Synergism and Antagonism of Proximate Mechanisms Enable and Constrain the Response to Simultaneous Selection on Body Size and Development Time: An Empirical Test Using Experimental Evolution

Goggy Davidowitz,^{1,*} Derek Roff,² and H. Frederik Nijhout³

1. Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona 85721; 2. Department of Biology, University of California, Riverside, California 92507; 3. Department of Biology, Duke University, Durham, North Carolina 27708

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ABSTRACT: Natural selection acts on multiple traits simultaneously. How mechanisms underlying such traits enable or constrain their response to simultaneous selection is poorly understood. We show how antagonism and synergism among three traits at the developmental level enable or constrain evolutionary change in response to simultaneous selection on two focal traits at the phenotypic level. After 10 generations of 25% simultaneous directional selection on all four combinations of body size and development time in *Manduca sexta* (Sphingidae), the changes in the three developmental traits predict 93% of the response of development time and 100% of the response of body size. When the two focal traits were under synergistic selection, the response to simultaneous selection was enabled by juvenile hormone and ecdysteroids and constrained by growth rate. When the two focal traits were under antagonistic selection, the response to selection was due primarily to change in growth rate and constrained by the two hormonal traits. The approach used here reduces the complexity of the developmental and endocrine mechanisms to three proxy traits. This generates explicit predictions for the evolutionary response to selection that are based on biologically informed mechanisms. This approach has broad applicability to a diverse range of taxa, including algae, plants, amphibians, mammals, and insects.

Keywords: simultaneous selection, antagonistic selection, synergistic selection, *Manduca sexta*.

Introduction

Body size and development time are two traits often correlated with fitness and as such are of much interest to biologists. Often, there is strong directional selection for body size to increase and development time to decrease (Kingsolver and Pfennig 2004; Kingsolver and Huey 2008). Natural populations of many organisms, however, are likely to encounter simultaneous selection favoring different antagonistic or synergistic combinations of body size and development time.

Traits such as body size and development time are the end products of complex genetic, physiological, developmental, and endocrine processes. How these underlying processes may constrain their simultaneous selection remains poorly understood. There is a growing interest in addressing this gap in our understanding by bringing these underlying developmental processes into a life-history and population-level context (e.g., Zera and Harshman 2001; National Research Council 2008; Gilbert and Epel 2009; Schwenk et al. 2009; Mykles et al. 2010; Flatt and Hyland 2011; Zamer 2011; Padilla et al. 2013; Martin et al. 2014). Incorporating mechanism into life history illuminates how traits can respond to selection and generates stronger and more nuanced predictions for why organisms and populations respond to selection the way that they do. Natural selection acts on whole organisms and therefore multiple traits simultaneously. No study to date has examined the underlying mechanisms of multiple traits under simultaneous selection.

Here we provide the first empirical test of how underlying proximate mechanisms enable and/or constrain the response of multiple traits to simultaneous selection. We focus on two major fitness-related traits, body size and development time, in the model species *Manduca sexta* (tobacco hornworm, Sphingidae). *Manduca sexta* is an ideal

* Corresponding author. Present address: Department of Entomology, University of Arizona, Tucson, Arizona 85721; e-mail: goggy@email.arizona.edu.
ORCID: Davidowitz, 0000-0001-5769-2552.

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model organism to address this question because natural populations are likely to encounter natural selection favoring different combinations of body size and development time. Furthermore, there is a great deal known about the regulation of body size and development time in this species (D'Amico et al. 2001; Davidowitz et al. 2003, 2004, 2005, 2012; Davidowitz and Nijhout 2004; Nijhout et al. 2006, 2010, 2013; Davidowitz and Helm 2015; Helm and Davidowitz 2015).

The geographic distribution and natural history of *M. sexta* suggest that different populations may experience simultaneous selection for different combinations of small or large body size and short or long development time. *Manduca sexta* has a broad geographic range, from southern Canada to Chile, and thus will encounter a broad range of biotic and abiotic conditions. Its diet is exceptionally narrow among Lepidoptera: larvae (the tobacco hornworm) feed almost exclusively on plants in the nightshade family (Solanaceae) but can also be found on devil's claw (*Proboscidea* spp., Martyniaceae; Mechaber and Hildebrand 2000).

In the laboratory, body size and development time in *M. sexta* are affected by both temperature and diet quality: larvae are larger on a higher-quality diet and at colder temperatures. In contrast, development time is shorter on a high-quality diet and under higher temperatures (Davidowitz et al. 2004). In nature, *M. sexta* body size, development time, survival, and reproductive success are all affected by differences in diet quality among (Mechaber and Hildebrand 2000; Mira and Bernays 2002; Diamond and Kingsolver 2010a, 2010b; Contreras et al. 2013) as well as within (Potter et al. 2012; Thaler et al. 2013) single host plants. Differential selection with consequences for body size, development time, and survival may also occur from the interactions of host-plant quality with parasitism (Mira and Bernays 2002; Diamond and Kingsolver 2010; Wilson and Woods 2015) and predation (Mira and Bernays 2002; Thaler et al. 2013). In nature, *M. sexta* also experiences a broad range of temperatures, both among populations and within a single population (Casey 1976; Potter et al. 2009, 2011). Such effects of temperature on body size, development time, survival, and fecundity in *M. sexta* can, however, reverse on low-quality versus high-quality host plants (Diamond and Kingsolver 2010). This increases the potential combinations of body size and development time on which natural selection may act in natural populations.

We imposed 10 generations of simultaneous directional selection on all four combinations of body size (big or small) and development time (short or long)—Big/Short, Big/Long, Small/Short, and Small/Long—to address three questions: (1) How does the underlying developmental mechanism enable or constrain the simultaneous response to selection of body size and development time? (2) Can the relationships among the developmental parameters accurately predict the

response to simultaneous selection of the two focal traits? (3) How does the physiology underlying the developmental traits respond to selection? Our results show how antagonistic and synergistic selection at the developmental-mechanistic-proximate level determines the response to simultaneous directional selection at the phenotypic ultimate level.

Regulation of Body Size and Development Time in M. sexta

Three mechanistic traits underlie body size and development time: the critical weight (CW), the interval to cessation of growth (ICG), and growth rate (GR; fig. 1). These traits are themselves regulated by a complex interaction of developmental, endocrine, physiological, and molecular processes (Nijhout et al. 2013; Gokhale and Shingleton 2015). In this study, we focus on the three mechanistic traits, rather than on the more complex processes underlying them, to facilitate explicit and testable predictions for how the underlying mechanism can constrain or enable the response to simultaneous selection on the two focal traits (Davidowitz 2016). For simplicity, we refer to the three mechanistic traits (CW, ICG, and GR) as “developmental traits” or “proximate mechanisms,” although it should be clear that they encapsulate complex interactions of the developmental, endocrine, physiological, and molecular processes that underlie them (Davidowitz 2016).

The Critical Weight (CW). A larva in its final developmental stage (instar) feeds and grows until it reaches a threshold mass (fig. 1), termed the “critical weight” (CW; Nijhout and Williams 1974). Attainment of the CW is the decision point for the life-history transition from the juvenile growth phase to the reproductive adult phase in *M. sexta* (as it is in a broad range of taxa; see Davidowitz and Helm 2015). At the CW, the corpora allata, the glands that synthesize and secrete juvenile hormone (JH), switch off (Nijhout and Williams 1974). At this point the circulating titers of JH drop precipitously (fig. 1; Baker et al. 1987; Riddiford 1994, 1995), as a result of catabolism by JH esterase. The CW in *M. sexta* is typically about 54% of peak larval mass (Davidowitz et al. 2004). The CW is not static but can evolve (D'Amico et al. 2001; Davidowitz et al. 2003, 2012); it is sensitive to diet quality but not to temperature (Davidowitz et al. 2003, 2004). Higher CWs result in larger peak larval sizes and longer development times, because the cascade of events that terminate growth occurs later and at a larger size (Davidowitz et al. 2005). The CW sets in motion an irreversible cascade of endocrine, developmental, and physiological events that ultimately lead to pupation and metamorphosis. Recent work has shown that the decision to transition from the juvenile to the adult stage is determined by the accumulation of a threshold amount of resources in the larval fat

Figure 1: Representation of the developmental traits that regulate body size and development time in the last larval instar of *Manduca sexta*. The diagonal blue line represents the growth trajectory of the larva. The thick blue line on the growth trajectory indicates when growth rate (GR) was measured. The green circle and dotted green dotted line indicate the critical weight (CW). The dotted and dashed teal lines indicate the interval to cessation of growth (ICG). The black rectangle represents the photoperiodic gate for prothoracicotrophic hormone (PTTH) secretion. The red circle indicates the cessation of growth, and the dashed red triangle represents the secretion of the ecdysteroids within the PTTH photoperiodic gate. The dashed orange line represents juvenile hormone (JH) titer, which drops dramatically after the CW.

body that are about 20% of larval biological mass, irrespective of growth and resource variation imposed by either diet or temperature (B. R. Helm and G. Davidowitz, unpublished data).

The Interval to Cessation of Growth (ICG). In the last larval instar, JH inhibits PTTH (prothoracicotrophic hormone). A number of days after the larva passes the critical weight and JH is cleared from the hemolymph, secretion of PTTH is disinhibited and in turn induces the secretion of ecdysteroids (fig. 1) from the prothoracic gland (Bollenbacher et al. 1979; Rountree and Bollenbacher 1986). The ecdysteroids, also called molting hormones, regulate molting and metamorphosis in insects. The secretion of PTTH is governed by a photoperiodic clock and can occur only during a well-defined window of time, called a photoperiodic gate (fig. 1), that reoccurs each day (Truman 1972). Secretion of PTTH occurs during the first photogate that follows complete clearance of JH from the hemolymph (Truman 1972; Truman and Riddiford 1974). Calculation of the ICG, therefore, requires knowledge of when PTTH is secreted within the photogate (see app. A for further details). The first pulse of ecdysteroids (fig. 1) leads to a cessation of larval feeding and growth. The larva then purges any remaining food from its gut and wanders in search of a pupation site (Dominick and Truman

1984, 1985). Thus, as in all other insects, the secretion of PTTH and ecdysteroids is the mechanism that stops growth (Nijhout 1994; Nijhout et al. 2013).

The time between attaining the critical weight and the secretion of PTTH and ecdysteroids is termed the “interval to the cessation of growth” (ICG; fig. 1). During the ICG, the larva feeds and grows normally and can more than double its size (Davidowitz et al. 2003, 2004). The ICG is sensitive to variation in temperature but not to diet quality (Davidowitz et al. 2004). Increasing the ICG allows a larva more time to feed, which results in a larger body size and a longer development time (Davidowitz et al. 2005).

The timing of the critical weight and the timing of the ICG together determine the duration of the growth period of the last (fifth) instar larva (fig. 1). The duration of the growth period of the fifth instar, together with the development time of the first four instars, determines the total development time of the *M. sexta* larva.

Growth Rate (GR). The third trait involved in the regulation of body size and development time is growth rate (GR; fig. 1). Growth rate is often considered a performance trait or even a life-history trait in itself. Here, however, we consider GR a mechanistic trait, because it interacts with the critical weight and the ICG to determine the final larval size and total de-

velopment time of the last larval instar. The GR is sensitive to both diet and temperature (Davidowitz et al. 2004). The GR determines how rapidly a larva will attain the critical weight but not the critical weight itself. The GR also determines the amount of mass accumulated during the ICG but not the duration of the ICG. Therefore, the GR comes into play in determining size only during the ICG, but it affects development time only in the time required to reach the critical weight (Davidowitz and Nijhout 2004). Growth itself is regulated by five signaling pathways, the most important of which are the insulin signaling (IIS) and the target-of-rapamycin signaling (TOR) pathways (Gokhale and Shingleton 2015). The IIS and TOR pathways are the intermediaries between nutrition and growth (Nijhout et al. 2013), although recent evidence shows that JH masks their effects in regulating body size in *M. sexta* (Hatem et al. 2015) and regulates GR by mediating growth through the ecdysteroid and IIS pathways in *Drosophila* (Mirth et al. 2014). We measured GR only in the last larval instar (fig. 1), as it is in this instar that more than 90% of growth occurs (Davidowitz et al. 2004) and is also when growth ceases.

Predictions for How the Underlying Mechanism Regulates the Response to Selection

Incorporating proximate mechanisms into our understanding of how traits evolve allows us to make explicit and testable predictions about how the underlying mechanism enables and/or constrains the response to simultaneous selection on the two traits (Davidowitz et al. 2005). For example, when body size and development time are both selected to increase (upper-right quadrant of fig. 2), the response to selection will be determined by an increase in CW and ICG, as both are under synergistic selection and both are expected to increase. It will be constrained by GR, which is under antagonistic selection (outside boxes in fig. 2), because one way for body size to increase is to increase GR, but at the same time GR decreases under selection for increased development time. We assume that, in a given direction of simultaneous selection on the two focal traits, the response to selection will be enabled primarily by the developmental traits that act synergistically. We also assume that the response to selection is constrained by the

Figure 2: Predictions for how the underlying mechanism regulates the response to selection of two focal traits, body size and development time. Quadrants (and arrows) represent the direction of simultaneous selection on body size and development time (e.g., in the lower-right quadrant, body size is selected to decrease while development time is selected to increase), with the selection line indicated at the top of each quadrant. “Big” and “small” refer to body size, and “short” and “long” refer to development time. The exterior boxes indicate how the developmental traits will change when each focal trait changes individually. The middle couplet indicates which of the developmental traits are under synergistic or antagonistic selection within each quadrant. The top line of the bottom triplet is the prediction for the response to selection of the developmental traits under synergistic selection. The two bottom lines of the triplet are the predicted signs of the genetic correlations between the developmental traits and the focal traits. GR = growth rate; ICG = interval to cessation of growth; CW = critical weight; dt = development time; two plus signs indicates positive genetic correlation; two minus signs indicate a negative genetic correlation. Modified from figure 3 of Davidowitz et al. (2005).

developmental traits that act antagonistically. It is important to emphasize, however, that the prediction for the response to selection must take into account all three developmental traits (GR, CW, and ICG), those that act synergistically as well as those that act antagonistically, as body size and development time are determined by the interaction of all three developmental traits (see eqq. [2], [3]). The framework of Davidowitz et al. (2005) makes predictions only with regard to those traits that act synergistically. The effect of those developmental traits that act antagonistically can be determined only from the genetic correlations following selection, which will be examined in a separate study.

The prediction for how the developmental trait under synergistic selection changes in response to simultaneous selection of the two focal traits is given in the top line of the bottom triplet in each quadrant in figure 2. We note that we do not make a quantitative prediction for how much the developmental traits will increase or decrease. Rather, we qualitatively predict how they will change relative to those in the population before selection (Davidowitz et al. 2005). The magnitude of the response depends on the genetic correlations among the focal and developmental traits within each genetic line (Davidowitz et al. 2012). These genetic correlations, as well as the **G** and **P** matrices among the developmental and life-history traits following selection, will be reported elsewhere.

In the initial presentation of the framework for the regulation of simultaneous selection on multiple traits (Davidowitz et al. 2005), we assumed that a positive correlation between the two focal traits was required for the predictions to hold. Since then, a deeper understanding of the system (Nijhout et al. 2006, 2010; Davidowitz et al. 2012; this study) has led us to modify this assumption. For the general predictions in figure 2 to gain support, the framework requires specific signs (negative or positive) of specific genetic correlations between the developmental traits and the two focal traits. When the two focal traits are both selected in the same direction (upper-right and lower-left quadrants in fig. 2), there should be a positive correlation between CW and ICG with both focal traits. When body size and development time are selected in opposite directions (upper-left and lower-right quadrants in fig. 2), growth rate should be correlated positively with body size and negatively with development time (Davidowitz et al. 2012).

Material and Methods

The Simultaneous-Selection Design

The initial colony of *Manduca sexta* before selection and the larva-rearing methods are described in Davidowitz et al. (2012). For each of 10 generations, about 240 pupae of each sex were subjected to 25% simultaneous directional selec-

tion in each combination (genetic line) of body size and development time (Big/Long, Big/Short, Small/Long, and Small/Short). The 25% simultaneous selection was performed by first randomly numbering all individuals. We then plotted pupal mass against development time for all individuals within a genetic line. A line was then drawn through the means of each trait, such that we had crosshairs centered on both means (JMP software, ver. 8.0). Twenty-five percent of all individuals were identified in the relevant quadrant (fig. 2) for a given genetic line. In a few instances there were not enough individuals in the relevant quadrant; in these cases, additional individuals closest to that quadrant were included. Males and females were selected for separately because of sexual size dimorphism (Stillwell and Davidowitz 2010); however, the sexes were combined within lines, as the focus of this study is differences among lines. There is no difference in development time between the sexes (Stillwell et al. 2014). Each line was replicated twice, once each at Duke University (Duke) and the University of Arizona (UA). Pupae in two control lines were selected at random with a random-number generator. Sixty pairs of moths from each selection line were mated in a communal 1-m³ mating cage and provided with 25% sugar water and oviposition platforms as in Davidowitz et al. (2012).

Measurement of the Developmental and Focal Traits

The three developmental traits (growth rate, critical weight, and the interval to cessation of growth) and the two focal traits (body size and development time) were measured directly before selection (Davidowitz et al. 2012) and in each of the 10 genetic lines (two controls and two replicates for each combination of body size and development time) after the 10 generations of selection. Body size was measured as pupal mass (Davidowitz et al. 2012). Development time was measured as the number of days from hatching to wandering. The first pulse of ecdysone (see above) induces the larva to purge its gut, climb off the host plant, and wander along the ground in search of a pupation site. In *M. sexta*, wandering is readily identified by a change in larval coloration, exposure of the dorsal aorta, and the larva burying itself under the remaining food in its container (Davidowitz et al. 2012). Growth rate was measured as the gain in larval mass in the 24-hour period between days 3 and 2 before peak larval mass (Davidowitz et al. 2012). Growth in holometabolous insects is typically sigmoid in shape (Nijhout et al. 2010), with slightly slower growth at the beginning and end of the instar (as the larva recovers from the molt and prepares for pupation, respectively) and linear growth in between (fig. 1). Because insect growth is exponential, more than 90% of larval growth occurs in the last larval instar. Thus, measuring growth rate during the linear phase of the last larval instar ensures that it captures the

majority (>90%) of the growth of the insect. The critical weight is defined as the minimal weight at which no further feeding is necessary for a normal time course to pupation and was measured as in Davidowitz et al. (2003). It occurs about halfway through the last larval instar and well within the linear phase of growth (fig. 1). The interval to cessation of growth was measured as in Davidowitz and Nijhout (2004), with a modification explained in appendix A.

Statistical Analysis

The framework for the regulation of simultaneous selection on body size and development time predicts only responses after selection relative to the population before selection (Davidowitz et al. 2005). Therefore, changes in the developmental traits and two focal traits were tested (Student's *t*-test, assuming unequal variances) between the line before selection and the genetic lines after selection (app. B). We calculated correlations among the focal traits, among the developmental traits, and among the focal traits and the developmental traits, using Pearson product-moment correlations, after box plots and the extreme Studentized deviate test (Grubb's test) revealed no outliers within the data. These correlations were calculated among all nine genetic lines (two replicates for each of the four directions of simultaneous selection and the before-selection line), among specific directions of selection on individual traits (big, small, short, long), as well as synergistic (Big/Long, Small/Short) and antagonistic (Big/Short, Small/Long) selection between body size and development time (fig. 2). When applicable, we corrected the *P* value for multiple comparisons (see below). As these correlations were calculated from mean values for each genetic line, they can be interpreted as genetic correlations in the broad sense (apps. B–D). We calculated the standard errors of the correlations as

$$SE_r = \sqrt{\frac{1 - r^2}{n - 2}}, \quad (1)$$

where SE_r is the standard error of the correlation, and *n* is the sample size as given in appendixes B–D.

We further examined the relationship between the two focal traits under simultaneous selection. Using mean values for each genetic line, we regressed body size on development time for short versus long directions of selection, big versus small directions of selection, and when the two focal traits were both selected either in the same direction (synergistic selection) or in opposite directions (antagonistic selection). In the regression analyses of the developmental traits and the timing of hormone titers, we halved the *P* values, because in all cases we predicted a positive (one-sided) relationship. We tested for specific signs (positive or

negative) of the genetic correlations (one-tailed test) by halving the *P* values in appendix D of these genetic correlations.

Test of Predictive Ability of the Developmental Traits

We tested how well the three developmental traits predicted the response to selection of the two focal traits by regressing observed development time or body size on predicted development time or body size. To ensure the mathematical independence of the observed and predicted estimates of body size and development time, the observed peak larval size (body size) and development time were measured directly on individuals not used to calculate the values of the developmental traits, as described above (before selection: *n* = 1,342; Big/Short: *n* = 717; Small/Long: *n* = 1,049; Small/Short: *n* = 463; Big/Long: *n* = 1,391). These excess individuals were separated from the individuals used to calculate the predicted body size and development time by 1–3 generations. Thus, the data set used to calculate the predicted estimates of body size and development time was independent of the data set of the observed measures. Predicted body size for each genetic line was calculated from the respective values of CW, ICG, and GR (as calculated above), as in D'Amico et al. (2001) and Davidowitz et al. (2012):

$$\text{predicted peak larval size} = \text{CW} + (\text{ICG} \times \text{GR}). \quad (2)$$

Predicted development time was calculated as

$$\text{predicted development time} = \text{dt14} + \text{ICG} + \frac{\text{CW}}{\text{GR}}, \quad (3)$$

where dt14 is the development time (in days) of the first four instars: before selection: 12.04; Big/Short: 12.57; Small/Long: 14.51; Small/Short: 12.1; Big/Long: 13.83. We note that in this selection experiment we selected on pupal mass as our measure of body size for logistical simplicity, whereas the mechanism for the regulation of body size (see above) refers to peak larval mass (the maximal size attained by the larva) as the measure of body size. Data on peak larval mass and dt14 were collected only for the UA lines, and thus the observed-versus-predicted test was performed only on these lines.

Test of the Physiology Underlying the Three Developmental Traits

We examined in more detail how the changes in GR may have occurred. To determine whether changes in GR were due to changes in diet conversion efficiency, we measured the efficiency of the conversion of ingested food (ECI;

Waldbauer 1968) of the control and the four selection lines after selection. In each line, 37–40 last-instar larvae were weighed and then given a preweighed amount of standard diet and allowed to feed for 6 hours in a 266-mL cup with a perforated lid for gas exchange. After 6 hours, both the larva and the diet were reweighed and the ECI was calculated as

$$\text{ECI} = \frac{\text{larva6} - \text{larva0}}{\text{diet0} - \text{diet6}} \times 100, \quad (4)$$

where the 0 and 6 refer to weights at times 0 and 6 hours, respectively. The ECI was measured on three consecutive days, and the mean ECI was calculated for each larva ($n = 156$ larvae, $n = 468$ total ECI measurements). Growth rate in this experiment was measured as

$$\text{growth rate} = \frac{\text{larva6} - \text{larva0}}{6}, \quad (5)$$

and the mean growth rate of each of the three daily measurements was calculated for each larva. A preliminary study showed that after 6 hours, diet mass did not decline significantly because of evaporation in the enclosed cups ($t = -0.534$, $P = .5957$, $n = 30$). We did not measure ECI before selection.

The timing of PTTH secretion is determined primarily by JH catabolism by JH esterase (JHE). We assayed JHE titers of the two lines in which we predicted that the response to selection would be determined primarily by the hormonal traits, Big/Long and Small/Short, as well as the lab colony as a control. The JHE assay used is described in McCutchen et al. (1995). Samples (10 μL) of hemolymph were assayed from three individuals at 1-g intervals (3.5–11.5 g). To test whether the increase in JHE secretion was correlated with the critical weight, we regressed the mass at which JHE titers increased on the critical weight in each of the three lines. We predicted that the size at which JHE begins to increase would increase with an increase in critical weight.

Body size and development time are ultimately determined by the mechanism that terminates feeding and growth. In *M. sexta*, as in all insects, the cessation of growth is caused by a pulse of ecdysone. To better understand how the mechanism regulating the cessation of growth may have evolved, in addition to estimating the timing of PTTH secretion, we measured the timing and titer of ecdysteroid secretion. We used the 2B antibody (a gift from W. Bollenbacher of the University of North Carolina, Chapel Hill), which has an equal affinity to ecdysone and 20-hydroxyecdysone (20E). We used 20E as a standard. The ecdysone radioimmunoassay was as described in Hatle et al. (2003). Three to five individuals were assayed every 2 hours and their 20E titer av-

eraged. Unfortunately, the raw data were lost because of a computer malfunction, so we report only average values. We used a moving average to smooth the data.

Developmental switches in insects that are controlled by the developmental hormones JH and ecdysone can occur through changes in titer thresholds as well as the timing of tissue sensitivity windows (Nijhout and Davidowitz 2009). Although we did not test for these directly, we indirectly tested whether the timing of sensitivity windows may have evolved by regressing the timing of peak ecdysone titers on the timing of PTTH secretion. The timing was measured as hours since lights-on within the photogate. This regression also tested our assumption that the timing of ecdysone secretion measured on the Duke lines and the timing of PTTH secretion measured on the UA lines are concordant with each other. To gain insight into whether changes in ecdysone thresholds may have evolved, we regressed ecdysone titers on the timing of PTTH secretion.

The perturbations of the developmental processes following selection may be reflected in differential mortality. We measured mortality as the proportion dead of all individuals after selection. An analysis of means (ANOM) for proportions ($\alpha = 0.05$) tested which genetic lines differed from the combined mean mortality. All possible pair combinations ($n = 15$) were tested by ANOM for proportion pairs, with a Bonferroni correction applied ($\alpha = 0.0033$). Sample sizes (in the form, Line: live pupae + dead pupae = total individuals) were as follows: Before: 2,873 + 51 = 2,924; Big/Long: 2,672 + 331 = 3,003; Big/Short: 826 + 45 = 871; control: 783 + 64 = 847; Small/Long: 1,314 + 343 = 1,657; Small/Short: 2,191 + 101 = 2,292, for a grand total of 11,594 individuals.

Results

Data for the following results are archived in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.p5067> (Davidowitz et al. 2016).

How Does the Underlying Developmental Mechanism Enable or Constrain the Simultaneous Response to Selection of Body Size and Development Time?

After 10 generations, body size and development time differed significantly from those in the initial population in all four combinations of simultaneous directional selection (fig. 3; app. B). In all cases, the change in body size was linear (or marginally so) over the 10 generations of selection, with the exception of the two control lines, which were under random selection and did not change after selection (fig. 3; table F1; tables F1 and F2 are available online). Only half of the lines showed linear changes in development time over the 10 generations of selection (fig. 3; table F1).

Figure 3: Response (mean $\pm$ SEM) to 10 generations of selection for body size (A, C) and development time (B, D) for the University of Arizona (A, B) and Duke University (C, D) replicates. “Big” and “small” refer to body size, and “short” and “long” refer to development time.

In contrast to the relatively linear evolution of the univariate traits, the multivariate response to selection was highly erratic (fig. 4). The Duke replicates appeared more erratic than the UA lines, likely because of smaller sample sizes.

The values for the growth rate, critical weight, and interval to cessation of growth (GR, CW, and ICG, respectively) before selection are given in the center box of figure 5. Their values after the 10 generations of selection are given in the boxes of their respective quadrants. Statistical tests of the evolution of the developmental traits after selection, relative to those in the population before selection, showed a significant response to selection of both focal traits and the three developmental traits (app. B), with three exceptions. ICG did not evolve in either replicate of the Small/Long line, and the ICG was marginally significant ($P = 0.066$) in the UA Big/Long line.

After selection, the two focal traits were genetically uncorrelated in all directions of selection after correction for multiple comparisons ($\alpha = 0.008$; app. C). The three developmental traits GR, CW, and ICG were largely uncorrelated genetically with either focal trait. CW and GR, however, showed strong genetic correlations ($r > 0.86$) with body size in most directions of selection, although some of these were not significant after correction for multiple comparisons (app. D). With one exception (CW-GR), none of the developmental traits showed significant genetic correlations with each other in any direction of selection (app. E).

There was a general lack of genetic correlation between body size and development time among genetic lines (app. C; correction for multiple comparisons, $\alpha = 0.008$), consistent with the lack of significant full-sib and parent-offspring genetic

correlations in the before-selection genetic line (Davidowitz et al. 2012). Appendix C also suggests that the genetic correlation between body size and development time was dependent on the relative direction of selection between the two traits. There was a very strong genetic correlation in lines that were selected for short development time (Big/Short, Small/Short; before selection: $r_g = 0.95 \pm 0.18$, $P = .0135$) but not in those selected for long development time (Big/Long, Small/Long; before selection: $r_g = -0.108 \pm 0.57$, $P = .8629$; fig. F1A; app. C; figs. F1–F4 are available online). Similarly, there was a strong genetic correlation between body size and development time when both focal traits were under synergistic selection (Big/Long, Small/Short; before selection: $r_g = 0.894 \pm 0.26$, $P = .0406$) but not when the traits were under antagonistic selection (Big/Short, Small/Long; before selection: $r_g = -0.814 \pm 0.34$, $P = .0935$; fig. F1B; app. C).

When both focal traits were under synergistic selection, CW had positive correlations with body size ($r_g = 0.92 \pm 0.23$, $P = .0128$) and development time ($r_g = 0.93 \pm 0.21$, $P = .0114$), as predicted (fig. 2). The ICG had positive correlations with body size ($r_g = 0.59 \pm 0.47$, $P = .1468$) and development time ($r_g = 0.30 \pm 0.55$, $P = .3121$), although these correlations cannot exclude 0. When body size and development time were under antagonistic selection, growth rate had a positive correlation with body size ($r_g = 0.96 \pm 0.17$, $P = .0055$) and a negative correlation with development time ($r_g = -0.82 \pm 0.33$, $P = .046$), as predicted (fig. 2).

The response of the three developmental traits to selection on the two focal traits is given in figure 5A. Ten of the

Figure 4: The response to all four combinations of simultaneous selection on body size and development time. A, University of Arizona replicates. B, Duke University replicates. All points are differenced from the controls. “Big” and “small” refer to body size, and “short” and “long” refer to development time.

12 predictions (83%) were supported (fig. 2; $\chi^2 = 10.8$, $P = .001$): growth rate increased in the Big/Short line and decreased in the Small/Long line, critical weight and the ICG both decreased in the Small/Short line, and critical weight increased in both replicates of the Big/Long line. Only in the Big/Long line did the results differ from the predictions: in both replicates, the ICG decreased instead of increasing, although the change in the UA line was marginally significant (app. B; $P = .066$). Figure 5B shows the relative changes of all five traits combined, the three developmental and the two focal traits in all five lines.

Can the Relationships among the Developmental Parameters Accurately Predict the Response to Simultaneous Selection of the Two Focal Traits?

When calculated from the developmental traits (fig. 5A), the predicted response to selection explained 100% of the observed response of body size (observed peak larval size = $0.9815 \times \text{predicted peak larval size} - 0.0616$, $n = 5$, $R^2 = 0.995$, $P < .0001$) and 93% of the observed response of development time (observed development time = $1.5823 \times \text{predicted development time} - 8.3772$, $n = 5$, $R^2 = 0.931$,

Figure 5: Response of the three developmental traits after simultaneous selection on body size and development time. *A*, Quadrants represent the direction of simultaneous selection on body size and development time, with the direction of selection given in each quadrant (e.g., in the lower-right quadrant, body size is selected to decrease while development time is selected to increase). “Big” and “small” refer to body size, and “short” and “long” refer to development time. Values of the developmental traits before selection are shown in the central box and those after selection in the respective quadrants. Predictions for how the developmental traits change after selection are given in blue within each quadrant. In the instances where the response to selection matches our prediction, it is highlighted in blue. Where the response does not match the prediction, it is given in red. An asterisk indicates where the two focal and three developmental traits have evolved significantly from those in the population before selection. Statistical tests of the response to selection and specific *P* values are given in table F1. CW = critical weight; ICG = interval to cessation of growth; GR = growth rate. *B*, Relative response of the four selection lines, along with the line before selection. The diagonals represent the growth rates, the diamonds body size and development time at the beginning and end of the last larval instar, and the circles the critical weights. The ICG is the difference in days from the circles to the right-hand diamonds. Data for *B* are taken from *A* and appendix B.

$P = .0079$) over all four combinations of simultaneous selection and the population before selection (fig. 6).

How Does the Physiology Underlying the Developmental Traits Respond to Selection?

The photogate for PTTH secretion evolved relative to the population before selection, but it did not differ among the four combinations of body size and development time after selection. The opening and closing of the gate were de-

fined as the 5% confidence intervals above the lower asymptote and below the upper asymptote, respectively. Thus, the pooled photoperiodic gate was 13 hours long, opened 3 hours after lights-on, and closed 16 hours after lights-on (fig. 7A). Although the timing of the photogate did not differ among the selected lines, secretion of PTTH within the photogate did (fig. 7B). The line before selection (Davidowitz et al. 2012) secreted PTTH at the beginning of the photogate, as did the Small/Long and Big/Short lines. Interestingly, in these lines the response to selection is determined primarily by GR and constrained by the develop-

Figure 6: Relationship of observed and predicted body size (A) and development time (B) of the four selection lines and the population before selection. Error bars of the observed traits are 1 SEM and in all cases are smaller than the symbol. Data used to calculate the predicted values are taken from figure 5 (see text) and therefore have no error bars.

mental hormones. The two lines in which body size and development time were selected in the same direction (Big/Long and Small/Short) and in which their response to selection was determined by the two developmental hormone proxies, CW and ICG, secreted PTTH toward the middle of the gate. The disinhibition and secretion of PTTH within the photogate induces the secretion of the molting hormone ecdysone (see above). In all cases, the onset of the increase in ecdysone titers occurred very close to the timing of PTTH secretion within the photogate, as expected (fig. 7).

Across all four lines, the timing of peak ecdysone titer could not be predicted by the timing of PTTH (timing of peak ecdysone titer = $10.86 + 0.26 \times \text{timing of PTTH}$, $n = 4$, $R^2 = 0.09$, $P = .7052$). Close examination of this regression (fig. F2A), however, shows that this result is dominated by the Small/Long outlier. Removing this outlier reveals a very strong relationship between these two measures, with a slope of 1.0: timing of peak ecdysone titer = $5.5 + 1.1 \times \text{timing of PTTH}$, $n = 3$, $R^2 = 0.999$, $P = .0108$. The timing of PTTH secretion explained 85% of the variation in peak ecdysone titers ($P = .0394$; fig. F2B). The mass at which JHE titers increased showed a positive and signifi-

cant relationship with the critical weight (fig. F3; mass at which JHE titers increased = $1.46 \times \text{critical weight} - 4.88$, $R^2 = 0.99$, $n = 3$, $P = .0290$).

Growth rate was significantly and positively correlated with ECI in all lines (table F1). The prediction that the Big/Short line would have a higher growth rate than the Small/Long line (fig. 2) was supported (t -test: $t_{1,78} = -12.24$, $n = 80$, $P < .0001$). Congruently, the ECI was also significantly higher in the Big/Short line than in the Small/Long line (t -test: $t_{1,78} = -2.29$, $n = 80$, $P = .0250$), suggesting that the change in growth rate may be due to a change in the ECI in the different selection lines.

The ANOM for proportions revealed that all lines differed significantly in mortality from the mean, except the control line. The highest mortality was in the Small/Long line (fig. F4). All pair combinations were significantly different from each other ($\alpha = 0.0033$), with the exception of control-Big/Short ($P = .0440$) and Big/Short-Small/Short ($P = .3443$) pairs.

Discussion

Studying the complex genetic, physiological, developmental, molecular, and endocrine processes that underlie traits can provide important insights into how they may respond to selection as well as what part of the underlying mechanism may constrain their evolution. Knowledge of the underlying mechanism can generate explicit and nuanced predictions for how and why traits respond the way that they do, beyond predictions based on the phenotypes alone. Here we have shown that the antagonistic and synergistic interactions in the timing of hormonal and signaling pathways can enable or constrain the response to simultaneous selection, depending on the direction of selection. The use of proxy traits that represent the complexity inherent in these signaling pathways may be a powerful tool to reduce endocrine and molecular complexity. This reduction in complexity may then enable explicit and testable predictions of how the endocrine system can enable or constrain evolutionary change (Davidowitz 2016).

Natural populations of *Manduca sexta*, a specialist hawkmoth with a broad geographic range, are likely to encounter simultaneous selection favoring different antagonistic or synergistic combinations of body size and development time in response to host-plant quality, temperature, precipitation, parasites, and predators. Using an experimental-evolution approach, we have shown here that three developmental proxy traits—critical weight, interval to cessation of growth, and growth rate—explain how such a response to simultaneous selection on body size and development time can occur.

Changes in *M. sexta*'s three developmental traits explain the response to selection of body size and development time exceptionally well: the plots of the observed and predicted

Figure 7: Timing of prothoracicotropic hormone (PTTH) secretion and ecdysone titers among lines within the PTTH photoperiodic gate. *A*, The sigmoid curve was calculated as the pooled photogate (solid line) and 95% confidence limits (dashed lines) of the four combinations of selection on body size and development time. The timing of the line before selection was shifted later by 1.5 hours to put all lines on the same timescale (see text). The three black crosses represent, from left to right, the opening, middle, and closing of the photogate. *B*, Ecdysone titers of the four selection lines ("big" and "small" refer to body size, and "short" and "long" refer to development time.) within the PTTH photoperiodic gate. The vertical lines indicate when, on average, each genetic line secreted PTTH within the photoperiodic gate, with the vertical black line representing the colony before selection (data from Davidowitz et al. 2012).

responses to selection explain 100% of body size response and 93% of development time response (fig. 6). Thus, the approach of using the synergistic and antagonistic interactions at the physiological and developmental levels provides a robust conceptual framework for how the underlying developmental physiology can be simplified to illuminate trait evolution.

Interestingly, even though the interval to cessation of growth did not respond to selection as predicted in either the Big/Long line or the Small/Long line (figs. 2, 4, 5; app. B) and despite the highly erratic response to simultaneous selection of the two focal traits (fig. 4), the three developmental traits still explained nearly all of the variation in the response to selection. This illustrates that a response to selection

can be understood only when variation due to those traits that enable the response to selection, as well as variation due to traits that constrain a response, is taken into account.

Regulation of the Response to Simultaneous Selection

Even though the responses to selection of body size and development time individually were generally linear in all lines (fig. 3; table F1), the response to selection on both traits simultaneously was very erratic (fig. 4). Simultaneous selection on morphological traits often results in relatively symmetric and linear responses to selection (e.g., Bell and Burris 1973; Beldade et al. 2002). In contrast, when development time is selected simultaneously with a morphological

trait, the response to selection is almost always erratic and asymmetric (e.g., Scheiner and Istock 1991; Zijlstra et al. 2003; this study; fig. 4). The approach used here can explain why this occurs. The underlying developmental traits (GR, CW, and ICG) cause the phenotypic landscapes of body size and development time to be orthogonal to each other over the natural range of values seen in this study (Nijhout et al. 2006). The simultaneous selection on the two focal traits pulls the developmental traits in different directions, such that they are antagonistic over much of the parameter space of the two focal traits, irrespective of the direction of selection. As a consequence, body size and development time cannot evolve easily in the same direction, resulting in an erratic response to simultaneous selection (Davidowitz et al. 2012). These erratic responses may be exacerbated by random differences in the strength of selection between the two traits and among generations.

The results of this study suggest a general pattern for mechanistic control of the response to simultaneous selection on body size and development time. When the two focal traits were selected in the same direction (figs. 2, 5, upper-right and lower-left quadrants), the response to selection was determined primarily by the endocrine system, JH (as measured by the CW) and PTTH and ecdysone (as measured by the ICG), and constrained by the nutrient signaling pathways that control growth rate. When the two traits were selected in opposing directions, one to increase and the other to decrease (figs. 2, 5, upper-left and lower-right quadrants), the response to selection was determined primarily by the signaling pathways that control GR (Gokhale and Shingleton 2015) and constrained by the endocrine regulation of developmental events. The implications are that selection acts on different components of the underlying developmental mechanism, depending on whether the focal traits themselves are under either synergistic or antagonistic selection.

This general pattern allowed us to make additional, more nuanced predictions with regard to how the underlying mechanism determines the response to selection of the two focal traits. When both focal traits were selected in the same direction, we predicted positive correlations between the CW and ICG and both focal traits (fig. 2), because all four traits should respond to selection in the same direction. This prediction was upheld for the CW but not for the ICG (app. D). From this we infer that when both traits were selected in the same direction, the response to selection was determined primarily by the CW. In other words, the response to simultaneous synergistic selection on body size and development time is determined primarily by the timing of the decision to transition from the juvenile growth phase to the reproductive adult phase. Such a life-history strategy seems reasonable. A life-history decision to escape poor conditions early at the cost of small size would have a selective advantage. Conversely, when conditions are benign, a life-history strat-

egy that favors postponing the decision to switch from the juvenile to the adult stage so as to maximize body size would also have a selective advantage.

Such life-history strategies are relevant to natural populations of *M. sexta*. In the southwestern United States, *M. sexta* population dynamics is strongly affected by summer rainfall patterns (Riffell et al. 2008; Contreras et al. 2013), which can be highly variable both within and among years (Davidowitz 2002). A single larva can eat the equivalent of 4,781 cm² of leaf area, which can be greater than that of an individual host plant (G. Davidowitz, unpublished data). In years of low rainfall, when host plants are relatively small, natural selection should favor small body size and short development time, so that the small host-plant size is sufficient to reach metamorphosis. In contrast, in years of high rainfall, when a single host plant is large enough to sustain a larva, natural selection should favor long development time and large body size, as large body size confers a fitness advantage in female fecundity and male spermatophore size (Levin et al. 2016; G. Davidowitz, unpublished data). This life-history strategy may be reinforced by the probability of parasitism. A still-growing larva that needs to descend from its defoliated host plant in search of a new plant is highly susceptible to parasitism by entomopathogenic soil nematodes. Normal larvae that have ceased growth and are wandering in search of a pupation site appear to be immune to these parasitic nematodes (Miranda et al. 2013).

When body size and development time were selected in opposite directions, we predicted a positive relationship between growth rate and body size and a negative relationship between growth rate and development time (fig. 2). These predictions were upheld for body size and growth rate but not for development time and growth rate (app. D). We interpret this to mean that the response to antagonistic selection on the focal traits is determined primarily by the relationship between growth rate and body size and less so by the relationship with development time. The implication is that when body size and development time are under antagonistic selection, the response to selection would be determined primarily by the nutrient signaling pathways that determine the quantity of resources accumulated and less by the rate at which these resources are accumulated (Nijhout et al. 2013; Gokhale and Shingleton 2015).

It is not clear why the ICG of the Big/Long line did not respond to selection as predicted. It is not likely that this was due to chance alone, because the direction of change for both replicates was the same (albeit in the direction opposite that predicted). Nor was it likely that this was due to a lack of genetic variation in the ICG in this direction of selection, because the ICG did evolve (app. B), just not in the direction we predicted. The relatively high mortality in this line (fig. F4) suggests that growth and development were significantly perturbed in this direction of selection.

It is possible that this perturbation affected the regulation of the ICG, leading to its unpredicted response in the Big/Long line.

It is generally accepted in the life-history literature that the longer an organism has to grow, the bigger it will be (Roff 1992). This implicitly assumes that all individuals follow the same growth trajectory (Roff 2000). Our results show that over all directions of selection, the genetic correlation between body size and development time is not significantly different from 0. This result is consistent with our previous work on laboratory strains (Davidowitz et al. 2012) as well as field work on wild *M. sexta* (Kingsolver et al. 2012). In contrast, there are strong positive genetic correlations between body size and development time when both are under selection for short development time (fig. F1A; app. C) and when both traits are under synergistic selection (fig. F1B; app. C). The implication is that the genetic correlations between development time and body size are dependent on their simultaneous directions of selection. A similar result was found in *Tribolium* by Englert and Bell (1969), who showed that the direction and strength of genetic correlations differed, depending on the direction of selection of the individual traits and on the past selection history of the different genetic lines.

Evolution of Hormonal Control

Truman (1972) and Truman and Riddiford (1974) first identified the photogate for PTTH secretion a few years after their laboratory colony was established from the wild in the late 1960s (Kingsolver 2007) and found it to be 8 hours long. The gating of PTTH is thought to ensure that the larvae wander in search of a pupation site at night, when they are less visible to predators (Chapman 1998). In our study, the photogate for PTTH secretion lasted 13 hours and did not differ among the four combinations of selection. It is likely that the near doubling of the photogate over the past 40 years is the result of relaxed predation pressure in the laboratory.

Secretion of PTTH within the photogate defines the end point of the ICG. It is interesting to note that the two selection lines under synergistic selection (Big/Long and Small/Short) whose response to selection was determined in part by the ICG both secrete PTTH near the middle of the photogate (fig. 7), whereas the other two lines (Big/Short and Small/Long) secrete PTTH at the onset of the photogate. It is possible that under synergistic selection, secreting PTTH toward the middle of the photogate provides increased flexibility in contracting or expanding the duration of the ICG as needed in response to selection. Together, these results indicate that the mechanisms that regulate the cessation of growth (duration of the photoperiodic gate and the timing of when PTTH is secreted within the gate) can both evolve

under selection. When PTTH secretion depends, in part, on whether the two focal traits are under synergistic or antagonistic selection. Two lines, Small/Long and Big/Short, appear to secrete PTTH before the gate opens (fig. 7). We think that this is likely due to small sample sizes at the beginning of the photogate.

The regressions of PTTH and ecdysone (fig. F2) indicate that the simultaneous response to selection in the timing of PTTH and ecdysone secretion can evolve independently only when perturbed sufficiently. It is likely that the developmental regulation of the two focal traits in the Small/Long direction of selection were perturbed to such a degree that mortality was at its maximum (fig. F4), resulting in the outlier for this line (fig. F2). More generally, however, the timing of PTTH secretion and that of ecdysone secretion are very tightly correlated (fig. F2), as we would expect from the control mechanism of these two hormones (see above). This tight correlation might impose a constraint on independent selection on PTTH or ecdysone, although the strong relationship between the timing of PTTH secretion and peak ecdysone titers (fig. F2) indicates that ecdysone threshold titers have also evolved in response to simultaneous selection on body size and development time. It is not clear whether they have evolved independently or as a correlated response to selection.

The timing of PTTH secretion was measured on the UA lines, and the timing of ecdysone secretion was measured on the Duke lines. Their tight relationship (fig. F2) validates our assumption that the hormonal controls were the same in both replicates.

The critical weight is a proxy trait for the hormonal regulation of the timing of the decision to switch from the juvenile growth phase to the adult reproductive phase. The interval to cessation of growth (ICG) is a proxy trait for the timing of the hormonal regulation of the cessation of growth. Growth rate is a proxy trait for the nutrient signaling pathways that regulate resource accumulation. This study demonstrates how these three developmental proxies themselves may, in turn, respond to selection. Growth rate is positively correlated with the efficiency of the conversion of ingested food, irrespective of whether it enables or constrains selection (table F1). Growth rate is regulated largely through the insulin signaling and TOR pathways (see above), although, to our knowledge, no study has explicitly examined whether these pathways also regulate conversion efficiency. The mass at which JHE activity increases is strongly correlated with the critical weight. Therefore, JHE activity can respond to selection in *M. sexta*, as has also been shown in crickets (Zera et al. 1996). Although not addressed in this study, the critical weight may respond to selection via the pathways that shunt nutrients to storage (as opposed to growth and maintenance), irrespective of critical-weight manipulation via temperature and diet (B. R.

Helm and G. Davidowitz, unpublished data). The termination of the ICG occurs with the secretion of PTTH and ecdysone. Our results indicate that PTTH secretion may respond to selection via the pathways that determine the duration of the photoperiodic gate as well as the pathways that regulate the timing of PTTH secretion within that gate (figs. 7, F2). The cessation of feeding caused by the secretion of ecdysone may respond to selection via the pathways that regulate both the timing of the tissue sensitive window to ecdysone and the pathways that regulate the threshold of ecdysone titer (figs. 7, F2).

The General Applicability of the Developmental Proxy Framework

A goal of biology is to understand how genotypes translate into phenotypes. Such genotype-phenotype ($G \rightarrow P$) mapping has to incorporate the complex interactions between external environmental effects (genotype $\times$ environment and phenotype $\times$ environment) with the internal environment of genetic architecture, physiology, and development to produce the phenotype (Lewontin 1974; Alberch 1991; Hansen 2006; Houle 2010; Houle et al. 2010; Pigliucci 2010). Phenomics, generally defined as the “acquisition of high-dimensional phenotypic data on an organism-wide scale” (Houle et al. 2010, p. 855), attempts to match the huge influx of genomic data with a parallel level of data at the phenomic level to enable $G \rightarrow P$ mapping. A major obstacle in $G \rightarrow P$ mapping is the complexity and high information content of the systems involved, particularly that of the phenome, which may be alleviated somewhat by identification of risk factors (in the case of disease) or key determinants with strong influence on the $G \rightarrow P$ map (Buchanan et al. 2006; Houle et al. 2010).

The regulation of body size and development time shown in this study may provide a method to simplify $G \rightarrow P$ mapping. Davidowitz (2016) gives the example of eight signaling cascades that regulate growth in insects. If we ignore the gene products that make up these signaling cascades, there are 40,320 possible permutations of interaction among these cascades. If we are to be extremely conservative and accept that 99% of these interactions are not biologically possible or relevant in the regulation of insect growth, we are still left with 403 possible permutations that would have to be parameterized in a predictive model of the mechanistic regulation of insect growth. In contrast, if we use the three main key events in determining body size and development time—CW, ICG, and GR—as in this study, there are only three possible combinations. This can greatly reduce the complexity of the $G \rightarrow P$ map of body size and development time.

This reduction in complexity can be taken a step further. Each of the three developmental traits is itself regulated by complex mechanisms (see above and app. A), each

of which may have only a few major determinants. It is possible, then, to build a hierarchy of $G \rightarrow P$ mapping with relatively few determinants (proxies) at each level, which can make the overall $G \rightarrow P$ map more tractable.

Is it possible to use such key determinants to possibly simplify $G \rightarrow P$ maps in other organisms as well? We believe so. Davidowitz and Helm (2015) describe a more general framework for the regulation of body size and development time that is derived from the *M. sexta* framework described here and can be identified in a diverse range of taxa, including green algae, flowering plants, insects, amphibians, and mammals. For example, the critical weight (CW) in *M. sexta* can be more generalized as the decision point to switch from the juvenile growth phase to the adult reproductive phase and can be initiated by either internal (green algae, insects, and mammals) or external (amphibians and plants) triggers. In green algae, there is a threshold size beyond which further growth is not necessary for cell division. In mammals, there is a negative feedback loop that is controlled by growth itself (rather than age) that causes the downregulation of cell proliferation genes, halting growth. In insects, there is a critical size (CW in *M. sexta*) that induces the transition from juvenile to adult and is likely regulated by insulin and TOR signaling pathways. The juvenile-adult transition in amphibians is initiated by external triggers transduced by the hypothalamo-pituitary-adrenal/thyroid axis. Finally, in plants, there are four genetic pathways that initiate flowering: the autonomous, vernalization, and gibberellin pathways; *SOC1* (suppressor of overexpression of *Constans1*) and *FT* (flowering locus T) integrate these three pathways to determine flowering. Similar generalizations can also be identified in other taxa for the cessation of growth, the time interval between the decision to switch from the juvenile to the adult phase, and the actual cessation of growth (both of these are part of the ICG in *M. sexta*) and growth rate (Davidowitz and Helm 2015).

This more general framework can help bridge the gap between too much detail, which can obscure our ability to make mechanism-based predictions about life-history evolution, and too little detail, which eliminates mechanism from life-history predictions altogether. A future challenge will be to develop similar frameworks to test mechanism-based predictions of life-history evolution in other taxa and other life-history traits and to test their efficacy in $G \rightarrow P$ mapping.

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APPENDIX A

Modification of ICG Measurement

The interval to cessation of growth (ICG), the time interval between when the larva attains the critical weight and the secretion of the ecdysteroid molting hormone, is determined by two traits, the time it takes to catabolize juvenile hormone by juvenile hormone esterase (JHE) and the secretion of PTTH. In fifth-instar *Manduca sexta* larvae, PTTH induces the secretion of the first pulse of ecdysteroids, which causes the larva to stop feeding and prepares the larval tissues for pupation. Secretion of PTTH and ecdysteroid is readily identifiable by a color change in the larva and the appearance of the dorsal vessel (larval heart). PTTH itself is inhibited by JH and cannot induce the pulse of ecdysteroids before JH is cleared from the hemolymph (Nijhout and Williams 1974; Bollenbacher et al. 1979; Rountree and Bollenbacher 1986). The secretion of PTTH is governed by a photoperiodic clock and can occur only during a well-defined window of time, called a photoperiodic gate, that reoccurs each day (Truman 1972). Thus, the ICG is not a continuous trait; rather it is discrete, which must be taken into account in calculating it (Helm and Davidowitz 2015). Secretion of PTTH occurs during the first photogate that follows complete clearance of JH from the hemolymph (Truman 1972; Truman and Riddiford 1974). Calculation of the ICG, therefore, requires knowledge of when PTTH is secreted within the photogate.

The ICG is calculated as the time (in days) until PTTH secretion after the critical weight is reached and is measured in the same experiment that measures the critical weight (D'Amico et al. 2001; Davidowitz et al. 2003; Davidowitz and Nijhout 2004). This is done by subtracting the time at which an individual larva was assigned to a treatment in the critical-weight experiment from the time at which PTTH was secreted. Identifying the timing of PTTH secretion requires measuring the photogate for PTTH secretion (see below). In measuring the ICG, Davidowitz and Nijhout (2004) previously assumed that, on average, PTTH was se-

creted in the middle of the photogate. This is a reasonable assumption for a single population. In this study, however, we are explicitly predicting that the ICG will evolve (fig. 2). We therefore could not assume that, after the 10 generations of selection, the photogate itself would not evolve or that PTTH secretion within the gate would not evolve.

We developed a bioassay to assess when PTTH was actually secreted. We measured the photogate for PTTH secretion for each of the Duke selection lines as in Truman (1972), with 6–30 individuals per 2-hour interval. We plotted a two-parameter (slope and inflection point) logistic sigmoidal curve for each genetic line (JMP, ver. 11.0.0). Analysis of means (ANOM) showed that no lines differed significantly from a mean slope ($\alpha > 0.05$) but that the lines did differ in their inflection points. An equivalence test (JMP 11.0.0) showed that the four selection lines did not differ ($\alpha > 0.05$) from each other in their inflection points (mean inflection point for the four selection lines: 9.2 ± 0.16 [SEM] hours), but all selection lines differed significantly in inflection point from the control line (7.7 ± 0.34 hours). We interpret this to mean that the four selection lines (Big/Long, Big/Short, Small/Long, and Small/Short) had the same photogate for PTTH release, which was 1.5 hours later than the photogate of the control line. We did not measure the photoperiodic gate for the population before selection.

We therefore focused on determining when, within the photogate, PTTH was secreted in each genetic line. For each of the UA genetic lines we measured the population-level critical weight and ICG (cw_p and icg_p , respectively), using the established method (Davidowitz et al. 2003, 2012; also see above). We separately reared 205–1,477 larvae (app. B) from each selection line. These larvae were originally reared to estimate the **G** and **P** matrices, which will be reported on elsewhere. Using these larvae, we calculated the individual-level critical weight (cw_i) as in Davidowitz et al. (2012). We iteratively moved the average secretion time of PTTH from the supposed midgate until cw_p equaled the average of cw_i (t -test against a mean, sample sizes as in app. B). Thus, we let the larvae reveal when they secreted PTTH. Finally, we calculated the ICG for each selection line by subtracting the bioassay-determined time of PTTH secretion from the time the larva was put into the critical-weight experimental treatment (Davidowitz et al. 2003). This provides the time, in days, to PTTH secretion from the critical weight separately for each genetic line. For logistical reasons we measured the timing of the photogate (slope and inflection point, as described in the paragraph above) on the Duke replicate lines and the timing of PTTH secretion within the photogate (as explained in this paragraph) on the UA replicate lines. We assume that the timing of PTTH secretion is the same for both replicates of each genetic line, and we provide a test of this assumption.

APPENDIX B

Summary Statistics of the Response to Selection

Table B1: Summary statistics and test (*t*-test) of the response to selection of the two focal traits and three developmental traits before and after 10 generations of 25% directional selection for each of the genetic lines

Trait, replicate	Mean	SD	Lower 95% CL	Upper 95% CL	Sample	<i>t</i>	df	<i>P</i>
Before selection (UA only):								
Size	6.60	.69	6.56	6.64	1,195			
DT	18.29	1.08	18.23	18.35	1,195			
GR	2.61	.47	2.58	2.63	1,195			
CW	7.0	.15	6.94	7.03	40			
ICG	1.92	.54	1.75	2.10	40			
Big/Long:								
Size:								
UA	7.96	.76	7.90	8.02	604	36.932	1,101.5	<.0001
Duke	7.70	.82	7.59	7.81	220	18.809	278.2	<.0001
DT:								
UA	20.15	1.28	20.05	20.26	604	30.817	1,047.5	<.0001
Duke	21.59	1.36	21.41	21.77	224	34.293	277.6	<.0001
GR:								
UA	3.17	.51	3.12	3.21	604	22.681	1,129.6	<.0001
Duke	2.38	.47	2.27	2.49	71	-3.925	78.7	.0002
CW:								
UA	8.5	.16	8.46	8.53	81	51.174	81.7	<.0001
Duke	8.5	.15	8.43	8.53	37	43.918	74.6	<.0001
ICG:								
UA	1.70	.74	1.54	1.87	80	-1.858	101.8	.066
Duke	1.51	.51	1.34	1.68	37	-3.485	75.0	.0008
Big/Short:								
Size:								
UA	8.44	.74	8.39	8.49	794	55.969	1,609.8	<.0001
Duke	7.04	.63	6.95	7.13	194	9.015	272.5	<.0001
DT:								
UA	18.54	1.02	18.47	18.61	794	5.188	1,761.1	<.0001
Duke	18.11	1.04	17.96	18.26	194	-2.221	264.1	.0272
GR:								
UA	3.11	.48	3.07	3.14	794	22.995	1,668.7	<.0001
Duke	2.77	.32	2.70	2.85	77	4.378	99.0	<.0001
CW:								
UA	10.5	.12	10.46	10.52	71	125.557	69.6	<.0001
Duke	8.5	.15	8.43	8.53	41	44.267	79.0	<.0001
ICG:								
UA	1.31	.93	1.10	1.53	71	-4.371	108.8	<.0001
Duke	.91	.71	.69	1.14	41	-7.209	74.7	<.0001
Small/Long:								
Size:								
UA	4.94	.55	4.87	5.02	205	-38.259	323.1	<.0001
Duke	5.03	.59	4.94	5.12	167	-31.558	234.0	<.0001
DT:								
UA	21.54	2.18	21.24	21.84	205	20.883	221.4	<.0001
Duke	20.33	1.30	20.13	20.53	168	19.450	200.7	<.0001
GR:								
UA	1.91	.63	1.82	1.99	205	-15.129	244.0	<.0001
Duke	1.54	.33	1.47	1.62	74	-26.082	92.2	<.0001

Table B1 (Continued)

Trait, replicate	Mean	SD	Lower 95% CL	Upper 95% CL	Sample	<i>t</i>	df	<i>P</i>
CW:								
UA	5.5	.16	5.43	5.51	63	−49.417	85.4	<.0001
Duke	5.5	.15	5.48	5.55	68	−49.436	81.6	<.0001
ICG:								
UA	1.84	1.10	1.57	2.12	63	−.497	96.1	.6203
Duke	1.78	.93	1.55	2.00	68	−1.033	106.0	.3037
Small/Short:								
Size:								
UA	5.46	.57	5.43	5.49	1477	−45.882	2,321.9	<.0001
Duke	4.73	.48	4.67	4.80	221	−49.189	406.2	<.0001
DT:								
UA	17.74	1.10	17.69	17.80	1477	−12.880	2574.3	<.0001
Duke	17.35	.69	17.26	17.45	223	−16.804	454.1	<.0001
GR:								
UA	2.48	.38	2.46	2.50	1477	−7.573	2,265.7	<.0001
Duke	2.05	.30	1.98	2.12	78	−15.165	102.9	<.0001
CW:								
UA	6.0	.15	5.98	6.05	64	−32.125	82.8	<.0001
Duke	6.5	.16	6.45	6.55	47	−14.707	83.9	<.0001
ICG:								
UA	1.68	.58	1.53	1.82	63	−2.190	87.6	.0312
Duke	.75	.42	.62	.87	47	−11.218	72.5	<.0001

Note: The experiments used to measure CW and ICG return a single value of the appropriate weight bin (see text), and the *t*-tests for CW and ICG represent a test of the individuals within those weight bins. Three values of ICG (Big/Long UA, Big/Short, Small/Short) deviate slightly (0.01–0.04 days) from those in figure 5A because of rounding errors. “Big” and “small” refer to body size, and “short” and “long” refer to development time. CL = confidence limit; size = pupal mass; UA = University of Arizona; DT = development time; GR = growth rate; CW = critical weight; ICG = interval to cessation of growth; *t* = *t*-statistic. Boldface indicates a significant correlation ($\alpha = 0.05$).

APPENDIX C

Correlations between Body Size and Development Time

Table C1: Pearson product-moment correlations between development time and body size among the different directions of selection

Category	Lines included	Sample	<i>r</i> ± SE	<i>P</i>
All lines	Before, Big/Short, Big/Long, Small/Short, Small/Long	9	.098 ± .38	.8019
Short	Before, Big/Short, Small/Short	5	.950 ± .18	.0135
Long	Before, Big/Long, Small, Long	5	−.108 ± .57	.8629
Big	Before, Big/Short, Big/Long	5	.361 ± .54	.5510
Small	Before, Small/Long, Small/Short	5	−.273 ± .56	.6569
Antagonistic	Before, Big/Short, Small/Long	5	−.814 ± .34	.0935
Synergistic	Before, Big/Long, Small/Short	5	.894 ± .26	.0406

Note: The Before line is common to the University of Arizona and Duke replicates. The selection lines include both replicates. “Before” refers to the initial colony before selection. “Big” and “small” refer to body size, and “short” and “long” refer to development time. “Antagonistic” and “synergistic” refer to selection on the life-history traits (see fig. 2). Because these correlations were calculated using mean values for each genetic line (as in app. B), they can be interpreted as genetic correlations in the broad sense. Boldface indicates significant correlations ($\alpha = 0.05$ for individual tests and 0.008 for multiple comparisons; see text).

APPENDIX D

Correlations among Focal and Developmental Traits

Table D1: Pearson product-moment correlations among the developmental traits and focal traits

Test	Sample	$r \pm SE$	P	Test	Sample	$r \pm SE$	P
Big (Before, Big/Short, Big/Long):				Small (Before, Small/Short, Small/Long):			
Size-CW	5	.879 $\pm$.28	.0496	Size-CW	5	.670 $\pm$.43	.2161
Size-ICG	5	-.163 $\pm$.57	.7938	Size-ICG	5	.544 $\pm$.48	.3430
Size-GR	5	.573 $\pm$.47	.3127	Size-GR	5	.746 $\pm$.38	.1481
DT-CW	5	-.003 $\pm$.58	.9961	DT-CW	5	-.743 $\pm$.39	.1504
DT-ICG	5	.268 $\pm$.56	.6625	DT-ICG	5	.556 $\pm$.48	.3307
DT-GR	5	-.328 $\pm$.55	.5897	DT-GR	5	-.635 $\pm$.45	.2500
Short (Before, Big/Short, Small/Short):				Long (Before, Big/Long, Small/Long):			
Size-CW	5	.924 $\pm$.22	.0251	Size-CW	5	.996 $\pm$.05	.0003
Size-ICG	5	.159 $\pm$.57	.7988	Size-ICG	5	-.591 $\pm$.47	.2940
Size-GR	5	.975 $\pm$.13	.0049	Size-GR	5	.869 $\pm$.29	.0555
DT-CW	5	.767 $\pm$.37	.1306	DT-CW	5	-.024 $\pm$.58	.9692
DT-ICG	5	.431 $\pm$.52	.4683	DT-ICG	5	-.631 $\pm$.45	.2536
DT-GR	5	.940 $\pm$.2	.0174	DT-GR	5	-.344 $\pm$.54	.5707
Antagonistic (Before, Big/Short, Small/Long):				Synergistic (Before, Big/Long, Small/Short):			
Size-CW	5	.984 $\pm$.10	.0024	Size-CW	5	.923 $\pm$.23	.0256
Size-ICG	5	-.619 $\pm$.45	.2658	Size-ICG	5	.592 $\pm$.47	.2935
Size-GR	5	.956 $\pm$.17	.0109	Size-GR	5	.728 $\pm$.40	.1634
DT-CW	5	-.737 $\pm$.39	.1551	DT-CW	5	.928 $\pm$.21	.0228
DT-ICG	5	.562 $\pm$.48	.3246	DT-ICG	5	.300 $\pm$.55	.6242
DT-GR	5	-.816 $\pm$.33	.0919	DT-GR	5	.390 $\pm$.53	.5164
Body size:				Development time:			
CW	9	.934 $\pm$.14	.0002	CW	9	-.093 $\pm$.38	.8114
ICG	9	-.031 $\pm$.38	.9362	ICG	9	.523 $\pm$.32	.1481
GR	9	.849 $\pm$.20	.0038	GR	9	-.283 $\pm$.36	.4613

Note: "Before" refers to the initial colony before selection. "Big" and "small" refer to body size, and "short" and "long" refer to development time. "Antagonistic" and "synergistic" refer to selection on the focal traits (see fig. 2). Size = pupal mass; DT = development time; GR = growth rate; CW = critical weight; ICG = interval to cessation of growth. Because these correlations were calculated using mean values for each genetic line (as in app. B) they can be interpreted as genetic correlations in the broad sense. Boldface indicates significant correlations ($\alpha = 0.05$ for individual tests, 0.008 for the developmental traits, and 0.017 for the life-history traits; see text).

APPENDIX E

Correlations among Developmental Traits

Table E1: Pearson product-moment correlations among the developmental traits

Test	Sample	$r \pm SE$	P	Test	Sample	$r \pm SE$	P
All lines:							
CW-ICG	9	-.339 $\pm$.36	.3724				
CW-GR	9	.823 $\pm$.21	.0065				
ICG-GR	9	-.131 $\pm$.37	.7374				
Big (Before, Big/Short, Big/Long):				Small (Before, Small/Short, Small/Long):			
CW-ICG	5	-.518 $\pm$.49	.3718	CW-ICG	5	-.249 $\pm$.56	.6859
CW-GR	5	.542 $\pm$.49	.3456	CW-GR	5	.751 $\pm$.38	.1434
ICG-GR	5	-.097 $\pm$.57	.8767	ICG-GR	5	.108 $\pm$.57	.8625

Table E1 (Continued)

Test	Sample	$r \pm SE$	P	Test	Sample	$r \pm SE$	P
Short (Before, Big/Short, Small/Short):				Long (Before, Big/Long, Small/Long):			
CW-ICG	5	$-.195 \pm .57$	.7536	CW-ICG	5	$-.655 \pm .44$	.2304
CW-GR	5	$.853 \pm .30$	.0663	CW-GR	5	$.834 \pm .32$	.0794
ICG-GR	5	$.261 \pm .56$	.6711	ICG-GR	5	$-.172 \pm .57$	.7818
Antagonistic (Before, Big/Short, Small/Long):				Synergistic (Before, Big/Long, Small/Short):			
CW-ICG	5	$-.704 \pm .41$	.1849	CW-ICG	5	$.237 \pm .56$	.7006
CW-GR	5	$.925 \pm .22$	.0245	CW-GR	5	$.537 \pm .49$	.3506
ICG-GR	5	$-.603 \pm .46$	.2820	ICG-GR	5	$.692 \pm .42$	.1952

Note: "Before" refers to the initial colony before selection. "Big" and "small" refer to body size, and "short" and "long" refer to development time. "Antagonistic" and "synergistic" refers to selection on the focal traits (see fig. 2). Size = pupal mass; CW = critical weight; ICG = interval to cessation of growth; GR = growth rate. Because these correlations were calculated using mean values for each genetic line (as in app. B), they can be interpreted as genetic correlations in the broad sense. Boldface indicates a significant correlation ($\alpha = 0.05$ for individual tests and 0.017 for multiple comparisons; see text).

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Larval growth of *Manduca sexta*, the tobacco hornworm. From left to right, the first five images are of eggs and first-, second-, third-, and fourth-instar larvae. The two larvae on the right are both in the fifth instar, one at the beginning and one at the end. More than 90% of growth occurs during the fifth (last) larval instar, where growth increases tenfold in just 5 days and when peak body size is attained. Development time is the time it takes from hatching from an egg on the left, to peak size on the right. Photo: Goggy Davidowitz.