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EVOLUTION IN A CONSTANT ENVIRONMENT: THERMAL FLUCTUATIONS AND THERMAL SENSITIVITY OF LABORATORY AND FIELD POPULATIONS OF *MANDUCA SEXTA*

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Adaptation to temporal variation in environmental conditions is widespread. Whether evolution in a constant environment alters adaptation to temporal variation is relatively unexplored. We examine how constant and diurnally fluctuating temperature conditions affect life-history traits in two populations of the tobacco hornworm, *Manduca sexta*: a field population that routinely experiences fluctuating temperatures; and a laboratory population (derived from this field population in the 1960s) maintained at a constant temperature for more than 250 generations. Our experiments demonstrate that diurnal fluctuations significantly alter body size and development time in both populations, and confirm that these populations differ in their responses to a mean temperature. However, we found no evidence for population divergence in responses to diurnal temperature fluctuations. We suggest that mean and extreme temperatures may act as more potent selective forces on thermal reaction norms than temperature variation per se.

KEY WORDS: **Fluctuating environments, rapid evolution, temperature adaptation, thermal reaction norms.**

In most natural environments, thermal conditions vary at multiple time scales. How organisms adapt to thermal environmental variation has been explored both theoretically and empirically (Lynch and Gabriel 1987; Huey and Kingsolver 1989; Gilchrist 1995). Theoretical models predict and comparative analyses suggest that taxa experiencing greater thermal environmental variation have relatively broader thermal niches (Huey and Bennett 1987, 1990; Huey and Kingsolver 1989). Long-term experiments with *Escherichia coli* show that evolution in an alternating temperature regime can improve fitness at both the low and high component temperatures (Leroi et al. 1994; Bennett and Lenski 1996). Conversely, laboratory experiments indicate that evolution in a constant thermal environment can reduce fitness at extreme low or high temperatures (Cooper et al. 2001; Krebs et al. 2001; Kingsolver and Nagle 2007). Whether evolution in a constant thermal environment can alter adaptation to temporal variation in thermal environments is relatively unexplored.

For terrestrial ectotherms, diurnal fluctuations in body temperature are ubiquitous in natural environments. Many insect studies document differences in growth and development rates between constant and diurnally fluctuating temperature regimes with the same mean temperature (Worner 1992). These differences can result both from the nonlinear effects of temperature, and from evolutionary adaptation to thermal variation (Ragland and Kingsolver 2008).

Here we explore whether evolution in a constant thermal environment may affect performance in diurnally fluctuating conditions, using the tobacco hornworm, *Manduca sexta*, as a model system. We compare growth and development in a field population

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of *M. sexta* that routinely experiences a wide range of thermal conditions, with a laboratory population that has been maintained at a constant temperature for more than 250 generations. A previous study showed that this laboratory population has significantly reduced tolerance when exposed to constant high temperatures (35°C) (Kingsolver and Nagle 2007). Our results confirm differences in thermal sensitivity between the field and laboratory populations in response to mean temperature, but not in response to diurnal fluctuations per se.

Materials and Methods

Our studies used *M. sexta* from two sources. The Field population was established from eggs collected in tobacco fields at the North Carolina (NC) State University Research Station in Clayton NC (Sampson County). This region was the original field source of the first large-scale rearing facility for *M. sexta* (the Yamamoto strain), established in Raleigh, NC at North Carolina State University in the 1960s (Yamamoto 1974; Yamamoto and Fraenkel 1960; Yamamoto et al. 1969). Two USDA facilities in Fargo ND and Beltsville MD were later established using material from the NC State colony. To our knowledge, all current major scientific laboratory colonies of *M. sexta*—including the one used in the present study—are ultimately derived, directly or indirectly, from the NC colony (Kingsolver 2007). The Laboratory population in our study was taken from a laboratory colony maintained under standard larval rearing conditions (artificial diet, constant 25˚C, 15L:9D photocycle) by L. Gilbert and colleagues at the University of North Carolina (UNC) for over 25 years. Under standard *Manduca* rearing conditions, this represents more than 250 generations in laboratory conditions since the colonies were first established from the field.

Within its geographical range in the southeast and southwest of North America, *M. sexta* larvae typically experience a wide range of environmental and body temperatures during growth and development, including maximal diurnal temperatures exceeding 35˚C during summer and subfreezing temperatures during winter (Casey 1976). In the southwest US, mean caterpillar body temperatures can range diurnally from 17°C to 40°C in the summer (Casey 1976). Temperature logger data for physical models of caterpillars in tobacco fields in piedmont North Carolina suggest that diurnal fluctuations in caterpillar body temperature exceeding 20°C are not uncommon during the summer months (Kingsolver 2000; J. G. Kingsolver, unpubl. data). A series of studies by Stamp and colleagues in the 1980s and 1990s with *M. sexta* (from a laboratory colony) demonstrated differences in growth and development time between constant and fluctuating temperatures, indicating the nonlinear effects of temperature on growth and its interaction with plant host quality (Stamp 1990, 1993, 1994; Stamp and Bowers 1990; Stamp and Horwath 1992).

The present experiments were initiated with newly hatched first-instar larvae during 2005–2006, and larvae were reared in individual petri dishes. Larvae were reared in environmental chambers (Percival model 36-VL, Percival Scientific, Perry, IA) on standard *Manduca* diet (Riddiford 1967) with the addition of small amount of dried tobacco leaf (8.3% by dry weight of diet) (Kingsolver 2007). Field-collected eggs were reared through one generation in the laboratory under standard conditions (25°C, 16hL:8hD, artificial diet) prior to use in the experiments, to reduce potential effects of parental environment (Gilchrist and Huey 2001).

At hatching, 25–30 caterpillars from each population were assigned randomly to one of four treatment groups, combining a Constant Temperature treatment (25°C or 30°C), and a Fluctuating Temperature treatment (30°C:15°C on a 16 h:8 h thermoperiod and photoperiod, or 35°C:20°C on a 16 h:8 h thermoperiod and photoperiod). Note that the 25°C and 30°C:15°C treatments experience a mean temperature of 25°C, whereas the 30°C and 35°C:20°C treatments experience a mean temperature of 30°C. Thus we can distinguish the effects of mean temperature (25°C or 30°C) from those of the type of diurnal temperature regime (Constant or Fluctuating) (Ragland and Kingsolver 2008). The growth trajectories of 20–30 individuals for each population and treatment were determined by recording body mass and age (from hatching) at the start of each larval instar, wandering, pupation, and adult eclosion. Following wandering, larvae were placed individually in plastic cups with soil at room temperature (∼25°C) until eclosion. Here we report results for mass and development time at pupation; qualitatively similar results are found for wandering and adult stages. We only report results for individuals that survived to adulthood; survival at these temperatures is typically quite high (>80%) (Kingsolver and Nagle 2007).

Fixed-effects analysis of variance (ANOVA) on pupal mass and on pupal development time were conducted, with sex, population (Field or Laboratory), mean temperature (25°C or 30°C), temperature type (Constant or Fluctuating), and their interactions (excluding interactions with sex) as fixed effects in the model using R. Significant interactions between population and mean temperature are interpreted as population differences in mean thermal responses; interactions between population and temperature type would indicate population differences in response to diurnal temperature regimes.

Results

There were significant effects of sex and population on pupal mass (Table 1), confirming the greater mean size of females compared to males and of the Laboratory compared to the Field population (Kingsolver 2007) (Fig. 1). Mean temperature and temperature type also had significant effects on pupal mass (Fig. 1). Mean

Table 1. ANOVAs for time to pupation and pupal mass of *M. sexta*.

		df	*Mean Sq*	*F*	*P*>*F*
Time to pupation	Population	1	176.967	31.378	<0.0001
	Mean temperature	1	1436.126	254.636	<0.0001
	Temperature type	1	14.249	2.527	0.1139
	Sex	1	77.160	13.681	0.0003
	Population × Mean temperature	1	238.089	42.215	<0.0001
	Population × Temperature type	1	0.034	0.006	0.9380
	Mean temperature × Temperature type	1	79.165	14.037	0.0002
	Population × Mean temperature × Temperature type	1	16.294	2.889	0.0911
	Error	165	5.640		
Pupal mass	Population	1	178,987,291	390.278	<0.0001
	Mean temperature	1	28,905,388	63.028	<0.0001
	Temperature type	1	3,050,781	6.652	0.0108
	Sex	1	6,207,476	13.535	0.0003
	Population × Mean temperature	1	568,790	1.240	0.2670
	Population × Temperature type	1	39,314	0.086	0.7701
	Mean temperature × Temperature type	1	333,569	0.727	0.3950
	Population × Mean temperature × Temperature type	1	254,046	0.554	0.4578
	Error	165	458,615		

pupal mass was greater at a mean temperature of 25°C than 30°C, reflecting the temperature-size rule found in *Manduca* (Davidowitz et al. 2004; Davidowitz and Nijhout 2004) and in many other insects (Atkinson 1994). Mean pupal mass was greater in constant than in diurnal fluctuating rearing conditions, although the magnitude of the effect was modest (Fig. 1). There were no significant interaction terms involving population in the model, suggesting that the populations did not differ significantly in their responses of pupal mass to mean temperature or diurnal temperature regime (Table 1).

Development time to pupation was significantly influenced by sex, population, and mean temperature (Table 1). There were also significant interactions between mean temperature and temperature type, and between mean temperature and population. Mean development times were longer for the Field than the Laboratory population at a mean temperature at 25°C, but these differences disappeared at a mean temperature of 30°C (Fig. 2). As indicated by the mean temperature by temperature type interaction, the effects of diurnal fluctuation depended on mean temperature: fluctuating temperatures shortened mean development times at a mean temperature of 25°C (especially in males), but this effect was eliminated or reversed at a mean temperature of 30°C (Fig. 2). Increasing mean temperature caused a greater decline in development time in the Field than in the Laboratory population, as indicated by the significant interaction between population and mean temperature (Fig. 2). However, there were no significant interactions between population and temperature type, suggesting that the populations did not differ significantly in their responses to diurnal temperature regime (Table 1). Post-hoc analyses of development time for each mean temperature separately showed significant effects of temperature type at each mean temperature, and of population at a mean temperature of 25°C, but at 30°C there was no significant interaction between population and temperature type at either mean temperature. These analyses further support the inference that diurnal temperature fluctuations affected mean development time, and that populations did not differ in their responses to such fluctuations.

Discussion

A previous study with these *M. sexta* populations revealed significantly reduced tolerance to high constant rearing temperatures (35°C) in the Laboratory population compared with the Field population (Kingsolver and Nagle 2007). Evolutionary reductions in heat tolerance during laboratory domestication at constant temperatures have also been documented in other study systems (Cooper et al. 2001; Krebs et al. 2001). Here we explore whether evolutionary responses to diurnal fluctuations have also occurred during laboratory evolution at constant rearing temperatures.

Diurnal fluctuations in rearing temperature have two main effects on pupal mass and development time in this study system. First, fluctuations caused a reduction in pupal mass relative to constant temperatures (Fig. 1). This effect is consistent with the nonlinear effects of temperature on growth rate and size, sometimes called the Kaufmann effect (Worner 1992) or more generally Jensen's inequality (Ruel and Ayres 1999). Due to Jensen's inequality, the expected consequence of temperature fluctuations

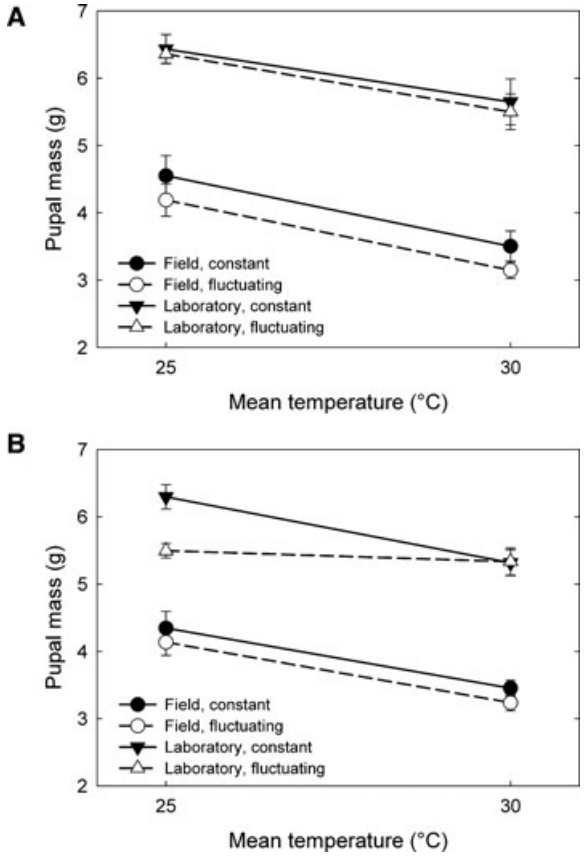

Figure 1. Pupal mass (mean ± 1 SE) as a function of mean rearing temperature for the Field (circles) and Laboratory (triangles) populations of *Manduca sexta*, under constant (solid line, filled symbols) and diurnally fluctuating (dashed lines, open symbols) temperature regimes. (A) Females (B) Males.

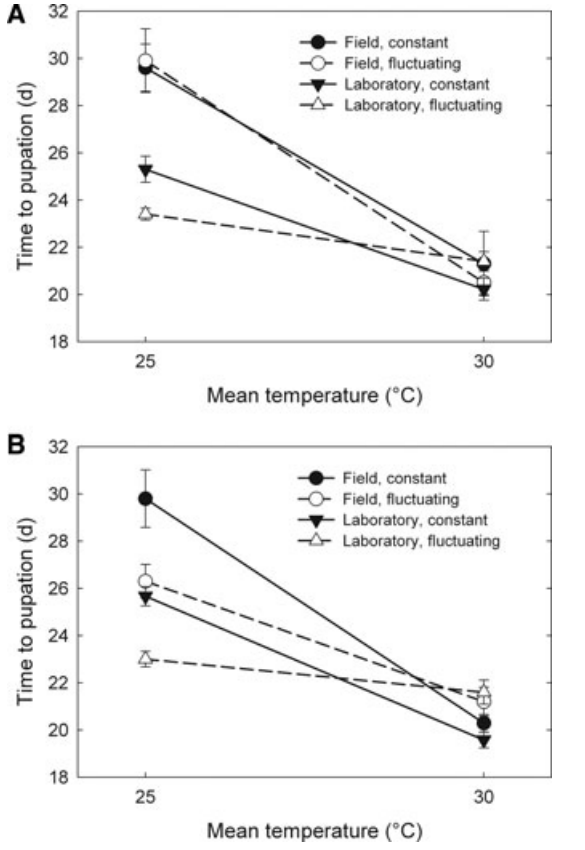

Figure 2. Development time to pupation (mean ± 1 SE) as a function of mean rearing temperature for the Field (circles) and Laboratory (triangles) populations of *Manduca sexta*, under constant (solid line, filled symbols) and diurnally fluctuating (dashed lines, open symbols) temperature regimes. (A) Females (B) Males.

depends on the curvature of the (nonlinear) thermal reaction norm (Ragland and Kingsolver 2008). For example, the relationship between (constant) rearing temperature and final mass (e.g., mass at wandering or pupation) has a negative (concave) curvature for temperatures between 20°C and 35°C (Kingsolver and Nagle 2007). As a result of this negative curvature, we would expect that diurnally fluctuating temperatures should reduce pupal mass, as observed here for *M. sexta*. Second, fluctuations caused a decrease in development time to pupation at a mean temperature of 25°C, but no or a reversed effect at a mean temperature of 30°C (Fig. 2). The relationship between (constant) rearing temperature and development time has a positive (convex) curvature for temperatures between 20 and 35°C, with the greatest curvature at lower temperatures (Kingsolver and Nagle 2007). Thus we would expect that diurnally fluctuating temperatures should increase development time—the opposite of the observed effect. In summary, the shortened development time we observe in response to diurnal fluctuations cannot be simply attributed to the nonlinear effects of temperature on developmental rates; transient (time-dependent) effects of temperature on development time must be involved (Ragland and Kingsolver 2008). Reduced development time in fluctuating temperature conditions has been reported in a number of insects (Brakefield and Kesbeke 1997; Magiafoglou and Hoffmann 2003; Ragland and Kingsolver 2008).

The Field and Laboratory populations differed significantly in their responses to mean temperature for development time, but not for pupal mass. This result is consistent with previous constant temperature studies that revealed a greater thermal sensitivity of development time for the Field than the Laboratory population over this temperature range (Kingsolver and Nagle 2007). However, we found no evidence for population differences in their responses to diurnal temperature fluctuations. Thus, evolution in a constant temperature environment for over 250 generations has not led to detectable divergence in the response of the Laboratory population to fluctuating temperatures. Similarly, geographic populations of the pitcher plant mosquito (*Wyeomyia smithii*) show genetic differences in development time in response to constant temperatures but not to diurnal fluctuations (Ragland and Kingsolver 2008). Experimental evolution with *E. coli* in an alternating (between generation) temperature regime can improve

fitness at both the low and high component temperatures (Leroi et al. 1994). These results suggest that mean and extreme temperatures may act as more potent selective forces on reaction norms than temperature variation per se.

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