctuating (b) temperatures and heat knockdown proportion (c) at fluctuating temperatures.

Trait	Effect	Df.	F value	P value
A. Chill coma rec.,	Pop	3	42.42	< 0.001
non-diapause	Temp	1	152.16	< 0.001
	Sex	1	30.10	< 0.001
	Pop*Temp	3	8.25	< 0.001
B. Chill coma rec.,				
non- and post-diapause	Pop	1	107.49	< 0.001
	Sex	1	5.44	0.020
	DM[*]	1	1.77	0.18
C. Heat knockdown	Pop	3	5.58	0.001
	Temp	2	2.19	0.140
	Sex	1	0.19	0.661

[*] Developmental mode: non- or post-diapause

similar frequencies of minimum temperatures at or below zero during the growing season (Fig. 4.2b).

Experiment I. Chill coma recovery

Population of origin, sex, and temperature significantly affected chill coma recovery time (Table 4.2a). Recovery times were faster at the lower rearing temperature for all populations (Fig. 4.3a), and averaged across temperatures, northern clade populations recovered significantly faster than southern clade populations ($F_{1,545} = 118.07$, $p < 0.001$). Females recovered significantly faster than males ($F_{1,545} = 30.10$, $p < 0.001$), but there were no significant interactions between sex and population or temperature. There was a significant interaction between population and temperature, and linear contrasts suggest that the slope of the recovery time vs. temperature relationship was steeper for northern clade populations than for southern clade populations ($F_{1,\ 545} = 21.72$, $p < 0.001$). Developmental mode (post- vs. non-diapause) had no effect on chill coma recovery time in the cool treatment (Table 4.2b). The open symbols in Figure 4.3a show mean ± SE recovery time of post-diapause FL and MAS adults relative to non-diapause adults: recovery times for each population were indistinguishable based on developmental mode.

Experiment II. Heat knockdown

Population of origin significantly affected heat tolerance as measured by 1-knockdown proportion, but sex and rearing temperature did not (Table 4.2c). In three of the four populations a higher proportion was knocked down at lower rearing temperatures, but the trend was non-significant (Fig. 4.3b). Southern clade populations did not exhibit consistently greater heat tolerance than did northern populations. Compared to the average value of the northern clade populations, one southern clade population (NC Coast) had a

statistically indistinguishable average heat tolerance ($F_{1,693} = 1.29$, $p = 0.257$) while the other had lower heat tolerance ($F_{1,693} = 16.34$, $p < 0.001$).

Discussion

Diapause timing and temperature

Geographic differences in diapause timing have clear implications for the way in which selection acts on thermal responses during the growing season in *W. smithii*. Winter diapause primarily influences the low end of the daily minimum temperature frequency distribution experienced by actively growing individuals. With the exception of the Massachusetts (MAS) population, frequencies of daily minimum temperatures experienced during the growing season reached zero at about -3º C and converged to similar values between -3 and +5º C (Fig. 4.2b). Moreover, compared to frequency distributions for the entire year, even the phenologically-corrected daily minimum temperature distribution for MAS reaches frequencies of sub-zero temperatures that are more similar to values for other populations. Direct selection on low temperature tolerance of non-diapause stages is thus more comparable across populations than simple and commonly used environmental proxies such as annual minimum temperature would suggest.

Divergence in phenology among geographic populations leads to a further interesting consequence: predictions about the relative evolutionary importance of selection at high and low temperatures may depend on the life history context. Considering only meteorological data, extreme winter minima decline more rapidly than extreme summer maximum temperatures with increasing latitude (Gaston and Chown 1999). This observation leads to the prediction that geographic populations along a latitudinal cline should be more divergent in cold stress tolerance than in heat stress tolerance (Addo-Bediako et al. 2000). Evolutionary

divergence in diapause timing does not affect the extreme cold temperatures experienced by a diapausing stage, nor does it affect the extreme hot temperatures experienced by non-diapause stages. In fact, when comparing heat and cold stress tolerance of active and overwintering stages, respectively, a number of species including *W. smithii* show greater geographic variation in cold compared to heat stress tolerance (Chown and Nicolson 2004; Zani et al. 2005). However, if phenology evolves to limit exposure to extreme cold temperatures in non-diapause stages then the thermal habitat of the growing season may exhibit similar geographic variation in extreme high and low temperatures. Figure 4.4 shows average annual minimum and maximum temperatures (estimated from the 35 years of weather data) for each of the geographic populations of *W. smithii* for the entire year and for the growing season alone. Clearly differences in diapause timing decrease geographic variation in annual minima and have no effect on annual maxima of the growing season. Direct selection on high and low temperature stress tolerance of non-diapause stages, therefore, should vary comparably across latitude and altitude in *W. smithii*.

Plasticity of tolerance

Colder rearing temperatures conferred enhanced cold tolerance in adult *W. smithii*, but undergoing diapause did not. In both northern and southern clade populations, adults reared at 18º C recovered faster from chill coma than those reared at 22º C. These results agree well with results from *Drosophila* showing a strong relationship between rearing temperature and cold tolerance (Gibert and Huey 2001). However, diapausing for 3 months at 18º C did not confer faster recovery times than non-diapause development at 18º C in one southern and one northern clade population. Physiological traits such as lower thermal threshold for development can vary between post- and non-diapause development

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Figure 4.4. Annual maximum temperatures (■) and annual minimum temperatures calculated for the entire year (▲) and for the growing season alone (♦) for each geographic site. Annual maxima occur during the growing season: thus, accounting for diapause timing does not change this value. Values are averages across 35 years of data as described in the methods; standard errors of the means are smaller than the symbols.
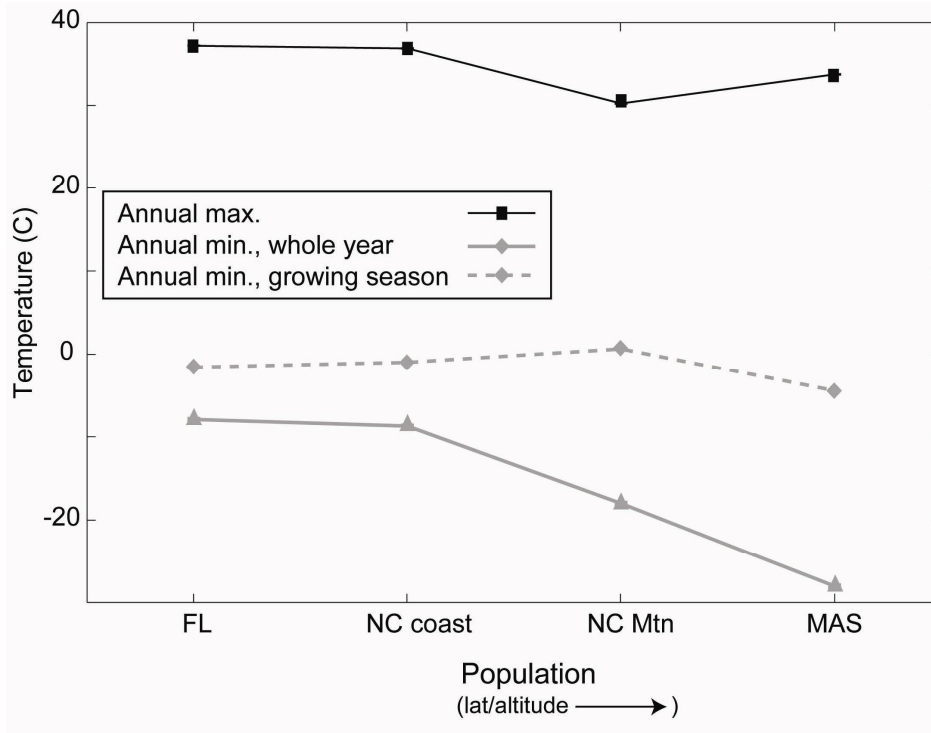
(Tauber and Tauber 1987), but this is a larval characteristic. If there are any differences in cold tolerance between post- and non-diapause larvae, they are uncorrelated with cold tolerance of the adult stage in *W. smithii*.

In contrast to chill coma recovery, heat tolerance was unrelated to rearing temperature. Studies of *Drosophila* suggest that higher rearing temperatures often confer increased heat tolerance (Hoffmann et al. 2005; Levins 1969), but this does not appear to be the case in *W. smithii*, even over a substantial (7º C) change in mean rearing temperature.

Geography of temperature tolerance

Northern clade populations had substantially faster chill coma recovery times than southern clade populations, suggesting that populations from colder environments have evolved enhanced cold tolerance. Further, northern clade populations exhibited a steeper relationship between rearing temperature and chill coma recovery time. The present study is the first report of intraspecific geographic variation in the plastic response to rearing temperature, but intraspecific variation for chill coma recovery at a single rearing temperature follows a similar pattern in *Drosophila melanogaster* (Hoffmann et al. 2002), *Drosophila serrata* (Hallas et al. 2002) and the isopod *Porcellio laeris* (Castaneda et al. 2005), where populations at higher latitudes exhibit faster recovery times. Latitudinal trends in chill coma recovery are also observed in interspecific comparisons. Gibert *et al*. (2001) found that out of 84 *Drosophila* species, 26 temperate species recovered significantly faster than 48 tropical species (the distributions of recovery times do not overlap), strongly suggesting that chill coma recovery is a climatic adaptation.

These geographic patterns makes good intuitive sense, but the mechanism of selection driving latitudinal trends in chill coma recovery remains unclear. In *Drosophila*

melanogaster, chill coma recovery is correlated with survival of stressful cold temperatures (Anderson et al. 2005), and overwintering survival is a plausible selective factor (Gibert et al. 2001). In *W. smithii*, however, diapausing larvae overwinter, so direct selection on the cold tolerance of the adult stage can only occur during the growing season. Thus, selection on overwintering survival would only produce the observed geographic patterns in *W. smithii* if cold tolerance were correlated across life history stages. Comparisons of temperature stress tolerance across life history stages are uncommon, but a study by Krebs *et al.* (1998) shows that expression of Hsp70, a common temperature stress-induced protein, is positively genetically correlated across life history stages in *Drosophila melanogaster*. If this pattern of correlation is a common phenomenon, geographic trends in cold tolerance in any life history stage may often reflect direct selection on overwintering rather than on active life history stages. Alternatively, for direct selection on adult cold tolerance to produce the observed geographic patterns, the geographic populations would have to differ in the frequency at which stressful cold temperatures are experienced during the growing season.

After accounting for phenological differences between populations, the temperature data partially support the hypothesis that correlated selection on overwintering survival drives geographic trends in adult chill coma recovery in *W. smithii*. Considering temperature distributions for the entire year, northern clade populations experience sub-zero temperatures much more frequently than do southern clade populations (Fig. 4.2a). Considering only the growing season, however, three of the four populations experience nearly identical frequencies of temperatures below about 5° C (Fig. 4.2b). The NC Mtn. population exhibits faster chill coma recovery than the two southern clade populations, but only diapausing larvae experience cold temperatures more frequently than do southern clade diapausing

larvae. Thus, selection on the diapause stage more adequately explains the enhanced cold tolerance of adults from the NC Mtn. population in comparison to southern clade populations.

Irrespective of the specific selective factors that determine cold tolerance, the joint geographic trends in cold and heat tolerance in *W. smithii* are consistent with the predictions of Addo-Bediako *et al.* (2000). Northern clade populations were significantly more cold tolerant than southern clade populations, but southern clade populations were not more heat tolerant as measured by the knockdown assay. Similarly, population replacement rate (R_0) after acute high temperature stress is unrelated to geography, while northern populations achieve higher R_0 than southern populations when a similar cold stress is applied (Zani et al. 2005). Survival of lethal high temperatures in both larval and adult stages is also unrelated to geography (Armbruster et al. 1999). When stressed to the brink of extinction in high temperature environments for multiple generations, southern populations do achieve higher yearlong replacement rates than northern populations (Bradshaw et al. 2004), but clearly populations diverge more in cold tolerance than in heat tolerance.

Winter minimum temperatures are more divergent than summer maximum temperatures among the study populations, but southern clade populations do experience higher frequencies of temperatures above 35° C than do northern clade populations (Fig. 4.2). Why, then, is there no evidence for high temperature adaptation? Artificial selection for increased intrinsic rate of increase (a composite fitness index) under chronically stressful high temperatures produced no evolutionary response in *W. smithii* (Armbruster et al. 1999), implying that the evolution of heat tolerance is somewhat constrained. Whether genetic constraint contributes to geographic trends in tolerance across a broad range of taxa is a

largely unanswered question. Natural populations of *Drosophila melanogaster* occasionally

demonstrate increased heat tolerance at lower latitudes (Hoffmann et al. 2002), but *D.*

melanogaster is also one of the only species in which the geography of temperature tolerance

has been explored in any great detail. More empirical studies in a number of insect taxa will

be necessary to address the contribution of genetic constraint to geographic patterns in

temperature tolerance.

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CHAPTER 5

THE EFFECT OF FLUCTUATING TEMPERATURES ON LIFE HISTORY TRAITS: COMPARISONS AMONG GEOGRAPHIC POPULATIONS OF *WYEOMYIA SMITHII*

Abstract

The relationship between phenotype and the mean value of an environmental factor is well studied, but the influence of environmental variation on the phenotype is often overlooked. In a comparative framework, effects of environmental variation may have important practical and evolutionary implications for experimental design and the study of adaptive divergence. Here I compare the effects of temperature variation on life history traits of geographic populations of the pitcher plant mosquito *Wyeomyia smithii*, to test 1) whether temperature variation affects life history traits within populations 2) if there is an effect of temperature variation, whether the magnitude and direction of the effect vary across mean temperatures, and 3) whether among-population differences in temperature variation effects confound population comparisons of mean temperature effects. I address these questions by measuring development time, pupal mass, and survival under both constant and fluctuating temperature conditions. Within populations I infer significant effects of temperature variation on all the measured traits, but the magnitude and direction of these effects depended on mean temperature. I detected no significant interaction between the effects of temperature fluctuation and population of origin, implying that in the range of temperatures examined in this study, population comparisons at constant temperatures accurately reflect population comparisons at fluctuating temperatures. However, the effects of temperature fluctuation

were most variable across populations for the trait (pupal mass) for which thermal reaction norms were most divergent among populations. These results suggest that population responses to temperature variation will tend to vary most with geography when the shapes of thermal reaction norms are highly variable among populations.

Introduction

Most organisms inhabit environments that vary within and across generations. A considerable number of theoretical and empirical studies have investigated the evolution of plasticity and acclimation in response to such environmental variability (*e.g.*, Bell 1997; Bennett and Lenski 1997; Gilchrist 1995; Huey and Kingsolver 1989; Kingsolver and Huey 1998; Levins 1963; Woods and Harrison 2002). The vast majority of these studies, however, focus on the relationship between phenotype and the mean value of an environmental variable, *i.e.*, the familiar form of a norm of reaction. Less well appreciated and less frequently addressed is the relationship between environmental variability and phenotype, *e.g.*, a norm of reaction for trait value across two environments with the same mean but different variance for a given environmental factor. Understanding the phenotypic effects of environmental variation can not only inform adaptive hypotheses, but may also be important to consider when evaluating mean effects.

Temperature is one of the most widely studied and physiologically influential environmental factors. Temperature-dependence of physiology is a fundamental concern in ecological and evolutionary studies of life histories, particularly for ectotherms. The effects of temperature are widely acknowledged and accounted for, but attention is generally focused on mean temperature. Effects of temperature variation are often marginalized or ignored, although the body of literature devoted to phenological models of insect emergence constitutes a notable exception (Hagstrum and Milliken 1991; Worner 1992). Unlike instantaneous rates of growth and development, even simple traits such as body size and development time are influenced by the long-term effects of temperature variation integrated over many underlying physiological processes (Kingsolver et al. 2004). Most terrestrial and

many aquatic ectotherms experience a broad range of developmental temperatures that vary diurnally and seasonally. Moreover, both mean temperature and temperature variation change with geography and often with microhabitat. Consequently, comparisons among geographic populations or among microhabitats that focus solely on mean temperature may ignore important geographic differences in response to temperature variation.

Whether thermal variability affects life history differences among populations is a question with important practical and evolutionary implications. From the perspective of experimental design, if phenotypic responses to temperature variation differ among populations, the choice of fluctuating temperature rearing environments may alter among-population comparisons of mean temperature effects. From an evolutionary perspective, geographic variation in the degree of environmental variability may contribute to geographic clines in temperature-sensitive traits. For example, the amplitude of diurnal and annual temperature fluctuations varies with altitude and latitude (Taylor 1981). Thus, geographic populations arrayed along latitudinal and altitudinal clines will experience different annual mean temperatures and different degrees of temperature variation. Numerous studies have explored population differences in the relationship between life history traits and mean temperature (*e.g.*, Berner et al. 2004; Blanckenhorn and Fairbairn 1995; Bradshaw et al. 2004; Burke et al. 2005; Castaneda et al. 2004; Fischer and Fiedler 2002; Hallas et al. 2002). In addition, a substantial number of studies have examined the effects of temperature variation within populations (*e.g.*, Behrens et al. 1983; Bradshaw 1980; Dallwitz 1984; Davis et al. 2006; Elliott and Kieckhefer 1989; Hagstrum and Milliken 1991; Kieckhefer and Elliott 1989; Lamb 1961; Petavy et al. 2004; Petavy et al. 2001), and at least one study has examined genotype by temperature variation interactions within a single population

(Brakefield and Kesbeke 1997). To our knowledge, however, no single study has directly tested for population differences in response to temperature variation.

Here I test for geographic variation in the effects of fluctuating temperatures among populations of the pitcher plant mosquito, *Wyeomyia smithii*. The three populations included in the study span a gradient in mean temperature and temperature variation in the eastern United States. Survival to, time to, and mass at pupation were measured in several temperature rearing environments. I applied two diurnally fluctuating temperature environments mimicking typical cool and warm diurnal temperature cycles measured in the field. In addition, I applied three constant temperature rearing environments, two equal to the means of the fluctuating environments. Comparison between the constant treatments and fluctuating treatments with the same mean provide a direct, commonly used test for the effects of temperature variation (Beck 1983; Hagstrum and Milliken 1991). I also include data from a relatively low constant rearing treatment with no comparable fluctuating treatment to illustrate the importance of duration of exposure and thermal threshold effects. Putative physiological mechanisms, implications for experimental design, and adaptive divergence among populations are discussed.

Methods

Study organism

The pitcher plant mosquito, *Wyeomyia smithii*, obligately oviposits into the leaves of the purple pitcher plant, *Sarracenia purpurea*. Both plant and mosquito range from the panhandle of Florida north to Newfoundland along the eastern seaboard and into the Great lakes region of North America, covering a broad range of thermal and seasonal habitats (Bradshaw et al. 2000). Larval hibernal diapause, or dormancy, is cued by photoperiod, and

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geographic populations demonstrate a cline in photoperiodic response. Bracketed between diapause termination and initiation, the length of the growing season declines with increasing latitude or altitude (Bradshaw and Lounibos 1977).

In the spring of 2004 I established separate laboratory colonies from collections of approximately 1000 larvae from each of three geographic populations (Table 5.1). Phylogeographic data suggest that FL and NC coast populations cluster together in a southern clade, while the NC Mtn. population falls into a more distantly related northern clade (Armbruster et al. 1998;W. Bradshaw, unpublished data). Compared to the NC coast and FL populations, the NC Mtn. population experiences lower daily mean temperatures for the entire year. All populations experience similarly variable temperature environments on this scale (see Chapter 3; similar standard deviations of the means, Table 5.1.). However, only temperatures experienced during active growth will affect life history traits during non-diapause development (*i.e.*, development that does not initiate from or terminate in a diapause stage). Thus, the thermal environment of the growing season is perhaps the most critical component of direct, temperature-mediated selection on life history traits of actively growing individuals (see Chapters 3 and 4). Using the critical photoperiod of each population to define the growing season as described in Chapter 3, I estimated average daily mean temperature and the standard deviation of this mean for the growing season alone. During the growing season, the NC Mtn. population experiences both the lowest mean temperature and least variable temperature conditions: the standard deviation of the mean is nearly 40% lower than the values for FL and NC Coast (Table 5.1). The FL and NC coast populations experience a similar thermal environment,

Table 5.1. Geographic and temperature data for study populations of *Wyeomyia smithii*. Temperature data include annual average daily mean ($\overline{T}_{ANN}$), standard deviation of the annual average ($\hat{\sigma}_{ANN}$), average daily mean of the growing season ($\overline{T}_{GRW}$), and standard deviation of the growing season average ($\hat{\sigma}_{GRW}$). Temperature units are ° C, and means and standard deviations were calculated from 35 years of weather data obtained from weather stations < 2 km from each site.

Population	Lat./lon.	Altitude (m)	$\overline{T}_{ANN}$	$\hat{\sigma}_{ANN}$	$\overline{T}_{GRW}$	$\hat{\sigma}_{GRW}$
FL	30°N 85°W	10	19.3	12.7	23.0	9.1
NC coast	34°N 78°W	20	17.5	14.4	23.5	9.8
NC Mtn.	35°N 83°W	900	11.2	14.0	18.0	5.9

whereas NC Mtn. experiences a cooler, less variable thermal environment during the growing season.

Field-collected larvae were reared under standard long-day conditions (16:8 LD) and a temperature regime fluctuating between 13 and 29° C. Food consisted of a standard suspension of 4:1 guinea pig chow to freeze-dried brine shrimp (Hard et al. 1992). Pupae were transferred to five gallon mating cages and once eclosion commenced each cage was supplied weekly with a freshly cut pitcher plant leaf for oviposition and a sponge moistened with honey-water for adult nutrition. Eggs were collected every three days and transferred to diapause-inducing conditions (8:16 LD, 20° C constant temperature) until all adults in a cage had died. Once all populations were synchronized in diapause I moved the larvae to standard long day conditions to initiate the next generation. I maintained breeding populations at a minimum of 500 individuals representing a constant proportion from each egg collection.

Constant temperature experiment

On the day of hatch 25 haphazardly selected first instar larvae (= one cohort) from the F_2 laboratory generation of each population were transferred to 150 X 25mm culture dishes with 170ml distilled water. To maintain *ad libitum* food conditions I transferred larvae to a new culture dish each weak, supplying the new dish with a fresh aliquot of 0.05g/ml standard food suspension. Cohorts were started with 1.00 ml food suspension, 1.75, 2.5, and 3.0ml were added for the next three weeks, and 2.5ml every week thereafter to simulate food capture in a pitcher plant (Bradshaw and Holzapfel 1986). Temperature rearing treatments included 16, 20, and 27° C constant. Cohorts were haphazardly assigned to temperature treatments for a total of 6 cohorts in each temperature treatment for each

population. For larvae surviving to pupation I recorded time to pupation, mass at pupation, and sex.

Fluctuating temperature experiment

Methods and results for fluctuating temperatures appear elsewhere (see Chapter 3) and are similar to those for constant temperatures. Briefly, cohorts of 25 larvae originating from the F_3 laboratory generation of each population included in the constant temperature experiment were randomly assigned to one of two temperature rearing treatments, each with equal variance and the same diurnal profile: 1) fluctuation from 16 to 32° C with a mean of 20° C, and 2) fluctuation from 23 to 39° C with a mean of 27° C. Actual temperature-time profiles were designed to mimic a cool summer day most typical of the NC Mtn. population and a hot summer day most typical of the NC coast or FL populations (see Chapter 3). Cohorts were maintained with the same feeding conditions described for the constant temperature experiment.

Statistical Analyses

In the constant temperature experiment survival was scored as either successful pupation (1) or failure to pupate (2), and these data were analyzed using mixed model logistic regression (implemented in SAS version 9.1, Proc Glimmix [SAS Institute 2004]) including cohort as a random effect and temperature and population as fixed effects. Time to pupation and mass at pupation scored for those individuals surviving to pupation were analyzed via separate mixed model ANOVAs (SAS Proc Mixed) with cohort as a random effect and population, temperature, and sex as fixed effects. Time to pupation was natural log-transformed to improve normality, while pupal mass was transformed as Ln(mass +1) to prevent negative values. Sex was excluded from the survival analysis because I could not

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sex individuals before pupation and was included in the other analyses because sex has a large influence on pupal mass and development time. Post-hoc pairwise comparisons were performed using linear contrasts and associated F statistics.

To examine constant vs. fluctuating temperature effects I compiled a data set that included all data from the fluctuating temperature experiment for the NC Mtn., NC coast, and FL populations and data from the 20 and 27° C temperature treatments from the constant temperature experiment. Survival, time to pupation, and mass at pupation were analyzed as above with the addition of a fixed effect for temperature fluctuation (constant or fluctuating). Since constant and fluctuating temperature experiments were performed at different times and on different generations, there was a potentially confounding temporal effect. However, temporal block effects were likely minimal because the same (powdered) stock food formulation was used in the same dilutions prepared in an identical manner for both experiments, experimental chambers held temperature at ± 0.1° C precision, and inbreeding in the lab stock colonies was minimized by maintaining large breeding populations. In addition, a temporal block effect would affect all populations equally, so it would not bias estimates of an interaction between temperature variation effects and population of origin.

Results

Constant temperatures

Survival to pupation was relatively high at 20 and 27° C rearing temperatures and declined precipitously at 16° C for all populations (Figure 5.1). Population, temperature, and their interaction significantly influenced survival to pupation (Table 5.2). At 27° C FL and NC Coast larvae successfully pupated at a significantly higher average (across populations) proportion compared to NC Mtn. larvae ($F_{1,45} = 22.78$, $p < 0.001$), whereas at 16° C FL

Figure 5.1. Mean ± SE percent survival across temperatures for each geographic population.
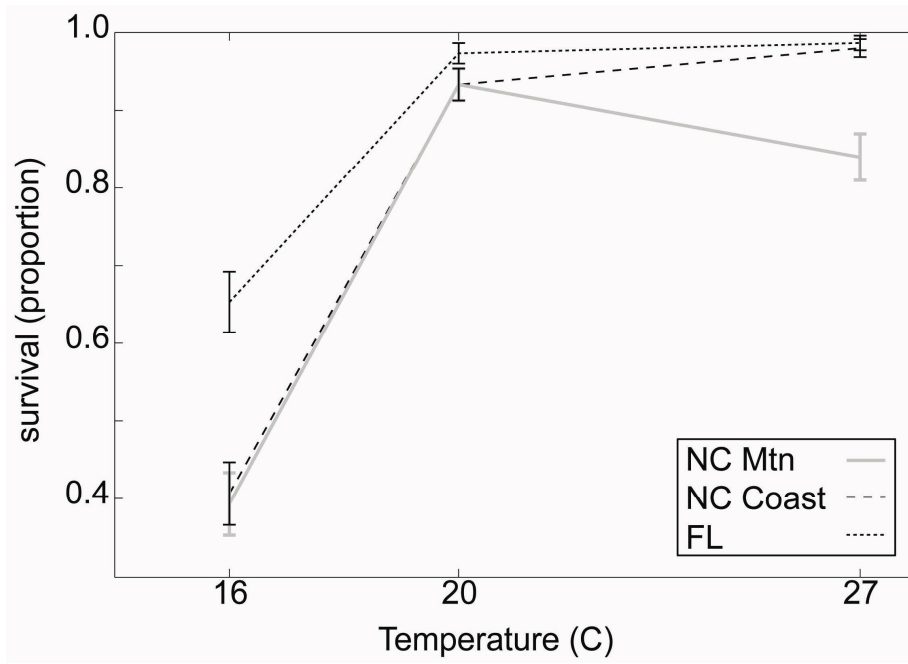
Table 5.2: Mixed model ANOVA table for analysis of constant temperatures.

Trait	Effect	DF	F-Value	p-value
Survival	$Temp_{mn}$	2, 45	112.29	< 0.001
	Pop	2, 45	12.74	< 0.001
	Pop*$Temp_{mn}$	4, 45	3.41	0.0161
Development time	$Temp_{mn}$	2, 45	1115.80	< 0.001
	Pop	2, 45	0.73	0.490
	Sex	1, 45	218.51	< 0.001
	Pop*$Temp_{mn}$	4, 45	5.23	0.002
	Sex*$Temp_{mn}$	2, 45	5.87	0.005
Pupal mass	$Temp_{mn}$	2, 45	351.02	< 0.001
	Pop	2, 45	3.47	0.0390
	Sex	1, 45	1670.83	< 0.001
	Pop*$Temp_{mn}$	4, 45	7.70	< 0.001
	Pop*Sex	2, 45	19.05	< 0.001
	Sex*$Temp_{mn}$	2, 45	3.69	0.0320

larvae survived to pupation at a higher proportion than the average proportion of NC Coast and NC Mtn. larvae ($F_{1,45}$ = 24.93, p < 0.001). No differences between populations were significant at 20° C. Larvae from all populations suffered about a 40 - 60% decrease in survival at 16° C compared to that at 20 or 27° C (Figure 5.1). All response variables measured on pupae are conditioned on these survival responses. Particularly at 16° C, development time and pupal mass reflect the surviving subset of the total number of individuals included at the outset of the experiment.

Increasing temperature led to decreasing development time and males developed faster than females at all temperatures (Fig. 5.2a,b). Mixed model ANOVA revealed significant main effects of population, temperature, and sex (Table 5.2). Population by temperature and sex by temperature interactions were significant (Table 5.2), indicating differences between sexes and populations in the temperature-development time relationship. All populations developed at similar rates (no significant pairwise differences) at 16 and 20° C, but at 27° C females and males from NC Mtn. develop more slowly than the average value of NC coast and FL (averaged across sexes: $F_{1,45}$ = 19.24, p < 0.001).

Females attained a larger mass at pupation than males, and all populations followed the temperature-size rule typical of most insects, decreasing in mass with increasing temperature in both sexes (Fig. 5.2c,d). There were significant effects of population, temperature, and sex (Table 5.2). Trends in the mass-temperature relationship were complex, with no consistent patterns among populations, sexes, or temperatures; the interaction effects population by sex, population by temperature, and sex by temperature were all significant (Table 5.2).

Figure 5.2. Mean ± SE development time (a,b) and pupal mass (c,d) for females (a,c) and males (b,d) at 20 and 27 C constant.
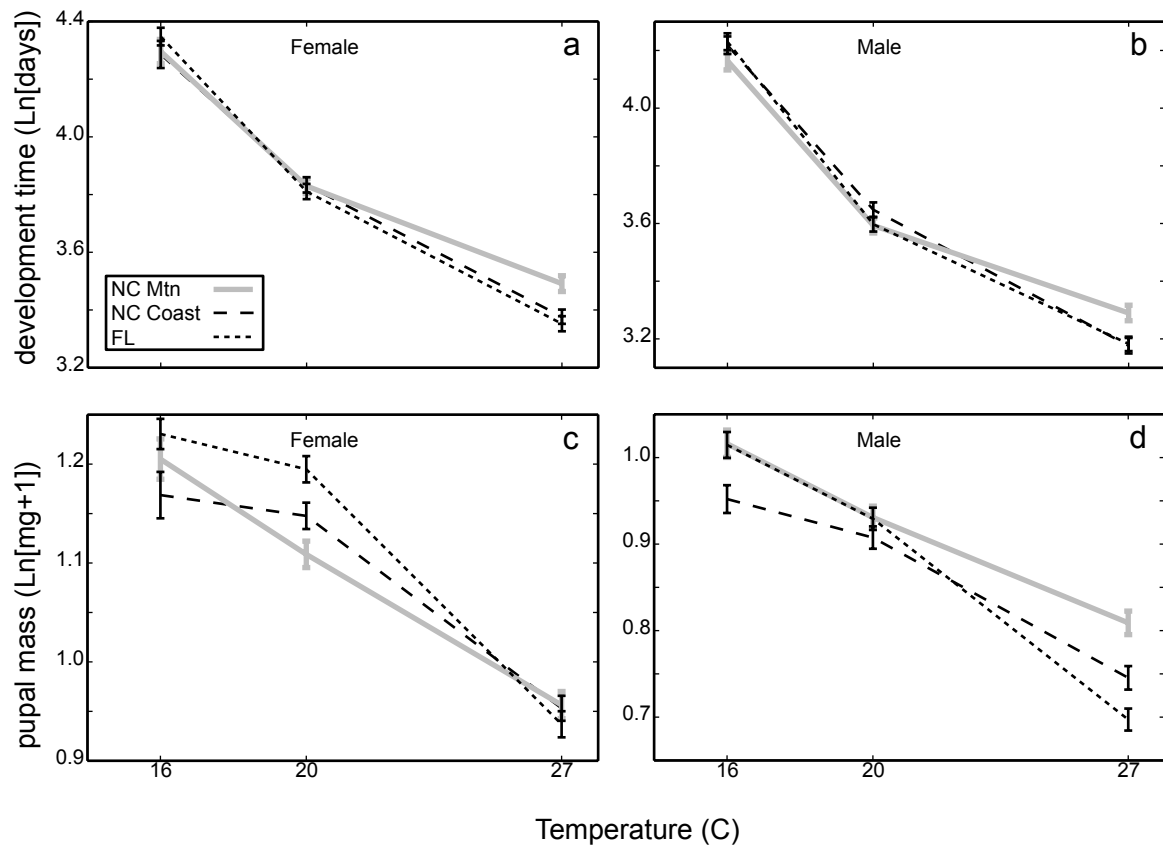
Constant vs. fluctuating temperatures

Temperature fluctuation had no detectable effect on survival (Table 5.3), and no interaction terms including temperature fluctuation in the ANOVA model were significant (excluded from model via AIC). Thus, when reared at the same mean temperature larval survival was similar in constant and fluctuating temperature conditions. Population effects were significant (Table 5.3), and in this model the differences between populations were the same as reported at 20 and 27° C in the analysis of constant temperatures alone (see previous section).

As in the analysis of constant temperatures, population, mean temperature, sex, and population by temperature interactions significantly affected development time (Table 5.3). Temperature fluctuation, the factor of primary interest, was significant as a main effect and also interacted with mean temperature (Table 5.3). No interaction terms including sex were significant, indicating that development time in males and females was similarly effected by temperature mean and fluctuation. Figure 5.3a,b plots the difference between development time at constant and fluctuating rearing temperatures (constant – fluctuating). Differences between fluctuating and constant temperatures were non-significant (not different from zero) at $T_{ave} = 20°$ C in both sexes and for all populations. At $T_{ave} = 27°$ C, however, development time was significantly shorter at the constant rearing treatment in both sexes and for all populations (Fig. 5.3a,b; corrections for multiple comparisons did not alter statistical significance). Temperature fluctuation by population interactions were non-significant in the ANOVA, suggesting that fluctuating temperatures had equivalent effects on all populations.

Figure 5.3. Difference between constant temperatures and fluctuating temperatures with a comparable mean (constant - fluctuating) for development time (a,b) and pupal mass (c,d) of females (a,c) and males (b,d). Presence of a symbol indicates a value significantly different from zero at the p < 0.001 (*) or p < 0.01 (●) level.
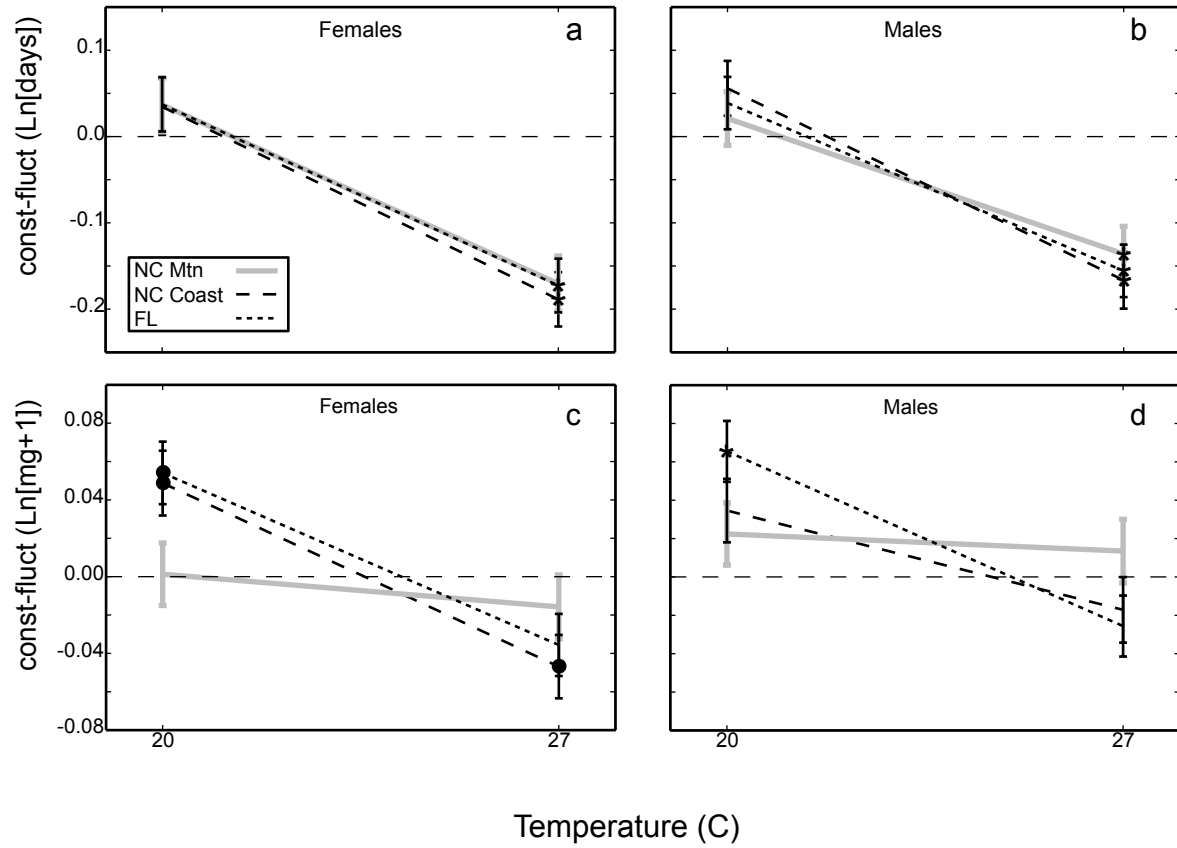
Table 5.3: Mixed model ANOVA table for analysis of constant and fluctuating temperatures. 'Temp$_{mn}$" and Temp$_{fl}$" are the effect of mean temperature and temperature variation, respectively.

Trait	Effect	DF	F-Value	p-value
Survival	Temp$_{mn}$	1,99	0.58	0.447
	Temp$_{fl}$	1,99	0.74	0.393
	Pop	2,99	5.20	0.007
Development time	Temp$_{mn}$	1,96	834.98	< 0.001
	Temp$_{fl}$	1,96	36.08	< 0.001
	Pop	2,96	14.28	< 0.001
	Sex	1,96	1009.12	< 0.001
	Temp$_{mn}$* Temp$_{fl}$	1,96	90.55	< 0.001
	Pop* Temp$_{mn}$	2,96	13.29	< 0.001
Pupal mass	Temp$_{mn}$	1,96	688.55	< 0.001
	Temp$_{fl}$	1,96	1.83	0.179
	Pop	2,96	3.55	0.033
	Sex	1,96	4564.89	< 0.001
	Temp$_{mn}$* Temp$_{fl}$	1,96	23.94	< 0.001
	Temp$_{mn}$* Pop	2,96	10.73	< 0.001
	Temp$_{fl}$ *Sex	1,96	4.85	0.030
	Pop*Sex	2,96	57.62	< 0.001

Population, mean temperature, and sex also significantly affected pupal mass, as did population by sex and mean temperature by population interactions (Table 5.3). In contrast to the results for development time, temperature fluctuation was not a significant main effect in the ANOVA but did significantly interact with mean temperature and sex. Both female and male pupae from FL and NC coast were generally larger at constant temperatures compared to fluctuating at 20° C mean temperature, whereas this relationship reversed at 27° C mean temperature (Fig. 5.3c,d). After correcting for multiple comparisons only the difference between constant and fluctuating temperatures for FL males at 20° C remained significantly different from zero, whereas all other differences that were formerly significant became marginally non-significant. Arguments about the relative merits of multiple comparisons aside, the general trends for the FL and NC coast populations are qualitatively similar (FL and NC Coast are also the most phylogenetically similar populations), and these trends are consistent across sexes. In contrast, pupa from NC Mtn. were roughly the same size at constant and fluctuating temperatures in both sexes and at both mean temperatures. These post-hoc comparisons suggest that interactions between mean temperature and temperature fluctuation vary across populations, but as with development time, ANOVA revealed no significant interactions including both population and temperature fluctuation.

Discussion

Temperature variation effects

In comparison to results from an analysis of fluctuating temperatures, universally low survival to pupation at 16° C constant implies a strong effect of duration of exposure. Larvae reared at temperatures fluctuating from 15.5 to 32° C and hovering at or below 16.5° C for more than 7 hours at night show 90% survival rates (fluctuating temperature regime about

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20° C mean; survival data not shown), while larvae reared at 16° C constant show 40 - 60%

survival (Fig. 5.1). Clearly 16° C is not an acutely stressful temperature for *W. smithii*, or

even chronically stressful on the scale of a diurnal temperature cycle. However, it appears

that long-term chronic exposure to this temperature is stressful enough to cause high

mortality. The lack of diurnal temperature fluctuation is not the sole factor responsible for

this result, as survivorship at means of 20 and 27° C was comparable between constant and

fluctuating temperatures (Table 5.3). Thus, an interaction between mean temperature and

temperature variation must have contributed to the observed levels of mortality.

Since 16° C is not acutely stressful, high mortality at 16° C suggests a physiological

mechanism that involves the thermal dependency of growth and development. Development

rate is determined by many underlying physiological processes that often vary in thermal

sensitivity and thermal thresholds below or above which these processes are strongly

inhibited (Beck 1983). Rearing at a constant temperature that surpasses a thermal threshold

of any underlying physiological process can thus result in developmental stagnation, and

eventually mortality (Beck 1983; Howe 1967; Lin et al. 1954), or partial mortality if there is

population variation for thermal thresholds that overlaps the constant rearing temperature.

Development time is greatly increased at 16° C compared to 20° C (Fig. 5.2a,b), indicating

that 16° C approaches the lower thermal threshold for development as measured at constant

temperatures. Moreover, I detected no significant effects of temperature fluctuation on

survival at 20 and 27° C mean temperature for any population, a trend also consistent with

thermal threshold effects that manifest only at lower temperatures. Davis *et al.* (2006) found

a similar pattern in green peach aphids in which survival was markedly lower in constant

compared to fluctuating conditions at a low mean temperature (15° C), but comparable to

constant conditions at intermediate mean temperatures (20 – 30° C). Collectively, these

results suggest that mean temperature by temperature fluctuation interactions for survival

may be driven by thermal threshold effects on development.

Differences in development time between constant and fluctuating temperatures agree

well with predictions based on the nonlinear relationship between development rate and

temperature. A wealth of empirical data from ectotherms, particularly insects, suggest that

development rate curvilinearly and asymmetrically declines on either side of a temperature

maximum such that the decline is more rapid at high compared to low temperatures (Sharpe

and DeMichele 1977; Fig. 5.4). Development time is the inverse of average development

rate, so rapid development rates translate into short development times. The development

rate-temperature curve predicts that where the curve is concave down, or decelerating (at

higher temperatures), development time will be shorter (faster average rate) at constant

temperatures compared to fluctuating temperatures about the same mean (Fig. 5.4, square

symbols). Where the curve is concave up, or accelerating (at lower temperatures), the

reverse will be true (Fig. 5.4, circle symbols). This property of the non-linear development

rate function, termed Jensen's inequality or the Kaufmann effect (Ruel and Ayres 1999;

Worner 1992), also predicts that the magnitude of the difference in development rate between

constant and fluctuating temperatures will be greater at higher rearing temperatures because

curvature at these temperatures is more extreme (Fig. 5.4, difference between open and

closed squares compared to difference between open and closed circles). Closely following

these predictions and consistent across populations and sexes, our data show that

development time is shorter at constant compared to fluctuating temperatures at a mean of

27° C , while the reverse is true a mean of 20° C (Fig. 5.3a,b). The difference between

Figure 5.4. Hypothetical function relating development rate to temperature. The equation:
$$d(T) = C \exp[-\alpha (T - d_{max}) - \exp[\beta (T - d_{max}) - 8]] + b$$
approximates the Sharpe-Schoolfield equation, modeling development rate as a function of temperature with an asymmetric decline from an intermediate maximum d_{max} (modified from Frazier et al. (2006)). The y-axis scale is arbitrary, dependant upon the constant C, T is temperature, α and β determine the steepness and symmetry of the decline from the optimum, and b is the y -intercept. For the function depicted here $\alpha = 0.008$, $\beta = 1$, and $b = -1.0 \times 10^{-4}$. Using this function to model development time at the mean rearing temperatures used in current experiment, the symbols represent the predicted values of average daily development rate for the constant (closed symbols) and fluctuating (open symbols) thermal profiles applied at daily means of 20 (circles) and 27° C (squares).
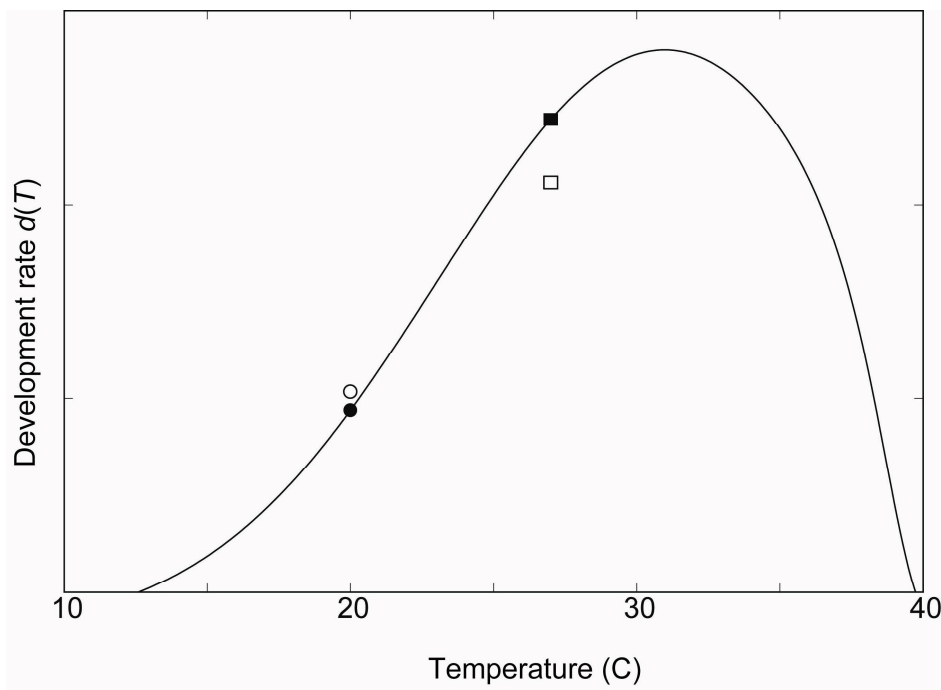
constant and fluctuating is lesser in magnitude and not significantly different from zero at a mean of 20° C, again consistent with predictions based on the less extreme curvature of the development time response curve at lower temperatures (Fig. 5.4).

In addition to adhering closely to theoretical predictions, our development time data also align well with results from other insects. In a survey of development time at constant compared to fluctuating temperatures for 17 species, Hagstrum and Milliken (1991) found that development time at constant temperatures was typically shorter above means of 25 – 30° C and longer at lower mean temperatures. This consistency of results across species implies that the approximate temperature ranges in which the temperature-development rate function is accelerating and decelerating are evolutionarily conserved.

Because the relationship between mass at maturity and temperature is the result of the interaction between growth rate and differentiation rate (Van Der Have and De Jong 1996), the shape of the mass-temperature relationship is often more linear than that for development time (*e.g.*, Gibert and De Jong 2001) and is likely more variable across species. Peteavy *et al.* (2001) found that across a range of mean temperatures from 12 to 32° C, *Drosophila melanogaster* adult body size was always smaller in fluctuating compared to constant temperature treatments. They suggested that these differences may have been driven by stress responses to extreme temperatures in the fluctuating treatments, but the observed trends are also predicted by the effects of nonlinearity (concave down across the entire range of measured temperatures). The significant mean temperature by temperature fluctuation interaction that I find in *W. smithii* suggests that the 'template' shape of the thermal reaction norm may be somewhat different than that observed across a similar range of temperatures in *Drosophila melanogaster*.

Geographic variation

For both development time and pupal mass, differences between constant and fluctuating temperatures were statistically indistinguishable among populations, suggesting that similar physiological process contribute to these differences across populations. However, the effects of temperature fluctuation appeared to be more consistent across populations for development time (Fig. 5.3a,b) than for pupal mass (Fig. 5.3c,d). Based on the landmark temperatures measured in this study and in a separate analysis of fluctuating temperatures (Chapter 3), the slope of the thermal reaction norm for development time appears to be much less variable among populations than that for pupal mass. Moreover, estimates of the difference between constant and fluctuating temperatures for pupal mass were most similar across mean temperatures and closest to zero (Fig. 5.3c,d) for the population with the flattest thermal reaction norm for mass (NC Mtn; Fig. 5.2c,d). With the statistical power available in our study, results suggest that comparisons between populations at constant temperatures do reflect differences in more natural thermal conditions. In fact, the rank order and pairwise differences among populations across constant temperatures for both pupal mass and development time are largely the same as those measured across fluctuating temperatures with the same mean (Chapter 3). That the effects of temperature variation appeared to be less consistent across populations for pupal mass than for development time suggests, however, that the effects of temperature fluctuation may vary most among population when the shapes of thermal reaction norms are most divergent.

Differences in thermal habitat among geographic populations of *W. smithii* appear to have driven divergence in response to mean temperature, but I did not detect population differences in response to temperature variation. As shown by the mean and standard

deviation values in Table 5.1, the FL and NC coast populations experience both a warmer and a more variable thermal habitat during the growing season than NC Mtn. integrated across the entire growing season. Southern clade populations (*e.g.*, FL and NC coast) exhibit increased yearlong replacement rate (Bradshaw et al. 2004) and decreased development time at high temperatures (Chapter 3; Fig. 5.2a,b) compared to northern clade populations, while northern clade populations exhibit enhanced survival of cold winter temperatures (Bradshaw et al. 2004) and increased fecundity (Chapter 3) at low temperatures. However, our data indicate no detectable concomitant changes in response to temperature variation, suggesting that physiological responses to temperature variation are relatively stable across geography, at least across the range of relatively benign environments used in this study.

Geographic differences in the effects of temperature variation are most likely to arise in thermal environments in which either thermal thresholds for any underlying developmental processes or thresholds for stress response induction are regularly surpassed. Applying thermal environments with extreme mean temperature or increasing the amplitude of diurnal temperature fluctuations increases the magnitude of temperature variation effects (Hagstrum and Milliken 1991; Petavy et al. 2001). Our results suggest that when thermal environments are relatively benign, population comparisons at constant and fluctuating temperatures lead to the same conclusions, but if there is among-population variation in thermal thresholds, differences in the effects of temperature variation will become increasingly apparent the more frequently temperatures beyond these thresholds are experienced. Caution should be taken when extrapolating population differences at relatively extreme constant temperatures to population differences in real, variable field conditions.

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CHAPTER 6

CONCLUSION

My results illustrate how the timing of life history stages or events critically determines selection on and evolution of those stages or events in seasonal environments. When environmental sensitivity can evolve independently across life history stages, selection should optimize environmental sensitivity of each stage for the environmental conditions experienced. Alternatively, high correlation of environmental sensitivity across life history stages should constrain adaptation to seasonal environments, since each life history stage cannot be independently optimized for the environment that it experiences. The interplay between selection on seasonal timing and the genetic architecture of environmental sensitivity will thus determine the course of life history evolution within a population and geographic variation among populations.

Mechanisms of selection on seasonality and environmental sensitivity remain unclear. Adaptation to periodically stressful environments (*e.g.*, cold winter temperatures) requires one of two possible strategies: deal with the stressor head-on without significantly changing life history schedules of growth and reproduction, or get out of the way. The former strategy is generally associated with physiological, morphological, or behavioral plasticity that allows growth and reproduction to continue, although perhaps not unabated. The latter strategy involves either migration or dormancy. Both strategies are viable solutions to the problems presented by stressful conditions, so in any given situation, why does one strategy evolve,

and not the other? If selection ultimately determines which strategy will evolve, there must

be some fundamental difference between the fitness costs/benefits of altering seasonal timing

and the costs/benefits of altering environmental fitness optima, tolerance range, etc. This is a

difficult question to rigorously address either empirically or theoretically. The ecological

dimensionality quickly becomes prohibitively large for any biologically realistic scenario that

is also sufficiently general. Intuitively, there is no apparent reason why selection would

generally favor one strategy over the other.

The role of constraint in determining the evolution of seasonal timing and

environmental sensitivity provides a more experimentally tractable and plausible explanation

for observed patterns of life history evolution. As discussed in chapter one, thermal

sensitivity of development appears to be relatively free to evolve, but this lability will

eventually be limited by physical, if not genetic constraints. For example, maintaining active

metabolism at sub-zero temperatures is nearly impossible, and likely represents an absolute

constraint on temperature conditions that are favorable for growth. In many cases, seasonal

timing of dormancy (and probably migration as well) may simply evolve to produce the

longest possible growing season given the absolute limits on performance at extreme

temperatures. Empirical tests of this hypothesis would be very feasible and would greatly

inform both evolutionary model of life history evolution and phenological models frequently

used in applied entomology and agronomy.

There are some clear examples of seasonal timing that does not evolve to the limits of

tolerance to abiotic factors, but these examples are typically limited to situations in which

there are seasonal changes between presence/absence of some critical resource. This is

common for herbivores with very specific host plant preferences, where the seasonal timing

of the herbivore is necessarily tied to the emergence of the plant. The degree of specialism, therefore, may actually be related to whether or not seasonal timing evolve to extend the growing season to an organism's physiological limits. Specialism may also be related to the evolution of tolerance to extreme environments. For example, an herbivore that obligately feeds on a host plant that emerges for only a few weeks in midsummer may have very low tolerance for cold temperatures when not in a diapausing or migratory life history stage. Ideally comparative, interspecific studies could address these questions, but there is also a wealth of data available on the seasonal phenology of insects, particularly crop pests. Linking these data with information on resource preferences and thermal physiology could prove informative.

Global climate change affects both changes in environmental mean values (rainfall, temperature) and the magnitude and timing of environmental fluctuations. Thus, the evolvability of both environmental sensitivity and seasonal timing may play an important role in determining how organisms respond to the changing environment. Understanding the selective and genetic factors that have shaped current life history strategies in seasonal environments will therefore have clear implications for projections of future ecosystem change and policies for management and preservation of biodiversity.

Appendix I:

SUPPLEMENTARY MATERIAL FOR CHAPTER 2

List of references, including full species names, as indexed in Table 2.1.

index	full species name	reference
1	*Chrysopa oculata*	Nechols et al, 1987
2	*Chrysopa oculata*	Tauber and Tauber, 1987
3	*Chrysopa carnea*	Tauber and Tauber, 1982
4	*Leptinotarsa decemlineata*	Tauber et al., 1988a
5	*Leptinotarsa decemlineata*	Tauber et al., 1988b
6	*Leptinotarsa decemlineata*	Hilbeck and Kennedy, 1998
7	*Hyphantrea cunea*	Gomi et al., 2003
8	*Hyphantrea cunea*	Gomi and Takeda, 1996
9	*Ostrinia nubilalis*	Calvin et al., 1991
10	*Ostrinia nubilalis*	Beck and Apple, 1961

Geographic locations of weather stations used in the analysis of latitudinal clines in thermal conditions.

Location	Latitude (°)	Altitude (m)
Pensacola, FL	30.5	34.1
Milton, FL	30.8	66.1
Bay Minette, AL	30.9	82.6
Alma, GA	31.5	58.8
Charleston, SC	32.9	12.2
Atlanta, GA	33.6	307.8
Columbia, SC	33.9	64.9
Wilmington, NC	34.3	10.1
Highlands, NC	35.1	1158.8
Raleigh, NC	35.9	126.8
Norfolk, VA	36.9	9.1
Richmond, VA	37.5	50.0
Glenn Dale, MD	39.0	45.7
Indian Mills, NJ	39.8	30.5
Philadelphia, PA	39.9	3.0
LaGuardia, NY	40.8	3.4
Providence, RI	41.7	45.5
Ithaca, NY	42.5	292.6
Birch Hill, MA	42.6	263.3
Portland, ME	43.7	13.7
Millinocket, ME	45.7	109.7

Regression of thermal conditions on latitude and altitude

The linear model for each regression:

$$y = \mu + \alpha + \beta + \varepsilon$$

where μ is the intercept, α and β are the regression coefficients for latitude and altitude, respectively, ε is the random error term, and y is either average day of last frost (number of days after January 1 until the last day with a minimum temperature below -1° C), average minimum temperature over the 50 days following the last frost, or average degree-day accumulation (sum of daily average temperatures) over those same 50 days. Adding an additional term for longitude did not significantly improve the fit of any of the models.

Coefficients of determination and estimated model parameters

Dependent Var.	Parameter	Estimate	p-value	r^2 of model
Last Frost	Intercept	-104.7	< 0.001	0.87
	Latitude	4.9	< 0.001	
	Altitude	0.030	0.003	
Average Min.	Intercept	14.2	< 0.001	0.42
	Latitude	-0.17	0.002	
	Altitude	0.00019	0.820	
Cumm. Deg-days	Intercept	1037.8	< 0.001	0.21
	Latitude	-8.6	0.043	
	Altitude	0.0026	0.972	

Since altitude significantly influenced the Dependent variable in at least one model, I calculated altitude-corrected latitude for each dependent variable as:

Alt.-corrected latitude = Latitude + (Altitude * β)/α

These corrected values appear as the x-axes in Figure 2.3.