Variation in growth and instar number in field and laboratory *Manduca sexta*

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The tobacco hornworm *Manduca sexta* has been an important model system for understanding physiological control of growth, development and metamorphosis of insects for more than half a century. Like all *Manduca*, *M. sexta* typically has five larval instars, with developmental commitment to metamorphosis occurring early in the 5th (final) instar. Here we show that *M. sexta* from a field population in North Carolina (USA) shows substantial intraspecific variation in the number of larval instars when feeding on a modified artificial diet. Individuals with six instars consistently exhibited slower growth rates during early larval development than individuals with five instars. The frequency of individuals with six instars decreased with increased rearing temperature. In contrast, *M. sexta* from a laboratory colony consistently had five instars, and had more rapid larval growth rates than *M. sexta* from the field. We identify a threshold body size at the start of the 5th instar that predicts whether an individual will have five (greater than 600 mg) or six instars (less than 600 mg). Variation in field populations in *Manduca* provides an important resource for understanding physiological control, developmental plasticity and evolution of growth rate, body size and instar number.

Keywords: body size evolution; developmental plasticity; growth rate; instar number; *Manduca sexta*

1. INTRODUCTION

The microevolution of development—how intraspecific variation in development leads to evolution at the population level—is a fundamental challenge for evolutionary biology (NSF Report 2005a). Identifying the causes and consequences of individual variation in development and developmental plasticity is key to understanding how phenotypic variation arises, is selected and evolves within species (NSF Report 2005b). Here we present results from a study of intraspecific variation in developmental plasticity in the tobacco hornworm, *Manduca sexta*. *M. sexta* has been a major model system in physiology and developmental biology for more than half a century, contributing importantly to our understanding of the physiology and hormonal control of moulting and metamorphosis (Nijhout 1994; Gilbert *et al.* 2000; Riddiford *et al.* 2003; Truman *et al.* 2006). These studies have been facilitated by *Manduca*'s pest status, rapid growth, large size and ease of laboratory rearing.

Laboratory colonies of *M. sexta* have been maintained continuously since at least the 1960s. To our knowledge, all current major scientific laboratory colonies of *M. sexta* are ultimately derived, directly or indirectly, from a colony in Raleigh, NC that was established in the 1960s from field collections in eastern Piedmont, NC (Clayton, NC; Yamamoto & Fraenkel 1960; Yamamoto *et al.* 1969; Yamamoto 1974). Under standard *Manduca* rearing conditions, this represents more than 240 generations in laboratory conditions since the colonies were first established. D'Amico *et al.* (2001) showed that maximal larval mass increased by approximately 50% in a laboratory population of *M. sexta* between 1971–1973 and 1999–2000, as a result of evolutionary changes in growth rate,

critical weight and the timing of hormonal secretion during the final larval instar. Recent studies demonstrate that field and laboratory populations of *M. sexta* have diverged in tolerance and sensitivity to high temperatures, and in critical photoperiod for diapause initiation (J. G. Kingsolver & A. M. Nagle 2006, unpublished data).

All *Manduca* species (including *M. sexta*) typically have five larval instars, although some sphingid genera (primarily in the subfamily Smerinthinae) have six larval instars (I. Kitching 2006, personal communication). Hormonal control of moulting and metamorphosis in *Manduca* is well understood (Nijhout 1994; Gilbert *et al.* 2000; Riddiford *et al.* 2003). Both larval and metamorphic moults are controlled by the steroid hormone ecdysone (Nijhout 1994). Juvenile hormone (JH) determines whether a moult is larval or metamorphic. The decline of JH during the early stages of the final instar switches the imaginal disks to pupal commitment; in the absence of JH, ecdysone causes the body wall epidermis to switch to pupal commitment (Kremen & Nijhout 1989, 1998; Riddiford 1994).

In this paper, we explore the differences in growth trajectories of *M. sexta* from field and laboratory populations feeding on a modified artificial diet, and document substantial variation in the number of larval instars within the field population. We describe variation in growth rate and instar number in field, laboratory and F1 crosses. We examine the effects of rearing temperature on growth rate and instar number, and the consequences of instar number for development time and final body size. By combining data across different rearing temperatures and different genetic lines, we identify a threshold size at the start of the 5th instar that predicts the ultimate number of instars for an individual. Our results illustrate how intraspecific variation in growth and instar number in

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field populations of *M. sexta* represents an important resource for exploring the physiological control of growth, development and size in this classic model system.

2. MATERIAL AND METHODS

The studies used *M. sexta* from two sources. The laboratory population was taken from a laboratory colony maintained under standard larval rearing conditions (artificial diet, constant 25°C, 15h L : 9h D photocycle) by L. Gilbert and colleagues at UNC for over 25 years. This colony is ultimately descended from the Raleigh, NC colony described above. The field population was established from eggs collected in tobacco fields at the NC State University Research Station in Clayton, NC (Sampson County). These two populations have been separated for more than 35 years, representing more than 240 in the laboratory and more than 70 generations in the field. After hatching, larvae were reared individually in Petri dishes in environmental chambers (Percival model 36-VL) on standard *Manduca* diet (Riddiford 1967), with the addition of a small amount of dried tobacco leaf (8.3% by dry mass of diet); this addition facilitated consistent feeding and growth by the field larvae (see §4). All experiments were conducted during the summer and autumn of 2005. Under constant temperature conditions between 20 and 30°C, we can achieve egg to adult survival rates exceeding 80% for both the laboratory and the field populations (J. G. Kingsolver & A. M. Nagle 2006, unpublished data).

Each experiment was initiated with newly hatched 1st instar larvae. For each experiment, the growth trajectories of 30–40 individuals from each population were determined by recording body mass and development time beginning with the start of the 3rd instar, for each of the following developmental stages: the start of each larval instar, immediately following moulting but prior to feeding; wandering or maximal larval size; pupation; and adult eclosion. Larvae were maintained under a long-day (16 h L : 8 h D) photocycle at one of the three constant rearing temperatures: 20, 25 or 30°C. Following wandering, larvae were placed individually in wooden pupation blocks at room temperature until eclosion. The results reported here only include those individuals that survived to eclosion.

We also measured growth trajectories at 25°C for 30 F1 progeny resulting from crosses between field and laboratory individuals. We considered crosses between field males and laboratory females and between field females and laboratory males; because we detected no effect of the direction of the cross the results presented here are combined.

We used analyses of variance (ANOVA) to test the effects of sex, population (laboratory or field) and instar number (five or six) on body mass and development time at start of the 5th instar, wandering, pupation and eclosion.

3. RESULTS

Mean sizes of eggs and hatchlings in the field and the laboratory populations were similar (mean (± 1 s.d.) hatchling mass: field, 1.17 ± 0.11 mg; laboratory, 1.10 ± 0.15 mg). At standard laboratory temperatures (25°C), larval growth rates for the laboratory and the field populations were approximately exponential (linear on a log scale; figure 1a). The laboratory population grew more rapidly throughout the larval development, and achieved significantly larger body masses at the start of the 5th instar, wandering, pupation and eclosion than did the field

Figure 1. Growth trajectories of *Manduca sexta* reared at 25°C. (a) Body mass (in ln(mg)) as a function of development time (d) for laboratory (squares) and field (diamonds) *M. sexta* with five (filled symbols) or six (open symbols) larval instars. Mean ± 1 s.d. is indicated. Stages represented: start of the 3rd, 4th, 5th, (6th) instars; wandering (maximum larval size); pupa; adult. (b) Body mass (in ln(mg)) as a function of development time (d) for F1 progeny of lab $\times$ field crosses, with five (filled symbols) or six (open symbols) larval instars. Mean ± 1 s.d. is indicated. Stages represented: start of the 3rd, 4th, 5th, (6th) instars; wandering (maximum larval size); pupa; adult.

population (ANOVAs: $p \ll 0.001$ in all cases). The mean times to wandering, pupation and eclosion were significantly shorter in the laboratory than the field population ($p < 0.003$ in all cases). There was greater variability in masses and development times at equivalent stages in the field population when compared with the laboratory population.

Laboratory individuals invariably had five larval instars. In contrast, 47% of the field individuals had six larval instars, with the remainder having five instars (figure 1). In the field population, development times to the start of the 3rd, 4th and the 5th instars were similar for five- and six-instar individuals, but individuals with six instars had slower growth rates. By the start of the 5th instar, the mean body mass was more than twofold greater for five-instar than for six-instar individuals ($p \ll 0.001$). As a result of the additional instar, mean times to wandering, pupation and eclosion were significantly delayed by 4–6 days in six-instar when compared with five-instar individuals ($p < 0.001$ in all cases). In addition, six-instar individuals showed greater variability in development times at each of these stages (figure 1). However, mean body masses at wandering ($p = 0.024$) and pupation ($p = 0.014$) were significantly greater in six-instar than in five-instar individuals.

Figure 2. Body mass (in $\ln(\text{mg})$) as a function of development time (d) for the field population of *M. sexta*, with five (filled symbols) or six (open symbols) larval instars. Mean ± 1 s.d. is indicated. Stages represented: start of the 3rd, 4th, 5th, (6th) instars; wandering (maximum larval size); pupa; adult. (a) Rearing temperature of 20°C. (b) Rearing temperature of 30°C.

F1 progeny of field $\times$ lab crosses yielded a mix of five-instar (52%) and six-instar (48%) individuals at 25°C (figure 1b). By the start of the 5th instar, mean body mass was more than threefold greater for five-instar than for six-instar individuals ($p \ll 0.001$). The mean times to wandering, pupation and eclosion were significantly delayed in six-instar when compared with five-instar individuals ($p \ll 0.001$ in all cases). The mean body masses at wandering, pupation and eclosion were significantly greater in six-instar than in five-instar individuals ($p < 0.003$ in all cases).

Not surprisingly, growth and developmental rates of the field population increased with increasing temperatures between 20 and 30°C (figure 2). Developmental trajectories of five- and six-instar individuals at 20 and 30°C (figure 2) were qualitatively similar to those at 25°C (figure 1). However, the proportion of larvae with six instars decreased dramatically with increasing temperature, with 69% at 20°C, 47% at 25°C and 17% at 30°C. At 20°C, times to wandering, pupation and eclosion were significantly delayed in six-instar when compared with five-instar individuals ($p < 0.003$ in all cases). The mean body masses at wandering, pupation and eclosion were greater in six-instar than in five-instar individuals, but this difference was only significant for wandering mass ($p = 0.035$). At 30°C, times to wandering and pupation were again significantly delayed in six-instar when compared with five-instar individuals ($p = 0.005$). In contrast to lower temperatures, the mean body masses at

Figure 3. Body mass (mg) and development time (d) at the start of the 5th larval instar for field (diamonds), laboratory (squares) and F1 progeny (circles) *M. sexta* that have five (filled symbols) or six (open symbols) larval instars. Rearing temperature of 25°C.

Figure 4. Body mass (mg) and development time (d) at the start of the 5th larval instar for field *M. sexta* that have five (filled symbols) or six (open symbols) larval instars. All rearing temperature treatments combined.

wandering, pupation and eclosion were not significantly affected by instar number at 30°C.

By combining data for the laboratory, field and F1 groups at 25°C, a clear pattern for instar number emerges (figure 3). At the start of the 5th instar, larvae with body masses above 600 mg had five instars (greater than 95%), whereas those below 600 mg had six instars (greater than 95%). For example, all laboratory individuals had masses above 1000 mg at the start of the 5th instar, and all metamorphosed during the 5th instar. By contrast, the development time to the 5th instar was not a useful predictor of the instar number.

Combining data across different temperature treatments for the field population reveals a similar pattern (figure 4). At the start of the 5th instar, larvae with body masses above 600 mg had five instars (greater than 95%), whereas those below 600 mg had six instars (greater than 95%). Again, the development time to the 5th instar was not a useful predictor of the instar number. These results suggest that there is a threshold size at the start of the 5th instar that determines the developmental status of the 5th instar (see below).

4. DISCUSSION

Under standard laboratory conditions, *M. sexta* from our laboratory population achieved more rapid growth rates and greater maximal sizes than those from the field

population (figure 1). D'Amico *et al.* (2001) showed that for a *Manduca* laboratory colony during 1971–1973 (soon after the initial establishment of the colony in the late 1960s), the mean maximum larval mass was 7.8 g—very similar to that observed for our current field population (mean = 7.9 g). This suggests that the divergence in size between laboratory and field populations during the past 40 years is primarily due to increases in growth rate and size in laboratory populations as a result of adaptation to laboratory conditions, as shown by the studies of D'Amico *et al.* (2001; J. G. Kingsolver & A. M. Nagle 2006, unpublished data). These changes are probably due to inadvertent selection in the laboratory for large size and rapid growth rates (D'Amico *et al.* 2001; Davidowitz *et al.* 2003, 2004, 2005; Davidowitz & Nijhout 2004).

A major finding of our studies was substantial variation in the number of larval instars within the field population of *M. sexta*. Variation in instar number in response to rearing temperature and nutrition has been documented in many insects, including other Lepidoptera (Wigglesworth 1972). In our study, instar number was strongly correlated with growth rate during early larval development: field individuals with more rapid growth had five instars, whereas those with slower growth had six instars (figures 1 and 2). By contrast, laboratory individuals consistently had rapid growth rates and five larval instars. F1 progeny from crosses between laboratory and field parents also showed a mix of five and six instars (figure 1). These results indicate that there are genetic differences among field, laboratory and F1 lines in the propensity to express additional larval instars during development in standard laboratory conditions.

Instar number in field individuals is also a developmentally plastic trait. For field individuals feeding on our artificial diet, the frequency of six-instar individuals decreased with increasing rearing temperatures between 20 and 30°C (figure 2). This response to rearing temperature suggests that more rapid growth rates at higher temperatures reduce the propensity to express additional instars. By contrast, when feeding on tobacco, larvae from the field population consistently have more rapid growth rates and five larval instars (S. Dyer *et al.* 2006, unpublished data). Similarly, many studies with laboratory colonies (including ours) show that *M. sexta* larvae consistently have five instars for a variety of different thermal conditions and food types (Reynolds & Nottingham 1985; Reynolds *et al.* 1986; Stamp & Horwath 1992; Stamp 1993, 1994; Petersen *et al.* 2000).

These results suggest that there is substantial variation in plasticity for instar number within our field population of *M. sexta*, whose expression is affected by food quality and rearing temperature, a pattern described in several other Lepidoptera (Wigglesworth 1972). The propensity to express additional instars in the field *M. sexta* was strongly associated with slower growth rate during early larval development. This variation in developmental plasticity in instar number has apparently been largely lost in laboratory populations (but see below). We propose that selection for rapid growth rate and large size during adaptation to artificial diet and laboratory conditions during the past 35–40 years has led to the loss of plasticity in laboratory colonies of *M. sexta*. Conversely, variation in growth rate and instar number in field populations of *M. sexta* represents an important resource for

understanding the development biology and physiological control growth, moulting and metamorphosis in this classic model system.

Early studies with *M. sexta* showed that additional larval instars can be induced experimentally in the laboratory (Nijhout 1975). For example, by starving 4th instar larvae at various weights, Nijhout (1975) showed that larvae less than approximately 600 mg at the start of the 5th instar (a head capsule width of 5 mm) could be induced to add an additional larval instar. Our studies indicate that a similar threshold exists for field and F1 larvae feeding on artificial diet (figures 3 and 4): individuals weighing more than 600 mg at the start of the 5th instar are committed to pupal development and have five instars, whereas those less than 600 mg add one (or potentially more) larval instar(s) before moulting into the final (metamorphic) instar. Because all individuals from laboratory colonies are greater than 600 mg at the start of the 5th instar, they consistently complete development in five instars.

These results suggest the following scenario for growth, development and instar number in *M. sexta* (Nijhout 1981). Different lineages of *M. sexta* are adapted for rapid growth on particular food resources, resulting in normal growth trajectories with five larval instars. The artificial diet represents a novel, low-quality food resource for *M. sexta* from field populations. This novel resource generates increased variation in growth trajectories among individuals, due to variability in feeding stimulation or nutritional responses. Growth rates are affected more than development rates, resulting in substantial variation in size at the start of the 5th larval instar. Individuals below a threshold size of 600 mg body mass at this stage initiate one (potentially more) additional larval instar, before moulting into the final (metamorphic) larval instar (Nijhout 1981).

The instar number has potentially important consequences for life history and fitness traits in *Manduca*. In our laboratory studies with field and F1 individuals, the instar number affected both development time and body size. For example at 25°C, the mean time from hatchling to pupation was 24% longer for six-instar when compared with five-instar individuals in the field population; but mean pupal mass was 20% greater for six-instar when compared with five-instar individuals (figure 1). Thus, the additional larval instar increased the total development time, but resulted in a larger pupal size (figures 1 and 2).

In field conditions, slow growth rate and longer development times can increase larval mortality due to natural enemies (Benrey & Denno 1997). Longer development times could also increase generation time and reduce the number of complete generations per year (*M. sexta* overwinters in a pupal diapause). Body size has many potential consequences for fitness (Kingsolver & Pfennig 2004). For example, in laboratory colonies of *M. sexta*, pupal mass is positively correlated with the number of mature eggs in the ovaries after eclosion, explaining 72% of the variation in egg number (Davidowitz *et al.* 2004, 2005). Thus, additional larval instars may decrease some fitness components (development rate and larval survival) and increase others (body size and fecundity). This raises the possibility that developmental plasticity in instar number may represent an adaptive response to novel food resources or slow growth conditions in *Manduca*, at

least at some temperatures. Whether adding instars is an adaptation or a maladaptive response to inadequate diets remains to be explored.

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