CAUSES AND CONSEQUENCES OF NOVEL HOST PLANT USE IN A PHYTOPHAGOUS INSECT: EVOLUTION, PHYSIOLOGY AND SPECIES INTERACTIONS

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ABSTRACT

Sarah Elizabeth Diamond: CAUSES AND CONSEQUENCES OF NOVEL HOST PLANT USE IN A PHYTOPHAGOUS INSECT: EVOLUTION, PHYSIOLOGY AND SPECIES INTERACTIONS
(Under the direction of Dr. Joel G. Kingsolver)

Understanding what determines host range—the number and type of different resources used by an individual, population, or species—is a fundamental question in biology. I explore ecological and evolutionary determinants of host plant range in the tobacco hornworm, *Manduca sexta*. I have taken advantage of both domesticated laboratory populations of *M. sexta,* and a recent host plant shift in wild *M. sexta* in the southern US, to examine how the recent evolutionary history, host plant quality, natural enemies and environmental temperatures impact the performance and fitness of *M. sexta*. Using laboratory experiments, I demonstrated severe reductions in performance and fitness associated with feeding on an evolutionarily novel host plant, devil's claw (*Proboscidea louisianica*): survival, growth and development rates, immune function, final body size, fecundity and total fitness were all reduced for *M. sexta* reared on devil's claw compared to their typical host plant, tobacco (*Nicotiana tabacum*). I found that these costs can be ameliorated under warmer thermal conditions, as the typical negative relationship between body size and rearing temperature was reversed on devil's claw. However, one of the greatest drivers of *M. sexta*'s adoption of devil's claw appears to be escape from an important braconid parasitoid natural enemy, *Cotesia congregata*. A field experiment demonstrated that the intrinsic costs of using devil's claw were offset by enemy release, resulting in comparable total fitness of *M.*

sexta feeding on devil's claw and tobacco. In general, domesticated laboratory populations of *M. sexta* exhibited qualitatively similar responses as wild *M. sexta*; however, there were a few key differences, *e.g.*, domesticated *M. sexta* exhibited relatively greater reductions in survival and fecundity on devil's claw. My research shows that the selective environment, and abiotic and biotic ecological factors are important components of host range, sometimes interacting in surprising ways to alter overall herbivore fitness and host plant us

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CHAPTER I

OVERVIEW

My dissertation research focuses on exploring the ecological and evolutionary determinants of host plant use in an important model organism, the tobacco hornworm, *Manduca sexta*. I have taken advantage of a relatively recent host shift in *M. sexta* to empirically examine how abiotic and biotic aspects of the environment, including plant quality, an important parasitoid natural enemy and temperature, impact the performance and fitness of *M. sexta*. It is evident from my dissertation work that herbivore fitness is the culmination of a number of different factors, which can interact in surprising ways to alter host plant use.

Compared to other plant-feeding insects, *M. sexta* is a dietary specialist, feeding on plant species from only one family (Solanaceae). However, *M. sexta* has adopted a novel host plant, devil's claw (Martyniacae), outside the Solanaceae. Through field observations, I have found that wild populations of *M. sexta* will lay eggs on devil's claw. In both field and laboratory experiments, I have empirically demonstrated severe performance and fitness consequences associated with feeding on this evolutionarily novel host plant: survival, growth and development rates, immune function, final body size, fecundity and total fitness (R_0) are all reduced for *M. sexta* reared on devil's claw compared to a typical solanaceous plant, tobacco.

Why then has *M. sexta* adopted devil's claw as a host plant? Through field

experiments, I found that the poor diet quality of devil's claw is offset by escape from an important braconid parasitoid natural enemy, *Cotesia congregata*: a high rate of parasitism on tobacco coupled with poor intrinsic performance on devil's claw resulted in comparable total fitness (R_0) of *M. sexta* reared on devil's claw and tobacco.

Similarly, abiotic environmental components can have equally important outcomes on host range. Through laboratory experiments, I found that the typical negative relationship between body size and temperature (temperature-size rule) is reversed on devil's claw. This reversal of the temperature-size rule apparently results from the stressful combination of low temperatures and low dietary quality. In addition to suggesting that the temperature-size rule occurs for a restricted range of non-stressful environmental conditions thereby limiting the robustness of this widespread pattern of phenotypic plasticity, these results also suggest that such reversals may impact seasonal and geographic patterns of host plant use in *M. sexta* and potentially other systems. For example, the adoption of novel host plants may therefore be facilitated in warmer environments, but retarded in cooler environments.

Clearly, the importance of assessing how abiotic and biotic components of the environment interact to determine herbivore fitness cannot be overstated. However, it is also necessary to understand how these aspects of the environment impact different components of herbivore performance and fitness. One aspect of performance that has received little attention in context of host plant use in herbivores is immune defense. In *M. sexta*, rates of infection are much greater when reared on devil's claw compared to the typical host, tobacco. Through laboratory experiments, I found that two aspects of immune response, melanization and cellular encapsulation, are significantly reduced for *M. sexta* reared on devil's claw compared to tobacco. This suggests that the novel host plant, devil's claw, not only directly

provides a diet of reduced nutritional quality, but secondarily results in weakened immune defenses for *M. sexta*. In general, examination of immune defenses across different environments may better inform our understanding of the consequences of host shifts in herbivores and other systems.

Furthermore, *M. sexta*'s status as a model organism in insect physiology has provided a unique opportunity to assess the impacts of laboratory domestication on this organism's performance and fitness. I have used population comparative methods to assess the degree to which results based on laboratory populations may be generalized to wild populations. Interestingly, I have found that laboratory domestication of *M. sexta* has contributed to reduced survival and fecundity (relative to wild populations) on the novel host plant, devil's claw, but not on tobacco, and not for all fitness components (growth, development and immune defenses).

In general, the results of my dissertation research demonstrate the importance of assessing multiple determinants of host use and evaluating their potential interactions to understand overall host use. In this regard, *M. sexta* and its natural host plants provide an excellent study system for continued research on the evolutionary ecology of host use. For example, *M. sexta* has independently adopted the novel host plant, devil's claw, in two distinct geographic locations (North Carolina and Arizona); *M. sexta* has a broad geographic distribution across the southern US, throughout which devil's claw occurs as a native or naturalized host plant. Has *M. sexta* adopted devil's claw in other locations? Are natural enemies the major driver in these locations as seems to be the case in North Carolina and Arizona? Further comparative work on the conditions under which devil's claw is adopted (or not adopted) by other populations of *M. sexta* across the US would yield a clearer picture

of the factors which either promote or retard host shifts. Related to this question, the determinants of host use such as resource quality, temperature, and natural enemies often vary over time (daily, seasonally, and annually): what are the consequences of such temporal variation for determining overall host use?

Finally, there are several open mechanistic questions regarding the physiological and biochemical bases for *M. sexta*'s ability to use devil's claw (a relatively toxic resource), and how *M. sexta* escapes parasitism from *C. congregata* in the field. For example, how much of the variation in performance on devil's claw is attributable to variation in detoxification enzymes? What physiological processes underlie *M. sexta*'s improved performance on devil's claw at warmer temperatures? How do host plant chemistry and host plant growth form contribute to *M. sexta*'s escape from parasitism on devil's claw? Comparative work across additional *Manduca* populations and study systems would elucidate the generality of such mechanisms.

CHAPTER II

EVOLUTIONARY DIVERGENCE OF FIELD AND LABORATORY POPULATIONS OF *MANDUCA SEXTA* IN RESPONSE TO HOST PLANT QUALITY[1]

Summary

The tobacco hornworm, *Manduca sexta*, has been an important model system in insect biology for more than 50 years. In nature, *M. sexta* successfully utilizes a range of host plants that vary in quality. The consequences of laboratory domestication and rearing on artificial diet for fitness of phytophagous insects on natural host plants have not been explored. We examine the evolutionary divergence of two domesticated laboratory populations and a field population (separated for more than 40 years, or >250 laboratory generations) of *M. sexta* with respect to performance and fitness on two natural host plants: a typical host plant, tobacco (*Nicotiana tabacum*) and a novel host plant, devil's claw (*Proboscidea louisianica*). For both field and laboratory populations, rearing on devil's claw resulted in animals with lower survival, smaller final size, longer development time, and reduced size-corrected fecundity, than animals reared on tobacco. Reductions in some fitness components (survival and fecundity) were greater for the laboratory population animals than the field population animals. When reared on tobacco, the laboratory population animals had similar or larger pupal masses and slightly shorter development times than when reared on artificial diet, suggesting that laboratory domestication on artificial diet has not greatly affected the ability of *M. sexta* to perform well on a typical natural host plant. Although field

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and laboratory populations exhibited qualitatively similar responses to host plant quality, *i.e.*, reduced performance on devil's claw, the magnitude of this reduction differed across populations, with the domesticated laboratory populations having greater reductions in performance than the field population. The use of domesticated populations as models for responses of field populations may therefore be more appropriate for considering environmental conditions that are relatively benign or near-optimal, than when exploring responses to extreme or stressful conditions.

Introduction

Most phytophagous insects utilize a range of host plants in nature that vary in nutritional quality. How insects respond and adapt to natural variation in host plant quality is an important question for understanding the evolution of host range and herbivore success (Ehrlich & Raven, 1964; Jaenike, 1990; Via, 1990; Agrawal, 2000; Cornell & Hawkins, 2003). In the laboratory, numerous economically or biologically important insects have been domesticated to feed on nutrient-rich artificial diets (Service & Rose, 1985; Economopoulos, 1992; Cacoyianni *et al.*, 1995; Harshman & Hoffmann, 2000; Matos *et al.*, 2000; Simoes *et al.*, 2007). Studies of such insects demonstrate that domesticated populations can rapidly evolve high fitness on artificial diets, relative to wild populations (e.g. Matos *et al.*, 2000). How does adaptation to artificial diets alter success on natural host plants that vary in nutritional quality? Because much of our knowledge of insect nutrition, growth and development comes from studies of domesticated model systems (Rose *et al.*, 1996), understanding how laboratory domestication has altered responses to natural host plants has important implications for the degree to which results from domesticated populations can be

generalized to natural populations.

The tobacco hornworm, *Manduca sexta* (L.), has been an important model organism in insect biology for more than half a century. Studies of *Manduca* have been motivated by its pest status and facilitated by its rapid growth and large body size, and ease of laboratory rearing on artificial diets. Laboratory colonies of *M. sexta* have been maintained continuously since the 1960s, and to our knowledge all major laboratory colonies of *M. sexta* are ultimately derived from mass-rearing facilities in Raleigh, NC, established in the 1960s from field collections in Clayton, NC (Yamamoto, 1960; Yamamoto *et al.*, 1969; Yamamoto, 1974). This represents more than 250 generations in the laboratory since colony establishment. The laboratory environment has selected for rapid growth and development and large body size in *M. sexta* during the past 40 years (D'Amico *et al.*, 2001). Evolutionary divergence of laboratory and field populations in developmental plasticity (Kingsolver, 2007), thermal sensitivity and diapause initiation (Kingsolver & Nagle, 2007; Kingsolver *et al.*, 2009) have been documented, but the consequences of domestication for host plant use have not been explored.

Like many phytophagous insects, *M. sexta* feed successfully on a range of host plants that differ in quality. Feeding by wild populations of *M. sexta* in nature is restricted largely to host plants in the family Solanaceae, although use of a non-solanaceous host plant, devil's claw (*Proboscidea* spp., family Martyniaceae) has recently been reported (Mechaber & Hildebrand, 2000; Mira & Bernays, 2002). Field observations by Mira and Bernays (2002) suggest that larval survival of populations of *M. sexta* feeding on naturally occurring devil's claw in the Southwestern US to which this plant is native, is lower than on solanaceous host plants. However, overall survival, growth, development time, and other aspects of

performance have not been explored for *M. sexta* reared on devil's claw through metamorphosis.

Here we explore the consequences for survival, growth, development, and fecundity of *M. sexta* reared on a typical host plant, tobacco, and a non-solanaceous host plant, devil's claw. We compare responses of two domesticated laboratory populations with a field population from the Southeastern US (Clayton NC) where devil's claw is a relatively recent introduction (Small, 1903). The laboratory populations of *M. sexta* were derived from this same field population over 40 years ago. In this study, we address three main questions. First, what is the overall performance and fitness of laboratory and field populations of *M. sexta* on a typical and novel host plant? Second, how has domestication of *M. sexta* altered performance on a typical host plant, tobacco, compared to its standard resource, artificial diet? Here we rely on data from previous experiments conducted on artificial diet (Kingsolver, 2007; Kingsolver, unpubl. data). Third, how do laboratory and field populations differ in their responses to a novel host plant?

Materials and Methods

Study organisms

The tobacco hornworm, *Manduca sexta* L. (Sphingidae) is distributed across tropical and temperate regions of the Nearctic (Rothschild & Jordan, 1903). Feeding is generally restricted to plants in the Solanaceae, but recently *M. sexta* has been shown to use non-solanaceous host plants (*Proboscidea* spp.) belonging to the family Martyniaceae, in the Southwestern US (Mechaber & Hildebrand, 2000; Mira & Bernays, 2002). The Solanaceae and Martyniaceae are not closely related (A.P.G., 2003), and the chemistry profiles of species

belonging to these two families are quite different (Gibbs, 1974; Sisson & Saunders, 1982; Harborne, 1984; Ihlenfeldt, 2004). For instance, *P. louisianica* (Martyniaceae) contains a novel phenylpropanoid glycoside, called martynoside, not found in solanaceous plants (Sasaki *et al.*, 1978).

Proboscidea spp. are commonly referred to as devil's claw, and are native to the Southwestern US, but have been introduced to other locations across the US via contaminated crop seed and have escaped from gardens where they are grown as ornamentals. One species of devil's claw, *P. louisianica* (Miller) Thellung, has been introduced relatively recently to the Southeastern US (approximately 100 years ago) and has since become naturalized in the region (Small, 1903). Although the status of devil's claw as a 'novel' host plant in the Southwestern US is unclear because the timing of *M. sexta*'s colonization of devil's claw is unknown (Mechaber & Hildebrand, 2000), we consider devil's claw to be a novel host plant in the current region of study (North Carolina) where the species was only recently introduced. Furthermore, we consider devil's claw a novel host plant for *M. sexta* relative to a typical North Carolina solanaceous host plant, tobacco. Although cultivated tobacco (*Nicotiana tabacum* L.) could be considered somewhat novel because of its likely hybrid origins (Ren & Timko, 2001), wild *Nicotiana* spp., as members of the Solanaceae, are considered typical host plants for *M. sexta* (Yamamoto, 1960).

Proboscidea louisianica is more patchily distributed across North Carolina than the predominant local solanaceous host plant, tobacco (*Nicotiana tabacum* L.) which is widely cultivated across the state (Radford *et al.*, 1968). Despite the limited distribution of devil's claw in North Carolina, our observations indicate field populations of *M. sexta* oviposit and feed on this host plant when grown adjacent to tobacco plants. In addition, preliminary

observations indicate that individual *M. sexta* females will oviposit on both of these host plants in the laboratory (Diamond, unpubl. data), suggesting that distinct 'host races' of *M. sexta* in this region are very unlikely.

In the current experiments, three genetic lines (populations) of *M. sexta* were used. The Duke laboratory population came from a colony maintained under standard larval rearing conditions (artificial diet, constant 25 °C, 15h L : 9 h D photocycle); this population was established by hybridizing long-term mass reared colonies from the University of Washington, University of Arizona and North Carolina State University in 2002. The UNC laboratory population came from a colony maintained under the same standard larval rearing conditions by L. Gilbert and colleagues at UNC for over 25 years. Animals in the laboratory colonies do not experience tobacco or other natural host plants at any stage of their life cycle. All major laboratory colonies of *M. sexta* are ultimately derived from mass-rearing facilities in Raleigh, NC. The source population for these laboratory strains, including the Duke and UNC colonies, came from field collections of eggs in Clayton, NC (NCSU Research Station) during the 1960s. Field population eggs used in the current experiments were offspring of adults that were collected as early instar larvae from tobacco plants grown at this same Clayton, NC field site, and reared through one generation on artificial diet in the laboratory to minimize parental effects.

Experiments

Eggs from each population (field and both laboratory colonies) were randomly assigned to tobacco and devil's claw leaves in the laboratory. Upon hatching, 40 larvae each from the field and UNC laboratory populations and 10 from the Duke laboratory population

were transferred to individual tobacco leaves; 70 larvae each from the field and UNC populations and 68 from the Duke population were transferred to individual devil's claw leaves. A greater number of larvae were transferred to devil's claw in anticipation of high mortality. However, relatively high survival of the field population on devil's claw required 14 larvae to be randomly removed from the experiment during their 3rd and 4th instars due to constraints on growth chamber space (making the initial n = 58 at 3rd and n = 56 at 4th instar and all subsequent developmental stages for the field population on devil's claw). Larvae were maintained under standard conditions (25 °C, 16 L : 8 D) in individual Petri dishes housed in environmental chambers (Percival 36-VL). Larvae remained in Petri dishes until 4th instar, when they were transferred to larger plastic enclosures (31 x 16 x 13 cm) with screened lids. Larvae were fed *ad libitum* on cut leaf material maintained in water picks. Leaf material was harvested from greenhouse-grown plants fertilized weekly with Peter's Pro Solution (15-16-17). We grew an excess of plants (over twice as many as larvae in the experiment) to minimize the effect of inducing secondary plant metabolites in response to repeatedly cutting leaves from individual plants. No pesticides were applied to these plants.

Larvae were maintained in growth chambers until reaching the wandering developmental stage (here defined as complete purging of the gut prior to pupation) when they were placed individually in wooden pupation chambers (maintained at 25 °C through metamorphosis). Pupae were transferred to plastic cups lined with soil and remained there until eclosion. We measured survival, development time, mass at 3rd, 4th, 5th, and (6th, for some individuals; see Kingsolver, 2007) instars, wandering, pupation, and eclosion. Analyses of growth and development were restricted to individuals that survived to eclosion.

Adults were frozen at -80 °C within 5 hours of eclosion. For adult females, estimates

of fecundity were obtained by dissecting out the ovarioles into Ringer's solution. The number of follicles at stage 6 (S6) and all subsequent stages of development (cf. Yamauchi & Yoshitake, 1984 for the staging of follicles) were counted with the aid of a dissecting microscope (Nikon SMZ-1B).

Statistical analyses

Population comparisons were based on linear contrasts between the field population and both laboratory populations (Duke and UNC). Survival to pupation was treated as a binomial variable and modeled using analysis of deviance with population (laboratory or field), host plant species (tobacco or devil's claw) and the interaction between population and host plant species as fixed effects. Development time to pupation and body mass at pupation (for individuals surviving to eclosion) were modeled using ANOVA with population, host plant species, sex and the interaction of population and host plant species as fixed effects. Sex was included in the models for growth and development to account for the sexual dimorphism present in *M. sexta* (on average, females are larger, but develop more slowly than males; Madden & Chamberlin, 1945). As there were no indications that population and host plant effects differed across the sexes, interactions between sex and host plant species and between sex and population were omitted from the models of growth and development. We focused on measurements of pupae because of the stability in body mass at this stage compared to other final developmental stages (*e.g.*, wandering and adult). A comparable analysis of development time to pupation and body mass at pupation using data from the current experiment for field and both laboratory populations reared on tobacco and data from earlier experiments for the same field and laboratory populations reared on artificial diet was

performed (Kingsolver, 2007; Kingsolver, unpubl. data). We used ANOVA with population, food type (tobacco or artificial diet), sex and the interaction of population and food type.

Estimates of fecundity were based on the number of follicles in ovarioles. Because body size has important effects on fecundity in *M. sexta* and other insects (Davidowitz *et al.*, 2004, 2005) and there are significant evolved differences in body size between field and laboratory populations (D'Amico *et al.*, 2001), we regressed pupal mass on fecundity, and used the residuals of the regression as a measure of size-corrected fecundity. Size-corrected fecundity was modeled using ANOVA with population, host plant species, and the interaction between population and host plant species as fixed effects. All statistical analyses were performed using R (version 2.8.1) statistical software.

Results

Survival

Field and laboratory populations had higher (> 80%) survival to pupation on the typical host plant, tobacco, than on the novel host plant, devil's claw (< 65%) (Figure 2.1). Overall, the field population had higher survival to pupation than the laboratory populations. Analysis of deviance detected significant effects of host plant species (tobacco versus devil's claw) ($\chi^2 = 39.7$, $P < 0.0001$, df = 1) and population (field versus both laboratory populations) ($\chi^2 = 5.36$, $P = 0.02$, df = 1), but no significant interaction between host plant species and population ($\chi^2 = 0.0008$, $P = 0.98$, df = 1) on the probability of survival to pupation. However, because most of the variation in survival to pupation across populations was found on devil's claw and host plant had a strong main effect, we performed post-hoc tests to further examine population differences in survival on each host plant separately. On

tobacco, no significant effect of population (field versus both laboratory populations) was detected for the probability of survival to pupation (χ^2 = 0.983, P = 0.322, df = 1). In contrast, on devil's claw, a significant effect of population was detected for the probability of survival to pupation (χ^2 = 4.38, P = 0.036, df = 1). Together, these results indicate that relative to the field population, the laboratory populations experienced a reduction in survival on devil's claw but not on tobacco.

Growth and development

Pupal mass was significantly greater on tobacco than on devil's claw, and in the laboratory populations than in the field population (Figures 2.2, 2.3a). Females had significantly greater pupal masses than males. ANOVA detected significant effects of host plant species ($F_{1,176}$ = 155, P < 0.0001), population ($F_{1,176}$ = 87.3, P < 0.0001), sex ($F_{1,176}$ = 13.3, P = 0.0003) and the interaction between host plant and population ($F_{1,176}$ = 8.53, P = 0.0039) on body mass at pupation. The significant interaction between host plant and population indicates field and laboratory populations responded differently to host plant quality. Post-hoc tests showed the laboratory populations had greater pupal mass than the field population on both host plants: tobacco ($F_{1,77}$ = 243, P < 0.0001) and devil's claw ($F_{1,98}$ = 69.6, P < 0.0001), however, the magnitude of this difference was greater on tobacco (Figure 2.3a).

Development time to pupation for the field and laboratory populations was significantly shorter on tobacco than on devil's claw, and for the field population than the laboratory populations (Figures 2.2, 2.3a). Males had significantly shorter development time to pupation than females. ANOVA detected significant effects of host plant species ($F_{1,176}$ =

185, $P < 0.0001$), population ($F_{1,176} = 42.8$, $P < 0.0001$), sex ($F_{1,176} = 9.33$, $P = 0.0026$) and the interaction between host plant and population ($F_{1,176} = 14.1$, $P = 0.0002$) on development time to pupation. Here again, the significant interaction between host plant and population indicates field and laboratory populations responded differently to host plant quality. Post-hoc tests showed that the laboratory population had longer development time to pupation than the field population on both tobacco ($F_{1,77} = 6.53$, $P = 0.0126$) and devil's claw ($F_{1,98} = 25.3$, $P < 0.0001$), but the magnitude of this difference was greater on devil's claw (Figure 2.3a).

Interestingly, the interaction between host plant and population (laboratory versus field) for development time was largely driven by the Duke laboratory population. Post-hoc analyses revealed that the Duke and UNC laboratory populations differed significantly in their development time across host plants (significant host plant and population [Duke versus UNC] interaction; $F_{1,103} = 27.1$, $P < 0.0001$). Specifically, the Duke laboratory population developed faster than the UNC laboratory population when reared on tobacco, but more slowly than the UNC population when reared on devil's claw (Figure 2.3a).

The long development times for the Duke laboratory population on devil's claw resulted in part from variation in instar number. When reared on tobacco, individuals from all three populations expressed the typical 5 larval instars. On devil's claw, 16.2% of the larvae that survived to eclosion in from the Duke laboratory population expressed an additional (6^{th}) larval instar (Figure 2.2). In contrast, the UNC laboratory population had no individuals reared on devil's claw that expressed an additional larval instar; although a few individuals had growth trajectories consistent with the expression of an additional instar, these larvae died prior to wandering. As a result, the Duke population's successful expression of

additional instars when reared on devil's claw contributed to the more prolonged development time of this population compared to the UNC population, which did not successfully express additional instars. The field population also had a small fraction (5.56%) of larvae that expressed an additional instar on devil's claw.

Comparisons with artificial diet

We also compared our results for performance on tobacco with previous results for the field and two laboratory populations reared on artificial diet (Kingsolver 2007; Kingsolver, unpubl. data) (Figure 2.3b). Body mass at pupation was significantly greater for the laboratory than the field population and for females, and on tobacco than on artificial diet. ANOVA detected a significant effect of food type (artificial diet or tobacco) ($F_{1,155}$ = 8.68, P = 0.0037), population ($F_{1,155}$ = 109, P < 0.0001), and sex ($F_{1,155}$ = 19.6, P < 0.0001) on mass at pupation, but no significant interaction between food type and population ($F_{1,155}$ = 0.301, P = 0.584) was detected.

Overall, development time to pupation was significantly shorter on tobacco than on artificial diet, and for the laboratory populations than the field population (Figure 2.3b). ANOVA detected significant effects of food type ($F_{1,155}$ = 88.9, P < 0.0001), population ($F_{1,155}$ = 29.9, P < 0.0001), and the interaction between food type and population ($F_{1,155}$ = 21.7, P < 0.0001) on development time to pupation, but no significant effect of sex ($F_{1,155}$ = 0.0036, P = 0.952) was detected. Importantly, the significant interaction between food type and population indicates that field and laboratory populations responded differently to artificial diet than to tobacco. In particular, the high rate of expression of additional instars of the field population reared on artificial diet (47% of individuals surviving to maturity)

contributed to longer development time to pupation in the field population than in the laboratory populations, which rarely express extra instars on artificial diet (Kingsolver 2007). Post-hoc analyses revealed significant effects of population, both on tobacco ($F_{1,77}$ = 6.53, P = 0.0126) and artificial diet ($F_{1,77}$ = 16.2, P = 0.0001): although the field population developed faster than the laboratory populations on tobacco, the field population developed more slowly than laboratory populations on artificial diet (Figure 2.3b).

Fecundity

Linear regression revealed a significant positive effect of pupal body mass on fecundity (number of follicles) (t_{76} = 6.84, P < 0.0001) (Figure 2.4). Pupal mass accounted for 37% of the variation in fecundity. Size-corrected fecundity was greater on tobacco than on devil's claw (Figure 2.4). ANOVA on size-corrected fecundity detected a significant effects of host plant species ($F_{1,74}$ = 17.5, P < 0.0001) and population ($F_{1,74}$ = 4.30, P = 0.0416), but no significant interaction between host plant species and population ($F_{1,74}$ = 1.42, P = 0.238) was detected. However, similar to the results for survival, most of the variation in size-corrected fecundity across populations occurred on devil's claw. Given the strongly significant effect of host plant, we analyzed the effect of population on size-corrected fecundity for each host plant separately. Post-hoc tests found no significant effect of population (field versus both laboratory populations) for size-corrected fecundity on tobacco ($F_{1,39}$ = 0.297, P = 0.589). In contrast, on devil's claw, a significant effect of population was detected for size-corrected fecundity ($F_{1,35}$ = 4.17, P = 0.0487). Together, these results indicate that compared to the field population, the laboratory populations experienced a reduction in size-corrected fecundity on devil's claw, but not on tobacco

(Figure 2.4).

Discussion

Performance on a novel host plant: tobacco versus devil's claw

Relative to *M. sexta*'s use of typical solanaceous host plants, *M. sexta* appears to be poorly adapted to using the non-solanaceous host plant, devil's claw. Both field and laboratory populations had reduced survival, longer development time, smaller pupal mass and reduced fecundity (indicated by significant main effects of host plant species) when feeding on a novel, non-solanaceous host plant (devil's claw), than they did on a typical host plant (tobacco). This supports and extends a previous study (Mira & Bernays, 2002) showing that *M. sexta* larvae had lower larval survival when feeding on devil's claw relative to a solanaceous host plant (*Datura wrightii*); our study shows that these deleterious effects extend to affect development time, final size and fecundity. In general, the relatively low frequency of occurrence of devil's claw and high dispersal ability of *M. sexta* probably limit adaptation to this host plant. In North Carolina and the southeastern US, tobacco is planted as a large monoculture and therefore more abundant relative to the devil's claw growing on the edges of these agricultural fields.

Consequences of laboratory domestication: tobacco versus artificial diet

The laboratory populations of *M. sexta* and others of which we are aware have been maintained on artificial diets that lack tobacco (or other host plant material) for more than 250 generations. How has domestication affected performance on tobacco compared to its standard laboratory resource, artificial diet? Our results indicate that relative to artificial diet,

both laboratory and field populations had similar survival (>80%), shorter developmental times, and similar or larger body sizes when reared on tobacco (Figures 2.2, 2.3b; see Kingsolver, 2007 for artificial diet results). This suggests that artificial diet may be a lower quality food resource than tobacco, even for domesticated populations, and that laboratory domestication has not detectably decreased performance or fitness on this typical solanaceous host plant.

It is evident, however, that domestication of *M. sexta* has clearly improved its performance on artificial diet (D'Amico *et al.*, 2001). The field population has much longer development times on diet than on tobacco, in part due to increases in the proportion of individuals expressing additional (supernumerary) larval instars on diet (Kingsolver, 2007). By contrast, despite a longer development time on tobacco, the laboratory populations develop considerably faster than the field population on artificial diet because they rarely express additional instars. Overall, the laboratory populations appear to be well adapted to artificial diet as a result of domestication, and perform better than the field population on this resource. However, although laboratory domestication has generated substantial evolutionary divergence in size, growth rates, thermal sensitivity and other characteristics (D'Amico *et al.*, 2001; Davidowitz *et al.*, 2003, 2004; Kingsolver, 2007; Kingsolver & Nagle, 2007), domestication and adaptation to artificial diet have not greatly affected the ability of *M. sexta* to perform well on a typical solanaceous host plant.

Consequences of laboratory domestication: devil's claw

In contrast to the results on tobacco, laboratory domestication appears to have strongly affected *M. sexta*'s performance and fitness on the novel host plant, devil's claw.

The reductions in body size and increases in development time that result from feeding on devil's claw were greater for the laboratory than for the field populations (Figures 2.2, 2.3a). Similarly, survival and size-corrected fecundity were significantly lower in the laboratory populations than the field population reared on devil's claw. Our results suggest that domestication has reduced the capacity of *M. sexta* to succeed on this novel host plant.

The field ancestors of the laboratory colony were likely exposed to devil's claw for many generations prior to domestication: the earliest records of *P. louisianica* in the Southeastern US are in the early 1900s (Small, 1903). This suggests the reduction in fitness of the laboratory populations on devil's claw is a consequence of domestication rather than further adaptation of the field population to devil's claw during the domestication of the laboratory populations. In addition, the laboratory populations of *M. sexta* are derived from the same field population used in this study, strongly suggesting that the observed differences between the field and laboratory populations in response to resource quality resulted from laboratory domestication rather than population variation. Certainly, the results of the current study are consistent with a scenario in which laboratory domestication contributed to poor performance on a novel host plant. However, further replication across additional field and laboratory populations would be required to completely distinguish laboratory domestication from alternative hypotheses.

One striking difference between the responses of the two laboratory populations to devil's claw is the developmental plasticity in instar number. A substantial fraction (16%) of the Duke population expressed an additional (6^{th}) larval instar when reared on devil's claw, whereas none of the UNC population did (Figure 2.2). This resulted in a large increase in development time for the Duke population on devil's claw (Figure 2.3a). Intraspecific

variation in the number of larval instars expressed has been demonstrated in many insect taxa, and adding larval instars is often a developmental response to poor diet quality (reviewed in Esperk *et al.*, 2007). Other studies suggest that the UNC laboratory population shows a reduced capacity for expressing additional larval instars in response to temperature and dietary quality, relative to the field (Kingsolver, 2007; Diamond & Kingsolver, in press) and Duke laboratory (Nijhout and Kingsolver, unpubl. results) populations. These results illustrate both the general and idiosyncratic consequences of domestication for different laboratory populations.

The genetic and physiological mechanisms underlying the reduction in fitness on devil's claw as a result of laboratory domestication also deserve further study. Does the fitness loss on devil's claw reflect mutation accumulation or antagonistic pleiotropy (Cooper & Lenski, 2000), and what aspects of *M. sexta*'s physiology do these changes affect? For example, domestication may contribute to relaxed selection on detoxification enzymes important in *M. sexta*'s ability to utilize different host plants (Feyereisen, 1999; Stevens *et al.*, 2000), or differences in how nutrients are metabolized relative to field populations (e.g. Mira & Raubenheimer, 2002; Warbrick-Smith *et al.*, 2006), which could result in the observed fitness loss on devil's claw.

Laboratory populations of *M. sexta* and other insect model systems are often used to study the effects of temperature, nutrition, host plant quality and other factors on performance and fitness (e.g. Stamp *et al.*, 1991; Krischik *et al.*, 1991; Yang & Stamp, 1995; Traugott & Stamp, 1997; van Dam *et al.*, 2000). Our results indicate that the impacts of domestication on growth, development and fitness are greater for host plants of lower dietary quality. This may not be surprising, given that tobacco and laboratory diets are nutrient-rich

in terms of dietary protein, water content, vitamins and other key nutritional components (Yamamoto, 1969; Bell & Joachim 1976; Knuckles *et al.*, 1979; Reynolds *et al.*, 1986; Timmins & Reynolds 1992; Timmins *et al.*, 1988), and importantly, lack the novel allelochemicals of devil's claw (Sasaki *et al.*, 1978). However, it remains an open question as to precisely which dietary component(s) contribute to the divergence of field and laboratory populations on devil's claw. In an analogous way, field and laboratory populations of *M. sexta* have comparable responses to rearing temperature at intermediate temperatures, but diverge dramatically in their tolerance to high temperatures (Kingsolver & Nagle, 2007). These observations suggest that use of domesticated populations as models to understand field populations may be well justified when considering responses to environmental conditions that are relatively benign or near-optimal. Conversely, domesticated populations may not accurately reflect important features of field populations when we consider more extreme, stressful or exotic environmental conditions.

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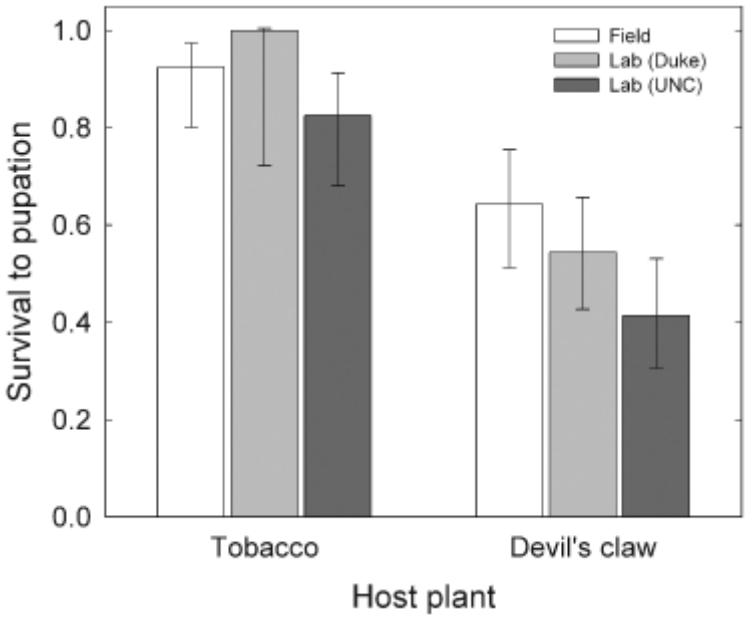

Figure 2.1. Mean survival from hatching to pupation for field (white bars), Duke laboratory (light gray bars) and UNC laboratory populations (dark gray bars) of *Manduca sexta* reared on tobacco (left) and devil's claw (right); 95% confidence intervals are indicated.

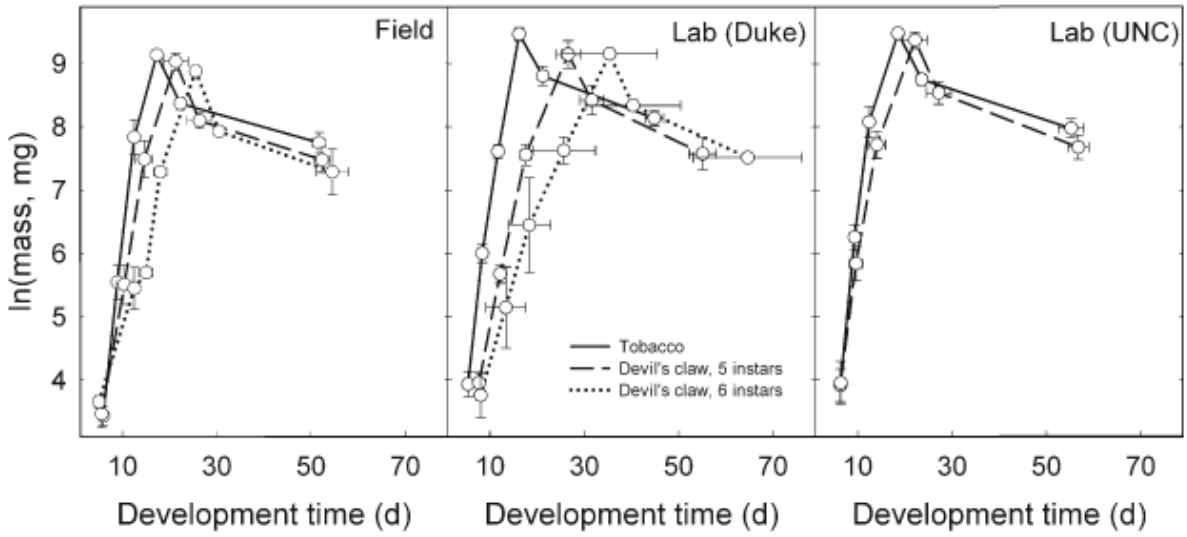

Figure 2.2. Body mass (in ln(mg)) as a function of development time (d) for field (left panel), Duke laboratory (middle panel) and UNC laboratory population (right panel) *M. sexta* females reared on tobacco and devil's claw. Males (not shown) exhibited qualitatively similar patterns of growth and development. For devil's claw, the mean growth trajectory for individuals with supernumerary (6) larval instars (dotted lines) is presented separately from the mean trajectory for individuals with 5 larval instars (dashed lines). All larvae reared on tobacco had 5 larval instars (solid lines). Mean ± 1 SD is indicated. Stages represented: start of the 3^{rd}, 4^{th}, 5^{th}, (6^{th}) instars; wandering (maximum larval size); pupa; adult.

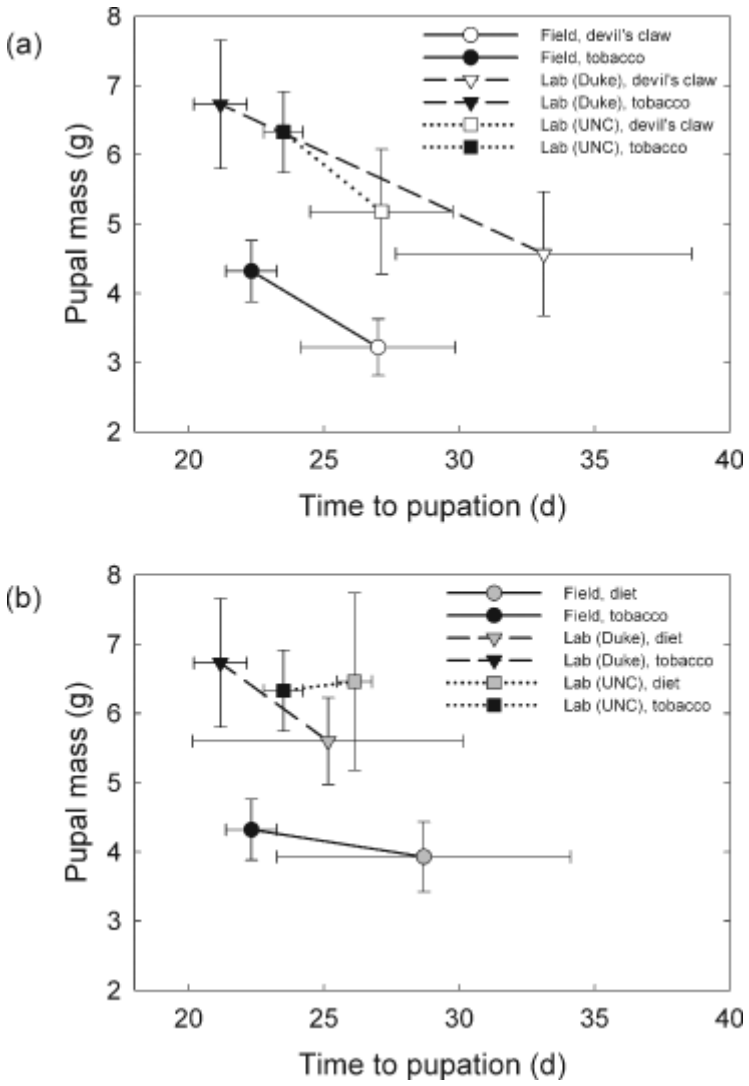

Figure 2.3. Pupal body mass (g) as a function of development time to pupation (d) for field (circles, solid line), Duke laboratory (triangles, dashed line) and UNC laboratory populations (squares, dotted line) of *M. sexta* females reared on (a) tobacco (black symbols) versus devil's claw (white symbols), and (b) tobacco (black symbols) versus artificial diet (gray symbols). Mean ± 1 SD is indicated. Males (not shown) exhibited qualitatively similar patterns of body size and development time to pupation.

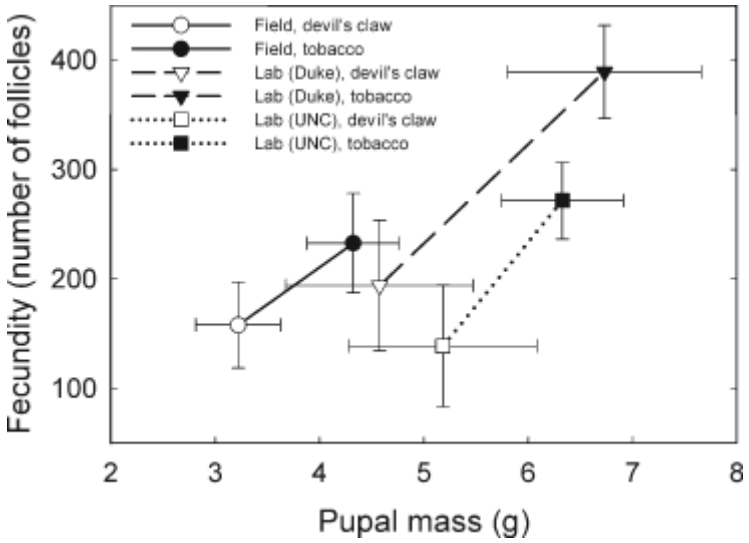

Figure 2.4. The number of follicles present in the ovarioles as a function of pupal body mass (g) for field (circles, solid line), Duke laboratory (triangles, dashed line) and UNC laboratory populations (squares, dotted line) of *M. sexta* reared on tobacco (filled symbols) and devil's claw (open symbols). Mean ± 1 SD is indicated.

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CHAPTER III

FITNESS CONSEQUENCES OF HOST PLANT CHOICE: A FIELD EXPERIMENT[1]

Summary

Determining the relative contributions of different ecological factors for herbivore fitness is one key to understanding the ecology and evolution of host plant choice by herbivores. Natural enemies are increasingly being recognized as an important factor: host plants of inferior quality for development may still be used by herbivores if they provide enemy-free space (EFS). Here we used the tobacco hornworm, *Manduca sexta*, to experimentally disentangle the effects of natural enemies from the potentially confounding factors of host plant quality, competition and microhabitat. We explored the consequences for both individual components of fitness and total fitness of *M. sexta* feeding on a typical high quality host plant, tobacco (*Nicotiana tabacum*) and a novel, low quality host plant, devil's claw (*Proboscidea louisianica*) in an experimental field environment in the presence of a parasitoid natural enemy, *Cotesia congregata*. Although early larval survival, development and growth rates, final body size and fecundity were all reduced for *M. sexta* feeding on devil's claw, a high rate of parasitism on tobacco and an absence of parasitism on devil's claw contributed to similar total fitness (net reproductive rate, R_0) across the two host plant species. Our results suggest *M. sexta* has adopted a novel host plant (devil's claw) outside its typical host range because this host plant provides enemy free space. In addition, oviposition

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behavior of adult female *M. sexta* appears to be well suited to exploiting the enemy-free space on devil's claw; oviposition by *M. sexta* on devil's claw appears to correspond with seasonal variation in parasitoid abundance.

Introduction

Many physiological and ecological factors can affect the fitness of herbivores feeding on different host plants (Ehrlich & Raven 1964, Mitter & Farrell 1991, Becerra 1997, Brower 1958, Hairston *et al.* 1960, Price *et al.* 1980). Nutritional quality, secondary metabolites and phenology of host plants can all affect whether a particular host plant species is used by an herbivorous insect population or species (Scriber & Slansky 1981, Wood & Keese 1990, Cornell & Hawkins 2003). Microhabitat differences between host plants can alter plant growth, nutritional quality and phenology. The abundance and species diversity of herbivores may differ across host plants, altering the fitness of herbivores of particular interest. Differences in natural enemy attack on different host plants can potentially impact the success of an herbivore on those plants (Dicke 1994, Bernays & Graham 1988). Determining the relative contributions of these different factors for herbivore fitness is one key to understanding the ecology and evolution of host plant choice by herbivores (Thompson 1988a).

The interaction between natural enemy attack and host plant quality has recently been of particular interest, because of its potential for increasing the breadth of host plants use by herbivores. For example, host plants of inferior quality may still be used by some herbivores because they provide "enemy free space" (EFS), in which the habitat or other characteristics of a species reduces its vulnerability to important natural enemies (Jeffries & Lawton 1984).

To assess the importance of EFS in determining host plant use, it is necessary to disentangle the effects of natural enemies from host plant quality, competition, microhabitat and other potentially confounding factors (Berdegue *et al.* 1996, Murphy 2004, Singer *et al.* 2004a,b). In addition, it is important to quantify the impact of host plant use on total fitness (e.g. intrinsic rate of increase (*r*) or net reproductive rate (R_0)), not just for individual components of fitness (e.g. survival or development rate) (Berdegue *et al.* 1996). This is because there may be tradeoffs or interactions among fitness components, such that individual components may not be strongly correlated with total fitness (Thompson 1988b). Although these factors have been recognized as critical components in examining the role of EFS in host plant use (Berdegue *et al.* 1996), only a few previous studies have controlled for major confounding explanatory variables to EFS (Murphy 2004), and none have obtained direct estimates of total fitness (e.g. R_0).

The tobacco hornworm, *Manduca sexta* L., has been an important study system for exploring insect herbivory and host plant use (e.g. Yamamoto & Fraenkel 1960, del Campo, *et al.* 2001, Kester, *et al.* 2002, Mira & Bernays 2002). This insect is largely restricted to feeding on host plants in the Solanaceae (Yamamoto & Fraenkel 1960). Recently, however, *M. sexta* was found to use a non-solanaceous host, devil's claw (*Proboscidea* spp., family Martyniaceae), even when it co-occurs with solanaceous host plants, in the southwestern US (Mechaber & Hildebrand 2000, Mira & Bernays 2002). Laboratory experiments and field observations with *M. sexta* suggest that survival and growth are significantly reduced on devil's claw (*Proboscidea* spp.) compared with typical solanaceous host plant species (Mira & Bernays 2002, Diamond *et al.* submitted).

Why then is devil's claw used as a host plant given the negative performance

consequences that result from feeding on this non-solanaceous host plant? An observational field study of southwestern US (Arizona) populations of *M. sexta* feeding on a typical solanaceous host plant species, *Datura wrightii* and a species of devil's claw, *Proboscidea parviflora,* suggests the use of devil's claw may be driven by enemy escape, as fewer predators and parasites were found on this host plant (Mira & Bernays 2002). In the field, survival to the final larval instar was greater on *P. parviflora* in association with decreased natural enemy attack on this host, despite high mortality of eggs and early instar larvae not attributable to natural enemies. Observational field studies take advantage of the natural variation in environmental factors to identify important ecological patterns in the field, in this case the positive association between host plant quality and natural enemy attack (Mira & Bernays 2002). Field experiments can complement observational studies by disentangling environmental factors that may potentially covary in nature, and allow us to distinguish the effects of natural enemies and host plant quality from competition, microhabitat and other factors affecting host use.

Here we describe a field experiment that examines the role of EFS in the adoption of a non-solanaceous host plant devil's claw, *P. louisianica* (a close relative of *P. parviflora*; Bretting 1981), by a southeastern US (North Carolina) population of *M. sexta* which typically feeds on solanaceous host plants such as tobacco. Our experimental design allowed us to exclude food limitation, herbivore competition and microhabitat variation as potential confounding factors, and to quantify the impact of host plant quality on herbivore success. In particular, we assessed both major fitness components (survival, growth rate, development rate, final body size and fecundity) and total fitness (net reproductive rate) for individuals reared on tobacco and devil's claw. Finally, to assess the ability of *M. sexta* to take advantage

of a potential enemy free space on devil's claw, we monitored levels of oviposition by *M. sexta* on these host plants before and after our field parasitism experiment (oviposition could not be measured during the experiment).

The major goal of this study was to test whether the adoption of a novel host plant by an herbivore was driven by EFS. If the herbivore, *M. sexta* has adopted the novel host plant, devil's claw, as a result of EFS, we should expect: 1) natural enemies significantly affect fitness in *M. sexta*, demonstrating natural enemies are important agents of natural selection in this system; 2) total fitness and fitness components of *M. sexta* are lower on devil's claw relative to tobacco for individuals not attacked by natural enemies, demonstrating devil's claw is intrinsically inferior in diet quality; 3) total fitness of all *M. sexta* (those attacked and not attacked by natural enemies) is at least comparable across devil's claw and tobacco, demonstrating escape from natural enemy attack offsets the intrinsically inferior diet quality of devil's claw. Our results indicate that although *M. sexta* reared on devil's experience severe reductions in fitness not attributable to natural enemies, this novel host plant provides escape from natural enemies for *M. sexta*. The trade-off between plant quality and natural enemy attack resulted in similar total fitness for *M. sexta* reared on tobacco and devil's claw, suggesting *M. sexta* has adopted devil's claw as a host plant because it provides enemy-free space.

Materials and Methods

Study organisms

Manduca sexta L. (Sphingidae) is distributed across tropical and temperature regions of the Nearctic (Rothschild & Jordan 1903). Larval feeding is generally restricted to plants in

the Solanaceae, but recently *M. sexta* has been documented to use non-solanaceous host plants belonging to the family Martyniaceae, in the Southwestern US (Mechaber & Hildebrand 2000, Mira & Bernays 2002). These plants (*Proboscidea* spp.) are commonly referred to as devil's claw, and are native to the Southwest, but have been introduced to other locations across the United States via contaminated crop seed and have escaped from gardens where they are grown as ornamentals. One species of devil's claw, *P. louisianica* (Miller) Thellung, has been naturalized to the Southeastern United States (Small 1903). *Proboscidea louisianica* is patchily distributed across North Carolina, compared to the predominant local solanaceous host, tobacco (*Nicotiana tabacum* L.) which is widely cultivated across the state (Radford *et al.* 1968). Despite the limited distribution of devil's claw in North Carolina, our observations indicate field populations of *M. sexta* oviposit and feed on this host when grown adjacent to tobacco plants.

Here we consider devil's claw a novel host plant for *M. sexta* relative to tobacco. Although cultivated tobacco (*Nicotiana tabacum*) may be considered somewhat novel because of its likely hybrid origins (Ren and Timko 2001), wild *Nicotiana* spp. and many other members of the Solanaceae are typical host plants for *M. sexta* (Yamamoto & Fraenkel 1960). Devil's claw, a native species to the southwestern US, is a relatively recent introduction (approximately 100 years ago) to the southeastern US (Small 1903). In contrast, tobacco has been cultivated in the area for at least several hundred years, and wild *Nicotiana* is native to the region (Radford *et al.* 1968).

An important source of mortality for *M. sexta* is the braconid wasp *Cotesia congregata* Say, which in North Carolina can parasitize more than 90% of *M. sexta* larvae during their second generation (*M. sexta* is bivoltine in North Carolina; Rabb 1971). *Cotesia*

congregata is a gregarious larval endoparasitoid of several species of sphingid moths including *M. sexta* (Krombein *et al.* 1979, Kester & Barbosa 1994). This parasitoid prefers to oviposit into third instar *M. sexta* larvae (Barbosa *et al.* 1991), although larvae at earlier and later developmental stages can also be parasitized (Alleyne & Beckage 1997). Like all parasitoids, *C. congregata* is lethal to its host; all successfully parasitized *M. sexta* larvae die before pupation.

Field experiment

We established a 12 x 20 m field plot at the Mason Farm Biological Reserve (Chapel Hill, NC). The plot was planted with 30 tobacco and 30 devil's claw plants that were randomized to grid locations spaced 2m apart, to control for microenvironmental variation. Tobacco and devil's claw plants were grown from seed in a greenhouse for 3 weeks before being transplanted to the field site in mid-July. Greenhouse plants were fertilized weekly with Peter's Pro Solution (15-16-17); no additional fertilizer was provided after being transferred to the field site. The plants were allowed to grow to a large size (approximately 1.25 m in height for tobacco and 0.75 m in height for devil's claw, as most of its growth is lateral) before *M. sexta* larvae were transferred to the plants. This was to ensure *M. sexta* larvae were not subject to food limitation or competition for food on either host plant species.

Other herbivores were removed from the plants every 1-4 days to maintain ample leaf material for the experimental *M. sexta* larvae and to reduce the production of induced defenses that would reduce leaf quality prior to the start of the experiment (Baldwin 2001). Naturally occurring *M. sexta* females laid eggs on the plants to be used in the experiment; we took advantage of this to explore oviposition preferences for tobacco versus devil's claw in

the field. We counted the number of *M. sexta* eggs laid on each host plant species and then removed them every 1-4 days beginning in mid-July and ending in mid-September, exclusive of the time during the parasitism experiment when the plants were covered with netting (see below), preventing oviposition by *M. sexta*. No pesticides were ever applied to the plants while in the greenhouse or at the field site.

We collected *Manduca sexta* eggs in early August from a field of tobacco plants in Clayton, NC. The eggs were brought into the lab and randomly assigned to cut leaves from greenhouse-grown tobacco and devil's claw plants; leaf water content was maintained by placing leaves in water picks. Eggs were maintained in growth chambers (Percival model 36-VL, 25 °C, 16 L : 8 D cycle) on their assigned host plant species until hatching. First instar larvae were transferred to new leaves and offered leaf material *ad libitum*. After molting into 2nd instar, larvae were brought out to the experimental field plot. Larvae were randomly assigned to plants in the field corresponding to the same host plant species on which they were reared in the lab. Three larvae were placed on each plant.

After *M. sexta* larvae were transferred to the field plants, the plants were covered with small gauge nylon netting. This allowed access by naturally occurring *C. congregata* parasitoids to the experimental *M. sexta* larvae, but prevented access by larger predators including birds, vespid wasps and predatory bugs. The netting also prevented potential competition between *M. sexta* larvae and other herbivores present at the field site.

The *M. sexta* larvae were checked daily to assess survival, developmental stage and whether they were parasitized. Larvae were allowed to feed on their host plants at the field site until: 1) death, 2) egression of larval parasitoids from their *M. sexta* hosts, or 3) mid-5th instar (a mass of approximately 3-4 g) for surviving non-parasitized *M. sexta* larvae. Upon

any one of these three events, *M. sexta* larvae were returned to the lab. Dead and parasitized *M. sexta* larvae were immediately frozen (-80 °C). Surviving non-parasitized *M. sexta* larvae had to be returned to the lab during 5th instar because at the end of the larval stage (the wandering stage), larvae purge their guts and burrow underground to pupate where they would be effectively lost from the experiment. The larvae returned to the laboratory were reared individually in plastic enclosures with screened lids. Larvae were fed on leaves cut from their host plant, and leaf water content was maintained by keeping the leaf material in water picks. The enclosures containing the larvae were maintained in growth chambers under standard rearing conditions (25 °C, 16 L : 8 D). Upon wandering, they were placed individually in wooden pupation chambers at room temperature (~25 °C) until pupation. Pupae were transferred to plastic cups lined with soil and remained there until eclosion. Adults were frozen at -80 °C within 5 hours of eclosion.

Mass and development time were recorded for each individual larva at wandering, pupation and at the adult stage within 5 hours of eclosion. For adult females, estimates of potential fecundity were obtained by dissecting out the ovarioles into Ringer's solution. The number of follicles at stage 6 (S6) and all subsequent stages of development (cf. Yamauchi & Yoshitake 1984) were counted with the aid of a dissecting microscope (Nikon SMZ-1B).

Larvae without external signs of parasitism that died prior to reaching 5th instar were dissected to search for internal indications of parasitism (e.g. punctures in the cuticle, *C. congregata* eggs or early-stage larvae). We were able to recover all *M. sexta* larvae (alive, dead or parasitized) from each host plant species, although four of the dead *M. sexta* larvae were severely desiccated and only a portion of each of these larvae were recovered from the field.

As a metric of total fitness, female net reproductive rate (R_0) was calculated as the product of survival to eclosion and potential fecundity. Females that survived to eclosion were scored as 1, and females that died prior to eclosion where no estimate of fecundity could be obtained, were scored as 0. Survival (1 or 0) was multiplied by potential fecundity (total number of follicles for an individual female that survived to eclosion, or 0 for females that died prior to eclosion) for each female to obtain R_0. We estimated the number of females that died prematurely because larvae that died in early developmental stages (either due to plant effects or parasitism) could not be sexed. Sex ratios for individuals surviving to eclosion were similar across host plant species ($\chi^2 = 0.033$, df = 1, $P = 0.8549$), so the total number of males and females surviving to eclosion were pooled. The overall ratio of adult males to females was statistically indistinguishable from unity ($\chi^2 = 0.373$, df = 1, $P = 0.5416$). Assuming the adult sex ratio reflects that of earlier developmental stages, i.e. that half of the larvae that died prematurely were male, we removed half of the total number of individuals that died prematurely on devil's claw and did the same for tobacco.

Data processing and statistical analyses

Parasitism and survival

We first assessed how host plant use of tobacco or devil's claw affected rates of parasitism by *C. congregata* or overall survival of *M. sexta*. Parasitism was considered a binomial variable and modeled using analysis of deviance with host plant species as a fixed effect. Because multiple larvae inhabited the same plant, we included the plant identification number as a random effect in the model to account for potential correlation among the results for a given plant.

Survival to pupation was treated as a binomial variable and modeled using analysis of deviance with host plant species (tobacco or devil's claw) and plant identification number as a random effect.

We also examined the covariate of final larval density of *M. sexta* (the number of larvae on a given plant at mid-5th instar, just prior to being returned to the laboratory) in the survival and parasitism models. Although starting densities of *M. sexta* larvae were equivalent, mortality varied across plant species and individual plants over the course of the experiment. We examined final *M. sexta* larval density and its interaction with the fixed effect of host plant species (where applicable) in these models to test for potential density-mediated survival and parasitism.

Body size, development time, fecundity and fitness

We assessed how host plant use impacted size, development time and fecundity of *M. sexta* that survived to adulthood. Development time and mass at pupation were modeled using mixed-model ANOVA (REML) with host plant species, sex and the interaction between host plant species and sex as fixed effects. Plant identification number was included as a random effect. We focused on performance at the pupal stage for comparison with previous results for performance on devil's claw and tobacco in the laboratory environment. We include only those individuals that survived to eclosion in our analyses.

Potential fecundity was modeled using mixed-model ANCOVA (REML) with host plant species as a fixed effect and plant identification number as a random effect. Because body size has important effects on fecundity in *M. sexta* and other insects (Davidowitz *et al.* 2004, Davidowitz *et al.* 2005), ln(adult mass) and the interaction of ln(adult mass) with host

plant species were included as fixed effects in this model. Here again, we also examined the covariate of final larval density of *M. sexta* and its interaction with the fixed effects of host plant species and sex (where applicable) in the models for body mass, development time and potential fecundity to test for potential density-mediated effects on *M. sexta* performance. We used a Mann-Whitney-Wilcoxon test to examine the consequences of host plant use for total fitness of *M. sexta*.

Patterns of oviposition

Finally, to assess *M. sexta*'s ability to take advantage of a potential enemy free space on devil's claw, we recorded egg counts on both host plant species over a total of 33 days prior to and following the time during the parasitism experiment when plants were netted and unavailable for oviposition by *M. sexta*. We divided this 33 day period into 3 smaller time intervals (A, B and C). The dates corresponding to each interval and the number of samples of eggs taken during each time interval were as follows: A (July 20-31; 4 samples), B (August 1-11; 7 samples), and C (September 8-17; 4 samples). Oviposition patterns of naturally occurring *M. sexta* were modeled using analysis of deviance. Whether an egg was laid on tobacco or devil's claw was considered a binomial response variable. Time interval was included as a fixed effect, and the sample was included as a random effect. All statistical analyses were performed using R (version 2.6.0).

Results

Parasitism and survival

Analysis of deviance detected a significant effect of host plant species ($\chi^2 = 31.619$, *P*

< 0.0001) on the probability of being parasitized. Parasitism exceeded 40% on tobacco (Figure 3.1), and all parasitized *M. sexta* larvae died prior to metamorphosis. Parasitism on tobacco had a significant effect on survival to pupation ($\chi^2 = 94.47$, $P < 0.0001$). In contrast, no larvae were successfully parasitized on devil's claw (Figure 3.1); specifically, no devil's claw-reared *M. sexta* larvae had *C. congregata* parasitoid larvae egress from them. Although unsuccessful parasitism (injection of parasitoid eggs that fail to develop) of *M. sexta* larvae on devil's claw is possible, post-mortem dissections of *M. sexta* larvae that died prematurely revealed no evidence of parasitism (parasitoid eggs or puncture wounds).

Survival of non-parasitized *M. sexta* larvae was significantly lower on devil's claw compared to tobacco (Figure 3.1). Analysis of deviance detected a significant effect of host plant species on the probability of survival to pupation for non-parasitized individuals ($\chi^2 = 15.466$, $P < 0.0001$). Results were qualitatively similar for the probability of survival to eclosion for non-parasitized individuals.

Analysis of deviance detected a marginal, but non-significant effect of host plant species (tobacco or devil's claw) ($\chi^2 = 2.786$, $P = 0.0951$) on the overall probability of survival to pupation, when survival of both parasitized and non-parasitized individuals was combined. Results were qualitatively similar for the probability of survival to eclosion ($\chi^2 = 2.304$, $P = 0.1290$). Although there were significant differences in daily survival on the two host plant species at earlier stages of development (e.g. at wandering, $\chi^2 = 4.417$, $P = 0.0356$), there was no significant difference in survival through metamorphosis (Figure 3.2).

The main effect of larval density and its interaction with host plant species (where applicable) were not significant in all parasitism and survival models (mean 5^{th} instar larval density ± 1 SE, on tobacco: 2.90 ± 0.06, on devil's claw: 2.13 ± 0.06).

Development time, size, fecundity and total fitness

ANOVA detected significant effects of host plant species ($F_{1, 52}$ = 125.885, P < 0.0001), and sex ($F_{1, 53}$ = 5.265, P = 0.0258) on time to pupation, but no significant interaction was found between host plant species and sex ($F_{1, 53}$ = 0.629, P = 0.4312). Development time to pupation was faster for *M. sexta* feeding on tobacco relative to devil's claw, and for males relative to females (Figure 3.3). Results for development time to eclosion were qualitatively similar.

ANOVA also detected significant effects of host plant species ($F_{1, 52}$ = 14.260, P = 0.0004) and sex ($F_{1, 53}$ = 14.369, P = 0.0004) on mass at pupation, but no significant interaction was found between host plant species and sex ($F_{1, 53}$ = 0.026, P = 0.8725). Body mass at pupation was greater for *M. sexta* feeding on tobacco relative to devil's claw; female pupal mass was greater than male pupal mass, reflecting sexual dimorphism in this species (Madden and Chamberlin 1945) (Figure 3.3). Results for body mass at eclosion were qualitatively similar.

ANCOVA revealed significant effects of host plant species ($F_{1, 36}$ = 80.114, P < 0.0001) and adult body mass ($F_{1, 9}$ = 17.919, P = 0.0022) on the number of follicles in the ovarioles (Figure 3.4). No significant interaction between host plant species and adult body mass was detected ($F_{1, 9}$ = 2.561, P = 0.1440). Larger females were more fecund than smaller females, and females reared on tobacco were significantly more fecund than females reared on devil's claw.

Again, the main effect of larval density and its interaction with the fixed effects of host plant species and sex (where applicable) were not significant in the models for body

mass, development time and potential fecundity.

Non-parametric analysis indicated median net reproductive rate for non-parasitized individuals was significantly greater on tobacco relative to devil's claw ($W = 100.5$, $P < 0.0001$). In contrast, non-parametric analysis indicated median net reproductive rate was not significantly different across host plant species when both parasitized and non-parasitized individuals are considered ($W = 757$, $P = 0.1834$). Mean net reproductive rates were similar on the two host plant species (mean ± SE: tobacco, 181.093 ± 27.735, N = 43; devil's claw, 150.238 ± 19.079, N = 42). As a result, when effects of both host quality and parasitism are considered, total fitness of *M. sexta* was similar on the two hosts.

Patterns of oviposition

Analysis of deviance revealed a significant effect of time interval on the probability of laying eggs on tobacco ($\chi^2 = 18.111$, $P = 0.0001$). Post-hoc analyses indicated the probability of laying eggs on tobacco was significantly different between time intervals A and B ($\chi^2 = 12.909$, $P = 0.0003$) and A and C ($\chi^2 = 16.761$, $P < 0.0001$), but not B and C ($\chi^2 = 0.011$, $P = 0.9166$). Patterns of oviposition at the field plot used in the parasitism experiment (see Materials and Methods) changed substantially during the period before and after the experiment (Figure 3.5). Earlier in the season (mid to late June) during time interval A, females almost exclusively laid eggs on tobacco. However, as the season progressed into July (time interval B), females began to lay eggs on devil's claw. Just prior to the parasitism experiment in mid July, females laid over twice as many eggs on devil's claw as on tobacco (Figure 3.5).

Discussion

Evaluating host plant use and Enemy Free Space in *Manduca*

The primary goal of this study was to test whether the adoption of a novel host plant by an herbivore was driven by enemy free space (EFS). To accomplish this, we experimentally disentangled the effects of natural enemies from the potentially confounding factors of host plant quality, competition, and microhabitat. Here we present evidence which strongly suggests the tobacco hornworm, *Manduca sexta*, has adopted a novel host plant outside its typical host range (devil's claw) because this host plant provides enemy free space from a parasitoid natural enemy, *Cotesia congregata*.

First, we found a striking pattern of differential parasitism of *M. sexta* across the two host plant species. Greater than 40% of the *M. sexta* larvae on tobacco were parasitized, all of which died prior to metamorphosis; however, no larvae were parasitized on devil's claw. This high rate of parasitism on tobacco and absence of parasitism on devil's claw (Figure 3.1) offset the high initial mortality on devil's claw through the first three larval instars (Figure 3.2). As a result, overall survival through metamorphosis of both parasitized and non-parasitized individuals combined was similar across the two host plant species. Importantly, parasitism contributed to significantly increased levels of mortality on tobacco, relative to the levels of background mortality of non-parasitized larvae (Figure 3.1). Together, these results demonstrate that the parasitoid, *C. congregata*, is an important agent of natural selection in this system, and that devil's claw can provide enemy free space from this parasitoid for *M. sexta*.

Second, we were able to eliminate host plant quality as a confounding factor to EFS in *M. sexta*'s use of devil's claw. Consistent with our results for *M. sexta* reared on tobacco

and devil's claw in the laboratory where natural enemies were absent (Diamond *et al.* submitted), the results of our field experiment demonstrate survival, growth, development time, and potential fecundity through metamorphosis (non-parasitized individuals) are significantly lower on devil's claw compared to tobacco (Figures 3.2-3.4). This is important, as host plant quality and EFS could both conceivably promote an herbivore's use of a novel host plant if there were no reduction in food quality on the novel host plant. In this case, it would be difficult to attribute the adoption of a novel host plant to either factor. Because we clearly show that devil's claw is of inferior quality for *M. sexta*, our results indicate that EFS is more important than host plant quality in *M. sexta*'s adoption of this novel host plant. Our experimental approach also allowed us to control for herbivore competition, food limitation, and microhabitat variation as additional alternatives to EFS for *M. sexta*'s use of devil's claw.

Third, to demonstrate that EFS on devil's claw is not an artifact of potential tradeoffs or interactions among fitness components (Thompson 1988b), we examined total fitness (R_0) of *M. sexta* on tobacco and devil's claw. Our result that R_0 of non-parasitized *M. sexta* is greater on tobacco compared to devil's claw is consistent with our results for individual components of fitness. This reinforces the conclusion that devil's claw is of inferior host plant quality for *M. sexta*. Similarly, our result that R_0 of both parasitized and non-parasitized individuals combined is comparable across host plant species is consistent with our result for survival through metamorphosis. Again, this strongly suggests EFS plays a major role in *M. sexta*'s adoption of the novel host plant, devil's claw.

Importantly, *M. sexta* appears to be well suited to take advantage of enemy free space on devil's claw. Our monitoring of oviposition patterns in the experimental garden before and after the field experiment indicate that females laid more eggs on tobacco earlier in the

season when parasitoid abundance was relatively low, but began to lay over twice as many eggs on devil's claw as tobacco later in the season (Figure 3.5). Peak abundance of the parasitoid, *C. congregata*, typically occurs from mid-July, into early August for populations in the southeastern US (Rabb 1971, Kester & Barbosa 1994), which corresponds well with *M. sexta*'s increased oviposition on devil's claw. This pattern appears to be common: we have observed increased oviposition of *M. sexta* on devil's claw at our field site in a subsequent field season (the site layout was comparable to one in the study described here, except that the plants remained free of netting for the entire field season; Diamond, unpubl.).

Selection of oviposition sites by adult females is a critical determinant of larval success in *M. sexta*. Naïve *M. sexta* larvae will accept several non-solanaceous host plant species (Yamamoto & Fraenkel 1960). However, solanaceous-reared *M. sexta* larvae have been shown to refuse non-solanaceous host plants, which arises from larvae developing a dependence on solanaceous host plant chemical to initiate and continue feeding (del Campo & Renwick 1999). Preliminary results indicate *M. sexta* larvae (instars 2-4) will not survive if switched from tobacco to devil's claw, and vice versa (Diamond, unpubl.). Because there are significant costs to switching host plant species, oviposition by *M. sexta* females is an important component of being able to take advantage of the enemy free space on devil's claw.

Most likely, these oviposition patterns across devil's claw and tobacco do not reflect the formation of host races in *M. sexta*. Laboratory experiments have shown that individual females lay eggs on both devil's claw and tobacco when given a choice between these two host plant species (Diamond, unpubl.). In addition, *M. sexta* adults are powerful fliers and highly dispersive, and allozyme data suggest little population differentiation within the

southeastern US (H.A. Woods, *pers. comm.*).

Differences in plant phenology can also affect seasonal patterns of oviposition in the field. In our experiment, however, plants of each species were germinated at the same time, and our measurements in the field took place during a time where both plant species were well-established and flushing new leaves. Thus, although phenological variation in host plant species could be an important driver of oviposition (and subsequently EFS), it was unlikely a major factor in our experiment. Interestingly, a similar pattern to the one documented here (increased oviposition on devil's claw later in the season) was found for *M. sexta* laying eggs on wild solanaceous plants, *Datura wrightii* and a species of devil's claw, *P. parviflora* in Arizona (Mechaber & Hildebrand 2000), suggesting this pattern may be relatively robust.

Our field experiments demonstrate that EFS is a critical determinant of *M. sexta*'s use of devil's claw in North Carolina. A previous field observational study with *M. sexta* in Arizona also reported reduced larval mortality due to natural enemies by use of devil's claw (*Proboscidea* spp.) (Mira & Bernays 2002). The differences between these two *M. sexta* systems are instructive. In Arizona, *M. sexta* experienced fewer natural enemies on endemic *Proboscidea* spp., relative to a native solanaceous host plant (*Datura wrightii*). In North Carolina, *M. sexta* achieved EFS on *P. louisianica*, a naturalized plant species from a relatively recent introduction (Small 1903), relative to a hybrid solanaceous host plant, cultivated tobacco, *Nictotiana tabacum* (Ren & Timko 2001). In our study, we focused on a single parasitoid natural enemy of major importance in North Carolina, and experimentally excluded social wasps, birds and other larger predators; Mira and Bernays (2002) considered the impacts of a diverse assemblage of natural enemies. These two complementary studies suggest escape from natural enemies by use of devil's claw, and perhaps other non-

solanaceous host plants, may be geographically widespread in *M. sexta*.

Because of the complexity and lability of multitrophic interactions (Thompson 1988a), the potential benefit of EFS that *M. sexta* gains on devil's claw is likely contingent on a number of environmental factors. For example, host plant abundance likely differs in nature, e.g. tobacco is often planted as a large monoculture (Radford *et al.* 1968), which may be particularly important if *M. sexta* females incur searching costs for devil's claw (Singer 1983). In addition, *M. sexta* is confronted with multiple natural enemies across several taxa that vary in importance and diversity across spatial and temporal scales (see Mira & Bernays 2002 for an extensive field survey). Clearly these factors could affect the overall suitability of devil's claw as a host plant for *M. sexta*, and are worth further study. More generally, spatial and temporal dynamics of plant-herbivore interactions have been shown to substantially impact EFS in some systems; the degree to which EFS may be characterized by a spatially and temporally varying mosaic is an interesting, but unresolved issue (Heard *et al.* 2006).

The mechanism by which *M. sexta* evade parasitism on devil's claw also deserves further study. We suspect either one or both of the following mechanisms may be involved: 1) the parasitoid wasps have a search image for caterpillar hosts on tobacco, a tall plant with broad, oval-shaped leaves, but cannot 'see' caterpillar hosts on devil's claw, a relatively short plant approximately half the height of tobacco with small, round-shaped leaves, and 2) the volatile compounds present in devil's claw are quite different from those in tobacco (see Sisson & Saunders 1982, and Riffle *et al.* 1990 for the biochemical profiles of these host plants) which may interfere with the parasitoid's attraction to or detection of caterpillar hosts on devil's claw.

Alternatively, *M. sexta* may not be entirely evading parasitism on devil's claw. Rather, the devil's claw allelochemicals may impair (or kill) the parasitoids, allowing the caterpillar hosts to survive without presenting symptoms of parasitism when feeding on devil's claw (see Singer & Stireman 2003 for an example of this mechanism). However, laboratory experiments in which *M. sexta* were reared on devil's claw and subsequently exposed to mated adult *C. congregata* females, revealed that all *M. sexta* larvae died within days of being parasitized (Diamond, unpubl.), suggesting this pharmacological mechanism is unlikely to occur in our system. Importantly, however, this highlights the fact that multiple mechanisms (e.g. resistance versus evasion of natural enemies, among several others; see Berdegue *et al.* 1996 for a complete list) underlie the production of enemy-free space, suggesting enemy-free space may be more likely to evolve.

Host use and Enemy Free Space in insect-plant systems

EFS can provide a mechanism by which insect herbivores can expand the set of host plants utilized to include host plants of inferior intrinsic quality. There are three necessary conditions for this to occur. First, there must be differences in attack rate by natural enemies across different host plants (Jeffries & Lawton 1984, Scheirs & de Bruyn 2002). Several reviews have found evidence for this condition in more than 80% of studies with insect-plant systems (Berdegue et al 1996, Heard *et al.* 2006). In most cases, however, these studies do not rule out alternative factors such as competition, host plant quality or microhabitat variation as drivers of host plant use by herbivores, which may require experimental manipulation (Mulatu *et al.* 2004).

Second, there must be a fitness cost to using the alternative host plant when natural

enemies are excluded. Exclusion studies to demonstrate fitness costs have become more common in the past two decades (Denno *et al.* 1990, Ohsaki & Sato 1994, Gratton & Welter 1999, Ballabeni *et al.* 2001, Zangerl *et al.* 2002, Zvereva & Rank 2003, Mulatu *et al.* 2004, Murphy 2004, Singer *et al.* 2004a,b; and the current study), and have detected significant costs in about half of these systems.

Interestingly, several of the studies showing positive support for EFS are in experimentally generated (Gratton & Welter 1999) or recent natural host shifts (this study; Mulatu *et al.* 2004, Murphy 2004). Negative fitness consequences (not due to natural enemies) associated with using a novel host plant may only be detectable during ongoing host range shifts or recent expansions because these costs may disappear following physiological adaptation to the novel host plant. This may make it more difficult to document EFS in extant plant-herbivore systems with relatively long histories of association.

The third condition is that fitness costs of using an inferior host plant are balanced by fitness gains via escape from natural enemies, such that total fitness is similar on the different host plants. This quantitative assessment is rarely done: often, only one fitness component (typically survival) is assessed. Even when multiple fitness components are measured, they are not always concordant (e.g. Denno *et al.* 1990, Ohsaki & Sato 1994). In the absence of estimates of total fitness, it is difficult to tell whether reductions in some fitness components but not others are indicative of a biologically relevant fitness cost, and therefore whether EFS is the predominant factor driving host plant use in that system. The results of our study underscore the importance of assessing both individual components of herbivore fitness and total fitness, to fully evaluate the role of EFS in host plant use.

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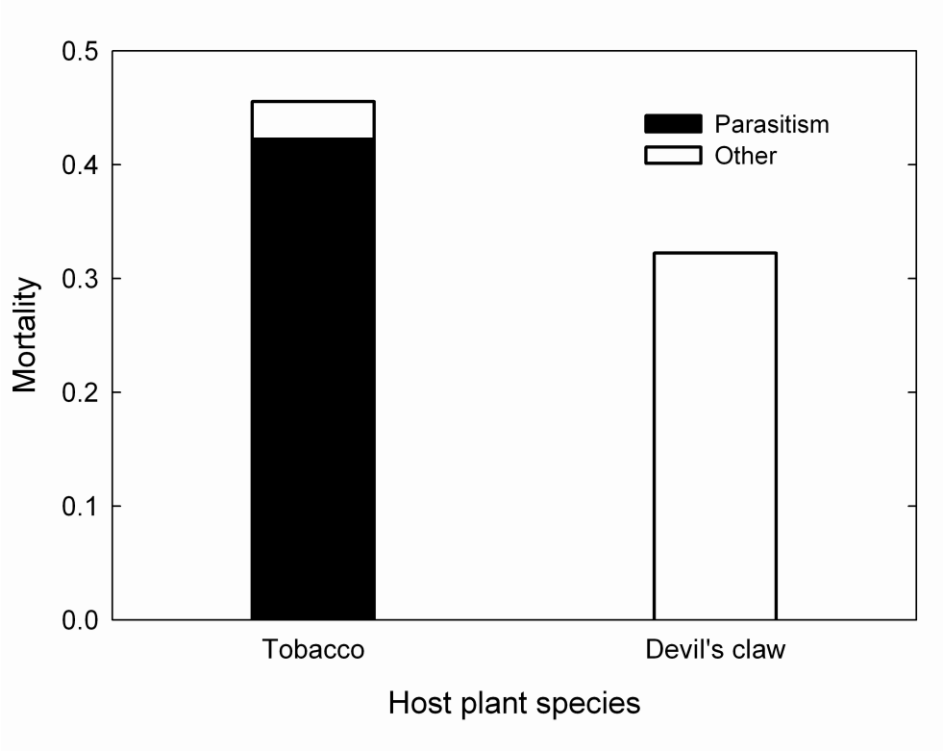

Figure 3.1. Cumulative mortality from 2nd instar to pupation due to parasitism (solid bar) and other non-parasitoid causes (open bar) for *Manduca sexta* feeding on tobacco and devil's claw in the field.

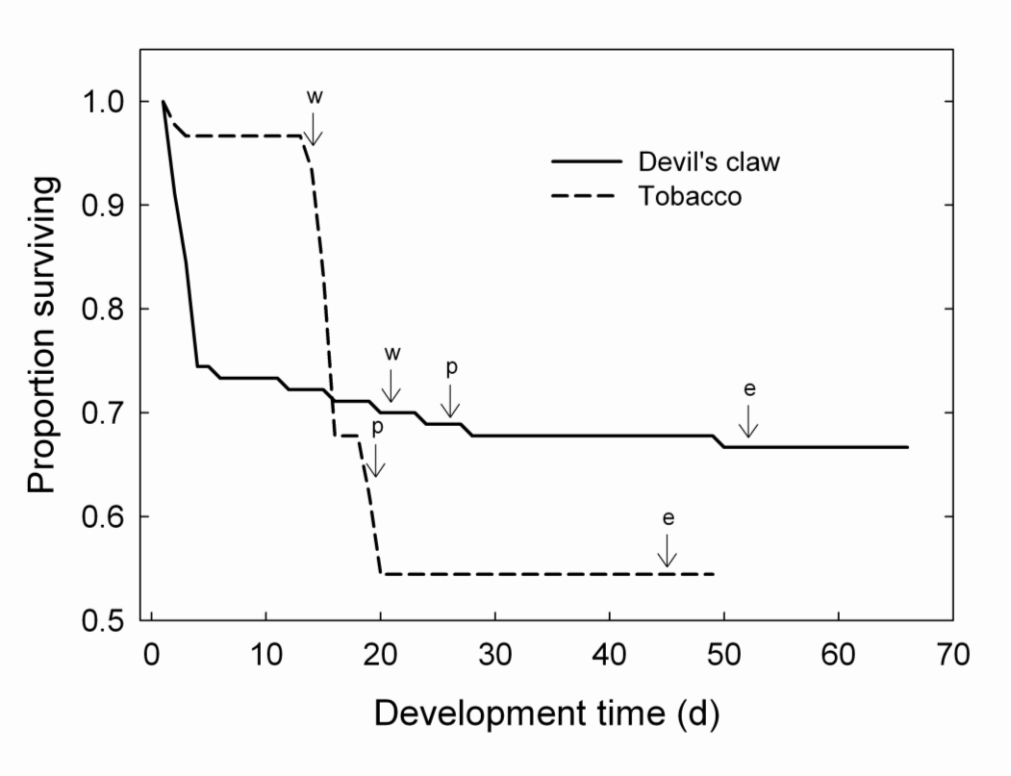

Figure 3.2. Mean daily survival from 2nd instar to eclosion for *Manduca sexta* feeding on tobacco (dashed line) and devil's claw (solid line); data are for survival until eclosion of the last individuals on tobacco and devil's claw. Mean times to reach wandering (w), pupation (p) and eclosion (e) are indicated for each of the two hosts.

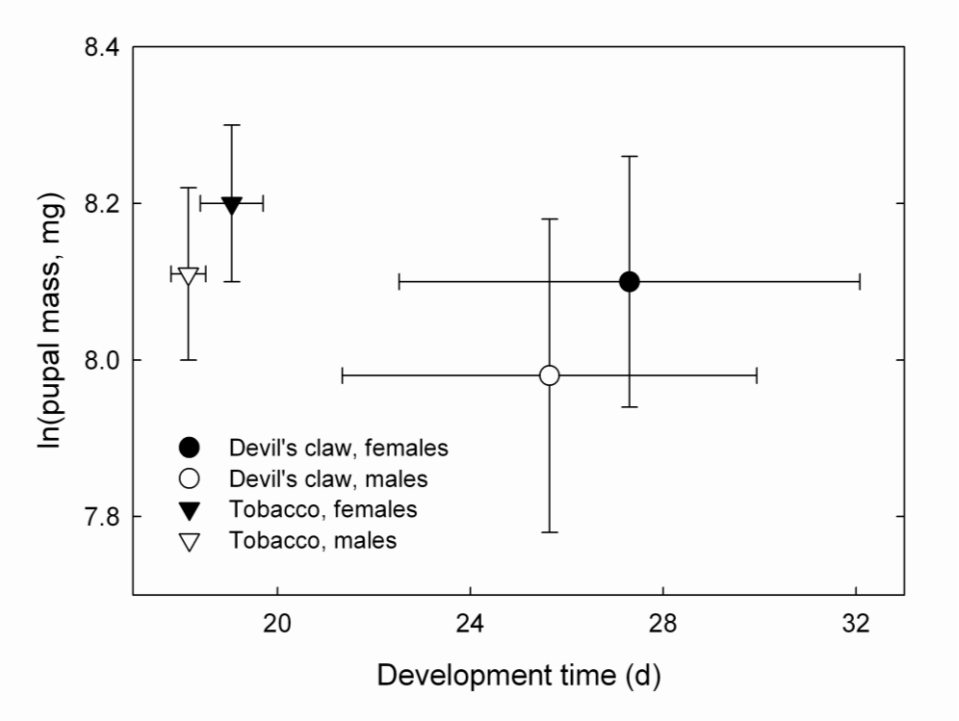

Figure 3.3. Body mass (in ln(mg)) at pupation as a function of development time (d) from 2nd instar to pupation for female (closed symbols) and male (open symbols) *M. sexta* feeding on tobacco (triangles) and devil's claw (circles). Mean ± 1 SD is indicated. Results for supernumerary instars are included with those for 5 larval instars.

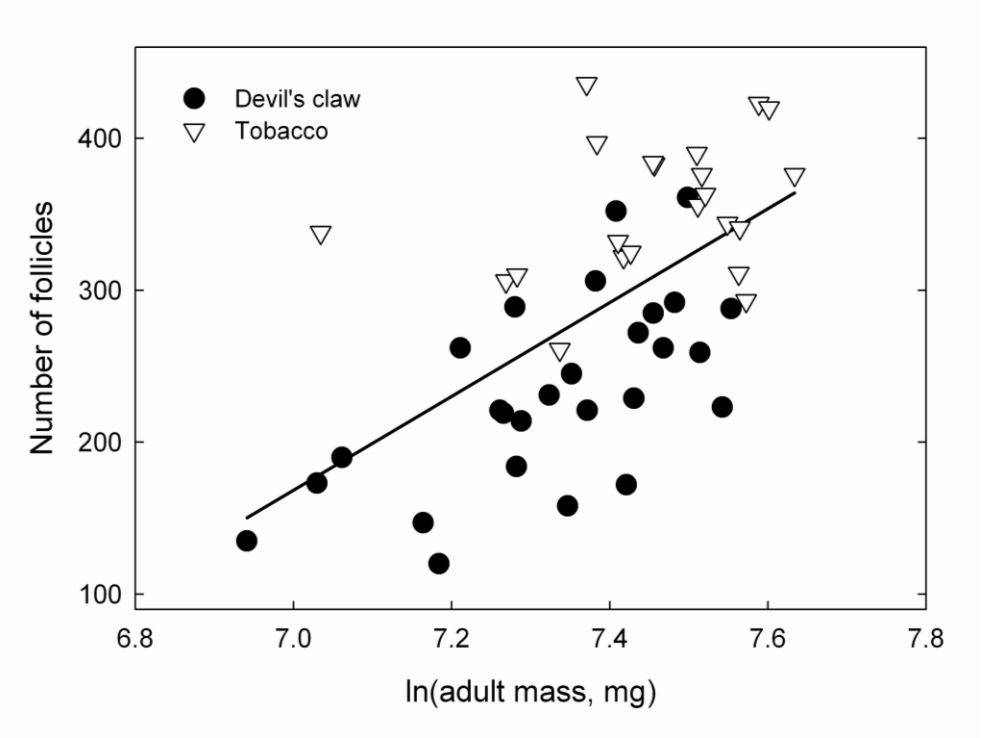

Figure 3.4. Potential fecundity (number of follicles) as a function of body mass (in ln(mg)) for adult females reared on devil's claw (closed circles) and tobacco (open triangles). The regression of fecundity on ln(adult mass, mg) is shown ($y = 309.02x - 1994.82$; $r^2 = 0.37$)

.

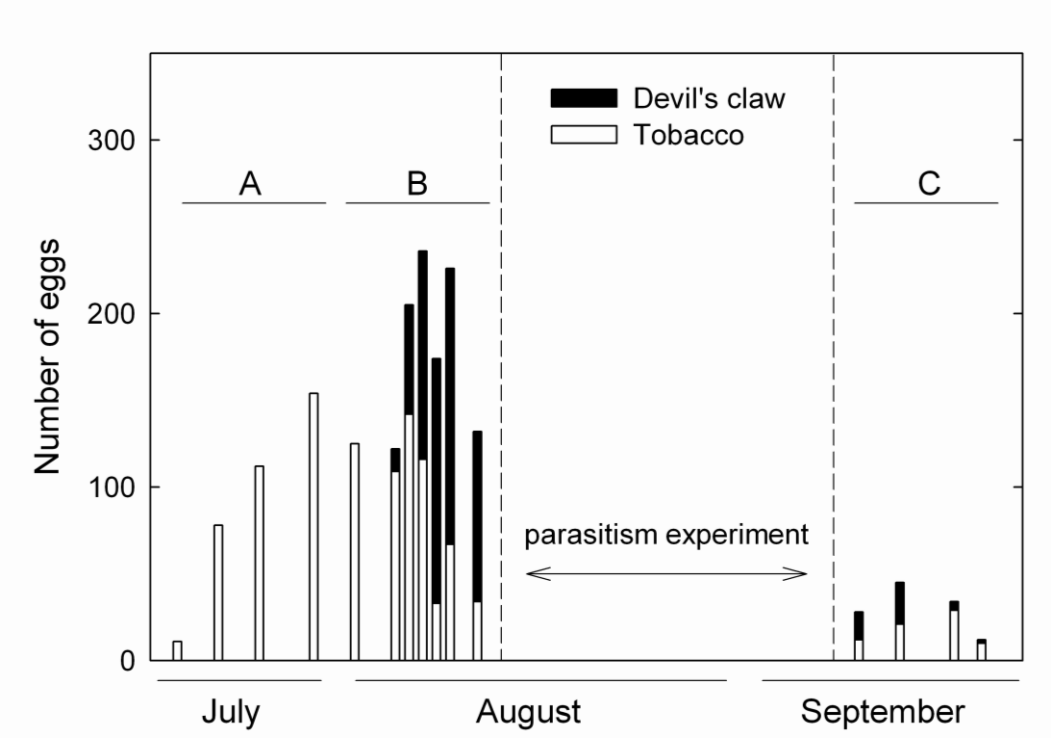

Figure 3.5. The number of eggs laid by naturally occurring *Manduca sexta* females on the randomized tobacco and devil's claw plants used in the parasitism experiment prior to and following the experiment. The three time intervals used in the statistical analyses are indicated: A (July 20-31), B (August 1-11), and C (September 8-17).

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CHAPTER IV

ENVIRONMENTAL DEPENDENCE OF THERMAL REACTION NORMS: HOST PLANT QUALITY CAN REVERSE THE TEMPERATURE-SIZE RULE[1]

Summary

The temperature-size rule, a form of phenotypic plasticity in which decreased temperature increases final size, is one of the most widespread patterns in biology, particularly for ectotherms. Identifying the environmental conditions in which this pattern is reversed is key to understanding the generality of the rule. We use wild and domesticated populations of the tobacco hornworm, *Manduca sexta*, and its natural host plants to explore the consequences of resource quality for the temperature-size rule. *M. sexta* reared on a high quality host, tobacco (*Nicotiana tabacum*), followed the temperature-size rule, with larger final size at lower temperatures. In contrast, *M. sexta* reared on a low quality host, devil's claw (*Proboscidea louisianica*), showed the reverse response. Wild and domesticated *M. sexta* exhibited qualitatively similar responses. Survival, growth and development rates, fecundity and final size decreased with decreasing temperature on devil's claw. We propose the reversal of the temperature-size rule results from the stressful combination of low temperatures and low dietary quality. Such reversals may impact seasonal and geographic patterns of host use in *Manduca* and other systems. Our results suggest the temperature-size rule occurs for a restricted range of non-stressful environmental conditions, limiting the robustness of this widespread pattern of phenotypic plasticity.

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Introduction

One of the most recognized forms of phenotypic plasticity is the relationship between body size and temperature (Atkinson 1994). In ectotherms, decreasing rearing temperature typically causes decreased growth and development rates, but larger final body size—a relationship that has been called the temperature-size rule. This generates a negative thermal reaction norm—the relationship between body size and rearing temperature—for final body size. Although substantial variation in thermal reaction norms has been documented among individuals, populations and species, the temperature-size rule is one of the most taxonomically widespread patterns in biology; more than 80% of ectothermic species studied to date follow the temperature-size rule (Atkinson 1994). The phenotypic pattern described by the temperature-size rule is mirrored at the evolutionary level in another widespread empirical generalization known as Bergmann's rule: cooler climates tend to have populations or species with larger body sizes (Partridge & Coyne 1997; Blanckenhorn & Demont 2004).

Both adaptive and mechanistic explanations for the temperature-size rule have been proposed (Berrigan & Charnov 1994; van der Have & de Jong 1996; Davidowitz *et al.* 2004; Nijhout *et al.* 2006), but it remains unclear whether this pattern is produced by natural selection or shared physiological constraints. Often, proposed mechanistic constraints and life-history optimality models seeking to explain the temperature-size rule suffer from a lack of generality (Angilletta & Dunham 2003; Angilletta *et al.* 2004; Angilletta 2009). How do we reconcile the lack of a satisfactory general explanation for the temperature-size rule with how commonly it occurs in nature?

Understanding exceptions to the temperature size-rule can be particularly informative (Angilletta & Dunham 2003; Walters & Hassall 2006; Kingsolver & Huey 2008). Exceptions

have been reported in several taxa, but the only documented reversals of the temperature-size rule (positive thermal reaction norms for final body size) have been in insects, most commonly in Lepidoptera and Orthoptera (Atkinson 1994; Mousseau 1997). Reversal of the temperature-size rule in one geographic population of the butterfly, *Pieris rapae*, has likely evolved within the past 160 years, since the colonization of North America by *P. rapae* (Kingsolver *et al.* 2007). Observed reversals of the temperature-size rule in grasshoppers can be produced by changes in the relative positions of the minimum temperature coefficients for growth versus development rates (Walters & Hassall 2006). In this case, the reversal of the temperature-size rule may emerge as a result of selection for a greater temperature threshold for growth versus development or, as a by-product of selection for greater thermal specialization in growth rate (Huey & Hertz 1984; Walters & Hassall 2006).

An important issue in exploring the temperature-size rule and it exceptions is how temperature may interact with other environmental factors that affect growth, development and final size (Berrigan & Charnov 1994). For example, numerous studies show that reductions in the resource (diet) quality result in reduced growth and development rates, delayed development times and smaller final body sizes (e.g. Stearns & Koella 1986); and resource quality is a major determinant of resource use in nature (Awmack & Leather 2002). In contrast, reduced rearing temperature typically results in reduced growth and development rates, delayed development time, but larger final body sizes—i.e. the temperature-size rule (Davidowitz *et al.* 2004). In some cases, changes in nutritional quality and secondary compounds can alter the slope of the thermal reaction norm for size (Stamp 1990; Stamp 1994; Yang & Stamp 1996; Kingsolver *et al.* 2006). If diet quality can alter thermal reaction norms, then this may impact food choice and resource use in different seasons or climatic

regions. How then do temperature and diet quality interact? Is the temperature-size rule robust to environmental variation in diet quality?

We explore these questions using the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), an important model system for the study of insect growth and development. When reared on an artificial diet, *M. sexta* follows the temperature-size rule across a range of thermal conditions (~20-35 °C; Davidowitz *et al.* 2004; Kingsolver & Nagle 2007). In nature, *M. sexta* feeds on a range of host plant species that vary in quality, but the consequences of this variation in diet quality for the shape of thermal reaction norms has not been explored. Here we focus on two food resources: a high quality host plant, tobacco (*Nicotiana tabacum*; family, Solanaceae), and a low quality host plant, devil's claw (*Proboscidea louisianica*; family, Martyniaceae). Previously, we have documented substantial fitness costs in *M. sexta*'s use of devil's claw: survival, growth rate, development rate, and fecundity are all reduced on this host plant species compared to tobacco (S.E. Diamond, S.D. Hawkins, H.F. Nijhout, J.G. Kingsolver, unpublished manuscript).

In the present study, we compare responses of two populations of *M. sexta*: a wild field population and a domesticated laboratory population. The domesticated population was derived from this same field population over 40 years ago, representing more than 250 generations in the laboratory. These populations have important differences in their thermal and dietary histories. The domesticated laboratory population has been maintained under a constant 25 °C thermal regime and reared on artificial diet since its introduction to the laboratory. In contrast, the wild field population experiences diurnally and seasonally fluctuating temperatures and feeds on a variety of natural host plants. As a consequence of laboratory domestication, the laboratory population exhibits greater thermal sensitivity,

particularly at warmer temperatures (35 °C), relative to the wild field population (Kingsolver & Nagle 2007; Kingsolver *et al.* 2009).

In this study, we experimentally examine whether the temperature-size rule is robust to environmental variation in diet quality. To accomplish this, we draw intraspecific comparisons, replicated across two populations, in which we explore the consequences of the interaction between temperature and diet quality for final body size. We address three major questions: 1) how do temperature and diet quality interact to affect survival, development time and fecundity, 2) does host plant quality affect the temperature-size rule, and 3) do population responses differ in the way host plant quality affects survival, development time, fecundity and the temperature-size rule? Here, fecundity provides a crucial link between body size and fitness: adaptive explanations for the temperature-size rule generally rely on a positive relationship between size and fecundity (Atkinson & Sibly 1997). Our results demonstrate that the combination of poor diet quality and low temperatures is stressful for *M. sexta*, contributing to prolonged development times and reductions in survival and fecundity. We show that the temperature-size rule can be reversed under these stressful conditions, reversing the slope of thermal reaction norms for final body size. This reversal may impact seasonal and geographic patterns of host use in *Manduca*, and perhaps in other study systems.

Materials and Methods

Study system

The tobacco hornworm, *Manduca sexta* L. (Sphingidae) is distributed across tropical and temperature regions of the Nearctic (Rothschild & Jordan 1903). Larval feeding is

generally restricted to plants in the Solanaceae, and *M. sexta* is an important agricultural pest on tobacco (*Nicotiana tabacum*: Solanaceae) in the southeast US.

Recently *M. sexta* has been documented to also use devil's claw (*Proboscidea* spp.: Martyniaceae) as a host plant in the Southwestern US (Mechaber & Hildebrand 2000; Mira & Bernays 2002). *Proboscidea* spp. are native to the Southwest, but have been introduced to other locations across the United States. One species of devil's claw, *P. louisianica* (Miller) Thellung, has been naturalized to the Southeastern United States (Small 1903). *Proboscidea louisianica* is patchily distributed across North Carolina, compared to the predominant local solanaceous host plant, tobacco (*Nicotiana tabacum* L.) which is widely cultivated across the state (Radford *et al.* 1968). Despite the limited distribution of devil's claw in North Carolina, our observations indicate field populations of *M. sexta* oviposit and feed successfully on this host plant when grown adjacent to tobacco plants (S.E. Diamond, S.D. Hawkins, H.F. Nijhout, J.G. Kingsolver, unpublished manuscript).

M. sexta have reduced survival, growth rate, development rate and fecundity when reared at intermediate temperatures (25 °C) on devil's claw compared to tobacco (S.E. Diamond, S.D. Hawkins, H.F. Nijhout, J.G. Kingsolver, unpublished manuscript). Here we consider devil's claw (*P. louisianica*) a low quality resource, and a low-alkaloid variety of cultivated tobacco (*N. tabacum*, var. LA Burley 21) a high quality resource. Tobacco seeds were obtained from the National Genetic Resources Program (USDA, ARS; Beltsville, Maryland).

In the current experiments, two genetic lines (populations) of *M. sexta* were used. The laboratory population came from a colony maintained under standard larval rearing conditions (artificial diet, constant 25-26 °C, 15h L : 9 h D photocycle) by L. Gilbert and

colleagues at UNC for over 25 years. This colony is ultimately derived from field collections of eggs in Clayton, NC (NCSU Research Station) during the 1960s. Field population eggs used in the current experiments were offspring of adults that were collected as early instar larvae from this same Clayton, NC field site, and reared through one generation on artificial diet in the laboratory to minimize parental effects.

Experiments

Eggs from each population (laboratory and field) were randomly assigned to tobacco and devil's claw leaves held under three different constant temperature regimes (20, 25, and 30 °C) in the laboratory. We employed a fully factorial design: there were 12 different treatment groups based on all combinations of population (field, laboratory), host plant species (devil's claw, tobacco) and temperature (20, 25, 30 °C). A previous study (at 25 °C) demonstrated greater larval survival on tobacco compared with devil's claw (S.E. Diamond, S.D. Hawkins, H.F. Nijhout, J.G. Kingsolver, unpublished manuscript). Therefore, upon hatching, 20 larvae from each population were transferred to individual tobacco leaves in each of the three temperature treatments, and a larger number of larvae (30-50) from each population were transferred to individual devil's claw leaves in each of the three temperature treatments.

Larvae were maintained individually in large Petri dishes (2.5 cm height x 14.5 cm diameter) housed in environmental chambers (Percival 36-VL) under a 16 L : 8 D photocycle. Larvae remained in Petri dishes until the fourth larval instar, when they were transferred to larger plastic enclosures (31 x 16 x 13 cm) with screened lids. Larvae were fed *ad libitum* on whole leaves cut from greenhouse-grown plants. Leaf water content was

maintained by placing the leaves in water picks. Leaves were changed every 2-3 days for early instar larvae, and every day for 5th instar larvae. Leaf material was harvested from greenhouse-grown plants of the same age (4 weeks old at the start of the experiment). These plants were fertilized weekly with Peter's Pro Solution (15-16-17). We grew an excess of plants to minimize the effect of inducing secondary plant metabolites in response to repeatedly cutting leaves from individual plants. No pesticides were applied to these plants.

Larvae were maintained in growth chambers until wandering when they were placed individually in wooden pupation chambers. After wandering, *M. sexta* larvae from all treatment groups were held under a constant 25 °C thermal regime until eclosion. Pupae were transferred to plastic cups lined with soil and remained there until eclosion. We measured survival, development time, body mass at the 2nd, 3rd, 4th, and 5th (6th in some cases) larval instars, wandering, pupation, and eclosion. Larval masses were recorded immediately following the molt into a given instar (indicated by slipping of the head capsule). Wandering masses were recorded following complete purging of the gut contents. Analyses of growth and development were restricted to individuals that survived to eclosion.

Adults were frozen at -80 °C at 24 hours post-eclosion. For adult females, estimates of potential fecundity were obtained by dissecting out the ovarioles into Ringer's solution. The number of follicles at stage 6 (S6) and all subsequent stages of development (cf. Yamauchi and Yoshitake 1984 for staging follicles) were counted with the aid of a dissecting microscope (Nikon SMZ-1B).

Statistical analyses

Survival to pupation was treated as a binomial variable and modeled using analysis of

deviance with population (laboratory or field), host plant species (tobacco or devil's claw), temperature (20, 25, 30 °C) and all two- and three-way interactions between these terms as fixed effects. Here (and in all subsequent analyses) temperature was treated as a continuous covariate; treating temperature as a factor did not substantially alter any of our results. Development time to pupation and body mass at pupation (for individuals surviving to eclosion) were modeled using ANCOVA with population, host plant species, temperature, sex and all two- and three-way interactions between these terms exclusive of those interactions with sex as fixed effects. We focused on measurements of pupae because of the stability in body mass at this stage. However, because all *M. sexta* were held at 25 °C after reaching the wandering stage regardless of larval rearing temperature, we also examine body mass and development time at the wandering and adult stages to confirm our results. We also evaluate the effects of temperature and plant quality on potential fecundity based on measurements of the number of follicles in ovarioles. Potential fecundity was modeled using ANCOVA with population, host plant species, temperature and all two- and three-way interactions between these terms as fixed effects. Because body size has important effects on fecundity in *M. sexta* and other insects (Davidowitz, *et al.* 2004, 2005), pupal mass was included as a continuous covariate. All statistical analyses were performed using R (version 2.8.1) statistical software.

Results

Survival

Relative to tobacco, mean survival was significantly reduced on devil's claw, particularly at cooler temperatures (Figure 4.1). Analysis of deviance detected significant effects of host plant species (tobacco or devil's claw) ($\chi^2 = 96$, $P < 0.0001$), temperature ($\chi^2 =$

32, $P < 0.0001$) and a marginally significant effect of population (laboratory or field) ($\chi^2 = 3.8$, $P = 0.052$) on the probability of survival to pupation. No significant two- or three-way interactions were found between host plant species, temperature and population (plant*population: $\chi^2 = 0.91$, $P = 0.34$; plant*temperature: $\chi^2 = 0.81$, $P = 0.39$; population*temperature: $\chi^2 = 0.18$, $P = 0.67$; plant*population*temperature: $\chi^2 = 0$, $P = 1$). In general, the laboratory population had slightly lower overall survival compared to the field population, as indicated by the significant main effect of population (Figure 4.1). Results were qualitatively similar for the probability of survival to adult eclosion.

To further explore the overall significant effect of temperature on survival, we performed post-hoc analyses: on tobacco, temperature ($\chi^2 = 0$, $P = 1$) was not significant (nor were population ($\chi^2 = 1.4$, $P = 0.24$) or the interaction between population and temperature ($\chi^2 = 0$, $P = 1$). In contrast, on devil's claw, temperature was significant ($\chi^2 = 33$, $P < 0.0001$), but population ($\chi^2 = 3.3$, $P = 0.067$) and the interaction of temperature and population ($\chi^2 = 0.28$, $P = 0.54$) were not significant. Therefore, the significant effect of temperature in the full model was driven entirely by the results for devil's claw in which survival decreased with decreasing temperature, rather than those for tobacco in which survival was relatively constant across temperature treatments.

Development and growth

Overall, there was greater variability in mean body mass and development time in the growth trajectories of *M. sexta* reared on devil's claw relative to tobacco (Figure 4.2; Appendix Figure 1). Larval growth trajectories on tobacco were approximately exponential (linear on a natural-log scale), with larvae generally growing at the same proportional rate

throughout larval growth. These patterns held for individuals within each temperature treatment, although mean trajectories for *M. sexta* reared at cooler temperatures were less steep than those reared at warmer temperatures (Figure 4.2, top; Appendix Figure 1, top). In contrast, growth trajectories for *M. sexta* reared on devil's claw were comparatively shallow, with growth slowing considerably during the final larval instar (Figure 4.2, bottom; Appendix Figure 1, bottom). These effects were especially pronounced at cooler temperatures.

Mean development time to pupation was significantly shorter on the high quality host plant, tobacco, and at warmer rearing temperatures. On each host plant species, mean development times to pupation of the two populations converge at warmer rearing temperatures (Figures 3.2, 3.3; Appendix Figures 1, 2). ANCOVA of development times to pupation detected significant effects of host plant species ($F_{1, 231} = 61$, $P < 0.0001$), temperature ($F_{1, 231} = 350$, $P < 0.0001$), the interaction of plant and temperature ($F_{1, 231} = 40$, $P < 0.0001$), the interaction of plant and population ($F_{1, 231} = 5.6$, $P = 0.018$) and the three-way interaction of plant, population and temperature ($F_{1, 231} = 5.3$, $P = 0.022$) on time to pupation, but no significant effects of population ($F_{1, 231} = 0.095$, $P = 0.76$), sex ($F_{1, 231} = 0.74$, $P = 0.39$), or the interaction of population and temperature ($F_{1, 231} = 0.23$, $P = 0.63$) were detected. In general, results for development time to wandering and eclosion were qualitatively similar (Appendix Table 1). The presence of significant interaction terms with population indicated population responses differed across host plant species and temperature. On devil's claw the slope for development time as a function of temperature is steeper (more negative) for the laboratory population compared to the field population. A similar pattern is found on tobacco, but the magnitude of the difference between the populations is greater: the

laboratory population develops more slowly than the field population at 20 °C (Figure 4.3, top; Appendix Figure 2, top).

Additional developmental responses differed across populations. Field and laboratory population individuals expressed 5 larval instars (the typical number of instars for *M. sexta*) on tobacco at all three rearing temperatures, and on devil's claw at the warmer temperatures (25 and 30 °C). In contrast, the induction of an extra (6^{th}) instar was seen in 30% of field population individuals surviving to eclosion that were reared on devil's claw under the coolest temperature regime (20 °C).

Mean pupal mass was significantly greater on the high quality host plant, tobacco, compared to devil's claw. As expected, greater mean pupal mass was achieved at cooler temperatures on tobacco. In contrast, greater mean pupal mass was achieved at warmer temperatures on the low quality host plant, devil's claw (Figures 3.2, 3.3; Appendix Figures 1, 2). ANCOVA on pupal mass detected significant effects of host plant species ($F_{1, 231} = 57$, $P < 0.0001$), temperature ($F_{1, 231} = 20$, $P < 0.0001$), sex ($F_{1, 231} = 52$, $P < 0.0001$), and the interactions of plant and population ($F_{1, 231} = 5$, $P = 0.027$), population and temperature ($F_{1, 231} = 4.7$, $P = 0.032$) and plant and temperature ($F_{1, 231} = 40$, $P < 0.0001$) on body mass at pupation, but no significant effects of population ($F_{1, 231} = 0.98$, $P = 0.32$) or the three-way interaction between plant, population and temperature ($F_{1, 231} = 1.5$, $P = 0.22$) were detected. In general, results for body mass at wandering and eclosion were qualitatively similar (Appendix Table 2). Here again, significant interactions with population indicated population responses in body size differed across host plant species and temperature. On tobacco, slopes for pupal mass as a function of temperature are similar across populations. In contrast, on devil's claw, the thermal reaction norm for body size is steeper (more positive) in the

laboratory population relative to the field population (Figure 4.3, bottom; Appendix Figure 2, bottom).

In the models for body size, the interaction term between plant and temperature was significant across all three later developmental stages (wandering, pupation and eclosion). This indicates that host plant quality alters the slope of thermal reaction norms for final body size (Figure 4.3, bottom; Appendix Figure 2, bottom). Post-hoc analyses for each host plant revealed that the slope of pupal mass as a function of temperature was significantly negative on tobacco (slope ± 1SE: -85 ± 27; t = -3.2, df = 117; $P = 0.0021$), but significantly positive on devil's claw (slope ± 1SE: 151 ± 24; t = 6.4, df = 119; $P < 0.0001$) when data were pooled across population and sex. Results were qualitatively similar for each combination of plant, population and sex. In summary, our analyses show that the temperature-size rule (negative thermal reaction norm for final body size) for *M. sexta* was followed on the high quality host plant (tobacco), but that the rule was reversed on the low quality host plant (devil's claw), with larger body sizes at warmer rearing temperatures.

Fecundity

In general, potential fecundity increased with larger body sizes. Females reared on tobacco were larger and more fecund at cooler temperatures; females reared on devil's claw were larger and more fecund at warmer temperatures (Figure 4.4). ANCOVA revealed significant effects of host plant species ($F_{1, 91} = 10$, $P = 0.0018$), population ($F_{1, 91} = 4.5$, $P = 0.036$), pupal mass ($F_{1, 91} = 9.9$, $P = 0.0022$), the two-way interactions between population and plant ($F_{1, 91} = 11$, $P = 0.0014$) and plant and temperature ($F_{1, 91} = 7.7$, $P = 0.0068$) and the three-way interaction between plant, population and temperature ($F_{1, 91} = 12$, $P = 0.0008$) on

the number of follicles in the ovarioles. No significant effects of temperature ($F_{1, 91}$ = 1.9, P = 0.17), or the two-way interaction between population and temperature ($F_{1, 91}$ = 3, P = 0.086) were detected. In general, results were qualitatively similar when adult mass was used as a covariate in place of pupal mass (Appendix Table 3). The significant three-way interaction between temperature, population and plant highlights an important exception to this pattern of fecundity generally increasing with larger body size. Laboratory population females reared at 20 °C on tobacco had severely reduced potential fecundity despite their large body size under these conditions (Figure 4.4). The abdomens of these females contained an abnormally high ratio of fat bodies to ovarioles.

Discussion

The primary goal of this study was to explore the generality of the environmental conditions under which the temperature-size rule is produced. Specifically, we examined the consequences of variation in food quality for the slope of thermal reaction norms, using two populations of the tobacco hornworm, *Manduca sexta*. Our results indicate that diet quality can qualitatively affect the slope of thermal reaction norms, leading to resource-dependent reversals of the temperature-size rule.

As expected, *M. sexta* reared on the lower quality host plant (devil's claw) experienced significant reductions in survival, growth and development rates, longer development times, and reduced final body size and fecundity (Figures 3.2, 3.3; Appendix Figures 1, 2). Presumably, reduced fitness on devil's claw results from the nutritional and allelochemical profile of devil's claw being evolutionarily novel for *M. sexta* (S.E. Diamond, S.D. Hawkins, H.F. Nijhout, J.G. Kingsolver, unpublished manuscript). Similarly, decreasing

temperature resulted in decreased growth and development rates and longer development times on both host plant species (Figures 3.2, 3.3; Appendix Figures 1, 2). However, the effects of rearing temperature on survival, final size and fecundity differed dramatically for the two host plants. Over the temperature range considered here (20-30 °C), survival on tobacco was uniformly high at all rearing temperatures (Fig 3.1). In contrast, survival on devil's claw decreased with decreasing temperature, suggesting that lower temperatures were stressful on the low quality but not the high quality host plant.

The different responses in final body size are of particular interest. On tobacco, *M. sexta* follow the temperature-size rule, with smaller final size (mass at wandering, pupation or eclosion) at increasing temperatures. This was primarily due to increases in body size during the last two larval instars (Figure 4.2 top; Appendix Figure 1, top). Previous results for *M. sexta* reared on artificial diet show a similar pattern (Davidowitz *et al.* 2004; Kingsolver and Nagle 2007). In contrast, *M. sexta* reared on devil's claw had positive thermal reaction norms for final size, with *larger* final size (mass at wandering, pupation or eclosion) at warmer temperatures, reversing the temperature-size rule (Figure 4.3, bottom; Appendix Figure 2, bottom). Our results demonstrate that reduced host plant quality can flip the sign of the thermal reaction norm for size, reversing the temperature-size rule. Importantly, both field and laboratory populations had positive thermal reaction norm slopes when reared on devil's claw. Despite substantial evolved differences in body size between the field and laboratory populations—inadvertent selection for rapid growth has contributed to a 50% increase in body size in the domesticated population (D'Amico *et al.* 2001)—the laboratory population exhibited qualitatively similar thermal reaction norms compared to the field population.

One interpretation of our results involves the impact of cold stress on growth and size. Experimental studies that consider a wide range of constant rearing temperatures, primarily with *Drosophila*, reveal that final size is maximized at an intermediate rearing temperature. Below this temperature, both survival and final size decrease as a result of cold stress; above this temperature final size decreases with increasing temperature (Moreteau *et al.* 1997; Karan *et al.* 1998). In this sense, the temperature-size rule can only apply to non-stressful thermal conditions (van der Have and de Jong 1996; Kingsolver and Huey 2008). The lowest temperature treatment in our study (20 °C) is likely not stressful for *M. sexta* on high-quality food resources such as tobacco or artificial diet, as survival to eclosion in such conditions is quite high (> 80%) (Figure 4.1). In contrast to tobacco, survival to eclosion was quite low in the coldest temperature treatment on devil's claw (< 35%). This suggests the reversal of the temperature-size rule on devil's claw is not a result of general cold stress. Rather, the specific interaction between cooler temperatures and low food quality may represent stressful conditions that lead to the reversal of the temperature-size rule.

Developmental and potential fecundity responses further support this interpretation. Extra (6^{th}) larval instars were induced only at the cooler temperature treatment on devil's claw. The induction of supernumerary instars is often a response to poor environmental quality (Nijhout 1994; Esperk *et al.* 2007). Interestingly, extra instars were seen only in the field population on devil's claw at 20 °C; however, the laboratory population apparently has a reduced developmental capacity for expressing additional instars in response to poor diet quality (S.E. Diamond, S.D. Hawkins, H.F. Nijhout, J.G. Kingsolver, unpublished manuscript). Similarly, fecundity was reduced for females reared at the coolest temperatures on devil's claw (Figure 4.4; and see below).

A key assumption in models for the evolution of age and size at reproduction, including models for the evolution of the temperature-size rule, is that body size is directly related to fitness (Stearns and Koella 1986; Atkinson and Sibly 1997); fecundity data provide an important link between body size and fitness. In general, our results for *M. sexta* support this assumption (see also Davidowitz *et al.* 2004, 2005). Potential fecundity was greatest under the specific dietary and thermal conditions that contributed to the largest body size: females were most fecund when reared at cooler temperatures on tobacco and warmer temperatures on devil's claw (Figure 4.4). An important exception to this pattern was found in laboratory population females reared at 20 °C on tobacco (Figure 4.4). Despite having the largest body size in all treatment groups, the bulk of the body cavity contained only fat bodies (see also Raguso *et al.* 2007), resulting in severely reduced potential fecundity at cooler temperatures. This reduced fecundity may represent a consequence of laboratory domestication at higher temperatures (25-26 °C), such that 20 °C may represent a stressful rearing temperature for this population. On the other hand, survival at 20 °C is quite high for this population on tobacco. It is unlikely the reduced fecundity we observed reflects a delay in reproduction at lower temperatures, as all *M. sexta* were maintained at 25 °C after wandering through metamorphosis when females develop ovarioles and follicles.

Previous work examining the interaction of diet quality and temperature in *M. sexta* has shown that diet quality can alter thermal reaction norms for body size. The effects of temperature on body size have been shown to vary with diet quality when quality was manipulated by the addition of plant allelochemicals to an artificial diet (Stamp 1990; Stamp 1994; Yang and Stamp 1996). Similarly, reductions in the protein content of artificial diet have been shown to alter the slope of thermal reaction norms for final body size in one

population of *M. sexta*: relative to full protein diet, the slope of the thermal reaction norm for body size at wandering became less steep (less negative) on a diet with a 39% reduction in protein content (Petersen *et al.* 2000). In comparison, it appears devil's claw is sufficiently stressful, due to its poor quality for development, to qualitatively alter the slopes of thermal reaction norms and to reverse the temperature-size rule. Severe reductions in the nutrient content of artificial diets may also lead to the reversal of the temperature-size rule in laboratory colonies of *M. sexta* (H. F. Nijhout, *pers. comm.*).

In *M. sexta,* the reversal of the temperature-size rule may be an important component of this species' adoption of devil's claw as a host plant. We have documented substantial fitness costs associated with *M. sexta*'s feeding on devil's claw (this study; S.E. Diamond, S.D. Hawkins, H.F. Nijhout, J.G. Kingsolver, unpublished manuscript). The primary reason for the adoption of devil's claw in spite of these costs appears to be escape from natural enemies (Mira & Bernays 2002; Diamond & Kingsolver, in press). However, if these fitness costs due to poor food quality can be ameliorated by warmer temperatures, as suggested by the present study, the warmer thermal conditions in the southwestern and southeastern U.S. where devil's claw is found may be an important component of *M. sexta*'s adoption of this host plant.

The frequency with which herbivores encounter low quality resources in nature may be relatively high: the lack of a relationship between oviposition preference and offspring performance is a widespread pattern in plant-insect interactions (reviewed in Mayhew 1997). The reversal of the temperature-size rule under poor food quality conditions may therefore be a common occurrence. Similarly, novel host plants are often of low quality for herbivores, presenting a barrier to host shifting (Price *et al.* 1980). This could suggest host shifts are

more likely to occur in warmer climates, where the reversal of the temperature-size rule would tend to benefit herbivores. Certainly, many additional factors can influence the probability of host shifting: for example, rates of natural enemy attack have been shown to covary with latitude (Hawkins *et al.* 1997). However, because host shifts and subsequent host race formation are important intermediate steps in the process of speciation (Abrahamson & Weis 1997), it is useful to consider how the reversal of the temperature-size rule in low quality dietary environments may mitigate the costs of switching to novel hosts in warmer climates, but may exacerbate these costs in cooler climates. Similarly, as thermal environments are being altered by global climate change (Deutsch *et al.* 2008), the interaction of temperature and food quality could have an appreciable impact on both current and predicted organismal responses to these altered thermal conditions. In general, the extent to which low quality dietary environments occur in nature, and the magnitude of the dietary reductions required to reverse the temperature-size rule are important, but unresolved issues.

Empirical and theoretical work examining the role of dietary resources in context of the temperature-size rule has focused on the effect of temperature on the acquisition, assimilation and allocation of a given resource (Atkinson 1996; Arendt 1997; Atkinson & Sibly 1997; Robinson & Partridge 2001; Karl & Fischer 2008). In addition, examination of the physiological mechanisms underlying the control of body size and the effects of variation in temperature and diet quality has provided further insight into the basis of the temperature-size rule (Davidowitz *et al.* 2004; Davidowitz & Nijhout 2004). This work has been important in understanding how the temperature-size rule is produced, but does not inform us about the range of environmental conditions over which the rule is followed. Here we have demonstrated that diet quality can qualitatively alter the slopes of thermal reaction norms for

final body size and reverse the temperature-size rule. As a result, the conditions under which the temperature-size rule is produced may be less robust than previously thought. Specifically, the temperature-size rule may only apply under conditions of high quality dietary resources, and when other environmental factors are not stressful. Environmental components such as diet quality may therefore be essential to understanding the robustness and limitations to the temperature-size rule.

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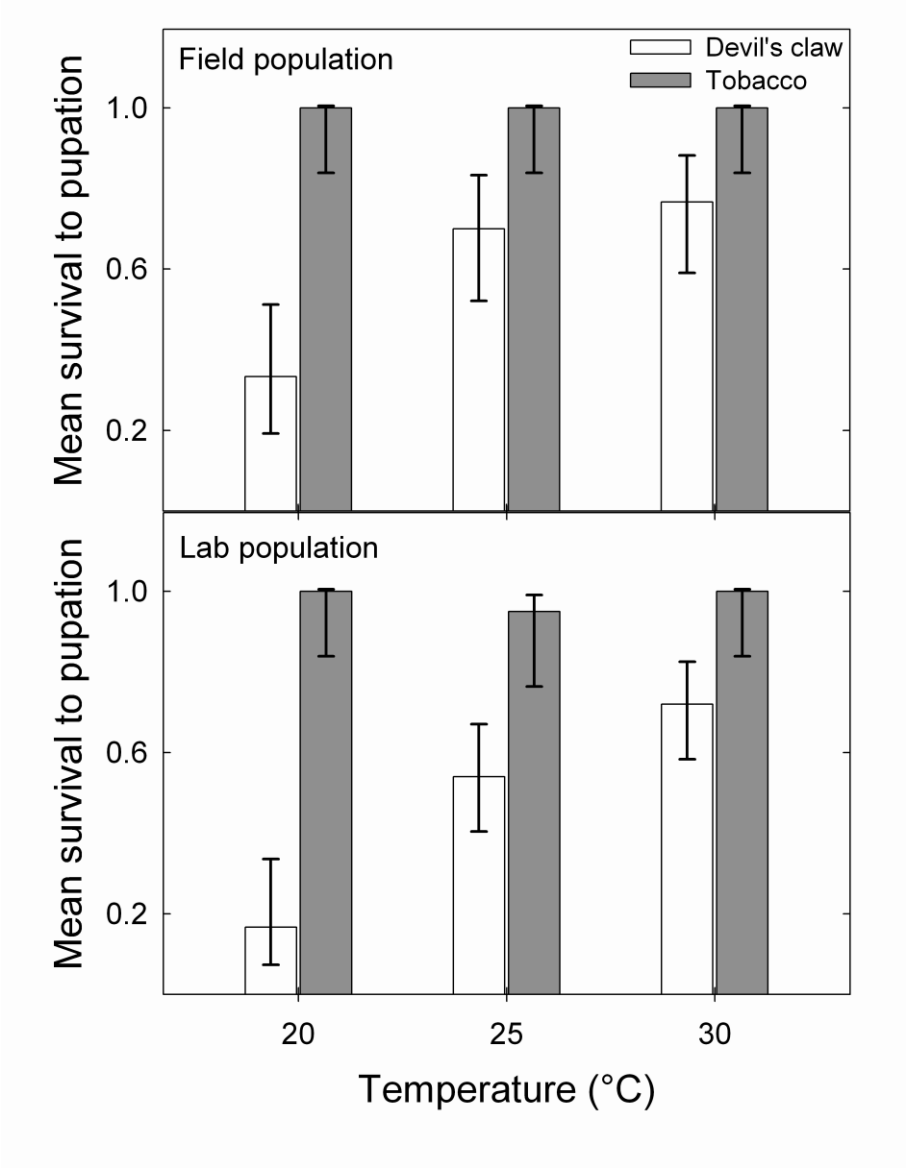

Figure 4.1. Mean survival to pupation ± 95% CI for field population (upper panel) and laboratory population (lower panel) reared on devil's claw (white bars) and tobacco (grey bars) at 20, 25, and 30 °C.

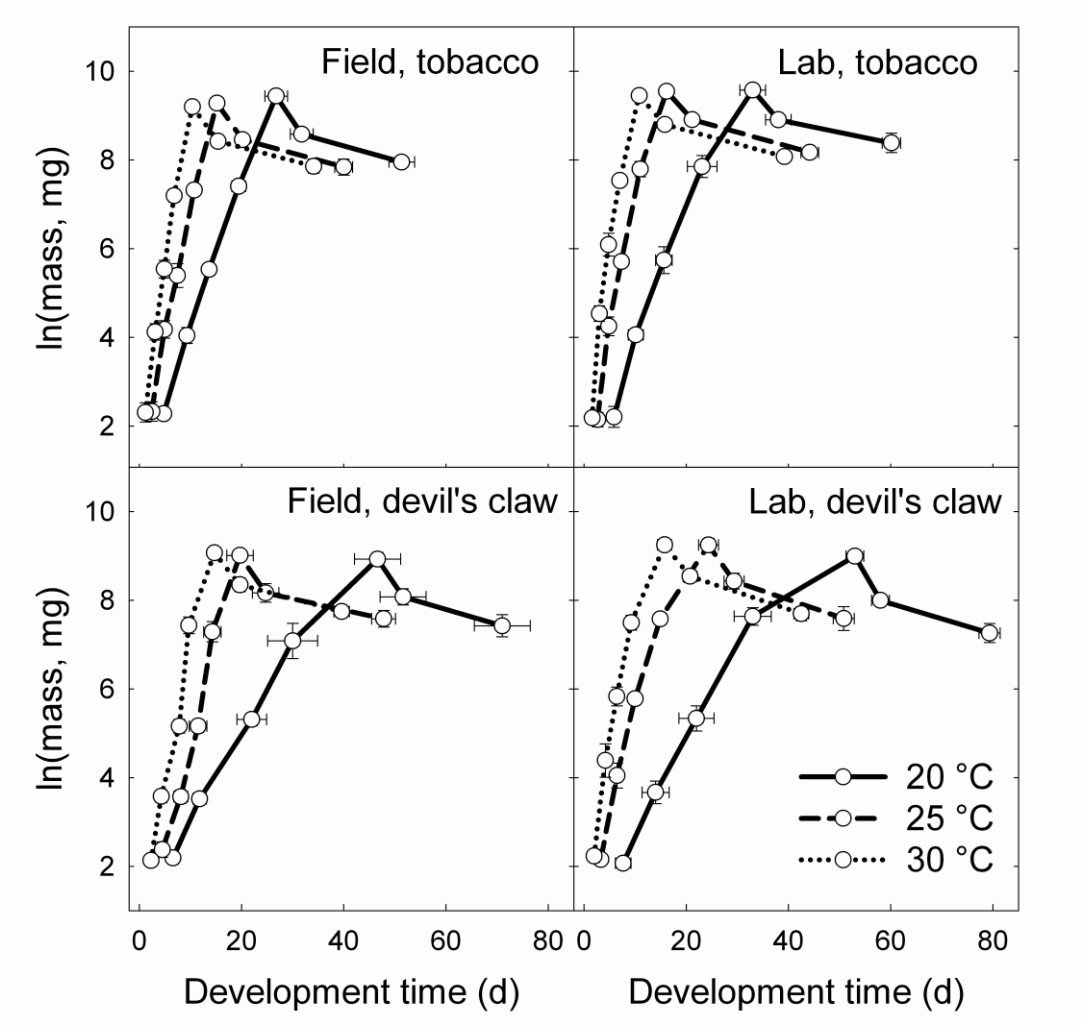

Figure 4.2. ln(mean body mass, mg) ± 1 S.D. as a function of mean development time (d) ± 1 S.D. at the 2nd, 3rd, 4th and 5th larval instars, wandering, pupation and eclosion for field population (left two panels) and laboratory population (right two panels) female *M. sexta* reared on tobacco (upper two panels) and devil's claw (lower two panels) at 20 (solid lines), 25 (dashed lines), and 30 (dotted lines) °C. Patterns for males were qualitatively similar (Appendix Figure 1). Individuals with extra (6th) instars were incorporated into the trajectories for individuals with 5 instars by omitting the 6th instar data points.

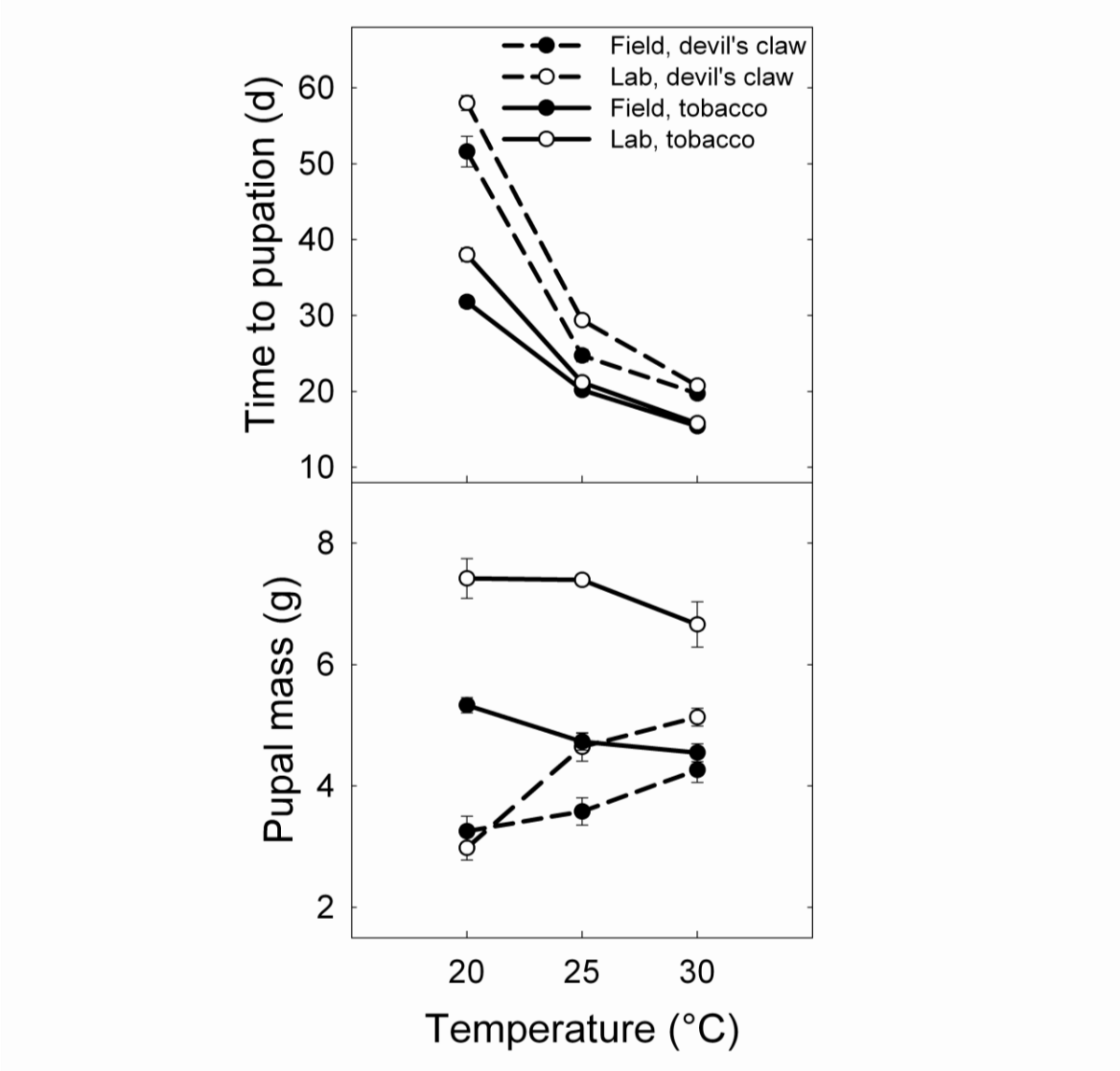

Figure 4.3. Development time to pupation (d) ± 1 S.E. as a function of temperature (°C) (upper panel), and pupal mass (g) ± 1 S.E. as a function of temperature (°C) (lower panel) for female *M. sexta* from the field population (closed symbols) and laboratory population (open symbols) reared on tobacco (solid lines) and devil's claw (dashed lines). Patterns for males were qualitatively similar (Appendix Figure 2).

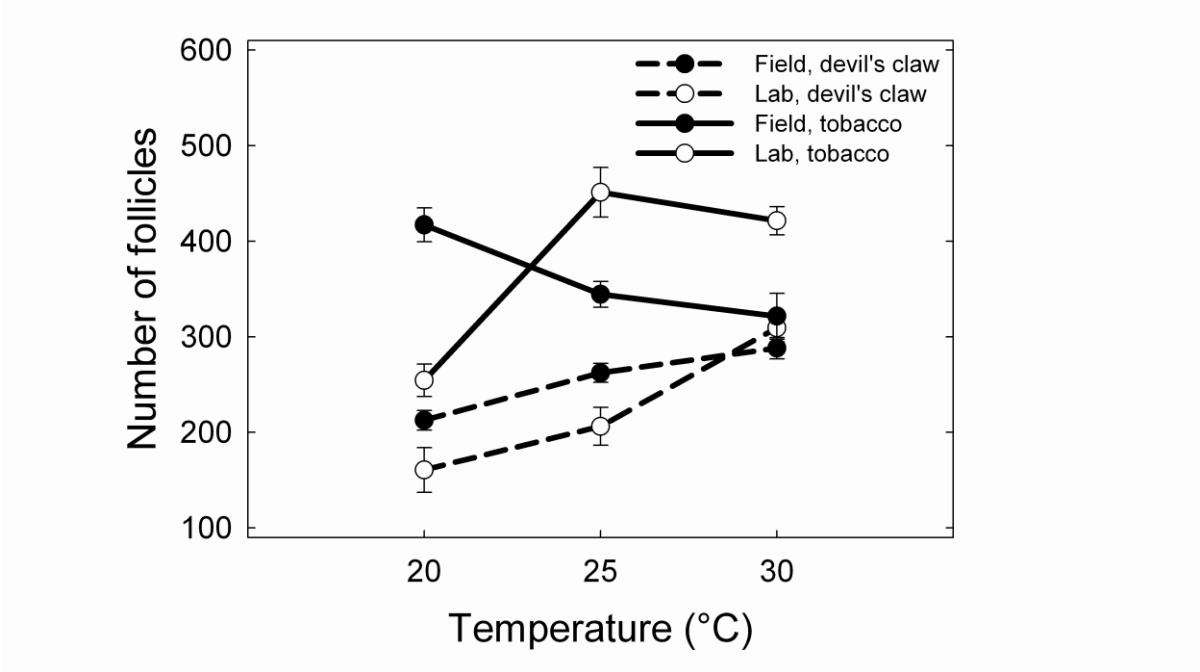

Figure 4.4. Mean number of follicles in the ovarioles ± 1 S.E. as a function of temperature (°C) for females from the field population (closed symbols) and laboratory population (open symbols) reared on tobacco (solid lines) and devil's claw (dashed lines).

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CHAPTER V

HOST PLANT QUALITY, SELECTION HISTORY AND TRADEOFFS SHAPE THE IMMUNE RESPONSES OF *MANDUCA SEXTA*

Summary

Immune defenses are an important component of fitness. Yet, susceptibility to pathogens is common, suggesting the presence of ecological and evolutionary limitations on immune defenses. Here, we use structural equation modeling to quantify the direct effects of resource quality and selection history, and their indirect effects mediated via body condition prior to an immune challenge on encapsulation and melanization immune defenses in the tobacco hornworm, *Manduca sexta*. We also investigate allocation trade-offs among immune defenses and growth rate following an immune challenge. We found considerable variation in the magnitude and direction of the direct effects of resource quality and selection history on immune defenses and their indirect effects mediated via body condition and allocation trade-offs. Greater resource quality and evolutionary exposure to pathogens had positive direct effects on encapsulation and melanization. The indirect effect of resource quality on encapsulation mediated via body condition was substantial, whereas indirect effects on melanization were negligible. Individuals in better condition prior to the immune challenge had greater encapsulation; however, following the immune challenge, greater encapsulation traded off with slower growth rate. Our study demonstrates the importance of experimentally and analytically disentangling the relative contributions of direct and indirect effects to understand variation in immune defenses.

Introduction

Pathogens (broadly defined *sensu* Stock *et al.* 2009) negatively impact host survival and reproduction, such that host immune defenses are an important determinant of overall host fitness (Schmid-Hempel 2003; Siva-Jothy *et al.* 2005). Although all plants and animals possess some form of immune defense (Hoffmann *et al.* 1999), susceptibility to pathogens is common (Lazzaro & Little 2009). Given the importance of immune defenses for host fitness, the prevalence of susceptible phenotypes seems paradoxical. Why is there variation in immune defenses?

Immune defense is an evolved trait. Although it is clear that selection imposed by pathogens, including bacteria, fungi, viruses and parasitoid natural enemies, has played an important role in shaping immune defenses over long evolutionary time scales (Schlenke & Begun 2003; Lazzaro 2008), the short-term dynamics of contemporary selection on host immune defenses are less clear. Because there is considerable spatial and temporal variation among populations in their exposure to pathogens (*e.g.*, Ingvarsson & Ericson 1998; Bensch & Åkesson 2003; Munster *et al.* 2007), the question becomes, to what extent are immune defenses limited by variability in exposure to agents of natural selection? If contemporary selection is important for maintaining immune defenses, what are the consequences of relaxing selection on immune defenses? Laboratory domestication of model organisms (Davis 2004) provides an ideal opportunity to address this question: selection on immune defenses is often relaxed for domesticated laboratory populations since they are relatively protected from pathogens compared to their wild population counterparts. Reduced immune defenses of domesticated versus wild populations would suggest contemporary selection is important for maintaining immune defenses.

In addition, ecological factors, operating within a generation, may limit immune defenses. Resource quality and availability largely determine maximum potential allocation to immune defenses, and immune defenses are generally improved with greater resource quality and availability (both in the laboratory: Feder *et al.* 1997; Siva-Jothy & Thompson 2002; Ojala *et al.* 2005; and in the field: Klemola *et al.* 2007). However, competing demands of growth, maintenance and reproduction prevent sole allocation of resources to immune defenses (Zuk & Stoehr 2002). As a result, resource allocation can impose ecological limits either on immune defenses or aspects of growth, maintenance and reproduction, depending on the current needs of the host (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Norris & Evans 2000). An important distinction is that while resources may directly affect immune defenses, resources may also act indirectly on immune defenses. In the latter case, the effects of resources on immune defenses are mediated via non-immune defense aspects of the host's biology. For example, resource quality can impact both immune defenses (see above) and body condition (Awmack & Leather 2002), but body condition can also impact immune defenses (König & Schmid-Hempel 1995; Benrey & Denno 1997; Siva-Jothy *et al.* 1998; Rantala *et al.* 2000; McKean & Nunney 2001; Rolff & Siva-Jothy 2002; Fedorka *et al.* 2004). Yet, very few studies are able to distinguish the direct effects of resources on immune defenses from their indirect effects mediated via other aspects of the host's biology. A recent exception is a study by Smilanich *et al.* (2009) on immune defenses in a nymphalid caterpillar, where structural equation modeling was used to reveal significant direct effects of plant allelochemicals on immune defenses and indirect effects mediated through host metabolism. The extent to which indirect effects of resources mediate overall immune defenses is largely unknown, but may provide important insight into ecological limitations on

immune defenses.

In this study, we examine the relative importance of the direct effects of resource quality and selection history (population-level differences in evolutionary exposure to pathogens), and their indirect effects mediated by body condition prior to an immune challenge and allocation trade-offs following an immune challenge on immune defenses in the tobacco hornworm, *Manduca sexta*. To accomplish this, we take advantage both of a recent host plant shift in *M. sexta* from a typical high quality host plant (tobacco; *Nicotiana tabacum*) onto a novel low quality host plant (devil's claw; *Proboscidea louisianica*), and the laboratory domestication of *M. sexta* over the past 35 years (> 260 generations) where we can directly compare two laboratory populations with the wild field population from which they were originally derived. We examine two major components of innate (non-specific) immune defenses—encapsulation, involving the layering of hemocytes (immune cells) around invaders to form a protective capsule, and melanization, involving the deposition of melanin, a cytotoxic molecule (Lavine & Strand 2002; Kanost *et al.* 2004)—in response to an abiotic immune challenge. Encapsulation and melanization represent functional consequences of immune activation, and are often strong predictors of survival and performance following an immune challenge: for example, in *M. sexta*, experimental reductions in melanization and encapsulation result in significantly lower survival against biotic immune challenges (Miller *et al.* 1994; Eleftherianos *et al.* 2007). Our experimental and analytical approach (see Figure 5.1 for a complete conceptual map), allows us to examine: 1) the direct effects of resource quality (tobacco versus devil's claw) and selection history (wild versus domesticated populations) on two immune defenses, melanization and encapsulation, 2) the indirect effects of resource quality and selection history on immune defenses as mediated by body condition,

estimated using growth rate prior to an immune challenge, and 3) allocation trade-offs between immune defenses and growth rate following an immune challenge.

Materials and Methods

Study organisms

The tobacco hornworm, *Manduca sexta* L. (Sphingidae) is distributed across tropical and temperate regions of the Nearctic (Rothschild & Jordan 1903). Feeding is generally restricted to plants in the Solanaceae, but *M. sexta* has adopted non-solanaceous host plants (*Proboscidea* spp.) belonging to the family Martyniaceae, in the Southwestern USA (Mechaber & Hildebrand 2000; Mira & Bernays 2002) where these plants are native, and the Southeastern USA (Diamond & Kingsolver 2010) where these plants have been recently introduced (Small 1903). Here, we use tobacco (*Nicotiana tabacum*) as a representative of a typical, high quality solanaceous host plant, and devil's claw (*Proboscidea louisianica*) as a novel, low-quality host plant. In our study area (North Carolina), devil's claw is a relatively recent introduction (Small 1903), and we have previously documented significant performance costs associated with feeding on this host plant (Diamond *et al.* 2010).

We used three different genetic lines (populations) of *M. sexta*, a wild field population and two domesticated laboratory populations, to assess the consequences of selection history for immune defenses. The field population was established with early instar larvae collected from tobacco plants at the North Carolina State University (NCSU) Research Station in Clayton, NC. To minimize parental effects, larvae were reared through one generation on artificial diet in the laboratory before use in the experiments. The Duke University (hereafter, Duke) laboratory population came from a colony maintained under standard larval rearing

conditions (artificial diet, constant 25 °C, 15h L : 9 h D photocycle) at Duke University; this population was established by hybridizing long-term mass reared colonies from the University of Washington, University of Arizona and North Carolina State University in 2002 (Nijhout *et al.* 2006). The University of North Carolina (hereafter, UNC) laboratory population came from a colony maintained under the same standard larval rearing conditions by L. Gilbert and colleagues at UNC for over 25 years. Animals in these laboratory colonies are not exposed to natural enemies or their naturally occurring host plants at any stage of their life cycle, and are reared on artificial diet containing antimicrobial and antifungal agents to reduce exposure to pathogens.

To our knowledge, all major laboratory colonies of *M. sexta* are ultimately derived from mass-rearing facilities in Raleigh, NC (Kingsolver 2007). The source population for these laboratory strains, including the Duke and UNC colonies, came from field collections of eggs from the NCSU Research Station in Clayton, NC (see above) during the 1960s.

Experiments and Measurements

Tobacco and devil's claw (tobacco, Coker var. 319; devil's claw, International Carnivorous Plant Soc., Inc., Pinole, CA) were grown from seed in the greenhouse and fertilized every two weeks with Peter's Pro Solution. The plants were 5 weeks old at the start of the experiment. No pesticides were ever applied to these plants. Eggs of each population (field and both laboratory colonies) were randomly assigned to host plant species (either devil's claw or tobacco). Initial sample sizes were sufficient to produce at least 15 viable larvae at the time of the immune challenge (see below); approximately 20-25 hatchling larvae from each population were assigned to the tobacco treatment, and approximately 30-

35 hatchling larvae from each population were assigned to the devil's claw treatment. Larvae were housed in growth chambers (Percival model 36-VL) under standard conditions (16 L : 8 D photocycle at a constant 25 °C). Larvae were reared singly in plastic enclosures (31 x 16 x 13 cm) with screened lids, and were fed on cut leaves kept in water picks until the beginning of the 4th larval instar.

We assessed two major components of innate immune defenses (non-specific defenses common to all plants and animals; Hoffmann *et al.* 1999), melanization and encapsulation, through the injection of Sephadex beads (see below) into the host hemocoel. The beads activate the deposition of both cytotoxic melanin and encapsulating layers of hemocytes (cf. Lavine & Strand 2002), similar to the responses against parasitoid eggs or larvae, and bacterial and fungal pathogens (reviewed in Vilmos & Kurucz 1998).

We chose the 4th (penultimate) larval instar for assessing growth rates and immune defenses because aspects of immune function such as hemocyte titer decreases immediately prior to and throughout metamorphosis in *M. sexta* (Beetz *et al.* 2008). All larvae in the experiment were therefore of the same developmental stage, but not necessarily the same age or size. To assay growth rates, we recorded development time to 4th instar and body mass both prior to and following the injection of the immune challenge for each individual larva. Pre-challenge larval growth rate was used to examine the potential condition-dependence of immune defenses, and defined as: ln(body mass at the beginning of the 4th instar) / development time to the 4th instar. Post-challenge growth rate was used to examine potential allocation trade-offs between immune defenses and growth rate, and defined as: ln(body mass after the immune challenge / body mass at the beginning of the 4th instar (when the immune challenge occurred)). The immune challenge interval was constant for all larvae (24

hours), so there was no age-dependence for post-challenge growth rates.

After molting into 4th instar (defined by slippage of the head capsule), larvae were weighed and then injected under the base of the left fourth proleg (via Hamilton 7000 series microliter syringe) with at least 15 (but not > 20) Sephadex beads (DEAE-Sephadex A-25, Sigma; beads were stained with a 0.1% Congo red solution and dried under UV light) suspended in 5 µl sterile Grace's insect medium (Sigma-Aldrich) (*sensu* Lavine & Beckage 1996). Beads were injected directly into the hemocoel. Because hemolymph loss was minimal following injections, the injection wounds were left unsealed. The injected larvae were returned to their respective host plants and allowed to feed for 24 hours. After 24h, body mass was measured (see above), and larvae were frozen at -80 °C. The Sephadex beads were extracted post-mortem, and mounted in glycerol (Sigma-Aldrich) on glass slides. During extraction, 10 beads from each individual were randomly selected for analysis, which mitigated potential biases arising from non-uniform encapsulation coupled with bead orientation on the slide. Final sample sizes of individuals from each treatment group of host plant-by-population (comprising 10 Sephadex beads from each individual) were 15, except for the domesticated UNC population which had 30 individuals each for tobacco and devil's claw.

Melanization was assayed as a binomial response variable for each bead: the presence or absence of melanin deposited either directly on the Sephadex bead or on hemocytes involved in the encapsulation of the Sephadex bead. Visualization of melanization was performed using brightfield microscopy.

Encapsulation (degree of hemocyte aggregation) was assayed as a continuous response variable, by subtracting the area of the Sephadex bead from the area enclosed by the

outermost edge of the encapsulating hemocytes. This yielded a measurement of the total encapsulation area. The encapsulated Sephadex beads were visualized using a combination of Nomarski differential interference contrast (DIC) and fluorescence microscopy (Zeiss LSM 510 confocal microscope). A fluorescent image of the Sephadex bead (the Congo red dye used to stain the Sephadex beads fluoresces) was overlaid on a DIC image of encapsulation. The fluorescent image of the bead allowed clear delineation of the bead edges, and the DIC image allowed clear visualization of cellular encapsulation (these surrounding hemocytes are largely transparent, requiring the use of DIC). The encapsulation area and bead area were measured using the Zeiss LSM Image Browser software. Encapsulation measurements were highly repeatable (based on 10 randomly sampled beads from each of the 6 treatment groups; $r = 0.98$, $P < 0.0001$), so measurement error was not incorporated into our statistical analyses.

Statistical analyses

We used two types of statistical analysis: linear models to test the effects of host plant quality (tobacco versus devil's claw) and selection history (wild versus domesticated: population differences in exposure to immune challenges) on pre- and post-challenge growth rates and melanization and encapsulation immune defenses; and structural equation models to quantify the direct and indirect associations among these variables. All statistical analyses were performed using R (version 2.10.1; R Development Core Team 2009). To explore the consequences of variation in host plant quality and selection history for melanization, we performed a mixed-model analysis of deviance with melanization (presence/absence) as the response, and host plant quality, selection history and their interaction as fixed factors. In most cases, population comparisons were based on linear contrasts between the wild field

population and both domesticated laboratory populations (Duke and UNC); we note deviations from these particular contrasts in the Results section when they occur. Bead area was included as a covariate to account for variation in bead size (mean bead size in μm ± 1 SD: 118 ± 24); importantly, bead area did not differ significantly across host plant quality-by-selection history treatment groups ($F_{5,1194} = 1.58$, $P = 0.164$). Similarly, to explore the consequences of host plant quality and selection history for encapsulation, we performed a mixed-model ANCOVA with encapsulation area as the response, and host plant quality, selection history and their interaction as fixed factors. Bead area was included as a covariate. The effects of host plant quality and selection history on pre- and post-challenge growth rates were also examined using ANOVA, with pre- or post-challenge growth rate as the response and host plant quality, selection environment and their interaction as fixed effects.

Host plant quality and selection history may affect immune defenses both directly, and indirectly where their effects on immune defenses are mediated via pre-challenge growth rate. Similarly, post-challenge growth rate may be affected directly by host plant quality, selection history and pre-challenge growth rate. Trade-offs in resource allocation following an immune challenge may further indirectly alter either immune defenses or post-challenge growth rate. In a strict sense, "direct" and "indirect" may represent relative differences in the complexity of the relationships between predictor and response variables: the "direct" effect, for example, of host plant quality on immune defenses may involve additional, unmeasured components. Here, we use "direct" and "indirect" to refer to the major structural relationships between the variables measured in our study, rather than hypothesized unmeasured variables.

We used structural equation modeling (SEM; also, path analysis) to quantify the relative contributions of the direct effects of host plant quality and selection history on

immune defenses and post-challenge growth rate versus their indirect effects mediated via pre-challenge growth rate (see Figure 5.1). Because strong multicollinearity among host plant quality and pre-challenge growth rate (variance inflation factor > 10 in both cases) violated the assumptions of traditional covariance-based SEM, we used component-based SEM (SEM using partial least squares; SEM PLS) (Chin & Newsted 1999; Monecke 2010). In SEM PLS, the predictor (exogenous) and response (endogenous) variables are reduced to principal components, and the predictor components are used to predict the scores on the response components. Host plant quality and selection history were included as exogenous variables. Pre- and post-challenge growth rates and immune defenses were included as endogenous variables; pre-challenge growth rate structurally mediated relationships between the exogenous variables and immune defenses and post-challenge growth rate. The standardized path coefficients estimated from the model are regression coefficients (beta weights) of standardized variables (mean = 0, SD =1). For indirect effects, individual path coefficients are multiplied along the path to obtain the total path contribution.

Results

Melanization was greater on the typical host plant relative to the novel host plant, and for the wild field population compared to the domesticated laboratory populations (Figure 5.2a). Analysis of deviance detected significant effects of host plant quality ($\chi^2 = 112$, $P < 0.0001$) and selection history ($\chi^2 = 26.2$, $P < 0.0001$), but not of the interaction between host plant quality and selection history ($\chi^2 = 0.433$, $P = 0.512$), indicating wild and domesticated populations had qualitatively similar responses to variation in host plant quality. Bead area was non-significant ($\chi^2 < 0.001$, $P = 1$).

Similarly, encapsulation was greater on the typical host plant relative to the novel host plant, and for the wild field population compared to the domesticated laboratory populations (Figure 5.2b). ANCOVA detected significant effects of host plant quality ($F_{1,116} = 18.3$, $P < 0.0001$) and selection history ($F_{1,116} = 9.76$, $P < 0.0001$), but not of the interaction between host plant quality and selection history ($F_{1,116} = 0.528$, $P = 0.469$), indicating wild and domesticated populations had qualitatively similar responses to variation in host plant quality. Bead area was non-significant ($F_{1,1079} < 0.001$, $P = 0.994$). We also secondarily explored differences among the two domesticated populations. Here, the most striking pattern was the greater mean encapsulation and melanization of the Duke laboratory population relative to the UNC laboratory population ($F_{1,88} = 5.31$, $P = 0.0214$; $\chi^2 = 6.75$, $P = 0.0342$, respectively).

Pre- and post-challenge growth rates (Figure 5.2c,d) were both improved on the typical host plant relative to the novel host plant ($F_{1,116} = 1250$, $P < 0.0001$; $F_{1,116} = 329$, $P < 0.0001$, respectively). Though pre-challenge growth rates were not significantly different between the wild and domesticated populations ($F_{1,116} = 1.64$, $P = 0.203$), post-challenge growth rates were greater for the domesticated populations compared to the wild population ($F_{1,116} = 32.9$, $P < 0.0001$). This reflects the greater growth (and final size) in the last instar in domesticated versus wild populations (D'Amico *et al.* 2001; Kingsolver 2007). The interaction between host plant quality and selection history was not significant in the analyses of pre- and post-challenge growth rates ($F_{1,116} = 0.0772$, $P = 0.782$; $F_{1,116} = 0.0473$, $P = 0.828$, respectively).

We used structural equation modeling (SEM) to quantify the direct and indirect associations among host plant quality and selection history, pre- and post-challenge growth

rate, and immune defenses (see above; Figure 5.1 and Table 5.1). The exogenous variables, host plant quality and selection history were included in the same SEM since there were no significant interactions between host plant quality and selection history from the ANCOVAs for encapsulation and melanization. The endogenous variables included encapsulation, melanization and pre- and post-challenge growth rates; pre-challenge growth rate structurally mediated relationships between the exogenous variables and encapsulation, melanization and post-challenge growth rate responses (path diagram is shown in Figure 5.1). The R^2 values for endogenous variables were relatively high (R^2 = 0.53, 0.83, 0.66, and 0.69 for encapsulation, melanization, pre-challenge and post-challenge growth rate, respectively), indicating the hypothesized SEM adequately fit the data.

SEM confirmed melanization and encapsulation were improved with greater host plant quality and for the wild population relative to the domesticated populations, and that pre- and post-challenge growth rates were improved with greater host plant quality. We further used SEM to quantify the relative magnitude of direct effects of host plant quality and selection history on immune defenses versus their indirect effects mediated via body condition (pre-challenge growth rate). The magnitude of the indirect effect of host plant quality on encapsulation (host plant quality to body condition path: 0.81 * body condition to encapsulation path: 0.90 = 0.73) was nearly twice the magnitude of the direct effect of host plant quality on encapsulation (0.39) (Figure 5.1). In contrast, while the magnitude of the direct effect of host plant quality on melanization was relatively high (0.72), the indirect effect was negligible (0.067). Selection history had moderate direct effects on encapsulation and melanization, but indirect effects of selection history mediated via body condition were quite small.

SEM also allowed us to explore potential allocation trade-offs by examining the relationships between each of the endogenous variables. Pre-challenge growth rate—a proxy for condition—had little effect on melanization, but was strongly positively related to encapsulation, and strongly negatively related to post-challenge growth rate (Figure 5.1). This indicates that individuals in better condition prior to an immune challenge have greater encapsulation; however, following an immune challenge, those individuals which allocate more resources to encapsulation, are able to allocate fewer resources to growth. Interestingly, this allocation trade-off between post-challenge growth rate and encapsulation was largely independent of melanization. The growth rate and encapsulation trade-off is further corroborated by larvae from the wild field population given a sham injection (5 µl sterile Grace's insect medium at the start of the 4^{th} instar; devil's claw-reared: n = 8, tobacco-reared: n = 6). Prior to injection, growth rates (residual growth rates removing the mean effect of host plant quality) of sham- and Sephadex-injected larvae were comparable ($t = 0.701$, $df = 42$, $P = 0.487$), but following injection, growth rates of sham-injected larvae were greater than those injected with an immune challenge ($t = 2.17$, $df = 42$, $P = 0.0354$).

Discussion

These studies explored how selection history, host plant quality, and allocation tradeoffs alter the immune defenses of the tobacco hornworm, *Manduca sexta*. Our analyses quantified the direct and indirect paths by which these factors contribute to immune responses in this system.

Several recent lines of evidence suggest selection has played an important role in shaping or maintaining immune defenses. For example, immune genes tend to have greater

rates of amino acid substitution than random samples of genes (Schlenke & Begun 2003; Lazzaro 2008), and signatures of natural selection on genes involved in immune defenses are concordant with variation among populations in their exposure to different suites of pathogens (Schlenke & Begun 2005). However, comparatively little is known regarding the short-term evolutionary dynamics of immune defenses (Lazzaro 2008). We used a population comparative approach to ask whether contemporary selection is important for maintaining immune defenses. The results of our study are consistent with this hypothesis: both components of immune defenses were reduced for each of the two domesticated laboratory populations of *M. sexta* compared to the wild field population from which they were derived over 35 years (> 260 laboratory generations) ago. However, we note that further replication, particularly at the level of the wild population, would be required to corroborate this pattern. The laboratory environments, in which *M. sexta* are protected from natural enemies and bacterial and fungal pathogens, relaxes selection on immune defenses. Indeed, observational studies of wild populations of *M. sexta* have shown that less than 2% of eggs laid survive to maturity, owing to the combined effects of predation, parasitism and pathogen infection (Mira & Bernays 2002). In contrast, survival of domesticated populations from egg to maturity typically exceeds 90% (Ahmad *et al.* 1989). The fact that there is greatly relaxed selection on immune defenses in domesticated *M. sexta*, coupled with the fact that two distinct domesticated populations of *M. sexta* (from Duke and UNC) had reduced immune defenses compared to their wild population ancestors, is consistent with our interpretation that selection is important for maintaining immune defenses. It also implies that caution should be exercised in generalizing immune defense results of domesticated laboratory populations to natural populations.

The results of our study provide strong empirical support for the hypothesis that immune defenses are improved with greater resource quality. We demonstrated that melanization and encapsulation immune defenses were both improved on the typical, high-quality host plant, tobacco, relative to the novel, low-quality host plant, devil's claw, for all three of our *M. sexta* populations (Figure 5.2a,b). Accumulating evidence suggests resource quality is an important determinant of immune defenses in an organism's current environment (Feder *et al.* 1997; Siva-Jothy & Thompson 2002; Ojala *et al.* 2005; Klemola *et al.* 2007). Yet, it seems likely that the relationship between resource quality and immune defenses may alter the probability of an organism invading and persisting in a novel environment. This is particularly valid for phytophagous insects, which tend to be relatively specialized on their host plant resources (Jaenike 1990). In the case of *M. sexta*, reduced immune defenses on the novel host plant, devil's claw, compared to the typical host plant, tobacco, may be one of the factors retarding the adoption of devil's claw as a host plant in the Southeastern (Diamond & Kingsolver 2010) and perhaps Southwestern (Mira & Bernays 2002) USA. In contrast, a recent study examining immune defenses of the autumnal moth, *Epirrita autumnata*, found that immune defenses were the same or better across typical and alternative host plant species, perhaps facilitating the adoption of alternative host plants in this species (Yang *et al.* 2008). The relationship between host immune defenses and resource quality may play a key role in determining the ability of hosts to invade and persist in novel environments.

Clearly, host plant quality and selection history impact immune defenses, but to what extent are these relationships driven by the indirect effects of host plant quality and selection history on immune defenses mediated via non-immune defense aspects of the host's biology?

Recent work by Smilanich *et al.* (2009) has demonstrated the utility of structural equation modeling for disentangling the contributions of direct and indirect effects to overall immune defenses. In this study, we used structural equation modeling to distinguish the relative contributions of the direct effects of host plant quality and selection history on immune defenses from their indirect effects mediated via host body condition prior to an immune challenge and allocation trade-offs following an immune challenge.

In general, we found substantial variation in the relative importance of direct and indirect effects on immune defenses. We emphasize that while "direct" and "indirect" effects refer to the major structural relationships between the variables measured in our study, in reality such direct and indirect effects may represent relative differences in the complexity of the relationships between predictor and response variables, due to potentially important unmeasured variables. In our study, the most important indirect effect was that of host plant quality on encapsulation mediated via body condition prior to the immune challenge, which explained nearly twice as much of the variation in encapsulation as the direct effect of host plant quality (Figure 5.1). In our study system, this result indicates that resource quality most strongly impacts immune defenses indirectly through body condition; however, the generality of this pattern is unclear, as most previous work on immune defenses cannot distinguish direct from indirect effects. The extent to which the accumulating evidence for positive effects of resource quality on immune defenses (see above) reflects intermediary effects of improved body condition is therefore an important open question.

In contrast, the indirect effect of selection history on encapsulation was quite small (Figure 5.1). This result is not surprising, as wild and domesticated populations of *M. sexta* have similar growth rates (our proxy for body condition) at the beginning of the 4th larval

instar when the pre-challenge growth rate was assessed (Kingsolver 2007; Diamond *et al.* 2010). Yet, this result does indicate that the reduced immune defenses of the domesticated laboratory populations relative to the wild field population are largely a direct consequence of domestication rather than intermediary effects on body condition.

In addition to examining the indirect effects of body condition prior to an immune challenge, we also used structural equation modeling to investigate indirect effects of allocation following an immune challenge. The positive association between pre-challenge growth rate and encapsulation coupled with the negative association between pre- and post-challenge growth rates suggests an allocation trade-off, in which encapsulation is maintained at the cost of slower growth. Much of the positive evidence for trade-offs with immune defenses comes from selection experiments (Lazzaro & Little 2009; for examples, see Boots & Begon 1993; Kraaijeveld & Godfray 1997), with relatively mixed support for such trade-offs based on standing genetic variation (*e.g.*, for positive support, see Hoang 2001; Freitak *et al.* 2003; McKean *et al.* 2008; for negative support, see Little *et al.* 2002; Altermatt & Ebert 2007; for mixed support within the same study system, see Stoehr 2010). Our result showing that encapsulation and post-challenge growth rate are related to pre-challenge body condition in opposite ways is consistent with an allocation trade-off. Particularly for the wild field population *M. sexta*, this lends further support for the importance of allocation trade-offs with immune defenses in natural populations.

Despite encapsulation being costly, the lack of association between pre-challenge growth rate and melanization suggests melanization is maintained without incurring observable costs, either due to investment in growth or other aspects of immune defense such as encapsulation. A possible explanation for this pattern is that the absolute amount of energy

required for encapsulation (a process involving the deposition of large numbers of hemocytes) may be greater than that for melanization (a process involving the enzymatic conversion and subsequent deposition of melanin) (Cerenius *et al.* 2008). An alternative, but non-mutually exclusive hypothesis is that the energetic costs of encapsulation and melanization may differ through ontogeny. In larval Lepidoptera, including *M. sexta*, circulating hemocytes—a general classification of various types of blood cells involved in immune defenses—originate from the proliferation of embryonically derived hemocytes already in circulation, and the production of hemocytes from hematopoietic organs (Nardi *et al.* 2003; Nardi 2004). Particularly in more advanced larval developmental stages, the hematopoietic organs are the main source of plasmatocytes, or hemocytes involved in encapsulation, whereas hemocytes already in circulation are the main source of oenocytoids, or hemocytes containing phenoloxidase, an enzyme necessary for the conversion of melanin precursors to active melanin (Strand 2008). The production of new plasmatocytes in response to an immune challenge may therefore be more energetically costly than recruiting oenocytoids already in circulation. Thus, at the time of the immune challenge in our experiments with *M. sexta*, the energetic costs of encapsulation may have been more prominent because they were more recent compared to the costs involved with melanization. There is a limited amount of empirical evidence demonstrating a pattern of highly divergent responses among different immune components (Moret & Schmid-Hempel 2001; Cotter *et al.* 2004; Freitak *et al.* 2007; Wilfert *et al.* 2007). The generality of this pattern and the underlying mechanisms are unclear but deserving of further study. Structural equation modeling may prove useful in this regard, and more generally in disentangling the relative contributions of physiological, ecological and evolutionary factors to variation in immune

defenses.

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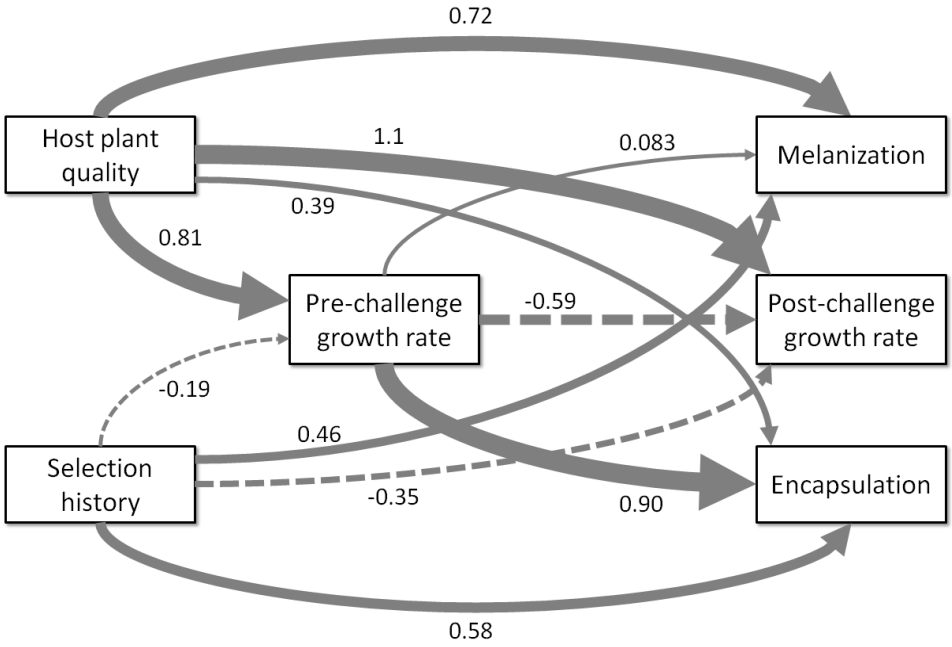

Figure 5.1. Structural equation model (path diagram) for the direct effects of host plant quality and selection history on melanization, encapsulation and post-challenge growth rates, and their indirect effects mediated through pre-challenge growth rate. The levels of the dichotomous variables, host plant quality and selection history were assigned such that devil's claw = 0, tobacco = 1, domesticated laboratory population = 0, and wild field population = 1. The width of the path corresponds with the magnitude of the effect. Positive relationships are indicated by solid lines, and negative relationships by dashed lines. See Table 1 for 95% confidence intervals of the path coefficient estimates.

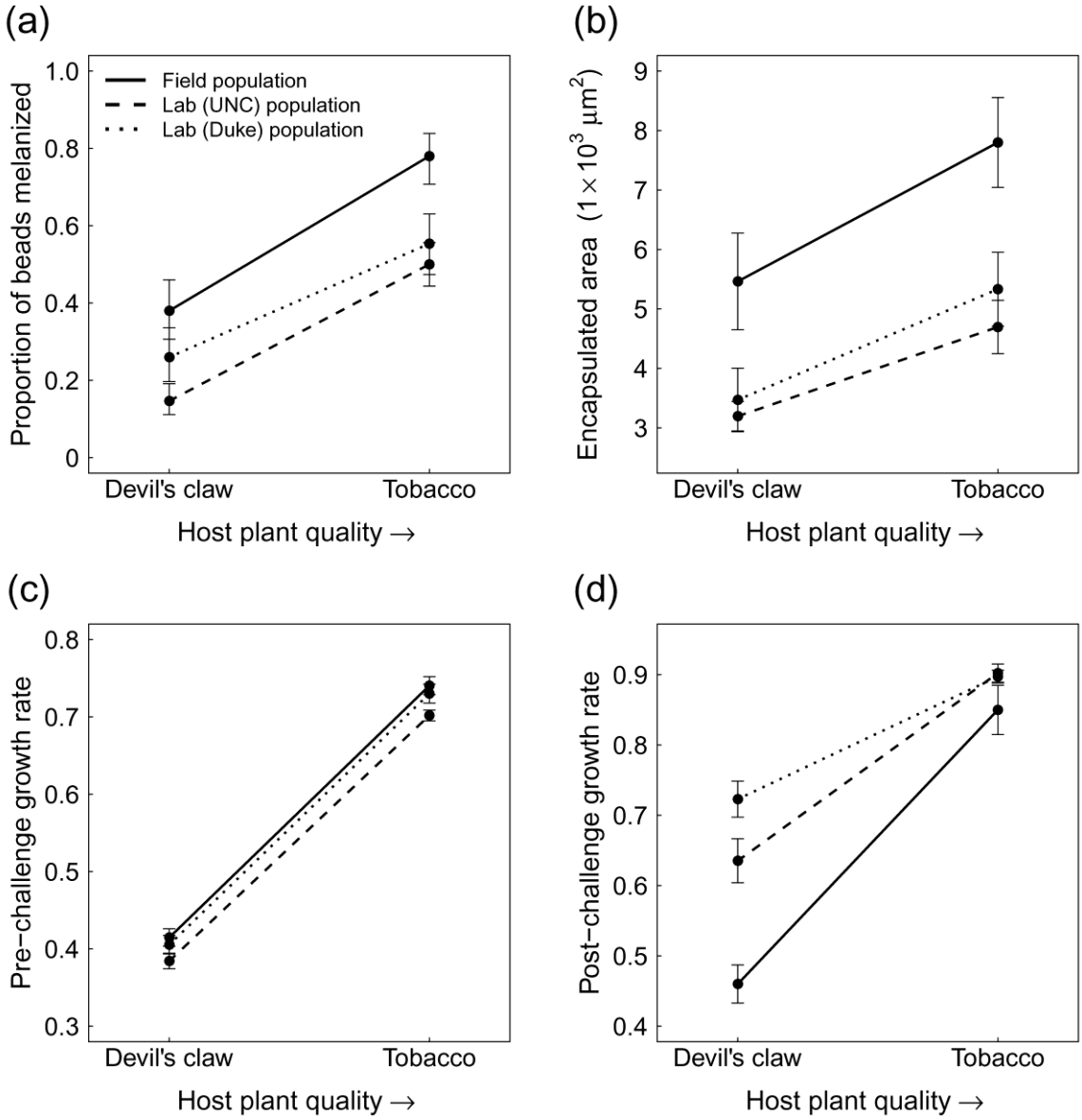

Figure 5.2. Mean immune responses and growth rates ± 1 S.E. (except panel *a*, where 95% binomial confidence intervals are indicated) for the wild field (solid line), UNC domesticated laboratory (dashed line) and Duke domesticated laboratory (dotted line) populations as a function of host plant quality. Specific responses include: (a) proportion of beads melanized, (b) encapsulated area, (c) pre-challenge growth rate, and (d) post-challenge growth rate.

Table 5.1. Standardized path coefficient estimates and 95% confidence intervals (obtained from n = 500 bootstrap replications) for the direct effects of host plant quality and selection history on melanization, encapsulation, and post-challenge growth rates, and their indirect effects mediated through pre-challenge growth rate.

Path	**Estimate**	**95% CI**
Host plant quality → encapsulation	0.39	0.19, 0.60
Host plant quality → melanization	0.72	0.59, 0.85
Host plant quality → post-challenge growth rate	1.1	1.0, 1.3
Host plant quality → pre-challenge growth rate	0.81	0.76, 0.86
Pre-challenge growth rate → encapsulation	0.90	0.69, 1.1
Pre-challenge growth rate → melanization	0.083	-0.043, 0.23
Selection history → encapsulation	0.58	0.44, 0.71
Selection history → melanization	0.46	0.40, 0.53
Selection history → post-challenge growth rate	-0.35	-0.51, -0.21
Selection history → pre-challenge growth rate	-0.19	-0.29, -0.09
Pre-challenge growth rate → post-challenge growth rate	-0.59	-0.77, -0.43

Note: Host plant quality and selection history are dichotomous variables, where 0 corresponds with devil's claw and the domesticated laboratory populations, and 1 corresponds with tobacco and the wild field population, respectively. Confidence intervals which do not contain 0 are significantly different from 0 at the p = 0.05 level.

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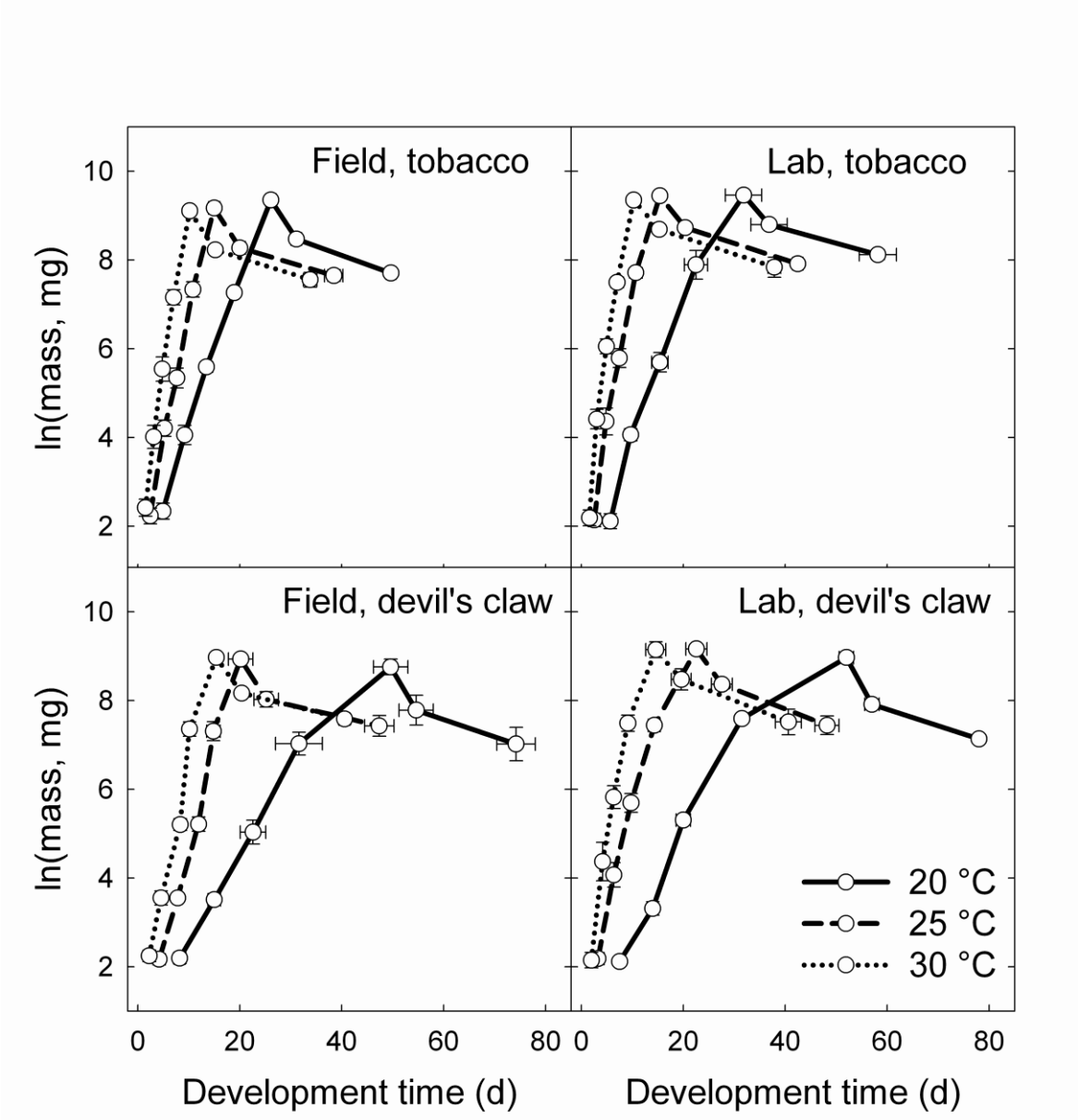
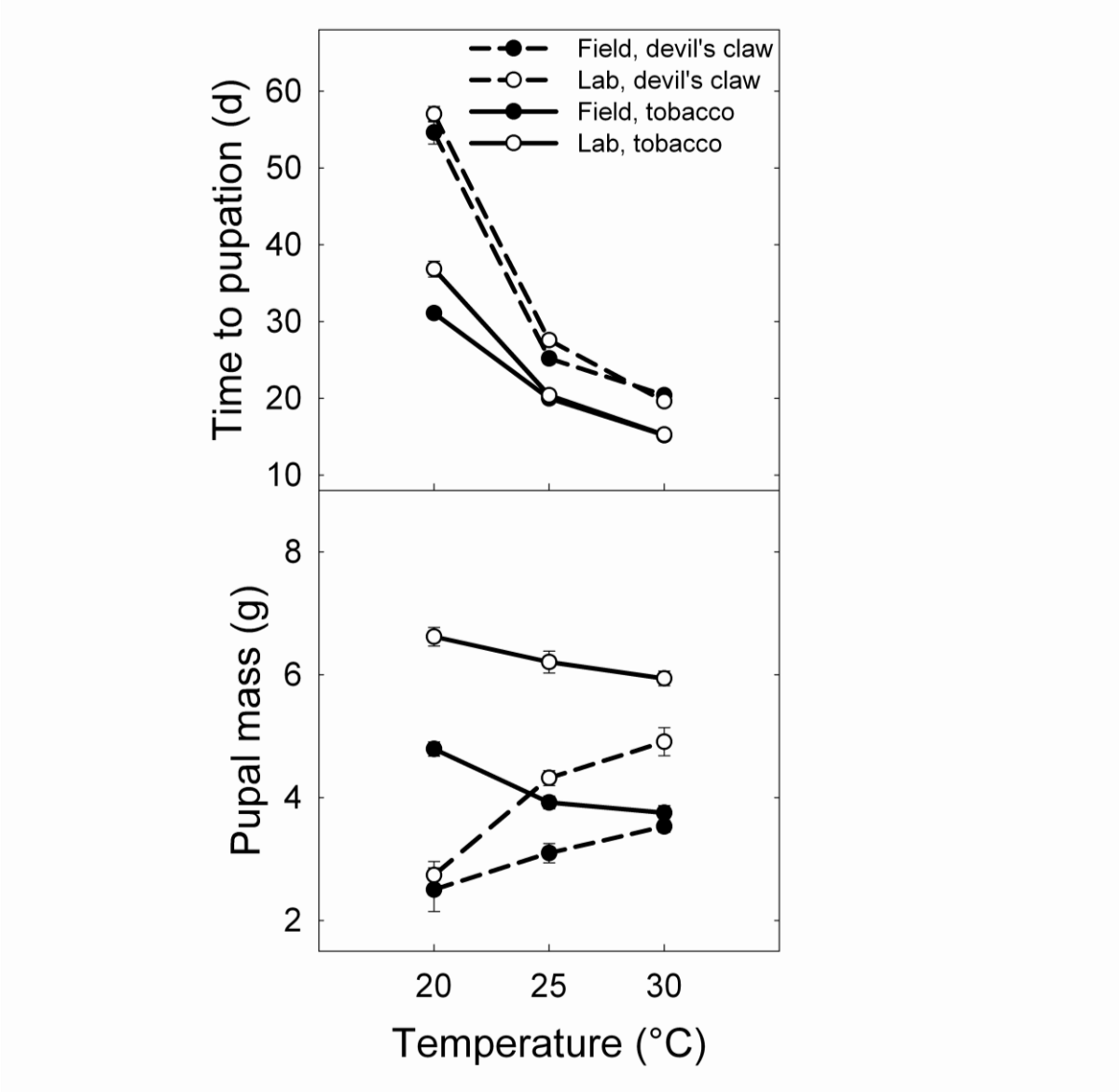
APPENDIX

Supporting figures and statistical analyses of the performance consequences of temperature and host plant quality (Chapter IV)



Appendix Figure 1. ln(mean body mass, mg) ± 1 S.D. as a function of mean development time (d) ± 1 S.D. at the 2nd, 3rd, 4th and 5th larval instars, wandering, pupation and eclosion for field population (left two panels) and laboratory population (right two panels) male *M. sexta* reared on tobacco (upper two panels) and devil's claw (lower two panels) at 20 (solid lines), 25 (dashed lines), and 30 (dotted lines) °C. Individuals with extra (6th) instars were incorporated into the trajectories for individuals with 5 instars by omitting the 6th instar data points.



Appendix Figure 2. Development time to pupation (d) ± 1 S.E. as a function of temperature (°C) (upper panel), and pupal mass (g) ± 1 S.E. as a function of temperature (°C) (lower panel) for male *M. sexta* from the field population (closed symbols) and laboratory population (open symbols) reared on tobacco (solid lines) and devil's claw (dashed lines).

Appendix Table 1. ANCOVA results for development time (d) to wandering, pupation, and eclosion.

Stage	**Term**	**SS**	**df**	***F***	***P > F***
Wandering*	Population	1.7	1	0.095	0.76
	Plant	1100	1	61	< 0.0001
	Temperature	6300	1	350	< 0.0001
	Sex	13	1	0.74	0.39
	Population * Plant	100	1	5.6	0.018
	Population * Temperature	4.2	1	0.23	0.63
	Plant * Temperature	710	1	40	< 0.0001
	Population * Plant * Temperature	95	1	5.3	0.022
	Residuals	4200	231		
Pupation*	Population	1.7	1	0.095	0.76
	Plant	1100	1	61	< 0.0001
	Temperature	6300	1	350	< 0.0001
	Sex	13	1	0.74	0.39
	Population * Plant	100	1	5.6	0.018
	Population * Temperature	4.2	1	0.23	0.63
	Plant * Temperature	710	1	40	< 0.0001
	Population * Plant * Temperature	95	1	5.3	0.022
	Residuals	4200	231		
Eclosion	Population	7	1	0.43	0.51
	Plant	1200	1	74	< 0.0001
	Temperature	6500	1	400	< 0.0001
	Sex	76	1	4.7	0.032
	Population * Plant	120	1	7.1	0.0082
	Population * Temperature	13	1	0.78	0.38
	Plant * Temperature	710	1	44	< 0.0001
	Population * Plant * Temperature	74	1	4.5	0.035
	Residuals	3800	231		

* results are statistically equivalent; development time from wandering to pupation was constant across groups

Appendix Table 2. ANCOVA results for body mass (mg) at wandering, pupation, and eclosion.

Stage	Term	SS	df	*F*	*P* > *F*
Wandering	Population	2200000	1	2.2	0.14
	Plant	95000000	1	96	< 0.0001
	Temperature	11000000	1	11	0.0011
	Sex	54000000	1	55	< 0.0001
	Population * Plant	1700000	1	1.7	0.19
	Population * Temperature	57000	1	0.058	0.81
	Plant * Temperature	61000000	1	62	< 0.0001
	Population * Plant * Temperature	2900000	1	3	0.087
	Residuals	230000000	231		
Pupation	Population	390000	1	0.98	0.32
	Plant	23000000	1	57	< 0.0001
	Temperature	7800000	1	20	< 0.0001
	Sex	21000000	1	52	< 0.0001
	Population * Plant	2000000	1	5	0.027
	Population * Temperature	1900000	1	4.7	0.032
	Plant * Temperature	16000000	1	40	< 0.0001
	Population * Plant * Temperature	610000	1	1.5	0.22
	Residuals	92000000	231		
Eclosion	Population	35000	1	0.19	0.66
	Plant	5400000	1	30	< 0.0001
	Temperature	3600000	1	20	< 0.0001
	Sex	15000000	1	82	< 0.0001
	Population * Plant	1800000	1	9.8	0.002
	Population * Temperature	42000	1	0.23	0.63
	Plant * Temperature	3900000	1	21	< 0.0001
	Population * Plant * Temperature	780000	1	4.3	0.04
	Residuals	42000000	231		

Appendix Table 3. ANCOVA results for fecundity (number of follicles) using pupal and adult body masses (mg) as covariates.

Stage (body mass covariate)	**Term**	**SS**	**df**	***F***	***P* > *F***
Pupation	Population	13000	1	4.5	0.036
	Plant	30000	1	10	0.0018
	Temperature	5500	1	1.9	0.17
	Pupal mass	29000	1	9.9	0.0022
	Population * Plant	32000	1	11	0.0014
	Population * Temperature	8700	1	3	0.086
	Plant * Temperature	22000	1	7.7	0.0068
	Population * Plant * Temperature	35000	1	12	0.00077
	Residuals	260000	91		
Eclosion	Population	17000	1	5.2	0.024
	Plant	70000	1	22	< 0.0001
	Temperature	16000	1	4.9	0.029
	Adult mass	280	1	0.089	0.77
	Population * Plant	21000	1	6.6	0.012
	Population * Temperature	14000	1	4.5	0.037
	Plant * Temperature	50000	1	15	0.00016
	Population * Plant * Temperature	29000	1	9	0.0034
	Residuals	290000	91		