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The developmental and physiological basis of body size evolution in an insect

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The evolution of body size is a dominant feature of animal evolution. However, little is known about how the underlying developmental mechanisms that determine size change as body size evolves. Here we report on a case of body size evolution in the tobacco hornworm *Manduca sexta* that occurred over a period of nearly 30 years. We take advantage of an extensive series of physiological studies performed in the early 1970s that established the parameters that regulate body size in this species and compare their values with those of modern individuals that are descendants of the same colony. We show that three of the five processes that determine adult body size changed during this period, while two remained constant. Changes in these three developmental processes completely account for the observed evolutionary change in body size.

Keywords: body size evolution; growth; *Manduca sexta*

1. INTRODUCTION

The evolution of body size is the most common type of evolution in many lineages of animals and has been widely studied in laboratory populations. Although numerous studies have demonstrated that body size can evolve in response to directional selection (Roff 1992; Stearns 1992) none have elucidated how the underlying developmental and physiological mechanisms that determine body size evolve. We have studied the evolution of body size in a laboratory colony of the tobacco hornworm *Manduca sexta*. We take advantage of an extensive series of experiments performed in the early 1970s that established the processes that regulate body size in this insect. We compare the parameter values of these processes with those of modern individuals and demonstrate that a subset of these processes completely accounts for the observed evolution of body size.

M. sexta is a large sphingid moth that has been maintained in the laboratory since the late 1960s and has become one of the principal model organisms for the study of insect developmental endocrinology. There is a close causal association in final (fifth)-instar larvae of *M. sexta* between somatic growth and the timing of various endocrine events that induce the onset of metamorphosis (Nijhout & Williams 1974*a*,*b*; Nijhout 1981). During the fifth instar, the secretion of prothoracicotropic hormone (PTTH) and ecdysteroids is inhibited by the presence of juvenile hormone (Nijhout & Williams 1974*b*; Rountree & Bollenbacher 1986). The circulating level of juvenile hormone is high during the first few days of the instar but drops precipitously when the larva reaches a specific critical weight (Nijhout & Williams 1974*b*). At the same time juvenile hormone esterases accumulate in the haemolymph and enhance the rate of degradation of juvenile hormone. Approximately 24 h after passing the critical weight juvenile hormone is fully cleared from the haemolymph and secretion of PTTH and ecdysteroids is disinhibited (Nijhout & Williams 1974*b*; Rountree & Bollenbacher 1986). Secretion of PTTH occurs during the first photoperiodic gate that follows after the clearance of juvenile hormone (Truman 1972; Truman & Riddiford 1974). The PTTH stimulates the secretion of ecdysteroids that cause the larva to stop feeding, induce the switch over to pupal commitment and, a few days later, the metamorphic moult (Riddiford 1985; Nijhout 1994). Larval growth thus stops when the sequence of events initiated by the critical weight culminates in the secretion of ecdysteroids. As with other insects, adults do not grow and the size that a larva attains at the time of metamorphosis completely defines the body size of the adult insect. *M. sexta* is one of the few organisms for which we understand the suite of processes that regulate body size. It is therefore possible to investigate which of these processes change as body size evolves.

2. METHODS

For ease of notation we will refer to the modern data as 1999 data (although they were taken in 1999 and 2000) and to the old data as 1972 data (although they were taken from 1971 to 1973). This time-interval is equivalent to *ca.* 220 generations. The colony of *M. sexta* we used for these experiments is a direct descendant of the colony used in the early 1970s. Larvae of *M. sexta* were reared at a constant temperature of 25 °C under a short day (12 L:12 D) photoperiod. The experimental data from 1972 that form the comparative baseline for the present studies were taken from Nijhout & Williams (1974*a*,*b*) and Nijhout (1981; H. F. Nijhout, unpublished results). Body size, as used in this paper, refers to the maximal weight of the last larval instar. Body size, initial size of the final larval instar, growth rate, critical weight, time for juvenile hormone decay and photoperiod gate were all measured using the same methods and under the same environmental conditions as the '1972' studies (Truman 1972; Nijhout & Williams 1974*a*,*b*; Truman & Riddiford 1974; Nijhout 1981).

The critical weight is functionally defined as the weight at which further growth is not necessary for a normal time-course to metamorphosis. Larvae that are starved at or above the critical weight metamorphose at the same time as larvae that

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continue to feed normally after passing the critical weight. In contrast, if larvae are starved before they have achieved the critical weight, metamorphosis is significantly (and variably) delayed compared to larvae that continue to feed. We measured critical weight by dividing last (fifth)-instar larvae into weight classes at 0.5 g intervals. Larvae within each weight class were either allowed to feed normally or starved (they were provided with a 2% agar block to prevent desiccation) and the time to PTTH secretion recorded. PTTH secretion can be indirectly but unambiguously identified because it initiates two readily observable physiological events: the deposition of pink ommochrome pigment along the dorsum and the exposure of the dorsal vessel due to a clearing of the tissues around the heart (Nijhout & Williams 1974*b*). The minimum weight in which there was no significant difference (*t*-test) between the starved and fed groups was determined as the critical weight.

Two types of artificial diet were used: a few initial studies were done with a diet recipe we obtained from Dr Lynn M. Riddiford (University of Washington) and which is the one used for normal colony maintenance. Because diet quality can affect larval growth rate and, presumably, final body size all experiments reported here were performed with larvae reared on a diet formulated to be identical to that used in 1972.

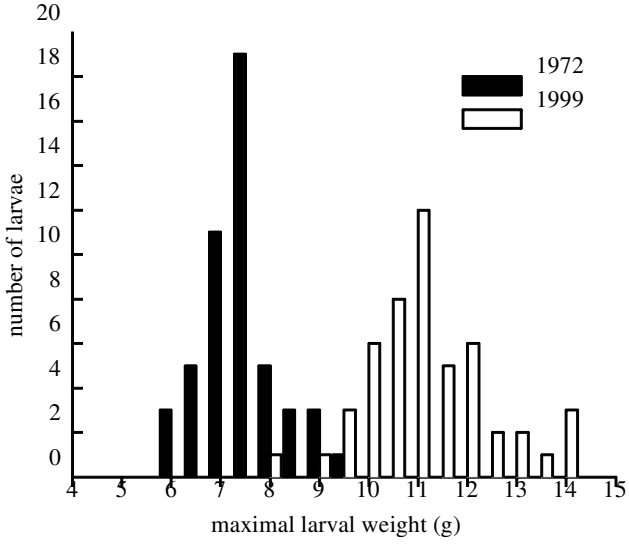

Figure 1. Size-frequency distributions of the maximal weights of fifth-instar larvae of *M. sexta* in 1972 and 1999. The difference between the means of these distributions is significant (*t*-test, $n = 50$, $t = 18.31$ and $p < 0.001$). These larvae were from the same colony, which has been kept in continuous culture in the laboratory without the introduction of new genetic material.

3. RESULTS

Two aspects of larval growth must be distinguished in order to understand the control of body size in *Manduca* (and other insects). First, at each larval moult, the size of the exoskeleton increases by a constant proportion. As a consequence, the size of a larva's exoskeleton increases exponentially from instar to instar. Second, a larva's mass also increases at an approximately exponential rate within each instar. Because growth is exponential, 88% of larval growth occurs in the final instar. The critical weight is achieved about the middle of the final larval instar. Hence, *Manduca* larvae nearly double their weight during the time between attainment of their critical weight and the onset of metamorphosis.

When the critical weight is reached, an irrevocable chain of events is set in motion that culminates in metamorphosis. How a larva assesses its critical weight is still unknown, except that the critical weight is proportional to the size of the larva's exoskeleton at the time of moulting to the final larval instar (Nijhout 1981). Thus, larvae that are larger at the outset of the final instar have a higher critical weight and, all things being equal, will metamorphose at a larger size. The final size that a larva attains is thus determined by five factors: the initial size of the final instar, the growth rate during that instar, the value of the critical weight, the time required for subsequent clearance of juvenile hormone and the timing of the opening of the subsequent photoperiodic gate for PTTH release.

The final sizes of the larvae in our laboratory colony of *Manduca* are substantially greater than those observed nearly 30 years ago (figure 1). In the early 1970s, larvae achieved a mean maximal weight of 7.8 g and a maximal weight of 9.5 g, whereas the larvae today have a mean maximal weight of 11.1 g and a maximal weight of 14.1 g, which is an increase of *ca.* 150%. Evidently, there has been a significant evolution of body size in this colony over a 30-year period. Because body size is determined by the five developmental and physiological factors outlined above, evolution of body size must be caused by evolutionary changes in one or more of these factors. An extensive series of physiological experiments in the early 1970s established the parameter value for the processes that control body size in *Manduca sexta* at that time. In order to ascertain the degree to which the body size of modern animals could be attributed to evolutionary changes in these parameters, we compare these values to those obtained from modern individuals that are direct descendants of this colony.

Growth curves for 1972 and 1999 larvae are shown in figure 2. The daily growth rate increased during the first 3 days and gradually decreased thereafter. The main difference in the growth trajectory occurred during the first 3 days of the instar when 1999 larvae grew significantly faster than those in 1972. After the third day, the growth rate of 1999 larvae ($2.4\,\mathrm{g\,day^{-1}}$) was only slightly greater than that of 1972 larvae ($2.1\,\mathrm{g\,day^{-1}}$). One possible cause for the difference in growth rate is a change in the formulation of the artificial diet. Hence, we prepared the diet according to the formula used in 1972 and found that growth on this diet did not differ significantly from that on our modern diet (figure 2). Nevertheless, all subsequent experiments were done with larvae reared on the original 1972 formula.

The initial size of the final (fifth)-instar larva in 1999 was identical to that found in 1972. This was a surprising finding because it seemed to us that the simplest way of increasing body size was to increase the growth increment between each moult. When a *Manduca* larva moults the size of the hard (sclerotized) portions of its exoskeleton increases by a constant factor. This results in an exponential increase in body size when measured over several moults. Exponential growth implies that most of the

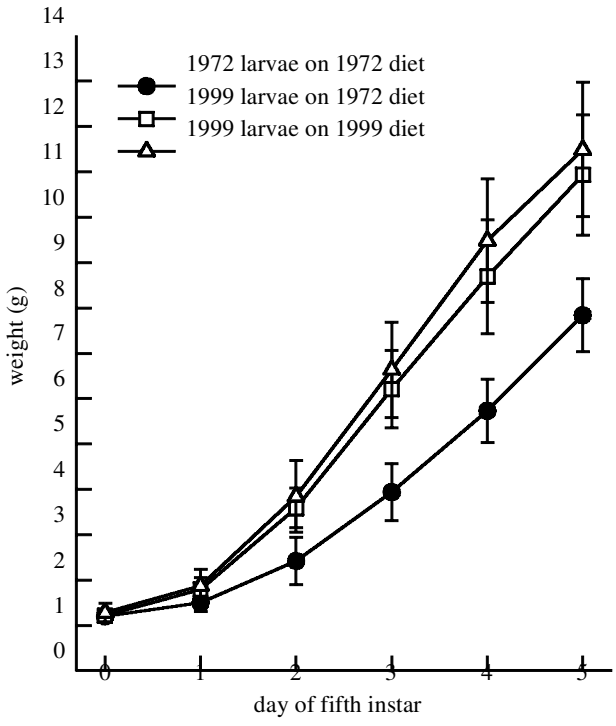

Figure 2. Growth curves of fifth (final)-instar larvae of *M. sexta*. The lower curve illustrates the mean growth rate of larvae in 1972 (Nijhout & Williams 1974*a*). The top two curves illustrate the mean growth rates of larvae in 1999 when fed on two different diets. The topmost curve is for 1999 larvae growing on the diet used in 1999 and the bottom curve is for 1999 larvae growing on a diet with the same formulation as that used in 1972. The peak weights of the larvae of our current (1999) colony reared on the two diets did not differ significantly (*t*-test, $n = 54$, $t = 1.93$ and $p > 0.05$). A repeated-measures ANOVA of the growth curves of larvae reared on the two diets showed no statistical difference in growth rates ($F = 1.5473$ and $p > 0.05$).

growth will occur in the last larval instar. In *M. sexta*, *ca.* 90% of the increase in body mass occurs in the final larval instar. Hence, the initial size of the final instar has a significant effect on the maximal size the larva can attain, as demonstrated by regression of the maximal larval weight (MLW) on last instar head capsule width (HCW), which is a measure of the general size of the exoskeleton: $\mathrm{MLW} = -24.8 + 6.2 \times \mathrm{HCW}$ ($F = 37.3$, $p < 0.0001$, $n = 21$ and $r^2 = 0.66$). In 1972 the initial weights of the fourth and fifth instars were 0.20 ± 0.02 g and 1.23 ± 0.15 g, respectively (all measures given as means ± standard deviations). In 1999 these weights were 0.20 ± 0.025 g and 1.28 ± 0.19 g, respectively. These means were found to be not significantly different (Games and Howell method) (Sokal & Rohlf 1981). In 1972 the head capsule sizes of fourth and fifth instars were 3.60 ± 0.35 mm and 5.85 ± 0.45 mm, respectively. In 1999 these sizes were 3.42 ± 0.10 mm and 5.70 ± 0.20 mm, respectively. These means were also found to be not significantly different (Games and Howell method) (Sokal & Rohlf 1981).

The critical weight in 1972 was 5.0 g (Nijhout & Williams 1974*a*). Two methods of estimating the critical weight (by starvation, as shown in figure 3 and by a decrease in the variance of the time to PTTH secretion,

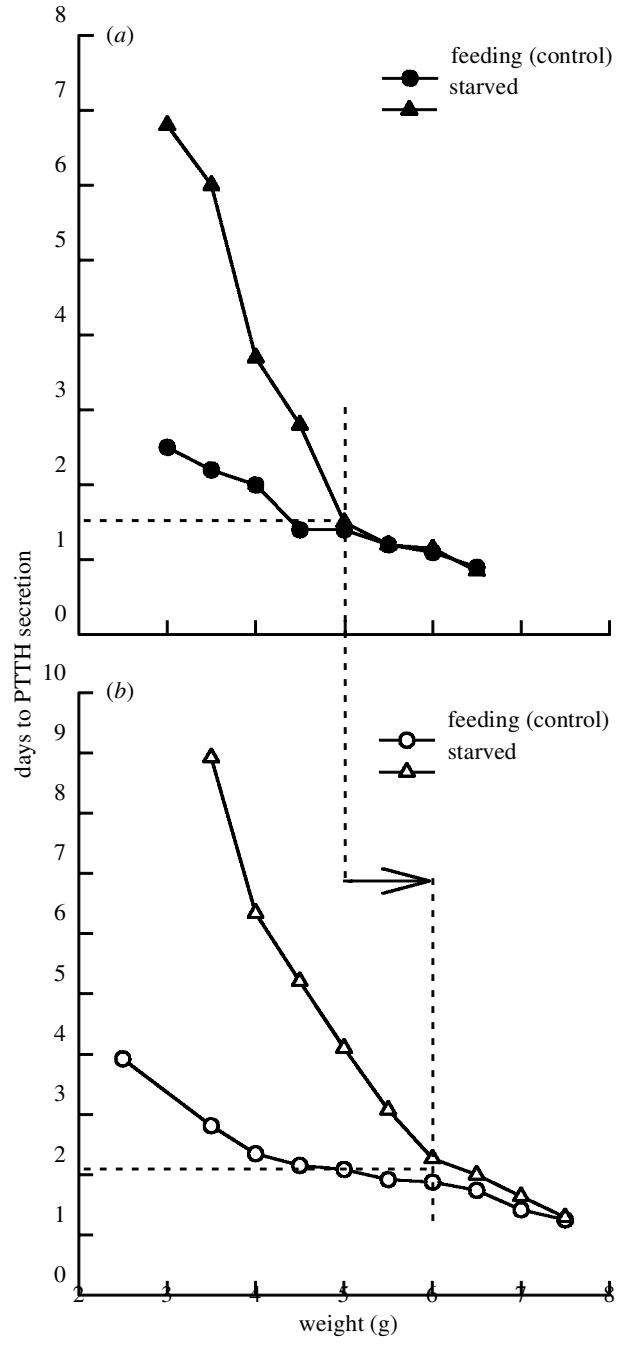

Figure 3. Determination of the critical weight. The critical weight is operationally defined as the minimal weight at which starvation no longer affects the timing of PTTH secretion (PTTH is a neurohormone that stimulates the secretion of ecdysteroid, which, in turn, causes the larva to stop feeding and initiates the physiological events that lead to pupation) (Nijhout 1994). Larvae that are starved at the critical weight or above release PTTH at the same time as larvae of the same weight that are allowed to continue feeding. The timing of PTTH secretion in feeding and starved larvae diverged at (*a*) 5 g in 1972 (Nijhout & Williams 1974*a*,*b*) and (*b*) at 6.0 g in 1999. The mean time between achieving the critical weight and PTTH secretion is indicated by the dotted horizontal line.

as shown in figure 4) show that, in 1999, the critical weight had increased to between 6.0 and 6.5 g. In addition, the delay between achievement of the critical weight and secretion of PTTH had increased from 36 ± 10 h in

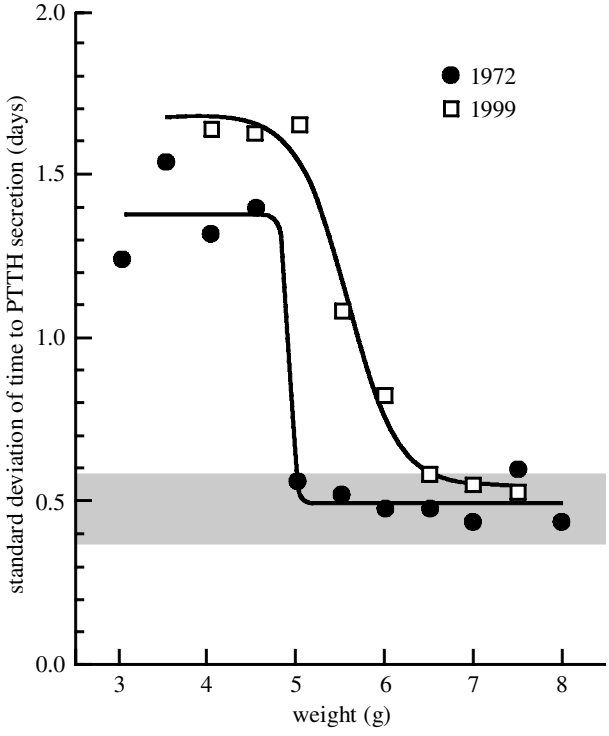

Figure 4. The timing of PTTH secretion in feeding larvae is well-synchronized. The horizontal grey bar indicates the standard deviations of the mean time to PTTH secretion for feeding larvae starting from a given weight. The curves indicate the standard deviations of the time to PTTH secretion of larvae starved at successively higher weights. When larvae were starved below their critical weight, the mean time to PTTH secretion increased (figure 3), as did the standard deviation of the mean time to PTTH secretion. In 1972, the standard deviations decreased abruptly in larvae starved above the critical weight of 5 g (Nijhout & Williams 1974*a*,*b*). In 1999 the standard deviations decreased more gradually and became equivalent to those of feeding larvae at weights of 6.5 g and above, suggesting a critical weight of 6.5 g.

1972 (Nijhout & Williams 1974*a*,*b*) to 50 ± 9 h in 1999 (figure 3). Part of the delay between achievement of the critical weight and PTTH secretion is due to the fact that, once a larva has achieved competence at secreting PTTH, it has to wait for a photoperiodic gate to open and, while a larva is 'waiting', it continues to feed and grow. In 1972 the daily photoperiodic gate was 8 h long (Truman & Riddiford 1974). If larvae become competent at secreting PTTH randomly during the day, approximately one-third of them will become competent while the gate is open and secrete PTTH immediately, while the remaining two-thirds will have to 'wait' until the next gate opens. The average larva in the population as a whole will thus wait *ca.* 5 h for the gate to open. One way of increasing body size would be to decrease the length of the photoperiodic gate so that larvae would, on average, have to wait longer for the gate to open and, thus, gain time for growth. We have determined the length of the photoperiodic gate using the methods of Truman (1972) and Truman & Riddiford (1974) and found that it is the same in length and timing in 1999 as it was in 1972. The gate is 8 h long, opens *ca.* 14 h after lights off and closes at *ca.* 22 h after lights off.

4. DISCUSSION

We have shown that body size in our colony of *M. sexta* increased by 50% during 30 years of laboratory culture. We do not know the selective force that has been at work, but two quite different possibilities come to mind. First of all it seems reasonable to assume that, in the normal course of colony maintenance, there is inadvertent selection in favour of bigger and healthier looking individuals (or against small individuals) for breeding. Alternatively, it is possible that, in nature, there is selection for rapid growth and early metamorphosis because of the high risk of predation and parasitoid load (Bernays 1997; Bernays & Woods 2000). Indeed, parasitization rates of *Manduca* larvae collected from the field in North Carolina range from 20% early in the summer to nearly 100% late in the summer (Rabb 1971). A selective pressure for early metamorphosis would be relaxed under laboratory conditions and this could also account for an evolutionary increase in body size in captive animals.

Perhaps the most intriguing finding of our studies was that only a subset of the processes that contribute to establishing the final body size of an individual changed during body size evolution. It is not clear why, for instance, the growth increment by which the larva increases as it moults from one instar to the next remained constant. Increasing the growth increment would result in a larger initial size of the final instar and, subsequently, a larger body size and would seem in principle to be the most effective way of increasing final body size. There is a substantial amount of phenotypic variation in this growth increment (as indicated by the standard deviations reported above), so it is a trait that could certainly be under selection. The absence of a response to selection could be due to a lack of additive genetic variance for the growth increment (in spite of a significant phenotypic variance) or because the growth increment is not genetically correlated with the trait under selection. The growth increment is likely to be more highly correlated with final body size than with time to metamorphosis, which suggests that relaxation of selection in favour of early metamorphosis may be the most likely cause of the observed evolution of body size.

It is worth inquiring to what degree the three factors that changed during body size evolution (growth rate, critical weight and delay between achieving the critical weight and secretion of PTTH) account for the change in body size. We can estimate the contribution of each of these factors to the final weight as follows. The 1999 growth rate after achieving the critical weight was *ca.* 2.4 g day^{-1} whereas in 1972 it was *ca.* 2.1 g day^{-1}. By multiplying the growth rate by the delay time and adding this to the critical weight, we obtain a prediction for body size. For 1972 this prediction is 2.1 g day^{-1} × 1.5 days (= 36 h) + 5.0 g = 8.15 g and for 1999 it is 2.4 g day^{-1} × 2.1 days (= 50 h) + 6.0 g = 11.04 g (taking the conservative estimate of 6.0 g for the new critical weight). These values are within 5% and 1%, respectively, of the empirically determined mean maximal weights of 7.8 g and 11.1 g (figure 1). These calculations suggest that evolutionary changes in these three factors almost completely account for the evolutionary increase in body size observed.

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REFERENCES

Bernays, E. A. 1997 Feeding by lepidopteran larvae is dangerous. *Ecol. Entomol.* **22**, 121–123.

Bernays, E. A. & Woods, H. A. 2000 Foraging in nature by larvae of *Manduca sexta* influenced by an endogenous oscillation. *J. Insect Physiol.* **46**, 825–836.

Nijhout, H. F. 1981 Physiological control of molting in insects. *Am. Zool.* **21**, 631–640.

Nijhout, H. F. 1994 *Insect hormones*. Princeton University Press.

Nijhout, H. F. & Williams, C. M. 1974*a* Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): growth of the last-instar larva and the decision to pupate. *J. Exp. Biol.* **61**, 481–491.

Nijhout, H. F. & Williams, C. M. 1974*b* Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): cessation of juvenile hormone secretion as a trigger for pupation. *J. Exp. Biol.* **61**, 493–501.

Rabb, R. L. 1971 Naturally occurring biological control in the Eastern United States, with particular reference to tobacco insects. In *Biological control* (ed. C. B. Huffaker), pp. 294–311. New York: Plenum Press.

Riddiford, L. M. 1985 Hormonal action at the cellular level. In *Comprehensive insect physiology biochemistry and pharmacology*, vol. 8 (ed. G. A. Kerkut & L. I. Gilbert), pp. 337–384. Oxford, UK: Pergamon Press.

Roff, D. A. 1992 *The evolution of life histories. Theory and analysis*. New York: Chapman & Hall.

Rountree, D. B. & Bollenbacher, W. E. 1986 The release of the prothoracocotropic hormone in the tobacco hornworm, *Manduca sexta*, is controlled intrinsically by juvenile hormone. *J. Exp. Biol.* **120**, 41–58.

Sokal, R. R. & Rohlf, F. J. 1981 *Biometry*. New York: W. H. Freeman.

Stearns, S. C. 1992 *The evolution of life histories*. Oxford University Press.

Truman, J. W. 1972 Physiology of insect rhythms. I. Circadian organization of the endocrine events underlying the moulting cycle of larval tobacco hornworms. *J. Exp. Biol.* **57**, 805–820.

Truman, J. W. & Riddiford, L. M. 1974 Physiology of insect rhythms. III. The temporal organization of the endocrine events underlying pupation of the tobacco hornworm. *J. Exp. Biol.* **60**, 371–382.