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Direct and indirect phenotypic selection on developmental trajectories in *Manduca sexta*

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Summary

1. Adult size and development time are the outcomes of growth and differentiation throughout the life of an individual organism – its developmental trajectory. Correlations among ages and sizes across different life stages may influence both direct and indirect components of selection on body size and age during development.

2. We used two field studies in experimental gardens in fall and summer to evaluate phenotypic selection on size and age across larval, pupal and adult stages in the tobacco hornworm, *Manduca sexta*, and how this may vary across seasons.

3. Rapid larval development was positively associated with survival to pupation and adulthood, in part because it allowed escape from larval parasitoids. Larval mortality owing to parasitoids was greater in the fall than in the summer. Egg production was positively correlated with adult mass, but not with development time.

4. Principal component analyses of size and age throughout development showed that adult size and development time were not negatively correlated, contrary to life-history expectations. As a result, selection favouring larger adult size (via female reproduction) and selection favouring rapid larval development (via juvenile survival) do not act in opposition in this system.

5. We discuss the physiological mechanisms that may underlie the independence of adult size and early larval development for holometabolous insects, and the implications for selection on body size and developmental trajectories.

Key-words: body size, developmental trajectory, growth rates, life history, *Manduca sexta*, natural selection, parasitoids

Introduction

Size and age at adult reproduction are key elements of life history (Stearns 1992; Roff 2002). In many organisms, greater adult size is associated with greater fecundity and potential reproductive output, and later, age at reproduction is associated with a lower probability of survival to reproduction. The resulting trade-off between adult size and age is central to models for the evolution of life histories (Roff 2000). In ectotherms, environmental temperatures and resource quality during development can alter adult size and age, affecting potential trade-offs and selection on these traits in variable environments (Atkinson 1994; Angilletta 2009).

Phenotypic selection describes how individual variation in phenotypic traits (e.g. size and age at some developmental stage) relates to variation in components of fitness (e.g. survival, reproduction and generation time). Adult size and age are the outcomes of growth and differentiation throughout the life of an individual organism. Here, we consider the developmental trajectory of an individual as its size and age at each key developmental stage or event during its life. As a result, there may be both direct and indirect components of selection on variation in developmental trajectories within a population (Stearns 1992; Roff 2002). First, adult body size may directly affect fecundity or adult survival, and age at adulthood may directly impact generation time. Second, survival to adult reproduction may be determined by size or age at earlier developmental stages and by the correlations of age and size at juvenile stages with adult age and size. For example, in insects, size and age in early larval stages may be important for survival and escape from natural enemies, and thus for survival to the adult stage. The phenotypic correlations of juvenile size and age with adult size and age will thus determine the net selection on developmental trajectories. Unfortunately, few studies have evaluated direct and

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indirect components of phenotypic selection on developmental trajectories in the field.

The holometabolous insect *Manduca sexta* provides an excellent study system for exploring these issues. After hatching, *M. sexta* larvae grow and develop through five (sometimes more) instars, feeding primarily on host plants in the family Solanaceae. Maximal body size towards the end of the final instar strongly determines pupal and adult body mass and potential fecundity (Nijhout 1975, 2003; Davidowitz, D'Amico & Nijhout 2003; Davidowitz, D'Amico & Nijhout 2004). In addition, survival to pupation is strongly influenced by larval mortality because of natural enemies, including several major larval parasitoids (Bernays 1997; Mira & Bernays 2002; Diamond & Kingsolver 2010b). As a result, both direct selection and indirect selection on developmental trajectories, and phenotypic correlations in size and age across life stages, are important for life-history variation in this system.

Here, we describe two longitudinal studies of selection in a field population of *M. sexta*. The two studies differ in seasonal environments and the effects of a major parasitoid on larval mortality. We quantify the relationship between developmental trajectories and juvenile survival, and the covariance structure of size and age across developmental stages. We use these data to explore direct and indirect components of phenotypic selection on adult size and age. Our results suggest that, contrary to life-history expectations, early larval growth is not strongly related to adult body mass, and net selection on adult size and development time may act quite independently in this system.

Materials and methods

STUDY SYSTEM

The tobacco hornworm, *M. sexta*, occurs in Central America and the southern United States, with eastern populations extending north into New York and Massachusetts. *M. sexta* larvae feed primarily on host plants in the family Solanaceae [but see (Mechaber & Hildebrand 2000; Mira & Bernays 2002; Diamond *et al.* 2010)]. In the south-eastern United States, including North Carolina, cultivated tobacco and tomato are dominant host plants for *M. sexta*, which can be an important agricultural pest in these systems. Our field studies used tobacco cultivars.

After hatching, *M. sexta* larvae grow and develop rapidly through five (occasionally more) larval instars, growing from ~1 mg to ~8–12 g in body mass in a few weeks under optimal conditions. Rates of larval growth and development are strongly influenced by environmental temperatures and host plant quality. Towards the end of the final instar, larvae stop feeding and wander off the host plant to pupate nearby in the soil. A facultative pupal diapause is determined by larval photoperiod, such that *M. sexta* populations have multiple generations per year in most areas (2–3 generations per year in North Carolina). Because pupae do not feed, maximum larval mass at wandering strongly determines pupal and adult size and the number of eggs (oocytes) produced by females.

For *M. sexta*, both host plant quality and larval mortality owing to natural enemies are important determinants of survival to adult reproduction. In the south-eastern United States including North Carolina, the larval parasitoid *Cotesia congregata* (Hymenoptera: Braconidae) is a major source of larval mortality, with parasitism rates of 50–90% in many populations. Rates of parasitism are often higher in late summer and fall. *C. congregata* typically attack and lay their eggs in 2nd instar larvae of *M. sexta*, but often do not kill the caterpillar until the 5th instar. Successful parasitism is easily detected by the presence of wasp pupae protruding from the external cuticle of the caterpillar. Thus, early larval growth and development may strongly determine survival to reproduction in this system.

FIELD STUDIES

The field selection studies were conducted in a cultivated tobacco garden in the Mason Farm Biological Reserve, Chapel Hill, NC. Before each study, the garden plot (12 m by 20 m in size) was tilled and fertilized (Peters Professional Soluble Plant Food, 20 : 20 : 20). To reduce the effects of host plant quality on variation in larval success, cultivated tobacco plants (*Nicotiana tabacum*, var. LA Burley 21) were grown from seeds in 10-cm pots in the greenhouse at UNC until they were ~30 cm tall and then transplanted to the field garden. Plants were placed 1 m apart with 1 m between rows, for a total of 60 plants in six rows in the study plot. A single buffer row of tobacco plants (kept at the 1-m spacing) was established around the perimeter of the study plot. The plants were allowed to establish for ~4 weeks prior to the start of a study; plants that did not thrive after transplanting were replaced. The garden was watered daily, and insect herbivores were removed weekly by hand from each plant. To minimize larval predation by birds and social wasps, plants were covered with bridal veil netting (mesh size ~3·5 mm diameter) just prior to the study. The netting allows parasitoids (including *C. congregata*) access to the caterpillars but excludes larger predators.

Our studies focus on how variation in larval growth and development determines variation in survival, mass and age at the pupal and adult stages, and in egg production. To initiate each study, *M. sexta* eggs were collected from tobacco plants grown without the use of pesticides at the NC State Agricultural Extension Farm, Clayton, NC, *c.* 100 km from Chapel Hill. *M. sexta* adults are highly dispersive with extensive gene flow among geographic populations in the eastern United States. Insect herbivores (including eggs and larvae of *M. sexta*) were removed from the garden plants prior to the study. The first study was initiated in September 2009. Eggs were allowed to hatch in the laboratory and maintained individually in an environmental chamber at 20 °C with a 14L/10D light cycle on tobacco leaves during the 1st larval instar. (This rearing temperature approximated mean ambient temperatures in the field during the study.) When larvae moulted into the 2nd instar, body mass and age were determined, and each larva was then randomly assigned to one of the tobacco plants in the field garden. Four larvae were placed on each plant, each on a separate leaf (total of 240 individuals on 60 plants in the study) towards the top of the plant, corresponding with the oviposition site preference of *M. sexta* females (Madden & Chamberlin 1945). Previous studies showed that this larval density avoids competition among larvae for food (Diamond & Kingsolver 2010b). In this system, 2nd instar larvae rarely move among leaves, so that individuals can be tracked over time. Starting with the moult into 3rd instar, the tip of the dorsal 'horn' of the larva was marked with water-based nail polish (Zoya nail polish), using different colours for different individuals on each plant. We used marking colours within the red spectrum of the colour palette to better match the caterpillar's natural reddish horn colour. Nail polish was reapplied after each subsequent moult. Preliminary studies detected no effects of marking on larval growth or survival. This enabled us to track individuals in the field throughout the study.

During daily field censuses, we recorded the presence or absence of each larva. Recapture probabilities (given alive) for larvae consistently exceeded 90% in each study. Previous laboratory and field studies show that larvae from this population moult during the morning hours when feeding on tobacco (Diamond & Kingsolver 2010b; Diamond *et al.* 2010), so field censuses and mass measurements were taken in the mornings. The start of an instar was defined by completion of head capsule slippage; if an individual was in the process of head capsule slippage during a census, we would return later during that census period when slippage was completed before measuring its mass. At the start of the 3rd, 4th and 5th instar, the mass and age of each larva were recorded. After measurement, each larva was returned to the underside of a leaf, with its prolegs clutching the leaf veins. When larval mass late in the 5th instar exceeded 5 g, larvae were removed from the field and reared individually in petri dishes on tobacco leaves in an environmental chamber (20 °C; 12L/12D photocycle) until wandering. Mass and age at wandering were recorded, and then each larva was placed in a wooden block (Yamamoto 1969) to pupate. Pupae were first checked at 5 days after wandering; pupal mass was measured at 7 days after wandering to ensure that the pupal cuticle hardened prior to handling. Pupae were placed in Solo® cups with moistened soil, kept at 20 °C and checked daily each morning until eclosion. In this first (September 2009) study, all individuals entered pupal diapause over the winter, extending the time to adult eclosion by more than 6 months (see Results).

The second field study (3–4 individuals per plant, for a total of 169 individuals on 44 plants), initiated in July 2010, used a similar approach to the first study, with four differences. First, larvae were reared in the laboratory until the start of the 3rd instar (rather than the 2nd instar as in the 2009 study). We predicted that this should decrease the rate of parasitism by *C. congregata* and larval mortality in the field. Second, none of the animals in this study entered pupal diapause, simplifying the interpretation of data for adult mass and development time. Third, warmer rearing conditions and a longer photocycle (16L/8D) were used in the laboratory components of the study to reflect the warmer field conditions during July rather than September. Hence, larvae were reared from hatching to 3rd instar at 25 °C; late 5th instars were reared to wandering at 26·7 °C: pupae were maintained at 25 °C. Fourth, estimates of potential fecundity for adult females were obtained by dissecting out the ovarioles into Ringer's solution, 48 h after eclosion. The number of follicles at stage 6 (S6) and all subsequent stages of development (Yamauchi & Yoshitake 1984) was counted with the aid of a dissecting microscope (Diamond & Kingsolver 2010b).

STATISTICAL ANALYSES

Phenotypic selection describes how individual variation in phenotypic traits relates to variation in components of fitness (e.g. survival, reproduction and generation time). One aim of our studies was to quantify directional selection on age and size at different larval instars. We focused on three distinct metrics of fitness: pupal mass (reflecting final body size), age at pupation (reflecting generation time) and the number of ovarioles (reflecting potential female reproduction). Because pupal mass and age at pupation are both outcomes of earlier growth and development (e.g. mass and age at different larval instars) and determinants of adult fitness (e.g. reproduction), we consider pupal mass and age at pupation as fitness responses in some analyses and phenotypic predictors in others (see Results). We focused on pupal rather than adult age and size for two reasons: (i) variability in pupal mass loss and the duration of pupal diapause in the 2009 study adds substantial noise and variability to adult mass and age, unrelated to larval growth and development, and (ii) pupal mass is more reliably measured than adult mass, because of rapid fluid loss during the eclosion process.

The specific goals of our analyses were to (i) determine how early age and mass (at the start of 2nd or 3rd instar) influenced survival, age and mass at the pupal and adult stages; (ii) quantify the correlation structure of developmental trajectories (ages and masses across the larval instars and pupal stages), using principal component analyses (PCA); and (iii) determine how developmental trajectories [using principal component (PC) scores from the PCA] influenced age and mass at eclosion (adult stage) and female egg production.

Directional selection is typically quantified using regression analyses (including logistic regression) that regress phenotypic traits onto fitness components (Lande & Arnold 1983; Arnold & Wade 1984; Janzen & Stern 1998). Accordingly, survival to pupation was modelled as a binomially distributed variable using logistic regression, with age and mass at 2nd instar as predictor variables. Similarly, age and mass at pupation (normally distributed) were modelled using multiple regression with age and mass at 2nd instar as predictor variables. Results for the 2009 and 2010 studies were analysed separately. For comparison with other selection studies (Lande & Arnold 1983; Kingsolver *et al.* 2001), we also estimated variance-standardized directional selection gradients (β) for age and mass at 2nd instar from these analyses.

Principal component analyses based on the correlation matrices were performed for age and mass for the following stages: start of the 2nd, 3rd, 4th and 5th instars; wandering; and pupa. Only individuals surviving to pupation were included in these analyses. The 3rd instar was not measured in the 2009 study, so is omitted in that analysis.

For individuals surviving to eclosion (adult stage), mass and age at eclosion (normally distributed) were modelled using multiple regression, with scores for the first two PCs (PC1 and PC2) as predictor variables. In the 2010 study, egg number was also modelled using multiple regression, with PC1 and PC2 scores as predictor variables. We also estimated variance-standardized directional selection gradients (β) for PC1 and PC2 scores from these analyses.

Plant was not included as a random effect in the statistical model because of the small number of individuals per plant (3–4 at the start of each study). Because of larval mortality, many plants had only 1–2 individuals that reached the wandering, pupal and adult stages, especially in the 2009 study. Preliminary analyses indicated that incorporating plant as a random effect even in the models of larval survival caused overfitting of the models and spurious correlations among model parameters (Pinheiro & Bates 2000). The random assignment of larvae to plants in our study design should minimize spurious associations between predictor and response variables.

Results

SURVIVAL, AGE AND MASS AT PUPATION

Larval mortality prior to pupation in the 2009 study was high (88%). Larval mortality owing to parasitism by *C. congregata* was 62% in this study, thus representing the single largest source of mortality. As a result, most mortality occurred during the 5th instar (when *C. congregata* larvae emerge to pupate on the host). In the summer 2010 study, larvae were first placed in the field at the start of the 3rd instar (rather than 2nd instar as in the 2009 study). Larval mortality in summer 2010 was much lower (23%) with only 2% mortality because of parasitism by *C. congregata*. This suggests that parasitoid

attack by *C. congregata* on *M. sexta* larvae may vary seasonally in this population (see Discussion).

In the 2009 study, earlier age at 2nd instar was significantly associated with higher survival to pupation ($P = 0{\cdot}045$). There was no significant association between mass at 2nd instar and survival to pupation ($P = 0{\cdot}710$). The variance-standardized directional selection gradients were $\beta = -0{\cdot}49$ (SE = 0·26) for age at 2nd instar and $\beta = -0{\cdot}06$ (0·12) for mass at 2nd instar. In the 2010 study, earlier age at 3rd instar was marginally associated with higher survival to pupation [$P = 0{\cdot}064$: $\beta = -0{\cdot}08$ (0·04)], but there was no significant association between mass at 3rd instar and survival to pupation [$P = 0{\cdot}956$: $\beta = 0{\cdot}000$ (0·004)]. The stronger selection on age in the 2009 study reflects the much higher mortality (and hence greater opportunity for selection) in that study.

In the 2009 study, earlier age at pupation was significantly associated with earlier age at 2nd instar [$P = 0{\cdot}029$: $\beta = -0{\cdot}059$ (0·027)], but not with mass at 2nd instar [$P = 0{\cdot}777$: $\beta = 0{\cdot}004$ (0·014)]. (Here and elsewhere, in assigning the sign to β, we define age at pupation, reflecting generation time, as inversely related to fitness.) There were no significant effects of age [$P = 0{\cdot}793$: $\beta = 0{\cdot}014$ (0·052)] or mass [$P = 0{\cdot}189$: $\beta = -0{\cdot}38$ (0·027)] at 2nd instar on pupal mass in this study. In the 2010 study, earlier age at pupation was significantly associated with earlier age at 3rd instar [$P < 0{\cdot}001$: $\beta = -0{\cdot}038$ (0·0004)], but not with mass at 3rd instar [$P = 0{\cdot}116$: $\beta = 0{\cdot}0006$ (0·0003)]. As in the 2009 study, there were no significant effects of age [$P = 0{\cdot}259$: $\beta = -0{\cdot}009$ (0·015)] or mass [$P = 0{\cdot}644$: $\beta = 0{\cdot}0006$ (0·013)] at 3rd instar on pupal mass in this study. These results suggest that rates of early larval growth and development may affect the age but not the size at pupation.

Developmental trajectories for the 2009 study (Fig. 1a) illustrate how ages and masses at different larval instars diverged between individuals that did or did not survive to pupation. By the start of the 4th instar, survivors were marginally younger ($P = 0{\cdot}073$) and significantly larger ($P = 0{\cdot}031$) than non-survivors. By the start of the 5th instar, survivors were significantly younger and larger compared with non-survivors ($P < 0{\cdot}001$ in both cases). Developmental trajectories for the 2010 study (Fig. 1b) also suggested divergence between individuals that did or did not survive to pupation. By the start of the 4th instar, survivors were significantly larger ($P = 0{\cdot}011$) but not significantly younger ($P = 0{\cdot}490$) than non-survivors. Similarly by the start of the 5th instar, survivors were significantly larger ($P = 0{\cdot}032$) but not significantly younger ($P = 0{\cdot}127$) compared with non-survivors. These results suggest that more rapid larval growth and development were associated with higher survival to pupation (see Discussion).

DEVELOPMENTAL TRAJECTORIES: CORRELATION STRUCTURE

Principal component analyses for the 2009 study showed that the first two PCs explained 62·5% of the variation in ages and masses across stages (2nd instar through pupa; see Methods). Loadings on the first PC (41·1% of variance) were dominated by positive values for age at all stages (Table 1a): higher PC1 scores were associated with later ages at all stages. In contrast, loadings on the second PC (21·4%) were dominated by positive values for mass at later stages (5th, wandering, pupa) and smaller negative values for mass and age at 2nd instar (Table 1a). The 2010 study revealed a similar correlation structure (Table 1b). The first two PCs explained 57·5% of the variation in ages and masses across stages. Loadings on the first PC (41·0% of variance) were dominated by positive values for age at all stages and smaller negative values for mass at younger ages (Table 1b): higher PC1 scores were associated with older ages at all stages, and perhaps smaller size at early larval stages. In contrast, loadings on the second PC (16·5%) were dominated by positive values for mass at

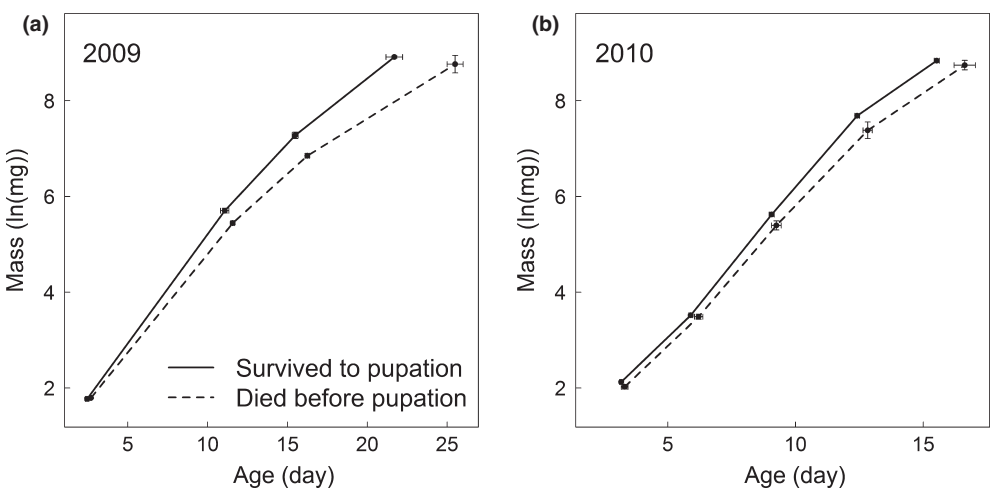

Fig. 1. Growth trajectories (body mass in ln(mg) as a function of age in days) for animals that survived to pupation (solid lines) and those that died prior to pupation (dashed lines) in the (a) 2009 and (b) 2010 study. Means ± 1 SE are represented at the following developmental stages: 2nd instar, 3rd (2010 study only), 4th, 5th and wandering. Note that in some cases, errors are sufficiently small to be obscured by the points representing means.

Table 1. PC loadings, (a) 2009 study and (b) 2010 study

PC1			PC2		
Stage	Age	Mass	Stage	Age	Mass
(a)					
2nd	0·345	0·146	2nd	−0·123	−0·271
3rd	na	na	3rd	na	na
4th	0·452	< |0·1|	4th	< |0·1|	0·152
5th	0·430	−0·214	5th	< |0·1|	0·316
Wander	0·452	0·152	Wander	< |0·1|	0·622
Pupa	0·443	< |0·1|	Pupa	< |0·1|	0·627
(b)					
2nd	0·345	−0·173	2nd	< |0·1|	< |0·1|
3rd	0·367	−0·121	3rd	< |0·1|	0·135
4th	0·416	< |0·1|	4th	< |0·1|	< |0·1|
5th	0·405	< |0·1|	5th	0·117	0·470
Wander	0·418	< |0·1|	Wander	< |0·1|	0·642
Pupa	0·421	< |0·1|	Pupa	< |0·1|	0·554

PC, principal component.

later stages (5th, wandering, pupa) (Table 1b). These results suggest two important patterns. First, developmental rates (ages) are strongly positively correlated across all developmental stages: individuals that are younger at one stage tend to be younger at all stages, including the pupal stage. In contrast, size at the later larval and pupal stages is relatively independent of age or size at earlier stages.

ADULT SIZE, AGE AND EGG PRODUCTION

All individuals in the 2009 study entered pupal diapause over the winter, increasing the duration and mass loss during the pupal stage. Adult mass was significantly associated with PC2 scores [$P = 0{\cdot}008$: $\beta = 0{\cdot}102$ (0·034)] but not with PC1 scores [$P = 0{\cdot}190$: $\beta = 0{\cdot}027$ (0·029)] (Fig. 2). Thus, larger size at later larval and pupal stages (PC2) was associated with larger adult size, but there was no significant association of larval developmental rates (PC1) with adult size (Table 1a). Age at adulthood was not significantly associated with either PC1 [$P = 0{\cdot}425$: $\beta = -0{\cdot}006$ (0·007)] or PC2 [$P = 0{\cdot}862$:

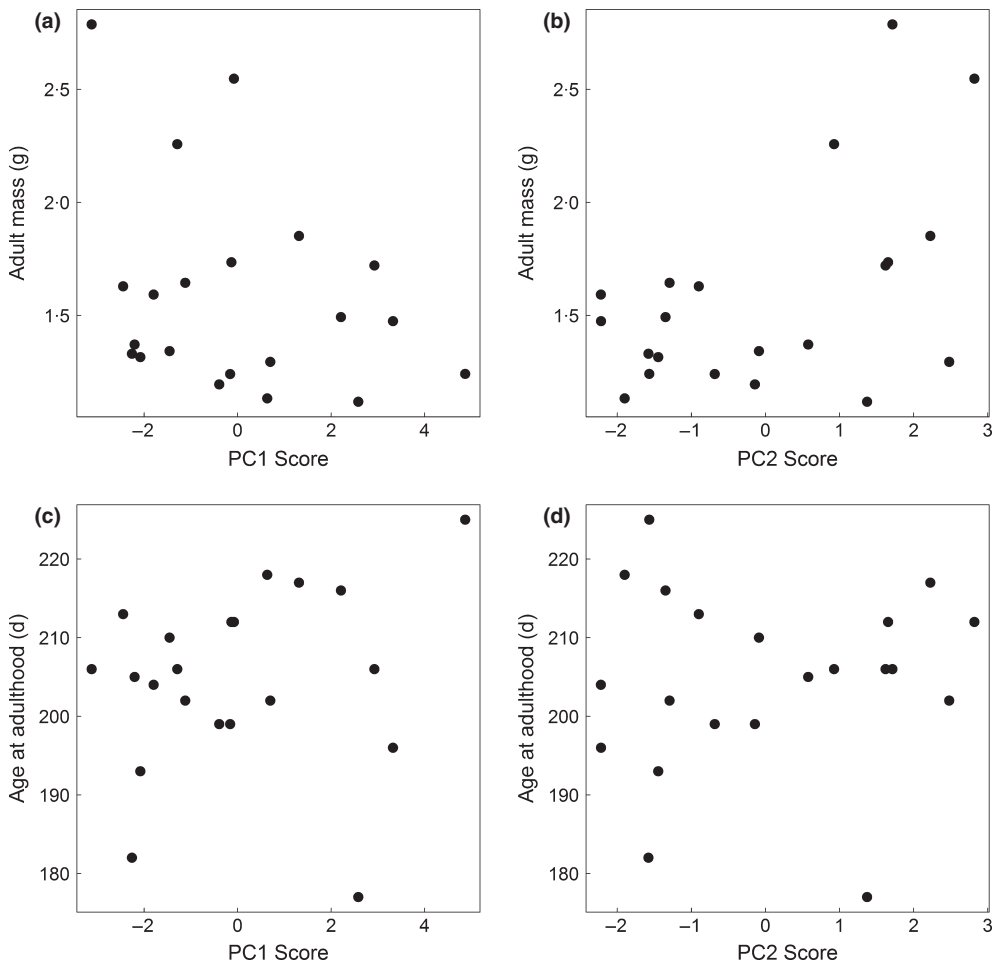

Fig. 2. Principal component (PC) analysis of variation in ages and masses across developmental stages for the 2009 study. PC1 scores reflect age at all larval stages (2nd to wandering) and at pupation (Table 1a); PC2 scores reflect mass at late larval stages (5th and wandering) and at pupation (Table 1b). (a) Adult mass (g) as a function of PC1 score, (b) adult mass (g) as a function of PC2 score, (c) age at adulthood (d) as a function of PC1 and (d) age at adulthood (d) as a function of PC2.

β = −0·001 (0·008)] (Fig. 2); this may reflect variability in the duration of pupal diapause in this study. Overall, pupal mass and age at pupation were uncorrelated (r = 0·058), but adult mass and age at adulthood were weakly correlated (r = 0·236); this resulted from the weak positive correlation between mass loss during pupation and the duration of the pupal stage (r = 0·18).

In the 2010 study, individuals did not enter pupal diapause prior to adult eclosion. In this study, adult mass was again strongly associated with PC2 scores [P < 0·001: β = 0·098 (0·009)] but not with PC1 scores [P = 0·126: β = −0·010 (0·006)] (Fig. 3). Thus, larger size at later larval and pupal stages (PC2) was associated with larger adult size, but there was no significant association of larval developmental rates (PC1) with adult size. Age at adulthood in this study was significantly associated with both PC1 [P < 0·001: β = −0·014 (0·001)] and PC2 scores [P < 0·001: β = 0·007 (0·002)]. Thus, earlier age at adulthood was associated with both more rapid development throughout the larval stages (PC1) and larger size at later larval and pupal stages (PC2). Again, pupal mass and age at pupation were essentially uncorrelated (r = −0·082), but adult mass and age at adulthood were weakly correlated (r = 0·264) because of the weak positive correlation between mass loss during pupation and the duration of the pupal stage (r = 0·164).

Egg production by females was measured in the 2010 study. There was no significant association of egg production with either age (P = 0·185) or mass (P = 0·164) at the 2nd instar. However, greater total egg production was significantly associated with larger pupal mass [P < 0·001: β = 0·086 (0·025)], but not with age at pupation [P = 0·686: β = −0·016 (0·021)] (Fig. 4). As expected, larger pupae (and adults) had greater fecundity in this study; but age or size at early larval stages was not significantly associated with fecundity.

Discussion

SURVIVAL AND VIABILITY SELECTION

Juvenile mortality in herbivorous insect populations is generally high, often exceeding 90% (Cornell & Hawkins 1985; Awmack & Leather 2002; Zalucki, Clarke & Malcolm 2002).

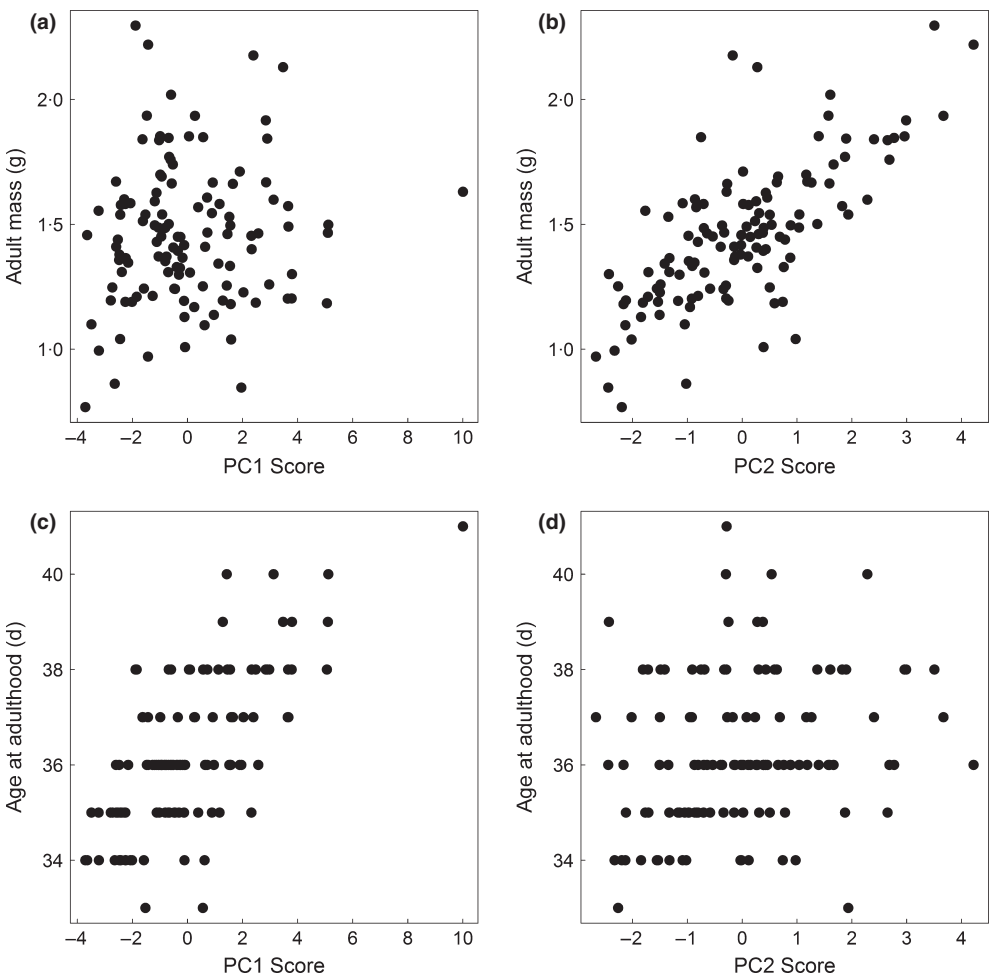

Fig. 3. Principal component (PC) analysis of variation in ages and masses across developmental stages for the 2010 study. PC1 scores reflect ages at all larval stages (2nd to wandering) and at pupation (Table 1a); PC2 scores reflect mass at late larval stages (5th and wandering) and at pupation (Table 1b). (a) Adult mass (g) as a function of PC1 score, (b) adult mass (g) as a function of PC2 score, (c) age at adulthood (d) as a function of PC1 and (d) age at adulthood (d) as a function of PC2.

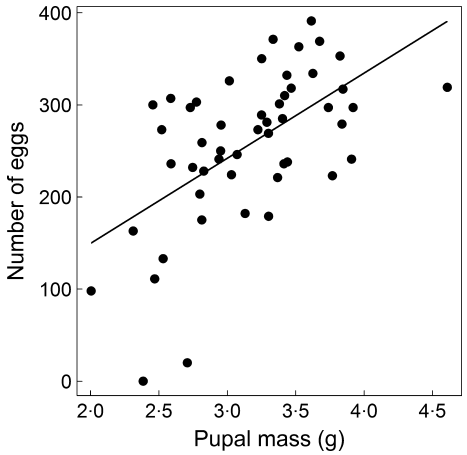

Fig. 4. Potential fecundity (number of eggs) as a function of pupal mass (g) for the 2010 study; the simple linear regression of fecundity on body size is indicated.

Juvenile mortality in the field typically results from a combination of weather, host plant quality and senescence, disease and natural enemies. By using field gardens of high-quality host plants, our studies focus on the potential impacts of natural enemies, especially larval parasitoids, on survival and viability selection in the field. Our results confirm that the larval parasitoid, *C. congregata*, can be an important factor in larval mortality of *M. sexta* in this population. In the fall 2009 study, parasitism rates exceeded 60%, with overall larval mortality (largely during the 5th instar, when the parasitoids emerge from and kill their host) of 88%. In the 2010 study, overall larval mortality was only 23%, with just 2% parasitism.

The difference in the rates of mortality owing to parasitoids between the two studies is likely due to two factors. First, *Cotesia* abundance varies seasonally, with lower parasitism rates of *M. sexta* in the first rather than in the later generations during the year. Previous studies at our field site suggest that parasitism can be quite variable, but may be greatest in late August and early September (S. E. Diamond, unpublished data). This may contribute to the higher rates of parasitism and mortality in the fall 2009 study (initiated in early September) than in the summer 2010 study (mid-July). Second, *C. congregata* primarily parasitize early (2nd–3rd) instar *M. sexta* larvae; larvae in the 2010 study were not exposed to parasitoids until the 3rd instar, reducing the time interval in which larvae were susceptible to parasitism (Beckage & Riddiford 1978; Walters *et al.* 2001).

The importance of natural enemies that specialize on early larval stages in this system suggests that rapid growth and development through these early instars could reduce the rates of larval mortality. In our studies, we placed larvae into the field at the start of the 2nd (2009) or 3rd (2010) instar. In the 2009 study, larvae that were younger at 2nd instar had significantly higher survival to pupation; and in the 2010 study, larvae that were younger at 3nd instar had marginally higher survival to pupation. Thus, the capacity for rapid larval development even at early instars may be important for escaping natural enemies, especially when rates of parasitoid attack are high.

In both studies, larvae that survived to pupation were younger and/or larger by the start of the 4th and 5th instars, compared with non-survivors (Fig. 1). Several factors may contribute to this. Infection and parasitism may slow growth and developmental rates, eventually contributing to death. Parasitism of 1st instar *M. sexta* by *C. congregata* can reduce rates of larval growth and development, but these reductions only emerge towards the end of the 3rd instar (Beckage & Riddiford 1978). In our studies, parasitism did not occur until 2nd instar or later, delaying the effects of parasitism on larval growth and development. As a result, the association of slower larval development with higher larval mortality in our studies cannot be explained entirely in terms of effects of parasitism itself on rates of larval growth and development (especially in the 2010 study with minimal parasitism).

These results suggest that rapid larval growth and development can allow larvae to escape natural enemies and increase survival rates. Benrey & Denno (1997) reported a similar finding for *Pieris rapae*: larvae that grew and developed more rapidly experienced lower juvenile mortality from the parasitoid *Cotesia glomerata*. Thus, parasitoids and other natural enemies may be an important factor in generating phenotypic selection on rapid growth and development in herbivorous insects (Bernays 1988; Benrey & Denno 1997). Alternatively, Bernays (1997) proposed that the presence of predators could select for slower rates of larval feeding and growth, decreasing visibility and exposure to potential predators. The experimental exclusion of potential predators (e.g. birds and social wasps) in our studies (see Methods) prevents us from evaluating this possibility.

THE STRUCTURE OF DEVELOPMENT TRAJECTORIES

The correlations among ages and sizes across developmental stages are key to predicting how selection on early larval stages may affect net selection and evolution of adult size and age at reproduction. Note that our PC analyses of developmental correlations here were for survivors, so the patterns cannot be explained in terms of potential differences between survivors and non-survivors. The first PC in both studies is dominated by positive loadings for ages across all developmental stages (Table 1), indicating phenotypic variation in overall developmental rate throughout the larval development. Masses at different stages do not load consistently on PC1. In contrast, the second PC is dominated by positive loadings for masses at later larval (5th instar and wandering) and pupal stages. However, masses at earlier larval stages have weak or even negative loadings for PC2 (Table 1). As a result, development rates in early larval instars are strongly associated with age at pupation. By contrast, size and growth rates in early instars are largely uncoupled from size at wandering and pupation. This correlation structure is also observed in laboratory studies of *M. sexta* in controlled temperature and food conditions (Gaydos 2007; Diamond & Kingsolver 2010a).

These results can be understood in terms of recent mechanistic studies of the physiological and developmental determinants of final size in *M. sexta* (Nijhout 1975, 2003; Davidowitz, D'Amico & Nijhout 2003, 2004; Nijhout, Davidowitz & Roff 2006). Maximal larval weight during the final instar is determined by several factors: a critical weight after which larvae begin to degrade juvenile hormone (JH); growth rate; and the time interval between degrading JH and secreting ecdysone (moulting hormone). Laboratory and modelling show that mass at wandering and pupation is largely determined by processes during the 5th (and to a lesser extent, 4th) larval instar, independent of size at earlier instars (Nijhout, Davidowitz & Roff 2006; Diamond & Kingsolver 2010a). As a result, pupal (and adult) mass and age are only weakly correlated phenotypically in this system, with the correlation depending in part on variation in the underlying developmental and physiological parameters (Davidowitz, Roff & Nijhout 2005; Nijhout, Roff & Davidowitz 2010). These results are also consistent with recent studies of *M. sexta* showing that egg temperatures affect early larval instars, but these effects dissipate at later instars (Potter, Davidowitz & Woods 2011).

These patterns are further supported by our regression analyses. In both studies, earlier age at 2nd instar was significantly associated with earlier age at pupation. In addition, larger mass at 2nd instar was significantly associated with earlier age at pupation. In contrast, neither age nor mass at 2nd instar was significantly associated with mass at pupation in either study. As a result, phenotypic variation in age and mass at pupation is largely uncoupled in this system. This has important implications for indirect and direct components of selection on age and size.

DIRECT AND INDIRECT SELECTION ON ADULT AGE AND SIZE

In both studies, larger adult mass was significantly associated with higher PC2 scores, but not with PC1 scores (Figs 2–3). The fact that mass at wandering and pupation (PC2) strongly determines adult mass is not surprising, because there is no food or water intake from wandering to adult eclosion. However, adult mass is not significantly influenced by development rates (PC1) or mass at early larval stages (Table 1). Data from the 2010 study showed that larger adult mass was significantly associated with greater egg production by females (Fig. 4). As in many field studies of phenotypic selection, our results indicate positive directional selection for increasing adult size via fecundity (Kingsolver & Pfennig 2004; Kingsolver & Diamond 2011). Conversely, in the 2010 study, earlier age at adulthood was significantly associated with earlier ages at all larval and pupal stages (PC1), as well as with smaller mass in the late larval and pupal stages (PC2). This relationship between PC1 and age at adulthood was not detected in the 2009 study, probably because of variability in the duration of pupal diapause over the winter (~150–190 days) in this study.

Collectively, our results identify three distinct aspects of phenotypic selection on developmental trajectories, including size and age at adulthood. First, female fecundity increased with adult female size. This result is consistent with numerous past empirical studies and with theoretical expectations. However, fecundity was not significantly associated with age at adulthood, or with mass or age at early larval stages. Second, rapid development by young larvae was associated with earlier age of adulthood, and thus shorter generation time. This may potentially influence overall fitness by allowing additional generations per year. Quantifying the effects of generation time on fitness is challenging because of the discrete number of generations per year (2 or 3) in this population (Roff 1980, 2002; Taylor 1980). However, age at adulthood was weakly correlated with adult body mass, and this correlation was unrelated to growth and development during the larval stages.

Third and most intriguingly, viability selection favoured faster rates of development (and perhaps growth) during the early larval stages. Viability selection in this population was due largely, but not entirely, to the larval parasitoid *C. congregata*. Because age at early larval instars is strongly correlated with age at adulthood, viability selection on larvae may indirectly influence changes in age at adulthood. However, because early larval development is not related to adult body mass, viability selection on larvae will not influence adult mass. In this sense, selection on adult size and selection on development rates operate independently in this system. The lack of significant negative correlations between adult size and age supports this conclusion: we found no evidence for the anticipated trade-off between body size and development rate, at least at the phenotypic level. Because larvae were assigned randomly to host plants in our studies, environmental covariation is unlikely to account for this finding (Rausher 1992; Stinchcombe *et al.* 2002).

Several lines of evidence support the apparent evolutionary independence of adult size and reproductive age in *Manduca*. Domesticated laboratory lines of *M. sexta* have evolved increased body mass at the wandering, pupal and adult stages, relative to ancestral and field populations (D'Amico, Davidowitz & Nijhout 2001). However, this increased body size has not been associated with substantial changes in development times, at least under good nutritional or host plant conditions (Diamond & Kingsolver 2010a, 2011; Diamond *et al.* 2010). In addition, substantial evolutionary changes in the size of eggs or early larval instars have not occurred during domestication (Kingsolver 2007; Kingsolver & Nagle 2007). Finally, artificial selection experiments and quantitative genetic studies with outcrossed, domesticated populations show that pupal body mass and development time can evolve independently in response to simultaneous selection on size and time (Davidowitz, Roff & Nijhout 2005; Nijhout, Roff & Davidowitz 2010).

These results contradict a critical assumption of many life-history models: a deterministic trade-off between adult size and development rate (Roff 2000). Our studies indicate both direct selection favouring increased adult size (via fecundity)

and direct selection favouring rapid larval development (via juvenile survival). But these do not result in indirect negative selection on adult size, because pupal size and larval development rates are not negatively associated in this system. More mechanistic approaches are needed to properly understand how developmental and physiological processes generate covariation among life-history traits in this system (Davidowitz, Roff & Nijhout 2005; Nijhout, Roff & Davidowitz 2010).

A trade-off between adult size and development rate is also central to most models for the evolution of thermal reaction norms for size and development time (Angilletta 2009). For example, adaptive models for the temperature–size rule predict net selection for smaller adult size under warmer conditions, reflecting a balance between direct fecundity selection on size and indirect viability selection on juvenile development rate (Berrigan & Charnov 1994; Berrigan & Koella 1994; Angilletta 2009). Our studies in both cool fall (2009) and warm summer (2010) field conditions do not support these predictions. How physiological mechanisms and phenotypic selection interact to determine thermal reaction norms and the temperature–size rule in insects remains to be established (Davidowitz & Nijhout 2004; Nijhout, Davidowitz & Roff 2006; Angilletta 2009; Diamond & Kingsolver 2010a).

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