Rapid Life History Evolution in an Invasive Butterfly

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ABSTRACT

SARAH ANNE SEITER: Rapid Evolution of Life History in an Invasive Butterfly
(Under the direction of Joel Kingsolver)

Invasive species are ideal for studying rapid evolution because by definition they are able to successfully adapt to new environments. Species which have been introduced to multiple regions are particularly useful because they are replicated natural experiments; by comparing several invasive populations we can learn if evolution follows a consistent pattern in new habitats, or whether it is context specific. My first two chapters compare the North American and Japanese populations of the cabbage white butterfly (*Pieris rapae*), a European invasive species. These experiments demonstrate that some traits (development time) evolve in a consistent pattern in response to climate, while others (body size, immune function) do not. I also tested whether selection on life history traits varied seasonally, and whether there was a correlation between body size and development time. Selection was consistent between seasons, whild development time and body size were decoupled, indicating that these traits may be evolve in relative independence. In chapter four I tested how reduction of lipids and protein affected thermal reaction norms in wild and domestic populations of *P. rapae*. I found that reducing protein disrupted reaction norms in the domesticated population but not the wild population, indicating that food adaptation can affect reaction norms. In my 5th chapter, I conducted a meta-analysis to develop new ways of measuring phenotypic plasticity and to evaluate whether phenotypic plasticity was related to mean trait value. We find that aspects of reaction norms may evolve independently of trait values.

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I. Introduction:

Temperature and seasonality are important selective forces for all organisms, particularly ectotherms. Populations of a species may experience radically different climatic regimes at different latitudes. For example, higher latitude populations may experience lower mean temperatures, greater annual temperature variability, and shorter seasons for active growth and development. Latitude may also generate important differences in biotic interactions, as natural enemies, competitors and food resources may become abundant at different times in different parts of the range. This latitudinal variation in climate and other ecological variables can result divergence in important morphological, developmental and physiological traits. Numerous studies have documented adaptive divergence in phenotype in populations from different parts of a species range (Hereford, 2009). For example, critical photoperiod in insects (the day length required to induce and maintain diapause) varies strongly with latitude in a variety of species (Tauber, 1972, Bradshaw et al. 2004). Plants show similar latitiduinal clines in growth, phenology, physiology and life history (Maron & Elmendorf, 2007, Colautti et al., 2008). Further, there is evidence that climate change has selected for adaptive shifts in latitudinally varying traits. For example, pitcher plant mosquitoes (*Wyeomia smithii*) have shifted in critical photoperiod over the last three decades: northern populations now diapause later in the year to take advantage of a lengthened growing season (Bradshaw & Holzapfel, 2001). Understanding how populations differentiate along latitudinal gradients is therefore important for understanding patterns of biological diversity and abundance in nature, as well as for predicting evolutionary responses to climate change.

Both mean trait values and phenotypic plasticity may experience selection along latitudinal gradients in environmental conditions. Theoretical models indicate that temporal environmental variation across generations can select for increasing phenotypic plasticity (Young, 2007). Populations in northern latitudes may experience not only more extreme environments, but also more variable environmental conditions, and therefore plasticity as well as trait value may follow a latitudinal gradient (Janzen, 1967). There is some experimental evidence for latitudinal gradients in plasticity: a study of 20 *Drosophila subobscura* populations from Europe and North Africa showed that not only did over all cold tolerance covary with latitude, temperate populations were more plastic in cold tolerance than tropical ones (Long, 1999). Presumably temperate populations experienced greater thermal variation than their tropical counterparts, selecting for increased plasticity. However, empirical evidence for latitudinal clines in plasticity is still limited.

Although clinal variation in traits is well documented in native species, less is known about how exotic species respond to selection along geographic gradients in climate. Biotic invasions are natural experimental systems for studying rapid evolution, because invasive species are exposed to novel environments, and thus experience strong selection. Exotic species that undergo a rapid range expansion over a latitudinal gradient of temperature and seasonality are particularly useful for studying the evolution of latitudinal clines (Colautti *et al.*, 2010). Responses to selection along latitudinal gradients are predictable, and patterns of traits in invasive populations can be compared those in the native range. Exotic species therefore offer a unique opportunity to test whether climatic gradients produce consistent patterns of adaptation, and how quickly such adaptation can occur. There is evidence that exotic species can rapidly evolve latitudinal patterns of traits in their new ranges. The invasive fruit fly *Drosophila subobscura* shows strong latitudinal cline in body size in its native range in Europe, and introduced populations in North and South America show the evolution of a parallel latitudinal pattern in

body size in the new range (Gilchrist & Huey, 2001). The fall webworm (*Hyphlantria cunea*), a north American moth species, re-established latitudinal clines in photoperiod and in developmental time in less than 50 years after its introduction to Japan (Gomi & Takeda, 1996).

However, introduced species may accommodate novel environments in several ways: through rapid evolution of traits, through phenotypic plasticity, or through evolutionary changes in reaction norms. Observed latitudinal clines may be the result of phenotypically plastic responses to geographic variation in the environment, local adaptation of phenotype, or a combination of both. The rapid evolution of plasticity may be important in explaining the success of introduced species, but has been infrequently investigated empirically (Richards et.al. 2006). Many studies argue that phenotypic plasticity should be beneficial to introduced species because plastic responses allow organisms to thrive in a broader range of environments. Adaptive phenotypic plasticity has been shown to facilitate colonization in multiple taxa (Yeh & Price, 2011, Donohue et al., 2001). However, evidence for the hypothesis that plasticity promotes successful invasion is inconsistent; in a review of 14 studies of invasion success in plants, only seven showed that plasticity was advantageous (Richards et.al. 2006). Even less is known about the maintenance or evolution of phenotypic plasticity in invasive species once they have successfully established in a new environment. Plasticity in gape size in snakes was beneficial in populations in the early stages of colonization, as snakes encountered novel prey. However, plasticity declined after became established; snakes evolved towards a canalized optimal phenotype matching prey size (Aubret & Shine, 2010). However invasive and native *Dropsophila subobscura* populations were similar in plasticity for body size and wing loading, indicating that plasticity was maintained by environmental variation in the new range (Gilchrist & Huey, 2004). Further experiments are needed to understand how quickly phenotypic plasticity can evolve after introduction, and what selective conditions are necessary to maintain or increase plasticity.

Understanding rapid evolution in novel environments is important not only for understanding patterns of geographic variation within and among species, but also for predicting the ecological and evolutionary responses of ectotherms in a changing climate. Evidence for local adaptation in invasive species raises several important questions:

1) How quickly can populations significantly diverge in important fitness related traits?

2) Are patterns of latitudinal adaptation general or regionally specific?

3) Do populations diverge in plasticity as well as in trait means?

4) What biotic and abiotic conditions promote adaptation in trait value and in plasticity?

Our recent studies on the invasive cabbage white butterfly (*Pieris rapae*) provide an ideal foundation for studying the evolution life history traits and trait plasticity in novel environments. *P. rapae* is native to Europe, but has become an invasive pest on every continent but Antarctica (J G Kingsolver, *et.al.* 2007). It was introduced to Canada in the 1860s, and rapidly colonized North America, quickly spreading over a wide latitudinal gradient (Scudder, 1887). It is not known precisely when *P. rapae* colonized Japan, but the butterfly has appeared in the descriptions and sketches by Japanese naturalists since the 1700s (Kawakatsu et.al., 2010). Because it is considered an agricultural pest it is the target of biological control programs in both North America and Japan using the imported European parasitoid wasp, *Cotesia glomerata* (Vos & Vet, 2004). *Cotesia glomerata* is the most important source of mortality for *P. rapae* larvae, and there is evidence that mortality due to *C. glomerata* varies by latitude, climate and seasonality (1.1, Appendix A). Previous studies of two North American populations of *P. rapae* document divergence in thermal reaction norms for body size (Kingsolver et al. 2007) , and suggest that larval diet can influence thermal reaction norms for some populations (Kingsolver et al. 2006). Because of the strong evidence for local adaptation in *P. rapae*, we believe it is an ideal experimental system for studying the selective forces that shape life history traits and plasticity.

My dissertation research utilized *P. rapae* to investigate the evolution of several important insect life history traits: developmental trajectory, immune function, adult body size

and fecundity. My studies examined divergence in thermal reaction norms, in both recently established North American population of *P. rapae* (Chapter 1) and in the older Japanese population (Chapter 2). We find that development time responds consistently to selection by climate; populations from regions with longer growing seasons develop faster and reproduce earlier, as earlier reproduction confers greater fitness. Some traits, such as immune function, varied by latitude in North America, but not in Japan. We did not find a relationship between body size on either continent. These results are consistent with other studies of invasive species; some traits respond to selection by climatic gradients. We also investigated the relationship between development time and body size within a single population (Chapter 3), and measured the strength and direction of selection on these traits at different life stages and different seasons. We found that in general there was selection for rapid development through survivorship, while large body size conferred advantage through fecundity. However, we found that body size and development time were largely uncorrelated at all ages and in all seasons. Fourth, we investigated the effect of protein and lipid reduction on reaction norms for body size and development time in wild and domesticated populations (Chapter 4). We found that for domestic populations, protein reduction altered the slope and direction of thermal reaction norms, while thermal reaction norms in wild populations were robust to protein reduction. Lipids had little effect on thermal reaction norms for body size and development time. Finally, we conducted a meta-analysis that explored whether genetic variation in trait means and phenotypic plasticity are strongly correlated within populations (Chapter 5). We found that there were correlations between mean trait values and plasticity, but this relationship depended on the type of trait, the taxonomic group studied, and the method used to measure plasticity. I describe these findings in greater detail in the chapters to follow, and discuss their implications for further evolutionary ecology research.

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II. Environmental determinants of population divergence in life history traits for an invasive species: climate, seasonality and natural enemies

Abstract:

Invasive species cope with novel environments both through phenotypic plasticity and evolutionary change. However, the environmental factors that cause evolutionary divergence in invasive species are poorly understood. We developed predictions for how different life history traits, and plasticity in those traits, may respond to environmental gradients in seasonal temperatures, season length, and natural enemies. We then tested these predictions in four geographic populations of the invasive cabbage white butterfly (*Pieris rapae*) from North America. We examined the influence of two rearing temperatures 20°C and 26.7°C) on pupal mass, pupal development time, immune function and fecundity. As predicted, development time was shorter and immune function greater in populations adapted to longer season length. Also, phenotypic plasticity in development time was greater in regions with shorter growing seasons. Populations differed significantly in mean and plasticity of body mass and fecundity, but these differences were not associated with seasonal temperatures or season length. Our study shows that some life history traits, such as development time and immune function, can evolve rapidly in response to latitudinal variation in season length and natural enemies, while others traits did not. Our results also indicate that phenotypic plasticity in development time can also diverge rapidly in response to environmental conditions for some traits.

Introduction:

Temperature and seasonality are important selective forces for all organisms, particularly ectotherms. Populations of the same species may experience radically different climatic regimes at different latitudes (Janzen, 1967). Higher latitude populations may experience lower mean temperatures, greater annual variability, and shorter seasons for active growth and development. Latitude may also generate important differences in biotic interactions, as the abundances of natural enemies, competitors and food resources may vary geographically (Kraaijeveld & Godfray, 2001; McKinnon *et al.*, 2010; Ardia, 2007). This latitudinal variation in climate and other ecological factors can cause evolutionary divergence in phenotypic traits. Numerous reciprocal transplant studies have documented adaptive divergence in phenotype in populations from different parts of a species range (Hereford, 2009). For example, critical photoperiod in insects (the day length required to induce and maintain diapause) varies strongly with latitude in a variety of species (Tauber, 1972; Bradshaw & Holzapfel, 2001). Plants show similar latitudinal clines in growth, phenology, physiology and life history (Colautti *et al.*, 2010a; Maron *et al.*, 2007).

Phenotypic plasticity as well as trait value is under selection by latitudinal gradients in climate. Theoretical models predict that for a population to maintain or increase phenotypic plasticity, there must be predictable environmental variation on the temporal scale of a generation (Young, 2007). Populations at high latitudes may experience more extreme and more variable environmental conditions (Janzen, 1967). Therefore plasticity as well as trait value may vary along latitudinal gradients (James *et al.*, 1997).

Although clinal variation in traits is well documented in native species, less is known about how exotic species respond to selection along geographic gradients in climate. Biotic invasions are natural experimental systems for studying the rapid evolution of traits and plasticity

because invasive species are exposed to novel environments, and thus experience strong selection. Exotic species that undergo a rapid range expansion over a latitudinal gradient of temperature and seasonality are particularly useful for studying the evolution of latitudinal clines (Colautti *etal.,* 2010b). Responses to selection along latitudinal gradients can be predictable, and patterns of traits arising in invasive populations can be compared those in the native range. Exotic species therefore offer a unique opportunity to test whether climatic gradients produce predicted patterns of adaptation, and how quickly such adaptation can occur. However, observed latitudinal clines in invasive species may be the result of phenotypically plastic responses to geographic variation in the environment, local adaptation of phenotypes, or a combination of both. The rapid evolution of plasticity may be important in explaining the success of introduced species, but has been infrequently investigated empirically (Richards *etal.,* 2006, Yeh & Price, 2011, Donohue *et al.,* 2001). Even less is known about the maintenance or evolution of phenotypic plasticity in invasive species once they have successfully established in a new environment. While some invasive species lose plasticity in fitness traits after introduction, ultimately reaching an optimum for their new environment, other invasive populations maintain or increase trait plasticity (Gilchrist & Huey, 2004; Aubret & Shine, 2010). In this study, we develop specific predictions about how climatic temperatures, season length and natural enemies may produce geographic differences among populations in life history traits and plasticity, and test these predictions in the invasive species *Pieris rapae*.

Methods:

Study System and Predictions:

We studied reaction norms for body size, development time, potential fecundity, and immune function in four populations of the invasive cabbage white butterfly (*Pieris rapae)*.

Pieris rapae was introduced to southeastern Canada in the 1860s, and rapidly colonized most of North America, spreading across the continent. Previous studies of *P. rapae* from North Carolina and Washington indicate that these populations have diverged in thermal reaction norms for body size and development time both from each other and from ancestral populations in Europe (Kingsolver *et al.* 2007)

Because it is an agricultural pest, *P. rapae* is the target of biological control programs using the imported European parasitoid wasp, *Cotesia glomerata* (Vos & Vet, 2004). *Cotesia glomerata* is the most important natural enemy of *P. rapae* larvae in many populations, and previous studies have suggested that mortality due to *C. glomerata* may vary with climate and seasonality (Ohsaki & Sato, 1994; Van Driesche, 1988). Rates of parasitism may also increase throughout the summer, as longer growing seasons allow for more generations and greater populations of parasitoids (Ohsaki & Sato, 1994). In this study we performed a meta-analysis of field studies of *P. rapae* to assess latitudinal patterns in parasitism rates. We then developed and tested predictions about how parasitism may vary by latitude, about how the population means of life history traits may vary geographically, and about how plasticity in these traits may also vary by latitude.

Latitude and Selection by Parasitoids:

To evaluate the relationship between climate and parasitoid prevalence we conducted a meta-analysis on field studies of parasitism in *P. rapae* from a range of latitudes. We tested for a relationship between parasitoid infection rate and latitude using prevalence values extracted from the literature (see appendix A). We predicted that *P. rapae* populations from high latitudes would have lower parasitoid prevalence, because the growing season is shorter in colder climates, allowing less time for parasitoid populations to grow.

To test for a relationship between parasitism rates and latitude, we performed a meta analysis. We compiled data from studies of parasitism rates of *Cotesia glomerata* and its

congener *Cotesia rubiclea* on *P. rapae* for field sites ranging from 35 N to 45 N (see appendix A) covering most of *P. rapae*'s range in the Northern hemisphere. We used the keywords "*Pieris rapae*", "Parasitoid", "*Cotesia glomerata*", "*Cotesia rubeclea*", "*Apanteles glomerata*" (a previous name for *C. glomerata*) and "natural enemies" as search terms. We located a total of nine studies appropriate for the meta-analysis. A majority of parasitoid surveys were carried out in late summer or early fall; for studies that sampled throughout the growing season we used data from August. We used a two-level linear model to test for a relationship between latitude and parasitism rate. We used parasitoid prevalence, logit transformed, as a response variable, latitude as a covariate and study as a random effect; responses were weighted by the square root of sample size for each study. For two studies where sample size was not available, we used mean sample size as a weight.

Predictions for population divergence in trait means:

We tested whether mean body size, development time, and potential fecundity have diverged in populations from four climatically distinct regions: North Carolina, Washington, Nova Scotia, and Michigan. The latter two populations are at similar latitudes but have very different mean temperatures and growing seasons (see Figure 2.1). In general, we predict that mean body size will have a negative relationship with mean summer temperature. We base these predictions on Bergman's rule, which states that populations at higher latitudes (with lower mean temperatures) tend to have larger body sizes (Fig. 2.1A). Egg production (potential fecundity) should follow a similar pattern, as fecundity and body size are often strongly related in insects (Fig 2.1A).

Mean development time can have either a positive or a negative relationship with growing season. In regions where there is no climatic limit to development time, species should optimize early reproduction over body size. In populations where the growing season is limited, some populations may reach reproductive maturity early and produce extra generations, while

others may best utilize the available frost-free period by delaying maturation until they are larger and have greater fecundity (Roff, 1980; Kivelä *et al.*, 2012). Like most insects, *P. rapae* faces the problem of optimizing either early reproduction (and thus producing more generations per year) or body size (and thus having greater potential reproductive fitness). Adding a second generation may increase fitness for individuals, but they must halve their development time in order to do so. Therefore *P. rapae* in regions with longer growing seasons might either develop more slowly because more frost-free days are available for growth, or alternately, long growing seasons might select for rapid maturity and more generations per year (Fig. 2.1 B)(Roff, 1980). However, models predict that early reproduction increases fitness more than body size, and we therefore predict that longer frost-free periods should favor early reproduction (Kingsolver & Huey, 2008).

Selection by parasitoids is hypothesized to be stronger in regions where parasitoid populations have more time available for population growth, we predict that mean immune function in *P. rapae* will increase with the length of the growing season (Kraaijeveld & Godfray, 2001). Larvae from populations with short growing seasons should have weaker immune systems, because the short frost-free periods of northern populations limit the growth of parasitoid populations (Fig. 2.1 B).

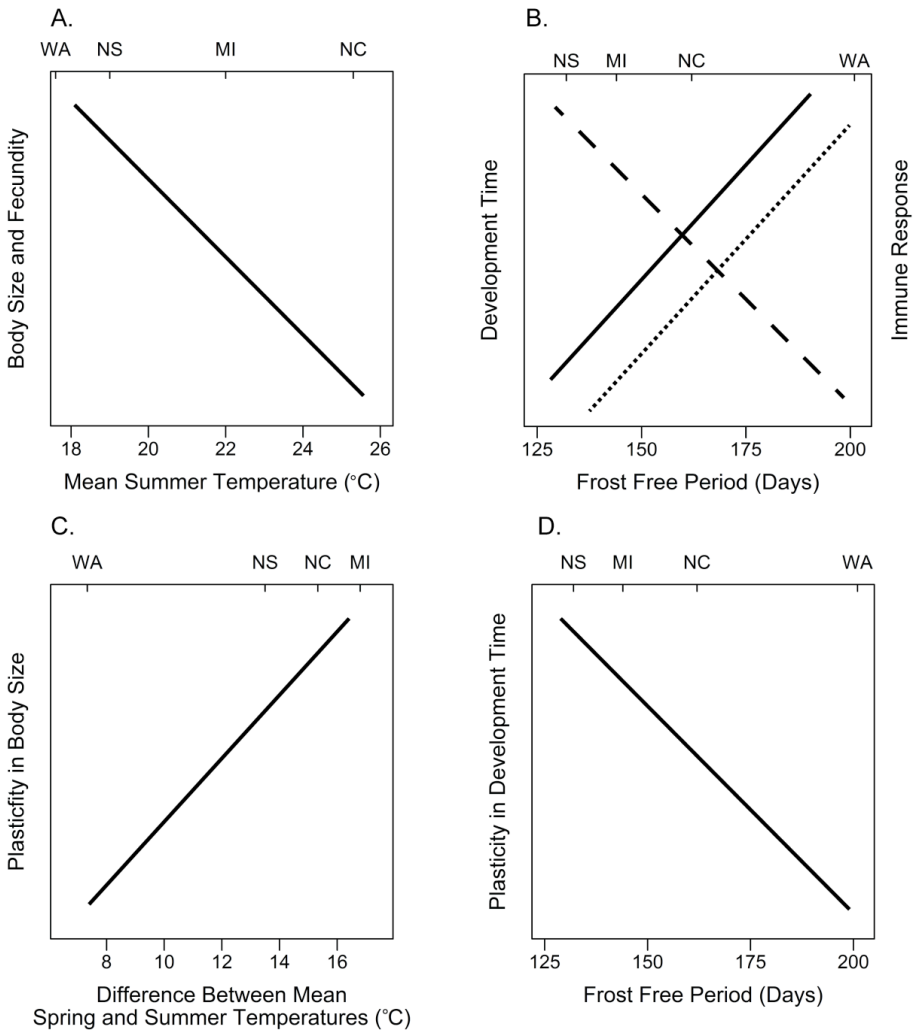
Predictions for population divergence in plasticity:

We also tested whether phenotypic plasticity for genotypes in a population (here defined as the difference in phenotypes between the warm and cool temperature treatments for each full-sib family) varied with climate (Stearns, 1992). Both phenotype and phenotypic range can undergo selection by environmental variation. Theory predicts that plasticity should be more likely to evolve in regions where there is significant environmental variation on the scale of a generation (Roff, 1980). Ectotherms may optimize their growth rate in response to temperature, and variation in growth rate typically translates into variation in body size (Yamahira & Conover,

2002). We therefore predicted that populations that experience greater differences between spring and summer temperatures should have greater plasticity in growth rate and therefore exhibit greater temperature dependent differences in body size (Fig. 2.1C) (Young & Badyaev, 2007; Snell-Rood, 2012).

We predict that plasticity in development time should also vary with climate. *P. rapae* varies in voltinism, ranging from two to three generations in the northern populations of Nova Scotia and Michigan, to more than six in North Carolina. Our predictions for plasticity in development time parallel those for development time themselves. Selection favors early reproduction and more generations in populations with long growing seasons. In populations with long seasonal windows, we predict that there should be less plasticity for development time (Kingsolver & Huey, 2008; Stearns, 1989; Stearns, 1992). We expect greater plasticity in populations with shorter growing seasons that must negotiate trade-offs between fecundity and development rate (Fig. 2.1 D). We tested these predictions using a series of laboratory experiments to see whether the four populations had diverged in these important life history traits, and whether those differences were in agreement with our predictions based on seasonal temperatures and season length (Fig. 2.1).

Figure 2.1. Predicted relationships of life history traits and plasticity to seasonal temperatures and growing season length. See text for details and rationale.
2A. We predict that both body size and fecundity will decrease in regions with higher mean summer temperatures.
2B. We predict that development time may either increase in regions with longer growing seasons (longer frost-free periods) if populations maximize body size (solid line); or decrease with longer growing seasons if early reproduction is favored to maximize the number of generations in the growing season (dashed line). We also predict that immune response will increase in regions with longer growing seasons (dotted line).
2C. We predict that phenotypic plasticity for body size should be greater in populations that experience a larger difference between spring and summer temperatures.
2D. We predict that phenotypic plasticity for development time should be greater in regions with shorterfrost-free periods.



Experimental Studies:

We collected *P. rapae* females from four populations during the summers of 2010 (Washington, Michigan, and Nova Scotia) and 2011 (North Carolina). Washington (WA) females were collected from farms outside Seattle (47.61° N), and from a system of community gardens within the Seattle metropolitan area. Michigan (MI) females were collected from organic farms near Ann Arbor (42.33° N) and Nova Scotia (NS) females were collected in community gardens in Halifax and from an organic farm near Wolfville (45.08° N). Animals were shipped live to the lab in Chapel Hill, NC using an overnight refrigerated shipping service. North Carolina (NC) females were collected from organic farms in Chatham County, NC (35.91° N) and did not require shipping. Females were kept in greenhouse conditions (~24 °C*,* 60-80% humidity, natural photocycle of 14L:10D*)* and provided with fresh collard leaves (*Brassica oleracea*) for oviposition. Eggs were collected daily. Collard leaves with eggs were placed in plastic containers and maintained in another environmental chamber (Percival 36-VL: Percival Scientific, Perry, IA, USA) at 25°C with 14L : 10D photoperiod until hatching. During the first instar (24-48 hours after hatching) caterpillars were transferred to artificial diet and placed in individual petri dishes. To quantify thermal reaction norms for growth, development time, and immune function we reared caterpillars from the four populations at two temperatures: 20°C and 26.7°C with a 14L:10D photoperiod. All populations experience these temperatures regularly during the course of a growing season. Our experiment included eight families of *P. rapae* caterpillars from North Carolina (N=303), seven families from Nova Scotia (N=336), five from Michigan (N=202), and nine from Washington (N=446). Caterpillars from each mother (sibship) were then assigned randomly to one of the two experimental temperature treatments. Caterpillars were fed *ad libitum* on artificial diet, (following the recipe from Papaj & Snell-Rood, 2009) and diet was changed three times per week to reduce bacterial growth and spoilage. Petri dishes were checked daily for mortality, and to identify individuals that had pupated. When an individual reached pupation, it was removed from the petri dish and weighed using standard gravimetric

techniques. Pupae were placed in plastic cups on a piece of damp filter paper, with a moist sponge to reduce desiccation, and returned to their experimental temperature. Cups were checked daily to determine the date of eclosion. Newly eclosed individuals were sexed, massed, and placed in a flight cage with individuals from their cohort for 48 hours to allow egg maturation. Adults were fed *ad libitum* on a 10% honey water solution and freeze killed after 48 hours. Abdomens of female butterflies were dissected in a glycerol solution and mature eggs (fully yolked with a developed chorion) were counted under a dissecting microscope.

Immune Assay:

We assessed immune function by injecting silica beads into the caterpillars' haemocoel (Sigma Aldrich Corporation, St. Louis, Missourri). The beads activate cellular encapsulation by hemocytes. This technique of assaying immune response by injection with a foreign body is commonly used in insect immunology, and has been shown to have a significant correlation with immune response to real parasites and pathogens (Diamond & Kingsolver, 2011a, Rantala & Roff, 2005, Smilanich *et al.*, 2009) Caterpillars were randomly selected for the injection assay at the beginning of the experiment but were reared in the same incubators using exactly the same methods as those not selected. Injections were administered during the first 24 hours of the 5th (final) instar, because immune function declines immediately prior to metamorphosis (Beetz *et al.*, 2008). However, because individuals developed at different rates, caterpillars were at the same physiological stage but not the same chronological age when the assay was administered.

Our immune assays follow those of Diamond and Kingsolver (2011a). We used DEAE Sephadex-A25 silica chromatography beads (Sigma Aldrich Corporation, St. Louis, MO). Beads were dyed with a 0.1mg/ml solution Congo Red Dye and allowed to dry completely before being stored in a freezer to sterilize and prevent contamination. Beads were mixed in a standard solution of 1g of dyed beads and 0.01L of sterile Grace's Insect Cell Culture Medium (Sigma Aldrich Corporation, St. Louis Missouri) to standardize the number of beads each individual received.

Caterpillars were injected with 5 ml of the bead solution to using a Hamilton 7000 series syringe with a 25-gauge tip (Hamilton Company, Reno, Nevada). On caterpillars received an average of 15.24 $\pm$0.52 beads from the injection. After injection, caterpillars were placed on fresh diet and returned to the appropriate temperature for 24 hours; after 24 hours they were freeze killed. Beads were extracted postmortem from caterpillars by dissection of the whole caterpillar and mounted on glass slides in a glycerol solution.

We measured encapsulation (area of hemocyte aggregation) as a continuous response variable using both differential interference contrast microscopy (DIC) which detects the cellular encapsulation area and the bead, and fluorescence microscopy which detects only the dyed bead (Zeiss LSM 510 confocal microscope) (Diamond & Kingsolver, 2011). Encapsulation and bead area were measured using the visualization program ImageJ (Abramoff *et al.*, 2004). We used an automated edge selection tool to determine the area of cellular encapsulation on the DIC image and a thresholding tool to select the area of the fluorescent bead from the fluorescence image. We determined the area of encapsulation by subtracting the area of the bead from the fluorescence image from the total area of the bead and encapsulation measured in the DIC image. Of 484 beads, three had a negative encapsulation value (the bead was larger than detected encapsulation area, while encapsulation should be bounded at zero) and these were excluded from the analysis.

Climatic Data:

We used the 1950-1980 Canadian Climate Normals (Canada Dept. of the Environment, 1982) to determine the 90% probability frost-free period for Nova Scotia. Because frost normals for Canada were not available after 1980, we also used 90% probability frost-free period from 1950 to 1980 for the U.S. field sites (Koss & Owenby, 1988). To determine the mean monthly temperature for June, July and August we used current 30 year averages (NOAA National Climatic Data Center 2011, Environment Canada 2010). We also confirmed that the current frost-free periods for North Carolina, Michigan and Washington (NOAA National Climatic Data

Center, 2011) did not differ substantially from the 1980 data. We calculated mean summer temperature by averaging the mean monthly temperature for June, July and August, and mean spring temperature by averaging monthly temperature means for March, April and May.

Statistical Analysis:

All statistical analyses were performed in R (v. 2.11.0). We ran a separate linear mixed-effects model (using R library nlme) for each of the four life history traits: pupal mass, pupal development time, egg production (ovariole number), and immune response (cellular encapsulation) (Pinheiro *et al*. 2012). For the immune response models we calculated the mean encapsulation area (log transformed and corrected for bead size) per individual for use as the response variable in our analyses, and included bead size as a covariate in the models. Family (sib-ship) was included as a random effect in all models. To test our predictions (Fig. 2.1), we considered models with rearing temperature, sex as fixed effects and a climatic variable as a covariate. Therefore we included mean summer temperature as the climatic variable in models for body size and egg production, and included frost-free period as the climatic variable in models for development time and immune response (Fig. 2.1 A-B). These models allow us to test whether specific climatic variables can explain the differences among populations in mean and plasticity of life history traits. However, populations might also differ in these traits in ways not predicted by these climatic variables. Therefore in cases where climate was not significant, we ran a second set of models in which the climatic covariate was replaced with population of origin as a fixed effect. This allowed us to test for possible differences among populations that are not associated with climate.

To test our predictions about differences in plasticity among populations, we calculated plasticity as the difference in mean phenotype between the two temperature treatments for each family in each population (Valladares *et al.*, 2006). Plasticity in both development time and body size was computed for each family; we used family as the experimental unit because we

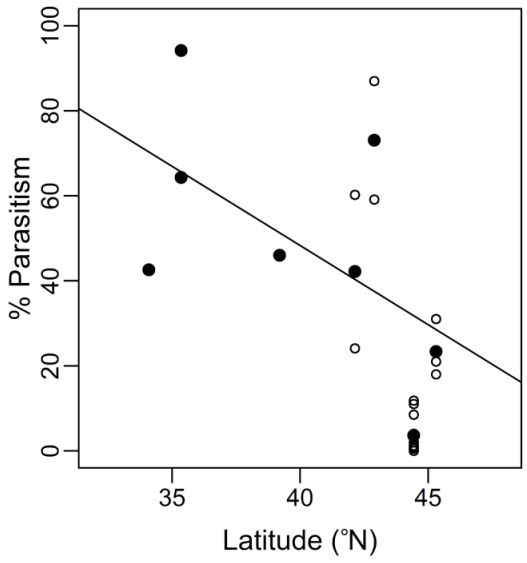
measured individual's phenotypes in either the warm or cool environment, and therefore computed plasticity at the genotype level. We then used linear models to test whether plasticity in development time is associated with frost-free period (Fig 2.1 D), and whether plasticity in body size is associated with the difference between mean temperatures in spring and summer (Fig 2.1 C). Because sample size (n) per family varied substantially within and among populations, we weighted our models by sqrt(n).

Results:

Meta-analysis:

Parasitism by *Cotesia spp*. was significantly greater at lower latitudes ($F_{1,14}$, F=5.29, p = 0.037) (Fig. 2). As a result, parasitoid prevalence was greater in regions with higher average temperatures and longer growing seasons.

Figure 2.2 Rate of parasitism of P. rapae larvae by Cotesia glomerata parasitoids as a function of latitude. Solid circles represent means, open circles indicate data from multiple years. Parasitism rate declined significantly with increasing latitude.



Pupal Mass:

Contrary to our prediction for pupal size (Fig. 2.1A), mean summer temperature did not predict mean body size ($F_{1, 464} = 0.053$, $p = 0.820$). We therefore ran a second model that included population of origin as a fixed effect (see Methods), and we report results from the population model below. We found that rearing temperature decreased body size ($F_{1, 460}= 35.195$, $p=<.0001$), and that males were larger than females ($F_{1, 460}= 38.867$, $p=<.0001$). Population had a significant effect on body size ($F_{3, 25}= 3.935$, $p= 0.0199$), and there was also a significant interaction between population and rearing temperatures ($F_{3, 460}= 7.768$, $p=<.0001$). Michigan, North Carolina, and Washington were all smaller at warm temperatures, while Nova Scotia was larger at warm temperatures (Fig 3 A and B). Mean body size in Michigan was smaller than in the other populations. There was also a significant interaction between rearing temperature and sex ($F_{1, 457}= 6.340$, $p=<.0121$); males were larger over all, and this difference was more pronounced at high temperatures. No other interaction terms were significant.

Potential Fecundity:

There was a significant effect of mean summer temperature on the number of eggs produced by females ($F_{1, 24}= 3.826$, $p= 0.0320$). In agreement with our predictions (Fig. 2.1A), females from populations with higher mean summer temperatures produced fewer eggs (Fig. 3C). However, these effects on fecundity were not mediated by differences in body size. There were no significant effects of pupal mass ($F_{1, 88}= 3.2862$, $p= 0.0733$) or rearing temperature ($F_{1, 88}= 0.0331$, $p= 0.8561$) on egg production, and no two way interaction terms were significant.

Development time:

Frost-free period had a significant effect on development time ($F_{1,27} = -10.42$, $p<0.001$). Butterflies developed more quickly in populations with long growing seasons, in agreement with our predictions (Fig. 2.1 B). Warmer rearing temperatures reduced development

time ($F_{1, 463}$= 8519.7, p<0.0001), but development time did not differ by sex ($F_{1, 463}$= 1.38, p= 0.24). We compared a model that treated population as a factor and a model that used climatic data as a continuous variable, in this case frost-free period, and included all possible two-way interactions in the models. There was little between-population variation in development time at warm temperatures, but at cool temperatures, Michigan and Nova Scotia were slower than North Carolina and Washington ($F_{1,463}$ = 99.07, p<0.001) (Fig. 2.4A). Females developed slightly faster than males ($F_{1,463}$ = 4.314, p<0.038)

Figure 2.3. Body size (A and B) or egg production (C) as a function of mean summer temperature (°C) for four study populations of P. rapae (MI = Michigan, NC = North Carolina, NS = Nova Scotia, WA = Washington. Open circles indicate individuals reared at cool temperatures; filled circle indicate those reared at warm temperatures.
A. Mean (+ 1SE) body mass (in g) of females.
B. Mean (+ 1SE) body mass (in g) of males.
C. Mean (+ 1SE) number of mature ovarioles at 48h.

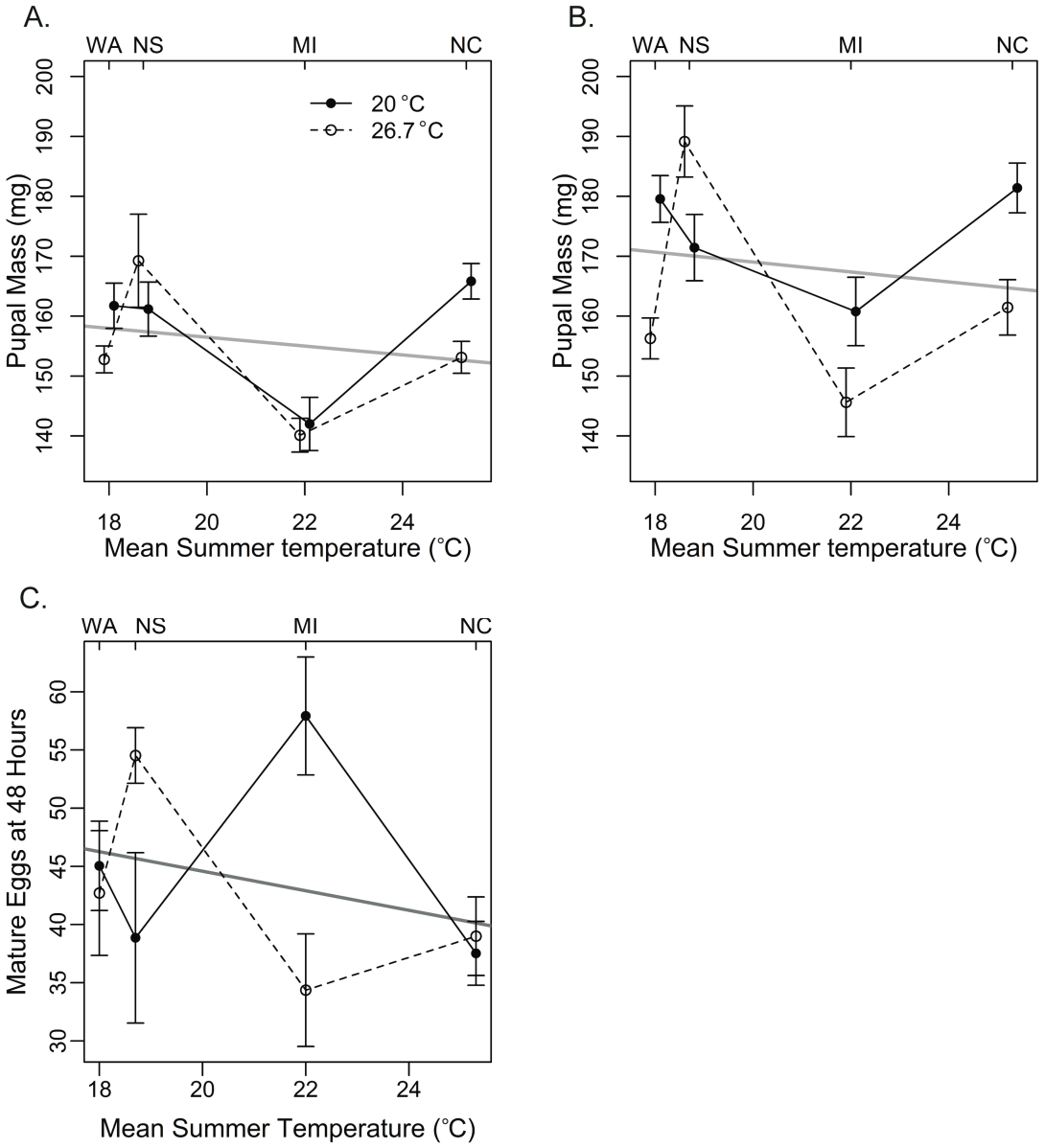
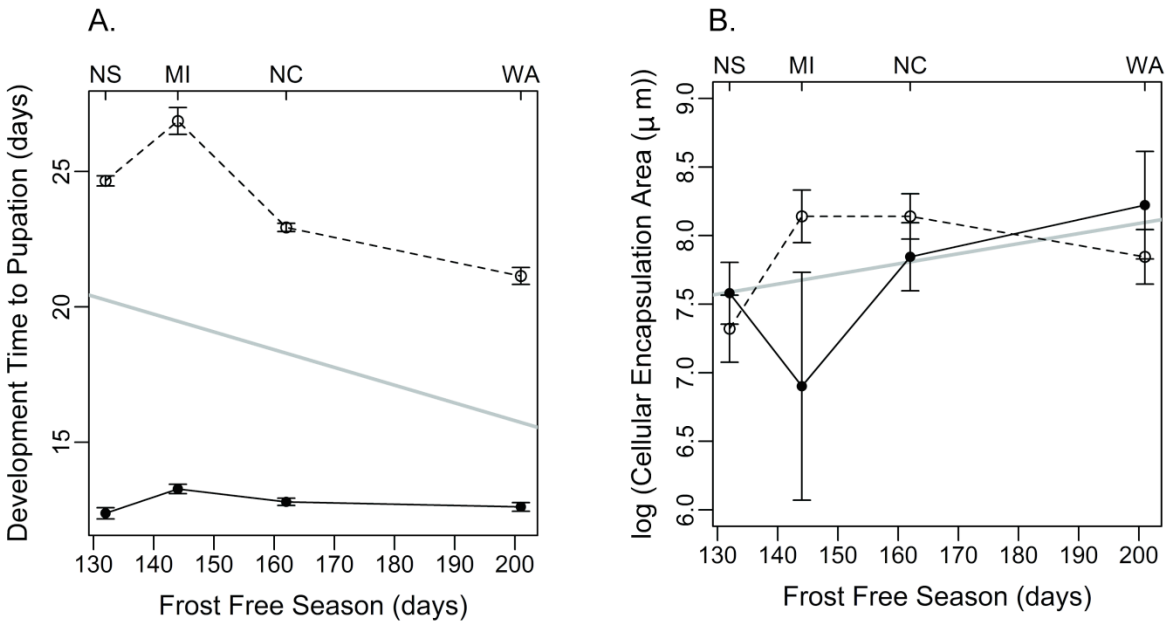


Figure 2.4. Development time (A) and immune function (B) as a function of frost-free season length (in days) for four study populations of P. rapae. Gray lines represent model coefficients. Symbols and abbreviations as in Figure 2.3.
A. Mean (+ 1SE) development time to pupations (in days).
B. Mean (+ 1SE) area of log transformed cellular encapsulation (in μm).



Immune response:

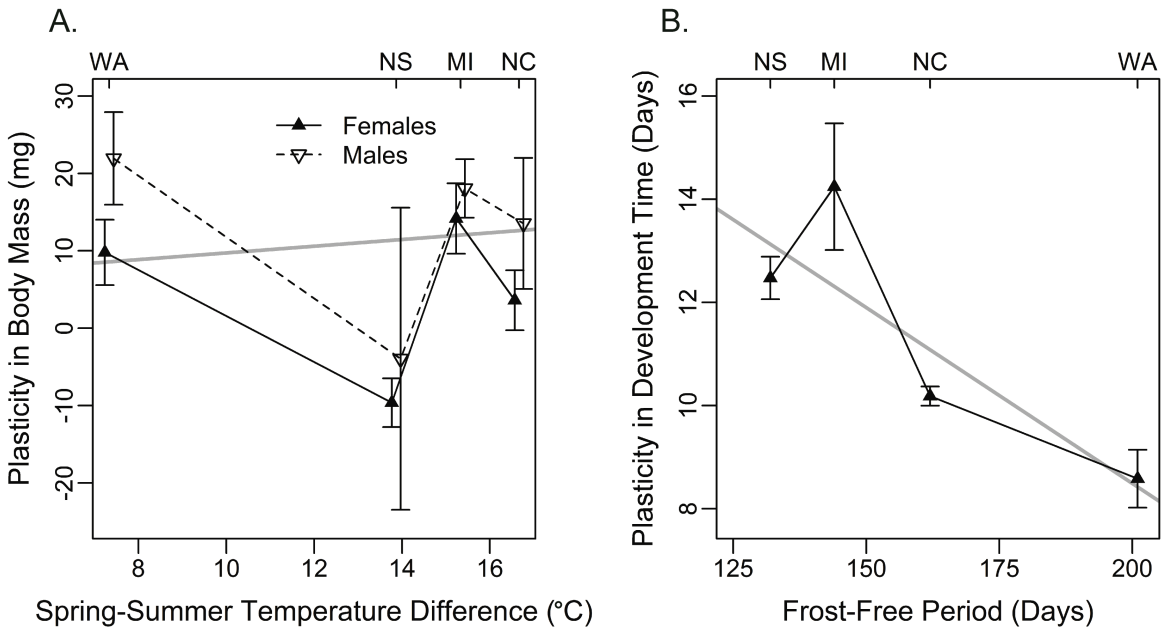
Frost-free period also had a significant effect on cellular encapsulation ($F_{1, 29}$= 4.378, p=0.0453). In agreement with our predictions (Fig. 2.1 B), encapsulation response was greater in populations with longer growing seasons (Fig. 2.4 B). Larger beads also produced greater encapsulation responses ($F_{1, 60}$=14.181, p=0.0004). There was no significant effect of rearing temperature on immune response ($F_{1, 60}$=0.397, p=0.5310), nor a significant interaction between temperature and frost-period period.

Phenotypic Plasticity :

In contrast to our predictions (Fig. 2.2C), there was no significant effect of seasonal temperature difference (between spring and summer temperatures) on plasticity in body size, so we used a model that included population as a fixed effect. However population was not

significant either (F_3=0.768, p=0.5181). Males were more plastic than females (F_1=12.372, p = 0.0010). However, plasticity in development time decreased significantly with increasing frost-free period (F_1=32.67, p<0.0000) (Fig. 1.5B), in agreement with our predictions. The sexes did not differ in plasticity for development time ($F_{1,}$ =0.41, p=0.52). Families from Washington and North Carolina had lower plasticity in development time, while those from Michigan and North Carolina were the most plastic (Fig. 1.5B). Note that this pattern of plasticity is largely due to differences at the lower rearing temperature (20 °C): mean development times were very similar among populations at the higher rearing temperature (Fig. 2.4A).

Figure 2.5 legend : Plasticity in body size as a function of spring summer temperature differences (A) and development time as a function of frost-free season length (in days) for four study populations of P. rapae. Gray lines represent model coefficients.
A. Plasticity in body mass. Open symbols are family plasticities for males and closed circles are for females
B. Plasticity in development time for each family, because there was no significant effect of sex in the model, males and females are pooled.



Discussion:

Evolution of Latitudinal Clines in Traits:

Latitudinal gradients are often thought to produce corresponding patterns in life history traits, and when invasive species colonize a new region, climatic gradients are expected to select for geographic clines in traits that parallel those in the native range. Some invasive species have re-established predicted patterns in traits after they are introduced; the invasive fruit fly *Drosophila subobscura* shows a strong latitudinal cline in body size in its native range in Europe, and introduced populations in North and South America show the evolution of a parallel latitudinal pattern in mean body size (Gilchrist & Huey, 2001). The fall webworm (*Hyphlantria cunea*), a North American moth species, re-established latitudinal clines in mean photoperiod and mean development time less than 50 years after its introduction to Japan (Gomi, 2007). In both cases, the geographic differences among populations were strongly associated with latitudinal gradients in average temperature and day length.

Our results with *P. rapae* indicate significant evolutionary divergence among populations in mean body size. However, in contrast to our predictions (Fig. 2.1A) these population differences are not explained by the differences in mean summer temperatures among the populations (Fig. 2.4A). This suggests that the evolutionary divergence in size is not the result of selection for adaptation to local thermal conditions. Gilbert (1984, 1988) studied population differentiation in body size for several European and Australian populations of *P. rapae*, and did not detect significant differences in reaction norms or means for body mass between populations, although he did note that growth was somewhat slow on his particular diet formula. Population differences in responses to host plant quality and to artificial diets can also influence patterns of growth rate and body size and may explain observed divergence (Kingsolver *etal.* 2006; and see below). Maternal effects can affect phenotype for both body size and development time (Fox *et*

al., 1995; Mousseau, 1998). However, a previous experiment in North Carolina and Washington *P. rapae* populations compared thermal reaction norms for body size and development time in individuals from wild-caught mothers and from mothers that were reared in the lab for a generation, and found only minor differences in development time due to maternal effects. It is therefore likely that maternal effects play a small role in the differences in reaction norms we find between North American populations. We also found that mean egg production decreased with mean summer temperatures (Fig. 2.3C). While this pattern is consistent with our prediction (Fig. 1A), it is not mediated by population differences in body size as we anticipated (Fig. 2.3A-B).

In contrast, population differences in mean immune function were significantly associated with the length of the frost-free period: populations with longer frost-free periods had greater immune function than those with short frost-free periods (Fig. 2.4B). Our meta-analysis of *P. rapae* populations (Fig. 2.2) showed a significant decline in the rate of parasitism by *C. glomerata* with increasing latitude. Parasitoid populations typically increase throughout the growing season, and therefore populations with long frost-free periods have the potential to reach greater peak parasitoid densities (Ohsaki & Sato, 1990). Our results support the hypothesis (Fig. 2.1B) that greater parasitoid abundance in areas with longer growing seasons has produced evolutionary divergence in immune responses among *P. rapae* populations. Latitudinal clines in immune function have been found in other invertebrate species, particularly those which are under selection by parasitoids. For example, Kraaijeveld & Godfray (1999) found that encapsulation response of European populations of *Drosophila subobscura* co-varied geographically with the virulence of the parasitoid wasps *Asobara tabida* and *Lepropolina boulardi* . Local differences in parasite prevalence were also found to be a good predictor of immune function for populations of the amphipod *Grammarus pulex* (Franceschi *et al.*, 2010). Other studies have found latitudinal clines in immune function to be the result of energetic tradeoffs with growth and development. DeBlocke *et al* . (2008) found that immune function in

damselflies was negatively correlated with development time and latitude; northern damselfly populations had longer development times and invested more to immune function. Our results do not support trade-offs between immune function and growth, but rather indicate that selection by natural enemies is an important determinant of immunocompetence for *P. rapae*. Theory predicts that invasive species should invest less in immune function because they are released from selection by the natural enemies of their native range. However our study shows that clines in immune function can re-evolve if invasives are re-introduced to natural enemies (Lee & Klasing, 2004; Llewellyn *et al.*, 2011).

Mean development time was also significantly associated with the length of the frost-free period: populations with longer frost-free periods had shorter development times than those with shorter frost-free periods (Fig. 2.4). The length of the growing season has both direct and indirect ecological effects on the fitness consequences of life history traits. First, longer growing seasons allow a greater number of generations per year in *P. rapae*. Our results suggest that shorter development times in regions with longer growing seasons may reflect selection for increasing the number of generations in these regions (Roff, 1980) in agreement with our predictions (Fig. 2.1). Second, higher rates of parasitism in regions with longer growing seasons (Fig. 2.2) may also select for more rapid development. Field studies of *P. rapae* in Maryland have shown that rapid development rate reduced the rate of mortality due to parasitism (Benrey & Denno, 1997); selection for rapid development to avoid parasitism by *Cotesia* wasps has also been demonstrated in *M. sexta* (Kingsolver *et al.* 2012). Our present results suggest that development time can evolve rapidly in response to growing season, as a consequence of climatic effects, biotic interactions or both.

Latitudinal Clines in Plasticity

While invasive species may accommodate novel environments through rapid evolution of traits, they may also cope with variation in their new environment through extant phenotypic plasticity *(*Donohue *et al.*, 2001; Yeh & Price, 2011). Our results indicate that North American populations of *P. rapae* have evolved significant differences in the slope and direction of reaction norms for body size and development time. Pupae and adults from Michigan, Washington and North Carolina were larger when reared at cooler temperatures, although the magnitude of this relationship varied between populations (Fig. 2.4). In contrast, Nova Scotia pupae were larger when reared at warmer temperatures and bigger overall (Fig. 2.4). Our results suggest that both the slope and direction of plasticity can evolve rapidly in natural populations, on time scales similar to those for the evolution of trait means. However population differences in the mean plasticity of body size were not significantly associated with seasonal temperature differences (Fig. 2.5). As a result, the evolutionary divergences in mean size and in plasticity of size are unlikely to represent adaptive responses to local thermal fluctuation.

Mean plasticity in development time was significantly associated with frost-free period: as we predicted (Fig. 2.1D), populations that experienced longer growing seasons had less plasticity in development time (Fig. 2.5B). This pattern of plasticity is largely due to differences at the lower rearing temperature (20° C), as mean development times showed little variation at the higher rearing temperature (26.7°C) (Fig. 2.3). Our results suggest that longer growing seasons may generate selection for and evolution of more rapid development especially at higher temperatures, allowing the completion of more generations per year (Roff, 1980; Taylor, 1981).

The rapid evolution of plasticity may be important in explaining the success of introduced species, but has been infrequently investigated empirically (Richards *et al.* 2006). Many studies argue that phenotypic plasticity should be beneficial to introduced species because plastic responses allow organisms to thrive in a broader range of environments. Adaptive

phenotypic plasticity has been shown to facilitate colonization in a variety of taxa (Yeh & Price, 2011; Donohue *et al.* 2001), but evidence for the hypothesis that plasticity promotes successful invasion is inconsistent. In a review of 14 studies of invasion success in plants, only seven showed that plasticity was advantageous (Richards *et al.* 2006). Even less is known about the maintenance or evolution of phenotypic plasticity in invasive species once they have successfully established in a new environment. Plasticity in gape size in snakes was beneficial in populations in the early stages of colonization, as snakes encountered novel prey. However, plasticity declined after the population became established; snakes evolved towards a canalized optimal phenotype matching prey size (Aubret & Shine, 2010). However, there is evidence that latitudinal variation may cause geographic gradients in plasticity in native species: a study of 20 *Drosophila subobscura* populations from Europe and North Africa showed that not only did cold tolerance co-vary with latitude, temperate populations were more plastic in cold tolerance than tropical ones (James *et al. 1997)*. Presumably temperate populations experienced greater thermal variation than their tropical counterparts, selecting for increased plasticity. However, empirical evidence for environmental clines in plasticity is still limited for both native and invasive species, and our results provide some of the first evidence that latitude may select for differences in plasticity during colonization.

There is previous evidence for geographic differentiation of life history in *P. rapae* (Kingsolver *et al.*, 2007). By design, we re-sampled the North Carolina and Washington populations and place them in the context of a wider range of climates. However, our results from the re-sampled North Carolina and Washington populations differ from this previous study in one important regard: experiments in 2003 and 2004 found that in North Carolina *P. rapae* reared at 20°C and 26°C, body size had a positive relationship with temperature. Our results for this population (based on experiments in 2010 and 2011) show a negative relationship between body size and temperature (Fig. 2.3). An important difference between the previous and current studies is the nutritional content of the artificial diets: our recent experiments used a diet with

more lipids (37% more cholesterol and 67% more linseed oil) than the diet used in previous experiments (following Snell-Rood & Papaj, 2009;Troetschler *et al.* 1985). Mean growth rates (pupal mass/pupal development time) for both populations were greater in the 2011 than is the 2003 study, especially at the higher rearing temperature, suggesting that higher lipid content increased growth rate. However, the different individual responses of pupal mass and development time in the two studies were complex, and depended on population and rearing temperature. Further studies of diet quality and thermal reaction norms are required to better understand the complex interactions between nutrition, temperature and growth rate. Diet affects thermal reaction norms in many insect species. Diamond and Kingsolver (2010) found that reaction norms for body size in *M. sexta* were highly dependent on diet, and our results indicate that adaptation to local host plants may result in variation of thermal reaction norms, which may play an important role in observed body size. Further experiments are needed to better understand the role of diet on adult body size and development time.

Organisms may adapt to environmental variation along latitudinal gradients through genetic adaptation of trait means as well as through the evolution of plasticity. Recent studies with invasive species have documented rapid evolutionary divergence along latitudinal gradients, but have not clearly identified the specific ecological factors underlying these patterns. Our results suggest that geographic differentiation in development time, immune response, and plasticity in *P. rapae* is more closely associated with latitudinal variation in season length and natural enemies than with variation in environmental temperatures. Understanding the environmental factors that cause population differentiation along latitudinal gradients is important for understanding patterns of biological diversity and abundance in nature, and for predicting evolutionary responses to climate in native species, or in invading exotics.

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III. Parallel Invasions May Not Produce Parallel Patterns of Adaptation: Rapid Divergence in an Invasive Insect

Abstract: Biotic invasions provide a natural experiment in evolution: when invasive species colonize new ranges, they may evolve new clines in traits in response to environmental gradients. Yet it is not clear how rapidly such patterns can evolve and whether they are consistent between regions. We compare four populations of the invasive cabbage white butterfly (*Pieris rapae*) from North America and Japan, independently colonized by *P. rapae* 150 years ago and 300 years ago respectively. On each continent we employed a northern and southern population to compare the effects of latitude on body mass, development rate, and immune function. For each population we used a split-sibling family design in which siblings were reared at either warm (26.7 °C) or cool (20 °C) temperatures to determine reaction norms for each trait. Latitudinal patterns in development time were similar between the two continents. In contrast, there were strong geographic differences in reaction norms for body size, but no consistent effects of latitude; there were no detectable effects of latitude or continent on immune function. These results implly that some life history traits respond consistently to selection along climatic gradients, while other traits may respond to local environmental factors, or not at all.

Introduction:

Latitudinal and altitudinal clines in traits are common in many populations and species, and there is significant evidence for genetic differentiation in response to climate (Mooney & Billings, 1961; Jonas & Geber, 1999; Caicedo et al., 2004). Consistent latitudinal clines across species and continents provide compelling evidence for the efficacy and predictability of natural

selection (Pegueroles et al., 1995; Gilchrist & Huey, 2001). But how rapidly can such clines evolve? The answer is important for understanding both the establishment of spatial patterns, and evolutionary responses to environmental change.

Introduced species provide a natural experiment to test whether evolution follows predictable patterns, particularly when an exotic species becomes established and rapidly colonizes a new region or continent (Baker & Stebbins, 1965; Parsons, 1983; Johnston & Selander, 1964). If traits evolve in introduced populations in latitudinal patterns parallel to those in the native range, it would support the hypothesis that environmental correlates of latitude dominate as a selective force on life history traits. However, if unique latitudinal patterns evolve after an introduction event, this would suggest that local biological communities and founder effects shape adaptation in traits. There is some evidence that clines in fitness traits can rapidly emerge after an invasion event (Maron et.al. 2007, Maron et.al. 2004, Gomi & Takeda, 1996). Studies of multiple, independent invasions or introductions that occur on different continents are particularly valuable in evaluating evolutionary predictability. For example, *Drosophila subobscura*, a European species introduced to both North and South America, developed clines in wing size on both continents similar to those of the European populations in within two decades of introduction (Gilchrist et al., 2001; Gilchrist & Huey, 2004). However, there have been few studies of multiple invasion events, where evolution in several invasive populations along environmental gradients can be compared.

In this paper we describe evolutionary divergence and plasticity of life history traits in the invasive cabbage white butterfly (*Pieris rapae*), along a similar latitudinal gradient in the eastern regions of two continents: North America (Canada and US) and Asia (Japan). *Pieris rapae* independently invaded and colonized North America and Japan during the past several centuries (see below). We focus on three life history traits—body size, development time, and immune function—and their phenotypic plasticity in response to rearing temperature. By comparing northern and southern populations of *P. rapae* at similar latitudes on the two

continents, we test whether geographic differences in life history are similar on the two continents, suggesting independent, parallel evolutionary adaptation during the past few centuries in these regions.

Methods:

Study populations:

The cabbage white butterfly, *P. rapae* is native to Europe, but was introduced to southeastern Canada in the 1860s and rapidly colonized North America, quickly spreading over a wide latitudinal gradient (Scudder, 1887). The butterfly has also appeared in descriptions and sketches by Japanese naturalists since the 1700s (Kawakatsu et.al., 2010), although the exact date of colonization in Japan from other parts of Asia is unknown. Japanese populations are now considered a distinct sub-species of *Pieris rapae* (*P. rapae crucivora*)(Obara et al., 2008; Fukano et al., 2012). Because it is an agricultural pest in many regions, *P. rapae* has been the target of biological control programs using the imported European parasitoid wasp, *Cotesia glomerata* (Vos & Vet, 2004), which now occurs in both Asia (including Japan) and North America.
Both climatic and biotic features of the environment may vary with latitude. For example in *P. rapae*, mean annual temperatures are higher and the growing season is longer in southern than in northern populations, in both North America (Seiter and Kingsolver, in press) and Japan (S. A. Seiter, unpubl. results). In addition, the frequency of parasitism by *C. glomerata* declines with latitude in *P. rapae* populations (Seiter and Kingsolver, in press). Many life history traits also exhibit marked phenotypic plasticity in response to temperature and other environmental factors (Angilletta 2009 book). Accordingly, we focus here on three life history traits—body size, development time, and immune function—and their phenotypic plasticity in response to rearing temperature. Previous studies in North America have documented population divergence in these life history traits and their reaction norms in *P. rapae* (Kingsolver et al 2007; Seiter and Kingsolver, in press). In North America, high latitude populations have weaker immune

responses, presumably due to reduced selection by *Cotesia glomerata;* we tested whether similar patterns of immune function exist in Japan. Phenotypic plasticity in body size has also diverged between populations. For example, in the ancestral population in Europe and in Washington North Carolina and Michigan, individuals were smaller when reared at high temperatures (a phenomenon known as the temperature size rule or TSR) (Seiter and Kingsolver, in press; but see Kingsolver et al 2007). By contrast, Nova Scotia individuals were larger at higher rearing temperatures, a reversal of the temperature size rule. By comparing *P. rapae* across similar latitudes on the two continents, we test whether geographic differences in life history are similar on the two continents, suggesting independent, parallel evolutionary adaptation during the past few centuries in these regions.

For these studies, we collected *P. rapae crucivora* during June-July 2011 from two areas in Japan: a northern population (Sapporo, latitude= 43.06° N) and a southern population (Kyoto, latitude= 35.00° N). In Sapporo, adult female *P. rapae crucivora* were collected from 1 - 15 July from agricultural fields in five locations near Sapporo University. Field caught females were shipped overnight to the Kyoto laboratory where they were housed in individual cages at 25 °C and given cabbage leaves (*Brassica oleracae*) to oviposit on. An additional sample of *P. rapae* was collected as pupae from two localities near Sapporo. These animals were also shipped overnight to Kyoto, where they were maintained in the laboratory at 25 °C until eclosion. Upon eclosion they were placed together in a flight cage for 48 hours to mate. After they were observed copulating, females were removed, placed in individual cages, and given cabbage leaves for ovipostion. *P. rapae crucivora* females from Kyoto were collected from the experimental gardens of the University of Kyoto from 26 June 26 – 15 July and their eggs collected using the same methods as the Sapporo population. Experiments on both populations were conducted in June-September of 2011. These two Japanese populations can be compared with two populations of *P. rapae rapae* in eastern North America (see Seiter and Kingsolver, in press) at similar

latitudes: a northern population at Nova Scotia (latitude = 45.08°N), collected in July 2010; and a southern population from piedmont North Carolina (latitude = 35.91°N), collected in April 2011. The two northern field sites and the two southern field sites have very similar mean monthly temperatures (Fig. 3.1).

Experiments

To facilitate comparisons, our experimental design mirrors that of Seiter and Kingsolver (in press) for North American populations of *P. rapae*. To compare thermal reaction norms for growth, development time and immune function, we reared caterpillars from the Sapporo and Kyoto populations at in warm and cool temperature treatments (20 °C and 26.7 °C). Eggs were collected daily from females by changing the cabbage leaves in their cages. Cabbage leaves with eggs were placed in plastic containers and maintained in environmental chambers at 25 °C until hatching. During the first instar (24-48 hours after hatching) caterpillars from each full-sib family were transferred to artificial diet and placed in individual petri dishes and assigned randomly to one of the two experimental temperature treatments (Snell-Rood & Papaj, 2009; Troetschler et al., 1985). Caterpillars were fed *ad libitum* on artificial diet (Troetschler et al., 1985; Snell-Rood & Papaj, 2009), and their diet was changed three times per week to minimize bacterial growth or spoilage. Petri dishes were checked daily for mortality, and to identify individuals that had pupated. When an individual reached pupation, it was removed from its petri dish and weighed using standard gravimetric techniques. Pupae were placed in plastic cups on a piece of damp filter paper, with a moist cotton ball to reduce desiccation, and returned to their experimental temperature.

Immune Assay

We measured immune function by injecting silica beads into the caterpillars' hemolymph. The beads activate the encapsulation response by hemocytes (insect immune cells). Assaying immune

response by injection with a foreign body is a common technique in insect immunology, and has been shown to have a significant correlation with immune response to real parasites and pathogens (Seiter and Kingsolver in press, Diamond & Kingsolver, 2011; Rantala & Roff, 2005; Smilanich, Dyer, Chambers, & Bowers, 2009). We randomly selected caterpillars for the injection assay at the beginning of the experiment but reared them in the same incubators using the same methods as those not selected. Because immune function in many insects declines immediately prior to metamorphosis (Beetz et al., 2008)injections were administered during the first 24 hours of the 5th (final) instar. Individuals developed at different rates in the two temperature treatments. As a result, caterpillars were at the same developmental stage but not the same chronological age when the assay was administered. We used DEAE Sephadex-A25 silica chromatography beads (40 -120um in diameter), from the Sigma Aldrich corporation (St. Louis Missouri, USA). We dyed the beads with a 0.1 solution Congo Red Dye and allowed them to dry completely and then were stored in a freezer to sterilize them and prevent contamination. Beads were mixed in a standard solution of 1g of dyed beads and 0.01 L of sterile Grace's Insect Cell Culture Medium to standardize the number of beads each individual received (Sigma Aldrich, St. Louis, MO, USA). Caterpillars were injected with 5 µl of the bead solution to using a Hamilton 7000 series syringe with a 25-gauge tip. After injection, caterpillars were placed on fresh diet and returned to the appropriate temperature and freeze killed after 24 hours. Beads were extracted postmortem from caterpillars by dissection and mounted on glass slides in a glycerol solution. Encapsulation and bead area were measured using the visualization program ImageJ (Diamond & Kingsolver 2011; Abramoff et al. 2004). Encapsulation (area of hemocyte aggregation) was assayed as a continuous response variable. Beads were photographed using both Nomarski differential interference contrast microscopy (DIC) and fluorescence microscopy (Zeiss LSM 510 confocal microscope). We used an automated edge selection tool (magic wand) to determine the area of cellular encapsulation on the DIC image and a thresholding tool to select the area of the fluorescent bead from the fluorescence image.

Statistical Analysis

All statistical analyses were performed in R (v. 2.11.0). Using R library nlme, we performed separate linear mixed-effects models for pupal mass and pupal development time, for individuals surviving to pupation. We included rearing temperature (Cool= 20 °C or Warm=26.7 °C), latitude (North = 43-45° N or South=35 °N), and continent (Japan or America) as fixed effects in our model; family was included as a random effect. To simply presentation and discussion of results, we refer to each population in terms of its continent and latitude: i.e., Sapporo = Japan-North; Kyoto = Japan-South; Nova Scotia = America-North; North Carolina = America-South. For the immune response models we calculated the mean encapsulation area (log-transformed and corrected for bead size) per individual for use as the response variable in our analyses. The immune analyses were done in R using the library lme4, which uses a maximum likelihood framework. Thus we performed a visual inspection of the residuals of the full model (which included latitude, continent, temperature, number of beads, and bead size and their interactions) to ensure good fit and then performed X^2-tests to compare the full model with models where each of these terms was omitted. We used generalized linear models (logit link function) to analyze the survival data; we included latitude, continent and temperature as fixed effects and family as a random effect. We performed X^2-tests to compare the full model (which included latitude, continent, and temperature, and their interactions) with models where each of these terms was omitted.

Results:

Development Time: We found strong effects of latitude, continent, and temperature on development time (Fig 3.2a). There was a significant three-way interaction between temperature, latitude and continent ($F_{1, 34} = 14.56$, $p = 0.0002$) (see Supplemental Table S1). In Japan, mean development time was shorter in the southern (Kyoto) than the northern (Sapporo) population at

both rearing temperatures. By contrast in North America, mean development time was shorter in the Southern population (North Carolina) than in the Northern population (Nova Scotia) at the cool rearing temperature, but the two populations were not significantly different at the warm temperature (Fig. 3.1b). Linear mixed effects models revealed significant single effects of temperature ($F_{1, 318} = 1142.17$ $p < 0.0001$), continent ($F_{1, 34} = 339.67$, $p < 0.0001$) and latitude ($F_{1, 34} = 12.74$, $p = 0.001$), and there were significant interactions between temperature and continent ($F_{1, 318} = 41.23$, $p < 0.0001$), and between continent and latitude ($F_{1, 34} = 5.23$, $p = 0.028$), but not between latitude and temperature ($F_{1, 318} = 1.16$, p 0.282) . Generally, development time was shorter in southern than northern populations, shorter in North American than Japanese populations, and shorter at warmer than cooler rearing temperatures.

Pupa Mass:

In contrast to development time, our analyses did not detect consistent latitudinal patterns of mean or plasticity of body size in North America and Japan (Fig. 3.2b). However, there was a strong and significant three-way interaction among continent, temperature and latitude ($F_{1, 34} = 18.70$, $p = .0001$) (Table S1). The main effects of rearing temperature ($F_{1, 319} = 0.89$, $p = 0.35$), continent ($F_{1, 34} = 3.35$, $p = 0.08$), and latitude ($F_{1, 34} = 3.32$, $p = 0.08$) on pupal mass were weak or non-significant (Table S1). The two-way interactions between temperature and latitude ($F_{1, 34} = 0.042$, $p = 0.84$), between continent and temperature ($F = 3.55$, $p = 0.061$), were non-significant or weak, although there was a significant interaction between continent and latitude ($F = 5.28$, $p = 0.0279$). In general populations from different continents and latitudes had similar masses at 20 °C, but not at 26.7 °C. However, the patterns at high temperature differed by continent and latitude. Specifically, 26.7 °C, mean pupa mass was larger for the Japan-South (Kyoto) and America-North (Nova Scotia) populations, but smaller for the Japan-North (Sapporo) America-

South (North Carolina) populations. In short, Japanese and North American populations had opposite latitudinal patterns of thermal reaction norms for body size.

Immune Function:

We did not find significant effects of temperature, continent or latitude on immune function, nor did we detect significant interactions among these factors (Table 3.1A). Individuals that received more beads as part of the assay mounted a significantly stronger immune response (Table 3.2A). We note that the standard errors on our estimates are quite large, making it difficult to detect any true patterns in these data (Fig. 3.2C).

Survivorship:

The generalized linear model demonstrated significant effects of population and temperature on survival to pupation (Table 3.1B). We found that temperature and continent had significant effects on survivorship but that latitude did not (Table 3.2B). Mean survivorship was greater for North American than for Japanese populations, and was greater at warm than cool rearing temperatures. Additional analyses indicated that two or three way interactions were not significant.

Figure 1: Mean monthly temperature (+ 1SE) for the four field sites from January 2001-January2012. Open symbols indicate animals from northern latitudes and filled symbols indicate animals from southern latitudes. North American populations are represented with squares, and Japan with circles.

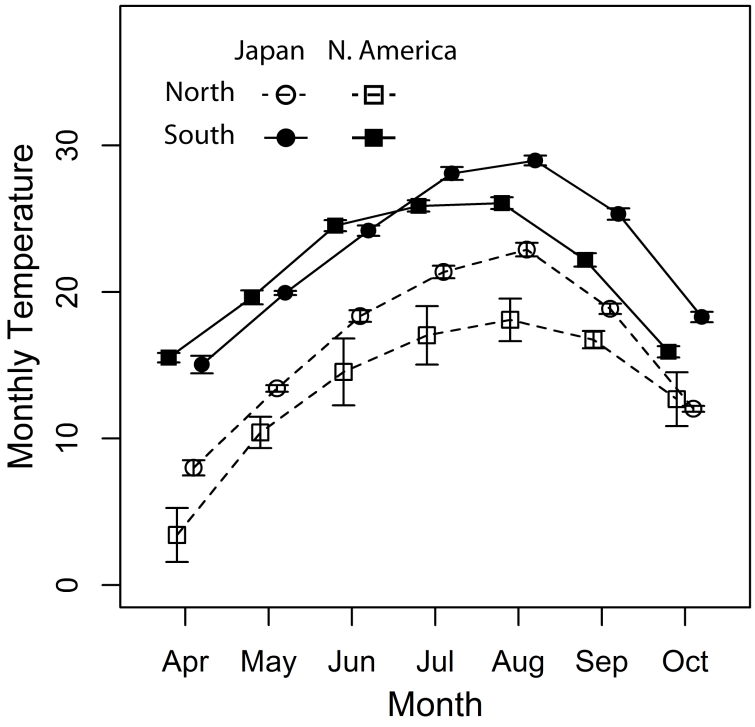
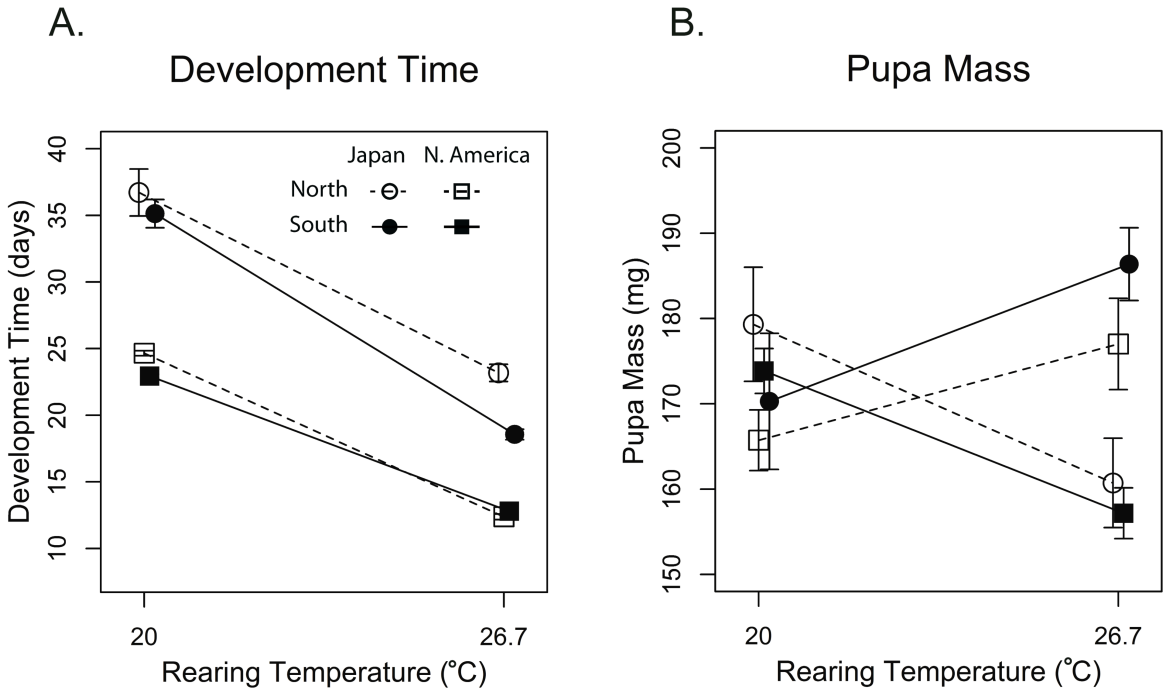


Figure 3.2 **Figure 2.** Development time (A) and Pupa Mass (B) and Immune Function (C) as a function of rearingtemperature . Open symbols indicate animals from northern latitudes and filled symbols indicate animals from southern latitudes. North American populations are represented with squares, and Japan with circles

a. Mean (+ 1SE) development time (in days)

b. Mean (+ 1SE) body mass at pupation (in mg).
c. Mean (+ 1SE) cellular encapsulation as a function of temperature.



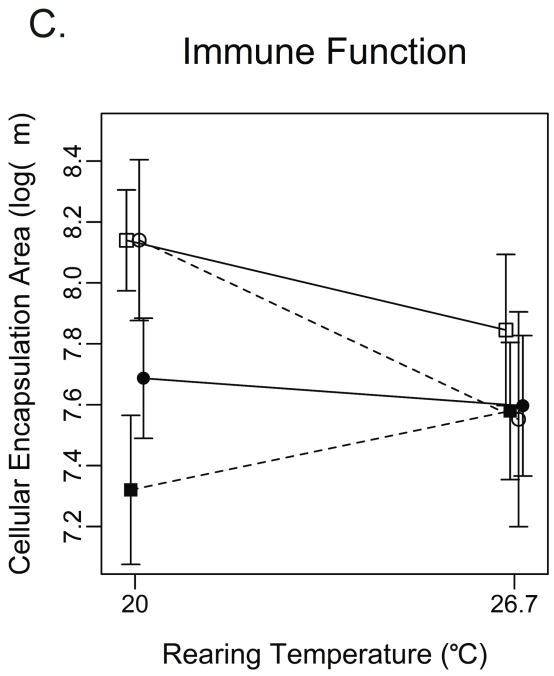


Table 3.1: the effect of temperature, continent, and latitude on immune function (a) and survivorship (b). The AIC for the full model for immune function was 177.9 and the log likelihood was -76.94. The AIC for the full model for survivorship was 1128.5 and the log likelihood was -555.28.

3.1a: Immune Function						
Model	**DF**	**ΔAIC**	**ΔLog Likelihood**	**X^2**	**X^2 d.f**	**p-value**
Full model (fixed effects: temperature, latitude, continent, bead number, bead size)	12	0	0			
Full model minus latitude	8	-4	-2.01	4.0268	4	0.4024
Full model minus continent	8	-4.94	-1.54	3.0864	4	0.5435
Full model minus temperature	8	-5.96	-1.03	2.0645	4	0.7239
Full model minus bead number	11	+39.65	-20.83	41.67	1	**<0.0001**
Full model minus bead size	11	-1.92	+0.05	0.1082	1	0.7422
2.1b: Survivorship						
Full model (fixed effects: temperature, latitude, continent)	9	0	0			
Full model minus latitude	5	-1.6	-3.17	6.34	4	0.1752
Full model minus continent	5	+25.3	-16.64	33.297	4	<0.0001
Full model minus temperature	5	+19.2	-13.56	27.133	4	<0.0001

Discussion:

There is abundant evidence for latitudinal clines in body size and other life history traits in numerous taxa (Blanckenhorn & Demont, 2004a). These clines are usually interpreted in

relation to climatic gradients, but some studies also emphasize potential changes in biotic interactions with predators and parasites that covary with latitude (Kraaijeveld & Godfray, 2001; McKinnon et al., 2010; Ardia, 2007; Corby-Harris & Promislow, 2008). Recent studies of exotic species show that such latitudinal patterns can evolve rapidly in a few decades, re-establishing latitudinal clines in traits that parallel those found in the native range. Field studies of invasive St. John's Wort in its native range in Europe and its introduced range in North America found parallel latitudinal clines in percent leaf nitrogen, but not for other traits such as leaf carbon and leaf area, while in controlled greenhouse conditions additional clines were detected in root and aboveground biomass (Maron et.al. 2007, 2004). Further, Maron et.al. found that clines could also change direction from year to year, due to differences in rainfall and temperature (2004).

Our studies with *P. rapae* also suggest that parallel clines evolved within a time scale of a few centuries for some life history traits but not in others. We found that *P. rapae* in Japan and North America had established parallel clines for development time, but not for body size or immune function. Southern populations on both continents developed faster than northern populations, with the exception of North America in the warm temperature treatment (Fig. 3.2, Table 3.1). This supports the hypothesis that longer growing seasons at lower latitudes selects for more rapid development, allowing more generations to be completed each year (Seiter and Kingsolver 2013). For example, in our *P. rapae* study populations, the mean number of generations per year varies from six (southern) to three (northern) in Japan, and from five (southern) to two (northern) in North America. This latitudinal pattern in development time has been documented in a number of temperate insects (Nygren et al., 2008; Blanckenhorn & Demont, 2004b Kingsolver and Seiter, in press), and may be particularly likely in systems in which most or all populations can potentially complete multiple generations each year. However, rapid growth and development may also occur in high-latitude and -altitude populations in order to complete a single generation over a short growing season (Roff 1980).

By contrast, we did not find consistent latitudinal patterns in body size (Fig. 3.2). For example, at the warm rearing temperature, mean pupal mass was larger in the northern than the southern population in North America; in Japan, mean pupal mass was larger in the southern than the northern population. In addition, there was a significant interaction among latitude, continental and temperature, suggesting population differences in thermal reaction norms for size. Comparative reviews have documented both Bergman clines (mean size increases with latitude) and reverse Bergman clines (mean size decreases with latitude) in insects and other ectotherms (Blanckenhorn & Demont, 2004a; Angilletta, 2001; Shelomi, 2012) Numerous factors can affect the evolution of body size in ectotherms, including diet quality and life history trade-offs among survival, fecundity, development time and body size Gilbert (1984, 1988) studied population differentiation in body size for several European and Australian populations of *P. rapae*, and found no differences in reaction norms or means for body mass between populations. The divergence in mean and plasticity in body size reported here likely reflects recent evolutionary responses to local conditions. Whether these size differences reflect adaptation to local environmental conditions, or indirect responses to selection on other, correlated traits, remains unknown (see below). Development time and body size are often positively correlated, although counter examples are abundant (Hu et al., 2012,(Stillwell & Fox, 2007; J.G. Kingsolver et al., 2007; J.G. Kingsolver et al., 2012; D. A. Roff, 2002; Shelomi, 2012); Roff, life history book) In this study we found no consistent relationship between body size and development time at the population level (Fig. 2.1). In addition, correlations within each population and temperature treatment group were generally small, ranging from -0.28 to 0.16. Weak correlations between body size and development time permit the independent evolution of these traits and may explain why development time follows a latitudinal pattern whereas body size does not (Shelomi, 2012).

Latitudinal clines in immune function have been demonstrated in some insect taxa, and are often the result of local adaptation to parasites (Kraaijeveld & Godfray, 2001; Corby-Harris &

Promislow, 2008; Kraaijeveld & Godfray, 1999, but see De Block et al., 2008). However in the current study we did not detect significant effects of latitude (or its interactions) on mean immune function: we find no evidence for parallel clines in immune function on the two continents (Fig. 2.2). It is possible that the lack of observed differences in immune function may reflect differences in the strength of selection by parasitoids on the two continents. While field surveys indicate that parasitoid prevalence is lower in northern Japan as it is in North America, there is evidence that *Cotesia glomerata* from Sapporo use a greater range of butterfly host species and are more resistant to encapsulation defenses (Sato, 1978; Tanaka et al., 2007; Sato, 1977). The observed variation in immune function may be the result of adaptation to different parasitoid host strains. Alternately, different abiotic and biotic factors may shape investment in immune function in Japan and North America.

Invasive species undoubtedly experience selection from novel abiotic and biotic environments. Results from our experiments with *P. rapae* demonstrate that for some traits, latitudinal patterns are maintained or can evolve rapidly within a few centuries. However, clinal patterns of adaptation may vary by trait, or by region. Further, some traits conform to predictions about climate adaptation, while others do not. Additional work is needed to understand which traits establish latitudinal clines during biotic invasions and which do not. Understanding this heterogeneous pattern of trait evolution in response to selection by climate gradients is important both in the study invasive species and for predicting how native species will respond to climate change.

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IV. Differental Phenotypic Selection on Body Size and Development Time in *Pieris rapae*

Abstract:

Body size and development time are both important fitness traits, and trade-offs between them are essential to many history models. However, selection on these traits can vary seasonally or by life stage in many organisms. There have been few field studies that measure selection on both development time and body size simultaneously, throughout the life span, and in different seasonal environments. We conducted two field studies in experimental gardens one in spring, and one in summer and measured phenotypic selection on body size and development time at larval, pupal and adult stages in the cabbage white butterfly. We quantified correlations between body size and development time at different life stages, and compared these relationships in spring and summer. Both principal components and linear mixed effects models revealed that development time was highly correlated across all life stages, but that body mass at early instars is uncorrelated with body size in adults. Further, we find that body size and development time were unrelated, implying that trade-offs do not constrain the evolution of these traits. Patterns of selection were consistent between spring and summer. We discuss the implications of our findings for the independent evolution of life history traits.

Introduction:

Understanding how selection acts simultaneously on development and body size during different life stages and in different seasons is critical to understanding the evolution of these important life history traits. Development rate and body size are both important fitness traits:

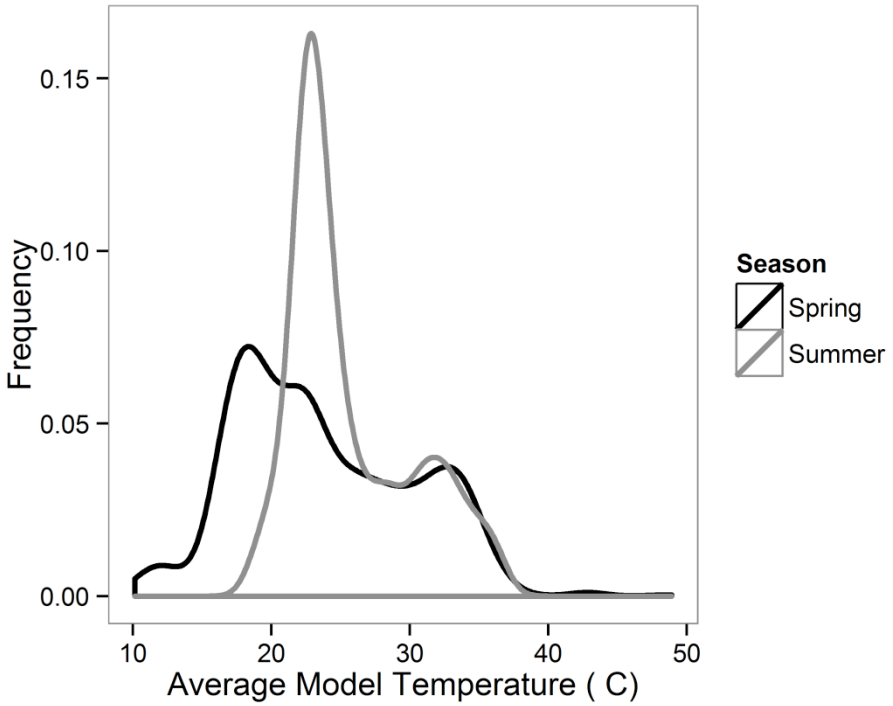
larger adult size is often associated with greater fecundity, while rapid development allows for faster generation time and higher survival to reproduction. As a result, many field studies have documented positive directional selection on adult size, or negative directional selection on development time to reproductive maturity (Kingsolver, 2000; Kingsolver, Diamond, Siepielski, & Carlson, 2012; Siepielski, DiBattista, & Carlson, 2009; Siepielski, DiBattista, Evans, & Carlson, 2011). Trade-offs between development time and body size are assumed to be widespread, and this assumption is the basis of many life history models. Indeed, divergence in these traits has been widely documented at the level of populations and species {add citations}. However, less is known about how covariation in these traits within populations influences fitness, particularly in natural field conditions. Given the expected positive correlation between body size and development time, measuring how selection acts on both traits in a range of environmental conditions is critical to understanding evolution of life histories. However, field selection studies have typically measured selection on either body size or development time, but not both, and have generally done so during only portion of the year (Kingsolver et al., 2012). Similarly, most field studies with animal populations only estimate selection for a single life stage, yet selection may vary depending on ontogenetic of the individual (e.g. Kingsolver et al., 2012); but see counterexamples).

Both biotic and abiotic factors may cause selection on development time and body size, but patterns of selection may differ seasonally, or vary by the life history stage of the individual. For ectotherms, the thermal environment strongly influences growth and development, and organisms developing during cooler parts of the year may experience radically different thermal environments (and developmental trajectories) than individuals in warmer parts of the year (Joel G. Kingsolver & Huey, 2008). Optimal development rate and body size may therefore differ depending on seasonal thermal conditions. However, biotic factors such as natural enemies can also be an important selective force on the life history of organisms. Natural enemies may pose a

greater risk during certain stages of development and organisms may grow or develop rapidly to reach a size or developmental refuge to escape attack by predators and parasites (Benrey & Denno, 1997; Paine, 1976; Werner, 1986; Werner & Anholt, 1993). Thermal environment and natural enemies may also interact in complex ways: parasite and predator abundance may vary seasonally, and seasonal differences in developmental trajectories may reflect the combined effects of the thermal environment and of mortality due to natural enemies (Kankeo, 2005; Stibor & Lampert, 2000, Diamond & Kingsolver, 2010; Kingsolver et al., 2012, Smith, Sibly, & Moller et.al., 2011). Finally age and body size at maturation may be the result of selection on correlated traits (Davidowitz et al., 2012; Davidowitz et al., 2005a). For example, in cases where natural enemies select for rapid development early in life, there may be increased development rates across the lifespan. However, the complex interplay between selection by abiotic and biotic factors, at different seasons and instars is still poorly understood.

In this study we consider phenotypic selection on development time and body size in the cabbage white butterfly (*Pieris rapae)* in two seasonal environments: spring summer (Figure 4.1). Although temperatures in spring are on average cooler than in the summer, there are important differences in natural enemies. Ohsaki and Sato found that parasitism by Cotesia glomerata varied seasonally, from over 90% in the summer, to just 15% in the spring. We used two field studies to test whether 1) development time and body size are correlated, 2) phenotypes in early development are correlated with adult phenotypes, 3) there is selection through viability and fecundity on body size and development time, and 4) these relationships vary seasonally. Correlations between body sizes and development times within and across different life stages can constrain the independent evolution of these traits (Roach, 1986). By measuring phenotype at multiple life stages, we can also examine whether there is consistent or varying selection on life history traits throughout the life span.

Figure 4.1: Frequency distribution of temperatures in the spring and summer studies. Data were collected using a Campbell Data Logger and 10 temperature sensors.



Pieris rapae is an excellent system for studying selection on body size and development time. Pupal mass and mass at late instars are strongly related to potential fecundity and females, and longer growth and development periods are required to achieve greater mass (Jones et al., 1982; Gilbert, 1984; Gilbert, 1988). However, longer development times also mean greater exposure to natural enemies, although this may vary from season to season. For example, slow growing amphibians are at greater risk from aquatic insect predators. In *P. rapae*, slow development has been shown to increase mortality from parasitoid attacks, and attack rates by *C. glomerata* also vary significantly by season (ranging from 15% in the spring, to 90% in the summer (Benrey & Denno, 1997; Ohsaki & Sato, 1994). In general we predict that there should always be selection for rapid development, but that selection for rapid development should be especially strong in late summer when parasitoids and other enemies are most abundant. We also predict a positive relationship between body size and potential fecundity (Gilbert, 1984,

1988; Jones et al., 1982). However, there may be trade-offs between rapid development (and increased survivorship) and body size: fast growing animals may enhance survivorship by reaching a developmental refuge earlier, escaping natural enemies but at the expense of body size. To test these predications, we measured age and mass at each instar, final body size, total development time, and fecundity. We also quantified the correlation structure of development time and body mass across instars, to determine whether traits could evolve independently at different life stages. Our results indicate that patterns of selection differ between spring and summer, and that some traits experience consistent patterns of selection in all seasons, while for other traits, selection is season dependent. We also find that development time is highly correlated between instars, while mass is relatively independent at different life stages.

Materials and Methods

Study System:

The cabbage white butterfly (*Pieris rapae*) is an invasive species that is native to Europe but now found on every continent except Antarctica. *P. rapae* larvae feed primarily on host plants in the family Brassicaeae; the most common host plants for *P. rapae* larvae are *Brassica* cultivars, and the butterfly is considered an agricultural pest in most of its range. Our field studies used one of the most common cultivars in the south-eastern United States: *Brassica oleracea (var. collards)*. *P. rapae* larvae develop through five larval instars and grow rapidly from ~1 mg at hatching to over 200 mg prior to pupation. Development and growth rate are strongly influenced by temperature and other environmental conditions (Kingsolver, Shlichta, & Ragland, 2006; Kingsolver, Massie, Ragland, & Smith, 2007) Although the number of generations per growing season varies by region, in North Carolina, there are 5-6 generations of *P. rapae* annually Kingsolver et al., 2007).

Field Studies:

Field selection studies were conducted in an experimental garden at the Mason Farm Biological Reserve, Chapel Hill, NC. The spring study was conducted from May 16th until May 29th, and the summer study was conducted from August 24 to September 8th. Prior to each study, the garden was tilled and fertilized (note fertilizer brand and concentration here). Collard plants (*Brassica oleracea*, variety: Flash) were grown from seed in the UNC greenhouse, and transplanted to the field after 6 weeks, and fertilized (Peters Professional Plant, whatever). For each study, a total of 240 plants were planted in rows 1 m apart, and collards were planted at half meter intervals in each row (20 plants per row over 12 rows). We also added a buffer row at the perimeter of the study plot so that all plants experienced the same density. The garden was watered daily using a drip irrigation system (Drip Depot, Medford, Oregon). To reduce larval predation by birds and social wasps, we covered each row with bridal veil netting (mesh size ~ 3.5 mm diameter) after planting. The netting allows parasitoids to pass freely, but excludes larger predators and some competitor herbivores.

During each field study we also quantified the thermal conditions experienced by larvae. *P. rapae* larvae do not actively regulate their body temperatures except to escape extreme high (>40 °C) temperatures, and previous field studies show that physical models can accurately ($\pm$ 2 °C) reflect body temperatures of *P. rapae* larvae in the field (Kingsolver 2000). We constructed cylindrical models with a length and diameter similar to a 5th-instar *P. rapae* larva, made of a moldable epoxy whose thermal conductivity is similar to that of water (Kingsolver 2000). Models were painted with acrylic (leaf green) paint, and a 0.5 mm-diameter copper-constantan thermocouple was imbedded in the center of each model along its long axis. During each field selection study, an array of 10 models were placed on hostplants in the experimental garden. Each model was placed on the shady underside of a collard leaf near the mid-vein; only one model was placed on any single plant. Temperatures of the models were measured every 15s, and the mean temperature of each model was output at 5-min intervals to a data logger (Campbell

CR3000). Model temperatures were monitored continuously throughout each field study. Here we consider the spatially-averaged (over the array of models) temperature as a function of time. We anticipate that larvae will experience lower temperatures in the spring than in the summer study.

There were a few notable differences between the spring and summer studies. First, plants were transplanted planted two weeks before the start of the spring study, but five weeks before the start of the summer study. Plants were also watered more frequently in the summer study to account for higher ambient temperatures (three times per day in summer, and two in spring). We anticipated that there might be seasonal differences in herbivory both by arthropods and rodents between the two studies, and we observed signficant herbivory only during the summer study. Some plants were entirely lost to rodent consumption; we replaced as many as possible 3 weeks before placing caterpillars on the plants, resulting in an experimental garden with 150 plants. We accounted for arthropod herbivory using a plant quality survey (see below). In addition to fertilizing with (fertilizer type), we also put blood meal on the experimental garden during the summer study, which deters rodents and acts as a nitrogen rich fertilizer (~13.25% nitrogen). Because plants were in the field for longer in the summer study and because some were replaced, we developed a rating system to quantify differences in plant quality. We scored the plants on three aspects of quality: number of leaves, leaf color, and presence of other herbivores. Principal components analysis showed that all three measures of plant quality were highly correlated, and the first principal component accounted for 84% of the variation in the data; we used this principal component as an index of plant quality in all subsequent analyses.

Study Populations

Our study contrasts patterns growth, development and survival in both spring and summer conditions in the field. Each study was initated with adult female *P. rapae* collected

from organic farms and community gardens within 35 km of our experimental garden. Because *P. rapae* is highly dispersive (Root & Kareiva, 1984), these females are sampled from a single genetic population. In each study, females were placed in individual flight cages in the UNC greenhouse *(~24 C,* 60-80% humidity, natural photocycle of 14L:10D)and given a single collard plant for oviposition; eggs were collected daily and allowed to hatch in an environmental chamber set to approximate field conditions (Percival Scientific 36VL, Perry, Indiana). For the spring study, the chamber was programmed with a 14L/10D photocycle where the temperature during the light cycle was 24 °C and 20 °C during the dark cycle, for an average temperature of 22.3 °C. In the summer study we also used a 14L/10D photocycle, but temperature during the light cycle was 28 °C and 24 °C during the dark cycle, to reflect the higher ambient temperatures in the field during the summer (see Fig. 1). Larvae were maintained through first instar in siblings cohorts grouped by the date they were laid. Hatch date was recorded for each larva. When individuals molted into 2nd instar, they were weighed individually and their age and family of origin were recorded. Larvae were then transported to the field, and each individual was assigned randomly to a plant in the experimental garden . If a larvae disappeared within 24 hours of being placed on a plant, it was replaced. In total 347 caterpillars were used in the spring study, and 186 were used in the summer study .

We conducted censuses of animals in the field each morning between 10:00 am and 12:00pm. We recorded the presence or absence of each individual and the developmental stage for all remaining animals. Transitions between instars were recorded when an individual had slipped its head capsule. We also measured the mass of each individual at the start of 5th instar. After the 5th instar weighing, individuals were returned to the plant for 48 hours during the May-June study and 24 hours during the August-September study. After the wandering period, larvae were returned to the lab, weighed again and reared in individual petri dishes in the environmental chamber (24 °C / 20 °C and 14L / 10D light cycle). Mass at pupation was recorded, and pupae

were placed in clear plastic cups on moistened laboratory filter paper, and provided a wooden stick to aid in eclosion. Mass and sex at eclosion were recorded and males were freeze-killed. To allow egg maturation and thus fecundity measurements, females were maintained in the environmental chamber for an additional 48 hours in individual cups and fed ad libitum on honey water. Females were then freeze killed, and estimates of potential fecundity were obtained by dissecting the ovarioles in glycerol solution. Mature eggs (those with a developed yolk and chorion) were counted with the aid of a dissecting microscope.

Statistical Analyses

A main goal of our studies was to quantify how size and age at early larval stages affects final body size, development time, and fecundity. We performed all statistical analyses in R (v. 2.11.0), and spring and summer studies were analyzed separately. For each study we performed separate linear mixed-effects models (library nlme) for pupal mass and pupal development time, for individuals surviving to pupation (cite Bates for Statistics Package). We selected pupal mass and development time as fitness responses for two reasons. First, they are the cumulative result of larval growth and development and are potentially important determinants of adult reproductive fitness. Second, pupal mass is the most reliable measure of body size in Lepidoptera because adults rapidly lose fluids during the eclosion process (ref, e.g. Davidowitz, Nijhout). For both the pupal mass and pupal development time models, we included mass and age at second instar and sex as predictors, and family as a random effect. For the summer analysis we also included the plant quality index (PC score) described above. We analyzed fecundity with similar linear mixed effects models, including pupal mass and development time as predictors of fecundity, and family as a random effect

We also considered how age and size in early larval stages affect survival to pupation. We used generalized linear models (lme4 library), treating survival to pupation as a binomial variable using a logit link function. For statistical testing we performed X^2-likelihood-ratio (c^2)

tests to compare the full model (which included age at second instar, mass at second instar, and the plant quality index in the summer analysis) with models where each of these terms was omitted. For all models we included family as a random effect.

We also performed a principal components analysis to characterize the correlations of size and age across different developmental stages. We based our principal components analysis on the correlation matrices of the age variables (age at the start of the 2^{nd}, 3^{rd}, 4^{th} and 5^{th} instars, and at pupation) and mass variables (mass at 2^{nd} instar, mass at 5^{th} instar, and mass at pupation). Only animals surviving to pupation were included in these analyses.

Results

Field temperatures:

Pupal Mass: We tested for effects age and mass at second instar and of sex on pupal mass in both studies; in the summer studies we also included the plant quality index as a predictor in our models. Males were significantly larger than females in both studies (Table 4.1 A). In the spring study, larvae that larger and younger at the start of 2^{nd} instar were significantly larger at pupation (Fig. 4.2A-B). By contrast in the summer study, age and mass at second instar were not significant predictors of pupal mass, nor was the plant quality index(Fig. 4.2A-B). To confirm that plant quality did not confound our results, we also ran a linear mixed effects model with plant quality as the only predictor and then extracted the residuals. We then performed analysis on the residuals testing for the effects of age and mass at second instar and sex. The analysis of the residuals agreed with our original model: only sex was a significant predictor of pupal mass in the summer study.

Pupal development time: In the spring study, age but not size at second instar was a significant predictor of pupal development time (Table 3.1. B, 4.2): younger larvae at 2^{nd} instar had significantly shorter development times. In the summer study, both younger age and smaller

mass at second instar were significantly associated with shorter development times (Table 1B; figure 4.2. C, D). In addition in the summer study, females had slightly but significantly longer development times than males; and poor plant quality scores were significantly associated with longer development time. Again we tested for a confounding effect of plant quality: we ran a model with only plant quality as a predictor of pupal development time and performed an analysis on the residuals. Here again we find that the results are not dependent on plant quality; in the residuals model, age and mass at second instar and sex were all significant predictors.

Fecundity and Survivorship: Pupal mass had a significant positive effect on egg production during the spring study but not in the summer study (although the relationship was positive, it was not significant) (1C). Development time had no effect on egg production for either study (Table 1D, Fig. 4.3). During the spring study, animals that reached third instar early were more likely to survive to pupation. Larval survivorship was similar in the two studies: survival to pupation was47.9% in the spring study, and 46.5%in the summer study. In the spring study, survival to pupation was significantly associated with age at 3rd instar, but not with age or mass at 2nd instar (Table 2): larvae that were younger at 3rd instar had higher survival in this spring. In contrast, in the summer study neither age nor mass at early larval instars was significantly associated with survival to pupation (Table 2).

Figure 4.2: The relationship between pupal mass and egg production.

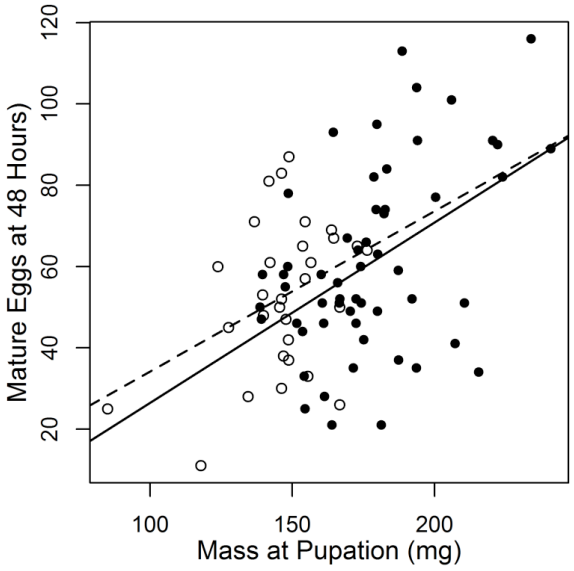
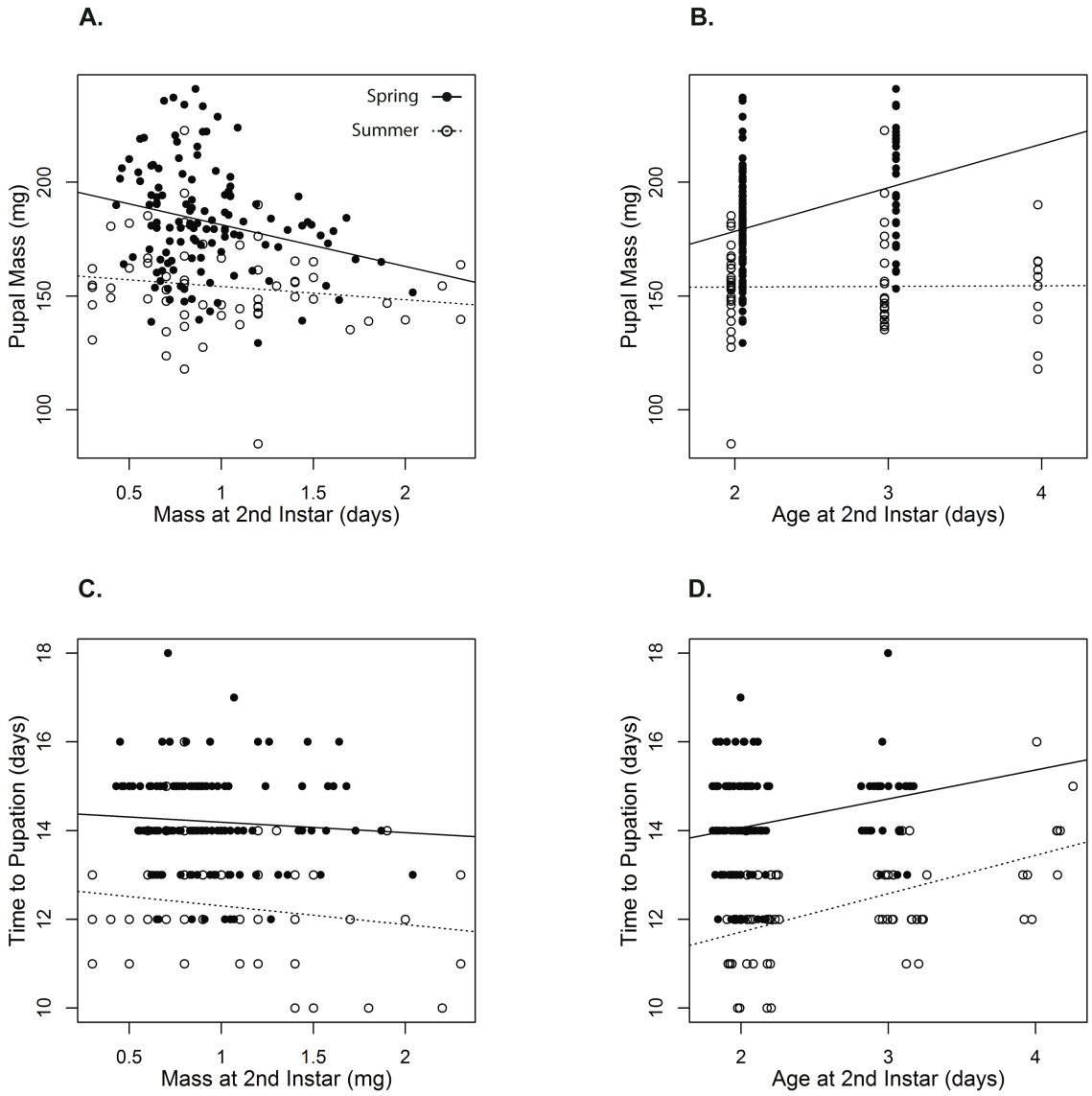


Figure 4.3: The relationship between pupal mass and mass (A) and age (B) at 2nd instar. The second two panels show the relationship between development time and mass at second instar (C) and age at second instar (D). Second instar values in panel D are jittered for display, but the age at second instar values are integers.



Correlation of Developmental Trajectories: Principal components analysis revealed positive correlations between ages at all developmental stages, and revealed that ages had negative correlations with mass at some but not at stages . These results were consistent between the spring and summer studies. The first two principal components (PCs) explained the majority of the variance -- 82% in the spring study and 87% in the summer study; thus we only report results for the first two PCs (Fig. 4.4). The first PC explained over half of the variance for both spring (61.9% and summer (71.7%) studies. In both studies, loadings on the first PC were dominated by negative values for age at all stages, and weaker positive values for mass at second instar and 5th instar (Fig. 4.4A). This PC indicates that individuals with (e.g.) younger ages at early instars also had younger ages at all stages, as well as relatively larger sizes at 2nd and 5th instars.). The second PC, explaining 20.7 to 15.8% of the variance in the spring and summer studies, respectively, exhibited loadings that were less consistent between seasons. In spring PC2 showed positive values for age at second instar and mass at pupation, but negative values for 5th instar, pupation, and mass at second instar (Figure 4.3B). In summer, patterns of correlation in PC2 were less complex: all the age values and mass at second instar had negative loadings while pupal mass had a large positive loading value (Figure 4.3B

Figure 4.4: Principal Components for Mass and Age in Each Season

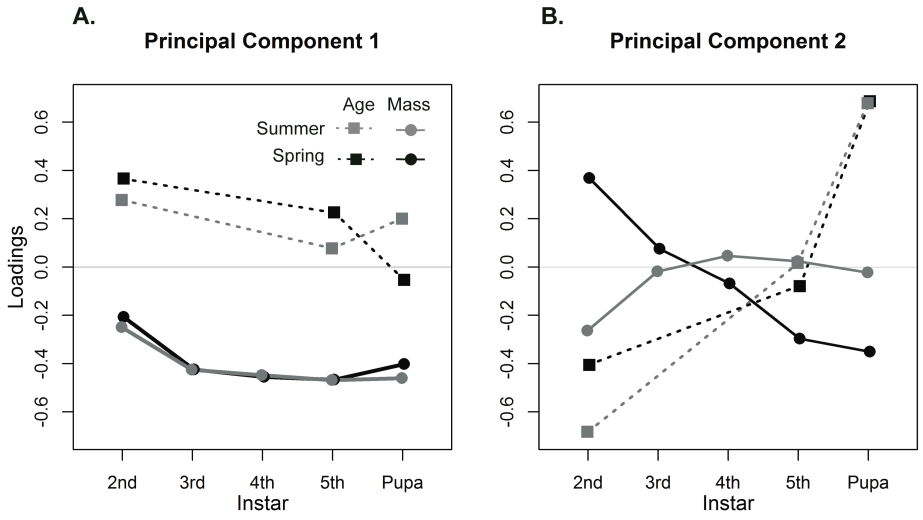


Table 1: Results from linear mixed effects models for for pupal mass (A) pupal development time (B) and fecundity (C for the spring (left columns) and summer (right columns) studies. Bolded values indicate a p-value of less than 0.05.

	Spring			Summer		
Models	**DF (Num, Den)**	**F-Value**	**p-value**	**DF (Num, Den)**	**F-Value**	**p-value**
A . Pupal Mass						
Age at 2nd Instar	**1, 111**	**10.58**	**0.002**	1, 53	0.01	0.930
Mass at 2nd Instar	**1, 111**	**2.90**	**0.031**	1, 53	1.61	0.216
Sex	**1, 111**	**11.20**	**0.001**	**1, 53**	**10.33**	**0.002**
Plant Quality Index	NA	NA	NA	1, 53	0.87	0.356
B. Pupal Development Time						
Age at 2nd Instar	**1, 110**	**6.147**	**0.015**	**1, 53**	**37.63**	**<0.0001**
Mass at 2nd Instar	1, 110	0.175	0.676	**1, 53**	**12.83**	**0.001**

Sex	1, 110	0.024	0.876	**1, 53**	**8.25**	**0.006**
Plant Quality Index	NA	NA	NA	**1, 53**	**15.89**	**0.0002**
C. Egg Production						
Pupal Mass	**1, 44**	**8.886**	**0.005**	1, 24	3.24	0.085
Development Time	1, 44	0.785	0.380	1, 24	0.01	0.941

Table 2: The effect of age and mass at second instar on survivorship . The AIC for the full model was 334.01 for the spring model and 181.47 for summer. The log likelihood was - 163.00 for the spring model and -85.73 for the summer model.

Survivorship	**DF**	**Δ AIC**	**Δ Log Likelihood**	**X^2**	**X^2 d.f**	**p-value**
Spring						
Full model (fixed effects: Age at 2nd instar, Mass at 2nd instar)	4	0	0			
Full model - Age at 2nd Instar	4	-1.43	-0.29	0.5676	1	0.4512
Full model – Age at 3rd Instar	**4**	**48.9**	**-25.45**	**50.898**	**1**	**9.73E-13**
Full model - Mass at 2nd Instar	4	0.65	-1.33	0.1034	1	0.1034
Summer						
Full model (fixed effects: Age at 2nd instar, Mass at 2nd instar, Plant Quality Index)	5	0	0			
Full model - Age at 2nd Instar	5	0.32	0.842	1.6832	1	0.1945
Full model – Age at 3rd Instar	5	1.26	0.371	0.7427	1	0.3888
Full model - Mass at 2nd Instar	5	-0.45	1.225	2.4495	1	0.1176
Full model – Plant Quality Index	5	1.16	0.422	0.8445	1	0.3581

Discussion:

In our study we contrasted selection on development rate and body size at different life stages and times of year. We find that selection is highly context dependent and may differ by trait, by life stage and season Mortality in juvenile insects can be very high, exceeding 90% in many populations, and can result from a combination of biotic and abiotic factors (e.g. host plant quality, temperature, and natural enemies) (Cornell and Hawkins 1985, Awmack & Leather 2002, Clarke and Malcolm 2002. Many studies indicate that selection on body size and development time is stronger during some larval instars than others; in particular, early instar larvae are at the greatest risk for mortality from natural enemies (Benrey & Denno, 1997; Mira & Bernays, 2002). Further, selection may differ seasonally based on changes in natural enemy abundance and abiotic conditions. Finally, different types of selection may dominate depending on life stage. Juvenile animals may be subject to viability selection, while selection on adult animals may occur through differences in mating success or fecundity. Selection at different life stages could be congruent; that is, positive selection on body size could improve survivorship in the juvenile stages and simultaneously confer greater fecundity in the adult stage. Conversely, there could be opposing selection on different life stages; large size might be beneficial in adulthood but have fitness costs early in life (Roff & Fairbairn, 2012). If phenotypes are highly correlated between life stages, then opposing selection at at different times in the life span may result in little net change in phenotype. However, if phenotypes are uncorrelated between life history stages, then selection can shape the optimal mass and development time independently at each instar.

Seasonal Patterns of Selection

Our results indicate that patterns of selection vary by season and by trait. In our analyses of pupal mass for the spring studies we found age at second instar was positively correlated with final body size (animals that took longer to reach second instar were larger), but mass at second

instar was negatively correlated with pupal mass . That is, caterpillars that were larger early in development were smaller as adults. Males were also significantly larger than females in the spring study. By contrast, only sex was a significant predictor of body size during the summer study. We found also found differences in seasonal patterns of selection in our analyses of development time. In spring caterpillars that developed quickly to second instar also became pupa sooner, but no other factors were predictive of development time. In the summer analysis however, animals that were younger at second instar also became pupa earlier, but and mass at second instar ,sex, and plant quality also had significant effects on development time. While age at second instar was the dominant predictor of development time in the spring study, other factors influenced development time in summer. Natural selection has been shown to shift in strength and direction depending on local environmental conditions (Grant & Grant, 2002; Kingsolver et al., 2012; Maron & Elmendorf, 2007; Maron, Vilà, Bommarco, Elmendorf, & Beardsley, 2004; Siepielski et al., 2009, 2011). Our results indicate that these shifts may be occur on short time scales, and that different life history traits may experience entirely different patterns of seasonal selection.

We predicted that fast development rate would have a positive effect on survivorship, and based these predictions in part on the assumption that there is strong selection at early instars by parasitoid wasps. Benrey and Denno found that there can be strong selection for rapid development by *Cotesia glomerata* in laboratory conditions, and field studies of *P. rapae* confirm that it is an important source of mortality (Ohaski and Sato 1994, VanDreische 1988a, b). Studies of a similar host-parasite system (*Manduca sexta* and *Cotesia congregata*) in North Carolina also indicate that there can be strong and seasonally varying selection by parasitoids, and that (Kingsolver et.al. 2012). While we did not observe any parasitism by wasps during either the spring or the summer studies, we did find that individuals that reached third instar sooner were more likely to survive to pupation in the spring study, but not the summer study. Mass at second

instar was not predictive of survivorship in either study. While parasitoids were not a source of mortality, rapidly reaching third instar may confer protection from other types of natural enemies that attack early in development (Mira and Berneys). Both viability selection and selection on fecundity may shape life history in *P. rapa*e:we find positive selection for rapid development at third instar through increased survivorship, and selection for greater body size at adulthood through greater egg production. Further, the lack of correlation between body mass and development time allows for the independent evolution of these traits. Pupal mass and total development time were weakly correlated (R= -0.051 in the spring study and -0.121 in the summer study).

Correlations of Life History Traits

Correlations between development time and mass at each instar are important for understanding how selection works simultaneously on both phenotypes. Our principal components analyses are based only on surviving animals, so we cannot address the role of developmental trajectories in survival. The first PC for both studies is dominated by negative loadings for age at all instars; however mass at each instar did not load consistently on PC1. This indicates that there is phenotypic variation in age at all developmental stages in both spring and summer. By contrast, the second PC in both spring and summer is dominated by strong positive loadings for mass at 5th instar and pupation, but by negative loadings for age at second instar. Loadings for age were weak for PC2. This correlation structure indicates that mass at early instars is decoupled from adult body mass, and that development time and body mass are largely unrelated. However, age at early instars is strongly related to total development time. These patterns are consistent with the results of our linear mixed effects models as described above: we found a positive relationship between age at second instar and time to pupation in both studies, but a negative correlation between mass at second instar and pupal mass. (Jones et al., 1982; Gilbert, 1984; Gilbert, 1988).

Field studies of North Carolina *M. sexta* revealed a similar correlation structure between age and body size: age at all developmental stages was highly correlated, while mass was uncorrelated between early development and pupation (J.G. Kingsolver et al., 2012). The physiological determinants of age and size at pupation are well understood in *M. sexta*. Final body size is determined during the 5th instar when caterpillars reach a critical weight, and initiate metamorphosis by degrading Juvenile Hormone, which is needed to sustain growth. Caterpillars continue feeding and growing during this period, and only stop eating and begin pupation when they begin secreting ecydosone, the molting hormone. The majority of larval growth occurs in the temporal window between these two endocrine events (Davidowitz et al., 2005b; Davidowitz et al., 2012; H Frederik Nijhout et al., 2010). Because so much growth occurs in a short period during late development, adult body mass is only weakly related to age and to mass at previous instars. The physiological mechanisms of metamorphosis are conserved across lepidopterans, and it is likely that the processes that allow independent size and age determination in *M. sexta* also determine these traits in *P. rapae* (Nijhout, 2003; Varjas et al., 1976)

These results provide a counterpoint to many life history models, which predict trade-offs between development time and body size (Abrams, Leimar, Nylin, & Wiklund, 1996; Angilletta & Dunham, n.d.; Berrigan & Koella, 1994; Scharf, Bauerfeind, Blanckenhorn, & Schäfer, 2010, but see Nijhout, Roff, & Davidowitz, 2010) . Further, many of these models assume that size or development rate at early instars is related to age at maturation or adult body size. While development time is strongly correlated across life stages, mass is not. Because there are weak correlations both between size and development time traits, and between traits at life stages, it may be possible for body mass and development time to evolve independently, or for size to evolve independently at different life stages. Although trade-offs in body size and development time are frequently observed between populations and species, it is notable that they are not strongly related within a population ,and that their relationship may vary based on environmental

conditions (Kingsolver et al., 2007; Scharf et al., 2010). If trade-offs between these traits either do not exist, or are transient within a single population, a closer examination of body-size development time trade-offs at the population or species level is necessary. If trade-offs occur only during some conditions, there may be greater independence in the evolution of these traits than previously suspected. Further investigation into concurrent selection on multiple traits is needed to better understand the evolution of life histories.

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IV: The effect of Protein and Lipid Reduction on Thermal Reaction norms for *Pieris rapae*

Abstract: For over 80% of ectotherms, increased rearing temperature results in smaller adult body size (known as the Temperature Size Rule). However, recent studies demonstrate that poor resource quality can reverse the Temperature Size Rule: the thermal reaction norm for body size may have a positive slope. However, populations may differ in their degree of evolutionary adaptation to poor quality resources, resulting in different reaction norms. We tested whether adaptation to reduced quality diets resulted in altered thermal reaction norms for body size and development time. We compared reaction norms for two populations of cabbage white butterflies (*Pieris rapae*): a domesticated population maintained in captivity for over 100 generations on high quality artificial diet, and a field caught population from Michigan that experiences variable host plant quality. We reared individuals from each population on three artificial diets: a standard diet, and diets that included 75% and 50% of the protein content of the standard. In the Michigan population, we find that protein reduction resulted in smaller body sizes, but that populations conformed to the temperature size rule regardless of diet. However, reaction norms for the domesticated population varied by diet and by sex. Domesticated females reversed the temperature size rule (had a positively sloped reaction norm) on the 75% protein diet, while males had negatively sloped reaction norms regardless of diet. We also tested for an effect of lipids on thermal reaction norms in an additional field population of *P. rapae*, but found only minor effects of lipids on development time, and no effect of lipid reduction on body size. Our results indicate that for some populations that have evolved on high protein diets, poor resource quality can cause

variation in the slope of reaction norms. In populations that experience variable diets, however, thermal reaction norms are not strongly influenced by diet.

Introduction: Many important aspects of insect growth, physiology and life history are strongly influenced by temperature. The relationship between temperature and phenotype, called a "thermal reaction norm", is well established for numerous traits in multiple taxa. Thermal reaction norms for body size in ecotherms are particularly well studied examples of phenotypic plasticity. While there is variation in thermal reaction norms for body size at the genotype, population and species level, a majority of ectotherms follow a consistent pattern in body size plasticity: individuals reared at warmer temperatures are smaller at maturity. This pattern, known as the "Temperature Size Rule" is observed in over 80% of all ecotherms studied (Atkinson & Sibly, 1997)

However, thermal reaction norms are not solely the result of genetic adaptation, environmental factors can also influence the relationship between rearing temperature and body size. Food resource quality can greatly influence growth, development and body size in herbivorous insects, but the effect of nutrition on growth can differ depending on temperature (Kingsolver, 2000; Nijhout, Roff, & Davidowitz, 2010; Stillwell & Fox, 2007). For example, Diamond and Kingsolver found that North Carolina *Manduca sexta* reared on tobacco leaves were larger at cold temperatures, consistent with the temperature size rule (Diamond & Joel G Kingsolver, 2010).However, when M. sexta caterpillars were fed a novel, poor quality host plant (Devil's Claw, *Proboscidea louisianica*) they were smaller at cold temperatures (and had lower mean body size at both temperatures). Experiments using artificial diets of varying quality confirm this pattern: poor quality diets reduce overall body size, but also may reverse the slope of the reaction norm (Diamond & Joel G Kingsolver, 2010) . Reversal of the temperature size rule on poor quality resources is typically the result of reduction in body size at cool temperatures, rather than increased body size at high temperatures, implying that diet quality has stronger

limiting effects in cold environments(Diamond & Joel G Kingsolver, 2010). Population differences in thermal reaction norms for body size may also reflect the degree of adaptation to resources. While *M. sexta* from North Carolina reversed the temperature size rule when reared on Devil's Claw, *M. sexta* from Arizona encounter the plant frequently and had typical reaction norms and were larger than North Carolina animals on Devils Claw. Stillwell et.al. found similar effects of host plant quality in bean beetles (*Callobruchs maculatus*): cool temperatures exacerbated differences in host plant quality, but the combined effect of temperature and diet was stronger in one population (Stillwell & Fox, 2007).

However, it is not clear what aspects of diet quality affect thermal reaction norms. There is substantial evidence that protein is an important and often limiting component of insect diets (Scriber & F Slansky, 1981; Frank Slansky & Feeny, 1977). Studies of *Manduca sexta* show that artificial diets with reduced protein limited growth rates and increased compensatory feeding (Kingsolver & Woods, 1998). Less is known about other dietary components, such as lipids. Stored lipids provide energy during the non-feeding pupal stage and in adulthood, and diets with inadequate lipids have been shown to cause metamorphic failure. Stockhoff (1993) found that gypsy moth caterpillars (*Lymantria dispar*) preferred low lipid - high protein diets early in development, but switched their preference to high lipid diets as they approached pupation (Stockhoff, 1993).

In this paper, we investigate whether resource quality can alter thermal reaction norms in the cabbage white butterfly, *Pieris rapae*. Studies on *P. rapae* caterpillars demonstrate that resource quality affects thermal reaction norm for short term growth rate, but it is not known how variation in response to growth rate translates into total development time and final adult body size (Kingsolver, Shlichta, Ragland, & Massie, 2006; Morehouse & Rutowski, 2010). Field studies have documented divergence of thermal reaction norms for body size and development time between populations, but these reaction norms also vary substantially within populations

(Kingsolver, Massie, Ragland, & Smith, 2007). Experiments on North Carolina and Washington *P. rapae* in 2003 revealed that North Carolina animals reversed the temperature size rule, but experiments in 2011 using a diet with higher lipid content demonstrated no difference in the thermal reaction norms between the populations (Kingsolver et.al, 2007). Individuals from both populations were smaller at pupation and developed faster on the high lipid diet in the 2011 experiments. Although other factors may have contributed to the difference in thermal reaction norms (i.e. adaptation, maternal effects), it is likely that adaptation to local food resources in *P. rapae* influences thermal reaction norms (Seiter and Kingsolver 2012, Seiter et.al. 2012).

We quantified the effects of protein reduction on thermal reaction norms in two populations of Pieris rapae, a field caught population from North Carolina, and a laboratory stock that had been in captivity for a decade. We measured thermal reaction norms for body size and development time on three diets with different protein levels. We also conducted a second experiment to measure the effects of lipids on thermal reaction norms in a second population from Michigan. We find that thermal reaction norms are significantly influenced by protein, but that these effects differ by population and by sex. We also find that lipids have comparatively minor, but significant effects on development time and no effect on body size.

Methods:

Study System:

The cabbage white (*Pieris rapae*) is a small white butterfly native to Europe that was introduced to North America in 1860. *P. rapae* lays small yellow eggs on the leaves and stems of plants in the mustard family *(Brassicaceae),* and goes through five developmental instars, before pupation and adulthood. Because larvae feed on cultivars, the cabbage white has become a common agricultural pest. From hatching to death, butterflies live about a month and in North Carolina there are 4-5 generations per year (Richards, 1940, Kingsolver et.al. 2006). Although *P.*

rapae is a generalist and is able to use a range of host plants, diet quality, particularly nitrogen content has been show to affect growth and development in the field (Slansky & Feeny, 1977). We wanted to evaluate how thermal reaction norms were influenced by protein in multiple populations of *P. rapae*, Studies of other lepidopterans indicate that domesticated populations can diverge rapidly in phenotype from wild populations. For our protein reduction experiments we used a domesticated population and a field caught population from Michigan. The domesticated population was a laboratory colony of *P. rapae*, that was maintained at North Carolina State University for the past 12 years. Originally derived from a wild population of animals in Ithaca, New York, the colony was maintained at 25.5 °C and fed Stoneville Artificial Diet (Southland Products, Lake Village, AK). We compared these animals with a wild population from Hastings, Michigan. Ithaca, NY and Hastings, MI are at similar latitudes and experience similar climates. More importantly, previous experiments in Michigan had demonstrated that the population normally conforms to the temperature-size rule, while the North Carolina population may reverse the temperature size rule. We wanted to evaluate the response of thermal reaction norms to protein reduction in a population that we knew to have typical responses to temperature (Seiter and Kingsolver, 2011. However, for the lipid reduction experiments, we chose the North Carolina population because we believed the variation in thermal reaction norms we observed between the 2003 and 2011 experiments is was due to differences in lipid content in the diets used.

Protein Reduction Experiments:

We compared thermal reaction norms for body size and growth on three different diets: a standard holdic diet used to rear *P. rapae* in the lab and two other diet treatments which contained 75% and 50% of the protein content of the standard (See Appendix A). Hatchling

caterpillars were assigned randomly to a diet and temperature treatment in a full factorial design. Animals were housed in environmental chambers set to the experimental temperatures. (Percival 36-VL: Percival Scientific, Perry, IA, USA). Diet was changed every 2-3 days to prevent spoilage. Individuals were weighed at the start of 5th instar (identified by the slipping of the head capsule), and at pupation using an electronic balance (Mettler Toledo model AT261 Delta-Range: Mettler Toledo Inc., Columbus, OH, USA) when they reached 5th instar, pupation, and eclosion. Individuals were sexed at eclosion, and then freeze killed.

There are a few notable differences between the laboratory and field experiments. In the first experiment, eggs were procured from the stock colony at North Carolina State University. Eggs were collected on parafilm placed next to a brassica leaf, collected daily, and housed an incubation chamber set to 20 C for 2-4 days (14:10 L:D cycle) until hatching. In this experiment we used three rearing temperatures (20, 25, and 30 C), which combined with the three diets yielded nine treatment groups. Additionally, in the first experiment, we used only half of the ascorbic acid called for by the diet recipe in the 50% diet group, due to a measurement error (see Appendix A).

We obtained animals for the second protein reduction experiment using a population of *P. rapae* from Michigan (the most readily available population available at the start of the experiment). Adult females were collected on organic farms in Barry County, Michigan on August 29, 2012 and Sept 6, 2012, and shipped overnight in a refrigerated container to the laboratory. Each butterfly was then placed in a flight cage in the greenhouse (~24 C, 60-80% humidity, natural photocycle of 14L:10D) with honey water and a collard plant for oviposition. After the eggs were laid they were placed in an incubation chamber at 25°C for 2-4 days (14L:10D cycle) until hatching. For this experiment we used the same protein reduced diets (Appendix A) but only two rearing temperatures (20 and 30 C), for a total of six treatment groups.

Lipid Reduction Experiments:

We also quantified the effects of lipid reduction on final adult body size and development time, using collected at local organic farms in Chatham County, North Carolina on June 13, 2012). Field caught females were housed in a greenhouse in flight cages (~24 C, 60-80% humidity, natural photocycle of 14L:10D), fed ad libitum on honey water and provided with fresh collard leaves for oviposition (*Brassica oleracea*). Eggs were collected daily and maintained in an environmental chamber (Percival 36-VL: Percival Scientific, Perry, IA, USA) at 25°C with 14L : 10D photoperiod until hatching. After hatching, the caterpillars were randomly placed onto experimental diet containing either a standard artificial diet, or a diet where lipids were reduced by either 75%, 50%, or 25% (see Appendix A, Snell-Rood & Papaj, 2009; Troetschler, Malone, Bucago, & Johnston, 1985). Hatchling caterpillars were then assigned to an environmental chamber at 20°C or 30°C at a 14L:10D photo cycle. The diet was changed every 2-3 days until the larvae reached pupation. The age of each individual was recorded at 5^{th} instar and at pupation, and caterpillars were weighed at these stages using an electronic balance (Mettler Toledo model AT261 Delta-Range: Mettler Toledo Inc., Columbus, OH, USA). Individuals were weighed and sexed at eclosion.

Statistical Analysis

Statistical analyses were performed using the software R, version 2.15.2 with the package nlme (Pinheiro et al., 2012). We used linear mixed-effect models to evaluate the effects of temperature and diet on a) pupal mass and b) development time to pupation. We used a similar model structure to analyze the data from all three experiments: we included pupal mass or development time as dependent variable, treated diet and temperature as covariates, and sex as a fixed effect. We also included all interaction terms between these variables. We also included cohort as a

fixed effect in the models where animals were started in the experiment on different dates (this occurred during both protein experiments). For the experiments using field caught females, we included family as a random effect to account for maternal and genetic effects.

Results

Protein Reduction Experiments

Pupal Mass: We found that protein reduction had a significant effect on pupal mass in both the domesticated and field caught populations, but that the strength and direction of this effect varied by both population and by sex. In the experiment using the domesticated population, we found that diet, sex, and cohort all had significant effect on pupal mass (Fig 1, C, D), but that temperature did not. Animals on the 50% protein diet were significantly smaller than those raised on the 75% and 100% protein diets. Males were larger than females at all temperatures. Females reared on the 75% diet were largest at the 30 C temperature treatment, a reversal of the temperature size rule, but this effect was not significant.

Field caught animals responded in a similar but not identical way to protein reduction compared to the laboratory population. Animals reared on the 100% protein diet were largest at pupation, animals reared on the 75% diet were intermediate in size and animals raised on the 50% diet were smallest, and this effect was independent of temperature. High rearing temperature reduced pupal mass in all diet groups (consistent with the temperature size rule), and males were larger than females regardless of diet or temperature. We found a significant interaction between temperature and sex; females responded more strongly to temperature, but in contrast to the domestic population, they decreased rather than increased body mass at high temperatures (Fig. 5.1 C, D).

Development Time: As predicted, cool rearing temperatures and low protein diets prolonged development in both domesticated and field caught populations. These two terms were the only

significant predictors of body size in the field population. In the domesticated population, we found that females were significantly slower than males. We also found significant effects of cohort (date laid) in the domesticated population. There was a significant interaction between diet and protein in the domesticated population as well, cold temperatures and reduced protein significantly prolonged development (Fig 5.1 A, B; Fig 5.2, A, B).

Figure 5.1: Development time (A and B) and body size (C, D) on diets containing three different levels of protein, using animals from a domesticated laboratory population**.**

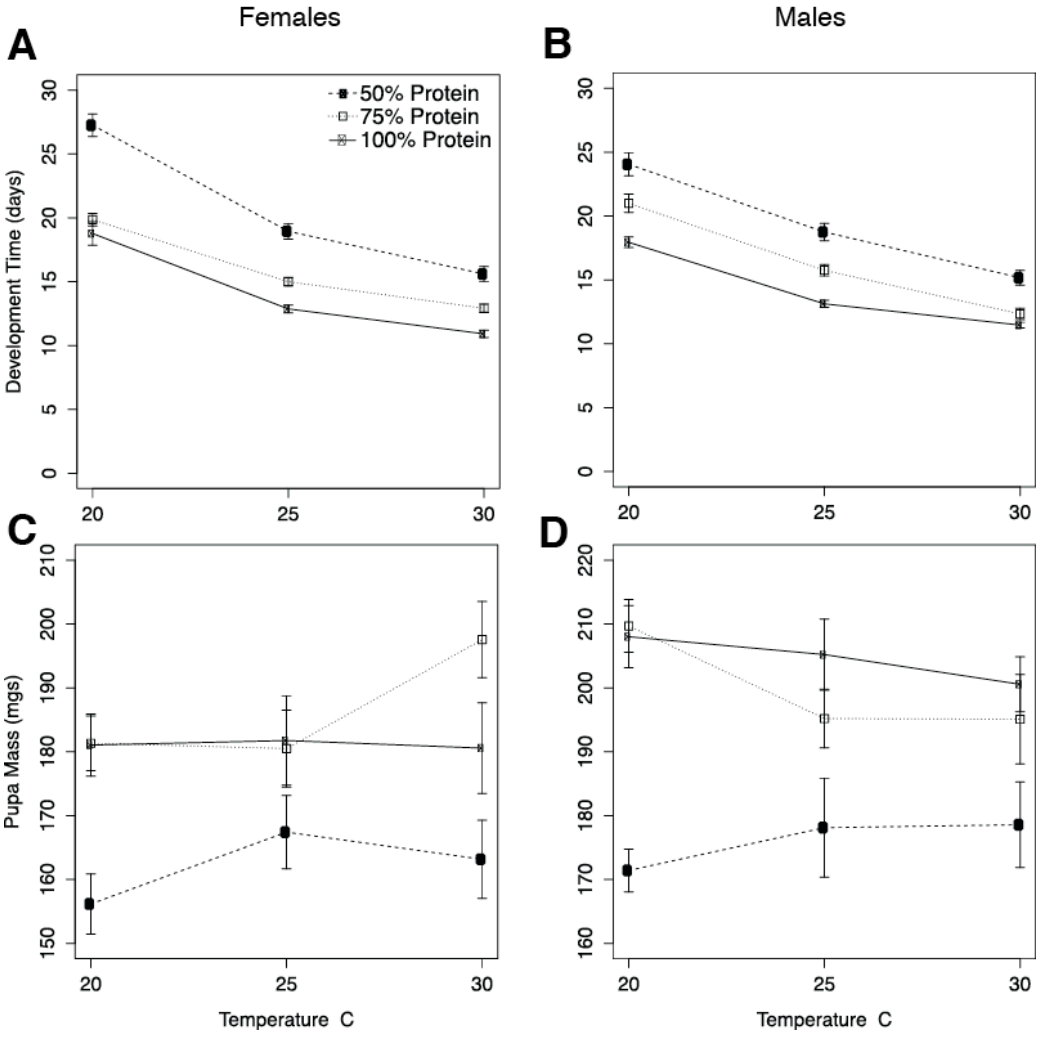

Figure 5.2: Development time (A, B) and body size (C, D) on diets containing three different levels of protein, using animals from a wild population from Michigan.

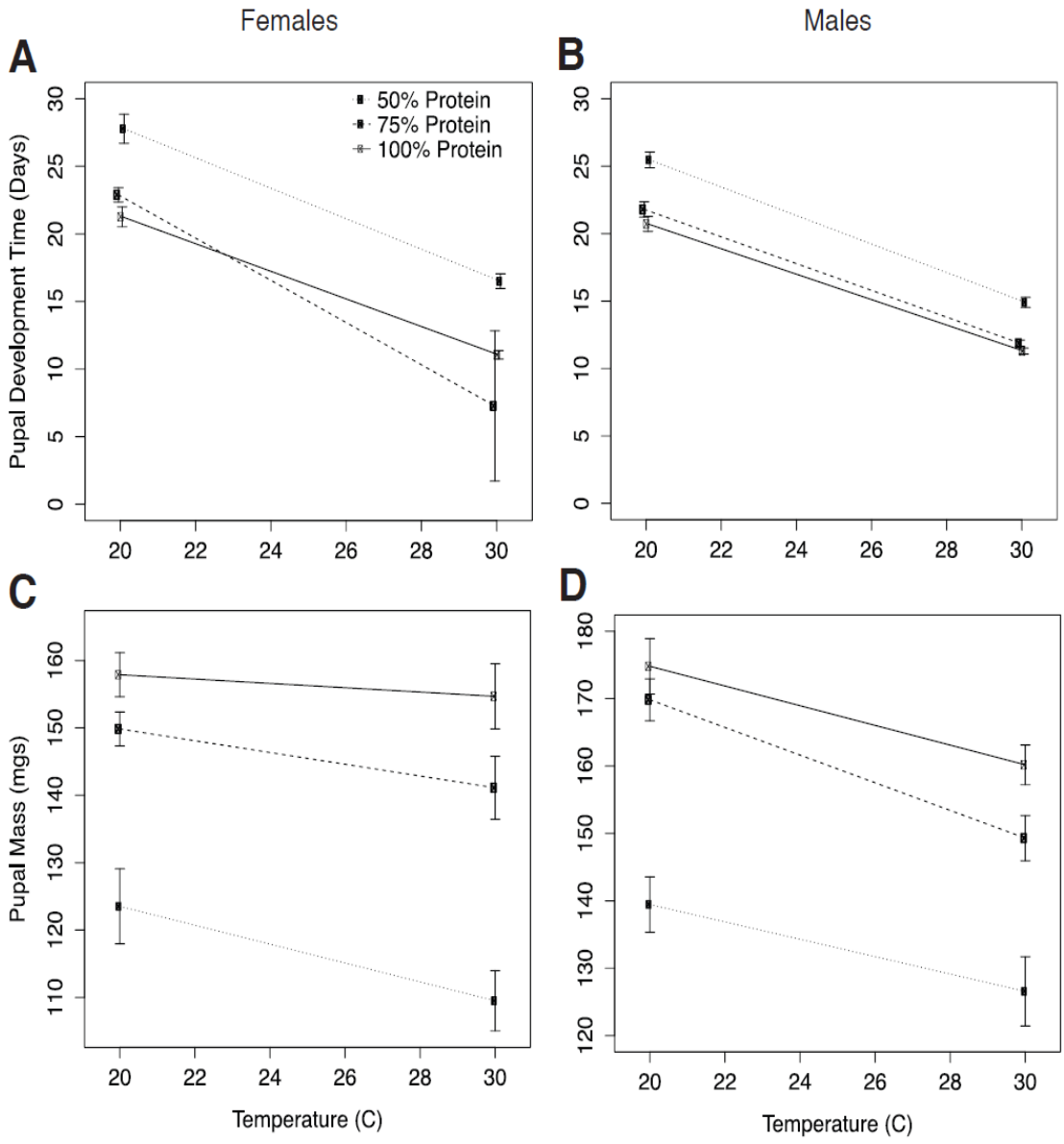

Figure 5.3: Development time (A, B) and body size (C, D) on diets containing three different levels of protein, using animals from a wild population from North Carolina.

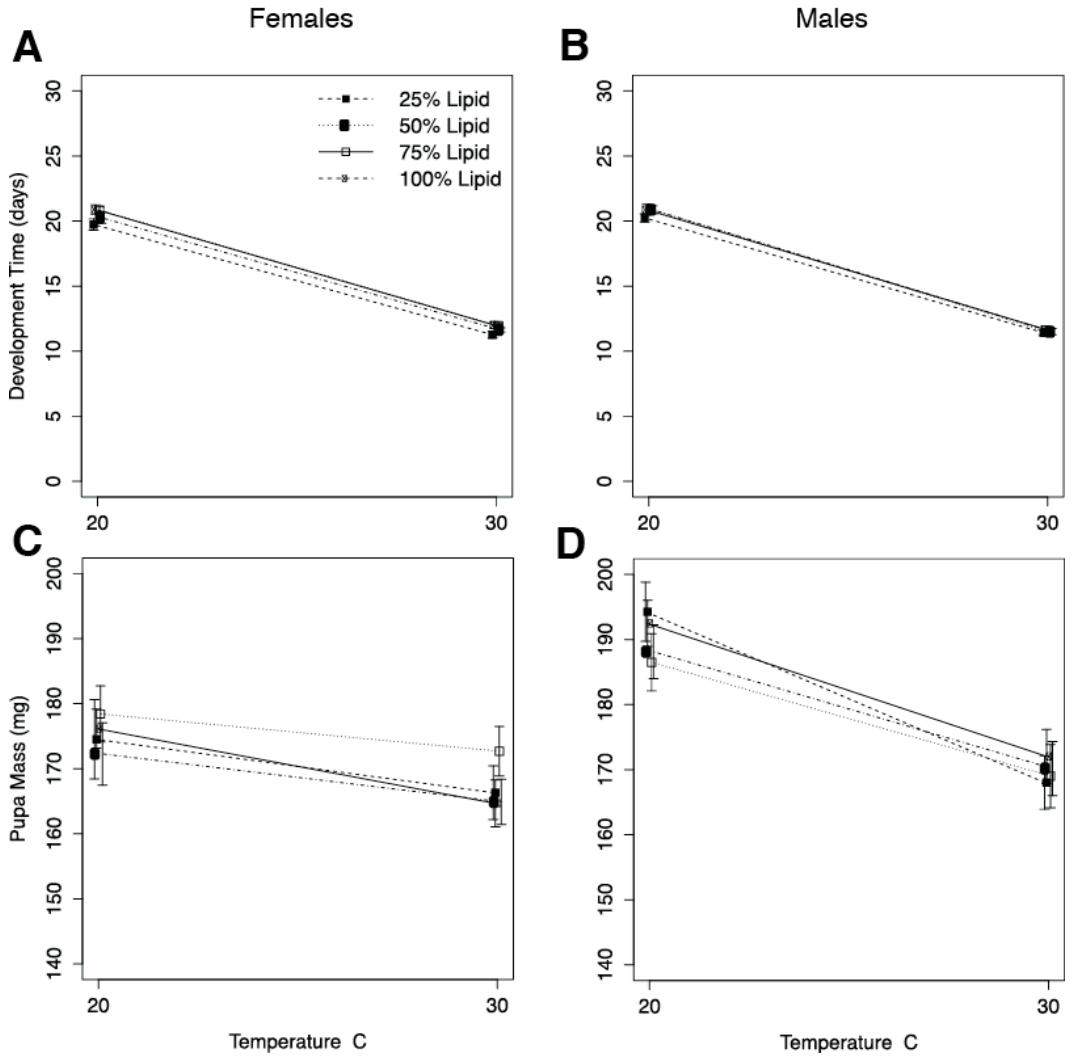

Table 5.1: Statistical models for the effects of protein reduction and temperature on development time and pupal mass in two populations of *Pieris rapae*, a domesticated population and a wild population from Michigan.

A. Pupa Mass

	Domesticated			**Field Caught**		
	DF	F	P	DF	F	P
Temperature	2	0.321	0.725	**1, 283**	**25.34**	**<.0001**
Protein	**2**	**37.12**	**<0.0001**	**1, 283**	**214.33**	**<.0001**
Sex	**1**	**41.14**	**<0.0001**	**1, 283**	**28.66**	**<.0001**
Temp * Protein	4	1.627	0.168	1, 283	0.01	0.925
Temp * Sex	2	1.609	0.202	1, 283	2.15	0.144
Sex * Protein	2	1.451	0.236	1, 283	0.10	0.748
Temp * Sex * Protein	4	0.993	0.412	**1, 283**	**4.798**	**0.029**

B. Development Time

	Domesticated			**Field Caught**		
	DF	F	P	DF	F	P
Temperature	**2**	**127.51**	**<0.001**	**1, 282**	**180.257**	**<.0001**
Protein	**2**	**127.51**	**<0.0001**	**1, 282**	**29.7633**	**<.0001**
Sex	**1**	**5.04**	**0.026**	1, 282	0.1361	0.7124
Cohort	1	38.24	<0.0001	NA	NA	NA
Temp * Protein	**4**	**4.053**	**0.003**	1, 282	1.2076	0.2727
Temp * Sex	2	2.376	0.095	1, 282	1.1310	0.2885
Sex * Protein	2	0.959	0.385	1, 282	0.1955	0.6587
Temp * Sex * Protein	4	1.017	0.399	1, 282	0.3425	0.5589

Lipid Reduction Experiment: Reducing lipids in the diet did not affect pupal mass, although all treatment groups were smaller when reared at higher temperatures. We also found that males

were larger than females in this experiment, and that this effect was significantly greater at low temperatures (Fig 5.3 C, D) . In contrast to our predictions, we found that greater lipid content actually prolonged development time; animals reared on the 25% lipid diet developed fastest (Fig 5.3, A, B). However, these effects were comparatively small. As expected, higher temperatures decreased development time. Sex did not have a significant effect on development time in this experiment, and there were no significant interactions between the other variables in this experiment.

Table 5.2:

	Pupal Mass			**Development Time**		
	DF	F	P	DF	F	P
Temperature	**1, 290**	**49.31**	**<.0001**	**1, 292**	**4418.31**	**<.0001**
Lipid	1, 290	0.004	0.948	**1, 292**	**11.641**	**0.0007**
Sex	1, 290	17.720	<.0001	1, 292	0.017	0.896
Temp * Lipid	1, 290	0.212	0.646	1, 292	0.891	0.346
Temp * Sex	**1, 290**	**8.973**	**0.003**	1, 292	2.852	0.092
Sex * Lipid	1, 290	0.474	0.492	1, 292	2.218	0.138
Temp * Sex * Lipid	1, 290	0.772	0.380	1, 292	0.004	0.953

Discussion:

Ectotherms most commonly develop faster and reach smaller adult sizes when reared in warm environments, and although this relationship is known as Temperature-Size rule, it is rule that is often broken. However, reversals of the temperature size rule can be instructive and permit a better understanding of the evolutionary and ecological factors that shape body size. Field and laboratory experiments demonstrate that diet quality can influence reaction norms; our results confirm that diet can affect thermal reaction norms, and that populations respond differently to low quality diets. We find that protein reduction has strong but not entirely consistent effects on

thermal reaction norms for pupal mass and development time. Numerous studies have linked reduction in dietary protein to behavioral and developmental responses, and to ecological relationships with other species in *P. rapae*. However, previous studies of *P. rapae* on artificial diets also indicated that developmental responses to protein might be influenced by temperature (Kingsolver et.al. 2007, Seiter and Kingsolver, 2012, Morehouse and Rutowski, 2012). In our study, only females from the domesticated colony reversed the temperature size rule, and both sexes had more variable reaction in the domesticated population. This increased variability in reaction norms may be the result of adaptation to standardized, high protein laboratory diets and selection for performance on low quality diets may be relaxed. By contrast, the Michigan population experiences natural variability in host plant species, quality, and availability, and there may be selection to maintain performance on low protein diets.

Adaptation to resources may determine whether reaction norms are robust to changes in diet. Evidence from *Manduca sexta* indicates that populations which are adapted to low quality diets can maintain typical reaction norms when reared on reduced protein or low quality diets. For example, *M. sexta* from Arizona populations commonly encounter the novel host plant Devil's Claw, and maintain typical reaction norms when reared on this lower quality food resource. *Manduca sexta* from North Carolina rarely encounter devil's claw and had body sizes and reversed the temperature size rule (Kingsolver and Diamond, 2012). Domestication has also been shown to affect thermal reaction norms in *M. sexta*. A comparison of two domesticated populations and a field population demonstrated that laboratory populations had adapted to artificial diet, but that one laboratory population had higher body size on diet, while the other performed better on tobacco plant. By contrast, the field population performed best on tobacco and had significantly reduced body size on artificial diet. When the three populations were reared on devil's claw, the field population performed best, indicating that domesticated populations experienced relaxed selection for growth on novel host plants (Kingsovler and Diamond 2010).

It is notable that males and females from the domesticated colony had different responses to protein reduction. Females on the 75% protein diet were larger at the high temperature, violating the temperature size rule. Male and female *P. rapae* experience different demands for resources. Males use protein in making pterin-based pigments that are important for attracting mates, and they also produce protein rich spermataphores that are transferred to females as a nuptial gift. Females on the other had invest heavily in egg production. The differing demands of reproduction on females and males may result in different patterns of resource allocation on reduced protein diets (Morehouse & Rutowski, 2010). Domestication may therefore affect males and females differently as one sex or the other may be more sensitive to protein limitation, and may adapt differently to the relaxed selection of laboratory diets.

By contrast, we find that lipids had no effect on pupal mass and very small but significant effect on development time. These results confirm research by Morehouse and Rutowski indicating that protein is the most important limiting factor in *P. rapae* development. In experiments on carbohydrate and protein reduction, they found that *P. rapae* could sucessfully compensate for carbohydrate reduction by increased feeding rates, but that rapid feeding was insufficient to offset protein reduction. Although we did not measure behavioral responses to diet reduction, we find that only protein had a strong effect on body mass or development time. Presumably differences in lipid content can also be compensated by increased feeding rate, or simply have little effect. Interestingly, reduced lipids actually shortened development time slightly, indicating that there may actually be fitness costs to high lipid diets.

While there is evidence for significant variation in thermal reaction norms in wild populations, it has been hypothesized that local adaptation to diet may drive these differences. While we found that reaction norms body size and temperature were relatively consistent between the domesticated laboratory and wild Michigan populations, there were some differences. Moorhouse et.al. provide evidence for genetic variation in responses to protein limitation (2010).

The substantial within population variation for reaction norms and this may permit the observed divergence in reaction norms between populations (Seiter and Kingsolver, Kingsolver et.al. 2007).

In addition to understanding how variation in thermal reaction drives population divergence, diet-dependent thermal reaction norms may have important ecological effects. Reduced development rate on poor quality diet result in increased parastoid prevalence. Slow developing larvae remain in earlier, more vulnerable developmental stages for longer, and are therefore susceptible to parasitoid attack (Benrey & Denno, 1997). Variation in host plant quality may also force individuals to forage in more exposed habitats where they are more exposed to predators (Stamp & Bowers, 2000; Stamp & Bowers, 1990). Final adult body size is also linked to fecundity in females, and diet quality may therefore affect population growth rates (Awmack & Leather, 2002). While investigations of thermal reaction norms have often been confined the laboratory, body size and development time have important consequences for population growth, fitness and trophic interactions. Future work is needed to determine how thermal reaction norms function in the context of ecosystems.

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VI: Limits to adaptive plasticity for continuous reaction norms: a meta-analysis

Abstract

Adaptive phenotypic plasticity is widespread in nature, but genetic correlations between plasticity and the overall mean trait value may potentially constrain the evolution of adaptive plasticity. While studies that use two environments necessarily define plasticity in terms of range or slope, studies of three or more continuous environments demonstrate that other aspects of reaction norm shape may be important. We conducted meta-analyses using data from 29 studies of continuous reaction norms that consider three or more environments; studies of thermal reaction norms for insects were highly represented in the dataset. We quantified three different metrics of plasticity: range, slope, and curvature. All three metrics were significantly positively correlated. Range and slope had the strongest correlation, while range and curvature reflected partially independent aspects of reaction norm shape. Trait means and range were significantly and positively correlated, whereas trait means and curvature were uncorrelated. The correlation between the grand trait mean (across all environments) and plasticity also depended on the type of trait measured: the correlation of mean with plasticity was near zero for life history traits, but strongly positive for functional/physiological traits. The independence of curvature and trait means suggests that some, but not all, aspects of plasticity may evolve independently of mean trait value.

Introduction

Phenotypic plasticity is the capacity of a genotype to reliably produce different phenotypes in different environments. Numerous studies have documented variation in plasticity at the level of genotype, population and species (Van Buskirk and Steiner 2009; Auld et al. 2010). Plastic trait expression can increase fitness by adjusting phenotype to match environmental conditions, resulting in adaptive plasticity. Given phenotypic and genetic variation for plasticity, variable environments can lead to selection and evolution of both means and plasticity of phenotypic traits.

However, there are important limitations to the evolution of adaptive plasticity. Physiological, developmental and genetic mechanisms may constrain the expression of different trait values in different environments such that the mean trait value (which we define here as the average trait value across all environments) and phenotypic plasticity in the trait may not vary independently (Flatt and Heyland 2011). At the population level, these mechanisms can generate genetic covariances in trait values across environments, constraining the independent evolution of trait means and plasticity (Via 1984; Via and Lande 1985). Correlations between overall trait means and plasticity can also complicate efforts to detect and quantify costs of plasticity (DeWitt et al. 1998; Auld et al. 2010). Indeed, the majority of studies that examine these costs have failed to detect them (Relyea 2002; Maughan et al. 2007; Van Buskirk and Steiner 2009)

These considerations suggest that a better quantitative understanding of the relationships between trait mean and plasticity is needed to understand the potential for and limits to evolution of adaptive plasticity (Via and Lande 1985; Via et al. 1995). In addition, the limits to adaptive plasticity may differ among different types of traits, or among different environmental factors (Flatt and Heyland 2011). For example, an analysis of thermal plasticity in insects suggested that

physiological and performance traits had relatively greater plasticity than morphological traits (Kingsolver et al. 2004). Whether this pattern holds for other taxa or other environmental factors has not been explored.

Constraints on phenotypic plasticity may be particularly important for continuous reaction norms, in which the expressed phenotype of a genotype varies with some continuous environmental factor (e.g. temperature, nutrient level, predator density) (Kirkpatrick and Heckman 1989; Gomulkiewicz and Kirkpatrick 1992). Characterizing variation for such reaction norms requires experiments where phenotypes are measured in three or more environments. Measuring plasticity in such studies can prove challenging, especially if reaction norms are nonlinear; currently there is no consensus on how best to quantify plasticity in this case. For example, Valladares et al. (2006) identified eighteen different metrics of plasticity used in the literature. It is not clear whether these metrics capture distinct aspects of plasticity, or whether they are all strongly correlated. Yet selecting appropriate metrics of plasticity is essential for addressing the relationship between phenotypic plasticity and overall trait mean.

In this study we use data on reaction norms for multiple genotypes within populations from multiple studies to address two main issues about patterns and limits to variation in continuous reaction norms. First, we evaluate the extent to which trait means and their plasticities are correlated, and whether this correlation varies with different metrics of plasticity. We also use meta-analysis to evaluate whether the correlation between trait mean and plasticity depends on the taxonomic group studied or on the type of trait being measured. Second, we consider three distinct metrics of plasticity and explore whether these metrics reflect different or similar aspects of plasticity (i.e. whether they are uncorrelated or strongly correlated). We use meta-analysis to evaluate whether the correlation between different metrics of plasticity varies depending on the type of trait studied or the study organism used.

Materials and Methods

To locate appropriate studies of reaction norms, we conducted a literature search on ISI Web of Science using the terms *"reaction norm"* and *"gene-by-environment interaction"*. The search, conducted on Jan. 26, 2011, yielded 426 publications. We identified and included experimental studies that exposed organisms to three or more environments and measured traits on related individuals from multiple genetic groups. We restricted our search to studies that exposed organisms to environments that could be ranked/ordered, excluding studies where this was not the case (e.g., host plant A vs. host plant B vs. host plant C). Further, we only included studies with 15 or more genetic groups (i.e., lines, families or clones). To obtain the raw data we contacted the authors of the selected papers. We also used data sets collected for a previous meta-analysis (Kingsolver et al. 2004).

Our final dataset included studies from 15 publications. Where publications included measures of multiple traits in the same experiment, we treated all independent traits as separate studies, for a total of 29 studies. To assure independence of traits we eliminated any composite traits; for example we excluded growth rate when measured as a composite of mass and development time. Morphological traits were included when they were independent of body mass. We categorized traits as morphological, growth-related, life history or functional/physiological. We defined growth-related traits as any measure of size or mass change over time. Traits pertaining to reproduction, dispersal, and development time were defined as life-history traits. Morphological traits measured variation in any physical structures of an organism. Functional/physiological traits were those that measured physiological processes or physical performance (e.g. locomotion). All told, 13 genera were represented in our data set. For the analysis we sorted these into three categories: arthropods (arachnids and insects), microbes (bacteria and viruses) and plants. Microbes differ slightly from the other organisms studied because these phenotypes were measured at the population level. However, reaction norms are

routinely measured at the population level for microorganisms, so we include them in this analysis (Forst and Inouye 1988; Remold and Lenski 2004; Kuemmerli et al. 2009).

Metrics of phenotypic plasticity

Although there are numerous approaches to quantifying plasticity described in the literature, we used three different metrics that may characterize different aspects of the reaction norm. For all metrics, we calculated the mean phenotypic value in each environment for all individuals of a given genotype (clone, family, etc.) to generate a genotype-level reaction norm. All plasticity metrics were calculated using these genotype-level reaction norms. Thus, for a given reaction norm measured in N environments, where phenotype is Z, and environment is E, we calculated the *"Range"* metric as the difference between the largest phenotypic value and the smallest phenotypic value from all environments sampled (i.e., max(Z) – min(Z)); see Fig. 6.1. Second, we calculated the slope (β_i) of the reaction norm between each consecutive environment:

$$\beta_N = \frac{Z_{j+1} - Z_j}{E_{j+1} - E_j}$$

We took the absolute value of each slope, and averaged these values for the genotype:

$$\text{Slope} = \sum_{i=1}^{N-1} \frac{|\beta_i|}{N-1}$$

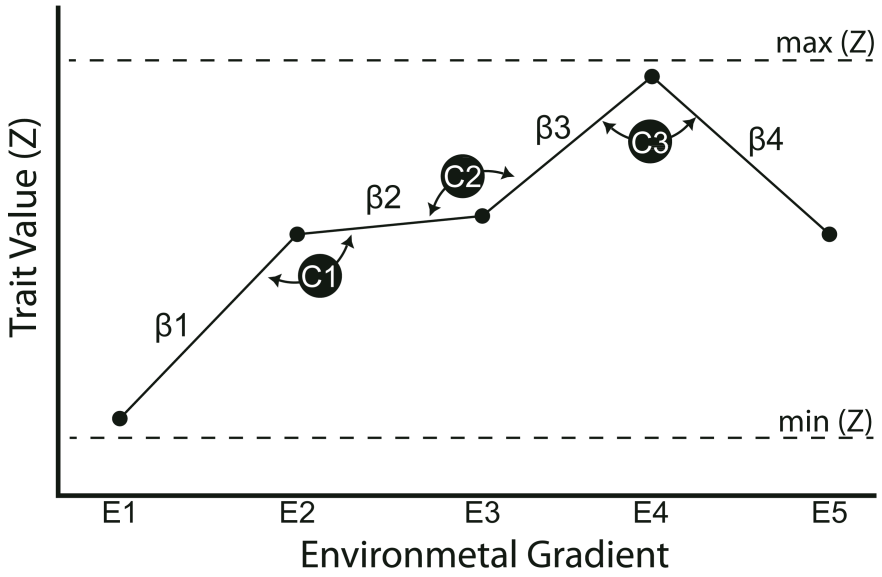
This "*Slope*" metric reflects the average slope of the reaction norm. Finally, we calculated the change in slope between adjacent line segments and averaged the absolute value of all curvatures for the genotype:

$$C_j = \beta_{j+1} - \beta_j$$

$$\text{Curvature} = \sum_{j=1}^{N-2} \frac{|C_j|}{N-2}$$

This "*Curvature*" metric reflects the average curvature or shape of the reaction norm. We also calculated mean trait value over all environments for each genotype by averaging the trait value for all individuals of the genotype over all environments. Note that for each study, correlations between plasticity and trait or between plasticity metrics were calculated within a given study, and the Pearson correlation coefficients (*r* values) are reported. Thus while traits might be measured on different scales, *r* values for correlations between trait and plasticity, or between plasticity metrics, are be scale-independent. At no point are raw trait means from different studies ever compared, rather we report the correlation coefficient for trait mean and plasticity, or between metrics for all genotypes within a given study and use the reported *r* values as our unit of analysis.

Fig. 6.1: A hypothetic reaction norm where phenotype (Z) was measured in five environments (E_1-E_5). Consecutive slopes (β_i) and curvatures (C_i) are illustrated; see text for further description.



Statistical analysis

All plasticity metrics and means were calculated using the *stats* package in the software R, version 2.11.0. The meta-analysis was conducted using the *meta* package in R, using the function *metacor*. The *metacor* function is specifically designed for meta-analysis of correlation coefficients, based on Fisher's *z* transformation. We used the correlation coefficients as measures of effect size, and trait type and taxonomic group as fixed effects in the models. In these models, effect sizes are weighted by sample sizes, and study identity is incorporated as a random effect.

Trait Means and Plasticity

We evaluated whether the mean trait value across all environment ("*Trait Mean*") and plasticity were correlated, and whether this correlation depends upon the type of trait, taxonomic group or the plasticity metric. If correlations between trait value and plasticity exist, then selection for trait value and plasticity cannot be independent; non-independent evolution of trait and plasticity may represent a potential limit to the evolution of plasticity. Conversely, selection for increased trait value could also result in increased plasticity regardless of whether such plasticity was adaptive. To test this relationship, we calculated the mean trait value across all environments for each genotype, and computed the Pearson product correlation (*r*) between mean trait value and each plasticity metric for the genotypes in each study. The *r* values for each plasticity metric (*Range, Slope* and *Curvature*) were analyzed separately. We conducted meta-analyses using *r* values as the measure of effect size, and taxonomic group and the trait type as fixed effects in each model as above.

Correlations Among Plasticity Metrics

We first tested whether the three plasticity metrics—Range, Slope and Curvature—were strongly correlated, or whether they captured distinct, uncorrelated aspects of the reaction norm. For each of the 29 studies, we calculated the three plasticity measures for every genotype in each

study, and computed the Pearson correlation (*r*) between each pair-wise combination of metrics (*Range* and *Slope*, *Slope* and *Curvature*, and *Range* and *Curvature*). Second, we used meta-analyses (*metacor* function in R) to test whether the magnitude of correlations among plasticity metrics depended on the type of trait or taxonomic group. We then used the *metacor* function to evaluate whether trait type or taxonomic group affected the strength of the correlation between plasticity metrics. For each model, we used the *r* values for a study as the measure of effect size and included either taxonomic group or trait type as fixed effects in the model. We considered nine models in total, one for each combination of plasticity metrics with trait type as a fixed effect, and a second set of three models, which tested the effect of taxonomic group for each pair of metrics. We selected studies with three environments or more because we wanted to avoid as much as possible the inherent correlations between slope and range, yet such correlations are in part inescapable. Therefore, we expected there to be some correlation between the three metrics (genotypes with greater ranges should also have greater slope), yet the degree to which these measures are independent is of interest to researchers measuring plasticity.

Results

The Dataset

Table 1 summarizes the data used in the analyses. Among taxonomic groups, more than half of the studies (62%) involved invertebrates, primarily insects, but plants (26%) and microbes (10%) were also represented. Among trait categories, growth traits made up 41% of the data set, morphological, and life history studies comprised 24 % of the data set each, and studies of functional traits made up the remaining 10% of the data set. Among environments, 79% of the studies involved temperature as the environmental variable, while studies that used light as the environmental variable made up 7% of the data set and studies of CO_2 made up 10%; there was a single study of nutrient levels also included. The number of environments measured ranged from

3 to 11, but 66% of the studies involved only 3 or 4 environments. As a consequence, our meta-analyses focused on taxonomic group and trait type, but did not consider models with multiple moderators or their interactions (see Methods).

Table 6.1. Number of studies for each trait type, taxonomic group, environmental variable, and number of environment treatments included in the experimental design. Not all combinations of experimental factors are present; for example all the microbe studies measured growth. For detailed information on the studies included see appendix A.

Taxonomic Group	**Number of Studies**
Plant	8
Microbe	3
Invertebrate	18
Type of Trait	
Morphology	7
Growth	12
Functional	3
Life History	7
Environmental Variable	
Nutrient	1
Light	2
CO2	3
Temperature	23
Number of Environments	
3	7
4	12
5	5
6	2
7	1
9	1
11	2

Trait Means and Plasticity

The correlation between mean trait value across environments (*Trait Mean*) and plasticity depended strongly on the metric of plasticity under consideration. For example the correlation between *Trait Mean* and *Range* was moderate (mean *r* for all studies: 0.395, 95% CI: [0.352, 0.437]), with a somewhat weaker correlation between *Trait Mean* and *Slope* ($r = 0.285$, 95% CI: [0.239, 0.331]). In contrast there was no significant correlation between *Trait Mean* and *Curvature* ($r = 0.026$ 95% CI: [-0.024, 0.076]), suggesting that *Trait Mean* and reaction norm shape can vary independently depending on how the reaction norm is quantified. The correlation of trait means with plasticity varied significantly among taxonomic groups, but the estimated differences among taxa depend on the plastic metric (Table 6.2B, Fig. 6.2C). This pattern is difficult to interpret. The correlation of trait means with plasticity also varied significantly among trait types (Table 6.2B). For example, the correlations of trait mean and plasticity were near zero for life history traits, but were strongly positive for functional traits (Fig. 6.2D). For growth and especially morphological traits, the correlation between mean trait and plasticity depended strongly on the plasticity metric. In contrast, correlations between *Trait Mean* and *Curvature* were lower for all types of traits, suggesting that overall mean trait values and reaction norm curvature vary quite independently.

Correlations Among Plasticity Metrics

All three plasticity metrics were positively correlated, but the magnitude of correlation varied among metrics. In general, *Slope* was strongly correlated with *Range* (*r* for all data sets = 0.862 (95% CI: [0.849, 0.875]) and with *Curvature* ($r = 0.809$ [0.791, 0.826]). *Range* and *Curvature* were more weakly correlated ($r = 0.603$ [0.570, 0.634]), suggesting that these two metrics do capture partially independent aspects of plasticity. However, the correlations between plasticity metrics varied significantly among taxonomic groups (Table 2A). For example, the

estimated mean correlation between *Range* and *Curvature* was lower for microbes and plants than for invertebrates (Fig. 6.2A). The correlations between plasticity metrics also varied significantly with the type of trait (Table 6.2A, Fig. 6.2B). *Range* and *Slope* were highly correlated ($r > 0.82$) for all trait types: these two metrics reflect similar aspects of plasticity regardless of the trait measured. In contrast, the correlations between *Range* and *Curvature* varied substantially with trait type. For example, these two metrics were highly correlated for morphological traits, moderately correlated for life-history and growth traits, but only weakly correlated for functional traits. There was a similar but weak pattern for the correlations between *Range* and *Slope*: the two metrics were highly correlated for growth, morphology, and functional traits, but were less strongly correlated for life-history traits.

Table 6.2: Correlations between plasticity metrics for different taxonomic groups and trait types (2a) and between mean trait value and plasticity (2b). Table 2a presents the results of the meta-analysis of the correlations between plasticity metrics. Table 2b presents the results of the meta-analysis of the relationship between mean trait value over all environments and plasticity (as measured by each of the three metrics). Between-group degrees of freedom are presented first, then within group. Tau^2 is an estimate of the between-study variance, and H indicates the impact of heterogeneity in the meta-analysis.

2 a. Correlations between plasticity metrics by taxonomic group and trait type

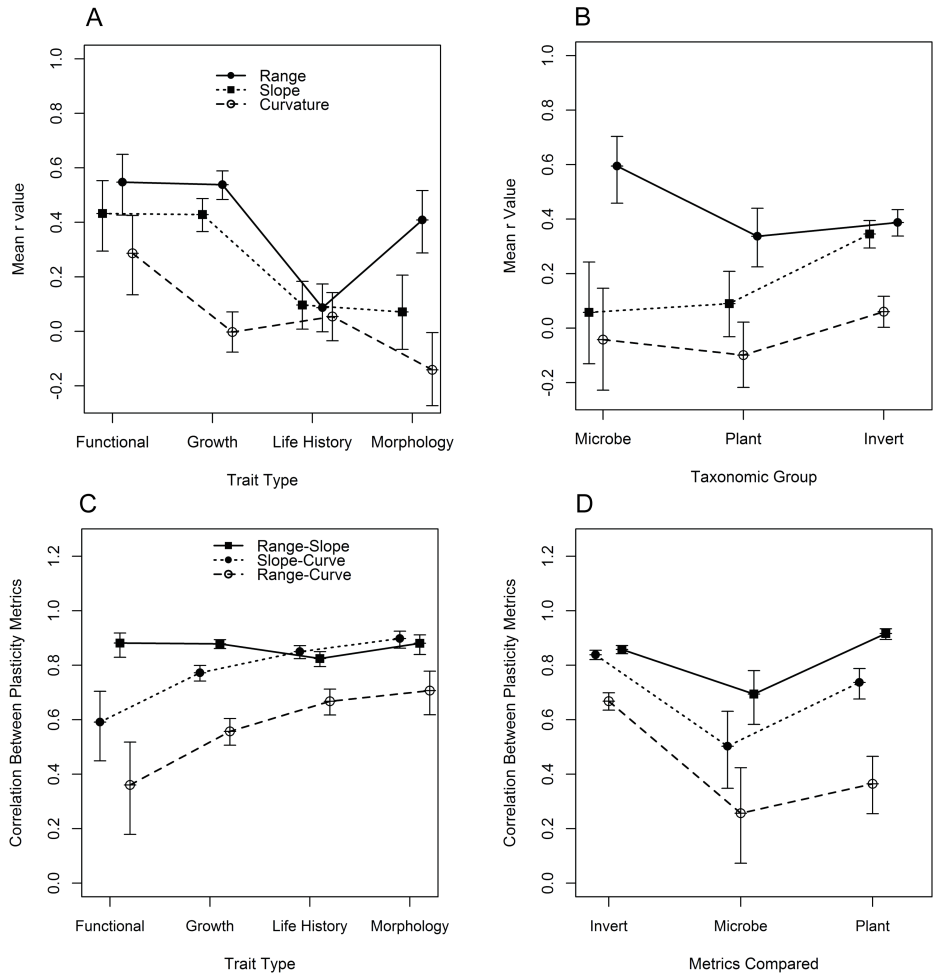
Taxonomic Group			
	Range-Slope	Slope-Curvature	Range-Curvature
P-value	< 0.0001	<0.0001	< 0.0001
Tau^2	0.7382	0.4970	0.3840
DF	2, 28	2, 28	2, 28
H	6.28 [5.73; 6.88]	5.197 [4.68; 5.75]	4.58 [4.1; 5.12]
Trait Type			
	Range-Slope	Slope-Curvature	Range-Curvature
P-value	0.0029	<0.0001	<0.0001
Tau^2	0.7382	0.4970	0.3840
DF	3, 28	3, 28	3, 28
H	6.28 [5.73; 6.88]	5.19 [4.68; 5.75]	4.58[4.1; 5.12]

2 b. Correlation between overall trait mean (in all environments) and plasticity (quantified by the three plasticity metrics)

Taxonomic Group

	Range	Slope	Curvature
P-value	0.0116	< 0.0001	0.0503
Tau ^{2}	0.3050	0.2200	0.1424
DF	(3, 26)	(2, 26)	(2, 26)
H	4.11 [3.65; 4.63]	3.53 [3.1; 4.02]	2.9 [2.51; 3.36]
Trait Type			
	Range	**Slope**	**Curvature**
P-value	<0.0001	<0.0001	0.0005
Tau ^{2}	0.3050	0.2399	0.1424
DF	(2, 25)	(2, 25)	(2, 25)
H	4.11 [3.65; 4.63]	3.536 [3.1; 4.01]	2.9 [2.51; 3.36]

Figure 6.2. Panels 2a and 2b show the average correlation between *Mean Trait* and plasticity (measured as *Range, Slope*, and *Curvature);* the strength of the correlation between *Mean Trait* and plasticity differs by trait type (a) and taxonomic group (b). Bars represent 95% confidence intervals. Panels c and d show the average correlation between each pair of plasticity metrics, sorted by taxonomic group (c) and trait type (d). In general, the correlation between plasticity metrics was strongest between *Range* and *Slope*, while the *Range* and *Curvature* metrics were the least correlated.



Discussion

A major goal of our study was to quantify the genetic covariation between overall trait mean and plasticity for sets of genotypes within populations. Genetic correlations between trait mean and plasticity can generate correlated evolutionary responses, potentially limiting or promoting the evolution of adaptive plasticity regardless of its contribution to fitness (Via and Lande 1985; Via 1993). Such correlations between trait and plasticity may be particularly important for continuous reaction norms (Kirkpatrick and Heckman 1989; Gomulkiewicz and Kirkpatrick 1992). A key result from our analyses is that the correlation between trait mean and plasticity for continuous reaction norms depends crucially on the metric of plasticity used. The correlations of trait mean with *Range* and *Slope* significantly positive (r = 0.395 and 0.285, respectively); in contrast, the correlation of trait mean and *Curvature* is near zero (r = -0.024). This indicates that trait mean and curvature may evolve independently, whereas other aspects of reaction norm shape (*Slope* and *Range*) show substantial positive genetic correlations with trait mean, potentially limiting the evolution of adaptive plasticity. The notion that different aspects of plasticity may differ in their potential for adaptive evolution warrants further attention.

Another key result is that the correlation between trait mean and plasticity depends significantly on the type of trait studied. The differences between life history and functional traits are particularly interesting (Fig. 3). The correlation of trait mean with plasticity for life-history traits was quite low ($r < 0.1$) for all plasticity metrics. In contrast, functional traits had large positive correlations, varying from 0.58 (*Range*) to 0.33 (*Curvature*). Because life-history traits are often strongly associated with fitness (Roff 1992), the apparent independence of trait means and plasticity for such traits has important implications for evolution of adaptive plasticity. Experiments that quantify genetic covariation in trait means and plasticity for different trait types in the same study systems would be particularly valuable for exploring this issue.

Most of the empirical data and theoretical models of phenotypic plasticity involve genotypes measured in only two environments. As a result, analyses of reaction norm slope (or equivalently, range) have dominated discussions of reaction norm evolution and costs of plasticity. But continuous reaction norms can take a variety of shapes that cannot be fully characterized by slope or range (Valladares et al. 2006). Here we have considered three different metrics for quantifying reaction norm shape. Our analyses show that the three metrics of plasticity we consider—*Range*, *Slope*, and *Curvature*—are generally positively correlated, but the magnitude of this correlation varies. In particular, *Slope* is highly correlated with both *Range* and *Curvature* (r^2 = 0.71 - 0.74), but the correlation between *Range* and *Curvature* is considerably smaller (r^2 = 36%). This suggests that our metrics of *Range* and *Curvature* represent relatively independent aspects of plasticity for continuous reaction norms. This reduced correlation may arise because the connection between *Range* and *Curvature* is quite different for nonlinear reaction norm shapes (e.g., sigmoid, monotonic, unimodal, etc.). The correlation among the plasticity metrics also depends on the trait of type measured, especially for the correlation between curvature and *Range*. In particular, the mean correlation between *Range* and *Curvature* is smaller for functional than for morphological or life-history traits. But these results do suggest that the choice of plasticity metric may result in different assessments of the magnitude and patterns of variation in plasticity for different types of traits and organisms. We recommend that future studies consider measuring both the *Range* and *Curvature* of reaction norms and evaluate the extent to which they are redundant.

An important limitation of this study concerns the underlying data available for analysis. Nearly 80% of the data consider temperature as the environmental factor, and over 60% involve invertebrates, primarily insects; vertebrates are completely unrepresented in the dataset (Table 1). This tempers the generality of the conclusions we can draw from our analyses, including the observed differences among different types of traits. Experimental studies of continuous reaction

norms for environmental factors other than temperature, and for taxa other than insects, are sorely needed.

A general theme of inquiry into the evolution of plasticity has been the topic of costs of plasticity (Van Tienderen 1991; DeWitt et al. 1998; Auld et al. 2010). Costs of plasticity are envisioned as a constraint (cost) on a plastic genotype that is incurred independent of the environment; a cost of plasticity is a cost that the genotype pays for the "ability" to be plastic. These costs, while theoretically important, have been empirically elusive (Van Buskirk and Steiner 2009). In a general sense, costs of plasticity are imagined to be constraints on the evolution of plasticity; they are a potential explanation for why we do not always observe perfect plasticity (i.e., exact, complete and ubiquitous phenotype-environment matching). The extent to which plasticity is correlated with the trait value is another form of constraint that may inhibit the evolution of "perfect plasticity". Most of the empirical literature on, and analytical methods for, quantifying costs of plasticity uses *Slope* or *Range* as the metric of plasticity (Van Tienderen 1991; DeWitt et al. 1998; Auld et al. 2010). Here we found that, particularly for morphological, growth and functional/physiological traits, the correlation of trait mean with *Range* or *Slope* averaged between 0.4 and 0.6. While not a complete correlation, this relationship may constrain the independent evolution of plasticity and trait means and as such represents an alternative explanation for the lack of costs of plasticity. That is, perhaps costs of plasticity are not often observed because the plasticity itself is correlated with the trait value. This hypothesis remains speculative and awaits theoretical evaluation as well as assessment with additional empirical data.

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VII. Conclusions

Climate differences are commonly framed as differences in abiotic factors such as seasonality, temperature and precipitation. However, climate may also indirectly select for differences in natural enemies and resources. In our study, observed clines in development time were likely the result of differences in season length. Our results indicate that some traits respond consistently to selection by climate, while others do not. We found that both the Japanese and North American populations of *P. rapae* established predicted clines for development time, but not for body size. Where growing seasons are long, it is possible for *P. rapae* populations to add produce additional generations of offspring. While large body size confers greater fecundity, early reproduction results in greater potential fitness. Faster development in long growing seasons may also be the result of selection by parasitoids; organisms may develop quickly to outgrow life stages in which they are vulnerable to natural enemies and to reach a developmental refuge. Our studies indicate that development time is under strong and consistent selection in both of our study regions. By contrast, geographic variation in immune function is likely the result of differences in selection by parasitoids (A. R. Kraaijeveld & H. C. J. Godfray, 1999; A. Kraaijeveld & H. Godfray, 2001). Parasitoid populations were larger in regions with longer growing seasons, resulting in stronger selection on immune function. Although both immune function and development time showed clinal patterns, they are the result of different selective forces. Some traits may be responding to patterns in biotic factors such as natural enemy abundance while others are responding to abiotic factors such as season length and temperature. Our results agree with other research on cline formation in exotic species, in that some traits

readily re-establish geographic clines, while others do not (Gilchrist *et al.*, 2001; Maron *et al.*, 2004; Maron & S. C. Elmendorf, 2007; Colautti *et al.*, 2008).

Life history theory predicts that there should be trade-offs between body size and development time. In our studies we found consistent latitudinal patterns in development time, but no clear patterns in body size. If body size and development time are intrinsically related, we would expect to observe concordant clines between the two traits, yet we did not. We also found no relationship between body size and development time within a single population: body size and development time were uncorrelated in both our spring and summer field experiments. These results provide a counterpoint to many life history models, which assume that body size and development rate are inversely related. Further, many of these models assume that size or development rate at early instars is related to age at maturation or adult body size. We find that development time is strongly correlated across life stages; animals that develop slowly early in life, take longer to reach maturity. However, there was no correlation between body size across life stages; smaller animals were actually likely to have a larger adult size. In lepidopterans the physiological determinants of body size are well understood: final adult size is determined primarily in the final larval instar before pupation, through a series of endocrine events regulating the cessation of growth (H.F. Nijhout, 2003; Davidowitz *et al.*, 2005; Diamond & Kingsolver, 2010; H Frederik Nijhout *et al.*, 2010). Final development time, on the other hand, is the result of the cumulative length of each developmental stage, and is therefore determined throughout the juvenile period. Because there are weak correlations both between size and development time, it may be possible for body mass and development time to evolve independently. However, it is not clear whether this decoupling of traits is specific to lepidopteran systems, or is a more generalizable to other taxa. A systematic review of body size - development time relationships would be a productive avenue of investigation for future researchers, and could be addressed effectively through meta-analysis.

We also found that diet could alter thermal reaction norms for body size, in particular in domesticated populations. Our results show that in wild populations of *P. rapae*, thermal reaction norms were robust to protein reduction, but that in domesticated populations reduced protein diets resulted in variation in reaction norms, and even a reversal of the temperature-size rule. In domesticated populations adapted to high quality diets, females experienced the greatest disruption of thermal reaction norms on reduced protein diets. Male and female *P. rapae* allocate protein towards different life history traits; males allocate protein towards pigmentation and spermataphore production, while females primarily invest protein in egg production. Domestication and adaptation to high protein diets may relax selection on the efficiency of protein assimilation. However male and female lepidopterans(Remes & Szelesky, 2010; McPherson & Chenoweth, 2012) may respond differently to domestication because they use protein differently, and may therefore not respond in the same way to protein manipulation (Boggs, 1981; Morehouse & Rutowski, 2010). A few studies have explored how domestication affects sexual dimorphism in vertebrates . However, sexual dimorphism is less well studied in insects, but they may prove a useful system for studying selection on sexual dimorphism during domestication. In vertebrates, sexual dimorphism in domesticated populations is often the result of selection for desirable secondary sex characteristics. However, domesticated insects are rarely selected for secondary sex traits, and changes in sexual dimorphism are likely to be the result of the differing resource requirements of males and females.

Our work on reaction norms raises questions about limits and constraints on the evolution of plasticity. Many studies have evaluated whether plasticity has costs, but less than half of them have detected them (Auld *et al.*, 2011). If plasticity is cost free, then there must be other types of constraints on its evolution. One potential limit to the evolution of plasticity is trait correlations. There is some evidence that genotypes that are more plastic might also have higher mean trait values. We used meta-analysis to test whether the average trait value for a given genotype is

correlated with plasticity. We evaluated this relationship in studies that measured phenotype in more than three environments. Further, we developed and compared several ways of measuring plasticity, each designed to capture different aspects of a reaction norm. We did find correlations between mean trait value and plasticity; highly plastic genotypes also had higher mean trait values, but this relationship depended on the type of trait measured and the taxon studied. However, selection on mean trait value may also constrain the evolution of plasticity. We also found that our metrics of plasticity captured different aspects of a reaction norm; specifically one of our metrics best captured the range of phenotypes produced, while another measured the shape of the reaction norm curve. In future studies, using multiple measures of plasticity may yield insights into how reaction norms evolve. Further, reframing discussions of plasticity as a larger conversation about the evolution of reaction norms may allow a more nuanced understanding of evolution. Our work demonstrates that some aspects of plasticity (range) may be constrained by correlations with mean trait value, but that others, like the shape of the reaction norm may evolve independently. Future workers may benefit from using multiple measures of plasticity in their analyses to detect what aspects of reaction norms are under selection (Valladares *et al.*, 2006).

Appendices

Appendix A: Studies used in parasitism and latitude meta-analysis

Study	Years	% Parasitism	Site	Latitude	Parasite
Parker (1970)	1970	42.57	Columbia, MO	34.1	*C. glomerata*
Ohsaki & Sato (1990)	1999	94.16	Kyoto, Japan	35.36	*C. glomerata*
Sato (1978)	1976	64.29	Kyoto, Japan	35.36	*C. glomerata*
Benrey and Denno (1994)	1995	46	Beltsville, MD	39.2	*C. glomerata*
Tanaka et.al. (2007)	2001, 2004	60.21 - 24.09	Sapporo, Japan	42.142	*C. glomerata*
Sato (1978)	1976	42.2	Sapporo, Japan	42.142	*C. glomerata*
Van Driesche & Bellows (1988)	1985-1986	86.98 - 59.13	Amherst, MA	42.9	*C. glomerata*
Wold-burkness *et.al* (2005)	1991-2003	2-11.8	Rosemount, MN	44.44	*C. glomerata*
Godin & Bovin (1998)	1993-1994	18 - 31	Montreal, QC	45.31	*C. rubecela*

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