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Host plant nitrogen enrichment has both positive and negative effects on the larval growth of a specialist butterfly

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Abstract. 1. The nitrogen limitation hypothesis posits that phytophagous insects benefit from nitrogen enrichment of their host plants through a reduction of the concentration of toxic compounds and an increase of free amino acids and proteins. However, species' response to nitrogen enrichment varies substantially and high nitrogen levels are associated with population decline, suggesting there are major costs to feeding on nitrogen-rich host plants.

2. To test the hypothesis that larval growth performance is maximal at intermediate nitrogen enrichment, nitrogen levels were measured in 18 populations of the host plant of *Lycaena helle*, a specialist butterfly inhabiting nutrient-poor wet meadows. The nitrogen content of host plants was then modified to mirror average natural nitrogen levels (C), highest field-recorded levels (T1), and levels higher than those observed across our study populations (T2).

3. Caterpillars fed with T1 leaves had a greater maximum body mass than caterpillars of the C group because of their improved food assimilation during the early stages of their development. Caterpillars of C and T2 groups had similar growth patterns but high nitrogen content had detrimental effects, as caterpillars fed with T2 leaves had a slower ingestion rate than C and T1 groups.

4. Quantifying the fitness consequences of these changes in growth performance is necessary to fully understand the implications of nitrogen enrichment for *L. helle* (rapid growth may result in fitness costs). However, conservation plans for this emblematic glacial relict species should also consider the preservation of its host plant quality to ensure its persistence.

Key words. Feeding behaviour, fitness, growth, habitat degradation, *Lycaena helle*, plant-insect interactions, pollution.

Introduction

Individuals' fitness is maximal within a range of conditions in which they optimise the allocation of resources to competing life-history traits (somatic growth, maintenance and reproduction; Stearns, 1992). Hence, species' habitats are best understood as the areas containing all the biotic and abiotic factors necessary to enable the successful completion of individuals' life cycle and the maintenance of self-sustained populations (Hall *et al.*, 1997; Dennis *et al.*, 2003). Conversely, habitat degradation results from the spatiotemporal variations in the factors associated with species' habitats, causing a decline in the fitness of individuals, which may result in higher extinction risks of local populations (Dennis & Eales, 1997; Thomas *et al.*, 2001). Therefore, understanding individuals' multifaceted needs during their entire life is key to predicting their response to rapid environmental changes.

Food quality is a fundamental aspect of habitat quality in phytophagous insects because they can only obtain the nutrients

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required to complete their life cycle through their host plant(s) (Scriber & Slansky, 1981). Numerous studies have shown that food quantity and quality strongly influence larval growth rate (Stamp & Bowers, 1990) which determines adult body size (Tammaru, 1998; D'Amico *et al.*, 2001), and hence individuals' fitness (e.g. Haukioja & Neuvonen, 1985; Honek, 1993; Hunter, 2001; Teder *et al.*, 2014). While nitrogen is a key nutrient that limits plant growth (Elser *et al.*, 2007), nitrogen is also an important element of food quality for phytophagous insects as nitrogen content of their tissues is much greater than that of their host plant (Mattson, 1980). Albeit limiting, there has been a widespread increase in organic nitrogen availability resulting from the assimilation of atmospheric nitrogen in soils (atmospheric nitrogen deposition), the use of nitrates in agriculture, and the release of large amounts of ammonia from farmed animal manure (Sutton *et al.*, 2009). The ensuing increased growth improves the palatability of the plants because of a rapid decline in concentration of defence metabolites (Herms & Mattson, 1992) and an increase in the concentration of soluble nitrogen compounds (e.g. free amino acids; Flückiger *et al.*, 2002). Therefore, the formulation of the 'nitrogen limitation hypothesis' (White, 1993) posits that a shortage in plant nitrogen reduces phytophagous insects' growth (e.g. Han *et al.*, 2014), and that increasing nitrogen contents result in shorter development times and greater maximum larval body mass (e.g. Cates *et al.*, 1987; Taylor, 1988; Clancy, 1992; Hunter & Mcneil, 1997; Inbar *et al.*, 2001).

However, increasing levels of nitrogen may also have deleterious effects on phytophagous insects through digestion costs (Stockhoff, 1991; Stevens *et al.*, 2004; Bobbink *et al.*, 2010; Turlure *et al.*, 2013; Tanner *et al.*, 2015). These costs may result from the higher cellulose content of nitrogen-rich plants (cellulose is generally not digested; Chown & Nicolson, 2004) and their higher concentration of toxic (nitrogen-containing) secondary metabolites (e.g. proteinase inhibitors or polyphenol oxidase; Tao & Hunter, 2012). Hence, there is probably a threshold beyond which the benefits of eating nitrogen-enriched food decrease, and further nitrogen enrichment may result in digestion costs (Stockhoff, 1991; Fischer & Fiedler, 2000). This hypothesis is supported by population-level studies showing that high nitrogen levels are typically associated with declines in population size (Weiss, 1999), and studies focusing on communities which found a decline in species diversity in nitrogen-rich areas (Öckinger *et al.*, 2006; Feest *et al.*, 2014), these effects being dependent on how phytophagous insects adjust their feeding behaviour and growth to these new conditions (Payne *et al.*, 2013; Chesnais *et al.*, 2016).

Better understanding of the digestion costs associated with an increase in plant nitrogen content requires: (i) measurement of the natural range of nitrogen content to which studied species are exposed; and (ii) understanding changes in the way food is processed (Chown & Nicolson, 2004). Indeed, there is substantial variation in the response of phytophagous insects to increasing plant nitrogen content and several studies have shown that nitrogen enrichment can also result in a decline in larval growth and fitness (Boersma & Elser, 2006; Castañeda *et al.*, 2010; Tao & Hunter, 2012). This can be due to differences in species' evolutionary history (specialist versus generalist; species inhabiting nutrient-poor versus -rich habitats), but this might also be due to a lack of knowledge of the natural range of plant nitrogen level experienced by the studied species. For instance, the exposure of individuals to unrealistically high nitrogen levels may result in negative effects on insect growth but such effects would have limited ecological and conservation relevance. Furthermore, the consequences of nitrogen enrichment in terms of food processing are poorly understood. The transformation of plant proteins into insect tissue can be divided into four steps: (i) the consumption of plant tissues; (ii) the lysis of plant proteins; (iii) the absorption of oligopeptides and free amino acids; and (iv) the synthesis of new insect proteins (Reynolds, 1990; Chown & Nicolson, 2004). Insects may modulate each of these steps to optimise the transformation of the food into tissues, and similar growth patterns may stem from fundamentally different ways of processing their food (Scriber & Slansky, 1981; Slansky, 1993). Indeed, caterpillars of many species can adjust the number and size of their meals according to the amount of nutrient contained in their food and/or may actively choose food items to compensate for unbalanced meals (Slansky, 1993). These pre-ingestion behaviours may enable individuals to ensure an optimal growth in spite of varying food quality, but may have major fitness consequences if changes in feeding behaviour lead to increased predation risks (Bernays, 1997). Similarly, there may be an optimal absorption rate if there is a trade-off between a rapid processing of the food and a thorough processing (Reynolds, 1990), and such a trade-off may also lead to fitness costs (e.g. Bunning *et al.*, 2016). Hence, full understanding of the effect of plant nitrogen content on the growth and fitness of phytophagous insects requires exposing them to plant nitrogen levels within the ranges of nitrogen experienced under natural conditions, and determining how phytophagous insects change their feeding behaviour and food processing means according to their host plant's nitrogen content.

The Violet Copper (*Lycaena helle*) is a critically endangered species listed in the European Red Data Book of the European Union (van Swaay *et al.*, 2010). In western Europe, this butterfly species is found in nutrient-poor wet meadows (Habel & Assmann, 2010), where caterpillars only feed on *Persicaria bistorta* leaves and adults feed on the nectar of a wide variety of plant species (Fischer *et al.*, 1999; Turlure *et al.*, 2009). During recent decades, populations of *L. helle* have rapidly declined across its European range (van Swaay *et al.*, 2010), and management plans have primarily focussed on preserving large patches of its larval host plant. Yet, natural (re)colonisation events seldom occur even in areas where large host plant patches occur in the vicinity of large populations (Bauerfeind *et al.*, 2009), suggesting that this species is also sensitive to changes in its host plant quality. Therefore, ensuring the long-term persistence of remnant populations of *L. helle* requires the quantification of how nitrogen enrichment changes its larval host plant composition, and hence growth patterns.

In this study, we conducted a laboratory experiment on *L. helle* caterpillars to test the hypotheses that: (i) an increase in nitrogen content of the host plant can improve larval growth at moderate

levels but becomes deleterious at higher levels; and (ii) these changes are underpinned by differences in food processing means. We first quantified the nitrogen levels of host plants collected in sites where *L. helle* is currently found or has recently been found in southern Belgium. We then grew host plants that we treated with a commercial fertiliser to produce plants with three nitrogen levels: the average nitrogen level found in our study area (control group, C); nitrogen-enriched plants (corresponding to the upper level of nitrogen content observed in our study area, T1); and substantially enriched plants (corresponding to nitrogen content beyond those observed across our study area, T2). We then collected eggs of *L. helle* from a large natural population, and caterpillars were fed with leaves coming from one of these host plant treatments. If the nitrogen limitation hypothesis held, we expected that the growth rate and maximum mass reached by the caterpillars before pupation would consistently increase with the amount of the fertiliser provided. If there was a threshold beyond which increasing nitrogen levels are no longer beneficial, we expected to observe the highest growth rate of the caterpillars in the T1 group. Finally, to better understand the processes underpinning caterpillar growth patterns, we measured the amount of food eaten and frass produced to determine whether host plant nitrogen content affected the way in which caterpillars processed their food.

Material and methods

Study site and leaf sampling

Lycaena helle is a habitat specialist butterfly species found exclusively in wet meadows with sheltered stands and bogs (see Habel *et al.*, 2011). We studied *L. helle* populations during 2009–2014 in 18 wet meadows located along three different valleys on the plateau des Tailles, Belgium (50°13′N, 5°47′E; study sites were spread over *c*. 20 km^2). In each site, we sampled 10–15 *P. bistorta* leaves; these leaves were sampled randomly with respect to their size and age and were collected within 3 days. Leaves were air-dried in the laboratory and ground into a fine powder (Retsch Crossed Beater Mill SK100, Haan, Germany). The leaves of each site were combined to enable us to obtain *c*. 10 mg of powder, which was then placed in a steriliser at 65 °C for 72 h. Leaf nitrogen and carbon compositions were subsequently measured using an organic elements analyser with Flash combustion (Thermo Fisher, Waltham, Massachusetts). The concentration of nitrogen and carbon were calculated per mass of dry leaf (mg g^{-1}). We present changes in host plant nitrogen content in terms of carbon:nitrogen (C:N) ratio because this ratio depended on leaf nitrogen concentration (Pearson's correlation: $r = -0.94$, $P < 0.01$, $N = 18$) and was unrelated to carbon concentration ($r = 0.17$, $P = 0.49$). We additionally present values of leaf nitrogen concentration to facilitate comparisons with other study systems.

Host plant treatment

In late April 2015, we dug out six samples of live *P. bistorta* at a site where pilot analyses indicated high natural C:N ratio levels (i.e. low eutrophication). Each sample consisted of 1 m^2 of soil (20 cm deep) containing *c*. 30–40 host plants. These samples were then placed in 1 × 1 m trays and reared outdoors. The samples were assigned to three treatments (two samples per treatment): control (C; no fertilisation), moderate fertilisation (T1) and high fertilisation (T2). Plants received 5 litres of water every 3 days in which we added 5 and 10 ml of NPK: 6-5-5 fertiliser (Substral universal fertiliser; Scotts Miracle-Gro Company, Marysville, Ohio) for the treatments T1 and T2, respectively. These treatments were derived from a pilot study undertaken in spring 2014, which enabled us to identify the amount of fertiliser required to mirror conditions experienced by the caterpillars in the wild (C and T1) and at greater nitrogen enrichment (T2). The host plant treatment took place between 28 April and 5 June and was stopped 1 week before the start of the experiment (the soil in the samples had accumulated the nutrients that would be subsequently used by *P. bistorta* during the experiment). To limit the risks of nutrient deficiency, 5 ml of fertiliser diluted in 5 litres of water were provided to all samples every 3 weeks. During this additional care treatment, we watered the roots of the plants from the sides of the trays and we did not use the leaves to feed the caterpillars for 2 days to minimise the risks of a direct exposure to the fertiliser. We determined the nitrogen content of the leaves in each of the six trays before and after the experiment using the method outlined previously and averaged the C:N ratio values for each treatment.

Caterpillar collection and rearing conditions

We collected 54 eggs from the largest *L. helle* population of our study area in early June. As this site currently holds a large *L. helle* population (*c*. 1500 adults; Turlure *et al.*, 2009) and females lay their eggs singly, the sampling of closely related individuals is unlikely. These eggs were placed in an incubator under 18:10 °C day:night, LD 14:10 conditions. In total, 42 larvae hatched, and the experiment was started 1–3 days after eggs had hatched. Caterpillars were transferred individually to a Petri dish by cutting a tiny piece of the leaf surrounding them. Caterpillars were then assigned randomly to one of the three treatments ($N_C = 12$; $N_{T1} = 15$; $N_{T2} = 15$). A small piece of host plant leaf (*c*. 100 mg of either C, T1 or T2 treatments) was placed on a disk of humid cotton (diameter 5.6 cm, 1.5 ml H_2O), and added to each Petri dish; caterpillars were fed *ad libitum*, and we increased the amount of food provided to *c*. 200 mg when they reached 10 mm in size.

Every 3 days, leaves that were eaten were replaced by fresh ones, frass was removed and Petri dishes cleaned. After each measurement, Petri dishes were placed randomly back in the incubator to avoid potential biases arising from any temperature variation that might exist within the incubator. Handling and measurements were stopped when caterpillars started pupating. Once caterpillars reached the size of 5 mm, we recorded their size (to the nearest 0.5 mm) and body mass (to the nearest 0.01 mg) every 3 days (all weight measures were performed on a Mettler Toledo XS204 scale, Columbus, Ohio). These measurements are non-invasive and reliable because *L. helle* caterpillars hardly curl up, giving very little variation over

repeated measures of body length (details not shown). We decided to keep a time step of 3 days throughout the experiment to minimise the manipulation of caterpillars.

Fresh and eaten leaves were weighed (to the nearest 0.01 mg) and photographed on a white background with a scale to quantify the proportion of the leaves eaten between two measurements. The surface of each leaf (fresh and eaten) was measured using IMAGEJ (Schneider *et al.*, 2012) and the caterpillars' fresh food intake was calculated as the proportion of surface eaten by a caterpillar multiplied by the mass of this leaf measured when fresh. Nitrogen fertilisation can affect both plant-specific leaf area (SLA, i.e. leaf thickness) and plant leaf dry matter content (LDMC) making it difficult to interpret differences in the amount of fresh food eaten based on the proportion of leaf surface eaten. There is no *a priori* reason to believe that the water content of the host plant would differ consistently between the treatment as the host plants were: (i) collected in the same location, (ii) kept in similar conditions, and (iii) provided with the same amount of water. There is a very strong relationship between plant fresh weight and area [$\beta = 19.00 \pm 0.83$ (SE)]. Our experimental treatment influenced this relationship ($F_{2,245} = 19.41$, $P < 0.01$) but primarily because of a slight decline in leaf mass at high leaf area values for T1 (Fig S1 in Supporting Information). Hence, our treatment is unlikely to have substantially increased the thickness of *P. bistorta* leaves. There was no significant relationship between the C:N ratio measured and the amount of water contained in the leaves across the 18 sites and experimental treatments (ANCOVA: $\beta = 0.06 \pm 0.08$, $P = 0.48$), suggesting that the bias due to an increased water content of nitrogen-rich leaves is limited.

When caterpillars reached 10 mm, frass samples were collected and weighed (to the nearest 0.01 mg) after being air-dried for 3 days; the amount of frass produced by smaller caterpillars could not be measured with satisfactory accuracy. In total, there were 531 observations, 431 measures of caterpillar body size and 333 measures of body mass. Pictures were taken for a total of 340 fresh and 295 eaten leaves (in 45 cases, caterpillars ate the entire leaf). As *L. helle* is an endangered and protected species, we only investigated larval growth and brought back pupae to the sampling site; measuring caterpillars' dry weight was therefore not possible. We found no relationship between the C:N ratio of *P. bistorta* leaves and their water content (see earlier), but we acknowledge that our study relies on the assumption that the caterpillars did not modify their water intake according to the treatment levels.

Statistical analyses

All analyses were run in R 3.3.2 (R Development Core Team 2016). As measures of body length and body mass were strongly correlated, we focused on the latter because of its greater accuracy. The analyses were conducted in three steps.

First, we tested whether the treatments influenced the overall growth characteristics of the caterpillars. ANOVAS were used to determine whether the treatments influenced caterpillars' time to pupation and maximum mass. When significant, *post hoc* (Tukey) tests were used to quantify differences between treatments. Assumptions of normality and equal variances were systematically checked. Only two caterpillars died during the experiment, therefore no test was carried out to examine the effect of the treatment on caterpillars' survival to pupation.

Secondly, we quantified the effect of the treatment on the caterpillars' growth patterns (Supporting Information, Fig S2). To this end, we used a mixed model with the repeated measures of caterpillar body mass as the dependent variable and the time elapsed since the beginning of the experiment (number of measurement) in an orthogonal polynomial form (time, time^2, time^3). Measurements of caterpillar body mass were log-transformed to satisfy the assumption of homoscedasticity. Caterpillars' ID nested within each time step was set as a random effect to model random intercepts and slopes. We used maximum likelihood estimations to compare models with different fixed effects (time, time^2, time^3 and treatment; random effects did not change). The performance of the full model was compared with more parsimonious models using corrected Akaike information criterion (AIC). The relative importance and model-averaged coefficient estimates with unconditional SE and unconditional 95% CI were calculated for models with differences in AICc lower than 4 using the R package 'MuMIn' v1.40.4 (Barton, 2017).

Finally, we tested the hypothesis that the differences in growth patterns resulted from the effect of the treatment on the way food is processed. To this end, we kept the variables describing the growth pattern (orthogonal polynomial time effects) to which we added the amount of food eaten and frass produced at each measurement in interaction with the treatment. Both the amount of food eaten and frass produced were scaled (mean = 0 and SD = 1) to enable direct comparisons with the orthogonal temporal variables (scaled variables are asymmetric because the original variables have a greater number of small values and a lesser number of large values). This analysis was undertaken on a subset of the data used to model the growth patterns as frass was collected when caterpillars reached 10 mm (Fig S2 in Supporting Information). As the amount of frass produced was strongly related to the amount of food eaten ($\beta = 0.822 \pm 0.043$, $t = 19.11$, $P < 0.001$), we used the residuals of this linear model to quantify the difference between the amount of frass produced and that expected given the amount of food eaten (we refer to this difference as the 'residual frass production'). The residual frass production is an estimate of caterpillars' food assimilation. In all analyses, FE will denote the amount of food eaten, and FR will denote the residual frass production. We first determined whether the treatment had an overall effect on FE and FR using a linear mixed model in which FE and FR were the dependent variables, the treatment was the explanatory variable and caterpillar ID was a random effect (to account for the non-independence of the repeated measures). We then tested the hypothesis that the treatment influenced caterpillars' food processing. In this analysis, models included caterpillar body mass as the dependent variable and the explanatory variables consisted in main effects of FE, FR, treatment, and their two- and three-way interactions. Again, caterpillar ID nested within each time step was used as a random effect. A significant FE × treatment interaction would indicate that caterpillar body mass gains per quantity of food eaten varied among nitrogen treatments (e.g. caterpillars eating

nutrient-poor food may have larger and more frequent meals, leading to high FE values). A significant FR × treatment interaction would indicate that the treatment altered the caterpillars' ingestion (e.g. caterpillars eating cellulose-rich food may have higher residual frass production). A significant FE × FR interaction would indicate that the body mass gains are dependent on both the amount of food eaten and residual frass production (e.g. caterpillars growing more eat more and have better assimilation). Finally, a three-way interaction FE × FR × treatment would indicate that the direction of the interaction FE × FR is dependent on the treatment level (e.g. in nutrient-poor food, the growth of caterpillars may result from a high amount of food eaten and a low residual frass production, while the growth of caterpillars eating nutrient-rich food may primarily depend on residual frass production). The use of orthogonal time variables and residual frass production effectively limits collinearity issues. All mixed-effects models were run in the R package lme4 (Bates et al. 2015) and *P*-values for each effect were estimated with the R package lmerTest (Kuznetsova *et al.*, 2017).

Data accessibility

All datasets are available in the Pangaea repository 888421 (https://doi.pangaea.de/10.1594/PANGAEA.888421).

Results

Across all sites, the average C:N ratio was 12.60 (range 10.46–15.04; Fig. 1) and the average nitrogen concentration was 3.63 mg g^{-1} (range 3.07–4.34). These values were close to the C:N ratio and nitrogen concentration measured in the control group (C:N ratio = 12.97; N concentration = 3.60). Our fertiliser treatment led to an increase in nitrogen concentration and a decrease in C:N ratio. Specifically, the treatment group T1 had a C:N ratio (10.18) consistent with the site presenting the lowest C:N ratio (a wet meadow surrounded by pastures), and T2 had a C:N ratio (9.20) clearly below the lowest ratio observed across all study sites (Fig. 1). Nitrogen concentrations of T1 and T2 host plants were slightly higher than those measured in the field site with the highest leaf nitrogen concentrations (4.58 and 5.08, respectively). Hence, our treatments overall mirrored host plant characteristics in an average site of our study area (C), in a nitrogen-enriched site (T1), and in a site with substantial nitrogen enrichment (T2).

The treatment tended to affect caterpillars' development time (ANOVA, $F_{2,37} = 2.199$, $P = 0.12$; Fig. 2a), and significantly influenced the caterpillars' maximum body mass ($F_{2,37} = 4.49$, $P = 0.02$; Fig. 2b). More specifically, T1 caterpillars had significantly greater maximum body masses than caterpillars of the C group (*post hoc* test, C – T1, mean difference = 11.19 mg; $P = 0.02$); while there was no significant difference between the maximum body mass of T2 and C caterpillars (*post hoc* test: mean difference = 8.60 mg; $P = 0.08$). There was no significant difference between the maximum body mass of T1 and T2 caterpillars (mean difference = 2.59 mg, $P = 0.75$; Fig. 2b).

The treatment influenced the growth patterns of the caterpillars. Overall, caterpillar growth over the entire experiment was

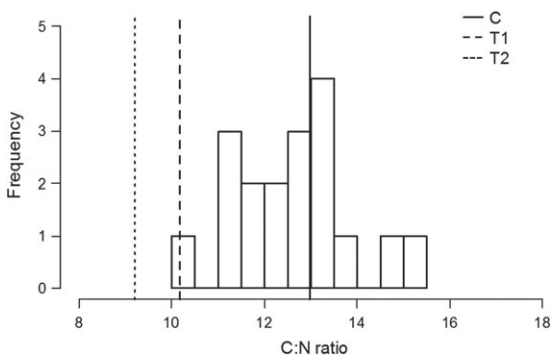

Fig. 1. Distribution of the carbon:nitrogen (C:N) ratio of *Lycaena helle*'s host plant (*Persicaria bistorta*) in our study area in southern Belgium. The C:N ratio of the leaves in the control group (C; no fertiliser; solid line) was close to the average C:N ratio measured across the 18 sites. The fertiliser treatment decreased the C:N ratio as the C:N ratio of the leaves of the T1 treatment (moderate amount of fertiliser) corresponded to the lowest value observed in our study area (dashed line), and the C:N ratio of the T2 treatment (substantial amount of fertiliser) was even lower (thin dashed line). Each treatment was sampled twice (before and after the start of the experiment), and the values presented here are averages of these measurements.

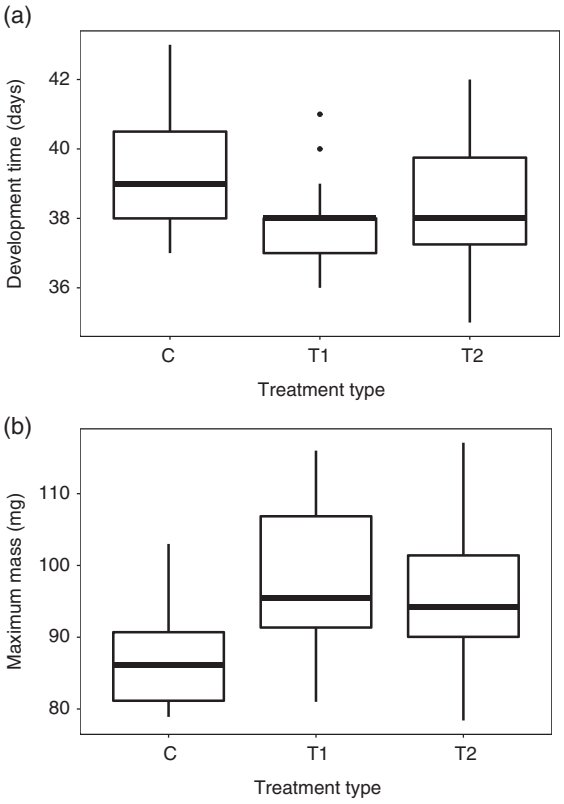

Fig. 2. Boxplots presenting the effect of the treatment on caterpillars' development time (a) and maximum body mass (b). C, control group; T1, caterpillars fed with leaves with a moderate fertiliser treatment; T2, caterpillars fed with leaves with a substantial fertiliser treatment. The dark line represents the median values for each treatment and boxes consist of first and third quartile values.

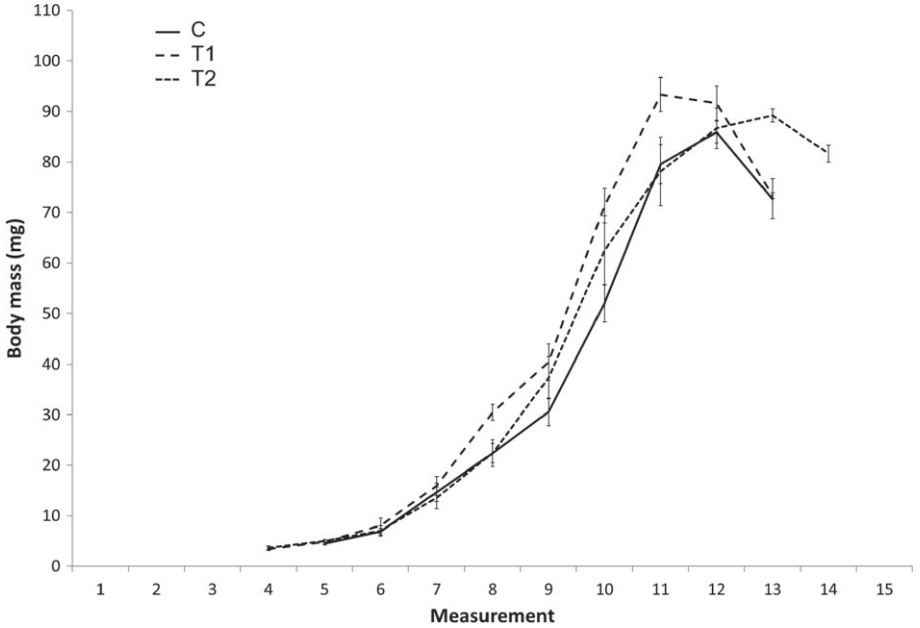

Fig. 3. Caterpillar growth over the entire experiment was best explained by a cubic polynomial term. Shown are body mass averages (± SE) at each measurement (*c*. 3 days) for caterpillars of the control group (solid line), the T1 group (dashed line), and the T2 group (thin dashed line).

Table 1. Relative performance of the models explaining the variance in caterpillar body mass.

Rank	Model	K	AICc	ΔAICc	w_i	Cum. w_i	LogLik	ER
1	(Time + Time^3) + (Time^2 × Tr)	12	87.69	0.00	0.55	0.55	−31.36	-
2	Time^3 + (Time + Time^2) × Tr	14	89.22	1.53	0.26	0.81	−29.95	2.15
3	(Time + Time^2 + Time^3) × Tr	16	91.28	3.59	0.09	0.90	−28.78	6.02
4	Time + Time^2 + Time^3 + Tr	10	92.19	4.50	0.06	0.96	−35.75	9.48
5	(Time^2 + Time^3) + (Time × Tr)	12	93.97	6.28	0.02	0.98	−34.50	23.06
6	(Time + Time^2) + (Time^3 × Tr)	12	94.71	7.02	0.02	1.00	−34.87	33.49
7	Time + Time^2 + Time^3	8	98.13	10.44	0.00	1.00	−40.84	185.06

K, number of parameters; AICc, Akaike's information criterion for small sample sizes; ΔAICc, difference in AICc values with the model having the lowest AICc; w_i, model weight; cum. w_i, cumulative model weight; LogLik, log likelihood; ER, evidence ratio. The variables Time, Time^2, Time^3 are orthogonal terms describing the time elapsed since caterpillars' hatching date; 'Tr' refers to the treatment effect (categorical variable).

best explained by a cubic polynomial term with a steep increase in body mass at *c*. day 27 (ninth measurement) followed by a maximum *c*. 6 days before pupation (Fig. 3). However, caterpillars of the T1 group had a faster early growth, as they had an earlier first inflection point (significant interaction between the treatment and T^2; Table 1; Supporting Information Table S1).

The effect of the treatment on the caterpillars' growth patterns was due to changes in their food processing means. Indeed, while the treatment had no influence on the amount of food eaten by the caterpillars ($\chi^2 = 3.60$, d.f. = 2, $P = 0.16$), caterpillars of T1 and T2 had consistently lower residual frass production than the control group, suggesting a better assimilation rate ($\chi^2 = 22.18$, d.f. = 2, $P < 0.01$, Estimate differences ± SE: C – T1, −0.45 ± 0.11, $P < 0.01$; C – T2, −0.49 ± 0.11, $P < 0.01$; T1 – T2, −0.05 ± 0.10, $P = 0.65$). Using these data to explain the differences in growth patterns clearly showed that the effect of the treatment on caterpillar growth arose from its opposing effect on FE and FR. More specifically, the two models that clearly better explained the data both contained the cubic polynomial temporal effects, and the significant negative interaction term FR × FE (Table 3). This interaction indicates that the relationship between FE and body mass declined when the FR increased (i.e. increasing FR produced led to lighter caterpillars for a given amount of FE). The effect of the treatment on caterpillar growth resulted from its effect on the interaction between FE and FR (i.e. the three-way interaction treatment × FR × FE) and on the interaction of the treatment both on the FE and FR (two-way interactions; Tables 2 and 3).

In the C group, the heaviest caterpillars were those with high FE and high FR (Fig. 4a; darker cells towards the upper right corner). The darker cells in the upper left corner indicate that heavy caterpillars had low FE and high FR, probably because they had started their preparation for pupation (the recorded maximum body mass might have been 1–2 days after the caterpillars' real maximum body mass as we measured caterpillar body mass every 3 days). T1 caterpillars had a similar pattern (heavier caterpillars had high FE and FR values; Fig. 4b),

Table 2. Relative performance of candidate models aimed at understanding the processes affected by the treatment.

Model	*K*	AICc	ΔAICc	w_i	Cum. w_i	LogLik	ER
(Time + Time^2 + Time^3) + (FC × FR) + (FC + FR) × Tr	17	−143.24	0.00	0.63	0.63	90.59	–
(Time + Time^2 + Time^3) + (FC × FR × Tr)	19	−141.55	1.69	0.27	0.90	92.26	2.33
(Time + Time^2 + Time^3) + (FC + FR) × Tr	16	−137.47	5.77	0.04	0.94	86.48	17.88
(Time + Time^2 + Time^3) + (FC × FR) + Tr	13	−137.27	5.97	0.03	0.97	82.78	19.80
(Time + Time^2 + Time^3) + (FC × FR)	11	−135.84	7.40	0.02	0.98	79.74	40.49
(Time + Time^2 + Time^3) + FC + FR	10	−130.93	12.31	0.00	1.00	76.14	471.73
(Time + Time^2 + Time^3) + FC + FR + Tr	12	−130.75	12.49	0.00	1.00	78.35	514.62
(Time + Time^2 + Time^3) + FC × Tr	13	−115.07	28.17	0.00	1.00	71.68	> 1000
(Time + Time^2 + Time^3) + FC	9	−109.93	33.31	0.00	1.00	64.52	> 1000
(Time + Time^2 + Time^3) + FC + Tr	11	−107.41	35.83	0.00	1.00	65.52	> 1000
(Time + Time^2 + Time^3) × Tr	16	−40.48	102.76	0.00	1.00	37.98	> 1000
(Time + Time^2 + Time^3) + Tr	10	−34.26	108.98	0.00	1.00	27.81	> 1000
(Time + Time^2 + Time^3) + FR + Tr	11	−32.16	111.08	0.00	1.00	27.90	> 1000
(Time + Time^2 + Time^3) + FR × Tr	13	−31.38	111.86	0.00	1.00	29.84	> 1000
(Time + Time^2 + Time^3) + FR	9	−30.47	112.77	0.00	1.00	24.79	> 1000

Tr, treatment; FE, amount of food eaten; FR, residual frass production (a measure of food assimilation); *K*, number of parameters; AICc, Akaike's information criterion for small sample sizes; ΔAICc, difference in AICc values with the model having the lowest AICc; w_i, model weight; cum. w_i, cumulative model weight; LogLik, log likelihood; ER, evidence ratio. Time, Time^2, Time^3 are orthogonal terms describing the time elapsed since caterpillars' hatching date.

Table 3. Averaged coefficient estimates obtained for the two best models (ΔAICc < 4) with their unconditional SE, 95% unconditional confidential intervals (CI), z- and *P*-values.

Variables	Estimate	Adjusted SE	CI	*z*-value	*P*
Time	1.45	0.11	1.23, 1.66	13.40	0.00
Time^2	−0.47	0.06	−0.58, −0.36	8.17	0.00
Time^3	−0.17	0.05	−0.27, −0.06	3.18	0.00
FE	0.17	0.02	0.13, 0.21	7.86	0.00
FR	0.18	0.04	0.11, 0.26	4.74	0.00
Treatment (T2)	−0.10	0.05	−0.20, 0.01	1.83	0.07
Treatment (C)	−0.20	0.06	−0.31, −0.08	3.31	0.00
FE × FR	−0.07	0.03	−0.13, −0.01	2.39	0.02
FE × treatment (T2)	0.06	0.02	0.01, 0.11	2.32	0.02
FE × treatment (C)	0.09	0.03	0.02, 0.15	2.62	0.01
FR × treatment (T2)	−0.12	0.06	−0.23, −0.01	2.12	0.03
FR × treatment (C)	−0.05	0.04	−0.13, 0.04	1.13	0.26
FE × FR × treatment (T2)	0.09	0.05	−0.01, 0.18	1.78	0.07
FE × FR × treatment (C)	0.03	0.05	−0.07, 0.12	0.52	0.60

FE, scaled amount of food eaten; FR, residual frass production; Tr, treatment. Time, Time^2, Time^3 are orthogonal terms describing the time elapsed since caterpillar hatching date. Parameter estimates and their significance levels for each of the two models are reported in Supporting Information, Tables 2 and 3.

but for a given amount of FE and FR, T1 caterpillars were significantly heavier than caterpillars of the C group (overall darker cells). In the T2 group, caterpillars' body mass was primarily influenced by their food intake but very weakly related to their FR (Fig. 4c; darker cells on the right hand side).

As the support for the model with the three-way interaction is weaker and fully assessing its biological importance would require collecting more data (Table S3 in Supporting Information), we examined in more detail the two-way interactions of the best model. This more parsimonious model clearly shows that the treatment influenced the way caterpillars processed their food. Indeed, the slope of the relationship between FE and body mass was smaller in T1 caterpillars (Table S3 in Supporting Information; estimated difference between C and T2 = −0.017, SE = 0.034, *t*-value = −0.51, *P* = 0.62; Fig. 5a) and the slope of the relationship between FR and body mass was smaller in T2 caterpillars than in T1 caterpillars (Supporting Information Table S2) and C caterpillars (estimated difference = −0.096, SE = 0.042, *t*-value = −2.31, *P* = 0.02; Fig. 5b). Therefore, T1 caterpillars were consistently heavier and needed to eat less food to grow than C caterpillars; T2 caterpillars were slightly heavier than C caterpillars during the early stages of their development but the absence of a relationship between FR and body mass in these caterpillars suggests that they experienced digestion costs (probably a slower digestion rate) resulting in a similar overall growth performance between T2 and C caterpillars.

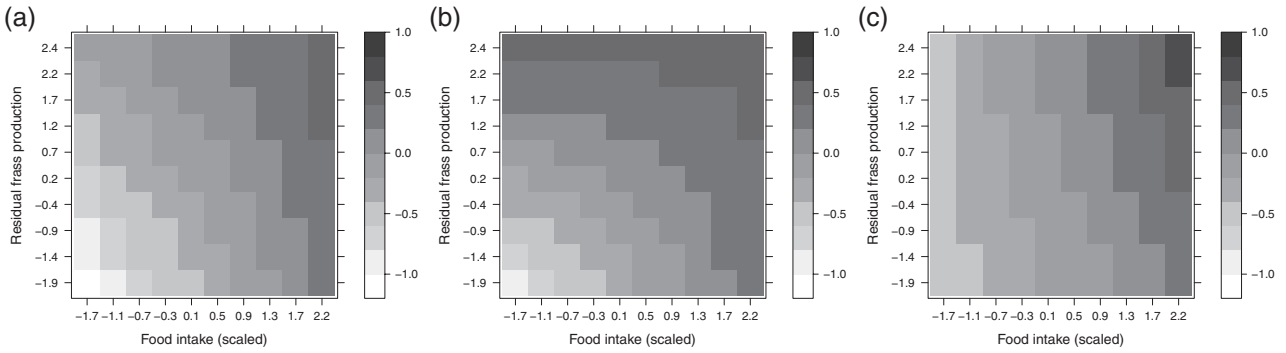

Fig. 4. Predicted values of caterpillars' body mass as a function of the amount of food eaten and residual frass production (a measure of food assimilation). Predicted values are presented for: (a) caterpillars of the control group; (b) caterpillars fed with host plants treated with a moderate amount of fertiliser (T1); and (c) caterpillars fed with host plants treated with a substantial amount of fertiliser (T2). Darker cells indicate larger predicted individual body mass. Parameter estimates were obtained by model averaging (Table 3).

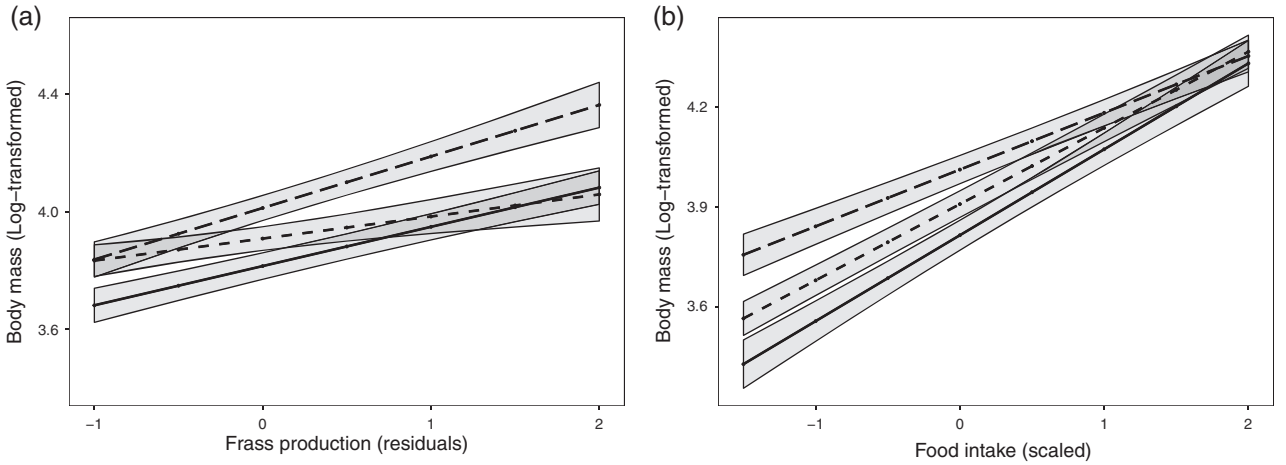

Fig. 5. Two-way interactions extracted from the best and most parsimonious model. (a) Effect of the treatment on the relationship between (scaled) food intake and caterpillars' body mass. (b) Effect of the treatment on the relationship between residual frass production (a measure of food assimilation) on caterpillar body mass. Solid lines, control group; dashed line, T1 group; thin dashed line, T2 group.

Discussion

The nitrogen limitation hypothesis posits that phytophagous insects benefit from nitrogen enrichment of their host plants (White, 1993). Our results suggest that this hypothesis might be true at moderate nitrogen enrichment levels (T1 caterpillars) and that substantial nitrogen enrichments may decrease caterpillars' growth performance (T2 caterpillars). In particular, T1 caterpillars had greater maximum body mass than caterpillars of the control group. This effect was due to a greater body mass gain early on in caterpillars' growth (Fig. 3), which continued throughout the experiment, resulting in higher maximum body mass before pupation (Fig. 2b). There was also a tendency for caterpillars of the T1 group to have a shorter development time (Fig. 2a). However, it is important to consider that our study design, in which signs of pupation were recorded every 3 days, makes it harder to identify changes in development time shorter than 3 days. These results are therefore consistent with previous studies that reported an improvement in growth performance with a small increase in nitrogen level (e.g. Cates *et al.*, 1987; Taylor, 1988; Clancy, 1992; Fischer & Fiedler, 2000; Li *et al.*, 2016), probably because nitrogen-enriched plants had lower concentrations of toxic allochemicals (e.g. Bobbink *et al.,* 2010; Larbat *et al.*, 2016) and higher amount of nutrients (Simpson & Raubenheimer, 1993).

To our knowledge, only two studies have simultaneously reported an increase and a decline in growth rate with increasing levels of nitrogen (Han *et al.*, 2014; Tao *et al.*, 2014) and our study is the first to identify a change in growth pattern and in assimilation rates. Indeed, our experiment showed that the better growth performance of T1 caterpillars was not due to an increase in food eaten but rather resulted from an improved assimilation rate (i.e. caterpillars of T1 had lower FR and a smaller relationship between FE and body mass). Further increasing the amount of nitrogen in the host plant reduced the growth performance of T2 caterpillars to a level similar to the C group; there was a tendency for a higher maximum body mass before pupation of T2 caterpillars (compared with C caterpillars) but this is probably due to a slightly longer development time (Fig. 3). These similar growth performances resulted from very

different ways of processing food, as the growth of caterpillars of the T2 group was greater early in the development of the caterpillars (compare Fig 4a and 4c: darker cells in the lower left parts of Fig. 4c) and was primarily influenced by FE. The decoupling between FE and FR in T2 caterpillars suggests that they had a slower digestion rate, possibly due to an increase in secondary compounds that require nitrogen and/or an increase in leaf cellulose content (Johnson *et al.*, 1989; Lou & Baldwin, 2004; Larbat *et al.*, 2012). Such changes in leaf characteristics might also explain why the leaf C:N ratio of the plants of the T1 and T2 treatments differed by only 10%, while the plants of the T2 treatment received twice the amount of fertiliser of those of the T1 treatment (see also Han *et al.*, 2014). Altogether these findings indicate that there is an optimum nitrogen level at which the growth performance of *L. helle* is maximised.

Working with a species under conservation concerns and attempting to mimic natural conditions inevitably bring some limitations, in terms of both sample size and study scope. Although the relatively small sample size of each treatment makes it difficult to find significant differences, other studies are clearly needed to strengthen the evidence of an optimal nitrogen enrichment. Our manipulation of the host plant was also carried out using a commercial fertiliser in which nitrogen, phosphorus and potassium are provided. Changes in the ratio of nitrogen to phosphorus concentrations may alter the performance of phytophagous insects (Tao & Hunter, 2012), but the fertiliser's concentrations of nitrogen and phosphorus were similar, making major changes in this ratio through our treatment unlikely. Phosphorus is also a limiting nutrient for plants (Vitousek *et al.*, 2010) but wet nutrient-poor meadows are more limited by nitrogen than by phosphorus because of their position in the soil chronosequence (Walker & Syers, 1976; Olde Venterink *et al.*, 2001). Hence, nitrogen concentration changes are more likely to explain the changes in palatability of *P. bistorta*.

Other studies measured the caterpillars' nutritional performance using gravimetric analyses based upon indices such as the efficiency of food conversion (EC; the ratio between the amount of dry weight gained and the amount of dry frass produced; see e.g. Scriber & Slansky 1981). These indices summarise growth patterns and food processing over the entire development and rely on the assumption that their temporal dynamics are similar during the development of caterpillars. Therefore, fine-scale effects may be unnoticed using this approach while younger caterpillars may be more sensitive to changes in plant composition, and caterpillars eating nutrient rich food may grow slowly in the early stages but have a compensatory growth subsequently. Our approach, based on the repeated measures of FE, FR and (wet) body mass gains in caterpillars, enables us to bypass this issue but was necessary to accommodate the constraint that individuals must be released in the wild after the experiment (no dry mass could be measured). Our use of fresh body mass gains might be biased if host plant leaves systematically differed in their water content, but the lack of a significant relationship between leaves' C:N ratios and their water content suggests that such a bias is unlikely to be strong in this experiment. Therefore, combining both approaches in future studies (using repeated measures of wet body mass gains on a set of individuals, and gravimetric methods using primarily dry weights in another set of individuals) would enable us to link subtle changes in food processing means and caterpillars' accumulation of organic matter.

What are the potential consequences of the enhanced growth performance of caterpillars feeding on host plants with moderate nitrogen enrichment? Levels of atmospheric nitrogen deposition have strongly increased during the last two centuries (Tipping *et al.*, 2017). These peaked in the 1990s and are currently slowly declining because of new environmental policies for atmospheric nitrogen. A similar trend is observed for nitrates and ammonia resulting from agricultural practices (Delbaere *et al.*, 2014). Increasing amounts of nitrogen may initially result in a better palatability of the host plants and hence caterpillars' growth improvement. If larvae were to reach a higher body mass before pupation, they may produce adults with higher fitness and better dispersal abilities (Haukioja & Neuvonen, 1985; Tammaru, 1998; Hunter, 2001). Yet, this positive bottom-up process may not be straightforward, as the increase in growth rate with plant nitrogen content led to lower pupa survival in a closely related species, the copper butterfly *Lycaena tityrus* (Fischer & Fiedler, 2000). Faster growth has also been associated with changes in wing shape and/or thorax size (Karl *et al.*, 2008), meaning that increased nitrogen content could also alter adult flight performance and reduce the emigration rate from eutrophicated sites. Moreover, it has been suggested that the nitrogen-rich frass produced by all the phytophagous insects may further facilitate the nitrogen absorption of their host plants (Belovsky & Slade, 2000). Such a feedback loop may therefore lead to high host plant nitrogen content and hence declines in caterpillar growth through digestion costs. Therefore, the very high level of nitrogen content observed until recently might have exerted a strong selection pressure acting on traits enabling caterpillars to feed on nitrogen-enriched food, and a decline in the magnitude of this selection pressure might benefit the populations of this glacial relict species adapted to nutrient-poor environments. Although quantifying the fitness consequences of changes in caterpillars' growth patterns is required to fully understand the ecological and evolutionary implications of nitrogen enrichment for *L. helle*, our results indicate that conservation plans for this emblematic glacial relict species should consider this species' host plant quality to increase its likelihood of long-term persistence.

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CL, CT, and NS conceived the ideas; CL, CV, and CT designed the experiment; CL and CV collected the data; CL and NS analysed the data; and CL led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Relationship between fresh leaf area and mass in each treatment group. Black, yellow, and brown dots represent the C, T1, and T2 treatment groups, respectively.

Figure S2. Hypothetical growth curve and the data used for fitting the temporal pattern (dashed line) and identifying the processes underpinning differences in growth patterns (shaded grey area).

Table S1. The averaged coefficient estimates for models measuring the effect of the treatment on the temporal growth patterns. Models with ΔAICc < 4 were averaged. Parameters are reported with their unconditional standard errors (SE), 95% unconditional confidential intervals (CI), *z*- and *P*-values.

Table S2. Output of the best model examining the processes underpinning the effect of the treatment on caterpillar growth. FE, scaled amount of food eaten; FR, residual frass production. As our hypothesis was that an intermediate nitrogen enrichment would lead to better growth performance, the T1 group was set as the reference group for the treatment variable to facilitate comparisons of this level with the C and T2 groups. Wald χ^2 values are provided for the terms including categorical variables.

Table S3. Output of the second best model examining the processes underpinning the effect of the treatment on caterpillar growth. FE, scaled amount of food eaten; FR, residual frass production. As our hypothesis was that an intermediate nitrogen enrichment would lead to better growth performance, the T1 group was set as the reference group for the treatment variable to facilitate comparisons of this level with the C and T2 groups. Wald χ^2 values are provided for the terms including categorical variables.

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