

ORIGINAL ARTICLE

High temperature slows down growth in tobacco hornworms (*Manduca sexta* larvae) under food restriction

Matthew B. Hayes^{1,†}, Lihong Jiao^{1,†}, Tsu-hsuan Tsao^{1,2}, Ian King¹, Michael Jennings¹ and Chen Hou¹

¹*Department of Biological Science, Missouri University of Science and Technology, Rolla, MO, 65409, USA, and* ²*Department of Biological Science, National Taiwan Normal University, Taipei, Taiwan, 11677, China*

Abstract When fed *ad libitum* (AL), ectothermic animals usually grow faster and have higher metabolic rate at higher ambient temperature. However, if food supply is limited, there is an energy tradeoff between growth and metabolism. Here we hypothesize that for ectothermic animals under food restriction (FR), high temperature will lead to a high metabolic rate, but growth will slow down to compensate for the high metabolism. We measure the rates of growth and metabolism of 4 cohorts of 5th instar hornworms (*Manduca sexta* larvae) reared at 2 levels of food supply (AL and FR) and 2 temperatures (20 and 30 °C). Our results show that, compared to the cohorts reared at 20 °C, the ones reared at 30 °C have high metabolic rates under both AL and FR conditions, but a high growth rate under AL and a low growth rate under FR, supporting this hypothesis.

Key words energy budget, food restriction, growth, hornworm, metabolism, tradeoff

Introduction

Ontogenetic growth, an energetically costly process, is fueled by metabolism (Wieser, 1994). Understanding the relationship between growth and metabolism has been a central theme in ecological physiology (Sibly & Calow, 1986; Karasov & del Rio, 2007), and it requires a framework of animals' energy allocation strategy. During growth, the energy assimilated from food, F , is partitioned between the energy deposited in new biomass, S , which is proportional to growth rate, and metabolic energy, B , which is dissipated as heat (Brody, 1945; Kooijman, 2000; van der Meer, 2006; Hou *et al.*, 2008), i.e.,

$$F = S + B. \quad (1)$$

For ectothermic animals, food availability and ambient temperature are 2 major environmental factors that largely

influence their energy budget (Atkinson, 1994; Miller *et al.*, 2009; Lee & Roh, 2010; Zuo *et al.*, 2012). When ectothermic animals are fed with unlimited food (*ad libitum* [AL]), high temperature induces an increased metabolic rate, B (Gillooly *et al.*, 2001). Along with metabolism, the growth rate increases with temperatures (Atkinson, 1994; Gillooly *et al.*, 2002; Zuo *et al.*, 2012). Thus, under AL condition the rates of metabolism and growth are positively correlated. The temperature-induced increase in the rates of metabolism and growth is known as the Q10 effect, referring to the increase in the growth and metabolic rate for a 10 °C increase in temperature, and usually takes on values between 2 and 3 (Gillooly *et al.*, 2001), but sometime below 2 (Hack, 1997; Chappell, 1983). The increased energy requirements are met by the increased food uptake rate until the capacity of an animal's digestive system reaches its limit (Hammond & Diamond, 1997). However, the correlation between metabolism and growth may not always be positive when temperature increases (Diamond & Kingsolver, 2010; Clissold *et al.*, 2013). When the food availability is limited and lower than AL level, Equation (1) ($F = S + B$) suggests an energy trade-off between growth, S , and metabolism, B (Hou *et al.*, 2011). For a given body mass, if F is limited, then any

Correspondence: Chen Hou, Department of Biological Science, Missouri University of Science and Technology, Rolla, MO 65409, USA. Tel: #1 573 341 6360; fax: #1 573 341 4821; email: houch@mst.edu

[†]These authors contributed equally to this study.

change in either S or B , due to environmental factors such as temperature, must cause a change in the other in the opposite direction. Since the metabolic rate of ectothermic species increases with ambient temperature, we hypothesize that in ectothermic animals fed with a fixed food supply lower than the AL level, high temperature will lead to a reduced growth rate. We use the 5th instar tobacco hornworms (*Manducasexta* larvae) as a model to test this hypothesis. The 5th instar hornworm grows from 1–2 g to 7–15 g in 6–10 d depending on the temperature and food level, making it an ideal model to study growth (Reynolds & Nottingham, 1985; Kingsolver & Woods, 1997).

Materials and methods

Animal rearing

In the summer of 2012, we raised approximately 100 tobacco hornworms (*Manduca sexta* larvae) from eggs obtained from Carolina Biological supply (NC, USA) AL on a long day cycle (17 h light : 7 h dark) at 25 °C until the 5th instar. On the first day of the 5th instar, we randomly separated the larvae into 2 incubators, which were set at temperatures 20 and 30 °C, respectively. At each temperature, we fed the larvae at 2 food supply levels, AL and food restriction (FR) (see below). We therefore had 4 cohorts of larvae (2 temperatures \times 2 food levels), which were labeled as 20 °C-AL, 20 °C-FR, 30 °C-AL, and 30 °C-FR. Each cohort consisted of \sim 25 larvae. Each larva was reared in an individual plastic clear vial (diameter: 5 cm; length: 12 cm).

Growth rate

We measured the body mass of each larva in every cohort at approximately the same time every day from the first day of the 5th instar to the nearest 0.1 mg, using a digital microbalance (Perkin-Elmer AD6; Downers Grove, IL, USA). We define the growth rate, in unit of gram/day, as the increment of body mass from 1 day to the next.

Food supply levels

After weighing the larval body mass, we fed the larvae with a wheat germ-based diet (hornworm medium bulk diet; Carolina Biological supply). The AL cohorts fed freely, and we measured the food uptake rate of every larva every day. During the experiment, no larva in the

AL cohorts ran out of food. For both FR cohorts at 20 and 30 °C, we fed each larva with the amount of food calculated from the equation $F = 0.5 \times m^{0.75}$, where F is the amount of food and m is the body mass, both in units of grams. Food supply was weighted to the nearest 1 mg. Our previous data on food uptake rate of AL larvae suggest that this FR level is well below AL for larvae reared at both 20 and 30 °C. The data from this study also confirm this. In this study, the food uptake rate of AL-fed cohorts scale with body mass as $F = 1.313 \times m^{0.74}$ ($R^2 = 0.76$) at 30 °C and $F = 0.622 \times m^{0.78}$ ($R^2 = 0.71$) at 20 °C. We used the same equation, $F = 0.5 \times m^{0.75}$, to feed both 20 °C-FR and 30 °C-FR cohort, because the FR level needs to be the same at both temperatures to test the hypothesis. During the experiments, every larva in the FR cohorts completely finished its food every day.

The higher temperature causes higher water loss in food. Although FR larvae at both temperatures obtain the same amount calories every day, the water content in diet affects the growth and metabolic rate of hornworms. Martin and Van't Hof (1988) have shown that the growth efficiency (body mass gain per food intake) is 12% lower, and metabolic rate is 16% higher, in the hornworms fed on a diet containing 65% water compared to the ones on an 82% water diet. To measure the water evaporation, at each temperature we prepared 5 food samples with the similar mass and shape as the food given to the larvae, and placed the samples in the vials that the larvae were reared in. We then calculate the percentage of water loss in diet after 12 and 24 h.

Metabolic rate

We used equipment from Sable Systems International (SSI; Las Vegas, NV, USA) to perform the flow-through respirometry with an incurrent flow measurement (Lighton, 2008). Before all trials, we calibrated a CA-10 CO₂ analyzer (SSI) with air run through a drierite/ascarite/drierite column and then spanned it with a gas of known CO₂ concentration (1 000 ppm; CO₂ in N₂ \pm 1). We then calibrated an FA-10 Oxygen analyzer (SSI) with water and CO₂ scrubbed air at 20.95% (Lighton, 2008). A baseline measurement was taken before, between, and after each experimental trial by running air scrubbed of water and CO₂ through an empty chamber and then into the respirometry system. We set flow rate at 60 mL/min using an SS-4 subsampler (SSI). This air was then sent to the larva or baseline chamber. Between the CO₂ and O₂ analyzers, we scrubbed the CO₂ produced by the larvae by a column of drierite/ascarite (II)/magnesium perchlorate so that the CO₂ concentration will not affect

the measurement of O_2 . Temperature was controlled using a pelt-5 temperature controller (SSI) that houses the respirometry and baseline chambers. Respirometry chambers for individual larvae were 60-cc syringe barrels fitted with rubber stoppers. We randomly chose 6 larvae from each cohort on the first day of the 5th instar, and used the same individuals for the respirometry measurement every day until the wandering stage. The rates of O_2 consumption and CO_2 production, \dot{V}_{O_2} and \dot{V}_{CO_2} , of each larva were measured for 7–10 min time interval every day after their body mass was measured.

We used SSI ExpeData software (SSI) to correct for drifts in CO_2 and O_2 concentration. The rates \dot{V}_{O_2} and \dot{V}_{CO_2} were calculated as $\dot{V}_{CO_2} = FR \times [CO_2]/100$, and $\dot{V}_{O_2} = FR \times (20.95 - [O_2])/(100 - [O_2])$, where FR is the flow rate, and $[CO_2]$ and $[O_2]$ are the concentration of CO_2 and O_2 in the respirometry chamber (Lighton, 2008). Each data point represents the average of the measurement taken during the time interval. The larval metabolic rate (in unit of watts) was calculated as $B = (43.25 - 22.5 \times RER)\dot{V}_{CO_2}/60$, where $\dot{V}_{CO_2}/\dot{V}_{O_2}$ is the respiratory exchange ratio (Blaxter, 1989; Withers, 1992).

Data analysis and statistics

Data on metabolic rate (B) was collected and analyzed every day for the same 6 larvae in each cohort from the first day of the 5th instar to the wandering stage. The data on food intake (F) and growth (S) was collected from all the larvae in each cohort that were alive at the end of the experiment. Mortality rate was between 10% and 20% among cohorts, so the data on F and S were from 20–23 individuals in each cohort every day. Larvae decrease their food intake and growth rate considerably as they approach the peak mass (Esperk & Tammaru, 2004; Sears *et al.*, 2012). Thus, we followed Sears *et al.* (2012) and restricted our analysis of the rates of food intake (F) and growth (S) to the “free growth period,” during which the increase in growth rate is positive. All 3 rates, F , S , and B , are expressed as scaling power laws of body mass (Greenlee & Harrison, 2005; Sears *et al.*, 2012), in the form of $R = a \times m^d$, where R is the rate of interest, a is the scaling coefficient, d is the scaling power, and m is the body mass. The scaling equation was logarithm transformed, $\text{Log}(R) = \text{Log}(a) + d \times \text{Log}(m)$, and the ordinary least square linear regression was used to estimate the scaling coefficients and powers. Statistical analyses were performed using SPSS 21. We performed

a full model ANCOVA with body mass as a covariate to test if there is significant interaction of 2 factors temperature \times food on the rates of growth and metabolism. We then conducted separate ANCOVA using temperature as a single factor to test if within the same diet regime temperatures have significant effects on growth and metabolism. Since we made multiple measurements on the same larvae repeatedly, we treated individual larvae as random factors to control for repeated measurements. We exclude the random factors from the model if their effects are insignificant ($P > 0.05$).

Results

Metabolic rate

For AL cohorts, the metabolic rate scales with body mass as $B_{30^\circ C-AL} = 0.00568 \times m^{0.77}$ ($R^2 = 0.80$) at $30^\circ C$, and $B_{20^\circ C-AL} = 0.00309 \times m^{0.83}$ ($R^2 = 0.82$) at $20^\circ C$ (Fig. 1A). For food restricted (FR) cohorts, the metabolic rate scales with body mass as $B_{30^\circ C-FR} = 0.00775 \times m^{0.39}$ ($R^2 = 0.39$) at $30^\circ C$ and $B_{20^\circ C-FR} = 0.00467 \times m^{0.46}$ ($R^2 = 0.43$) at $20^\circ C$ (Fig. 1B). The full model ANCOVA shows that there is no significant interaction of temperature \times food on metabolic rate ($F_{1,210} = 0.135$, $P = 0.714$). Within the same diet regime, different temperatures have no significant effect on the slopes of the metabolic rates (ANCOVA, $F_{1,131} = 1.574$ and $P = 0.212$ for AL cohorts; $F_{1,82} = 0.009$ and $P = 0.598$ for FR cohorts). But within the same diet regime, the intercept of the metabolic rate significantly increases at high temperature. In AL cohorts, $B_{30^\circ C-AL}$ is about 1.70 fold higher than $B_{20^\circ C-AL}$ (Q10 = 1.70, ANCOVA, $F_{1,131} = 126.31$, $P < 0.001$); and in FR cohorts, $B_{30^\circ C-FR}$ is 1.50 fold higher than $B_{20^\circ C-FR}$ (Q10 = 1.5; ANCOVA, $F_{1,82} = 69.39$, $P < 0.001$).

Growth rate

For growth rate, there was a significant interaction of temperature \times food (ANCOVA, $F_{1,258} = 122.042$, $P < 0.001$). Within the same diet regime (AL or FR), temperature has no significant effect on the slope of growth rate (ANCOVA, $F_{1,117} = 0.556$ and $P = 0.457$ for AL cohorts; $F_{1,143} = 1.824$ and $P = 0.179$ for FR cohorts). For AL-fed animals, Figure 2A shows that the growth rate of the cohort $30^\circ C-AL$ scales with body mass as $S_{30^\circ C-AL} = 0.909 \times m^{0.64}$ ($R^2 = 0.51$), 2.43 fold higher than the cohort $20^\circ C-AL$ $S_{20^\circ C-AL} = 0.386 \times m^{0.62}$ ($R^2 = 0.71$) (ANCOVA, $F_{1,117} = 118.063$, $P < 0.001$).

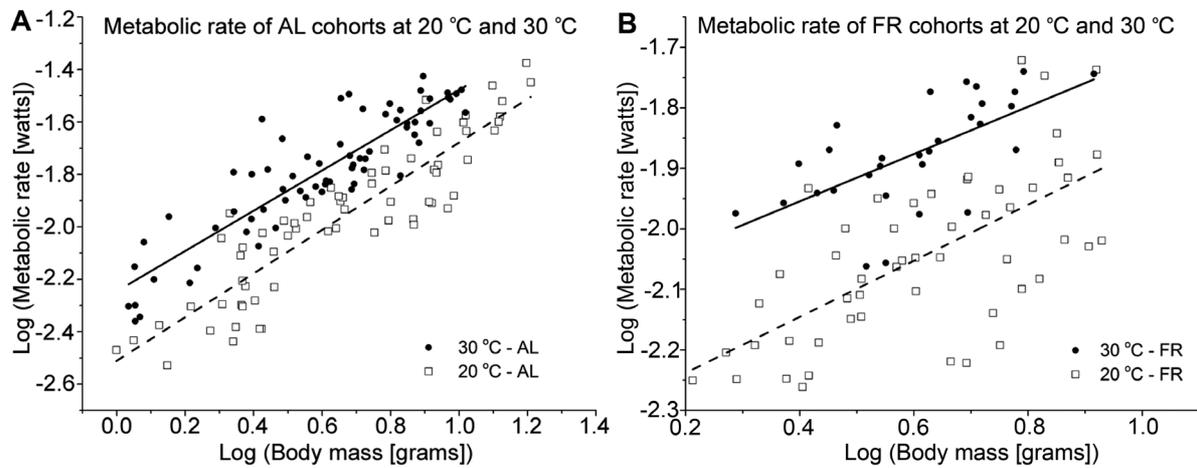


Fig. 1 The effects of temperature on metabolic rates in *ad libitum* (AL) and food restricted (FR) *M. sexta* larvae. Within the same diet regime, the slopes of metabolic rate are the same at different temperatures (ANCOVA, $P > 0.05$), but the intercept is higher at the higher temperature (ANCOVA, $P < 0.05$). There is no interaction of temperature \times food (ANCOVA, $F_{1,210} = 0.135$, $P = 0.714$).

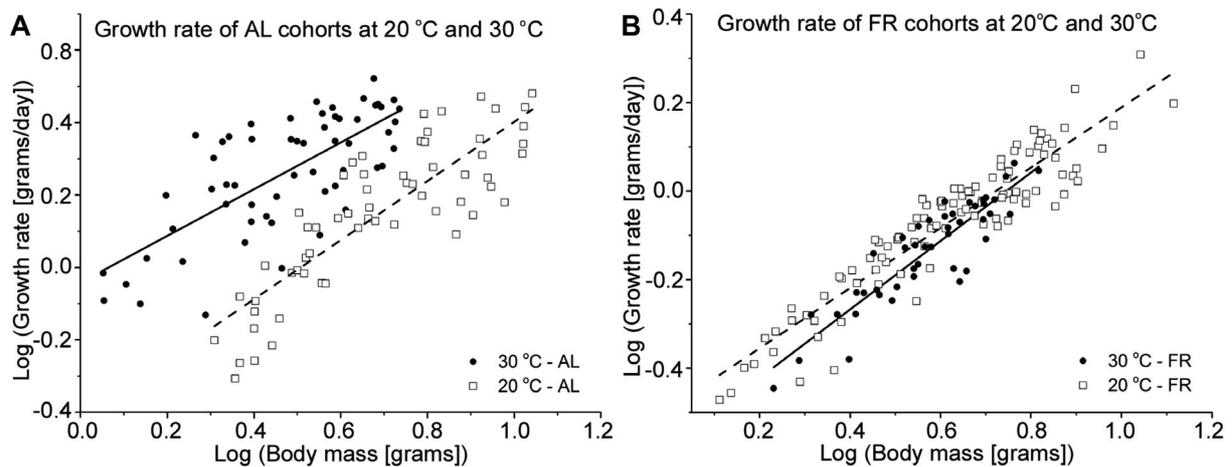


Fig. 2 The effects of temperature on growth rates in *ad libitum* (AL) and food restricted (FR) *M. sexta* larvae. With the same regime, the slopes of growth rate are the same at different temperatures (ANCOVA, $P > 0.05$). The intercept is higher at higher temperature under AL (Panel A), whereas it is lower at higher temperature under FR (ANCOVA, $P < 0.05$) (Panel B). There is a significant interaction of temperature \times food (ANCOVA, $F_{1,258} = 122.042$, $P < 0.001$).

However, opposite to what is observed in the AL-fed cohorts, Figure 2B shows that the growth rate of the 20°C-FR cohort, scaling as $S_{20^{\circ}\text{C-FR}} = 0.323 \times m^{0.68}$ ($R^2 = 0.87$), is 1.07 fold higher than the 30°C-FR cohort (ANCOVA, $F_{1,143} = 10.61$, $P < 0.001$), which scales as $S_{30^{\circ}\text{C-FR}} = 0.265 \times m^{0.77}$ ($R^2 = 0.80$).

The percentages of water loss after 12 h are $3.11\% \pm 0.66\%$ and $6.55\% \pm 2.10\%$ at 20 and 30°C, respectively. After 24 h, the water losses are $4.43\% \pm 0.42\%$ and $9.81\% \pm 2.41\%$ at 20 and 30°C, respectively. The sample size is 5 at each temperature.

Discussion

In this study we are interested in how increasing temperature affects the rates of growth and metabolism of food restricted hornworms fed with the same food supply level. In AL larvae, 10°C increase in temperature leads to a 1.7 fold increase in metabolic rate (Fig. 1), in agreement with the general Q10 effect (Chappell, 1983; Hack, 1997; Gillooly *et al.*, 2001). With the increasing temperature, the larvae increase food uptake by 2 fold, obtaining more energy to meet the increased metabolic requirement. The

similar temperature-induced increase in the food uptake rate in AL *M. sexta* larvae has been observed previously (Reynolds & Nottingham, 1985; Kingsolver & Woods, 1997). In AL larvae, the higher temperature also leads to a higher growth rate (Fig. 1B) as expected (Atkinson, 1994; Gillooly *et al.*, 2002).

In food restricted (FR) larvae, the 10 °C increase in temperature also causes an increase in metabolic rate to a lower degree—1.5 fold. However, under FR condition, the high temperature induces a 1.07 fold lower growth rate. Statistically, there is a significant temperature \times diet interaction for growth rate (ANCOVA, $F_{1,258} = 122.042$, $P < 0.001$), so that at high temperature, growth increases under AL condition, but decreases under FR condition. The interaction of temperature and diet is insignificant for metabolism (ANCOVA, $F_{1,210} = 0.135$, $P = 0.714$), and rising temperature increases metabolic rate regardless of diet regimes. Our hypothesis predicts the insignificant temperature \times diet interaction for metabolism, as well as, the significant interaction for growth. The metabolic rate of ectotherms always increases with the ambient temperature (Gillooly *et al.*, 2001). The higher metabolic rate comes with a high cost in terms of resources and energy from food. With a fixed food supply, it is inevitable that less resource and energy is available for growth. Thus, this tradeoff results in a slower growth rate at higher temperature (Fig. 2).

The tradeoff between growth and metabolism and the consequential suppression of growth at high temperature may also be enhanced by the prolonged starvation time at high temperature. The higher metabolism leads to faster food intake. We do not have accurate data on feeding behavior to conduct a rigorous statistical comparison on the feeding times between FR cohorts at different temperatures. But, FR larvae at 30 °C finished their food less than 8–10 h on average, whereas the ones at 20 °C spent more than 17–18 h. So, FR larvae at 30 °C experienced a longer starvation time than the ones at 20 °C during every 24-h period. Prolonged starvation may cause mobilization of reserves accumulated in fat bodies, and mass loss. Thus, the retarded net growth in the FR larvae at 30 °C (body mass gain – body mass loss during the 24-h period) is aggravated by the longer starvation. In this case, the tradeoff between growth and metabolism reach an extreme degree, that is, larvae not only allocate less energy to growth, but also have to mobilize biotissue (negative growth) to provide energy to match the increased metabolism when the energy from food is limited.

The differences in the growth rate between the FR cohorts at 2 temperatures are not likely caused by the difference in water losses in food at the different temperatures. Our results show that the FR larvae at 30 °C finished food

less than 8–10 h, and the water loss in 12 h at 30 °C is $6.55\% \pm 2.10\%$; the FR larvae at 20 °C spent 17–18 h on feeding, and the water loss at 20 °C in 24 h is $4.43\% \pm 0.42\%$. Thus, the difference between the water losses in the food that was consumed by the larvae at both temperatures is about $6\% - 4\% \approx 2\%$. In Martin and Van't Hof's study on hornworms (1988), 17% difference in water contents in diet causes 12% and 16% differences in growth and metabolism respectively. So, we believe that the 2% difference in our study is negligible.

The energy tradeoff between growth and metabolism has been observed in other insect species. Lee and Roh (2010) analysed the interactive effects of temperature and nutrition on growth rate in the final instar beet armyworm (caterpillar of *Spodoptera exigua*), which were reared at 1 of 3 temperatures (18, 26, and 34 °C), and received 1 of 6 diets differing in their ratio of protein and carbohydrate (P : C). They found that for rates of food intake and growth there was a significant temperature \times diet interaction, so that the difference in these rates between temperatures was greatest on moderate P : C diets and least on the most extreme diets (extremely high and low P : C), which are considered severe deficiencies of carbohydrate and protein, respectively. The authors stated “the mechanisms remains to be elucidated but severe energy (carbohydrate) and protein deficiency resulting from eating these diets seem likely.” We believe that the tradeoff between growth and metabolism revealed by our study can explain Lee and Roh's results. At balanced diet (moderate P : C diet), the food intake rates of armyworm are relatively high at all temperatures, which is similar to free-feeding in our study. Thus, growth increases with temperature, as also seen in our study, and the authors observed large differences in growth rate between temperatures. When diet has deficiency of either carbohydrate or protein (imbalanced P : C ratio), the food intake of armyworm is low at all temperatures, similar to the FR in this study. Because of the high metabolism at high temperature, relatively less resources and energy was allocated to growth in armyworms at high temperature, so that growth is suppressed at high temperature, and authors observed the smallest difference in growth rate between temperatures. The authors proposed: “this situation is expected to be aggravated when metabolic rate increases as a function of temperature,” but they did not measure the metabolic rate of the caterpillars. By measuring rates of growth and metabolism, our study explicitly reveals the tradeoff between them, and therefore supports Lee and Roh's speculation.

With a different purpose, a study of Miller *et al.* (2009) indirectly showed the tradeoff in locusts fed AL. The authors measured thermal preferences in migratory

locust (*Locusta migratoria*) and investigated growth efficiency (conversion of ingesta to body mass) at different temperature and diet regimes. Locusts were fed with diets of high-protein, high-carbohydrate, or a choice between both. The authors found that locusts placed in a thermal gradient selected temperatures near 38 °C, maximizing rates of weight gain. But at this temperature protein and carbohydrate were poorly converted to body mass, compared to the intermediate temperature (32 °C). The authors concluded “body temperature preference thus yielded maximal growth rates at the expense of efficient nutrient utilization.” Within the framework developed in our study, the growth efficiency (or nutrient utilization efficiency) is equivalent to S/F , the ratio of growth to food intake, which is equal to $(F - B)/F$ by the virtue of Equation (1). The observation that growth is higher, but the efficiency is lower at higher temperature in free-feeding locust indicates that as temperature increases, the percentage increase in metabolic rate, B , is faster than the percentage increases in food intake rate, F , so that the ratio $(F - B)/F$ is lower at the high temperature. The temperature induced mismatches between the rates of metabolism and food intake (faster increase in B but slower increase in F as temperature increases) have been seen in many free-feeding ectotherms (Kearney & White, 2012; Lemoine & Burkepile, 2012). Analysing the mechanisms underlying the mismatch is beyond the scope of this paper, and we refer to the recent publication of Lemoine and Burkepile (2012) for detailed discussion. In our study, the growth rate and growth efficiency in free-feeding larvae both increase as temperature. Using our data on the rates of growth and food intake of free-feeding larvae at 20 and 30 °C, we found that the growth efficiency (S/F) is about 57% at 20 °C on average, and increases to 61% at 30 °C, opposite to Miller *et al.*'s study on locusts. The reason that we did not observe the mismatch between the rates of metabolism and food intake is because it usually occurs at extremely high temperatures. In a study on hornworms, Kingsolver and Woods (1997) investigated the thermal sensitivity of growth and feeding with a temperature range from 14 to 42 °C. When temperature is above 34 °C (higher than that in our study), the mismatch was observed. In Miller *et al.*'s study (2009), the temperature, at which mismatch was seen, was 38 °C, also higher than that in our study.

In conclusion, through a simple experiment we show that due to the tradeoff between growth and metabolism, when food supply is fixed and below AL level, growth rate is negatively correlated to ambient temperature in hornworm, opposite of what has been observed in free fed insect larvae.

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Disclosure

The authors declare that they do not have any conflict of interest.

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