Increased temperature causes protein limitation by reducing the efficiency of nitrogen digestion in the ectothermic herbivore *Spodoptera exigua*

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Abstract. For insect herbivores, rising temperatures lead to exponentially higher metabolic rates. As a result, basic nutritional demands for protein and carbohydrates can be altered at high temperatures. It is hypothesized that temperature-driven increases in metabolic nitrogen turnover will exacerbate protein limitation by increasing metabolic nitrogen demand. To test this hypothesis, the present study examines whether metabolic nitrogen turnover at higher temperatures causes protein limitation of a generalist herbivore, the beet armyworm *Spodoptera exigua* Hübner (Lepidoptera : Noctuidae). Third-instar *S. exigua* larvae were reared at 25 and 30 °C on three artificial diets of varying protein : carbohydrate ratios (23 : 26, 17 : 26 and 6 : 26 %P : %C, respectively) and their growth rates, metabolic nitrogen demand and consumption rates were measured. Warming was found to lead to temperature-induced protein limitation of the *S. exigua* larvae by increasing metabolic nitrogen demand at the same time as reducing nitrogen digestion efficiency. Because climate change is increasing atmospheric temperatures rapidly worldwide, it is suggested that a better understanding of how temperature change can influence metabolic demands will provide key information for predicting herbivore growth rates and foraging strategies in the future.

Key words. Climate change, metabolism, nitrogen budget, Noctuidae, *Spodoptera exigua*.

Introduction

Temperature regulates a number of basic physiological processes (Savage *et al.*, 2004; Dell *et al.*, 2011; DeLong *et al.*, 2013). Metabolism, growth and reproduction increase exponentially with warming in almost all insect species, which, in turn, accelerates cellular energetic demands (Gillooly *et al.*, 2001; but see also Englund *et al.*, 2011; Lemoine & Burkepile, 2012). These altered metabolic requirements may reshape an insect's demands for specific macronutrients, such as carbohydrates or protein, driving changes in herbivore behaviour and feeding rates as the insects attempt to maintain nutritional homeostasis at higher temperatures (Rall *et al.*, 2010; Vucic-Pestic *et al.*, 2011; Clissold *et al.*, 2013; Lemoine *et al.*, 2013, 2014a). Given that climate change is rapidly increasing atmospheric temperatures

(IPCC, 2007), understanding the interactive effects of temperature and diet quality on herbivore performance and feeding behaviour remains an important research topic (Cross *et al.*, 2015).

Survival and growth of insect herbivores depend upon dietary quality, often in the form of protein : carbohydrate (P : C) ratios (Simpson *et al.*, 2015). The P : C ratios for optimal performance vary between orders and families of insects and are often influenced by an animal's life history. For example, generalist orthopteran herbivores need to ingest more carbohydrates than protein to maintain the lipid reserves needed for higher levels of activity (Raubenheimer & Simpson, 2003; Simpson *et al.*, 2015). By contrast, high P : C ratio diets are required for optimal performance of larval lepidopterans, which suffer from lower survival, longer developmental times and reduced pupal mass on protein-deficient diets (Lee *et al.*, 2002, 2004; Merkx-Jacques *et al.*, 2008; Simpson *et al.*, 2015). By altering metabolic demands, rising temperatures might significantly shift the dietary requirements of insect herbivores, which, in turn, can

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have significant consequences on herbivores that cannot switch diets accordingly. Although numerous studies have assessed the impact of warming temperatures on the energy requirements of insect herbivores (Kingsolver & Woods, 1997, 1998), there is a relative paucity of work exploring the physiological interactions between temperature and macronutrients such as protein (Lee & Roh, 2010).

Temperature interacts with diet quality in numerous ways to determine insect herbivore performance (Clissold & Simpson, 2015). Caterpillar growth rates increase with temperature but do so more rapidly on protein-rich diets (Kingsolver & Woods, 1998; Kingsolver *et al.*, 2006; Lee & Roh, 2010). This is probably because proteins denature more rapidly at higher temperatures, which, in turn, requires greater rates of protein synthesis and repair to maintain basic cellular function (Hachiya *et al.*, 2007). Increased maintenance requirements at high temperatures may occupy a greater fraction of available nitrogen, a crucial and often limiting component of protein synthesis in insects (Mattson, 1980; Somero, 2011). Preliminary evidence indicates that this is indeed the case. Growth and consumption rates increase exponentially with temperature for some herbivore species only on nitrogen-rich plants (Lemoine *et al.*, 2013, 2014a). Other herbivores increase their consumption of low nitrogen diets to maintain constant nitrogen intake (Williams *et al.*, 1994). Still other herbivores decrease their diet breadth at high temperatures by preferentially feeding on a smaller, more nutritious set of host plants (Lemoine *et al.*, 2013). However, it remains unknown whether changes in herbivore performance in relation to dietary nitrogen content are a product of higher nitrogen turnover and therefore increased protein demand.

The present study assesses warming-induced changes in herbivore metabolic nitrogen demands using the beet armyworm *Spodoptera exigua* Hübner as a model organism. *Spodoptera exigua* and its congeners require a dietary P : C ratio of at least 28% : 14% to achieve maximum growth and survival, although growth performance suffers on more heavily protein-biased diets (Lee *et al.*, 2002, 2004; Lee & Roh, 2010). The optimal P : C ratio does not change with temperature, although differences between the worst- and best-performing diets are exacerbated at high temperatures (Lee & Roh, 2010). Previous work reports *Spodoptera litura* final-instar larvae being allowed to self-select the optimal P : C diet at various temperatures (Lee *et al.*, 2015). However, in nature many insects spend their entire lives on a single host plant, potentially restricting their ability to obtain diets of mixed quality. Therefore, in the present research, mid-instar *S. exigua* were reared using no-choice feeding assays to assess how insects restricted to fixed quality diets respond to warming. Third-instar *S. exigua* larvae were reared on artificial diets of varying quality to test two hypotheses. First, that the growth rates of mid-instar *S. exigua* will be more strongly protein limited at higher temperatures. This would manifest as a significant temperature × diet interaction, where diet quality has stronger effects on growth at high temperatures. Second, that the temperature × diet interaction will be driven by increased metabolic nitrogen demand and therefore higher maintenance nitrogen requirements of early-instar larvae.

Table 1. The protein : carbohydrate (P : C) ratio, nitrogen concentration, casein concentration and cellulose concentrations of three diets used to compare growth rates of third-instar larvae of *Spodoptera exigua*.

P : C	Nitrogen (%)	Protein (% of diet)	Cellulose (% of diet)
6 : 26	1.8	2.9 g (6.4%)	14.1 g (30.9%)
17 : 26	2.8	5.4 g (11.8%)	11.7 g (25.5%)
23 : 26	4.8	10.4 g (22.8%)	6.6 g (14.5%)

Note that wheat germ is 23% protein, such that the 0% casein diet still contains some protein.

Materials and methods

All feeding experiments were conducted at the Smithsonian Environmental Research Center (SERC) in Edgewater, Maryland. *Spodoptera exigua* eggs were purchased from a commercial supplier (Bio-Serv, Flemington, New Jersey) and raised to the third instar at 25 °C on a standard lepidopteran diet (for diet composition, see Supporting Information, Table S1). Third-instar larvae were used for the feeding trials because earlier instars were too small to provide accurate estimates of consumption and later instars reached pupation too quickly at high temperatures.

Feeding experiments

Larvae were reared on three artificial diets of differing P : C ratios. Diet quality varied by replacing casein with nondigestible cellulose at the same time as holding all other sources of digestible carbohydrates (i.e. sucrose, fructose and wheat germ) constant. This allowed a constant water content to be maintained between diets because dietary water content is an important component of caterpillar growth. The replacements of casein resulted in diets of 23 : 26, 17 : 26 and 6 : 26 %P : %C (Table 1). Final instars of *S. exigua* larvae grow slowest on diets of 7 : 36 %P : %C and best when the P : C ratio is between 1 : 1 and 1 : 2 (Lee & Roh, 2010). The diets used in the present study therefore encompassed the range of quality needed to both minimize and maximize growth of *S. exigua* larvae. Dietary nitrogen concentration was determined via elemental analysis using eight replicate subsamples from each diet (FlashEA 1112 Series CHN analyzer; Thermo Electron Corporation, Waltham, Massachusetts) (Table 1). The substitutions of casein resulted in diets of 1.8%, 2.8% and 4.8% N. The 1.8% and 2.8% N diets approximated the nitrogen content found in natural plant tissues (Lemoine *et al.*, 2014b).

Upon reaching the third instar, *S. exigua* individuals were weighed, randomly assigned to one of the three diet treatments and sealed in a plastic container with approximately 1 g of the respective diet. Larvae from each diet were then randomly assigned to either 25/20 °C or 30/25 °C day/night temperature treatments (*n* = 13 per diet per temperature). Temperatures were maintained in climate-controlled growth chambers under an LD 14 : 10 h photocycle. The 25/20 °C temperature regime represented the average conditions during the mid-late spring at SERC, Edgewater, Maryland, and the 30/25 °C temperature

cycle simulated a severe but probable increase in spring temperatures as a result of climate change (IPCC, 2007).

Larvae were allowed to feed *ad libitum* for 36 h. Directly after the feeding trials, the larvae and frass from each container were weighed to calculate growth rates and frass production. The frass was dried to a constant weight at 50 °C for 24 h, ground to a powder and weighed to estimate total dry frass production over the trial period. As a result of variable and rapid evaporation rates during pre-trial weighing of diets, estimates of consumption (the difference in pre- and post-trial diet dry mass) were unreliable. Therefore, consumption rates were estimated using indigestible cellulose content in frass (Barboza *et al.*, 2009). To determine cellulose content, the acid detergent fibre protocol of the National Forage Testing Association was adapted to smaller samples. Approximately 5 mg of ground, dried frass was placed in a 20-mL glass vial and covered with 2–5 mL of acid detergent (1 L of 1 N H_2SO_4 and 20 g cetrimonium bromide). Vials were capped tightly with aluminium foil to prevent evaporation, heated to boiling and allowed to simmer for 45 min. The solution was filtered through a pre-weighed glass fibre filter and both the vial and filters washed twice with acetone. Filters were then dried at 60 °C for 24 h and reweighed. Cellulose content (mg) was calculated as the difference between post- and pre-wash filter weights. Excreted indigestible cellulose was converted to total dry matter consumption using the known percentage of cellulose in each diet (Table 1). The total nitrogen intake was then calculated by multiplying the estimated dry matter consumption rates by the known dietary nitrogen content (Table 1).

To test whether nitrogen metabolism increased at higher temperatures, the amount of excreted nitrogen was quantified for each individual. Although insects excrete nitrogen in a variety of forms (e.g. amino acids), the uric acid component in frass comprises the bulk of metabolized nitrogen and therefore provides a proxy for metabolic nitrogen turnover (Martin & Van't Hof, 1988; Karowe & Martin, 1989). First, the total nitrogen content of larval frass was determined via elemental analysis (FlashEA 1112 Series CHN analyzer; Thermo Electron Corporation). The uric acid concentrations in the frass of each larva were determined using a modified spectrophotometric analysis (Bhattacharya & Waldbauer, 1969; Martin & Van't Hof, 1988; Karowe & Martin, 1989). For each sample, approximately 5 mg dry frass powder was combined with 1 mL of 0.6% lithium carbonate solution in a 1.5-mL microcentrifuge tube. Samples were vortexed and then rocked for approximately 1 h. The samples were then centrifuged at 514***g*** for 5 min, the supernatant separated into a 15-mL falcon tube and the extraction repeated. This yielded approximately 2 mL of sample extract. Each sample was then diluted to 3 mL using 0.6% lithium carbonate solution. A serum-based uric acid assay kit (Amplex Red Uric Acid Kit; Life Technologies, Carlsbad, California) was used to determine the concentration of uric acid (μM) spectrophotometrically in each sample. The uric acid percentage was multiplied by total frass weight to yield total uric acid production. Because uric acid is 33% nitrogen by mass, total uric acid production was multiplied by 0.33 to convert uric acid to metabolized nitrogen.

Statistical analysis

To standardize initial starting mass, data from any *S. exigua* larvae with initial sizes >0.03 g were excluded, thereby restricting caterpillar mass to 0.007–0.030 g, although most larvae were of similar initial size (mean starting mass = 0.018 ± 0.007 g). This was necessary because larval growth rates decline with increasing body size, such that the growth capacity of larger caterpillars is reduced compared with smaller individuals. Excluding large third-instar larvae removed only six individuals from the analysis.

The first analysis used multivariate analysis of covariance to consider how diet quality and temperature may affect larval final mass, dry matter consumption, nitrogen intake and metabolic nitrogen production interactively. The model included diet and temperature as crossed categorical predictors. Because insect final mass and initial mass are often not linearly related, analysis of relative growth rates [i.e. ln(final mass/initial mass)] is discouraged (Raubenheimer & Simpson, 1992). Therefore, final mass was analyzed using initial mass as a quantitative covariate (Raubenheimer & Simpson, 1992). Rather than assume equal slopes for all treatments, interactions between the caterpillar initial mass and the two categorical predictors were also included. For simplicity, point estimates of each response for a caterpillar of average mass from the model are presented, which is conceptually identical to adjusted least-squares means for each treatment from an analysis of covariance (ANCOVA). All four response variables and caterpillar initial mass were standardized prior to analysis.

A detailed nitrogen budget was then constructed for *S. exigua* at both temperatures. First, nitrogen balance was calculated as the difference between nitrogen intake and total nitrogen excretion (including nitrogen from both egestion and uric acid). Nitrogen balance therefore estimates the surplus nitrogen available for processes beyond basic cellular respiration, such as growth. The relationship between larval final mass and nitrogen balance was modelled using ANCOVA regression. This regression included nitrogen balance as a quantitative predictor, as well as main effects and interactions of temperature treatment. Additionally, caterpillar initial mass was included as a covariate (Raubenheimer & Simpson, 1992). This model allowed an assessment to be made of how nitrogen availability translated into final caterpillar mass and whether the relationship between nitrogen balance and final caterpillar mass varied between temperatures. Temperature-induced protein limitation would manifest as a significant interaction between nitrogen balance and temperature, such that there is a stronger relationship between nitrogen balance and final mass at higher temperatures (i.e. excess nitrogen leads to more rapid growth at high temperatures).

Maintenance nitrogen requirement (MNR) is an estimate of the minimum nitrogen intake necessary to perfectly balance baseline nitrogen demands. Although often calculated as the *x*-intercept when regressing nitrogen balance against nitrogen intake (Bradshaw & Bradshaw, 2001), this may not accurately portray the nitrogen needs because not all nitrogen consumed is biologically available. A more accurate estimate of MNR uses truly digestible nitrogen (TDN) in place of nitrogen intake. To

calculate TDN, faecal nitrogen (egested nitrogen not bound in uric acid) was first regressed against diet quality at the same time as including temperature treatment and caterpillar mass as covariates to calculate metabolic faecal nitrogen (MFN). The MFN is the *y*-intercept of this regression, which denotes estimated nitrogen egestion in the absence of dietary nitrogen (Bradshaw & Bradshaw, 2001). The TDN was calculated as nitrogen intake minus faecal nitrogen plus MFN, which describes the nitrogen intake that is biologically available to an individual (Bradshaw & Bradshaw, 2001). The MNR was then calculated by regressing nitrogen balance against TDN, again including an interaction with temperature and caterpillar mass, to provide the most accurate estimate of MNR.

Temperature can also affect nutrient use efficiency of caterpillars by increasing ingestion rates, which reduces gut residence time and digestion of food (Kukal & Dawson, 1989). Nitrogen digestibility was calculated as TDN/total nitrogen intake (Bradshaw & Bradshaw, 2001).

All analyses were conducted using Bayesian methods to incorporate prior information to avoid overestimating effect sizes. Small sample sizes, as used in the present study, can lead to overestimates of the true effect sizes when using traditional analytical methods and have been a persistent problem in biological research (Button *et al.*, 2013; Gelman & Carlin, 2014). Bayesian methods allowed informative priors $N(0, 1)$ to be placed on all parameters, which shrinks parameter estimates towards 0 and helps prevent type M errors during the analysis of small sample sizes (Button *et al.*, 2013; Gelman & Carlin, 2014). Thus, the estimates of effect sizes are conservative based on the current data, although they may more accurately reflect the underlying process and potentially avoided erroneous overestimates of effects.

All regressions were allowed 25 000 burn-in iterations, followed by 25 000 sampling iterations. Every 10th sample from four concurrent Markov Chain Monte Carlo runs was saved. Calculations of MNR used all posterior draws, which allowed full uncertainty to be provided in these parameters. All response variables, as well as caterpillar initial mass, were standardized prior to analysis. Assumptions of normality and heteroscedasticity were examined using residual plots. All models were run using STAN, version 2.5 accessed via PYS-TAN (https://pystan.readthedocs.org*)*. Statistics are reported as 'probability of an effect' (*Pr*); for example, $Pr(30° > 25°)$ denotes the probability that a given response was greater at 30 than at 25 °C. Data analyses were conducted in PYTHON, version 2.7, using the *numpy*, *pandas* and *scipy* modules (Jones *et al.*, 2001; McKinney, 2010; van der Walt *et al.*, 2011).

Results

Growth rates of third-instar *S. exigua* larvae were higher overall at 30 °C $[Pr(30° > 25°) = 1.00]$ and caterpillar mass increased more rapidly with increasing dietary P : C at 30 than at 25 °C $[Pr(\text{Interaction}) = 0.989]$. At 25 °C, *S. exigua* mass increased by $29.9\% \pm 20.0\%$ between the 6 : 26 and 17 : 26 %P : %C diets $[Pr(17 : 26 > 6 : 26 | 25 °C) = 0.931]$ but did not increase further on the highest quality diet $[Pr(23 : 26 > 17 : 26 | 25 °C) = 0.749]$

(Fig. 1A). By contrast, at 30 °C, final caterpillar mass increased by $20.4\% \pm 13.4\%$ between the 6 : 26 and 17 : 26 %P : %C diets $[Pr(17 : 26 > 6 : 26 | 30 °C) = 0.952]$ and continued to increase by an additional $33.8\% \pm 12.3\%$ on the highest quality diet $[Pr(23 : 26 > 17 : 26 | 30 °C) = 1.00]$ (Fig. 1A). Thus, at 25 °C, *S. exigua* achieved maximum growth on the 17 : 26 %P : %C diet, after which increasing the P : C ratio had no effect, whereas at 30 °C, growth continued to increase on the highest quality diet (23 : 26 %P : %C) (Fig. 1A), supporting the initial predictions of temperature-induced protein limitation.

Consumption rates did not vary with dietary P : C $[Pr(\text{Diet Effect}) < 0.909]$ but did with temperature. *Spodoptera exigua* reared at 30 °C consumed $72.8\% \pm 26.6\%$ more dry matter than those reared at 25 °C $[Pr(30° > 25°) = 1.00]$ (Fig. 1B). Because consumption did not vary with dietary protein content, nitrogen intake was $170.7\% \pm 92.9\%$ higher on the 23 : 26 %P:%C diet compared with the two lower quality diets $[Pr(23 : 26 > 17 : 26, 6 : 26) = 1.00]$ (Fig. 1C). Interestingly, despite higher consumption at 30 °C, protein content in diets was sufficiently low that the increased consumption of dry foodstuff did not increase nitrogen intake rates substantially $[Pr(30° > 25°) = 0.854]$ (Fig. 1C).

Nitrogen catabolism was $152.2\% \pm 32.2\%$ higher at 30 °C compared with 25 °C $[Pr(30° > 25°) = 1.00]$ (Fig. 1D). This effect varied slightly among treatments, as indicated by an interaction between temperature and diet quality $[Pr(\text{Interaction}) = 0.956]$. On the lowest P : C diet, the higher temperature increased metabolic nitrogen production by 149.5%, whereas, on the highest P : C diet, it increased metabolic nitrogen production by 234% (Fig. 1D).

Increased nitrogen surplus was positively correlated with caterpillar mass. Importantly, the relationship between nitrogen balance, which is the surplus nitrogen available for processes beyond cellular respiration, and final caterpillar mass varied between temperatures $[Pr(\text{Interaction}) = 0.982]$, although this pattern appears to be leveraged by two influential points at 25 °C. At 25 °C, final caterpillar mass increased marginally as nitrogen balance increased $[Pr(\text{Slope} > 0 | 25°) = 0.940]$, indicating that increased nitrogen availability only slightly stimulated growth rates and that other nutrients may be limiting at this temperature (Fig. 2). At 30 °C, growth increased much more rapidly with nitrogen surplus $[Pr(\text{Slope} > 0 | 30 °C) = 1.00]$ (Fig. 2). Alleviation of nitrogen limitation therefore results in more rapid increases in growth at 30 °C than at 25 °C, indicative of stronger nitrogen limitation at higher temperatures.

Estimates of MFN were greater at 25 °C than at 30 °C $[Pr(25° > 30 °C) = 0.971]$ (Table 2), suggesting that exogenous nitrogen losses during digestion actually decline at higher temperatures. However, neither the slopes between TDN $[Pr(\text{Interaction}) = 0.568]$ and nitrogen balance $[Pr(\text{Interaction}) = 0.568]$, nor the intercepts $[Pr(30° > 25 °C) = 0.616]$ differed between temperatures. These similarities yielded similar estimates of MNR at each temperature (Table 2).

Nitrogen digestibility, or the fraction of ingested nitrogen assimilated by *S. exigua* larvae, was significantly lower at 30 than at 25 °C $[Pr(25° > 30 °C) = 1.00]$. Warming reduced nitrogen digestibility by $21.1\% \pm 2.7\%$, indicating that *S. exigua*
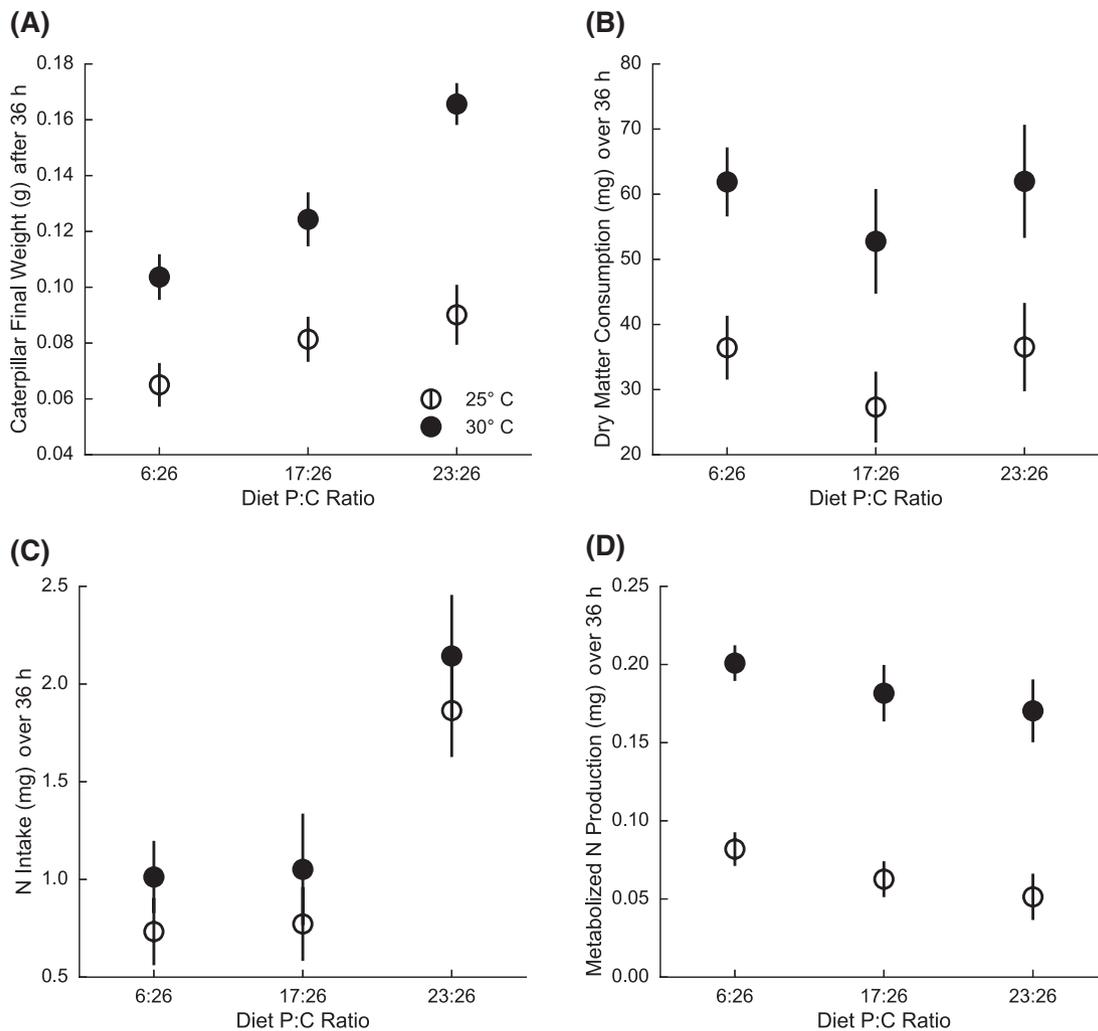
Fig. 1. Relationships between temperature, diet quality and four metrics of *Spodoptera exigua* third-instar larva developmental performance and feeding. (A) Caterpillar final mass, (B) estimated dry mass consumption, (C) estimated nitrogen (N) intake and (D) metabolic nitrogen production of caterpillars fed each of three different diets [varying in protein : carbohydrate (P : C) ratio] and at two different temperatures (25 and 30 °C). Points and lines indicate mean ± SE estimates of each developmental response for a third-instar larva of *S. exigua* of average mass.

absorbs substantially less nitrogen per unit food intake under warmed conditions.

Discussion

Confirming the initial predictions, the strength of protein limitation for *S. exigua* third-instar larvae increased with temperature as a consequence of increased metabolic nitrogen turnover, despite a similar MNR at both temperatures. Instead, protein limitation stems from reduced nitrogen digestibility at high temperatures. These results provide a mechanism for the widespread temperature × diet interactions of insect growth among studies using both artificial diets (Kingsolver *et al.*, 2006; Lee & Roh, 2010) and natural plant tissue (Lemoine *et al.*, 2013, 2014a). Accordingly, increased metabolic rates at higher temperatures may be a strong driver of herbivore feeding

behaviour and performance (Lemoine *et al.*, 2013) and may drastically alter plant–herbivore interactions in future climates.

Higher temperature increased protein turnover rates of *S. exigua* probably because warming causes proteins to denature more rapidly. As a result, protein repair and replacement occupy a larger share of the overall nitrogen budget at high temperatures (Hachiya *et al.*, 2007; Somero, 2011). Because the low-quality diets of the present study mimick typical plant nutritional quality, rising temperatures may increase metabolic nitrogen turnover by approximately 150%. Yet despite increased nitrogen turnover and stronger protein limitation of growth at high temperatures, MNR remained constant with temperature (Table 2). This suggests that protein limitation at high temperatures does not arise from increased maintenance nitrogen requirements.

Instead, protein limitation at high temperatures probably arises from the impact of rising temperatures on respiration
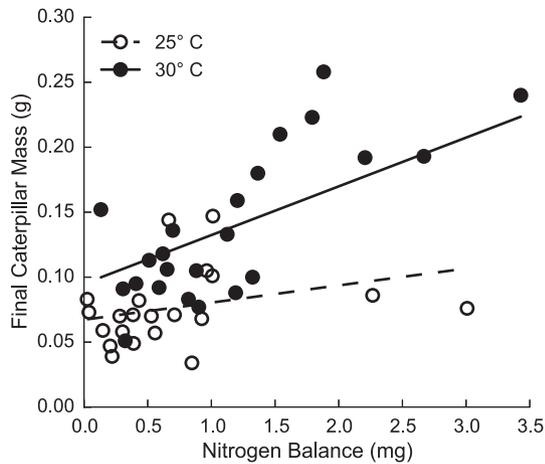
Fig. 2. Increased temperatures alter the relationship between nitrogen balance and caterpillar growth rate for third-instar larvae of *Spodoptera exigua*. Lines give the posterior median predictions for a third-instar larva of *S. exigua* of average mass.

Table 2. Nitrogen budget for *Spodoptera exigua* at two temperatures (25 and 30 °C).

N-Budget Component	25 °C	30 °C	$Pr(30\,°C > 25\,°C)$
MFN	0.139 ± 0.038 mg N	0.027 ± 0.046 mg N	0.029
MNR	0.206 ± 0.012 mg N	0.198 ± 0.015 mg N	0.348

Specifically, metabolic faecal nitrogen (MFN) and maintenance nitrogen requirements (MNR) are calculated from truly digestible nitrogen. $Pr(30\,°C > 25\,°C)$ denotes the probability that a given response was greater at 30 than at 25 °C.

rates. Oxygen consumption and CO_2 respiration increase exponentially with temperature (Kukal & Dawson, 1989; Lemoine & Burkepile, 2012; Parsons & Joern, 2014). To meet these increased energetic demands, the congener species *S. litura* maximizes carbohydrate intake by increasing feeding rates on carbohydrate-rich foods (Lee *et al.*, 2015). Lepidopteran larvae have plug-flow reactor guts, and increased food intake rate is associated with reduced gut passage time (Kukal & Dawson, 1989; Karasov & Martínez del Rio, 2007), which, in turn, leads to lower food assimilation rates and reduced nitrogen digestibility (Kukal & Dawson, 1989) (Fig. 3). Indeed, nitrogen digestibility by *S. exigua* declined by almost 25% at 30 °C compared with 25 °C. Reduced nitrogen digestibility, coupled with increased cellular division and somatic growth rates, is therefore probably responsible for the observed strengthening of protein limitation at high temperatures.

Temperature × diet interactions such as those reported in the present study appear to be ubiquitous (Kingsolver *et al.*, 2006; Lee & Roh, 2010; Lemoine *et al.*, 2013, 2014a). For example, temperature stimulates the growth rate of adult *Popillia japonica* beetles only on high nitrogen plants (Lemoine *et al.*, 2013). Lepidopteran larvae may be particularly vulnerable to environmental warming because their rapid growth rates demand high protein foods (Lee *et al.*, 2002, 2004; Merkx-Jacques
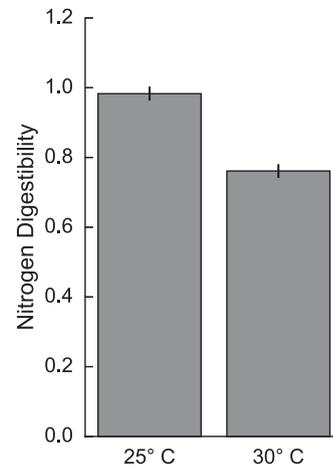
Fig. 3. Nitrogen digestibility in *Spodoptera exigua* third-instar larvae, calculated as truly digestible nitrogen/nitrogen intake. Nitrogen digestibility is significantly lower at 30 than at 25 °C.

et al., 2008). High protein diets enable the tobacco hornworm, *Manduca sexta* to grow more rapidly with warming than on low protein foods (Kingsolver & Woods, 1998). Similarly, the growth of tulip-tree beauty caterpillars *Epimecis hortaria* increases exponentially with temperature on high nitrogen spicebush (*Lindera benzoin*) but has no relationship with temperature on low nitrogen Americam sweet gum (*Liquidambar styraciflua*) (Lemoine *et al.*, 2014a). Growth rates of *S. exigua* also increase with temperature but do so more rapidly on high P:C foods (Lee & Roh, 2010). The results of the present study confirm this pattern but suggest that such patterns do not necessarily stem from increased minimum nitrogen requirements at high temperatures and, instead, derive from the combination of increased metabolic rates, increased food intake and reduced nitrogen digestibility.

The amount of food in the caterpillar guts is not accounted for in the present study, which might result in overestimating growth rates. However, because dry matter consumption does not vary between diets (Fig. 1B), estimates of growth should be biased similarly among diets within a temperature. That is, the overestimation of growth would be constant among diets, such that the qualitative patterns would be similar if food in the gut had been accounted for. Interestingly, *S. exigua* larvae did not offset rising nitrogen catabolism by increasing the consumption of low-quality diets to maintain relatively high growth rates. Total dry matter consumption did not vary among diet treatments (Fig. 1B), such that nitrogen intake was highest on the high nitrogen diets (Fig. 1C). Many herbivores do not change feeding rates on low nitrogen diets, suffering stunted growth, longer development times and reduced fitness on low nitrogen plants at higher temperatures (Lemoine *et al.*, 2013, 2014a). This often occurs in animals where post-ingestive processes, such as digestion, absorption or excretion, limit consumption (i.e. Type II functional response) (Karasov & Martínez del Rio, 2007). In addition, natural plant tissues contain secondary compounds that can prevent the compensatory feeding required to offset temperature-induced nitrogen limitation (Cruz-Rivera & Hay, 2003). Concomitant increases in atmospheric CO_2

concentrations along with climate warming will probably increase concentrations of secondary defences in plant tissues (Zvereva & Kozlov, 2006), which can either limit the ability of a herbivore to compensate for low-quality diets or reduce the assimilation efficiency of ingested foods (Williams *et al.*, 1994). In either case, the combination of reduced nitrogen digestibility as a result of warming and reduced food quality as a result of increased atmospheric CO_2 may have severe consequences for plant–herbivore interactions.

Previous work demonstrates opposite patterns compared with those reported in the present study; Lee *et al.* (2015) report that *S. litura* select lower P : C ratio foods when reared at warmer temperatures, indicating that carbohydrates become limiting at higher temperatures. These contrasting results can be explained by a number of methodological differences between the present study and that of Lee *et al.* (2015). First, the experiments in the present study are conducted over actual time (3 days), rather than physiological time (e.g. stadium duration) (Lee & Roh, 2010; Lee *et al.*, 2015). A delayed development on sub-optimal diets can actually increase total macronutrient intake over the course of development despite reduced daily intake rates (Lee & Roh, 2010). However, daily nutrient intake may still not sufficiently offset daily metabolic demands, even if total intake over the development time increases. Second, although P : C ratios vary among the diets used in the present study, the carbohydrate concentrations are held constant, rather than both protein and carbohydrate concetrations varying simultaneously (Lee *et al.*, 2002, 2004, 2015). Therefore, the present study cannot assess whether carbohydrate or protein become limiting at higher temperatures; however, the present study aims to characterize whether protein concentrations can become limiting at high temperatures.

Although diets consisting of above-optimal protein concentrations may be deleterious to caterpillar performance (Lee *et al.*, 2002, 2004, 2015), in many cases, these diets contain unrealistically high protein : carbohydrate ratios (i.e. 42% protein, 0% carbohydrate; Lee *et al.*, 2015). Such unrealistic diets can invoke pathological responses in insects (Simpson & Raubenheimer, 1995). Therefore, the diets in the present study were designed to mimic ecologically realistic nitrogen and carbohydrate concentrations in host plants (Lemoine *et al.*, 2014b). Finally, in previous studies, *Spodoptera* caterpillars are provided with multiple food choices, allowing them to self-select the appropriate P:C ratio (Lee *et al.*, 2002, 2003, 2015). However, many insect herbivores spend their entire life cycle on a single host plant and cannot feed selectively among multiple hosts. The current no-choice experiments were designed to replicate this scenario.

As described above, carbohydrate concentrations in the artificial diets were held constant, at the same time as allowing protein concentrations to vary among diets. As a result, diets have varied P : C ratios (6 : 26, 17 : 26, 23 : 26) and total nitrogen concentrations (1.8%, 2.8%, and 4.8% N) simultaneously. The observed responses cannot be definitively attributed to either reduced protein concentrations relative to carbohydrate or, more simply, reduced nitrogen content overall. Indeed, low foliar nitrogen content often suppresses insect growth and development (Williams *et al.*, 1994; Lemoine *et al.*, 2013). As

with the present study, it is difficult to ascribe such patterns solely to low foliar nitrogen content because foliar nitrogen covaries with numerous other plant nutritional components, including phosphorus (Lemoine *et al.*, 2014b), specific leaf area and toughness (Lind & Parker, 2010), and carbohydrates (Tjoelker *et al.*, 1999). The results of the present study may therefore accurately describe how temperature affects the feeding behaviour of specialist insects even if a more detailed mechanism cannot be identified. Second, protein composition changes markedly among diets; casein contains more leucine, phenylalanine, lysine and valine (Cohen, 2015). The growth performance of *Spodoptera eridania* declines when low-quality proteins are mixed (Karowe & Martin, 1989), making it difficult by inference to distinguish between reduced protein concentrations or altered amino acid composition as a driving mechanism for the patterns reported in the present study.

The present study demonstrates that rising temperatures can increase the metabolic nitrogen demands of ectothermic herbivores, thereby leading to increased baseline nitrogen demands and potential protein limitation of herbivore growth. Such changes could have profound implications for future plant–herbivore interactions. As warming increases the potential for nitrogen limitation, consumers will be forced to either alter their feeding behaviour or suffer reduced growth, development and fitness. Thus, potential protein limitation at higher temperatures will probably be a major determinant of herbivore feeding behaviour in the future.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI: 10.1111/phen.12138

Table S1. List of ingredients and amounts in g (or mL for dietary water) in the standard lepidopteran diet.

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