Alpine Insects: Physiology and Evolution in Cold, Thin Air

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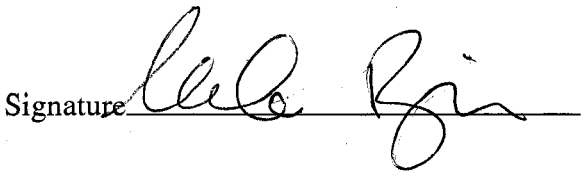
Signature 

Date Aug 1-07

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Abstract

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Biology

The dramatic environmental changes that occur along an altitudinal gradient and the harsh living conditions at high altitude profoundly affect the evolution and physiology of organisms. Organisms living along a mountain must possess specialized adaptations to succeed in their local environment or else, they must be able to succeed in a wide range of conditions. In Chapter 1, I use weather balloon data to characterize the high altitude environment, and then review the literature to explore how these physical changes may affect the physiology and evolution of insects. In Chapter 2, I present data from a comparative study on patterns of intraspecific insect body size across altitudinal gradients. The probability that intraspecific body size will increase or decrease along a mountain is influenced by the life history and environment of the species. In Chapter 3, I explore both the direct and interactive affects of air density and temperature on the feeding rates of larval *Drosophila melanogaster*. Feeding rates were slower at low temperatures and in hypoxia. In Chapter 4, I ask how beneficial plasticity may help flying insects cope with cold environments. Cold temperatures cause increased flight failure and reduced motivation to fly. However, *D. melanogaster* reared in cold temperatures are better able to initiate take-off flight at cold temperatures than flies reared at warm temperatures. The primary mechanism that improved flight performance in cold temperatures was reduced wing-loading. In Chapter 5, I study the evolutionary limits of adaptation to cold environments. For ectotherms, bio-

logical processes slow down as temperatures get colder. However, it was unclear whether insects that have evolved in cold environments are able to evolutionarily compensate such that their biological rates match those of warm-adapted species (biochemical adaptation hypothesis). According to a phylogenetically controlled comparative study, cold-adapted insects have much slower rates of population increase than warm-adapted insects, suggesting that thermodynamics, more than evolutionary compensation, establishes maximum population growth rates.

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DEDICATION

This work is dedicated to my family.

INTRODUCTION

About 20% of global land area is mountainous, making mountains a dominant feature of our landscape and home to many organisms. One of the most striking characteristics of mountains is the range of environmental conditions that occur over short geographic distances. For example, the distance from the base of the Sierra Nevada to the peak of Mount Dana (3,979 m, Yosemite Park, CA) is a mere 60 miles. Yet, the mean temperature drops approximately 24°C - the equivalent of traversing over 3,500 miles in latitude! The change in air temperature is accompanied by a 37% decrease in air density as well as changes in resources, ultraviolet exposure, humidity, and predators. The dramatic changes that occur along an altitudinal gradient, and the harsh living conditions at high altitude, profoundly affect the evolution and physiology of organisms (reviewed in chapter 1), and this is the basis of my dissertation research. My dissertation explores three general themes of insects living along mountains: 1) what are the large scale patterns of insect body size along altitudinal gradients (chapter 2); 2) How do reduced air density and temperature influence insect physiology, (chapter 3); 3) Can insects compensate for the cold environmental temperatures that characterize high altitudes using beneficial plasticity (chapter 4) and evolutionary adaptation (chapter 5)?

In chapter 2, we explore patterns of insect body size along altitudinal gradients. The strong links between organism body size and life history and physiology have prompted much research on geographic variation in body size, and on the underlying mechanisms which drive this variation. The first goal of this study was to determine whether within species body size tends to increase or decrease with altitude for insects. Insects are generally predicted to be larger in colder climates (i.e. high latitudes and altitudes, Bergmann's

rule) due to the developmental and evolutionary effects of cold temperatures on body size. However, when we compiled published data on the body size trends of 50 insect species along altitudinal gradients (males and females were counted separately when possible), only 30 of the 79 cases were consistent with the prediction, contradicting the generality of Bergmann's rule. Given this result, our second goal was to explore how life-history and environmental variables may influence intraspecific body size clines along altitudinal gradients. The body size cline an insect species expresses along a latitudinal gradient has been hypothesized to depend on the length of the species' generation time relative to its growing season. Species with relatively long generation times and relatively short growing seasons run the greatest risk of running out of time or resources. To avoid this, populations living in colder environments are more likely to reduce development time by maturing at smaller body sizes. In support of this hypothesis, we found that insect species with longer development times and short growing seasons are more likely to be smaller at high altitudes. Insects living along mountains do not express simple, general patterns of body size; instead body size is likely determined by the interplay of many variables including temperature, development time, and seasonality.

In chapter 3, I examine how reduced air density and temperature influence insect physiology. Studies of hypoxia on insect physiology and behavior generally have two shortcomings that make them difficult to apply to insects living along altitudinal gradients. First, the effects of hypoxia are typically only measured at a single temperature, despite evidence that hypoxia's influence on insect physiology is crucially dependent on temperature. Second, the standard method for generating hypoxia in the laboratory involves replacing oxygen with nitrogen (while maintaining sea-level air density). This differs from hypoxia found at high altitude which is caused by a reduction in total air density. For insects, these two forms of hypoxia may not be physiologically equivalent. At low air density, the diffusion coefficient of oxygen increases and in theory compensates for the reduction in P_{O_2} during gaseous diffusion. To better understand the challenges insects face at high altitudes, I designed an experiment that addresses these problems. I measured the feeding rate of larval

Drosophila melanogaster in a series of temperatures and atmospheric air treatments, including high altitude hypoxia (reduced air density), standard laboratory hypoxia (oxygen replaced with nitrogen), and normoxia. I found that hypoxia (equivalent to air at 5,800 m) reduced feeding rates at warmer temperatures, but not at cooler temperatures. Furthermore, the standard method of generating hypoxia in the laboratory overestimates the effects of low P_{O_2} at high altitudes by about 10%. Accordingly, hypoxia at high altitudes may not be as stressful as often assumed because colder air temperatures may ameliorate, at least in ectotherms, the negative effects of hypoxia at altitude. However, to understand the limitations and constraints imposed by hypoxia at high altitudes we need more data on the body temperatures of insects living along altitudinal gradients. Furthermore, laboratory studies of hypoxia should reduce total air density, so as not to overestimate hypoxia's influence on insect physiology.

The last two chapters examine the ability of insects to compensate for the cold temperatures that characterize high altitude environments using beneficial plasticity (chapter 4) and evolutionary adaptation (chapter 5). According to the beneficial plasticity hypothesis, organisms have a competitive advantage in the environment in which they develop. For insects living along altitudinal gradients, beneficial plasticity would be an ideal mechanism to promote survival in a wide range of environmental conditions. For flying insects, flight is crucial to their ability to find food, mates, and avoid predators. However, flight ability is compromised by cold temperatures; for example, at around 16°C, *D. melanogaster* are unable to generate adequate lift to fly. To explore whether insects use beneficial plasticity to improve flight performance in cold temperatures, we reared *D. melanogaster* in a series of temperatures and tested their free-flight performance at cold temperatures. If beneficial plasticity helps insects compensate for cold temperatures, we predicted that the cold-reared flies would perform better than warm-reared flies at cold flight temperatures. Our results supported the beneficial plasticity hypothesis. At 16°C flies from cold-rearing temperatures were more than twice as likely to fly as flies from warm-rearing temperatures. We also looked at a variety of traits that might contribute to the improved flight performance

of cold-reared flies, including wing loading, flight muscle mass, relative wing length, and wing beat frequency. The only variable that explained the improved flight performance of cold reared flies was a dramatic decrease in wing-loading. At least in terms of flight ability, beneficial plasticity may be an important mechanism that helps insects compensate for the increasingly cold temperatures along altitudinal gradients. Indeed, temperature dependent changes in wing area may be more important than genetic differences between low- and high-altitude flies. In a recent study, high-altitude flies had wings that were about 10% larger than their low-altitude counterparts when reared in a common garden. This is a small difference compared to the 40% increase in wing area due to developmental plasticity we observed in response to cold temperatures.

In chapter 5, we examine the ability of insects to evolutionarily adapt to their thermal environment. At cold temperatures, the rate processes of insects (and ectotherms in general) are slowed, resulting in slower biological rates such as: metabolic rate, feeding rate, population growth rate, and growth rate. Given this, we wanted to know whether insects in colder environments have a slower rate of living, or whether they evolutionarily adapt such that their rates match, or nearly match, those living in warmer environments. To answer this, we did a comparative study of insect species comparing their optimal temperature to their maximum rate of population growth. We predicted that if insects evolutionarily compensate for slowed biological reaction rates at cold temperatures, then warm- and cold-adapted species would have equivalent rates of population growth at their respective optimal temperatures (i.e. biochemical adaptation hypothesis). However, if thermodynamics drives maximum biological reaction rates, then warm-adapted species (those with higher optimal temperatures) would have greater maximum rates of population growth than cold-adapted species (i.e. thermodynamic constraint hypothesis). We observed a strong correlation between optimal temperatures and maximum rates of population growth, supporting the thermodynamic constraint hypothesis. By comparing our results with predictions from a recent thermodynamic model, we found no evidence that insects use biochemical adaptation to compensate for the depressing effects of cold temperatures on biological reaction

rates. Although insects evolutionarily adapt to their thermal environment by shifting their optimal temperature, they do evolutionarily compensate for the depressing effects of cold temperatures on biological reaction rates. Given this, insects living at high altitudes probably do live their lives at a slower rate than insects living at low altitudes.

Chapter 1

INTO THIN AIR: LIFE AT HIGH ALTITUDE[†]

1.1 Summary

Numerous physical parameters that influence insect physiology vary substantially with altitude, including temperature, air density, and oxygen partial pressure. Here, we review existing literature and present new empirical data to better characterize the high-altitude environment, and then consider how this environment affects the physiology and evolution of insects. Using weather balloon data from fifty-three sites across the globe, we estimate a mean altitudinal temperature lapse rate of 6.0°C/km. We also present empirically determined lapse rates for P_{O_2} and air density. The temperature decline with elevation may substantially compromise insect thermoregulation at high altitude. However, heat-transfer models predict that lower air density at elevation reduces convective heat loss of insects by to a surprisingly large degree. This effect combined with behavioral thermoregulation and the availability of buffered microhabitats make the net thermal consequences of high altitude residence strongly context-specific. The decline in P_{O_2} with elevation may compromise insect development and physiology, but its effects are difficult to predict without simultaneously considering temperature and air density. Flying insects compensate for low air densities with both short-term responses, such as increased stroke amplitude (but not wingbeat frequency), and with long-term developmental and/or evolutionary increases in wing size relative to body size. Finally, in contrast to predictions based on Bergmann's rule, a literature survey of thirty-six insect species suggests that those living in colder, higher altitudes do not tend to have larger body sizes.

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1.2 The high altitude environment

Rapid changes in the physical environment can profoundly alter biological communities across altitudinal gradients. For example, mean temperature drops by approximately 24°C from the base of the Sierra Nevadas (200 m) to the peak of Mount Dana (3979 m) in Yosemite National Park, California. The altitudinal temperature change associated with this short horizontally projected distance (97 km) is roughly equivalent to traveling over 4500 km in latitude (Hopkins, 1938). This rapid temperature reduction is likely a primary factor underlying the striking altitudinal changes in plant and animal communities. However, other physical factors such as oxygen, air density, humidity, and solar radiation may also shape high-altitude insect communities, but have been largely ignored. In this review, we discuss the rapid changes in the physical factors that characterize high altitude, and explore how these gradients influence the physiology and evolution of arthropods, and particularly of insects, living at high altitude.

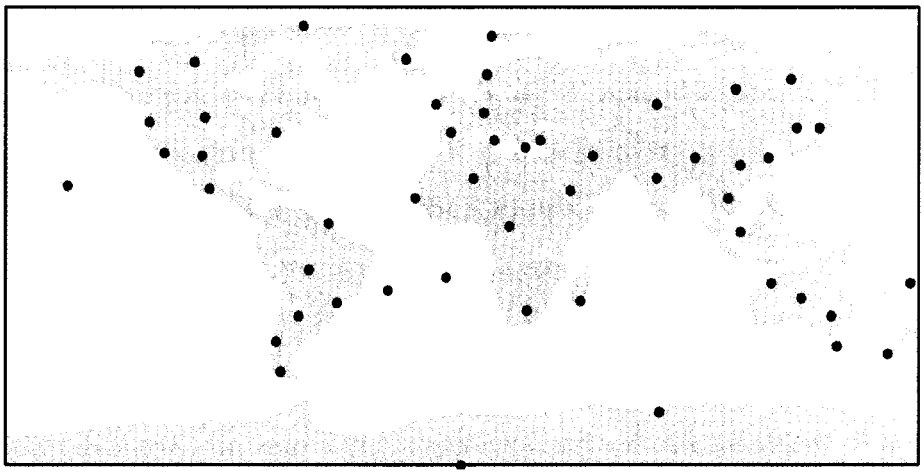

Figure 1.1: Locations of 53 weather balloon release sites used in the present analysis. Sites were chosen for their broad spatial distribution and based on availability of data. Map courtesy of L. M. McCoy.

Air temperature profoundly influences organismal physiology. It is therefore critical to know the rate of change of air temperature with altitude. Theoretically, dry air cools

adiabatically at a rate of 9.88°C/km (MacArthur, 1972). However, air is seldom completely dry; and when moist air rises and cools water condenses, thereby releasing heat. For several temperate and tropical mountains, moist adiabatic lapse rate (the change in temperature with altitude of a saturated parcel of air) is between 6.0 and 6.5°C/km (Körner, 1999); but we could not find a published global assessment of altitudinal temperature lapse rate.

The demand for precise weather prediction has generated a substantial data set which can be used to empirically determine moist adiabatic lapse rates globally. Weather balloons, released twice daily from sites worldwide, collect detailed climate data as they rise (http://weather.uwyo.edu/upperair/sounding.html). We analyzed climate data collected by weather balloons released on two different days (summer and winter solstices of 2003) from 53 sites distributed over the globe (Fig. 1.1).

For any given site, air temperature decreased linearly with altitude, as predicted by theory (Fig. 1.2A). The temperature lapse rate was steeper in summer than in winter (Fig. 1.2B; paired t-test, $t_{51} = -2.88$, $P = 0.006$, summer mean slope = -6.2°C/km, winter mean slope = -5.8°C/km). Among-site variation in temperature lapse rate was substantial (Fig. 1.2B) and could not be explained by non-linearity in the data (all linear regressions were strongly supported with $r^2 > 0.60$; see inset of Fig. 1.2B). Further analyses could determine the extent that factors such as latitude, land-type (continental, coastal, or island), ground temperature and humidity underlie the variation in temperature lapse rate (as Körner, 1999 hints is the case). However, such analyses much first address the multicollinearity among variables and the non-normally distributed data.

A caveat to analyzing weather balloon data is that it may not precisely represent temperature changes along a terrestrial altitudinal gradient, as balloon data are not affected by landscape (Board and Panel, 2000; Kalnay and Cai, 2003; Douglass et al., 2004). Local terrain can change wind speed and direction and alter solar heating. Proximity to open water and vegetation can also affect local air temperature. Accordingly, comparisons between temperatures measured by weather stations and weather balloons will be necessary to address this issue.

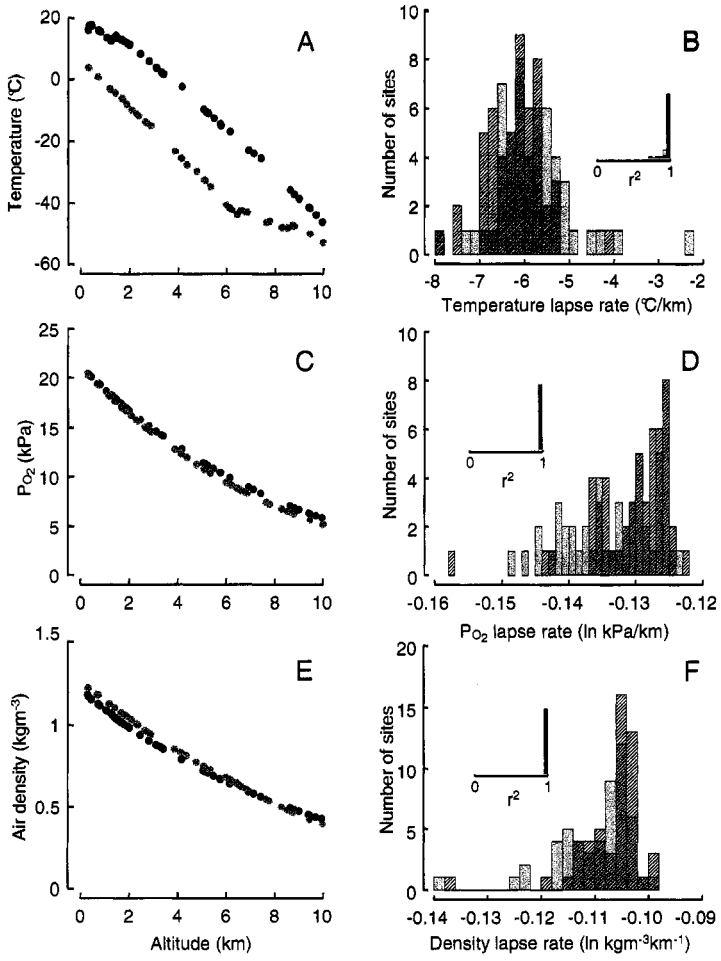

Figure 1.2: Air temperature (A), oxygen partial pressure (C), and air density (E) from weather balloons released from Stuttgart, Germany during summer solstice (black circles) and winter solstice (gray circles). Frequency distributions of lapse rates (slopes of regressions relating air temperature, ln P_{O_2}, and ln air density to altitude) for temperature (B), P_{O_2} (D) and air density (F). Frequency distribution of r^2 values for regressions are given in the inset. Hashed bars indicate summer data and gray bars indicate winter data. Lapse rates of temperature, P_{O_2}, and air density varied significantly with season.

Weather balloons measure ambient atmospheric pressure in addition to air temperature, allowing us to empirically derive global altitudinal lapse rates for oxygen partial pressure (P_{O_2}, estimated as 20.9% of atmospheric pressure; Fig. 1.2C). P_{O_2} lapse rates were significantly steeper in winter than in summer, indicating significant seasonal variation in oxygen availability at high altitudes (paired t-test, $t_{51} = 2.92$, $P = 0.005$; summer: $P_{O_2} = e^{3.07-0.131 altitude}$; winter: $P_{O_2} = e^{3.07-0.133 altitude}$; Fig. 1.2D). Although the effects of this variation on humans have been discussed elsewhere (Ward et al., 2000, p. 28; West, 1996), the significance for insects of seasonal variation in P_{O_2} is unexplored.

Weather balloon measurements of temperature, atmospheric pressure, and relative humidity allow us to determine altitudinal variation in air density (Fig. 1.2E). Air was denser at low altitudes in the winter relative to summer (significantly different intercepts; paired t-test, $t_{51} = -3.67$, $P = 0.005$; Fig. 1.2F). However, the density lapse rate was steeper in the winter than in the summer (paired t-test, $t_{51} = 3.42$, $P = 0.001$). Overall, water vapor pressure had a negligible influence on density (0.002 kgm^{-3} averaged across altitude). The reduction in temperature with altitude had large effects on air density. For example, if you neglect to account for the drop in temperature with altitude, your estimate of air density at 6000 m would be equivalent to the true air density at 7500 m.

1.3 Physiological challenges of high altitude

1.3.1 Thermoregulation

The decrease in mean air temperature with altitude does not necessarily result in equivalent changes in arthropod body temperature (Stevenson, 1985). Body temperature is determined by multiple heat transfer processes that depend on characteristics of the microhabitat as well as organism size, shape, and behavior (Porter and Gates, 1969; Bakken, 1976; Casey, 1992). Microhabitats vary due to factors such as proximity to and thickness of the boundary layer, radiative heating, and even heat from other organisms or structures (Mani, 1968; Somme, 1989; Walsberg, 1992). Behavioral changes in diurnal and seasonal activity with

altitude may also reduce exposure to temperature extremes (Kingsolver, 1983; Somme, 1989; Huey, 1991; Dahlgaard et al., 2001). These factors, and the relatively small size of arthropods, make it difficult to assess the temperatures experienced in the field. However, Jones et al. (1987) used a novel technique to determine developmental temperatures of *Drosophila melanogaster* across an altitudinal gradient (40-1000m). They released at different altitudes mutant flies for which adult eye color was determined by ambient temperatures experienced by the pupae. The eye color of adult flies collected from the field revealed the temperature experienced during development. Although mean air temperature dropped by 4°C from the low- to high-elevation site, mean developmental temperature of the flies was reduced by only 1°C. Similarly, grasshoppers (*Xanthippus corallipes*) collected at 3500 m maintained body temperatures only 7.8°C less than grasshoppers living at 1600 m despite a 10.6°C difference in air temperature and a 17.0°C difference in ground temperature between these sites (Ashby, 1997). These studies highlight the importance of behavior and microclimate in buffering the dramatic altitudinal drop in temperature (see also Huey, 1991).

Many climatic factors other than temperature have been extensively studied and shown to influence body temperature of arthropods (reviewed by Mani, 1968; Somme, 1989). For example, at higher altitudes, drier air may augment evaporative heat loss whereas reduced atmospheric scattering of incident radiation may increase solar heat load. One factor that has received little attention, however, is the effect of reduced air density on convective heat transfer (but see Huey et al., 2001).

Convection is often the dominant mode of heat transfer for small arthropods and is estimated by (Monteith and Unsworth, 1990; Porter and Gates, 1969; Casey, 1992)

$$Q_c = h_c A(T_b - T_e) \tag{1.1}$$

where Q_c is convective heat loss, h_c is the convective heat transfer coefficient, A is surface area, and T_b and T_e are temperatures of the body and environment, respectively. It is well

documented that convective heat loss is affected by environmental temperature and body size (influencing $T_b - T_e$ and A, respectively; Stevenson, 1985). However, the effects of altitude, *per se* on h_c for insects remain unexplored.

To calculate h_c under equilibrium conditions we model insects as geometrically similar cylinders and use the empirically derived dependence of the Nusselt number (Nu) on the Reynolds number (Re) for a cylinder in the correct Re range (40-4000; Monteith and Unsworth, 1990):

$$Nu = 0.62Re^{0.47}. \tag{1.2}$$

Nu describes the ratio of convective to conductive heat transfer and is estimated by

$$Nu = \frac{h_c L}{k}. \tag{1.3}$$

Re is a non-dimensional number indicating the ratio of convective to viscous transport of momentum in the fluid,

$$Re = \frac{\rho U L}{\mu} \tag{1.4}$$

where L is a characteristic length (calculated as $mass^{\frac{1}{3}}$), k is the thermal conductivity of the fluid, ρ is the fluid density, U is the fluid velocity, and μ is the dynamic viscosity of the fluid (Denny, 1993; Monteith and Unsworth, 1990; Campbell, 1977). Altitudinal variation in air density (ρ; Fig. 1.2E, F), thermal conductivity (k), and dynamic viscosity (μ) all potentially alter h_c and therefore convective heat exchange (Q_c). We therefore incorporated altitudinal variation in these properties of air into an analysis of convective heat loss (equations 1.1– 1.4) for an idealized homeothermic insect (approximated by a cylinder) with body temperature (T_b) regulated at 40°C either by endothermic heat production (e.g. bumblebees and moths; Heinrich, 1975) or by behavioral thermoregulation (e.g. grasshoppers; Ashby, 1997; butterflies Kingsolver, 1983). For this analysis, we used a sea-level air temperature (T_e) of 25°C, a temperature lapse rate of 6 °C/km and two wind speeds (1 and 6 m/s).

Reduced air density significantly reduces convective heat loss at altitude (Fig. 1.3). At 6000 m, actual convective heat loss is 26% less than it would be for a constant density atmosphere (Fig. 1.3). This difference is equivalent thermally to increasing ambient air temperature at 6000 m by 10°C). Altitudinal variation in thermal conductivity and in dynamic viscosity have smaller effects on convective heat transfer (unpublished data, Michael E. Dillon).

Although this model estimates heat loss for large homeothermic insects, the general result (that convective heat transfer is reduced at high altitude) holds for any insect. The altitudinal reduction in convective heat transfer will make it easier for insects to warm up and stay hot and more difficult for them to cool down. However, a full biophysical analysis that includes altitude has yet to be done for any organism. As such, we do not know the potential implications of this phenomenon for insect thermoregulation at high altitude.

1.3.2 Hypoxia

Unlike operative temperature, which insects may manipulate to varying degrees, the partial pressure of atmospheric oxygen is beyond an insect's control. Low P_{O_2} can acutely affect human performance, but it is unclear how it affects the physiological and life history traits of insects at high altitude.

Many studies have shown that short-term exposure to ecologically realistic P_{O_2} levels has little or no effect on insect metabolic rate or survival (reviewed by Hoback and Stanley, 2001; Greenlee and Harrison, 2004). However, at high altitude, insects experience constant low oxygen. Chronic exposure of hawkmoth (*Manduca sexta*) eggs to low but ecologically realistic P_{O_2} causes reduced metabolic rates, longer hatching times, and greater mortality (Woods and Hill, 2004). Similarly, chronic exposure to low P_{O_2} slows development, reduces survival, and results in smaller body size in both mealworms (*Tenebrio molitor*; Loudon, 1988; Greenberg and Ar, 1996) and fruit flies (*Drosophila melanogaster*; Frazier et al., 2001). The effects of chronic exposure to low P_{O_2} should be studied in conjunction with temperature, because temperature dramatically alters the physiological effects of re-

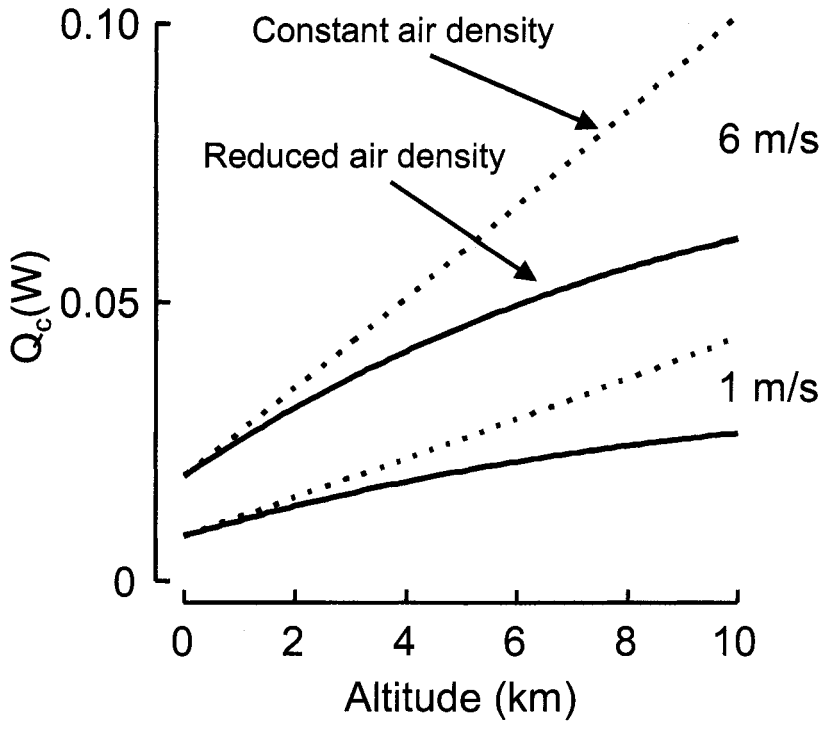

Figure 1.3: Instantaneous convective heat loss across altitude for an idealized insect (approximated by a cylinder) at two wind speeds assuming an initial body temperature of 40°C, a sea level air temperature of 25°C and a 6°C/km decrease in air temperature with altitude. See text for equations and details. Solid lines represent the estimate of true convective heat loss taking into account the empirical decrease in air density with altitude; dotted lines indicate convective heat loss predicted for a constant-density atmosphere.

duced P_{O_2} on ectotherms (Sibly and Atkinson, 1994; Atkinson and Sibly, 1996; Woods, 1999; Pörtner, 2001). High temperatures exacerbate the effects of low P_{O_2} because, in ectotherms, the metabolic demand for oxygen increases exponentially with temperature (Gillooly et al., 2001) while oxygen diffusion rates increase only modestly (about 5% per 10°C; Fig. 1.4; Denny, 1993). Several studies support the prediction that insects have more difficulty meeting tissue oxygen demands at warmer temperatures. For hawkmoth eggs, metabolic rate, survival, and hatching time were most affected by low P_{O_2} at high temperatures; in fact, at the highest rearing temperatures (32-37°C), sea level P_{O_2} proved limiting (Woods and Hill, 2004). *Drosophila melanogaster* reared in hypoxia and cool temperatures were 10-15% smaller than those reared in sea level P_{O_2}, whereas flies reared in hypoxia and warm temperatures were 30-40% smaller (Frazier et al., 2001). Growth rate and development time of this species showed similar trends. Evidence of interactive effects between temperature and oxygen delivery are also observed in other ectothermic organisms (Keister and Buck, 1961; Bryan et al., 1984; Hicks and Wood, 1985; Dupre and Wood, 1988; Schurmann and Steffensen, 1992; Donahaye et al., 1996; Frederich and Portner, 2000). To understand the effects of low P_{O_2} on alpine insects, we need to better characterize both the chronic effects of low P_{O_2} and insect body temperatures along altitudinal gradients. One prediction is that the effects of low P_{O_2} at altitude are mitigated by the concurrent decline in mean air temperature (Fig. 1.2A). However, endothermic insects that maintain high body temperatures, such as many bees, large flies, and moths may be particularly affected by low P_{O_2}. Ectothermic insects may also be strongly affected by low P_{O_2} at altitude because mean air temperature often does not represent the body temperature of ectothermic organisms. In addition, maximum air temperatures at high altitudes can meet or exceed those of lower altitudes; and microhabitats can be prohibitively hot, even at high altitudes.

It is important to note that in aforementioned studies, partial pressure of oxygen was manipulated by reducing the percentage of oxygen using nitrogen replacement within a normobaric gas mixture; air density thus remained close to the sea-level value. However, increasing altitude causes an absolute reduction in air density as well as in partial pres-

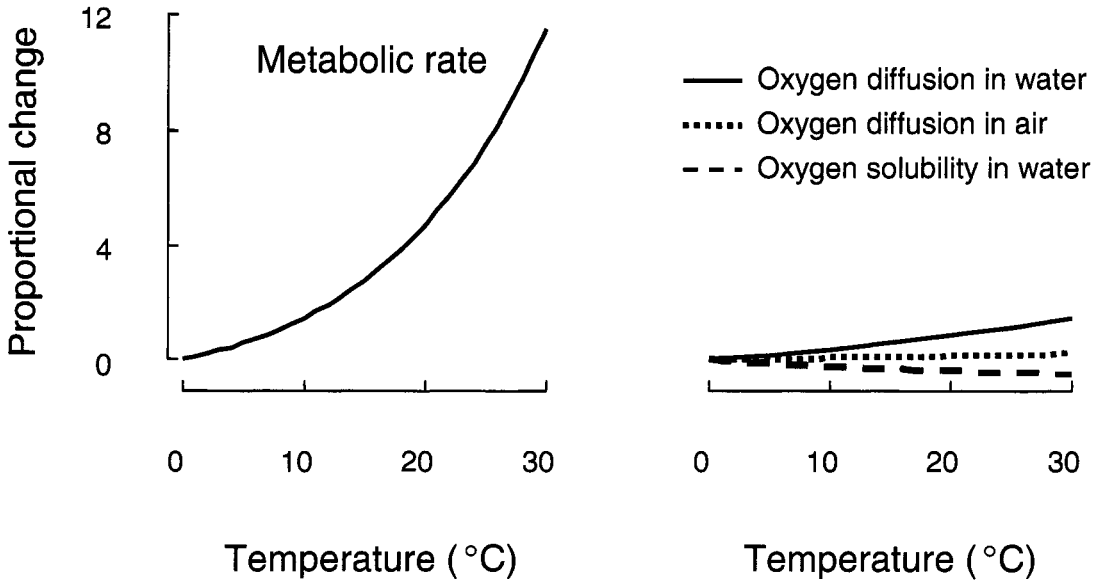

Figure 1.4: (A) Proportional change in ectothermic metabolic rate (Wg^{-1} body mass) as a function of body temperature (Gillooly et al., 2001). (B) Proportional change in the physical processes of oxygen delivery to tissues as a function of temperature; solid line represents diffusion coefficient of oxygen in water (*Dm*, cm^2s^{-1}; see Denny, 1993); dotted line represents diffusion coefficient of oxygen in air (*Dm*, cm^2s^{-1}; Denny, 1993); dashed line represents solubility of oxygen in water (μmol L^{-1}; see Benson and Krause, 1984).

sure of oxygen. Although reduced oxygen partial pressure decreases the driving force for oxygen diffusion according to Ficks Law, the concomitant reduction in air density theoretically lowers the mediums resistance to gaseous diffusion (i.e., larger diffusion coefficient) exactly compensating for the reduction in partial pressure (Denny, 1993). For insects in which air-based diffusion is the limiting step of oxygen delivery, the high-altitude environment may not compromise tissue oxygen delivery even if there is no physiological compensation for reduced P_{O_2}. The combined effects of temperature, oxygen, and air density on respiration and development are still unstudied. Factorial experiments that decouple temperature, oxygen, and air density using variable-density gas mixtures (see Dudley and Chai, 1996) would allow for measurement of both the direct and interactive effects of these factors on insect development.

If reduced P_{O_2} at high altitude challenges tissue oxygen delivery, insects may adaptively respond by altering the respiratory system. The insect respiratory system delivers oxygen to tissues via a network of highly branched tubes (trachea) that terminate at body tissues (Wigglesworth, 1972; Ghabrial et al., 2003). To compensate for low oxygen, insects increase both the number of terminal tracheal branches (Wigglesworth, 1954; Jarecki et al., 1999) and the diameter of primary trachea (Locke, 1958; Loudon, 1989; Henry and Harrison, 2004). In larval *D. melanogaster*, tracheolar density responds to tissue oxygen needs with remarkable precision and rapidity. Cells experiencing hypoxia release a local molecular signal, Branchless (Bnl), that induces sprouting in nearby terminal tracheal branches and directs them to the tissue (Jarecki et al., 1999). Using the GAL4-UAS reporter system (Brand and Perrimon, 1993), Jarecki et al. (1999) demonstrated that extensive proliferation of terminal tracheal branches can occur within one hour. Reduced oxygen also triggers a developmental increase in the diameter of primary trachea in mealworms (Locke, 1958; Loudon, 1989) and in fruit flies (Henry and Harrison, 2004), as well as heritable increases in primary trachea size in *D. melanogaster* following several generations in low oxygen (Henry and Harrison, 2004).

Given the developmental and evolutionary plasticity of the insect tracheal system, high

altitude environments may not typically compromise oxygen delivery. However, if thorax volume remains constant, increased tracheal volume will reduce the body volume available for other structures such as flight muscle or ovaries. Among species, differences in the relative allocation of muscle fibers, mitochondria, tracheae, and sarcoplasmic reticulum within a limited thoracic volume result in significant trade-offs in flight performance (Conley and Lindstedt, 2002). If high-altitude insects replace muscle, mitochondria, or sarcoplasmic reticulum with trachea or air sacs, their flight performance, which is already challenged at high altitudes (see below), may be further compromised.

1.3.3 Flight physiology

Insects should have reduced flight performance at altitude because of reduced mean air temperatures and changes in the physical properties of air. Low temperatures can compromise flight by altering metabolism and muscle physiology (Scaraffia and DeBurgos, 2000; Josephson, 1981; Hosler et al., 2000). Reduced air density at high altitude reduces both Reynolds numbers and aerodynamic forces generated by insect wings (Vogel, 1994; Dudley, 2000). High altitude may therefore compromise flight because a greater downward flux of air is required to maintain the constant momentum flux necessary to offset body weight (air viscosity also determines forces but varies little with altitude; Dudley and Chai, 1996). The power required to produce this momentum flux, the induced power, therefore also increases at high altitude (Dudley, 2000).

Despite the aerodynamic challenges, insects in the laboratory and on mountains are able to fly in reduced density air. Mosquitoes fly at air densities only 20% of the normobaric value (Galun and Fraenkel, 1961). Orchid bees (Apidae: Euglossini) in normoxic but hypodense gas mixtures can hover at air densities 36% of sea-level values (Dudley, 1995), and honeybees fly in hypobaric air corresponding to about 4500 m, or 65% of normobaric density (Withers, 1981). To fly in these low air densities, these insects must be changing the three dimensional motions of their wings (i.e. the wing-beat kinematics). Kinematic data for insects flying in hypodense air are available only for orchid bees and bumblebees

in the laboratory; the primary means of compensation is an increase in stroke amplitude (the angular extent of wing motion; see Dudley, 2000), whereas wing-beat frequency remains constant (Dudley, 1995; unpublished data, Michael E. Dillon). Orchid bees exhibit a similar response during maximum load-lifting tests, albeit in normobaria (Dillon and Dudley, 2004). Whether insects flying at high altitudes exhibit similar changes in wing-beat kinematics remains untested.

On a developmental or evolutionary time scale, flying insects may compensate for reduced air density by altering wing or body morphology. Insects with longer wings relative to body size reduce loss of momentum from the tip of the wing, decreasing induced power requirements of flight (Dudley, 2000). Insects with greater wing area relative to body size decrease the induced velocity necessary to sustain flight, thereby reducing induced power expenditure (Dudley, 2000). High altitude insects may therefore minimize induced power by changing wing length and/or wing area relative to body size. Some morphological data support this hypothesis. Wing length of *Drosophila robusta* is greater at higher elevations whereas thoracic dimensions remain constant (Stalker and Carson, 1948). Similarly, mountain honeybees have longer wings and greater wing area but invariant body mass relative to their lowland counterparts (Hepburn et al., 1998).

We further tested this hypothesis with bumblebees which are prominent alpine pollinators. We studied museum specimens of *Bombus festivus*, a species found across a wide altitudinal range (400 to 5200 m) and present in large numbers in the collections of the Institute of Zoology, Beijing, and the National Museum of Natural History, Washington D.C. Wet mass was not available for museum bumblebees, so we instead measured the width of the thorax at the lateral margins of the wing bases (the interalar width). We also measured the length of a flattened forewing for each specimen. For *Bombus festivus* queens, wing length increases with altitude (ANOVA, $F_{1,236} = 9.89$, $P = 0.002$; Fig 1.5A, open circles), but wing length relative to a reliable proxy for body mass (i.e., the cube of interalar width; based on unpublished data, Michael E. Dillon and the standard scaling relationship that mass length$^{1/3}$) does not change (ANOVA, $F_{1,236} = 0.364$, $P = 0.567$). For workers of this

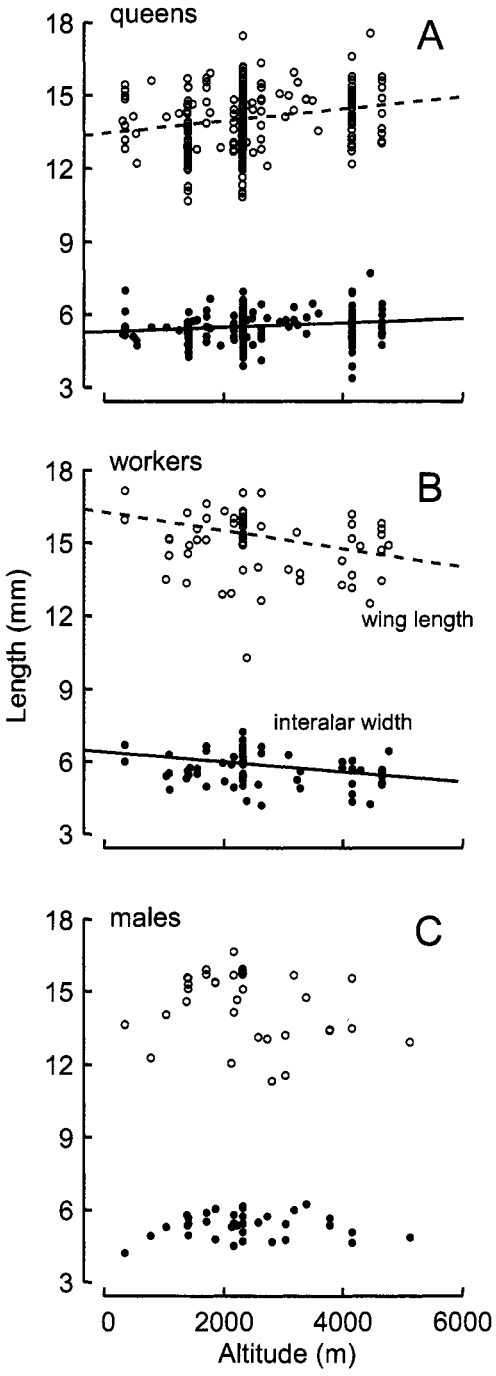

Figure 1.5: Altitudinal variation in interalar width (IW; filled points) and wing length (WL; open points) for *Bombus festivus*. (A) queens (n=238): IW ($P = 0.03$, $r^2 = 0.02$) and WL ($P = 0.002$, $r^2 = 0.04$), (B) workers (n=61): IW ($P = 0.03$, $r^2 = 0.08$) and WL ($P = 0.04$, $r^2 = 0.06$), and (C) males (n=34): IW ($P = 0.91$) and WL ($P = 0.17$). Only significant regression lines are shown.

species, wing length decreases with altitude (ANOVA, $F_{1,60} = 4.06$, $P = 0.048$; Fig. 1.5B, closed circles), but wing length relative to our proxy for body mass increases with altitude (ANOVA, $F_{1,60} = 4.15$, $P = 0.048$). For male *B. festivus*, neither wing length (ANOVA, $F_{1,33} = 2.00$, $P = 0.167$; Fig. 1.5C, open circles) nor wing length relative to our proxy for body mass (ANOVA, $F_{1,33} = 0.683$, $P = 0.415$) changes significantly with altitude. For this bee species, only the flight morphology of workers changes to help offset the increased induced power requirements of flight at high altitude. It is tempting to speculate that this morphological difference is related to caste differences in foraging behavior (only workers carry loads throughout their lifespan), but more data is necessary to test this hypothesis.

Altitudinal changes in wing-beat kinematics and morphology have important consequences for flight performance. In hummingbirds, systematic interspecific variation in morphology and kinematics with elevation is accompanied by a systematic decline in the power margin (the ratio of maximum power when load-lifting relative to unloaded hovering; Altshuler and Dudley, 2003; Altshuler et al., 2004). Hummingbirds at higher elevations operate closer to their maximum power availability during normal hovering, leaving less room for supplemental modulation of force and power production during escapes or sexual displays. A similar result might be expected for insects across elevational gradients, but this hypothesis has not been tested. Of particular interest for insects is whether low oxygen availability interacts with reduced air density to limit flight. Also, increased oxygen demand, which may characterize flight at high altitudes, can increase water loss in insects (Lehmann, 2001). Future experimental manipulations might include total density reduction under both normoxic and hyperoxic conditions to determine whether increased oxygen availability augments maximum performance. In particular, effects of air density, oxygen partial pressure, and air temperature can either be experimentally decoupled or analyzed in concert (Dudley and Chai, 1996), thus allowing biomechanical effects of reduced air density on flight aerodynamics to be identified independent of aerobic constraints on flight.

1.4 Patterns along altitudinal gradients

1.4.1 Intraspecific body size clines

The observation that individuals of many species are larger at higher latitudes has long intrigued biologists (Chown and Klok, 2003; Angilletta and Sears, 2004; Blanckenhorn and Demont, 2004). Because latitudinal clines in body size are developmentally (Atkinson, 1994) and evolutionarily (Partridge et al., 1994) linked with temperature, biologists have also searched for body size variation along elevational gradients. Specifically, the question we address is: for a given insect species are populations at colder high-altitude sites larger than those living at warmer low-altitude sites? We use two approaches to answer this question. First we evaluate in detail the available data for a bumblebee species (Hymenoptera: Apidae, genus *Bombus*) which occurs across a wide altitudinal gradient. Second, we summarize existing data on body size clines of other insects across elevational gradients.

Our analysis of *Bombus festivus* body size clines presents a mixed pattern: queens of this species are larger at higher altitude (Fig. 1.5A, filled circles; ANOVA, $F_{1,237} = 4.86$, $P = 0.03$); workers are smaller (Fig. 1.5B, filled circles; ANOVA, $F_{1,60} = 4.90$, $P = 0.03$); and males show no significant change in body size (Fig. 1.5C, filled circles; ANOVA, $F_{1,33} = 0.01$, $P = 0.91$). These among-caste differences in body size clines may reflect both behavioral and developmental differences.

We also reviewed published studies of intraspecific variation in body size with altitude deriving both from phenotypic plasticity during development and from adaptation across elevational gradients. The prediction that insects will be larger at colder high-altitude sites is clearly contradicted. Among twenty-nine species from four insect orders, we did not observe increased body size clines more often than predicted by chance ($\chi^2_{2,25} = 2.6$, $P = 0.27$; Table 1.1: field-caught). Similar results were obtained for an alternative analysis that excluded nine species that showed no significant body size cline, because this result could be due to either a lack of statistical power or a true lack of an altitudinal body size cline (field-caught: $\chi^2_{1,17} = 0.6$, $P = 0.44$). Evolutionary forces may select for genetically larger insects

at high altitude, but body size may still be reduced during development because of resource limitation. In a few studies, insects from different elevations were reared in common conditions to distinguish between phenotypic plasticity and adaptive differences in body size along elevational gradients. The data for these six species do not support the prediction that larger body size has evolved in high-altitude insects ($\chi^2_{2,2} = 0.4$, $P = 0.82$; data with no significant body size trend removed: $\chi^2_{1,1} = 0.33$, $P = 0.56$; Table 1.1: laboratory-reared), but the sample size is limited. This should be regarded as a preliminary analysis because these species do not represent independent samples, and should be analyzed with methods that control for phylogenetic history.

Several hypotheses may explain why altitudinal trends in body size do not mirror those observed along latitudinal gradients. Shorter geographic distances across altitudinal gradients may lead to high gene flow, limiting the possibility for development of a cline (GarciaRamos and Kirkpatrick, 1997; Kirkpatrick and Barton, 1997; Doebeli and Dieckmann, 2003). Also, altitudinal gradients include a systematic reduction in P_{O_2} which can result in smaller body size. An alternative hypothesis is that altitudinal and latitudinal gradients actually *are* functionally equivalent, and the apparently positive correlation between latitude and body size in ectothermic organisms is spurious. Although many studies have shown intraspecific increases in insect body size along latitudinal gradients, others have found opposite trends (reviewed by Chown and Gaston, 1999; Blanckenhorn and Demont, 2004).

1.4.2 Biodiversity

Given the pronounced changes in temperature, air density, and oxygen availability with elevation, as well as their direct physiological consequences, what are the broader implications for arthropod diversity? As with most metazoan taxa, species richness (i.e., the absolute number of species) and species density (number species per unit habitat area) tend to peak at intermediate elevations, typically between 1000 and 2000 meters (Janzen, 1973; Rahbek, 1995; Lomolino, 2001; Sanders, 2002). Diverse factors have been implicated in

Table 1.1: Insect body size clines along altitudinal gradients from published studies for species caught in the field or reared in a common laboratory environment, see Dillon et al. (2006) for data used in this table.

	Field-caught				Laboratory-reared			
Order	-	0	+	?[a]	-	0	+	?[a]
Coleoptera	13	4	5	0	-	-	-	-
Diptera	0	5	0	0	1	2	2	1
Neuroptera	2	0	0	0	-	-	-	-
Orthoptera	0	0	1	1	-	-	-	-
Total	15	9	6	1	1	2	2	1

[a] Different studies on the same species had conflicting results

such patterns, including variation in available habitat area at different elevational bands, changes in plant primary productivity, climatic limitations, and the source effect of lowland species contributing to mid-elevation faunas (Lawton et al., 1987; McCoy, 1990; Sanders, 2002).

Overall, faunistic changes with elevation typically parallel those seen in latitudinal species gradients, with reduced diversity at climatic extremes. One general ecological effect influencing both tropical and mid-elevational diversity may be the mid-domain effect (Colwell, 2000), whereby an upper limit to species range reduces richness at the ends of a geographical distribution, and correspondingly enhances diversity towards the geographical center. For insects across altitudinal gradients, tests of the mid-domain hypothesis are scarce and present mixed results. The mid-domain effect explains very little of variation in species diversity of Nevada butterflies (Fleishman et al., 1998). For Madagascar butterflies, the mid-domain effect explains as much as 75% of an altitudinal diversity gradient,

but underestimates species diversity at low elevations and predicts a peak in species diversity at a lower altitude than the empirically observed peak (Lees et al., 1999). For ants in Colorado, Nevada, and Utah, the strength of the mid-domain effect on species diversity varies considerably with state (13-90% of the variation in species diversity explained), but in all states it predicts a peak in species diversity higher than the observed peak (Sanders, 2002). More studies are clearly needed to determine the importance of geometrical constraints (i.e., habitat area, mid-domain effect) in determining altitudinal gradients in species diversity.

Many features of insect altitudinal distributions may derive from correlated variation in floristic composition and associated nutritional resources. Such effects may be particularly pronounced in flower-pollinator mutualisms. Lower air temperatures and increased aerodynamic costs of flight with elevation must impose increased energetic costs on volant insects (although this has not been explicitly tested), for which flowers in turn might provide greater nectar rewards (Heinrich, 1975). Surveys of floral nectar production across elevational gradients (e.g., Cruden et al., 1983) yield no systematic pattern, and no published data exist on sugar concentrations and overall nectar availability for those flowers actually visited by pollinating insects at different elevations. However, behavioral adaptations and taxonomic specialization among suites of pollinators clearly enable the maintenance of effective pollination regimes at high elevation (e.g., Arroyo et al., 1982; Bingham and Orthner, 1998). Potential linkage between mechanistic constraints on insect behavior and performance and ecological interactions remains an interesting prospect for future studies of alpine pollination systems.

Chapter 2

PATTERNS OF INTRASPECIFIC BODY SIZE ACROSS ALTITUDINAL GRADIENTS

2.1 Summary

To determine if insect body size tends to be larger at high altitudes (as predicted by Bergmann's rule), we compiled studies measuring intraspecific body size along altitudinal gradients. We found data for 50 insect species, with males and females analyzed separately when possible (making $N = 79$) because they often showed different trends. In 38% of cases, insect body size increased with altitude, and in 62% of cases, body size decreased with altitude, contradicting the generality of Bergmann's rule. The body size cline an insect species expresses along a latitudinal gradient has been hypothesized to depend on the length of the species' generation time relative to growing season. Species with relatively long generation times and relatively short growing seasons run the greatest risk of running out of time or resources. To avoid this, populations living in colder environments are more likely to reduce development time by maturing at smaller body sizes. Based on this hypothesis, we predicted that: 1) insect species with longer development times are more likely to be smaller at high altitudes (i.e., negative body size cline); and 2) insects living in regions with short growing seasons are more likely to be smaller at high altitudes. We used both conventional and phylogenetically corrected analyses to test these predictions. Overall, there was support for both predictions.

2.2 Introduction

Body size influences nearly every aspect of an organism's biology (Pennycuick, 1992; Schmidt-Nielsen, 1995) including metabolic rate (Gillooly et al., 2001), development time

(Gillooly et al., 2002), flight energetics (Dudley, 2000), and population growth rate (Savage et al., 2004). The strong links between organism body size and life history and physiology have prompted much research on geographic variation in body size, and on the underlying mechanisms which may drive this variation. The first goal of this study was to determine whether, within species, insect body size tends to increase or decrease with altitude. Our second goal was to explore how life-history and environmental variables may influence intraspecific body size clines along altitudinal gradients.

In 1847, Carl Bergmann proposed one of the first, and most well-known, hypotheses for global patterns of organismal body size (Blackburn et al., 1999). He predicted colder, high latitude environments will tend to harbor larger species because a reduced surface area to volume ratio helps animals maintain warmer body temperatures (Bergmann, 1847). Although originally formulated to explain interspecific body size variation in endotherms– and specifically mammals and birds (Blackburn et al., 1999; James, 1970)– Bergmann's rule has since been applied to non-endotherms (including insects) to explain both inter- and intraspecific geographic variation in body size. Because the small size of most insects precludes regulation of body temperatures above ambient (Stevenson, 1985), it is unlikely that the originally proposed mechanism for Bergmann's rule generally applies to insects.

Nonetheless, insects may generally follow Bergmann's rule due to mechanisms other than thermoregulation. Increasing body size clines along altitudinal gradients may be explained by the dependence of ectotherm body size on developmental temperatures. Insects that develop at colder temperatures tend to be larger (Atkinson, 1994), for reasons that are unclear and vigorously debated (for example: van der Have and de Jong, 1996; Van Voorhies, 1996; van Voorhies, 1997; Partridge and Coyne, 1997; Berrigan and Charnov, 1994; Perrin, 1995; Angilletta and Dunham, 2003; Angilletta and Sears, 2004; Atkinson and Sibly, 1996, 1997; Reeve et al., 2000; Blackburn et al., 1999; Walters and Hassall, 2006). Furthermore, some studies have shown genetic divergence in insect body size in response to thermal environment, with insect species living in colder environments evolving larger body sizes (Karan et al., 2000; Bryant, 1977; Stalker and Carson, 1947; James

et al., 1995, 1997). These body size clines can evolve quickly. A European fruit fly (*Drosophila subobscura*) that invaded North America, and quickly expanded its geographic range, evolved larger body sizes at higher latitudes after only 20 years (Huey et al., 2000). Laboratory populations of *D. melanogaster* maintained at low and high temperatures for many generations possess genetically based differences in body size (Partridge et al., 1994). For these reasons, insects are predicted to follow Bergmann's rule along latitudinal and altitudinal gradients.

In addition to testing for general patterns in body size along altitudinal gradients, we also tested the hypothesis that an insect's body size cline depends, in part, on its generation time and the length of its growing season (Roff, 1980; Chown and Gaston, 1999; Mousseau, 1997; Blanckenhorn and Demont, 2004). Insect species with long generation times relative to the local growing season risk running out of time (Blanckenhorn and Demont, 2004; Chown and Gaston, 1999), or alternatively, running low on resources (Chown and Klok, 2003) prior to completing development. Insects may overcome this limitation by increasing their growth rate (thereby developing more quickly) and/or by maturing at smaller body sizes. If an insect is unable to fully compensate via an increase in growth rate, they may be forced to mature at a smaller body size to ensure that they complete development before the end of the growing season. This trade-off between development time and body size leads to the prediction that insects with long development times relative to the growing season are more likely to be smaller at high altitudes. Conversely, species with short generation times relative to the growing season aren't constrained by this trade-off, so are more likely to be larger at higher altitudes due to the developmental and evolutionary effects of cold temperatures on body size.

To test these hypotheses, we compiled data from studies measuring within-species changes in body size along altitudinal gradients. If anything, decreasing body sizes were observed more frequently along altitudinal gradients than were increasing body sizes, contradicting the generality of Bergmann's rule. To determine whether body size clines depend on an insect species' generation time and the length of their growing season, we estimated

the development time of the species and the latitude (a proxy for season length) where they were collected. We predicted that insects with longer development times and those living at higher latitudes would be more likely to decrease in size with altitude (i.e. show a negative body size cline). Overall, both conventional statistical methods and methods controlling for phylogenetic history supported this hypothesis.

2.3 Methods

2.3.1 Data collection

We compiled published and unpublished measurements of body size across altitudinal transects for 50 insect species from 5 orders (Hemiptera = 1; Neuroptera = 1; Diptera = 3; Hymenoptera = 11; Coleoptera = 34; Table 2.1). For one species (*Coccinella septempunctata*), we included data from two independent studies because the results were conflicting. For 27 species, males and females were both included; for 15 species, males and females were lumped (we do not know if the researchers did not identify gender, or whether they ultimately combined males and females because they did not statistically differ); for 2 species, only males were included; for 6 species, only females were included. In some cases, males and females of a species showed different patterns. Therefore we used both genders in our analyses when possible, resulting in a maximum sample size of $N = 79$.

Body size clines were based on linear measurements of body size (elytra length, body length, etc) of field-caught insects. We did not include studies that used wing length as a proxy for body size because this trait can vary independently of body size in response to temperature (Gilchrist and Huey, 2004). We analyzed the cline data in two different ways. First, we ignored the statistical significance of reported trends as non-significance could either reflect biological reality or a lack of statistical power, and analyzed body size cline as a categorical variable with two possible outcomes: positive body size cline (larger at higher altitude) or negative body size cline (smaller at higher altitudes). We also calculated for each case (insect/gender) the percent change in body size per 1000 m relative to the

body size at the lowest altitude. We used relative rather than absolute change in body size because the features used to estimate body size varied among studies.

2.3.2 Latitude and development time

To address the hypothesis for the mechanisms underlying body size clines we estimated latitude (a proxy for season length) and development time for each insect. Latitude was either provided by the clinal study or determined by locating named sites on Google Earth. Development time was estimated from insect order and body length. We used body length because it was the most commonly used measure of body size in the clinal studies. When alternative measures of body size, such as thorax length, were provided, we estimated body length based on photographs of the insect species and the scaling relationship between the measured trait and body length. For the Hymenoptera data we converted field mass into a body length measurement using equations from Studier and Sevick (1992) and Ganihar (1997). As a last resort, we found measures of body length from other sources. Although studies relating development time to animal body size exist (Gillooly et al., 2002), they were too general to meet our needs. Given this, we analyzed the relationship between body size and development time for an independent dataset of 65 insect species from 7 insect orders (see Frazier et al., 2006, for species used in this analysis). In these studies, the egg to adult development time was measured at 3 to 9 temperatures (mean 4.7 measurements per species); we used each insect's fastest development time in the analysis. We performed a multivariate least squares regression that included insect order and body length (mm) as independent variables and the fastest egg to adult development time (days) as a dependent variable. Both insect order ($F_{6,57} = 13.78$, $P < 0.0001$) and body length ($F_{1,57} = 86.69$, $P < 0.0001$) explained most of the variation in development time ($R^2 = 0.78$). Using the coefficients of this regression Table 2.2 and mean body size (from clinal studies), we calculated development time for the insects in the body size cline study.

Table 2.1: Insect body size clines along altitudinal gradients from published studies of field caught insects.

Species (gender)	cline[†]	body size	feature	lat/long	reference
Coleoptera					
Abax ater (MF)	-16.12^{*}	17	elytra l.	51,7	(Thiele and Kirchner, 1958)
Adesmia metallica (F)	-30.77^{*}	12.5	elytra l.	31,35	(Krasnov et al., 1996)
Adesmia metallica (M)	-25.53^{*}	11	elytra l.	31,35	(Krasnov et al., 1996)
Agonum assimile (MF)	-3.59^{*}	10.7	elytra l.	51,7	(Thiele and Kirchner, 1958)
Anatolica paphia (MF)	-20.81^{NA}	11.3	body l.	36,73	(Mani, 1968)
Apotomopterus porrecticollis (F)	$+4.14$	37.0	body l.	36,138	(Sota, 1996)
Apotomopterus porrecticollis (M)	$+14.6^{*}$	34.0	body l.	36,138	(Sota, 1996)
Bembidion LATR (MF)	-19.6^{NA}	10.5	body l.	28,87	(Mani, 1968)
Blaps caraboides (MF)	-7.03^{NA}	19.7	body l.	40,73	(Mani, 1968)

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Table 2.1 continued

Species (gender)	cline†	body size	feature	lat/long	reference
Bothrometopus brevis (MF)	−14.91*	4.0	body l.	-53,71	(Chown and Klok, 2003)
Bothrometopus elongatus (MF)	+24.91*	3.1	body l.	-47,38	(Chown and Klok, 2003)
Bothrometopus gracilipes (MF)	−10.2	4.7	body l.	-53,71	(Chown and Klok, 2003)
Bothrometopus parvulus (MF)	+25.13*	4.4	body l.	-47,38	(Chown and Klok, 2003)
Canonopsis sericeus (MF)	−20.66*	10.3	body l.	-53,71	(Chown and Klok, 2003)
Carabus problematicus (F)	−2.71	25.0	elytra l.	53,-4	(Sparks et al., 1995)
Carabus problematicus (M)	−4.36	23.0	elytra l.	53,-4	(Sparks et al., 1995)
Cariabus albrechti (F)	−7.09	21.0	body l.	36,138	(Sota, 1996)
Coccinella septempunctata (F)	+0.98	7.2	body l.	51,16	(Ceryngier, 2000)
Coccinella septempunctata (F)2	+0.22	5.8	body l.	50,14	(Zhou et al., 1995)
Coccinella septempunctata (M)	+3.8*	6.7	body l.	51,16	(Ceryngier, 2000)
Coccinella septempunctata (F)2	−1.0	5.3	body l.	50,14	(Zhou et al., 1995)

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Table 2.1 continued

Species (gender)	cline†	body size	feature	lat/long	reference
Ectemnorhinus marioni (MF)	+13.98*	5.0	body l.	-47,38	(Chown and Klok, 2003)
Ectemnorhinus similis (MF)	+48.74*	6.9	body l.	-47,38	(Chown and Klok, 2003)
Ectemnorhinus viridis (MF)	−69.23*	5.6	body l.	-53,71	(Chown and Klok, 2003)
Erodius edomitus (F)	−20.2*	10.0	elytra l.	31,35	(Krasnov et al., 1996)
Erodius edomitus (M)	−20.2*	10.0	elytra l.	31,35	(Krasnov et al., 1996)
Gyrinus seiceolimbatus (F)	+5.33*	4.9	elytra l.	-5,140	(Svensson, 1991)
Gyrinus seiceolimbatus (M)	+4.49*	4.4	elytra l.	-5,140	(Svensson, 1991)
Leptocarabus arboreus (F)	−10.00*	22.0	body l.	36,138	(Sota, 1996)
Leptocarabus arboreus (M)	−8.93*	20.5	body l.	36,138	(Sota, 1996)
Leptocarabus procerulus (F)	−9.62*	29.0	body l.	36,138	(Sota, 1996)
Leptocarabus procerulus (M)	−8.47*	28.0	body l.	36,138	(Sota, 1996)
Micipsa philistina (F)	−2.03	23.9	elytra l.	31,35	(Krasnov et al., 1996)

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Table 2.1 continued

Species (gender)	cline†	body size	feature	lat/long	reference
Micipsa philistina (M)	−25.57*	16.2	elytra l.	31,35	(Krasnov et al., 1996)
Nicrophorus investigator (F)	+8.59*	26.5	elytra l.	39,-107	(Smith et al., 2000)
Nicrophorus investigator (M)	+8.59*	26.3	elytra l.	39,-107	(Smith et al., 2000)
Notiobia cupripennis (F)	−3.57	13.0	body l.	-27,-109	(Desender and Baert, 1996)
Notiobia cupripennis (M)	+0.28	12.5	body l.	-27,-109	(Desender and Baert, 1996)
Pimelia canescens (F)	+9.07	18.0	elytra l.	31,35	(Krasnov et al., 1996)
Pimelia canescens (M)	+9.98	17.3	elytra l.	31,35	(Krasnov et al., 1996)
Platyscelis margellanica (F)	-10.47^{NA}	11.8	body l.	36,73	(Mani, 1968)
Platyscelis margellanica (M)	-13.57^{NA}	11.3	body l.	36,73	(Mani, 1968)
Prosodes alaiensis (F)	$-NA^{NA}$	23.0	body l.	40,64	(Mani, 1968)
Prosodes alaiensis (M)	$-NA^{NA}$	23.0	body l.	40,64	(Mani, 1968)
Prosodes costifera (F)	$-NA^{NA}$	22.0	body l.	40,73	(Mani, 1968)

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Table 2.1 continued

Species (gender)	cline†	body size	feature	lat/long	reference
Pterostichus madidus (MF)	*−NA**	14.4	elytra l.	51,7	(Thiele and Kirchner, 1958)
Pterostichus vulgaris (MF)	−47.22*	15.2	elytra l.	51,7	(Thiele and Kirchner, 1958)
Sepidium dathan (F)	−25.16	15.2	elytra l.	31,35	(Krasnov et al., 1996)
Sepidium dathan (F)	+37.04*	13.5	elytra l.	31,35	(Krasnov et al., 1996)
Trachyderma philistina (F)	−6.16	23.7	elytra l.	31,35	(Krasnov et al., 1996)
Trachyderma philistina (M)	−2.63	23.7	elytra l.	31,35	(Krasnov et al., 1996)
Zophosis complanata (F)	−28.00*	11.0	elytra l.	31,35	(Krasnov et al., 1996)
Zophosis complanata (M)	−27.89*	10.9	elytra l.	31,35	(Krasnov et al., 1996)
Diptera					
Drosophila buzzatii (F)	+0.33	2.9	thorax l.	-26,-66	(Dahlgaard et al., 2001)
Scathophaga stercoraria (F)	+2.56	8.5	hind tibia l.	47,9	(Blanckenhorn, 1997)
Scathophaga stercoraria (M)	−1.52	9.8	hind tibia l.	47,9	(Blanckenhorn, 1997)

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Table 2.1 continued

Species (gender)	cline†	body size	feature	lat/long	reference
Sepsis cynipsea (F)	+0.92	2.7	head w.	47,9	(Blanckenhorn, 1997)
Sepsis cynipsea (M)	+1.37	2.4	head w.	47,9	(Blanckenhorn, 1997)
Hemiptera					
Dalbulus maidis (F)	+6.23*	3.0	interocular w.	-17,-46	(De Oliveira et al., 2004)
Dalbulus maidis (M)	+1.43	2.5	interocular w.	-17,-46	(De Oliveira et al., 2004)
Hymenoptera					
Bombus bifarius (F)	−3.1	17.0	thorax w.	37,-119	Dillon, unpub.
Bombus bifarius (M)	−7.6	15.7	thorax w.	37,-119	Dillon, unpub.
Bombus californicus (F)	+2.6	22.9	thorax w.	37,-119	Dillon, unpub.
Bombus californicus (M)	−6.0*	24.6	thorax w.	37,-119	Dillon, unpub.
Bombus edwardsii (F)	−8.8	21.7	thorax w.	37,-119	Dillon, unpub.
Bombus edwardsii (M)	+0.6	21.9	thorax w.	37,-119	Dillon, unpub.

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Table 2.1 continued

Species (gender)	cline†	body size	feature	lat/long	reference
Bombus fervidus (M)	−3.0	23.7	thorax w.	37,-119	Dillon, unpub.
Bombus flavifrons (F)	+0.4	18.2	thorax w.	37,-119	Dillon, unpub.
Bombus insularis (M)	−3.8*	19.2	thorax w.	37,-119	Dillon, unpub.
Bombus lapponicus (F)	+9.5*	16.7	thorax w.	37,-119	Dillon, unpub.
Bombus lapponicus (M)	−2.2	18.7	thorax w.	37,-119	Dillon, unpub.
Bombus melanopygus (F)	−18.3*	22.6	thorax w.	37,-119	Dillon, unpub.
Bombus mixtus (F)	−3.00*	19.8	thorax w.	37,-119	Dillon, unpub.
Bombus mixtus (M)	+23.0*	20.0	thorax w.	37,-119	Dillon, unpub.
Bombus vandykei (F)	−0.1	19.3	thorax w.	37,-119	Dillon, unpub.
Bombus vandykei (M)	−2.7	18.5	thorax w.	37,-119	Dillon, unpub.
Bombus vosnesenskii (F)	+5.6*	23.9	thorax w.	37,-119	Dillon, unpub.
Bombus vosnesenskii (M)	+0.3	21.8	thorax w.	37,-119	Dillon, unpub.

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Table 2.1 continued

Species (gender)	cline†	body size	feature	lat/long	reference
Myrmeleon immaculatus (F)	−11.48*	NA	intereye w.	38,-84	(Arnett and Gotelli, 1999)

† value based on feature, sign is direction of cline, $^*P < 0.05$, NA indicates data was not provided.

Table 2.2: Relationship between body length and development time in insects, based on a multivariate regression model of 65 insect species

Variable (N)	Estimate (95% CI)	*SEM*
intercept	10.822 (7.855, 13.790)	1.4837
length (mm)	2.322 (1.823, 2.821)	0.2495
insect order		
coleoptera (14)	6.505 (2.988, 10.022)	1.7586
collembola (1)	16.374 (6.105, 26.643)	5.1347
diptera (6)	-14.831 (-9.865, -19.797)	2.4828
hemiptera (20)	-6.998 (-3.820, -10.176)	1.5890
hymenoptera (13)	-0.969 (-4.733, 2.795)	1.8818
lepidoptera (6)	0.778 (-4.143, 5.688)	2.4549
thysanoptera (5)	-0.859 (-5.351, 3.633)	2.6090

2.3.3 Tree construction

For the phylogenetic analyses we constructed a phylogenetic tree (Fig. 2.1 using the Integrated Taxonomic Information System (http://www.itis.gov), the NCBI Taxonomy Browser (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html), and primary literature. Phylogenetic relationships between orders follow Grimalde and Engel (2005). Deeper branches were inferred from primary literature. Within Coleoptera: Carabidae follow Sota and Ishikawa (2004) to genus and Will et al. (2000) to tribe; Tenebrionidae follow Doyen (1993) to tribe; Curculionidae follow Kuschel and Chown (1995) to genus. Species relationships within Drosophilinae from Robe et al. (2005), and Sciomyzoidea and Muscoidea after McAlpine (1989). Where trees were not available, relationships were left as poly-

tomies. Females and males of a species were on different tips with short branch lengths. Node depth was calibrated based on estimated divergence times (Grimalde and Engel, 2005; Pons et al., 2004; Regier et al., 2004; Wiegmann et al., 2003) and branch lengths are proportional to time.

2.3.4 Statistical analysis

To determine whether development time and season length influenced intraspecific body size clines of insects along altitudinal gradients we used both conventional and phylogenetically controlled analyses. To statistically control for the non-independence of species due to their shared evolutionary history (Felsenstein, 1985; Harvey, 1991) we used phylogenetic generalized least squares (PGLS) (Grafen, 1989; Martins and Hansen, 1997; Garland and Ives, 2000; Halsey et al., 2006), performed in R using the APE (Analysis of Phylogenetics and Evolution) package (Paradis et al., 2004) and modifications of code written by R. P. Duncan. PGLS incorporates the predicted covariance among species, based on their phylogenetic relatedness, into a statistical model fit by generalized least squares (GLS).

The covariance matrix used by the PGLS model is derived from the phylogenetic tree, and is based on a Brownian motion model of evolution. However, deviations from Brownian motion can be incorporated into the model by modifying λ, which is multiplied by the off-diagonal elements of the covariance matrix. Lambda (λ) varies between 0 and 1, indicating either phylogenetic independence (i.e. star phylogeny, conventional analysis), or evolution of traits via Brownian motion (i.e. no change to the covariance matrix derived from the phylogenetic tree). The model of evolution that best describes the relationships between the variables in the statistical model can be determined by fitting the PGLS model with a series of λ values ranging from 0 to 1. The λ that results in the highest model maximum likelihood can then be used to obtain model estimates and significance levels. This λ value also provides an estimate of the magnitude of the phylogenetic signal in the data (Freckleton et al., 2002; Halsey et al., 2006). We chose the λ value to use in the analyses based on AIC, using modifications of code originally written by R. P. Duncan.

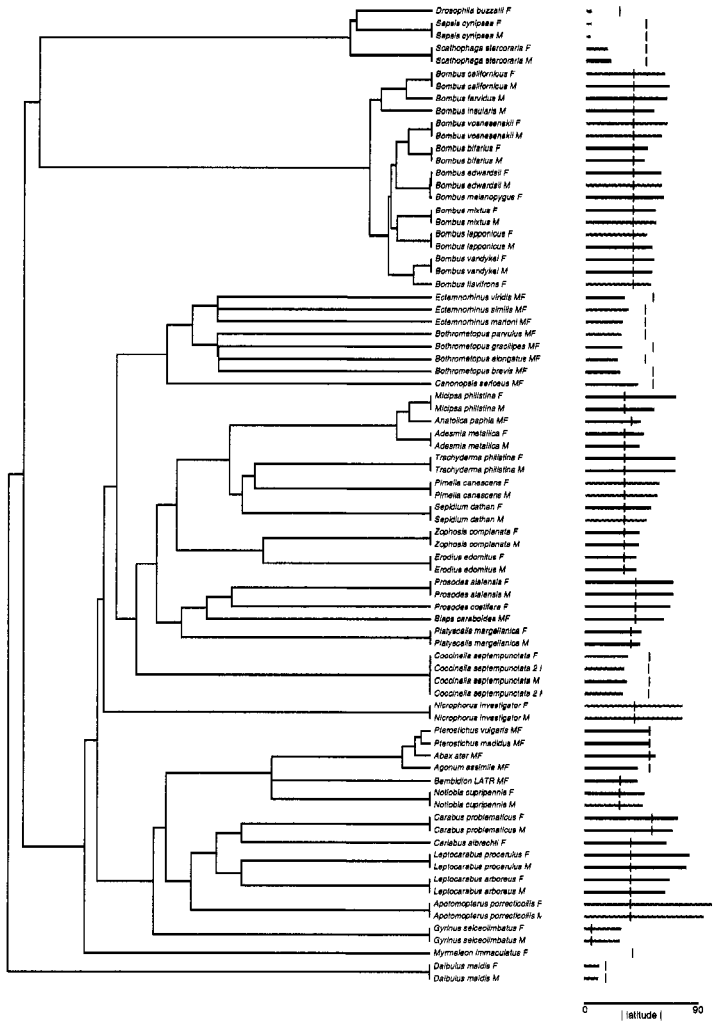

Figure 2.1: Phylogenetic tree used in PGLS analyses. Gender codes are located next to the species names (M=males; F=females; MF=gender lumped). The length of the bar next to each species represents development time, and color represents direction of the body size cline (green represents positive and red represents negative body size clines). Black tick marks indicate the absolute latitude of data collection.

Alternatives to simple Brownian motion can also be incorporated into PGLS analysis by using an Ornstein–Uhlenbeck (O-U) model, which constrains the Brownian motion 'random walk' towards some central point (Martins, 1994; Butler et al., 2000), representing stabilizing selection. The strength of the constraint is set by alpha (α), which also varies from 0 (no constraint or equivalent to simple Brownian) to 1 (strongly constrained Brownian motion). As with λ, we empirically estimated α by finding the value which maximized the log-likelihood of the PGLS model.

2.4 Results

2.4.1 Altitudinal clines in body size

Most insects were smaller at higher altitudes (49 out of 79 cases; $\chi^2 = 4.5696$, $df = 1$, $P = 0.03254$), contradicting Bergmann's rule (Table 2.1). If we excluded the 34 cases in which body size did not show a statistically significant cline, we found no significant trend in body size over altitude – body size increased with altitude in 15 cases and decreased with altitude in 23 cases ($\chi^2 = 1.6842$, $df = 1$, $P = 0.1944$).

2.4.2 Effects of development time and season length on body size clines

Conventional analyses

In our first analysis, cline was treated as a categorical variable, with species body size clines categorized as either positive (i.e. larger at higher altitudes) or negative (i.e. smaller at higher altitudes), regardless of the reported significance of the cline. Development time significantly affected the probability of an insect species expressing a positive or negative body size cline (ordinal logistic regression; $P = 0.0246$; odds ratio = 8.74). Specifically, species with longer development times were more likely to be smaller at high altitudes (Fig, 2.2A). The latitude where the species was found (proxy for season length) had no effect on the probability of expressing a particular body size cline (ordinal logistic regression; $P = 0.1908$; odds ratio = 0.97).

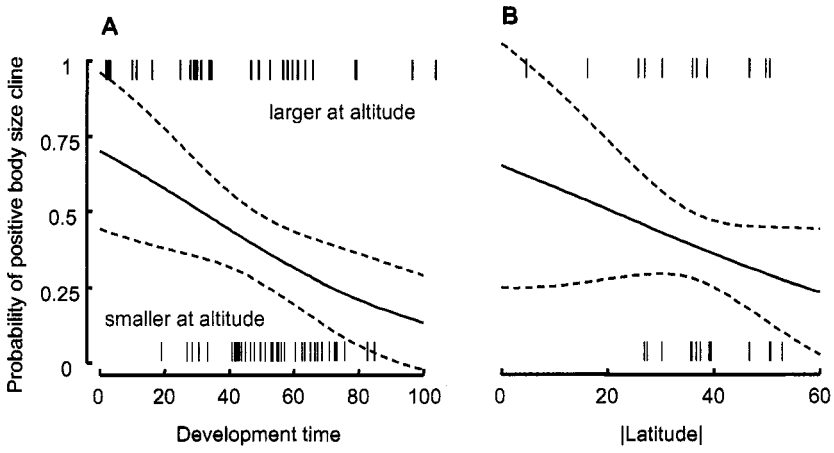

Figure 2.2: The probability an insect species will have a positive body size cline (i.e. larger at high altitudes) based on development time (A) and absolute latitude (B). Insects with longer development times are more likely to be larger at high altitudes, and absolute latitude had no significant effect. Green tick marks at the top of the figures represent cases with positive clines, and red tick marks represent cases with negative clines. These figures are based on a conventional (i.e. non-phylogenetic) logistic regression analysis. Dotted lines are 95% confidence intervals.

We further analyzed the data by including body size cline as a continuous variable (percent change per 1000 m) in a multivariate regression model. In this case, neither development time ($F_{1,71} = 0.02$; $P = 0.8765$) nor absolute latitude ($F_{1,71} = 0.23$; $P = 0.6276$) significantly influenced the magnitude of the body size cline. There are several possible explanations for why this analysis conflicts with the previous analysis in which the body size cline was analyzed as a categorical variable. First, the analyses may reflect a biological reality that development time influences the direction, but not the magnitude, of an insect's body size cline. Alternatively, weaknesses in the continuous data may obscure the relationship between development time and the magnitude of the body size cline. One such weakness is evident when the percent change in body size is plotted against the altitudinal range (Fig. 2.3); studies that spanned less than 1000 m had much greater variation in the magnitude of the calculated cline than did studies using a larger altitude range. Additionally, a wide variety of body parts served as the estimates of body size (i.e. body length, elytra length, tibia length, etc.). Using the percent change in body size assumes that these body parts scale linearly with one another, which may not be the case.

Phylogenetic generalized least squares (PGLS) analyses

To control for the statistical non-independence of the cline data due to shared phylogenetic history, we used PGLS analysis. We incorporated our estimate of the phylogenetic relationships among the species (Fig. 2.1) and two models of evolution (unconstrained Brownian motion and Ornstein–Uhlenbeck) into a generalized least squares model to analyze the effects of development time and latitude on body size clines.

When body size cline was treated as a categorical variable (i.e. positive or negative), the PGLS analyses generally supported the hypothesis that development time and latitude influence the direction of insect body size clines along altitudinal gradients. PGLS does not support the use of categorical dependent variables, so we had to switch the dependent and independent variables in our analyses, such that cline was considered the independent variable. This gives the same statistical results, but limits the analysis to a single independent

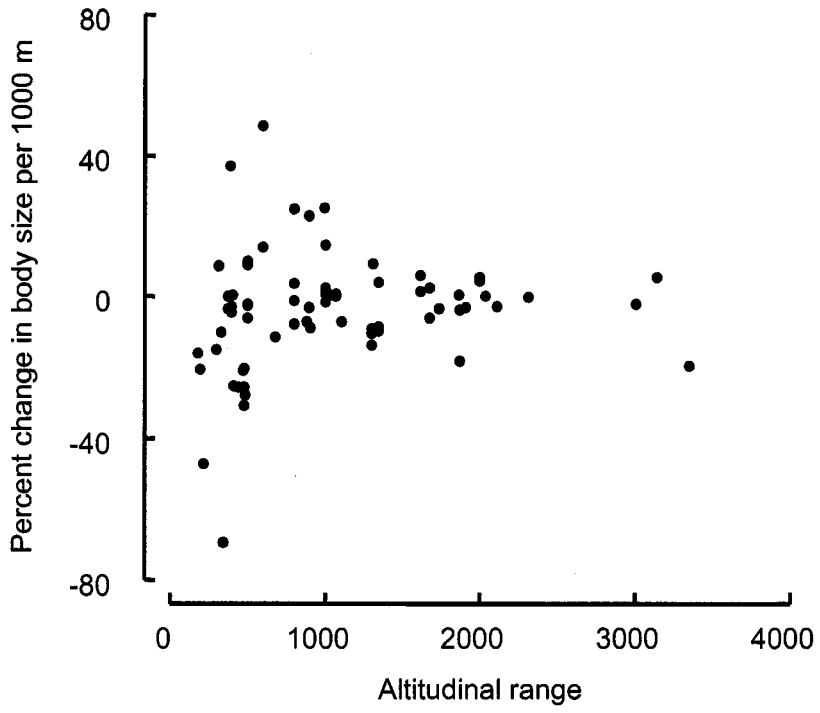

Figure 2.3: Percent change in body size is plotted against the altitudinal range. Studies that spanned less than 1000 m had much greater variation in the magnitude of the calculated body size cline. This suggests that percent change in body size is an unreliable measure in studies that used a smaller altitudinal range.

variable. Therefore development time and latitude had to be analyzed separately. Given the directional predictions made for the effects of development time and latitude on body size clines (increases in either variable should increase the probability of a negative body size cline), we report one-tailed *P*-values. However, we also provide the two-tailed 95% confidence intervals for comparison, and to allow the reader to draw their own conclusions.

For the PGLS analysis of development times, selecting the appropriate λ (recall, λ is a measure of phylogenetic signal in the data) was important because the conclusions were strongly influenced by this value. For example, the development time of insects with positive body size clines ranged from 11 days shorter ($\lambda = 1$) to 10 days longer ($\lambda = 0$) than insects with negative body size clines (Fig. 2.4A). However, AIC was very low across all but the highest values of λ, and the slope estimates for most λ values were significantly negative. To select the λ that best fit the tree and data, we used both the minimum AIC and weighted AIC values (Halsey et al., 2006). To obtain these values, we ran the PGLS analysis 100 times using the same statistical model, but varying λ from 0 to 1. The model with the minimum AIC score had a λ of 0.44 (Fig. 2.4, red points). At this λ, insects with positive body size clines had significantly shorter development times (Table 2.3, $P = 0.023$). Although this technique is useful when AIC reaches a well defined minimum – i.e. a strong trough in the AIC curve – it may be less meaningful to choose a minimum when AIC is similarly low across a wide range of λ. In these cases, an 'AIC-weighted slope' – simply the average of all slopes weighted by their AIC values – may be more appropriate (Fig. 2.4, indicated by blue point; Halsey et al., 2006). In this case, the AIC-weighted slope was similar to the minimum AIC, however it was marginally non-significant ($P = 0.053$).

We further asked whether changing the model of evolution from Brownian motion to Ornstein–Uhlenbeck (a type of constrained Brownian motion) changed the outcome of the analysis. In this model, the parameter α describes the degree to which Brownian motion is constrained. As with λ, we determined the best fit α by re-running the PGLS analysis with α values ranging from 0 to 1, and used the minimum AIC α value to obtain associated parameter estimates and $P-$values. Incorporating an O–U model of evolution did not change

Table 2.3: Phylogenetic generalized least squares (PGLS) analysis of the influence of development time and absolute latitude on changes in body size along altitudinal gradients. Body size is analyzed as a categorical variable (i.e. positive or negative). These analyses represent variations on the Brownian motion model of evolution. Parameter estimates and $P-$values are from the minimum AIC values of λ (for Brownian) or α (for O–U).

Variable	Estimate (95% CI)	min AIC	λ or α^a	P-value
A. Development time (d)				
Unconstrained Brownian	-9.12 (-0.18, -18.07)	693.0	0.44	0.023
Ornstein and Uhlenbeck	-8.00 (-16.78, 0.78)	702.1	0.01	0.037
B. Absolute latitude				
Unconstrained Brownian	-3.63 (-7.91, 0.64)	583.9	0.30	0.047
Ornstein and Uhlenbeck	-3.78 (-8.03, 0.48)	592.9	< 0.01	0.041

[a] λ is used in unconstrained Brownian motion models and α is used in Ornstein and Uhlenbeck models.

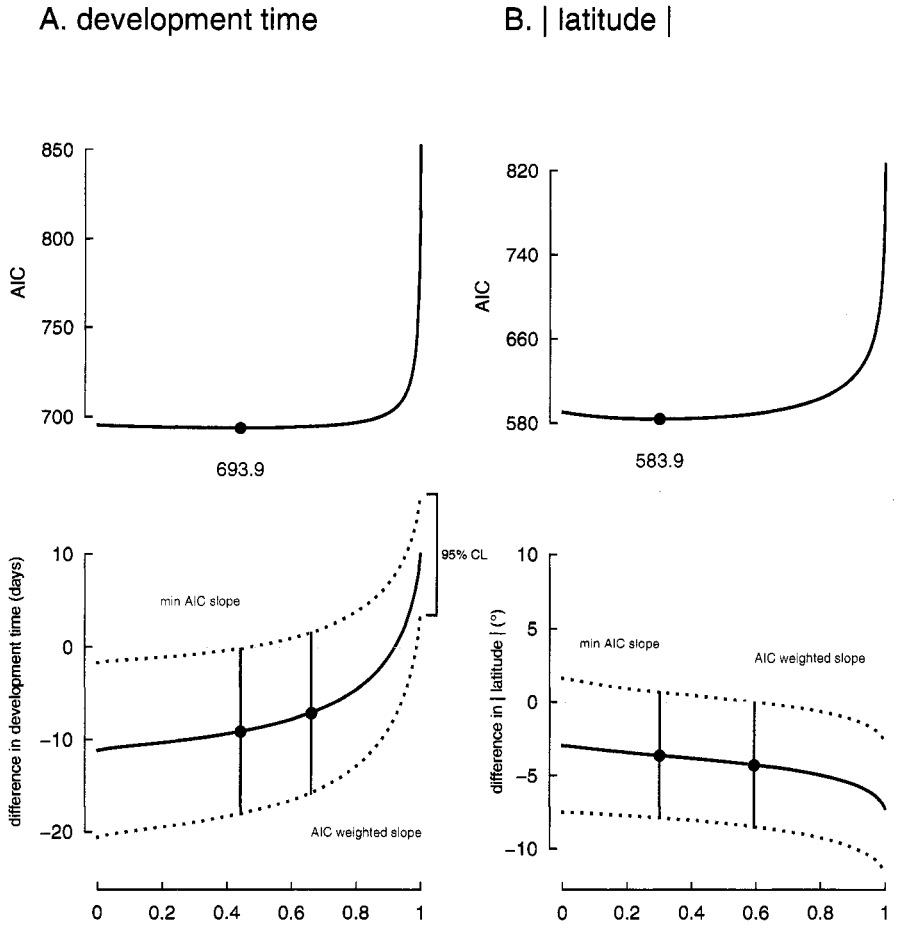

Figure 2.4: Effect of development time (A) and absolute latitude (B) on intraspecific body size clines along an altitudinal gradient based on a phylogenetically informed analysis (PGLS). Variations in the Brownian motion model of evolution (the null prediction), can be incorporated into a PGLS analysis by altering the value of λ, top plots. Lambda can vary from 0 to 1, with 0 indicating that the trait values are not correlated with phylogeny, and with 1 indicating that the trait values reflect evolution via Brownian motion. The statistical estimates for both the minimum AIC (red points) and weighted AIC (blue points) are presented (see Table 2.3). Insect species with shorter development times and those that live at higher latitudes are more likely to express Bergmann body size clines (i.e. larger body sizes at higher altitudes).

the conclusion: insect species with shorter development times were more likely to be larger at higher altitudes ($P = 0.037$).

Using the same approach we used for the development time analyses, we next asked whether our proxy for season length (absolute value of latitude) predicted the direction of altitudinal clines in body size. At the minimum AIC (λ=0.30), the parameter estimate was positive, suggesting that insects with positive body size clines lived closer to the equator (i.e. live at lower absolute latitudes; Fig. 2.4B, Table 2.3; $P = 0.047$). The weighted-AIC slope ($\lambda = 0.30$) was also significantly negative ($P = 0.024$; Fig. 2.4, indicated by blue point). The model AIC values were uniformly low across a wide range of λ, and the parameter estimate was significantly negative except at the lowest λ. The O-U model also supported this pattern at the minimum AIC (Table 2.3, $P = 0.041$).

As in the conventional analysis, when we analyzed body size cline as a continuous variable (percent change in body size per 1000 m), the results conflicted with the results from the categorical analyses. When cline was analyzed as a continuous variable, we found no significant influence of either development time or absolute latitude on this variable ($P > 0.30$; unconstrained Brownian motion and O-U models). We can not say whether this conflict is due to 1) weaknesses in the continuous data (see "conventional analyses" above; Fig. 2.3), or 2) whether the direction of the body size cline, but not the magnitude, is influenced by development time and absolute latitude. The analyses and figures presented below are based on the categorical analysis, because this analysis seems less susceptible to bias.

2.5 Discussion

Within a species, insects are not generally larger at higher altitudes, as would be predicted by Bergmann's rule. In fact, in 49 of the 79 cases included in this study, insects were actually smaller at higher altitudes, contradicting Bergmann's rule (Table 2.1). Similarly, a recent study of insects along latitudinal gradients provided no support for Bergmann's rule: 19 showed a positive body size cline and 29 showed a negative body size cline, a result no

different than that predicted by chance (Blanckenhorn and Demont, 2004). Taken together, data from 98 insect species across both latitudinal and altitudinal gradients suggest that insects are equally likely to get larger or smaller in colder climates.

This study supports the hypothesis that insect body size clines are influenced by the length of the insect's generation time relative to local season length (Roff, 1980; Chown and Gaston, 1999; Mousseau, 1997; Blanckenhorn and Demont, 2004). Both conventional and phylogenetic analyses generally supported the prediction that insect species with longer development times were more likely to be smaller at higher altitudes. On average, species that became smaller with increasing altitude took about 25% longer (8 days) to develop than did species that became larger were larger (Figs. 2.1, 2.2A, 2.4A; Table 2.3). Similarly, Blanckenhorn and Demont's (2004) study of insects along latitudinal gradients found that species with longer development times tended to have smaller intraspecific body sizes at higher latitudes. Furthermore, insect species experiencing shorter growing seasons (i.e. higher latitudes) were more likely to be smaller at higher altitudes according to the phylogenetically informed analyses (Figs. 2.1, 2.4B; Table 2.3), but not a conventional analysis (Fig. 2.2B). The results from the phylogenetic analyses were consistent with Chown and Klok's (2003) observations of beetle species along altitudinal gradients, although they suggest that resource limitation, rather than time, is the primary mechanism driving this pattern.

These studies support the hypothesis that insect populations living in the colder regions of a species' range must hasten development to complete a generation during their growing season, especially if they have long generation times, or live in an area with shorter growing seasons. Otherwise, the insect runs the risk of running out of time or resources prior to completing development. An insect species can speed its generation time by either maturing at a smaller adult body size or by increasing growth rate. Insects can increase their growth rate to some extent, but such increases may be limited by thermodynamic constraints (Gillooly et al., 2002). The insects in these studies appear to speed development by maturing at smaller body sizes, suggesting that the necessary reduction in development

time can not be accomplished solely by increasing growth rates. It would be interesting to explore the relative contributions of decreased body size and increased growth rate to faster development times, in order to determine the evolutionary constraints on these important fitness related variables.

Considering insect phylogeny revealed some interesting patterns in the traits of interest. First, the direction of body size clines in insects appears to be a labile trait, given that both positive and negative trends are well distributed across the phylogenetic tree (Fig. 2.1). The insect orders with data for more than one species (Diptera, Hymenoptera, and Coleoptera) included both positive and negative body size clines. Furthermore, the PGLS analysis confirms that the traits examined in this study are correlated with phylogeny, according to the λ values obtained by AIC. When $\lambda = 0$, then traits are not correlated with phylogeny, and the conventional analysis is most appropriate; when $\lambda = 1$, then traits are evolving through Brownian motion, and closely related individuals share a high degree of trait similarity. For development time, the model in which AIC was minimized had a λ of 0.44; and for latitude λ equaled 0.30 (Fig. 2.4, minimum AIC estimates indicated by red points). Given this, the phylogenetically informed analyses are likely more meaningful than the conventional analyses, because the analyzed traits are correlated with phylogeny.

Another factor that may influence body size clines is the number of generations an insect species completes in a single growing season. If an insect species has a univoltine life-history along their entire altitudinal range, then all else being equal, body size is predicted to decrease at higher altitudes. If a species has a multivoltine life-history along their entire altitudinal range, then body size is predicted to increase at higher altitudes. The pattern is more complicated when the number of generations changes with altitude. For example, if a species goes from two to a single generation, then body size is predicted to have a 'sawtooth' cline at the transition point (Mousseau and Roff, 1989; Gomi and Takeda, 1996; Trudgill and Perry, 1994; Mousseau, 1997). In this case, body size is predicted to be larger at the altitude where the transition occurs, because the time constraints for development will have relaxed somewhat for the univoltine population. Given this, including voltinism

would enhance future analyses, but this data was unavailable for this study. However, in collecting the data for this study, we found that the relationship between body size and altitude was linear in most cases.

Overall, the general patterns of body size along altitudinal gradients are not well predicted by Bergmann's rule. Instead, it appears that the relationship between environmental temperature and body size is more complex and may depend on the life-history and environment of insect species. This study, as well as others (Blanckenhorn and Demont, 2004; Chown and Klok, 2003), suggest that species faced with greater time constraints for development and reproduction are more likely to have smaller body sizes in colder environments (i.e. converse Bergmann cline); and species that are less restricted by time are more likely to follow Bergmann's cline. The body sizes of the insects in these experiments were measured in the field, making it impossible to know whether the body size clines reflect genetic differences between populations or developmental plasticity. Common garden rearing experiments can be used to tease apart the genetic and environmental contributions to body size along altitudinal gradients (Partridge and Coyne, 1997), and possibly provide a better understanding of the underlying causes of these body size patterns.

Chapter 3

EFFECTS OF AIR DENSITY, OXYGEN PARTIAL PRESSURE, AND TEMPERATURE ON FEEDING RATES OF LARVAL *DROSOPHILA MELANOGASTER*

3.1 Summary

Hypoxia is one of the defining characteristics of high altitude environments, and is due to a reduction in total air density. Hypoxia likely challenges insects living on mountains, given that they need oxygen to meet their aerobic demands. However, the influence of high altitude hypoxia on insect physiology and behavior are not well understood. Furthermore, most studies of hypoxia are difficult to apply to insect's living at altitude for two reasons. First, the effects of hypoxia are typically measured at a single temperature, despite evidence of strong interactive effects of temperature and oxygen on insect physiology. Furthermore, instead of reducing total air density, most studies simulate high altitude conditions by replacing oxygen with nitrogen, while maintaining sea level, or near sea-level, air densities. There are theoretical reasons to believe that these two forms of hypoxia are not functionally equivalent to insects. To gain a better understanding of how hypoxia at high altitude affects insects, I measured the effects of temperature (15, 20, 25, 27.5, and 30°C) and hypoxia (50% sea level P_{O_2}, equivalent to $\sim 5,800$ m altitude) on larval feeding rates of *Drosophila melanogaster*. Furthermore, I generated hypoxia using both the standard laboratory method and by reducing total air density. I found that hypoxia slowed larval feeding rates at temperatures $\leq$20°C, but failed to have a statistically significant affect at cooler temperatures. This supports the prediction that ectotherms have more difficulty meeting tissue oxygen demands at warmer temperatures. Our data also suggest that standard laboratory methods of generating hypoxia overestimate the effects of high altitude hypoxia by about 10%. These

results indicate that we need better natural history data for insect body temperatures along altitudinal gradients to understand the true consequences of hypoxia at altitude. Furthermore, laboratory hypoxia needs to be generated by reducing total air density if researchers want to apply their results to insects living at altitude.

3.2 Introduction

Insects experience both hypoxia and low temperatures at high altitude (Mani, 1968; Hoback and Stanley, 2001). For example, fruitflies in the genus *Drosophila* are found as high as 5000 m in the Indian Himalayas (Khare et al., 2002), where air density is 55% normal sea level and temperatures are ~30°C lower than at sea level. Although high altitude environments are common and insect diversity is often high there, the physiological affects of hypoxia and cold at altitude are not well understood. This is partially because the impacts of hypoxia and temperature are strongly interactive. Nonetheless, the influence of temperature and of hypoxia on ectotherm physiology is generally studied in isolation, even though insect behavior and physiology are strongly influenced by both temperature and hypoxia (Woods, 1999; Pörtner, 2001; Frazier et al., 2001; Woods and Hill, 2004). Furthermore, most laboratory studies simulate hypoxic environments (i.e., low P_{O_2}) by replacing O_2 with N_2, while holding barometric pressure at sea level values. Such simulated environments may not have the same physiological effect as the reduction in total air density that characterizes high-altitude hypoxia. Here I ask how temperature and low air density – independently and in concert – influence feeding rates of larval *Drosophila melanogaster*.

The effects of low P_{O_2} on ectotherm physiology depend on temperature (Sibly and Atkinson, 1994; Atkinson and Sibly, 1996; Woods, 1999; Pörtner, 2001). In general, high temperatures exacerbate the effects of low P_{O_2} because tissue oxygen demand increases exponentially with temperature (Gillooly et al., 2001) but oxygen diffusion rates increase only a modest 4% for every 10°C increase in temperature (Denny, 1993). Interactions between temperature and O_2 have been observed in several ectotherms. Metabolic rate, survival, and hatching time of hawkmoth eggs (Woods and Hill, 2004) were most affected by hypoxia at

high temperatures. Similarly, final adult body size and growth rate of *D. melanogaster* were strongly affected by hypoxic rearing conditions at warmer temperatures, but only modestly so at cooler temperatures (Frazier et al., 2001). Other interactive affects of temperature and P_{O_2} have also been observed in other ectotherms (Keister and Buck, 1961; Bryan et al., 1984; Hicks and Wood, 1985; Dupre and Wood, 1988; Schurmann and Steffensen, 1992; Donahaye et al., 1996; Frederich and Portner, 2000). These studies suggest that hypoxia should be studied in the context of temperature, given the large interactive effects of these variables.

To understand how high-altitude conditions affect insect physiology and behavior, laboratory experiments must also accurately reproduce high-altitude hypoxia. In nature, hypoxia at high altitude is caused by the overall reduction in air density. In contrast, hypoxia is typically generated by replacing O_2 with N_2, while maintaining air densities near sea-level (normobaric). Whether this treatment is functionally equivalent to high altitude hypoxia is questionable, but theoretical reasons suggest it may not be the same for insects. A decrease in the partial pressure of O_2, caused either by N_2 replacement or by reduced barometric pressure, reduces the driving force for O_2 diffusion. However, when air density is also reduced, as occurs at altitude, resistance to gaseous diffusion is also lowered (i.e., larger diffusion coefficient). This will increase diffusive flux sufficiently to compensate for - at least in theory - the reduction in oxygen partial pressure (Denny, 1993).

Given this, high-altitude environments may not compromise O_2 delivery in insects as much as traditional laboratory studies would suggest. Whether this is the case depends on the relative strength of the barriers to oxygen delivery posed at each step of the insect respiration system. To obtain oxygen, insects rely on both convective and diffusive mechanisms. Most species use some sort of convection to move bulk air through the body via abdominal pumping (Greenlee and Harrison, 1998), unidirectional air flow (Weis-Fogh, 1967), and tracheal compression (Westneat et al., 2003). Insects also rely on diffusion of respiratory gases through air-filled trachea. At the terminal trachea, diffusion occurs across the air/fluid interface (Wigglesworth, 1983). If gaseous diffusion is a major barrier to respiration in in-

sects, then reduced diffusion resulting from low P_{O_2} at high altitude will likely be mitigated to some extent by the reduction in air density (Harrison et al., 2006). However, the relative contributions of convection, gaseous diffusion, and air/fluid diffusion to oxygen delivery are currently unknown and probably vary among insect species and during ontogeny.

In this experiment, I use a factorial experimental design to decouple O_2 partial pressure, air density (Fig. 3.1; Dudley and Chai, 1996), and temperature to study the independent and interactive effects of these variables on the feeding activity of larval *Drosophila melanogaster*. Feeding rate is an important fitness related trait (Bakker, 1961, 1969), and measured as the rate of scooping movements of the larval mouth parts, also known as cephalogpharyngeal contractions (described by Green et al., 1983). This provides a reliable measure of actual feeding rate (Ruiz-Dubreuil et al., 1996), and has been linked to larval growth rate (Ruiz-Dubreuil et al., 1996; Burnet et al., 1977), adult body size (Ruiz-Dubreuil et al., 1996; Burnet et al., 1977), viability (Ohnishi, 1979), and competitive ability (Joshi and Mueller, 1988, 1996; Santos et al., 1997). This experiment addresses: 1) how low P_{O_2} and temperature independently and interactively influence larval feeding rates and 2) whether hypoxia achieved by reducing percent oxygen is functionally equivalent to that achieve by reduced barometric pressure.

3.3 Methods

Experimental *D. melanogaster* were obtained from a laboratory colony of flies originally collected from an apple orchard near Wenatchee, WA, USA (47°30′N, 120°17′W) in June 2005 (donated by M. E. Dillon). The colony was created by combining 100 isofemale lines, using about 25 males and 25 females from each line. The colony was maintained at 25°C (12:12 L:D) with a population of 1000 to 3000 flies each generation. To maintain standard rearing conditions for colony flies, eggs were collected approximately every 10 days, larvae were reared outside of the colony under relatively low densities (50 to 100 eggs/vial; 25°C; 12h:12h L:D) on a diet of cornmeal, molasses, yeast, agar, tegosept. Newly emerged adults were transferred to the colony. Adult food bottles (150 *mL*) were replaced every 3 to 5

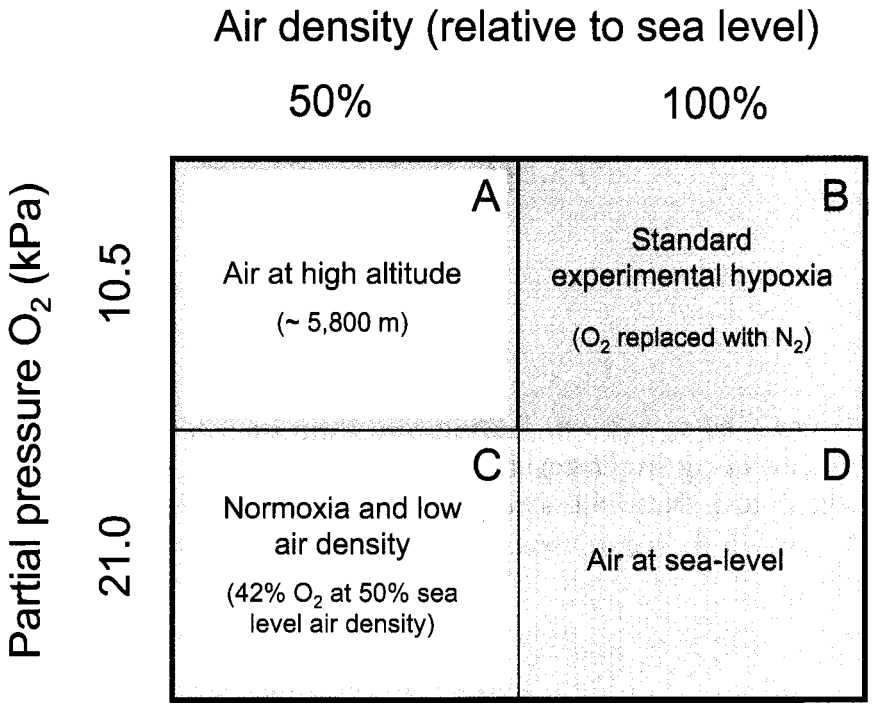

Figure 3.1: Experimental treatment groups used to decouple the effects of reduced oxygen partial pressure versus air density on feeding rates of larval *D. melanogaster*. Pink boxes are atmospheric treatments with low P_{O_2}, and blue boxes are treatments with standard sea level P_{O_2}. White filled boxes are treatments with reduced air density, and solid boxes are treatments with sea level air density. Boxes A and D represent natural atmospheric conditions found at high altitude and at sea level, respectively. Box B represents the most common method of reducing P_{O_2} in the laboratory (i.e., replacing O_2 with N_2). Box C determines whether reduced air density influences feeding rates at standard sea level P_{O_2}.

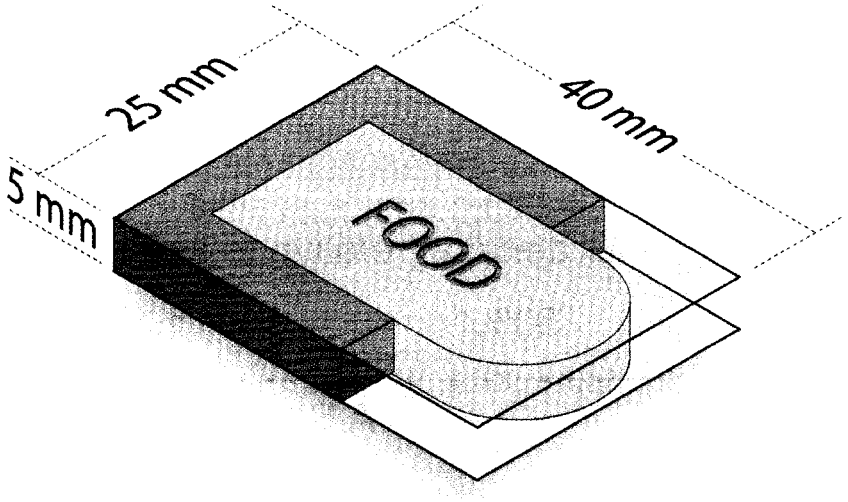

Figure 3.2: Arena used to measure feeding rate of larval *D. melanogaster*. The arena was made by sandwiching food media between two halves of a standard microscope slide, sealing the bottom and sides with a strip of silicone rubber, and covering the top with surgical cloth tape (not shown) to prevent larval escape but to allow air transfer.

days.

To obtain larvae for experiments, I collected eggs during 6 *h* laying periods. These eggs were then individually transferred into feeding arenas, made by sandwiching a thin layer of fly media (1.5 *mL*) between two microscope slides (Fig. 3.2). I placed 40 eggs in each feeding arena. [Note: preliminary experiments indicated that this density did not lead to overcrowding, as abundant food remained even after the larvae began pupating.] Larvae developed in their feeding arenas in an incubator (22 C; 12:12 L:D). To prevent the media from drying out, the arenas were placed inside plastic boxes containing a damp piece of felt. This system was advantageous because larvae could be observed feeding on normal media without disruption, even while embedded in the food. Larvae spent most of their time visibly feeding along the glass walls of the feeding arena, and the small size of the

arena was amenable to viewing under a dissecting scope.

Larval feeding rates increase to the 3rd instar, and then decline (Santos et al., 1997). Accordingly, I measured feeding rates on day 6, when most larvae had reached early or mid 3rd instar. I measured feeding rate (see below) using a factorial experimental design with two P_{O_2}'s (10.5 and 21.0 *kPA*), two air densities (50% and 100% sea level), and 5 temperatures (15, 20, 25, 27.5, 30°C), for a total of 20 treatment groups. The experiment was replicated 3 times during October 2006, and the order of treatments was altered for each replicate.

These treatment groups decouple the effects of low P_{O_2} and low air density on insect physiology (see Fig. 3.1). Two of the atmospheric treatments were similar to natural conditions (Fig. 3.1, A and D). One treatment simulated air at about 5,800 m altitude (Fig. 3.1A, hypodense hypoxia), a high, but not ecologically unrealistic altitude for insects (Hoback and Stanley, 2001). Another treatment replicated air at sea level (Fig. 3.1D, normodense, normoxia). Two treatments were purely experimental conditions, not readily found in the natural world (Fig. 3.1, B and C). One replicated the standard method of creating hypoxia in the laboratory and replaced O_2 with N_2, and thus, maintaining sea-level air densities (Fig. 3.1B, normodense, hypoxia). For the other, air density was reduced, but the partial pressure of oxygen was kept at normal sea level values by increasing the percentage of O_2; this treatment reveals whether low air density *per se* influences feeding rate when oxygen is not limiting.

Feeding arenas (Fig. 3.2) were placed in a 200 *mL* Plexiglas chamber, through which gas mixtures could flow and in which barometric pressure could be altered. The bottom of the chamber was made of aluminum, and was placed on a stainless steel plate, that continually circulated temperature-controlled water. To monitor the temperature experienced by larvae, a calibrated thermocouple thermometer (Physitemp Bat-12, Baily Instruments Inc., Saddlebrook, NJ, USA) was inserted into the food of the feeding arena. To alter atmospheric conditions, I used a hand pump to remove air from the chamber and simultaneously monitored air pressure (Honeywell Model SA Pressure Transducer PN 9306490; Acton,

MA 01720 USA, calibrated using a U-tube manometer). Premixed tanks (10.5% and 42% O_2) were also used to obtain various atmospheric treatments. To measure feeding rate, I placed two feeding arenas onto the aluminum floor of the atmospheric control chamber. The chamber was then flushed with the appropriate mix of air at a rate of 350 to 400 *mL* per minute for 5 minutes. This amount of time was adequate for the food to reach the target treatment temperature. The chamber was then sealed, and air pressure was adjusted as appropriate. Larvae were given 10 *m* to adjust. I then filmed larval feeding behavior (Sony Handycam model #DCR-HC36 video camera attached to a dissecting scope). I circled around each feeding arena one time to avoid recording individual larvae more than once. The average recording time for each pressure/temperature treatment was 4.6 min $\pm$ 1.7 *sd*. The average absolute deviation from the target temperature was 0.37°C $\pm$ 0.29 *sd* (calculated by subtracting the start and end temperature from the target temperature for each run and taking the absolute value). The absolute percent deviation from the 50% sea-level air density treatment was 1.75% $\pm$ 0.9 *sd*.

While replaying the video clips, I scored feeding rate as the number of scooping movements of larval mouthparts per second. Larval *Drosophila* often feed continuously for periods, but then stop for short intervals (Ruiz-Dubreuil et al., 1996). I measured feeding rate only for larvae for which 20 to 30 *s* of continuous feeding was recorded, with no significant breaks (less than one or two seconds). At low test temperatures, I scored feeding rates of video clips played at normal speeds. At higher test temperatures, I slowed the clips to half speed, as feeding rates were too high to score accurately.

To analyze feeding rate, I used a multivariate regression model with the three experimental replicates included as a random effect. Within treatments, feeding rate appeared normally distributed as 18 of the 20 treatment groups were not statistically different from a normal distribution, based on the Shapiro-Wilk goodness of fit statistic (for the two that were significantly different, $P = 0.048$ and 0.008; these did not appear to have any significant outliers or be skewed in a predictable fashion). Therefore, feeding rate was not transformed in any analyses. The atmospheric treatments were analyzed as nominal traits

(see Fig. 3.1), and coded with dummy variables. I included squared terms in the model to fit observed curvilinearity in the response variable. Analyses were performed in JMP.

3.4 Results

Feeding rates were measured for 474 larvae, and sample sizes for the 20 treatment groups ranged from 15 to 35 individuals (Table 3.1; mean 24 ± 5.3 *sd*). According to a least-squares regression model, hypoxia ($P < 0.0001$) and low temperatures ($P < 0.0001$) reduced larval feeding rates (Fig. 3.3). The effects of temperature on feeding rate were non-linear, according to Akaike's Information criterion for model selection (although the P-value of Temp^2 was $P = 0.1192$ and therefore not statistically significant at $\alpha < 0.05$). The AIC interpretation is also consistent with the wealth of studies showing that most biological reaction rates do not scale linearly with temperature (Gillooly et al., 2001). Effects of hypoxia were more pronounced at warmer temperatures (Fig. 3.3), as indicated by a significant interaction between atmospheric treatments and temperature ($P < 0.0001$). To compare the P_{O_2} and air density treatments, I performed a second, more comprehensive, multivariate regression analysis that incorporated dummy variables into the model (Table 3.2). Based on this analysis, reduced P_{O_2} slowed larval feeding rates (Fig. 3.3; Table 3.2, atmospheres A & B vs. C & D, $P < 0.0001$); and the effects of low P_{O_2} were exacerbated by warmer temperatures (Table 3.2, temp x atmosphere). At 30°C, feeding rate was about 35% faster in 21 vs. 10 *kPA* O_2 (combined affects of low air density and low percent oxygen). At 15°C, however, hypoxia did not significantly effect feeding rate ($P > 0.05$, Tukey pairwise comparison).

High altitude hypoxia (reduced total air density) slowed feeding rates less than the standard laboratory method of generating hypoxia (O_2 replaced with N_2, normobaric), even though P_{O_2} was the same in both treatments (Fig. 3.3; Table 3.2, atmospheres A vs. B, $P = 0.0081$). Across all temperatures, feeding rate was about 10% faster in hypobaric hypoxia than in normobaric hypoxia. Temperature did not significantly influence the relative effects of these two atmospheric treatment groups on feeding rate, but this interaction effect

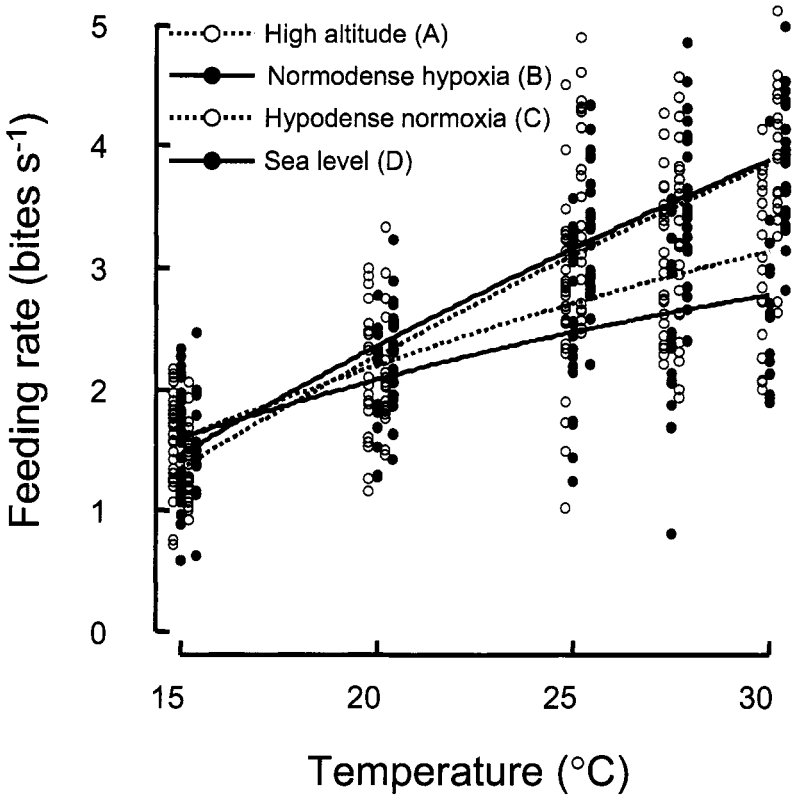

Figure 3.3: Effects of temperature and atmospheric treatment on feeding rates of larval *D. melanogaster*. As temperature increased feeding rate also increased. Hypoxia (A and B) significantly slowed feeding rate, mainly at high temperatures. The standard laboratory method of simulating high altitude (B; replacing O_2 with N_2 and thus keeping air density near sea level values) reduced larval feeding rate significantly more than the reduction in air density at high altitude (A). Reduced air density did not have any effect on feeding rate as long as sea level P_{O_2}s were maintained (C, obtained by using a 42% O_2 gas mixture). Points from different atmospheric treatments are staggered for viewing.

Table 3.1: Feeding rate (bites sec^{-1}) of *D. melanogaster* at each temperature and atmosphere treatment.

Temperature °C	Atmosphere[a]	N	Mean±*SD*
15	A (hypodense hypoxia)	35	1.66±0.38
15	B (normodense hypoxia)	32	1.63±0.45
15	C (hypodense normoxia)	27	1.39±0.29
15	D (normodense normoxia)	18	1.48±0.46
20	A	25	2.13±0.52
20	B	16	2.02±0.44
20	C	23	2.08±0.46
20	D	24	2.29±0.41
25	A	29	2.76±0.70
25	B	27	2.64±0.62
25	C	24	3.46±0.75
25	D	24	3.30±0.54
27.5	A	27	3.06±0.64
27.5	B	16	2.50±0.76
27.5	C	26	3.30±0.77
27.5	D	20	3.56±0.65
30	A	19	2.98±0.70
30	B	15	2.69±0.65
30	C	22	3.88±0.58
30	D	25	3.86±0.49

[a] see Fig. 3.1 for description

Table 3.2: Effects of temperature, P_{O_2} and air density on the feeding rate of larval *D. melangaster*. The coding for atmospheric conditions is as follows: A) hypodense hypoxia (i.e. high altitude); B) normodense hypoxia (O_2 replaced with N_2); C) hypodense, normoxia (created by using a 42% O_2 air mixture at 50% sea level air density); and D) normdense normoxia (sea level). The analysis includes three comparisons between these atmospheric treatment groups to answer the following questions: 1) Are feeding rates slower in reduced P_{O_2} vs. sea level P_{O_2} (A & B vs C & D)? 2) Is a total reduction of air density at high altitude physiologically equivalent to the standard laboratory method of generating hypoxia (A vs. B)? 3) At sea level P_{O_2}, does reduced air density influence feeding rate (C vs. D). Feeding rate data was collected during three experimental replicates. Test replicate was included as a random variable in the model and explained 18.9% of the total model variance. Model $R^2 = 0.69$, $N = 474$.

Variable	DFDen[a]	$F-ratio$	$P-value$
temperature	464.3	644.7	< 0.0001
atmosphere (A & B vs C & D)	464.0	64.3	< 0.0001
atmosphere (A vs. B)	464.3	7.1	0.0081
atmosphere (C vs. D)	464.0	0.8	0.3623
temp x atmosphere (A & B vs C & D)	464.2	63.9	< 0.0001
temp x atmosphere (A vs. B)	464.1	3.6	0.0579
temp x temp	464.4	2.6	0.1109

[a] Degrees freedom corrected for models including random effects.

was only marginally non-significant (Table 3.2, temp x atmospheres A vs. B, $P = 0.0579$). At sea level values of P_{O_2}, reduced air density had no deleterious or beneficial effects on feeding rate (Fig. 3.3; Table 3.2, atmospheres C vs. D, $P = 0.3623$).

3.5 Discussion

Fly larvae are basically eating machines, so environmental conditions that influence their feeding rates are likely to have profound effects on fitness (Bakker, 1961, 1969; Mueller, 1988). Hypoxia and body temperature are two variables that likely influence feeding rate of *Drosophila melanogaster* and other insects living at high altitude. In this experiment, I used a factorial experimental design to decouple oxygen partial pressure and air density (Fig. 3.1; Dudley and Chai, 1996). This allowed me to address two questions. First, how do low P_{O_2} and temperature independently and interactively influence larval feeding rate? And second, are the standard laboratory methods of generating hypoxia (reduced percent oxygen) functionally equivalent to high-altitude hypoxia (reduced air density)?

The feeding rates of 474 larvae were measured at 5 temperatures (15, 20, 25, 27.5, and 30°C), two P_{O_2}'s (10.5 and 21.0 *kPA*), and two air densities (50% and 100% sea level), for a total of 20 treatment groups. The low P_{O_2} treatments (10.5 *kPa*) are equivalent to air at about 5,800 *m* altitude, a high, but not ecologically unrealistic altitude for insects (Hoback and Stanley, 2001). There were 4 different atmospheric treatments that are labeled as (Fig. 3.1): A) hypodense hypoxia (standard high altitude); B) normodense hypoxia (common experimental treatment where oxygen is replaced with nitrogen; C) hypodense normoxia (achieved by using a 42% oxygen concentration at 50% air density); and D) normodense normoxia (standard sea level).

3.5.1 Affects of low P_{O_2}

Reduced P_{O_2} generally slowed larval feeding rate (Fig. 3.3, lines A & B vs. C & D; Table 3.2), but only at higher temperatures. For example, at 30°C, feeding rate was about

27% slower in 10.5 *kPA* vs. 21 *kPA* O_2; however at 15°C, feeding rate was not affected by hypoxia. This interaction between temperature and oxygen is likely due to the differential effects of temperature on ectotherm metabolic rate, diffusion rates, and solubility of gases in fluids (Sibly and Atkinson, 1994; Atkinson and Sibly, 1996; Woods, 1999; Pörtner, 2001). Ectotherm metabolic rates increase exponentially with temperature (Gillooly et al., 2001) but diffusion rates increase only about 5% per 10°C increase in temperature (Denny, 1993), thus exacerbating the challenge of supplying tissue oxygen at elevated temperatures (Woods, 1999). Further complicating O_2 delivery is the reduction in the solubility of gases at warmer temperatures. For both reasons, insects appear to have smaller safety margins (Harrison et al., 2006) for oxygen delivery at warmer temperatures. At warm enough temperatures, even sea level atmospheric oxygen negatively influences the development of *D. melanogaster* larvae (Frazier et al., 2001) and of hawkmoth eggs (Woods and Hill, 2004).

Based on the interaction between temperature and oxygen, the impact of hypoxia at high altitudes may be mitigated by the decrease in temperature. Whether this is the case depends on the body temperatures insects maintain in the field, which is difficult to assess (Dillon et al., 2006). Body temperatures along an altitudinal gradient may not differ as much as predicted by the change in mean environmental temperature due to behavioral differences and the availability of microhabitats (Huey, 1991). Indeed, the developmental temperatures of *D. melanogaster* along an altitudinal gradient (using mutants with temperature sensitive eye color), were less varied than predicted based on the differences in mean environmental temperature (Jones et al., 1987). A similar result was observed in grasshoppers (Ashby, 1997).

Insects are often considered relatively impervious to low P_{O_2} because many studies show that insect metabolic rate, survival, and many behaviors are not affected until extraordinarily low P_{O_2}'s are reached (i.e. less than 5% P_{O_2}; reviewed by Hoback and Stanley, 2001; Greenlee and Harrison, 2004). However, under certain circumstances, the safety margin for oxygen delivery may be smaller than suggested by these experiments. For example, long-term exposure to hypoxia may be more stressful than short-term exposure

(Dillon et al., 2006). Mealworms (Loudon, 1988; Greenberg and Ar, 1996) and fruitflies (Frazier et al., 2001) that develop in low hypoxia take longer to develop, and have reduced survival. Furthermore, hypoxia may affect some stages of insect development more than others, especially when convective O_2 delivery is limited, such as in eggs (Woods and Hill, 2004) and in larvae (Keister and Buck, 1961). Metabolically demanding activities are also more likely to be affected by low P_{O_2}. In this case, larval feeding is likely to require a great deal of oxygen given the frequency of the behavior (3.3 bites per second at 25°C) and the large fraction of the larval body that participates in the activity. The high oxygen requirements of feeding behavior are further supported by the extensive tracheation of the larval mouthparts.

For insects living at high altitude, the physiological effects of hypoxia may be ameliorated by adaptation or beneficial plasticity. Insects have an arsenal of short- and long-term methods to help regulate internal P_{O_2} in response to challenging environments (reviewed by Harrison et al., 2006; Dillon et al., 2006). For example, one hour after exposure to hypoxia, *D. melanogaster* begin growing more tracheae (Jarecki et al., 1999). Tracheae have also been shown to increase in diameter both through developmental plasticity (Loudon, 1989; Henry and Harrison, 2004) and evolution (Henry and Harrison, 2004). Given this, insects that actually live at high altitude may be less affected by hypoxia than short-term exposure would suggest. However, there are trade-offs to investing resources and space to respiratory structures versus other body tissues (Conley and Lindstedt, 2002; Dillon et al., 2006).

3.5.2 P_{O_2} vs. air density

At high altitude, hypoxia results from an overall reduction in air density. In most laboratory experiments, however, hypoxia is generated by replacing oxygen with nitrogen, while maintaining total air densities near sea-level. Whether these conditions are functionally equivalent is unknown, and to my knowledge have not specifically been compared in insects. Theoretical reasons suggest that the effects of reduced P_{O_2} may be mitigated by increased diffusion at high altitude. Although the reduction in P_{O_2} along an altitudinal gra-

dient reduces the driving force for diffusion, the concomitant drop in air density increases the diffusion coefficient because air molecules can travel further before encountering another molecule. In theory, these forces perfectly compensate for one another (Denny, 1993).

High-altitude environments may not compromise tissue oxygen delivery in insects to the extent predicted by most laboratory studies. Results from this present study suggest this is indeed the case. Feeding rates were faster in high altitude hypoxia than in standard laboratory hypoxia (Fig. 3.3; Table 3.2, A vs. B, $P = 0.0081$). Averaged across all temperatures, feeding rate was about 10% faster when low P_{O_2} was due to reduced air density. However, at sea level values of P_{O_2}, reduced air density had no deleterious or beneficial effects on feeding rate (Fig. 3.3; Table 3.2, C vs. D, $P = 0.3623$).

This study helps illuminate one aspect of basic insect physiology. To obtain oxygen, most insect species rely on a combination of convection and diffusion through air and across the air/fluid interface. However, the major barrier to oxygen delivery is uncertain. Because the effects of low P_{O_2} appear to be mitigated when air density is reduced, gaseous diffusion appears to be a significant barrier to oxygen delivery for the tracheal system of *D. melanogaster*. Whether this also applies to insects that appear to depend more on convective oxygen delivery will require further research.

3.5.3 Conclusions and other notes

Differences in gross feeding rate could be due to changes intrabout duration, number of feeding movements within each bout, and the duration of intervals between bouts (Ruiz-Dubreuil et al., 1996). I measured intrabout feeding rate because it is the best predictor of gross feeding rate (Ruiz-Dubreuil et al., 1996). Furthermore, it correlates positively with larval growth rate (Ruiz-Dubreuil et al., 1996; Burnet et al., 1977), adult body size (Ruiz-Dubreuil et al., 1996; Burnet et al., 1977), viability (Ohnishi, 1979), and competitive ability (Joshi and Mueller, 1988, 1996; Santos et al., 1997). Whether the other components of feeding rate show the same sensitivity to temperature and oxygen remains to be determined.

Insects living at high altitude must deal with hypoxia and temperature. This present

study suggests that hypoxia and temperature have both direct and interactive effects on the feeding capacity of larval *D. melanogaster*. This implies that experimental studies of insect performance at altitude require factorial manipulations of both hypoxia and temperature. Ultimately, understanding how high altitude hypoxia affects insects in the field will require more natural history data on the temperatures experienced by larvae, pupae, and adults (Dillon et al., 2006). This information may facilitate the development of more accurate models to predict the impacts of global climate change on insects living on mountains. Furthermore, this study suggests that the normodense hypoxic conditions most often used in experiments probably provide a decent, but biased estimate, of the effects of high-altitude hypoxia on insect physiology. Specifically, many laboratory studies may overestimate the influence of hypoxia at high altitudes on insect physiology and behavior.

Chapter 4

TEMPERATURE-SENSITIVITY OF MORPHOLOGICAL DEVELOPMENT AND FLIGHT PERFORMANCE IN *DROSOPHILA*: EVIDENCE FOR BENEFICIAL PLASTICITY[†]

4.1 Summary

The alpine environment is likely to challenge insect locomotion because of low mean temperatures and reduced barometric pressure. In this study, we measured the direct and interactive effects of these factors on walking and flight performance of wild-caught *Drosophila melanogaster* Meigen. We found that decreased temperature and decreased air pressure both reduced walking speed and flight performance. Flies walked more slowly at 18°C and in the lowest air pressure treatment (34 kPa). This treatment, equivalent in air pressure to the top of Mount Everest, was the only air pressure that significantly reduced fly walking speed. Therefore, walking performance in the wild is likely limited by temperature, but not oxygen availability. In contrast to walking performance, low but ecologically realistic air pressures dramatically reduced overall flight performance. The effects of reduced air pressure on flightperformance were more pronounced at colder temperatures. Reduced flight performance in high altitude conditions was primarily driven by an increased reluctance for flies to initiate flight rather than outright failure to fly. Such reluctance to fly in high altitude conditions may in part explain the prevalence of aptery and brachyptery in high altitude insects. The observed interactive effects of temperature and air pressure on flight performance confirm the importance of simultaneously manipulating both of these factors when studying the impact of altitudinal conditions on insect physiology and behavior.

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4.2 Introduction

Insects rely on flight to find mates, interact socially, evade predators, and obtain resources; when flight performance is compromised these fitness-related traits will be impaired. Cold temperatures challenge the flight performance of ectothermic insects; yet many flying insect species maintain healthy populations in cold environments. To fly in these challenging environments insects must compensate with adjustments in morphology and physiology via the processes of local genetic adaptation, acclimatization, and developmental plasticity. In this study, we test the "beneficial plasticity" hypothesis (i.e. that adult organisms have a competitive advantage in the environment in which they develop) using a factorial experimental design (Huey and Berrigan, 1996; Huey et al., 1999). Specifically, we tested whether *Drosophila melanogaster* Meigen that develop in colder environments have better free-flight performance at cold temperatures than those that develop in warmer environments. To determine which traits could be contributing to the improved cold flight performance of flies that develop at cold temperatures, we examined the thermally dependent variation of several traits, including body mass, wing area, wing loading, wing length, relative flight muscle mass, and wing beat frequency.

Evolutionary physiologists often hypothesize that developmental plasticity – the physiological and morphological responses of a single genotype to an organism's environment during development (Stearns, 1992) – is beneficial. However, many experiments that have explicitly tested for beneficial plasticity or acclimation have either rejected this hypothesis (Blanckenhorn, 2000; Gibbs et al., 1998; Gibert et al., 2001; Huey et al., 1995; Leroi et al., 1994; Wilson and Franklin, 2002; Woods, 1999; Woods and Harrison, 2001; Zamudio et al., 1995; Zwaan et al., 1992) or have had mixed results (Bennett and Lenski, 1997; Carter and Wilson, 2006; Deere and Chown, 2006; Deere et al., 2006; Stillwell and Fox, 2005), suggesting that alternative hypotheses, such as "colder/hotter is better," or "optimal developmental temperature" may be evolutionarily more important than beneficial plasticity and acclimation (Huey et al., 1999). This otherwise intuitive hypothesis may lack exper-

imental support for a number of reasons. These include potentially high costs of plasticity, unreliable or insensitive cues triggering plasticity, evolutionary constraints, and long-term negative effects of non-optimal conditions on organisms (DeWitt et al., 1998; Wilson and Franklin, 2002; Woods and Harrison, 2002).

Flying at cold temperatures is challenging for insects. As ectotherms, their physiology is strongly determined by temperature (Gillooly et al., 2001; Huey and Kingsolver, 1989), and flying insects have the highest metabolic requirements measured for any organism (Harrison and Roberts, 2000). Therefore any cold-induced reduction in metabolism is likely to limit their ability to fly. Cold temperatures impair the contractile properties of muscle (Josephson, 1981), resulting in lower wing-beat frequencies and reduced power output during tethered (Curtsinger and Laurie-Ahlberg, 1981) and free flight (Lehmann, 1999). As temperatures decline, fruitflies become less motivated to initiate flight and more likely to experience flight failure (Dillon and Frazier, 2006). Potentially compounding the problem, most ectothermic species mature at larger body sizes when they develop in cold temperatures (Atkinson, 1994), and therefore must generate more power to support their extra body weight during flight (Dillon and Dudley, 2004).

Insects may compensate for reduced flight performance in cold temperatures by altering the physiology of flight muscles, which generate power, or the morphology of wings, which generate aerodynamic forces. Flight muscle performance could be improved through changes in biochemistry (Hochachka and Somero, 2002; Laurie-Ahlberg et al., 1985), or by increasing the mass of flight muscle relative to body mass (Marden, 1987). These changes could translate into increased force and power production through changes in gross kinematics (wing-beat frequency and stroke amplitude) or more subtle changes in the three dimensional motions of the wings (Dickinson et al., 1999; Dudley, 2000; Sane, 2003). Insects from cold environments could also improve flight performance by increasing wing area relative to body mass (i.e. decreasing wing loading, body weight $\times$ wing area^{-1}, Nm^{-2}), which should reduce induced power requirements and increase lift production (Dudley, 2000). Insects that develop in cold temperatures tend to have lower wing loading due to

both evolutionary and plastic responses (Azevedo et al., 1998; David et al., 1994; Gilchrist and Huey, 2004; Loeschcke et al., 1999; Morin et al., 1999; Norry et al., 2001; Petavy et al., 1997; Starmer and Wolf, 1989). Changes in wing shape may also improve flight performance. For example, elongating the wing, while maintaining the same wing area, should theoretically improve some aspects of flight performance because the higher translational velocity of the wing tips (at the same angular velocity) yields greater aerodynamic forces (Ellington, 1984; Pennycuick, 1968). To test the main prediction of the beneficial plasticity hypothesis, that adult performance is enhanced in the environment in which the organism develops, we reared *D. melanogaster* at three temperatures and tested free-flight performance at cold and moderate temperatures. We chose a range of developmental temperatures within those commonly experienced by this species in the field and lab to avoid possible stressful effects (Wilson and Franklin, 2002). We also examined body, wing and muscle morphology to determine how thermally dependent variation in these traits may contribute to flight success in cold temperatures. Specifically, we predicted that flies developing in cold environments would have increased wing beat frequencies, relatively larger flight muscles, decreased wing loading, and longer wings (after controlling for wing area).

4.3 Methods

4.3.1 Experimental animals

The *Drosophila melanogaster* used in this experiment were collected as eggs from an Ives line maintained in the laboratory at room temperature (~24°C, see Frazier et al., 2001, for colony and egg collection information). The eggs were placed 20 per vial (9.5 cm long, 2.2 cm diameter, ~9 mL dextrose diet) to control population density. These flies were then reared at 15, 23 or 28°C (T_{dev}) in temperature controlled incubators under a 14L:10D light cycle. As flies began emerging, we transferred the adults to fresh food vials every 8 *h* to control for age. Males and females were separated to prevent mating. We allowed the flies to mature for 48-72 *h* before starting flight assays because wing beat frequency and power

output increases until 2 days of age and then remains constant from 2 to 8 days of age (Curtsinger and Laurie-Ahlberg, 1981). During the adult maturation period, all flies were held at 22°C to ensure that any developmental effects were due to beneficial plasticity rather than reversible, short-term acclimation after emergence ("phenotypic flexibility," Piersma and Drent, 2003).

4.3.2 Flight assay

To evaluate flight performance at cold temperatures, flies were randomly assigned to one of three flight test temperatures: 14, 16, or 18°C (T_{test}; respective means$\pm s.d.$: 14.5$\pm$0.29, 16.02$\pm$0.22, 18.16$\pm$0.20; based on the mean start and end temperature recording for each flight test). Individual flies were aspirated into a covered 500 *ml* water-jacketed beaker (Konte, Vineland, New Jersey, USA), the jacket of which was continually flushed with temperature controlled water. This flight chamber was housed in a temperature-controlled incubator. The temperature inside the flight chamber was monitored throughout the experiment using a calibrated thermocouple thermometer (Physitemp Bat-12, Bailey Instruments Inc., Saddlebrook, NJ, USA) to ensure that the temperature did not significantly deviate from the T_{test}. The water-jacketed beaker successfully buffered the temperature inside the flight arena, across all treatments, the difference between the highest and lowest temperature during a flight test averaged 0.40°C$\pm$0.33 *s.d.* After a one-minute thermal equilibration period, we encouraged escape behavior by chasing the fly with the tip of a fine, thermally-equilibrated paintbrush inserted into the beaker through an opening in a piece of rubber covering the top of the beaker. We scored the flight performance of each fly, placing it in one of three categories: those that could take off and fly the full width of the beaker (8 cm) were categorized as performing a "flight"; those that flew $>$ 5 cm but $<$ 8 cm, stereotypically a take-off followed by an arching loop ending on the chamber floor, were categorized as generating "lift" (these flies were unable to sustain flight, but we considered this behavior distinct from "failed" flight because they traveled further than the maximum jumping distance observed in preliminary experiments of wingless flies); flies that traveled

$<$ 5 cm, stereotypically were unable to generate any lift and do little more than jump off of the bottom of the chamber, were "failed" fliers. We continued chasing the fly until it performed a flight or 5 minutes had passed.

4.3.3 Morphological and physiological data

We immediately weighed each fly after the flight assay, on a Cahn C-33 microbalance (± 2 μg; Cahn Instruments, Inc., Cerritos, CA, USA) and then preserved the fly in 70% ethanol. For measures of wing morphology, both wings were removed and mounted on slides. Total wing area and wing length for each fly was quantified to the nearest 2 μm using a computer-controlled microscope-mounted digital camera and Scion Image software (Scion Corporation, Frederick, Maryland, USA). We estimated flight muscle ratio (*FMR*) as the ratio of dry thorax mass to dry body mass. The head, thorax, and abdomen were separated and dried for 24 *h* at 55°C, and then immediately weighed using the Cahn microbalance. The thorax primarily houses flight muscle and thus provides an index of flight muscle mass.

We measured the wing-beat frequencies (WBF) of the flies that performed a flight or generated lift with an optical tachometer, which converted fluctuations in light due to wing beats into a sound recording on tape (Unwin and Ellington, 1979). A battery-powered light was wrapped with a white piece of paper and positioned directly behind the flight chamber. This provided diffuse lighting and a high-contrast background that was optimal for operating the tachometer. The optical tachometer recordings were digitized and visualized using the SpectraPLUS sound analysis program (Pioneer Hill Software, Poulsbo, Washington, D.C.) as previously described (Roberts, 2005, 1998; Roberts et al., 2004). Each recorded sequence contained 6-10 clearly distinguishable, uninterrupted wing beats. For a given fly, WBF was determined to the nearest 0.2 Hz by dividing the number of clearly distinguishable, uninterrupted wing beats in the sequence by the duration of the sequence (measured to the nearest 0.0001 s).

4.3.4 Data analysis

To analyze the effects of flight temperature (T_{test}) and developmental temperature (T_{dev}) on flight performance, we used an ordinal logistic regression model because our metric of flight performance was an ordered categorical response variable. We included in the model appropriately centered interaction terms to test for beneficial acclimation ($T_{test} \times T_{dev}$) and squared terms to fit observed curvilinearity in the response variable. Statistical analyses were done in R (R Development Core Team, 2005). We used ordinary least squares regression to analyze morphological and physiological variation of traits. For all analyses, we compared partial deviances (χ^2 tests) of models with different combinations of main effects, interactions, and squared effects to obtain the final model. Type I error was set at 0.05.

4.4 Results

4.4.1 Flight performance

Flies developed in one of three temperatures (T_{dev}: 15, 23, 28°C), and we categorized the flight performance of each fly at a single air temperature (T_{test}: 14, 16, 18°C), for a total of 9 treatment groups. There were 30-35 flies in each treatment group and 282 flies in the overall experiment (Fig. 4.1).

We used ordinal logistic regression to assess the effects of test temperature, development temperature, and gender on flight performance (Table 4.1). The final model fit the flight performance data well (model likelihood ratio $\chi^2 = 243.57$; $df = 6,282$; Nagelkerke $R^2 = 0.658$), and accurately predicted flight performance based on measures of association (Goodman-Kruskal $\gamma = 0.82$; Somers' $D = 0.796$; Kendall's τ-α rank correlations between predicted probabilities and observed responses $= 0.509$), and the average sensitivity over all possible specificities was high (c-index $= 0.898$, i.e. area under ROC curve, Swets, 1988).

Test temperature had the largest effect on *D. melanogaster flight* performance (Fig. 4.1;

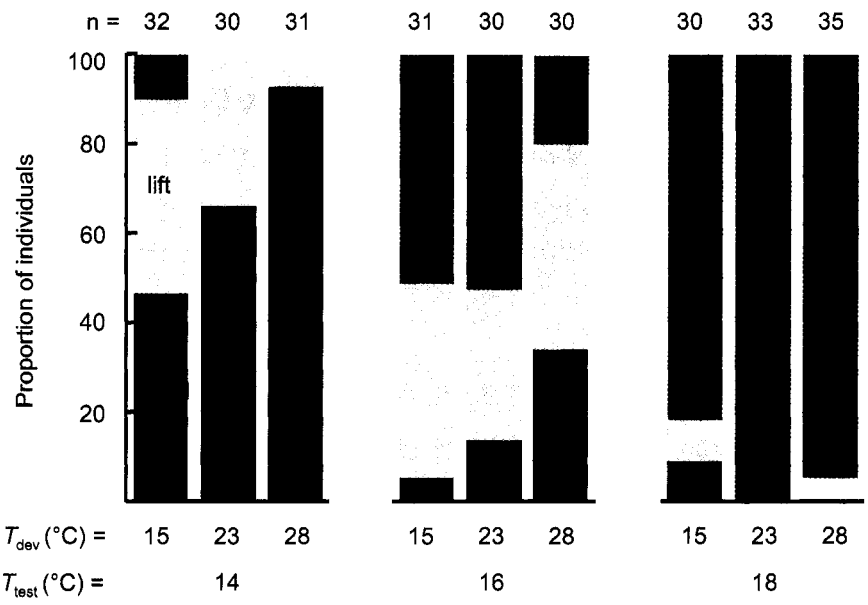

Figure 4.1: Effects of test temperature (T_{test}) and development temperature (T_{dev}) on flight performance of *D. melanogaster*. Green indicates proportion of flies that were able to perform a flight, yellow indicates lift generation (but not flight); red indicates flight failure (see methods for details). Flies that developed in colder temperatures had significantly better flight performance in colder temperatures (see Table 4.1), indicating beneficial plasticity.

Table 4.1: Ordinal logistic regression assessing the effects of gender, test temperature (T_{test}), developmental temperature (T_{dev}), and their interaction (i.e. beneficial plasticity) on fruit fly performance.

Variable (df)	Coefficient	SE	χ^2	P-value	Odds ratio
T_{test} (1)	1.526	0.146	109.05	< 0.0001	4.602
T_{dev} (1)	-0.122	0.035	11.79	0.0006	0.885
$T_{test} \times T_{dev}$ (1)	0.089	0.024	13.15	0.0003	1.093
${T_{test}}^2$ (1)	0.010	0.079	0.02	0.08961	1.010
${T_{dev}}^2$ (1)	-0.029	0.010	8.73	0.0031	0.971
gender (1)	-0.242	0.289	0.70	0.4018	1.274

Table 4.1, T_{test}, $P < 0.0001$). At the lowest test temperature (14°C), only 3% of the flies were able to fly, whereas, at the highest test temperature (18°C) nearly all flies were able to fly ($\sim$ 93%). Developmental temperature also influenced the flight performance of *D. melanogaster*. Flies that developed at 15°C had the highest probability of flying at the coldest test temperature, indicating beneficial plasticity (Fig. 4.1; Table 4.1, $T_{test} \times T_{dev}$, $P = 0.0003$). At 14°C, cold-reared (15°C) flies failed 47% of the time, whereas warm-reared (28°C) flies failed 94% of the time. A similar trend was observed at 16°C; flies reared at 15°C failed 6% of the time, whereas flies reared at 28°C failed 33% of the time. Development temperature also had significant curvilinear effects (squared terms) on flight performance; flight performance declined more rapidly as developmental temperature went from 28 to 23°C than it did from 23 to 16°C (Fig. 4.1; Table 4.1, ${T_{dev}}^2$, $P = 0.0031$).

4.4.2 Temperature effects on morphology and WBF

D. melanogaster were larger when they developed in cold temperatures (Fig. 4.2; Table 4.2A, T_{dev}, $P < 0.001$). Females were also significantly larger than males (Fig. 4.2;

Table 4.2A, gender, $P < 0.001$), and there was a significant interaction between gender and development temperature on body mass (Table 4.2A, $T_{dev}\times$ gender, $P < 0.001$), suggesting that developmental temperature affected males and females differently. Specifically, male body size increased relatively more than did female body size when developmental temperature shifted from 23 to 16°C.

Wing area was larger in cold-reared flies (Fig. 4.2A), and more than compensated for the increase in body size. As a result, flies developing at cold temperatures had the lowest wing loading (Fig. 4.2B; Table 4.2B). The scaling relationship between body mass and wing area depended on whether the variation in these two variables was due to development temperature or other factors. When the variation in body size and wing area was due to developmental temperature, then wing area scaled with $\text{mass}^{2.41}$ (based on a regression analysis using the mean body mass and mean wing area for each developmental temperature and gender, hollow black points on Fig. 4.2A; 95% confidence interval: 1.32 to 3.50, $N = 6$). This scaling relationship favors the flight performance of the larger, cold-reared flies, which was unexpected given empirical and theoretical scaling relationships between body size and wing area at a set rearing temperature. Indeed within each developmental temperature, wing area scaled with $\text{mass}^{0.32}$ (calculated from the mean of the slopes from each T_{dev} and gender group, i.e. the solid lines in Fig. 4.2A, $N = 6$).

Another potential mechanism insects may use to improve flight performance in cold temperatures is increasing the proportional length of their wings. To determine if flies that developed in cold temperatures had relatively longer wings, we analyzed residuals from wing length regressed on wing area to control for the fact that wings with greater areas were also longer (Pearson's Correlation Coefficient $= 0.94$, $N = 228$). We found that residual wing length was not statistically influenced by developmental temperature ($P = 0.631$), insect mass ($P = 0.236$), or gender ($P = 0.332$).

Flying insects could also increase their flight muscle ratio (FMR, mass thorax $\times$ mass body^{-1}) to generate more power for flight. We predicted that flies from cold developmental temperatures would have a higher FMR. On average, thorax dry mass was $\sim 50\%$ ($\pm 8.3\%$

Table 4.2: Factors affecting: (A) mass, (B) wing loading, and (C) wing beat frequency in *D. melanogaster*. The F, P, and R^2 statistics are provided for each model, followed by estimates and statistics for each variable within the model.

Variable	Estimate (95% CI)	*SE*	*t*-value	*P*-value
A. ln*mass* (mg): $F_{4,277} = 437.7, P < 0.001, R^2 = 0.86$				
T_{dev}	-0.0084 (-0.0108, -0.0061)	0.0012	-7.10	< 0.001
${T_{dev}}^2$	-0.0010 (-0.0015, -0.0005)	0.0003	-4.12	< 0.001
gender (males)	-0.1835 (-0.2579, -0.1091)	0.0378	-4.86	< 0.001
$T_{dev}\times$ gender	-0.0062 (-0.0094, -0.0029)	0.0017	-3.74	< 0.001
B. wing loading (Nm^{-2}): $F_{3,254} = 616.9, P < 0.001, R^2 = 0.88$				
T_{dev}	0.0772 (0.0731, 0.0814)	0.0021	36.664	< 0.001
mass (*mg*)	1.9250 (1.7077, 2.1424)	0.1104	17.444	< 0.001
gender (males)	0.2855 (0.2045, 0.3665)	0.0411	6.941	< 0.001
[a]C. ln*wingbeat frequency* (s^{-1}): $F_{5,102} = 84.18, P < 0.001, R^2 = 0.80$				
T_{dev}	0.0096 (0.0037, 0.0156)	0.0030	3.202	0.002
T_{test}	0.0399 (0.0337, 0.0460)	0.0031	12.892	< 0.001
ln*mass* (mg)	0.2405 (0.0873, 0.3937)	0.0772	3.114	0.002
ln*wing area* (mm^2)	-0.1794 (-0.4150, 0.0563)	0.1188	-1.510	0.134
gender (males)	0.0482 (0.0042, 0.0923)	0.0222	2.173	0.032

[a] Includes flies from both the flight and lift categories of performance, but not failed flight. Flies that performed a flight or generated lift are grouped in the analysis because there was no significant difference in *WBF* between these flies (when flight performance was included as a variable in the above analysis, $P = 0.65$).

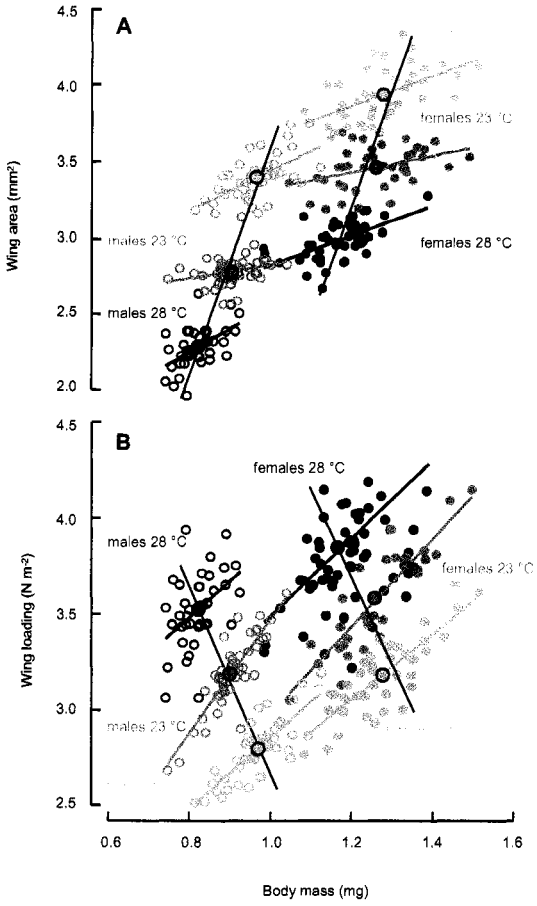

Figure 4.2: Effects of body mass, development temperature, and gender (females, closed points; males, open points) on: (A), wing area, and (B) wing loading of *D. melanogaster*. When wing-loading was compared within a single developmental temperature larger flies had greater wing-loading (solid regression lines indicate relationships within T_{dev} and gender treatments). However, this scaling relationship was dramatically altered when variation in wing area and body size was due to development temperature, such that flies from colder temperatures had much lower wing loading (dashed black lines indicate relationships across developmental temperatures). The black outlined points indicate mean body mass and wing area/loading for each treatment group.

sd, $N = 282$) of total body dry mass and this ratio was not correlated with developmental temperature ($P = 0.416$), mass ($P = 0.236$), or gender ($P = 0.332$).

Although the percentage of thorax (and presumably flight muscle) remains constant, insects developing in cold environments might have increased wing beat frequencies (WBF) due to changes in muscle physiology. For this analysis, we included flies that performed a "flight" and those that generated "lift" (those that failed to fly were excluded); we combined these two groups in the analysis because they did not have significantly different WBFs ($P = 0.65$). WBF declined with decreasing flight temperature (Fig. 4.3; Table 4.2C, T_{test}, $P < 0.001$), as observed in other studies (Curtsinger and Laurie-Ahlberg, 1981; Laurie-Ahlberg et al., 1985; Lehmann, 1999; Stevenson and Josephson, 1990; Unwin and Corbet, 1984), and males had significantly faster WBFs (Fig. 4.3, open points; Table 4.2C, gender, $P = 0.032$). Flies that developed in cold temperatures had significantly lower WBFs than flies that developed in warm temperatures at all test temperatures (Fig. 4.3, red vs. orange and blue points; Table 4.2C, T_{dev}, $P < 0.002$). These data demonstrate that cold-reared fruitflies do not compensate for colder flight temperatures by increasing wing beat frequency.

4.5 Discussion

D. melanogaster exhibited beneficial developmental plasticity in flight performance in response to cold development temperatures (Fig. 4.1, Table 4.1). As previously demonstrated (Lehmann, 1999), temperatures less than 18°C impeded the flight performance of *D. melanogaster*. At 16°C, only $\sim 42\%$ of the flies we tested were flight capable and only 3% could fly at 14°C. However, flies reared in colder temperatures had a significant flight advantage in comparison to flies reared in warmer temperatures. For example, at 16°C, 52% of cold-reared flies (18°C) were able to fly, whereas only 20% of warm-reared flies (28°C) could fly. At 18°C test temperatures, there were no statistically significant differences in the flight performance of warm- and cold-reared flies (nearly all flies could fly). The large number of recent studies that have rejected beneficial plasticity and acclimation (Blanckenhorn,

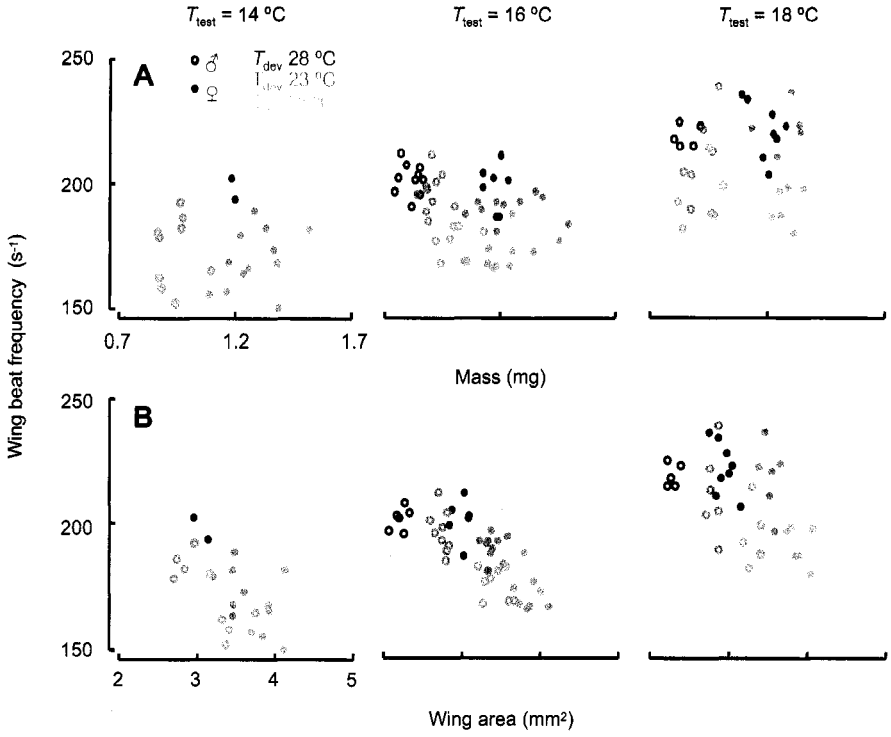

Figure 4.3: Wing beat frequency (s^{-1}) of *D. melanogaster* as a function of flight temperature (T_{test}, °C), developmental temperature (T_{dev}, °C), body mass (A), and wing area (B). As test temperature increased, wing beat frequency significantly increased (plots from left to right). Flies developing at cold temperatures (blue points, 18°C) had significantly lower wing beat frequencies at every test temperature in comparison to flies developing at intermediate temperatures (orange points, 23°C) or warmer temperatures (red points, 28°C). Males (open points) had faster wing beat frequencies than females (filled points). Heavier flies tended to have faster *WBF*s (A; Table 4.2C), and wing area did not significantly influence *WBF* (B; Table 4.2C) after statistically controlling for T_{rear}, T_{test}, and gender.

2000; Gibbs et al., 1998; Gibert et al., 2001; Huey et al., 1995; Leroi et al., 1994; Wilson and Franklin, 2002; Woods, 1999; Woods and Harrison, 2001; Zamudio et al., 1995; Zwaan et al., 1992), suggest that these processes may be evolutionarily insignificant for compensatory responses of organisms to their environments. However, other studies have demonstrated that at least some traits exhibit beneficial plasticity or acclimation (Ayrinhac et al., 2004; Barnes and Laurie-Ahlberg, 1986; Fischer et al., 2003; Li and Wang, 2005; Seebacher and Wilson, 2006; Wilson and Franklin, 1999). Our results suggest that beneficial plasticity of wing area may help *D. melanogaster* occupy a wide range of thermal environments, particularly cooler ones.

The only explanation we found for the improved flight performance of cold reared flies at cold temperatures was a dramatic increase in wing area relative to body mass (i.e. decreased wing loading; Fig. 4.2; Table 4.2B). The other traits we measured either did not vary with developmental temperature (wing length, flight muscle ratio), or varied in a way that would not benefit flight performance (wing beat frequency).

Similar to most other ectotherms (Atkinson, 1994), flies that developed in cold environments had larger overall body sizes (Fig. 4.2; Table 4.2A); but their wing area increased disproportionately such that they had dramatically lower wing loadings than flies that developed in warm environments (Fig. 4.2B; Table 4.2B), giving them an aerodynamic advantage at cold flight temperatures. In order to aerodynamically compensate for the increase in body size (assuming all else remains equal) the wing area of flies from cold environments must scale isometrically with body mass (wing area $\propto$ mass^1) because lift is directly proportional to wing area (Denny, 1993; Dudley, 2000). In fact, when variation in wing area and body mass was due to developmental temperature, then wing area scaled with mass$^{2.41}$ (black, dashed lines in Fig. 4.2A). This scaling relationship is surprising given the expectation that the scaling coefficient of wing area to body mass should be ~ 0.66 based on dimensional analysis predictions (Pennycuick, 1992) and < 1 based on empirical data from comparative studies both within and among species (Casey and Joos, 1983; Dillon and Dudley, 2004; Dillon and Frazier, 2006; Dudley, 2000; Gilchrist and Huey, 2004; Starmer

and Wolf, 1989). Indeed, in this study we found that *within* a developmental temperature, wing area scaled with mass$^{0.32}$ (average of the 6 solid regression lines in Fig. 4.2A). These data suggest that the scaling relationship, and thus the developmental pathway, between body mass and wing area depends on whether the variation in these traits is due to developmental temperature or other variables, such as food supply during larval development or genetic variation in body size.

Decreased wing loading in *Drosophila* spp. in response to cold developmental temperatures has been observed in other studies (Barnes and Laurie-Ahlberg, 1986; David et al., 1994; Gilchrist and Huey, 2004; Petavy et al., 1997). Due to the theoretical advantages of reduced wing loading for generating lift during flight (Dudley, 2000) and increasing mechanical power output (Barnes and Laurie-Ahlberg, 1986), this response has been hypothesized to be adaptive for flight (Loeschcke et al., 1999; Norry et al., 2001; Starmer and Wolf, 1989), and has been demonstrated in some cases (Roberts et al., 2004; Stalker, 1980; but see: Dillon and Dudley, 2004; Dillon and Frazier, 2006; Marden, 1987). The association between wing loading and flight performance in these flies reared at different temperatures indicates a specific pathway of beneficial developmental plasticity.

In response to cold developmental temperatures, physiological changes in the flight muscle could improve flight performance (Barnes and Laurie-Ahlberg, 1986; Hochachka and Somero, 2002; Rogers et al., 2004). Changes in the biochemistry of flight muscles may translate into increased lift via effects on wing-beat kinematics. Contrary to our predictions based on the beneficial plasticity hypothesis, flies that developed in cold temperatures had significantly lower wing beat frequencies than flies that developed at warm temperatures at all test temperatures (Fig. 4.3; Table 4.2C). This result is consistent with Barnes and Laurie-Ahlberg (1986), and may be explained by the fact that the flies that developed in cold temperatures were heavier and had larger wings; and larger insects tend to have reduced wing beat frequencies (Dillon and Dudley, 2004; Dudley, 2000) due to resonance issues and an increase in the induced power required to move a larger wing.

Given that WBF is predicted to decline with larger body and wing sizes (Dudley, 2000),

flies from cold developmental temperatures could have been partially compensating by beating their wings faster than expected given their larger body and wing size. However, we did not see any evidence of compensation because cold-reared flies had slower *WBF*s than similarly sized flies from warmer developmental temperatures (Fig. 4.3A); furthermore, based on our data, heavier flies actually appeared to have faster wing beat frequencies than smaller flies (Table 4.2C, ln*mass*) and there was no significant influence of wing area on *WBF* (Fig. 4.3B; Table 4.2C, ln*wingarea*) after statistically controlling for developmental temperature, test temperature, and gender.

D. melanogaster that develop in cold environments could still have physiological mechanisms that improve flight muscle performance that we did not measure. Flies could increase force production during flight at cold temperatures by adjusting other wingbeat kinematics, such as stroke amplitude, the timing of wing rotation, the wing angle of attack, or the inclination of the stroke plane (Fry et al., 2005; Sane, 2003; Sane and Dickinson, 2001).

Flying insects could also compensate for cold temperatures by increasing the ratio of flight muscle to body mass (*FMR*). Marden (1987) found that flight muscle ratio was a strong predictor of take-off performance among 61 species of birds, bats, and insects. However, fruit flies from cold developmental temperatures did not have a greater flight muscle ratio than fruit flies from warm developmental temperatures, nor did *FMR* vary with insect mass, or gender.

The relative contributions of genetic adaptation, plasticity, and acclimatization, to the success of species distributed across thermal gradients are generally unknown. Genetic adaptation plays some role given that numerous studies have documented local genetic differences for insect populations across environmental gradients for traits such as wing size (Azevedo et al., 1998; David et al., 1994; Gilchrist and Huey, 2004; Loeschcke et al., 1999; Morin et al., 1999; Norry et al., 2001; Petavy et al., 1997; Starmer and Wolf, 1989) and chill tolerance (Ayrinhac et al., 2004). Developmental plasticity and/or acclimatization may also be important, especially given that local genetic adaptation may be hindered by extensive gene flow, especially in mobile insect species with large geographic ranges, such as fruit

flies. Although several recent studies suggest that beneficial plasticity or acclimation may not be an evolutionarily important mechanisms, there is evidence that it may contribute to the ability of *D. melanogaster* to occupy a wide range of thermal environments. For example, Ayrinhac and colleagues (2004) showed that the recovery of *D. melanogaster* from chill coma was due more to phenotypic plasticity (explaining 80% of the variability in this trait) than to genetic differences between high and low latitude populations (explaining 4% of the variability of this trait). For wing-loading, phenotypic plasticity may also be more important than population level genetic differences for fruitflies in cold environments. Gilchrist and Huey (2004) showed that high latitude (Denmark, 56°0′) populations of female *D. subobscura* had 9% greater wing areas than low latitude populations (Spain, 36°45′); but flies that developed at 15°C had 36% greater wing areas than flies at 25°C. In this study, we documented strong developmental plasticity of flight performance in response to cold developmental temperatures, due to decreased wing loading.

Chapter 5

THERMODYNAMICS CONSTRAINS THE EVOLUTION OF INSECT POPULATION GROWTH RATES: "WARMER IS BETTER" †

5.1 Summary

Diverse biochemical and physiological adaptations enable different species of ectotherms to survive and reproduce in very different temperature regimes, but whether these adaptations fully compensate for the thermodynamically depressing effects of low temperature on rates of biological processes is debated. If such adaptations are fully compensatory, then temperature-dependent processes (e.g., digestion rate, population growth rate) of cold-adapted species will match those of warm-adapted species when each is measured at its own optimal temperature. Here we show that cold-adapted insect species have much lower maximum rates of population growth than do warm-adapted species, even when we control for phylogenetic relatedness. This pattern also holds when we use a structural-equation model to analyze alternative hypotheses that might otherwise explain this correlation. Thus, although physiological adaptations enable some insects to survive and reproduce at low temperatures, these adaptations do not overcome the "tyranny" of thermodynamics, at least for rates of population increase. Indeed, the sensitivity of population growth rates of insects to temperature is even greater than predicted by a recent thermodynamic model. Our findings suggest that adaptation to temperature inevitably alters the population dynamics of insects. This result has broad evolutionary and ecological consequences.

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5.2 Introduction

Body temperature profoundly affects the physiology, performance, and fitness of ectotherms, which include most organisms on earth. Diverse biochemical and physiological adaptations allow different ectotherms to survive and reproduce in temperature regimes ranging from polar oceans to thermal vents (Cossins and Bowler, 1987; Hochachka and Somero, 2002). Nevertheless, whether such adaptations are able to compensate for the rate-depressing effects of low temperature on biochemical reaction rates of cold-adapted species is debated.

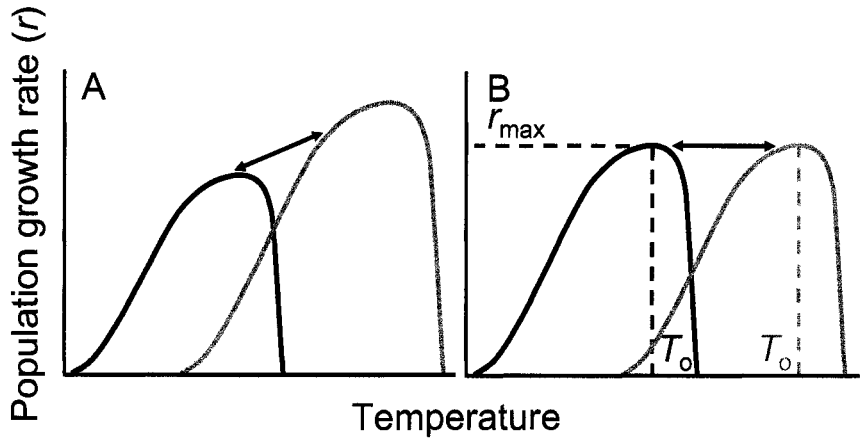

Figure 5.1: Two competing hypotheses predict how the maximum population growth rate of ectotherms evolves in response to temperature adaptation. Here we present extreme versions of each. (A) The thermodynamic hypothesis ("warmer is better") predicts that species adapted to warm body temperatures (gray curve) will have relatively high maximal rates of population growth (r_{max}) at their optimal temperature (T_o). (B) The perfect-compensation hypothesis predicts that biochemical adaptation can overcome the rate-limiting effects of low temperature, so that r_{max} will be independent of T_o.

Two opposing hypotheses dominate these debates. The "thermodynamic-constraint" hypothesis argues that low temperature slows rates of biochemical reactions and that adaptation is unable to overcome this fundamental thermodynamic depression (Hamilton, 1973; Heinrich, 1977). Consequently, cold-adapted species (even at their optimal temperatures) will inevitably have lower rates of locomotion (Garland et al., 1993), metabolism (Gillooly

et al., 2001), development (Gillooly et al., 2002; Charnov and Gillooly, 2003), and population growth (Savage et al., 2004) than will warm-adapted species at their thermal optimum. This hypothesis is sometimes referred to as "warmer is better" (Bennett, 1987; Huey and Kingsolver, 1989; Fig. 5.1A). The 'perfect-compensation' hypothesis (Clarke, 2003) counters that a suite of biochemical adaptations (Hochachka and Somero, 2002) can circumvent the temperature dependence of reaction kinetics: if these adaptations are fully compensatory, then cold-adapted ectotherms will achieve biological reaction rates that match (Clarke, 2003) those of warm-adapted species (Fig. 5.1B) in their respective optimal thermal environments.

Evaluating these competing hypotheses is important to our understanding of the nature of – and constraints on – physiological and biochemical adaptation to temperature. Moreover, these evaluations are relevant to population and community ecology: if warmer is better, then adaptation to warmer (or colder) temperatures will alter maximum rates of population growth as a correlated evolutionary response (Arnold, 1987) to selection on thermal sensitivity *per se*.

To test these competing hypotheses, we compiled and analyzed data from studies that measured the intrinsic rate of population growth (r) at several temperatures in the laboratory. The intrinsic rate of population growth describes the exponential population growth rate per day for an individual with unlimited resources (Birch, 1948; Charlesworth, 1994) and is an important component of fitness (Charlesworth, 1994). Rates of population growth– as do many other physiological rates – increase with body temperature (T_b) to some optimal temperature (T_o) and then rapidly decline with further increases in T_b (Huey et al., 2001). If thermodynamics constrains physiological adaptation to temperature, then the maximum rate of population growth, or r_{max} (r measured at T_o), of warm-adapted insects will be higher than that of cold-adapted insects (Fig. 5.1A). In contrast, if adaptation circumvents thermodynamics, then r_{max} will be independent of temperature adaptation (Fig. 5.1B).

In this study, we find that r_{max} increased with T_o; this result is qualitatively consistent with the thermodynamic model (Charnov and Gillooly, 2003; Savage et al., 2004).

However, this observed correlation might be spurious if both r_{max} and T_o were evolving independently to some common environmental variable. For example, selection in cold environments might favor organisms that not only are cold-adapted but also are thermal generalists, as cold terrestrial environments generally have high daily and seasonal temperature variation (Janzen, 1967). If so, r_{max} might decrease in cold-adapted species not because of thermodynamics but rather as a correlated response to a trade-off between thermal breadth and maximal population growth rate (Levins, 1968; Huey and Hertz, 1984; Gilchrist, 1995; Pörtner, 2004).

Alternatively, the lower r_{max} of cold-adapted species may reflect a down-regulation of population growth in response to reduced resource availability (e.g., net primary productivity, NPP) in colder environments (Clarke, 1983). To evaluate these alternative hypotheses, we were able to compile data on mean environmental temperatures (T_{mean}), seasonal temperature variation (T_{season}), and NPP for many of the sampled species. Then we used a structural equation model analysis to evaluate the relationships among r_{max}, T_o, body mass, NPP, T_{mean}, and T_{season}. This additional analysis suggested that r_{max} was directly influenced by T_o but not by NPP or T_{season}, and thus it supported only the thermodynamic model (Charnov and Gillooly, 2003; Savage et al., 2004).

5.3 Methods

5.3.1 Data collection

We compiled data from laboratory studies of insects for which r was measured at four or more constant body temperatures (T_b) and for which an optimum temperature (T_o) was evident. By surveying the literature for insect species meeting the above criteria, we collected data for 65 insect species from eight orders (25 from Homoptera [includes Hemiptera], 13 from Hymenoptera, four from Lepidoptera, six from Diptera, 13 from Coleoptera, three from Thysanoptera, and one from Collembola). The sampled species were exclusively terrestrial, and most were agricultural pests or control agents. For a few species, multiple data

sets were available; to avoid pseudoreplication, we analyzed the study that provided the most complete data (i.e., the most temperature intervals, specified locations of collection, body size, etc.). Raw data and references are provided in Appendix A of Frazier et al. (2006).

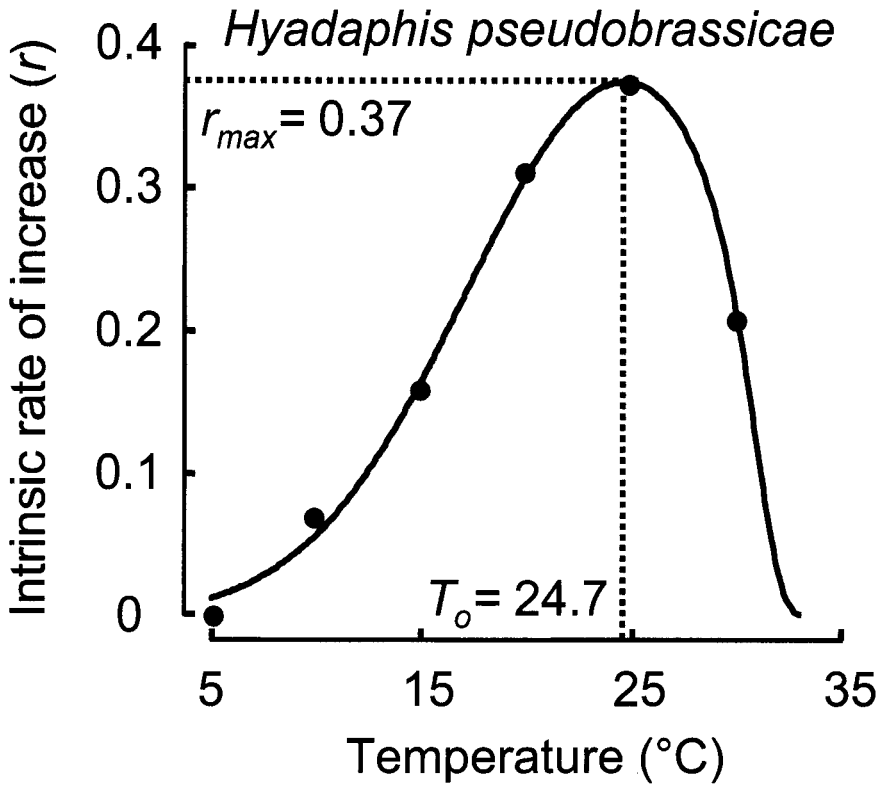

Figure 5.2: Example of a curve fit of population growth rate (r) to body temperature °C estimated from a Gaussian times a Gompertz function (see 'Methods') for the aphid *Hyadaphis pseudobrassicae* (DeLoach, 1974). Thermal optimum (T_o) and maximum rate of population growth (r_{max}) are indicated.

For each species, we fitted population growth rate ($r(t)$) to body temperature (T_b) using a Gaussian times a Gompertz function to accommodate the nonlinear nature of this

relationship (see Fig 5.2):

$$r(t) = r_{max} e^{-e^{[\rho(T_b - T_o) - 6]} - \sigma(T_b - T_o)^2} \quad (5.1)$$

From this equation, we estimated the r_{max} and T_o of each species (σ represents the increasing part of the population growth rate curve, and ρ represents the declining part of the curve). This function provided a reliable fit as long as data were relatively monotonic; in some cases, we had to remove r values that were anomalously lower than predicted based on surrounding values before the curve fitting (van Berkum, 1988). We used S-Plus, version 6, to estimate curve fits.

5.3.2 Phylogenetic analysis

To control for phylogenetic relatedness, we used standardized independent contrasts (Felsenstein, 1985) computed with Phenotypic Diversity Analysis Programs (Garland et al., 1993, 1999; Garland and Ives, 2000). The phylogenetic hypothesis for our sampled species was based on several sources (Fig. 5.3). Relationships among the eight orders follow Wheeler et al. (2001). The Tree of Life Project (Maddison and Schulz, 2004) was generally referenced throughout. We used various sources for the insect orders. Thysanoptera (Sorensen et al., 1995); Hemiptera (deeper branching patterns of the Sternorrhyncha, Cimicomorpha, Pentatomomorpha [Sorensen et al., 1995]; family groupings of Aphidoidea [von Dohlen and Moran, 2000; Martinez-Torres et al., 2001]; relationships within Aphididae [C. D. Von Dohlen, C. A. Rowe, and O. E. Heie, unpublished manuscript]); Coleoptera (Lawrence and Newton, 1982; Howland and Hewitt, 1995); Hymenoptera (superfamily branching [Dowton, 2001]: families in Chalcidoidea appear to be non-monophyletic, so we collapsed these nodes; Ichneumonoidea [Smith et al., 1999]; Muscidifurax species [Taylor et al., 1997]); Lepidoptera (Minet, 1991; Weller et al., 1992); Diptera (superfamily branching [McAlpine, 1989]; Tephritoidea relationships [Muraji and Nakahara, 2001, 2002])

Initial branch length estimates were obtained from several sources (Kukalova-Peck,

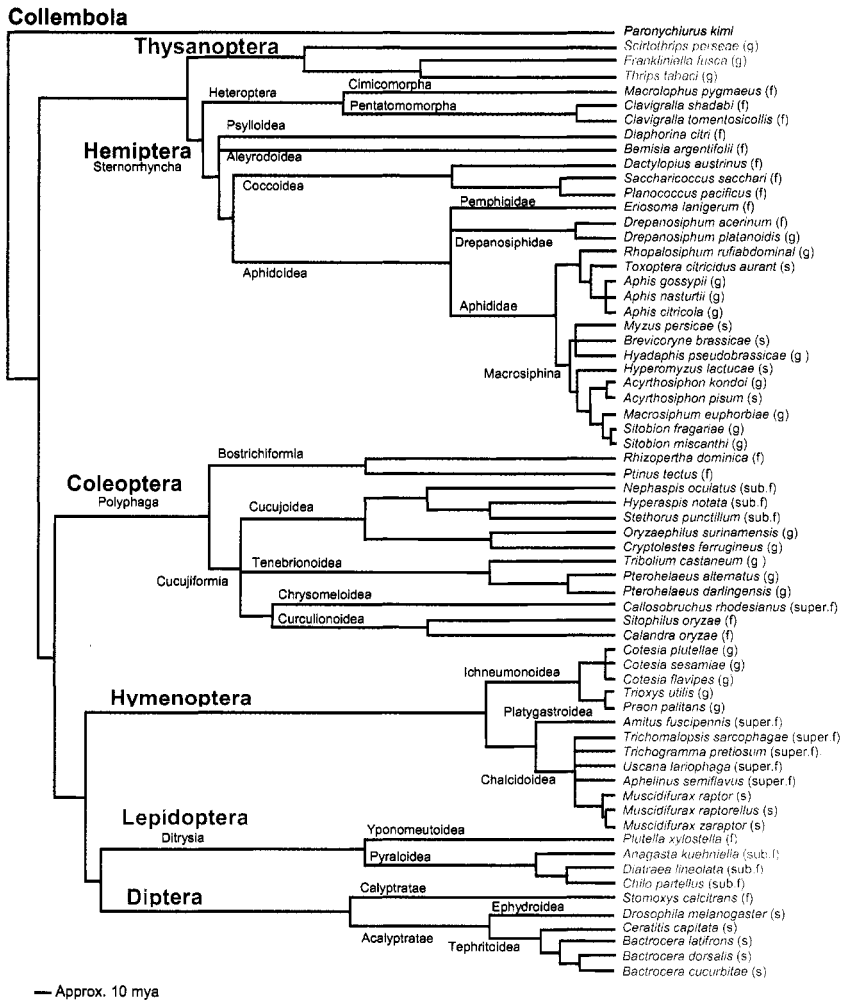

Figure 5.3: Estimated phylogeny of insect species used to generate independent contrasts. Parenthetical information indicates the taxonomic level used to place each insect species (s=species; g=genus; f=family). A higher taxonomic level than species indicates that the clade was assumed to be monophyletic.

1991; Labandeira, 1998; Gaunt and Miles, 2002) and were necessarily approximate. Ultimately, independent contrasts were computed after transforming all nonzero branch lengths to 1, as this was necessary to eliminate correlations between the absolute values of the independent contrasts and their standard deviations (Garland et al., 1992). This transformation assumes a punctuational model of evolutionary change. To determine whether our results were robust to other branch length transformations, we redid one of the primary analyses (Table 5.1), using three alternative branch length transformations (power of 0.1, Nees arbitrary method, and log_{10}). For T_o, we tested only one alternative transformation (power of 0.1) because the two others resulted in contrasts that were correlated with their standard deviations. The results for all transformations were consistent with the patterns found with unit branch length transformations and were therefore robust: the only observed difference was that *ln* dry mass became marginally non-significant ($P = 0.087$) when T_o and r_{max} branch lengths were transformed to exponent 0.1 and log_{10}, respectively.

To obtain standardized units of body mass, we converted length measures (a commonly reported measure of insect size) into dry mass, using equations from Ganihar (1997) for Coleoptera, Collembola, Diptera, and Hemiptera; from Hodar (1996) for Hymenoptera and Thysanoptera; and from Sample et al. (1993) for Lepidoptera. Population growth rate (r) is inversely correlated with body mass (Gaston, 1988; West et al., 1997), so we accounted for interspecific variation in size either by using *ln* dry mass as a covariate or by analyzing residuals from *ln* r_{max} on *ln* dry mass.

We estimated regression slopes using reduced major axis (RMA), which is less biased than ordinary least squares (OLS) estimates when the independent variable has error variance (McArdle, 1988; Garland et al., 1992). Nevertheless, RMA estimates are still likely to be biased because the error variances of the independent and dependent variables are assumed to be proportional to the total variance of each variable. This assumption is likely wrong (McArdle, 2003). Nonetheless, the RMA slope estimates should be less biased than OLS estimates, which assume that the independent variable has no error variance, and are also less biased than major-axes estimates, which are inappropriate when variables have

different units of measure and thus unequal error variances (Sokal and Rohlf, 1995). In any case, we present also the estimates from OLS analyses for comparison.

Normally distributed data are an important assumption in regression and in structural-equation model analyses. In all analyses, we used the natural logarithms of r_{max} and of dry mass because these transformations not only improved normality but also allowed us to test quantitative predictions of the thermodynamic model (Savage et al., 2004). The variables r_{max} ($P > 0.05$), T_o ($P > 0.05$), and ln dry mass ($P = 0.037$) did not significantly differ from a normal distribution when these critical P values were corrected for multiple comparisons (Shapiro-Wilk normality tests). For the structural-equation model, we used AMOS (Arbuckle, 2003) to evaluate whether the distributions of our variables of interest had significant skew and kurtosis. The distribution of T_{mean} (skew $= -1.044$, critical ratio $= -2.858$) was significantly skewed (i.e., critical ratio $> |2.0|$), and those of T_{season} (kurtosis $= 2.334$, critical ratio $= 3.197$) and T_{mean} (kurtosis $= 3.356$, critical ratio $= 4.596$) had significant kurtosis (i.e., critical ratio $> |2.0|$). Because of these significant, though fairly modest, departures from normality, we used bootstrapping to estimate P values (bias corrected, 2,000 iterations) and to evaluate model fit.

To determine whether evolutionary changes in r_{max} were related to net primary productivity (NPP) or seasonal temperature variation (T_{season}), we used a structural equation model to analyze these and other variables. Environmental data for NPP, T_{season}, and mean environmental temperatures (T_{mean}) from near the collection site were available for a subset of the insect species ($n = 46$). Climate data (New et al., 1999) were based on mean monthly temperatures for 1961–1990. Using these data, we compiled yearly mean temperatures (T_{mean}, the average of the mean monthly temperatures from all years of data collection) and an index of seasonal temperature variation (T_{season}, the average of the mean temperatures from all years of data collection for the warmest month minus that for the coldest month). These estimates are based on annual climate data and thus are not the actual body temperatures (means or variances) that insects experience in the field during their activity season. However, our estimates are likely correlated with body temperatures experienced by the in-

sects. Values of net photosynthetic accumulation of carbon by plants (*NPP*) were obtained for 1982–1998 and are based on the NASA-CASA (National Aeronautics and Space Administration, Carnegie-Ames-Stanford approach) model, with a spatial resolution of 0.5° latitude/longitude (Potter et al., 2003). These estimates are based on entire communities and are for a full year, and thus they will probably not represent the actual *NPP* available to a single species during its growing season, but our estimate is likely correlated with available *NPP*.

ANCOVA and regression analyses were performed using the R statistical package, version 2.0.0 (R Development Core Team 2004). We used AMOS, version 5, to generate and compare structural-equation models (Arbuckle, 2003).

5.4 Results

A phylogenetically corrected comparative analysis of 65 insect species showed that insects with high T_o (i.e., warm-adapted insects) had significantly faster maximum rates of population growth than did insects with low T_o (Fig. 5.4A; Table 5.1, analysis 1). For our primary analysis (Table 5.1, analysis 1B), we used standardized independent contrasts to control for phylogenetic history (Felsenstein, 1985; Garland et al., 1992), and we estimated the regression slope as the reduced major axis (RMA; see Methods).

The positive correlation between contrasts for $ln\ r_{max}$ and for T_o (Fig. 5.4A) qualitatively supports the thermodynamic constraint hypothesis. Nevertheless, some compensatory evolution of r_{max} could still have occurred and would be evident if the observed slope of r_{max} on 'inverse body temperature' $(1/kT_b)$ was less steep than that predicted by a recent thermodynamic model (Savage et al., 2004). That model explicitly predicts that body size corrected population growth rates (r) scale inversely with inverse body temperature according to $e^{-E/kT}$, where k is Boltzmanns constant ($eV\ K^{-1}$), T is absolute temperature (K), and E is the average activation energy of rate-limiting biochemical reactions of metabolism (eV). Thus, the slope of $ln\ r_{max}$ on $1/kT_o$ should equal E, which is estimated to range between -0.6 and -0.7 eV (Gillooly et al., 2001, 2002; Charnov and Gillooly, 2003; Savage

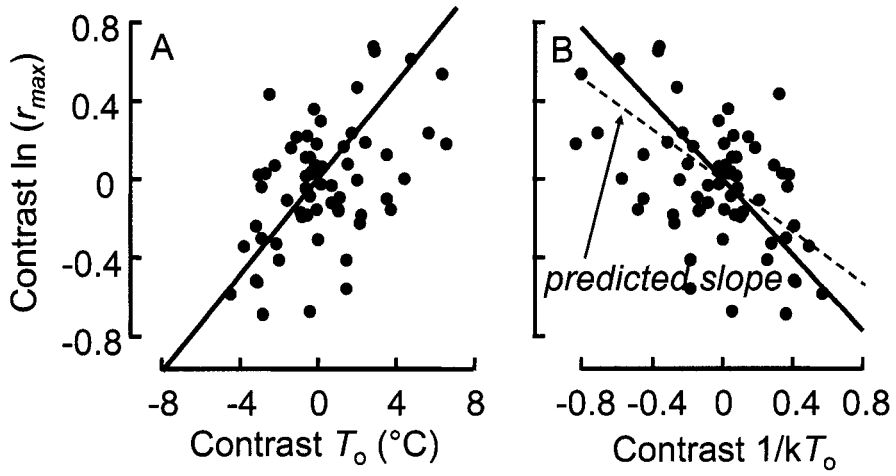

Figure 5.4: Analysis of maximum intrinsic growth rate (r_{max}, female offspring per female per day) versus optimal temperature (T_o, ∘C) for 65 insect species, using standardized phylogenetically independent contrasts. (A) Analysis of body size-corrected residuals of $ln\ r_{max}$ versus T_o is consistent with the "warmer is better" hypothesis (see also Table 5.1). (B) Analysis of body size-corrected residuals of $ln\ r_{max}$ and $1/kT_o$ (eV^{-1}) to test the quantitative predictions of the thermodynamic hypothesis (see text for details). The observed slope (solid line) was significantly steeper than the thermodynamically predicted slope (dashed line).

Table 5.1: Analyses of population growth rate versus optimal temperature, using phylogenetically independent contrasts (Garland et al., 1992).

Source of variation	Estimate (95% CI)	Standard error	t	P
Analysis 1, $ln\ r_{max}$ versus T_o				
A. Ordinary least squares regression[a]				
T_o	0.059 (0.031, 0.086)	0.014	4.35	< 0.001
ln dry mass	-0.076 (-0.147, -0.004)	0.036	-2.11	0.039
B. Reduced major axis regression[b]				
T_o	0.123 (0.099, 0.154)			
Analysis 2, $ln\ r_{max}$ versus $1/kT_o$, (i.e. Thermodynamic model)				
A. Ordinary least squares regression[a]				
$1/kT_o$	-0.459 (-0.675, -0.243)	0.108	-4.24	< 0.001
ln dry mass	-0.076 (-0.148, -0.004)	0.036	-2.12	0.038
B. Reduced major axis regression[b]				
$1/kT_o$	-0.966 (-0.776, -1.204)			

[a] $F = 10.67, df = 2,62, P < 0.001, R^2 = 0.25$

[b] Performed on residuals from $ln\ r_{max}$ on ln dry mass

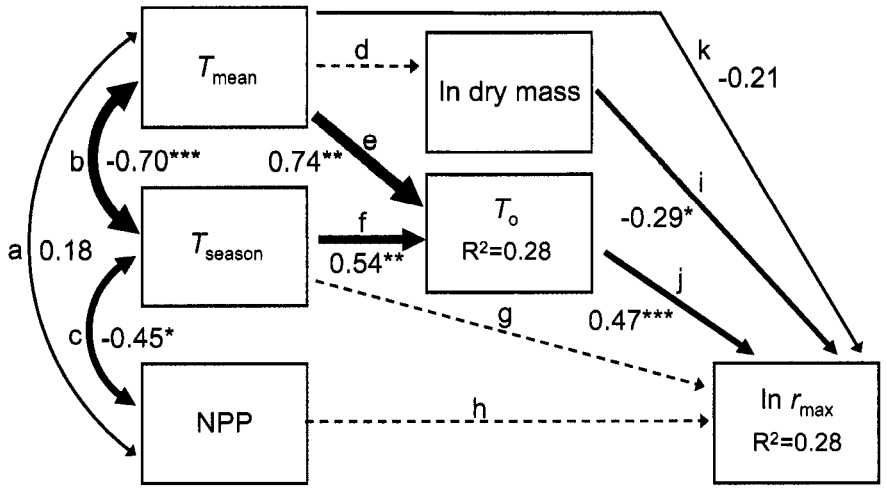

Figure 5.5: Using a structural-equation model with phylogenetically independent contrasts to test alternative models. The analysis ($n = 45$ contrasts) shows the relationships between the maximum rate of population increase ($ln\ r_{max}$) and optimal temperature (T_o °C), mean environmental temperature (T_{mean}, °C), seasonality (T_{season}, °C), yearly net primary productivity (NPP, gC^2year^{-1}), and dry body mass (ln dry mass, mg). Warm-adapted insects had significantly higher maximum population growth rates (j), supporting "warmer is better." Alternative hypotheses, such as seasonal temperature variation (g) and NPP (h), were not supported. Arrow widths are proportional to the strength of the relationship, and dashed arrows represent paths that were excluded from the final model because they failed to improve model fit, according to Akaikes Information Criterion. Numbers beside arrows are standardized coefficient estimates based on maximum likelihood for correlations (double-headed arrows) or hypothesized causal relationships (single-headed arrows). Asterisks indicate bootstrapped P values of $<.001$ (three asterisks), $<.01$ (two asterisks), or $<.05$ (one asterisk).

et al., 2004).

To estimate the slope of $1/kT_o$ on $ln\ r_{max}$, we used standardized phylogenetically independent contrasts and RMA regression (Table 5.1). The estimated RMA slope (-0.97; Fig. 5.4B, solid line) was steeper than that predicted by the thermodynamic model (Fig. 5.4B, dashed line). Furthermore, the 95% confidence interval (CI) of the observed slope (-0.78 to -1.20, calculated using the method of Jolicoeur and Mosimann McArdle, 1988) did not overlap the range predicted by the thermodynamic model (E = -0.6 to -0.7). Thus, r_{max} seems even more sensitive to T_o than is predicted by the thermodynamic model (Savage et al., 2004); this result is the opposite of what would be expected if compensatory evolution had occurred.

Table 5.2: Matrix summary of Pearson correlation (r) estimates. Estimates are based on phylogenetically independent contrasts. Means and standard deviations are included for each variable.

	T_{mean}	T_{season}	NPP	ln dry mass	T_o	ln r_{max}
T_{mean}	1.000					
T_{season}	-0.703	1.000				
NPP	0.178	-0.452	1.000			
ln dry mass	-0.034	-0.010	0.092	1.000		
T_o	0.364	0.017	-0.031	-0.124	1.000	
ln r_{max}	-0.063	0.154	-0.135	-0.362	0.427	1.000
SD	5.451	6.306	96.453	1.121	2.344	0.314
mean	-0.859	0.806	7.238	-0.118	-0.014	-0.044

We next considered hypotheses alternative to "warmer is better" that might underlie the observed correlation between T_o and r_{max}. As described above (see the introduction to this chapter), that correlation could be spurious if both traits were evolving independently in

Table 5.3: Statistics from the best-fit structural equation model. Variables include maximum rate of population increase ($ln\ r_{max}$, female offspring per female per day), optimal temperature (T_o, °C), mean environmental temperature (T_{mean}, °C), seasonal temperature variation (T_{season}, °C), yearly net primary productivity (NPP, $gC^{-2}year^{-1}$), and dry body mass (ln dry mass, mg). Statistics based on maximum likelihood are presented for correlations (*double-headed arrows*) or hypothesized causal relationships (*single-headed arrows*). Because data were slightly nonnormal, the *SE*, 95% confidence intervals (CI), and *P* values are bias corrected by bootstrapping (2,000 iterations).

Relationship	Estimate	95% CI	SE	*P*
$T_o \rightarrow \ln r_{max}$	0.063	0.030, 0.114	0.021	0.002
$T_{mean} \rightarrow T_o$	0.317	0.106, 0.481	0.099	0.007
$T_{season} \rightarrow T_o$	0.201	0.063, 0.369	0.078	0.007
ln dry mass $\rightarrow ln\ r_{max}$	-0.081	-0.171,-0.011	0.040	0.020
$T_{mean} \rightarrow ln\ r_{max}$	-0.012	-0.030, 0.007	0.009	0.139
$T_{mean} \leftrightarrow T_{season}$	-23.61	-42.12, -8.15	6.192	0.001
$NPP \leftrightarrow T_{mean}$	91.58	-63.63, 275.92	78.715	0.285
$NPP \leftrightarrow T_{season}$	268.62	-268.62, -19.72	-268.615	0.028

response to some common environmental variable, such as seasonal temperature variation (T_{season}) or net primary productivity (NPP). To evaluate whether these variables were influencing r_{max}, we compiled data on climate and NPP near the collection sites of a subset ($n = 46$) of the sampled species. We used a structural-equation model with standardized independent contrasts (Bauwens et al., 1995) to evaluate the relationships among six variables (Fig. 5.5; Tables 5.2,5.5). We used the Akaike Information Criterion (AIC) to evaluate the proposed models (Table 5.4), to determine which combination of T_o, T_{mean}, NPP, and T_{season} was most likely to influence r_{max}.

The overall fit of our final structural-equation model was good, based on the bootstrap Bollen-Stine statistic for nonnormal data ($P = 0.874$; a non-significant P value indicates a good fit). Other indexes of model fit were also positive (likelihood ratio $\chi^2 = 5.594$, $df = 10, P = 0.848$; root mean square error of approximation $= 0, 90\%$, CI$= 0.000 - 0.092$; comparative fit index $= 1.000$; see Kline, 2005 for overview). Nevertheless, because input data had significant departures from normality, these and other goodness-of-fit estimates may be suspect, as normality is an assumption of structural-equation models. However, patterns based on bootstrapped values were very similar to those using non-bootstrapped values, suggesting that our results were robust to the observed departures from normality.

The AIC best-fit model (Table 2; Fig. 5.5) supported the thermodynamic hypothesis: maximum population growth rate (r_{max}) was correlated with T_o (Fig. 5.5, path j) but not with NPP (Fig. 5.5, path h) or seasonal climatic variability (Fig. 5.5, T_{season}, path g). In fact, the combined probability that the best model for the observed data does not include T_o is only 4.1%. Overall, this analysis reinforces the view that T_o affects r_{max} and that NPP and T_{season} have little, if any, influence.

The structural-equation model analysis also revealed other interesting relationships. For insects, thermal environment (T_{mean} and T_{season}) positively influenced r_{max} indirectly via its effects on T_o (Fig. 5.5, paths e, f). According to the best-fit AIC model, T_{mean} negatively influenced r_{max} directly (Fig. 5.5, path k). This effect was modest compared to the indirect effect of T_{mean} on r_{max} (via T_o) and was nonsignificant ($P = 0.139$); nonetheless, it improved

Table 5.4: Akaike Information Criterion (AIC) for alternative nested SEM models. Akaike weights evaluate the probability that a particular model is the best model for the observed data, given the candidate set of models. Of our candidate models, the best model includes an effect of T_o on r_{max} but no effect of either net primary productivity (NPP) or seasonal temperature variation (T_{season}). AIC values are calculated from *ln* transformed model likelihood estimates, so small differences in AIC values can represent large differences in model support. The Akaike weight is calculated with values that are normalized across all candidate models to sum to 1.

Candidate models	Parameters (df)	AIC	Akaike weight
[a] T_o, $T_{mean} \rightarrow \ln r_{max}$	17 (10)	39.594	0.246
$T_o \rightarrow \ln r_{max}$	16 (11)	39.998	0.201
T_o, $T_{season} \rightarrow \ln r_{max}$	17 (10)	41.117	0.115
T_o,NPP, $T_{mean} \rightarrow \ln r_{max}$	18 (9)	48.077	0.107
T_o,NPP, $\rightarrow \ln r_{max}$	17 (10)	41.302	0.105
T_o,T_{season}, $T_{mean} \rightarrow \ln r_{max}$	18 (9)	48.262	0.097
T_o,T_{season}, NPP, $T_{mean} \rightarrow \ln r_{max}$	19 (9)	42.730	0.051
T_o,T_{season}, $NPP \rightarrow \ln r_{max}$	18 (9)	42.890	0.047
None	15 (12)	46.058	0.010
$T_{season} \rightarrow \ln r_{max}$	16 (11)	47.263	0.005
$NPP \rightarrow \ln r_{max}$	16 (11)	47.385	0.005
$T_{mean} \rightarrow \ln r_{max}$	16 (11)	47.955	0.004
T_{mean}, $NPP \rightarrow \ln r_{max}$	17 (10)	49.030	0.002
T_{season}, $T_{mean} \rightarrow \ln r_{max}$	17 (10)	49.067	0.002
NPP, $T_{mean} \rightarrow \ln r_{max}$	17 (10)	49.349	0.002
T_{season}, NPP, $T_{mean} \rightarrow \ln r_{max}$	19 (9)	50.913	0.001

[a] best model

the overall model fit according to the AIC, which was the criterion we adopted to determine the final model (Johnson and Omland, 2004).

5.5 Discussion

Our analyses suggest that an insects maximum rate of population growth (r_{max}) is strongly influenced by thermodynamics, a pattern that is consistent with the thermodynamic model (Savage et al., 2004). Specifically, the evolution of a low T_o (Fig. 5.4; Fig. 5.5, path j) seems to cause a decrease in r_{max} as an evolutionarily correlated response. The magnitude of the response is nontrivial. For every 1°C drop in T_o, r_{max} will decline by an average of 8 to 12%. (Note that these estimates are approximate and will vary with the starting value of T_o.) Because population growth is exponentially related to r_{max}, a decline of this magnitude should profoundly influence insect population dynamics.

Savage et al. (2004) previously reported that rates of population growth (corrected for body size) of ectotherms scale negatively with inverse body temperature, as predicted by their thermodynamic model (Gillooly et al., 2001, 2002; Charnov and Gillooly, 2003). Our analyses here, which find a similar pattern, are complementary to those in Savage et al. (2004). Nevertheless, our approach is different from theirs in several ways. First, we focused on determining whether r_{max} co-varies evolutionarily with T_o and thus analyzed only one pair of data (r_{max} and T_o) for each species. In contrast, Savage et al. (2004) examined the general relationship between r (not r_{max}) and body (not optimal) temperature (T_b), and they analyzed multiple estimates for each species (r at various T_b, where all $T_b \leq T_o$). Thus, both analyses focus on complementary but somewhat different issues. Second, we used independent contrasts to control for phylogenetic history, whereas Savage et al. (2004) did not correct for phylogeny. Third, we analyzed data only for insects: Savage et al. (2004) analyzed data for insects ($n = 5$ species) as well as several other ectotherm taxa (see Fig. 2 in Savage et al., 2004. Fourth, we considered competing hypotheses to the thermodynamic model (see below).

The positive correlation between T_o and $ln\ r_{max}$ (fig. 5.4A) and the inverse correlation

between $1/kT_o$ and $ln\ r_{max}$ (Fig. 5.4B) support the thermodynamic-constraint hypothesis (Gillooly et al., 2001, 2002; Charnov and Gillooly, 2003; Savage et al., 2004) and are inconsistent with the perfect compensation hypothesis (Fig. 5.1). Moreover, even a "partial-compensation" hypothesis is seemingly contradicted by the fact that the observed slope of $ln\ r_{max}$ on $1/kT_o$ is steeper than that predicted by the thermodynamic model; as noted above, partial compensation should reduce that slope. Even so, the unexpectedly steep slope of $ln\ r_{max}$ on $1/kT_o$ (Fig. 5.4B) challenges – at least quantitatively – the thermodynamic model (Gillooly et al., 2001, 2002; Charnov and Gillooly, 2003; Savage et al., 2004). We can suggest several possible reasons for this discrepancy: first, the models estimates of average activation energies of enzymatic reactions might be too low; second, organismal- level processes (e.g., r_{max}), which reflect interactions of many biochemical reactions, might be more sensitive to temperature than are enzymes themselves (contrary to the assumption in Savage et al., 2004; or third, the evolution of processes such as r_{max} is sensitive to many environmental factors and not just to optimal temperature *per se*.

Although our data and those of Savage et al. (2004) qualitatively support the thermodynamics hypothesis, we were concerned that the correlation between r_{max} and T_o could be spurious if both traits were evolving independently in response to some common environmental factor. Consequently, we used a structural-equation model (Fig. 5.5; table 2) to evaluate two competing hypotheses. First, a lower r_{max} of cold-adapted species is a by-product of selection for thermal generalization in cold, thermally variable terrestrial environments (Levins, 1968; Huey and Slatkin, 1976; Gilchrist, 1995; Pörtner, 2004). Second, a lower r_{max} of cold-adapted species reflects selection for the down-regulation of population growth in response to reduced resource availability (e.g., net primary productivity [*NPP*]) in cold environments (Clarke, 1983). The structural-equation model analysis supported the crucial role of T_o on r_{max} (Fig. 5.5, path j) and contradicted any involvement of *NPP* (Fig. 5.5, path h) or seasonal temperature variation (Fig. 5.5, T_{season}, path g). Thus, this analysis supported only the thermodynamic model and not the alternative hypotheses.

The structural-equation model also supported other relationships. Not surprisingly, T_o

was positively correlated with mean environmental temperature (Fig. 5.5, path e). Interestingly, T_o was also positively correlated with seasonal temperature variation (Fig. 5.5, path f). (In fact, T_{season} was almost as good a predictor of T_o as T_{mean}.) Perhaps countergradient selection (Levins, 1968, 1969; Conover and Schultz, 1995) favors a high T_o in relatively seasonal environments, as high-T_o species will potentially have relatively high r_{max} and thus be able to "make hay while the sun shines." On the other hand, a high T_o in such environments might simultaneously further reduce the activity season, which will already be short.

Overall, our analyses suggest that an insects maximum rate of population growth (r_{max}) is strongly influenced by thermodynamics (Hamilton, 1973; Heinrich, 1977; Bennett, 1987; Garland et al., 1993; Savage et al., 2004). Moreover, we find no evidence that physiological compensation ameliorates the effect of thermodynamics on r_{max}. If compensation did occur, the slope of $1/kT_o$ on $ln\ r_{max}$ should be less steep than that predicted by the thermodynamic model; in fact, the observed slope was steeper than predicted. Nevertheless, T_o accounts for only a fraction of the observed variance in r_{max}, and large differences in r_{max} among the insect orders cannot be explained by differences in body size (tables 1, B1); and so perhaps compensatory adaptation accounts for part of this residual variance.

The lack of evidence for compensatory adaptation of r_{max} seems strikingly and paradoxically inconsistent with the wealth of studies that convincingly demonstrate physiological and biochemical adaptation to temperature (Brett, 1970; Cossins and Bowler, 1987; Huey and Kingsolver, 1989; Hochachka and Somero, 2002). Can this inconsistency be resolved? We think so. Obviously, physiological and biochemical adaptation to temperature occurs and enables insects and other ectotherms to shift their thermal fitness curves up or down along a temperature axis and thus to invade new thermal environments. Indeed, T_o is strongly correlated with mean environmental temperature for insects (Fig. 5.5, path e). Nevertheless, biochemical adaptation seems unable to overcome the 'tyranny' of thermodynamics, at least for r_{max} of insects. In other words, although physiological adaptation to cold allows organisms to invade cold environments, it is seemingly incapable of compen-

sating for reduced rates of maximal population growth.

Several other comparative studies generally support the thermodynamic-constraint hypothesis: field and laboratory growth rates of unicellular algae (Eppley, 1972), locomotor stamina of lizards (Garland et al., 1993; Bauwens et al., 1995; Bennett and Lenski, 1997), and growth rates of scallops (Heilmayer et al., 2004) and trees (Rehfeldt et al., 2002). Thus, evidence from diverse taxa demonstrates that adaptation of ectotherms to cold temperatures seemingly reduces maximum performance and maximum population growth rates. Thus, thermal evolution has not fully escaped the 'tyranny' of thermodynamics (Barcroft, 1934; Clarke and Fraser, 2004). For insects, and possibly for other ectotherms, "warmer is better" (Bennett, 1987; Huey and Kingsolver, 1989; Savage et al., 2004).

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VITA

Melanie Frazier was born in Cottonwood, AZ where she spent her childhood exploring the ditch by her house and whacking her way through weeds. She entered Yavapai College in 1992, receiving an A.A. degree in Liberal Arts. From there, she attended Arizona State University, obtaining a B.S. degree in Biology in 1997 and a M.S. degree in Biology in 2000. While at ASU, she worked in Jon. F. Harrison's lab studying acid-base loading in insects (undergraduate research) and the interactive affects of temperature and oxygen on insect development (graduate research). Melanie was accepted into the Biology graduate program at the University of Washington in 2000, where, in addition to the dissertation work described here, she developed and taught middle school science curricula. When not parked in front of her computer, Melanie enjoys catching up on news, sewing, reading, and lounging with her husband and friends.