Warming and nutrient-depleted food: Two difficult challenges faced simultaneously by an aquatic shredder

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Abstract: Global warming and changes in nutrient supply can alter the functioning of headwater stream ecosystems, where leaf-shredding invertebrates are pivotal for the transfer of nutrients through the food web. However, little is known about how warming and nutrient-depleted leaf litter interact and potentially exacerbate the already large elemental imbalance between shredders and their food. We hypothesized that: 1) shredders cannot increase their feeding rate to compensate for the effects of both factors simultaneously, and 2) shredders will increase assimilation efficiency of limiting nutrients, especially N, by decreasing excretion, increasing nutrient bioavailability in the gut, or both by adaptations of digestive enzymes. We performed a microcosms experiment with a caddisfly species subjected to 3 temperatures and 2 diets that differed in their N and P content. Overall, our results suggest that the combination of increasing temperatures and decreasing diet nutrient content lowered the capacity of larvae to acquire nutrients, either by decreasing nutrient assimilation, increasing nutrient excretion, or both. However, the trypsin enzyme maintained functionality under the range of temperatures we tested (5–15°C), which likely allows larvae to ensure N availability. Moreover, we detected reduced excretion and improved apparent assimilation of N relative to P. However, larval survival still decreased significantly at the highest temperature (15°C) when larvae were fed the low-nutrient diet. These results indicate that regulation mechanisms for reducing elemental constraints from poor food quality are less effective at higher temperatures. If these effects also occur in other detritivores, nutrient recycling and ecosystem function in headwater streams might be compromised as the climate warms.

Key words: homeostasis, elemental imbalance, enzyme kinetic, nutrient assimilation, egestion, excretion, artificial diets

Headwater stream ecosystems in temperate regions are largely heterotrophic and subsidized by terrestrial inputs of senescent leaves (Wallace et al. 2015). Thus, leaf-litter stoichiometry influences the nutrient stoichiometry of streams (Frost et al. 2002). Both invertebrate detritivores (i.e., shredders) that feed primarily on this allochthonous material and microbial decomposers are pivotal for nutrient cycling in stream ecosystems (Graça 2001, Wallace and Eggert 2015). Two of the most important drivers of global change—climate warming and altered nutrient supply—may affect nutrient cycling by changing how and at what rates organisms acquire, store, and mobilize elements in ecosystems. However, little is known about how warming and altered nutrient supply interact to influence ecological processes (Cross et al. 2015), despite broad recognition that disturbance interactions can have complex effects and lead to ecological surprises (sensu Paine et al. 1998).

Increased atmospheric carbon dioxide concentration ([CO₂]) is the main cause of global warming. Increases in both temperature and CO₂ can reduce the nutritional quality of leaf-litter to stream detritivores, potentially exacerbating the already large elemental imbalance between shredders and their food (Frost et al. 2006). Experimental studies have found that elevated [CO₂] increases the C:N ratio of tree leaves and causes greater accumulation of secondary chemicals (Stiling and Cornelissen 2007), which might adversely influence riverine shredders (Tuchman et al. 2002). Further, gradient studies at regional (Chen et al. 2013, Salinas et al. 2018) and global (Yuan and Chen 2009, Boyero et al. 2017) scales predict that warming could significantly reduce leaf N and P (or just P). Some of these studies also point to warming-derived changes in other leaf traits of riparian vegetation, such as increasing silicon content and toughness (Boyero et al. 2017, Salinas et al. 2018), which can interfere with the feeding and digestive
activities of shredders (Massey and Hartley 2009, Graça and Cressa 2010).

The Metabolic Theory of Ecology predicts that metabolic rates of ectotherms should increase exponentially with rising temperatures (Gillooly et al. 2001), which should drive matching increases in other rates, such as consumption (Brown et al. 2004). However, most empirical results indicate a mismatch between temperature scaling of metabolic and consumption rates (Lemoine and Burkepile 2012). This mismatch results in a temperature threshold above which increasing consumption cannot compensate for the increasing metabolic costs, causing growth to decrease (Iversen 1979, González and Graça 2003). Thus, even the modest temperature increases forecasted for streams (average 2–3°C; Morrill et al. 2005) may have adverse outcomes for organism fitness and population dynamics (Lemoine and Burkepile 2012), especially for cold-adapted stenotherms (Woodward et al. 2010). If this mismatch is concomitant with decreased nutrient content of leaf litter, deleterious effects on cold-adapted shredders could be magnified, particularly in species unable to develop compensatory feeding because of poor-quality food (Santonja et al. 2018). However, recent studies of the combined effect of warming and poor food quality on shredder performance shows conflicting results. Some studies suggest that warming may aggravate the adverse effects of nutrient-poor litter (Correa-Araneda et al. 2015, Mas-Martí et al. 2015), whereas others indicate greater effects of elevated temperature than depleted litter quality (Ferreira et al. 2010) but no clear joint effects of both factors (Landeira-Dabarca et al. 2019). These discrepancies could arise from shredder idiosyncrasies, the varying range of temperatures tested, or from the use of litter species that differ in leaf traits (i.e., secondary chemicals) other than elemental nutrient content. These differences could act as confounding factors given their varying and often critical roles in feeding deterrence, digestion, and assimilation (e.g., Frost and Tuchman 2005).

Studies on joint effects of warming and depleted litter quality on shredder performance have addressed the fundamental strategies shredders use to optimize their nutrient content: regulation of feeding, assimilation and excretion rates, and nutrient allocation. Shredders use enzymatic processes in nutrient digestion and metabolism to maintain homeostasis, but these processes are not well understood. Ectotherms facing warming might adapt the functionality of hydrolytic enzymes involved in food processing by: 1) changing the amount of enzyme produced, 2) producing different isoenzymes that maximize functionality under different thermal parameters (Somero 2004, Gelman et al. 2008), or 3) a combination of both responses. The production of different isoenzymes is linked to changes in the molecular conformation of the enzymes, which can result from 1 to a few amino acid substitutions (Somero 2004) and modifies the kinetic parameters of enzymes. Production of different isoenzymes is well known for intracellular enzymes of ectotherms (Somero 2004) and hydrolases produced by soil microbiota (German et al. 2012, Frey et al. 2013). Indeed, recent studies suggest that global warming may trigger selection for hydrolytic enzymes that have relatively-constant affinity for the substrate ($K_	ext{m}$, the substrate concentration at which the rate of substrate conversion equals half of the maximum rate), which would allow the enzymes to maintain high levels of activity (Blagodatskaya et al. 2016). Thus, the kinetic parameters of digestive enzymes in riverine shredders may be a promising tool to assess the potential of shredders to deal with climate change.

Here we hypothesized that: 1) shredders cannot compensate for the joint effects of warming and nutrient-depleted litter by increasing feeding rate (likely caused by constraints in handling and gut residence times) when the combination of both temperature and nutrient depletion exceed a certain threshold, and 2) shredders compensate for the joint effects of warming and nutrient depletion by increasing the assimilation efficiency of limiting nutrients. This latter change could be accomplished either by regulating excretion to decrease nutrient release, increasing nutrient bioavailability in the gut, or both. Increasing the bioavailability of nutrients in the gut could be done by modifying the kinetic parameters of enzymes involved in the acquisition of C, N, and P. We tested these hypotheses in a microcosm experiment with the caddisfly Allogamus mortoni Navás (Trichoptera:Limnephilidae), a species endemic to the Iberian Peninsula that is widespread and locally abundant in headwater streams. We evaluated how food consumption, egestion, excretion, and the kinetic parameters of key digestive enzymes ($\beta$-glucosidase, trypsin, and alkaline phosphatase) responded to the combined effects of increasing temperature and decreasing food quality.

**METHODS**

We performed a laboratory-based microcosm experiment to assess effects of the interaction between warming and diet quality on shredder performance. Our full factorial experimental design considered 3 levels of temperature and 2 levels of diet. Response variables included food consumption, survival, egestion, excretion, digestive enzyme activity, and body stoichiometry.

**Pre-experimental setup and analyses**

We collected detritivores and leaf litter from 3 low-order streams in the Sierra Nevada Natural Park (Andalusia, Spain), where alder (Alnus glutinosa L. Gaertner) and willow (Salix spp.) dominate riparian vegetation. In these streams, the mean ($\pm$SE) annual temperature between 2013 and 2016 was $11.0 \pm 1.6°C$, the winter mean was $3.5 \, °C$ (range = 0.9–8.0°C), the spring mean was 9.6°C (range = 2.4–14.2), and the summer mean was 14.1°C (range = 10.8–16.8). This annual range was used as a reference to set our experimental temperature range. Additional environmental information about this region is given in Casas et al. (2011). We collected senesced alder leaves from the riparian forest during autumn...
2017, air-dried them for 2 wk, and stored them until we used them in this experiment. We collected intermediate-size (dry mass range: 0.006–0.051 g) A. mortoni larvae in April 2018 and transported them to the laboratory in a cool box with stream water and sand. Upon arrival to the lab, 3 subsets of individuals were made. The 1st subset of larvae (n = 9) were starved for 24 h to clear their digestive tracts to measure initial body nutrient content. The 2nd group was used to build a predictive model of size-body dry mass (n = 138). The 3rd group (n = 108) was used for the experiment. They were randomly assigned to 3 experimental temperature groups (5, 10, and 15°C) in separated aerated aquariums with sand and gravel under a 12:12 (light:dark) photoperiod. Individuals were acclimated for 2 wk prior to the experiment. During the 1st wk of acclimation they were fed the experimental diet, larvae were fed leaf litter from their stream of origin, and during the 2nd wk of acclimation they were fed the experimental diet (see below).

We prepared 2 diet qualities that used alder leaf litter as a main component. We leached leaves for 48 h in distilled water, dried them at 60°C for 72 h, and then ground them to obtain a powder of particle size <1 mm. The high-quality diet contained 80% weight/weight (w/w) leaf-litter powder, whereas the low-quality diet contained 40% w/w leaf-litter powder + 40% cellulose powder. All the ingredients were mixed thoroughly with agar (20% w/w) suspended in hot distilled water, then spread on a tray to form a thin layer and allowed to coagulate. After the material cooled, we cut it into discs (12-mm Ø) that we dried at 60°C for 72 h, weighed, and froze until we used them in our experiments. We measured toughness of the hydrated discs (n = 9/diet type) as the critical mass required to penetrate the disc with a texturometer (TA.XT2 Plus; Stable Micro Systems, Godalming, UK) (Graça and Zimmer 2005), which was equipped with a cylindrical steel sounding line with a puncture surface of 0.38 mm². Carbon and nitrogen of diets were determined using a Leco TruSpec CN elemental analyzer (LECO Corporation, St Joseph, Michigan), and phosphorus concentration was determined by the ascorbic acid method on incinerated aliquots (incinerated at 550°C for 5 h). We used t-tests to compare physical and chemical characteristics of the 2 artificial diets. The stability in water of discs was assayed previously (see Table S1).

Experimental setup
The experimental setup consisted of eighteen 35 × 35 × 20-mm ice buckets (6 treatment combinations replicated 3 × each). We placed 6 buckets in each of 3 temperature-controlled incubators simultaneously (5, 10, and 15°C), and we randomly assigned each of the 6 buckets in each incubator to 1 or 2 food treatments (high-quality and low-quality food). Each bucket contained filtered stream water with forced aeration and sand (1–2-mm particle size). A 0.5-mm mesh septum, placed at the bottom of each bucket, separated larvae from their feces, which we collected daily and froze until analysis (3 pooled samples from 6 randomly-chosen ind/treatment). We fed larvae 2 pre-weighed discs of the assigned diet, then collected the partially-consumed discs every 3rd d (see Table S1), dried them at 60°C for 72 h, and reweighed them. We used the final product to estimate feeding rates. We also put a pair of discs in buckets (n = 7) with no larvae as a control to evaluate mass loss from factors other than consumption. The experiment lasted only 11 d because of the high mortality observed in some treatments.

Consumption, survival, egestion, and excretion
We estimated the relative feeding rate (RFR) as follows:

\[ \text{RFR} = \frac{(\text{DM}_i - \text{DM}_f)}{(\text{DM}_{\text{larva}} \times t)} \]  

(Eq. 1)

where DMₖ is dry mass of the disc at the end of the period of exposure to the larva, DMᵢ is dry mass of discs at the beginning corrected by changes in weight not caused by shredder consumption, t is exposure time to consumption, and DMₗₐᵢᵣᵥᵃ is the final dry mass of the larva. At the end of the experiment, we used SigmaScan® Pro software (version 5.0; Systat Software, Inc, San Jose, California) to measure individual head width from photographs to indirectly estimate DMₗₐᵢᵣᵥᵃ. We developed a head width–body DM (dried at 60°C for 72 h, weighed to the nearest 0.1 mg) regression for A. mortoni (without case): DM (g) = 2.3391 × 10⁻⁴ e².1437 × head width (cm) (R² = 0.90, root mean square error = 0.23, n = 138). We used a 2-way analysis of variance (ANOVA), with diet quality and temperature as factors, to test for differences in RFR among treatments, followed by Tukey’s post-hoc test. We log-transformed data to ensure homoscedasticity of variances (Levene’s test). We recorded individual mortality daily during the experiment and used Cox proportional-hazards regression models to compare survivorship among treatments.

Collected feces were oven dried (60°C for 72 h) and then analyzed for C, N, and P content (as for diets, see above). We then used a 1-way ANOVA with resampling (ANOVA-r) to assess if nutrient stoichiometry differed between each diet and feces egested at a given temperature. Resampling is recommended when replication is low, as it was here (n = 3), which consists of reordering data randomly many times to run successive ANOVAs (see Howell 2009). After carrying out an ordinary ANOVA, we randomized each response variable 5000 × and calculated the F statistic based on this randomization. The p-value is the proportion of randomized F exceeding observed F (Bärlocher 2005). We accounted for multiple comparisons with the post-hoc Dunnett’s test (Holm–Sidak correction of p-values).

We quantified N and P excretion rates at the end of the experiment by transferring surviving larvae to sterile beakers. We used 3 beakers/treatment (n = 3), and each beaker contained between 3 and 6 individuals depending on survival in the treatment. Each beaker also contained distilled water that had been oxygenated and tempered for 12 h before we measured N and P at the treatment temperature.
After 4 h (enough time to ensure confident measurements but to avoid starvation [Devine and Vanni 2002]), we analyzed ammonium ($\text{NH}_4^+$) and phosphate ($\text{PO}_4^{3-}$) with the salicylate and the ascorbic acid methods, respectively (APHA 2005). Excretion rates were expressed as $\mu$g $\text{NH}_4^+$-N or $\text{PO}_4^{3-}$-P/g DM for each larva/d. We used a permutation test for factorial analysis of variance (f-ANOVA-r; see above), followed by post-hoc Dunn’s test, with diet and temperature as factors, to assess if shredders could potentially reduce excretion of limiting nutrients (N and P).

After we measured excretion rates, we starved 3 ind/treatment for 24 h to clear their digestive tracts and then analyzed the elemental nutrient concentration (as for diets, see above) of each individual. Pre-experimental nutrient concentrations of individuals ($n = 9$, see above) were measured in the same way. All elemental ratios were expressed as molar ratios. We used f-ANOVA-r, followed by a post-hoc Dunn’s test, to assess if diet quality and temperature affected C:N, C:P, and N:P of individuals exposed to a given temperature and diet treatment were tested by ANOVA-r. We estimated the net gain in N and P as the difference between ingested and egested amounts. We estimated the net gain in the same way. All elemental ratios were expressed as moles.

**Digestive enzyme activities**

We measured enzyme activity at 5, 10, and $15^\circ$C to assess the effect of temperature on the kinetic parameters ($K_m$ and maximum enzyme activity [$V_{\text{max}}$]) of 3 digestive enzymes involved in the hydrolysis of C-, N-, and P-containing substrates ($\beta$-glucosidase, trypsin, and alkaline phosphatase). Assays at each temperature were run with larvae from the corresponding temperature treatment. We performed kinetic assays by measuring activities at 9 substrate concentrations that spanned realistic ranges of each substrate (0–526 $\mu$mol/mL for $\beta$-glucosidase activity, 0–53 $\mu$mol/mL for trypsin activity, and 0–132 $\mu$mol/mL for alkaline phosphatase activity). These concentrations represent the real substrate concentrations at the reaction mix, not the stock substrate concentrations. For each temperature treatment, 3 replicates (i.e., pooled samples, see below) were incubated in duplicate (analytical replicates). We performed preliminary assays to determine the saturation concentrations of fluorescent substrates. Kinetic analyses were performed to ensure linear increase of fluorescence over time during the assay.

We prepared the enzyme extracts used in the assays by pooling (n = 3) individuals from the same treatment in a glass homogenizer and then centrifuging them at 12,000 × g for 15 min. We used the substrates 4-methylumbelliferyl-b-Dglucopyranoside (MUF-G) for $\beta$-glucosidase, Boc-Gln-Ala-Arg-7 amido-4 methyl coumarin for trypsin, and 4-methylumbelliferyl-phosphate disodium salt for alkaline phosphatase. Reaction mixtures consisted of 20 $\mu$L of enzymatic extract, 120 $\mu$L borate buffer (50 mM, pH 7.5), and 10 $\mu$L of each substrate solution in a 96-well microtiter. Fluorescence was measured at an excitation-emission wavelength of 355 to 460 nm. Enzyme activity was expressed as relative fluorescence units (min$^{-1}$ mg$^{-1}$).

We measured the apparent kinetic parameters of the enzymes with the Michaelis–Menten equation,

$$V = \frac{(V_{\text{max}} \times [S])}{(K_m + [S])} \quad \text{(Eq. 2),}$$

where $V_{\text{max}}$ is the maximum enzyme activity, $K_m$ is the half-saturation constant, which is the substrate concentration at which the reaction rate equals $V_{\text{max}}/2$, and S is the substrate amount. We estimated the enzymatic parameters $V_{\text{max}}$ and $K_m$ for each experimental replicate (n = 3) with the Michaelis–Menten equation by fitting means of the 2 analytical replicates. We then used ANOVA-r to discern whether $K_m$ differed among temperatures and 2-way ANOVAs by resampling (f-ANOVA-r) to test the effects of diet quality and temperature on $V_{\text{max}}$. Both ANOVAs (ANOVA-r and f-ANOVA-r) were followed by post-hoc Dunn’s tests. All statistical analyses were done with the computing environment R (version 3.6.2; R Project for Statistical Computing, Vienna, Austria).

**RESULTS**

**Artificial diets**

Diets differed in toughness and stoichiometry except for N:P ratio (Table 1). The low-quality diet contained half of

| Characteristic     | High quality | Low quality | t-value | p-value |
|--------------------|--------------|-------------|---------|---------|
| %C                 | 47.1 ± 0.1   | 44.1 ± <0.1 | 28.56   | <0.001  |
| %N                 | 2.4 ± <0.1   | 1.2 ± <0.1  | 37.84   | <0.001  |
| %P                 | 0.029 ± <0.001 | 0.015 ± <0.001 | 46.36   | <0.001  |
| C:N                | 22.8 ± 0.3   | 42.5 ± 0.5  | −37.34  | <0.001  |
| C:P                | 4195.0 ± 15.8 | 7499.5 ± 107.8 | −34.83  | <0.001  |
| N:P                | 184.4 ± 2.6  | 176.5 ± 2.5 | 2.14    | 0.069   |
| Toughness (g)      | 47.9 ± 20.3  | 24.3 ± 1.9  | 8.09    | <0.001  |

Table 1. Characterization of diets used to feed *Allogamus mortoni* during the experiment. All ratios are molar. Results of t-test analysis (t- and p-values) for each characteristic are also shown.
the N and P of the high-quality diet, and the C:N and C:P ratios of the low-quality diets were twice as high as the high-quality diet. Trials of diet-disc stability in water produced similar results for both diets, with discs being highly stable in water for 72 h (remaining mass > 95%; Table S1). We used this result to set the exposure time of the diet to the larvae.

**Feeding and survival**

Feeding rates (Fig. 1, Table S2) did not change with diet quality \( (F_{1,102} = 0.40, p = 0.53) \), but they increased with temperature \( (F_{2,102} = 26.58, p < 0.001) \), being \( 3.7 \times \) higher at 15 than at 5°C. There was no evidence of an interaction between diet quality and temperature \( (F_{2,102} = 0.33, p = 0.72) \).

Survival of larvae was higher in the high-quality diet treatments than in the low-quality diet treatments (log-rank statistic = 20.79, \( p < 0.001 \); Fig. 2). Individuals fed the low-quality diet had a higher probability of dying (Hazard’s ratio, \( \beta = 10.25 \pm 1.06 \) as temperatures increased (log-rank statistic = 9.05, \( p = 0.013 \); Fig. 2), but this trend was not as apparent for individuals fed the high-quality diet (log-rank statistic = 4.12, \( p = 0.13 \)).

**Comparison of C:N:P stoichiometry between food and feces**

The ability of *A. mortoni* to digest C, N, and P was influenced by both diet quality and temperature (Table S3).

With the high-quality diet, the C:N ratios of feces did not appear to differ from those of the food \( (\text{ANOVA-}r, p = 0.329) \). However, the C:N ratios in the low-quality food were higher than those in the corresponding feces \( (\text{ANOVA-}r, p < 0.001) \), especially at 5°C at which food C:N was \( 1.5 \times \) higher than in feces (Fig. 3A). Fecal C:P and N:P ratios were \( 1.8 \) and \( 1.6 \times \) lower on average, respectively \( (\text{ANOVA-}r, \text{ both } p < 0.001) \) than those in the corresponding diet in all temperature treatments (Fig. 3B and 3C, respectively; Table S3). P-enrichment of feces was most obvious at 5°C, whereas it tended to be lower for animals reared at 10 or 15°C on either diet.

**Excretion**

The amount of excreted NH\(_4\)\(^+\)-N varied among temperatures \( (\text{f-ANOVA-}r, p = 0.0012) \) and between diets \( (\text{f-ANOVA-}r, p = 0.011) \). The effect of temperature on excretion depended on diet \( (\text{f-ANOVA-}r, p = 0.002; \text{ Fig. 4A, Table S4}) \). When larvae fed on the low-quality diet, NH\(_4\)\(^+\)-N excreta was 3 and \( 2 \times \) higher at 15°C compared to 5 and 10°C, respectively (post-hoc test, \( p = 0.004 \) and 0.011, respectively), but differences were negligible among temperatures when larvae fed on the high-quality diet (post-hoc test, all \( p > 0.054 \)). Thus, at 15°C the excretion of N was \( 2.3 \times \) higher in the low- than in the high-quality diet (post-hoc test, \( p = 0.016; \text{ Fig. 4A} \)). Excreted PO\(_4\)\(^3-\)-P increased with increasing temperatures \( (\text{f-ANOVA-}r, p < 0.001) \), regardless of diet quality \( (\text{f-ANOVA-}r, \text{ Fig. 4B, Table S4}) \), but the only apparent difference among temperatures was between 5 and 15°C, with excreted P \( 4 \times \) higher on average at 15°C (post-hoc test, \( p = 0.012 \)).

**Enzyme assays**

\( \beta \)-glucosidase activity decreased nearly 400 relative fluorescence units as temperatures increased from 5 to 15°C \( (\text{f-ANOVA-}r, p = 0.047) \), regardless of diet quality \( (\text{f-ANOVA-}r, p = 0.135; \text{ Fig. 5A, Table S5}) \). However, temperature did not influence the activity of trypsin or phosphatase \( (\text{f-ANOVA-}r, p = 0.356 \) and 0.082, respectively; \text{ Fig. 5B, C, Table S5}) \). Diet quality did not affect enzymatic activities \( (\text{f-ANOVA-}r, \text{ all } p > 0.063; \text{ Fig. 5A–C, Table S5}) \). The effect of temperature on \( K_m \) differed among enzymes. Apparent \( K_m \) (sensu Wallenstein et al. 2010) of \( \beta \)-glucosidase was doubled from 5 to 10°C \( (\text{ANOVA-}r, p < 0.001; \text{ Fig. 5D, Table S5}) \). However, \( K_m \) could not be estimated accurately at 15°C because of the high variability in the data that resulted from the high mortality of individuals exposed to that temperature. Apparent \( K_m \) of trypsin was not influenced by temperature \( (\text{ANOVA-}r, p = 0.36) \), although it appeared to decrease as temperatures rose \( (\text{Fig. 5E, Table S5}) \). Apparent \( K_m \) of phosphatase activity increased as temperatures increased \( (\text{ANOVA-}r, p = 0.013) \), notably \( (1.7 \times) \) between 5 and 15°C \( (\text{ANOVA-}r, p = 0.013; \text{ Dunn's test } p = 0.017; \text{ Fig. 5F, Table S5}) \).
Changes in body stoichiometry and nutrient net gain

Insect body C:N ratios did not differ across diets or temperatures (f-ANOVA-r, $p = 0.926$ and 0.837, respectively), nor did they differ from the initial values in any treatment (ANOVA-r, all $p > 0.247$; Fig. 6A, Table S6). Differences in body C:P and N:P across diets were small (f-ANOVA-r, $p = 0.077$ and 0.057, respectively), and there was no temperature or interaction effect. However, when pre- and post-experiment body stoichiometry were compared, larvae fed on the high-quality diet had $2.2\times$ higher C:P and N:P ratios at the end of the experiment relative to the beginning of the experiment at $10^\circ$C (ANOVA-r, $p = 0.008$ and 0.038, respectively) and $2.3\times$ higher at $15^\circ$C (ANOVA-r, $p = 0.025$ and 0.016, respectively) (Fig. 6B, C).

Individuals fed the high-quality diet assimilated $2.5\times$ more N than those fed the low-quality diet (f-ANOVA-r, $p < 0.001$).
Figure 4. Mean (±SE) excretion rates (µg nutrient larva⁻¹ d⁻¹) of Allogamus mortoni fed on 2 diets differing in nutrient content (high and low) and exposed to 3 different temperatures. Larvae from each treatment were pooled into 3 replicates. Box plots show median values (central line), 25th and 75th percentiles (box), and the ranges (whiskers). Different letters indicate significant differences in ammonium (NH₄⁺) excretion across temperatures (f-ANOVA-r, p = 0.0012), diets (f-ANOVA-r, p = 0.011), and interaction (f-ANOVA-r, p = 0.002) (A); and phosphate (PO₄³⁻) excretion across temperatures (f-ANOVA-r, p < 0.001) (B).

Figure 5. Mean (±SE) maximum enzyme activities (Vₘₐₓ) for β-glucosidase (A), trypsin (B), and alkaline phosphatase (C) of Allogamus mortoni acclimated to different temperatures when fed on 2 diets differing in nutrient content (high and low). Mean (±SE) effects of temperature on apparent Michaelis–Menten constant (Kₘₐₓ) for β-glucosidase (D), trypsin (E), and alkaline phosphatase (F). The Kₘₐₓ of β-glucosidase could not be estimated confidently at 15°C. Larvae from each treatment were pooled into 3 replicates. Box plots show median values (central line), 25th and 75th percentiles (box), and the ranges (whiskers). Different letters indicate significant differences in Vₘₐₓ and Kₘₐₓ (f-ANOVA-r and ANOVA-r, respectively; all p < 0.05).
Temperature also affected N net gain (f-ANOVA-r, \(p < 0.001\)), with a steeper increase with rising temperature when fed on high- compared to low-quality diet (Fig. 7A, Table S7). Phosphorus net gain was \(19\) higher when individuals were fed the high-quality diet than the low-quality diet (f-ANOVA-r, \(p < 0.003\)) and increased with temperature (f-ANOVA-r, \(p < 0.0013\)), particularly with the high-quality diet, which was \(3.8\) higher on average at 10 and 15°C than at 5°C (f-ANOVA-r, \(p < 0.001\)) (Fig. 7B, Table S7).

**DISCUSSION**

Understanding how climate change drivers interact (synergies, antagonisms, or additive effects) to affect ecological
processes is vital for enacting appropriate management actions in the face of forecasted changes (Côté et al. 2016). Delterious effects on shredder fitness are expected in headwater streams because of synergies between warming and reduced nutritional quality of leaf litter. These synergies produce a mismatch between temperature scaling of metabolic and consumption rates (Lemoine and Burkepile 2012), which hinder compensation of metabolic costs when temperatures rise. However, studies addressing this issue show conflicting results. Some studies show that low-quality diets boost the temperature sensitivity of shredders (Villanueva et al. 2011, Correa-Aranda et al. 2015), but others indicate that warming reduces nutrient limitation to some extent, with non-additive and unpredictable effects (e.g., Landeira-Dabarca et al. 2019). These differing observations could be related to: 1) the heterogeneity in traits other than elemental nutrient content, such as secondary chemicals of the leaf-litter species used in experiments; 2) the different assimilation strategies of shredders; or 3) changes in nutrient demand at different temperatures. Moreover, it is unknown whether thermal adaptation of hydrolytic digestive enzymes is possible in detritivores. Consideration of the adaptive response of enzymes is necessary if we hope to understand how global-change drivers interact to affect ecological processes (Cross et al. 2015). We aimed to fill this gap in knowledge and avoid potential unknown confounders in diets by simultaneously studying the effects of warming and nutrient-depleted food on the shredder A. mortoni. To do this, we measured digestive enzyme activity when shredders were fed artificial diets at different temperatures. Artificial diets allow high-control feeding tests (Kampfraath et al. 2012, Crenier et al. 2017).

Our results showed that A. mortoni were negatively affected (decreasing survival and nutrient net gain) by both rising temperature and decreasing food quality. Both diet qualities were ingested at a similar rate, which increased with temperature. Thus, we found no evidence of stoichiometric regulation through compensatory feeding or assimilation efficiency, even though these pathways have been described previously (Plath and Boersma 2001). Factors that determine the capability of a shredder to address low-quality food by compensatory feeding are poorly understood but have been suggested to relate to the way species interact with leaf-litter traits other than limiting nutrients, such as toughness or lignin (Flores et al. 2014, Frainer et al. 2016). Our results suggest that the lack of compensatory feeding is an idiosyncrasy of A. mortoni because the lower-quality food was softer than the high-quality food, which could have made it easier to consume (Graça and Cressa 2010). In the absence of compensatory feeding, the low-quality diet was unable to support survival, particularly at the highest temperature, probably because of the high cost of maintenance with a misbalanced food.

To maintain relative constant homeostasis, shredders can change the rate at which they lose or gain limiting nutrients by increasing assimilation rates, reducing excretion rates, or both (Sterner and Elser 2002, Evans-White and Halvorson 2017). Organisms usually adjust either P (Danger et al. 2013, Fuller et al. 2015) or N (Evans-White et al. 2005, Frainer et al. 2016) assimilation or excretion rates. Overall, our results indicate that A. mortoni balances N more efficiently than P. Larvae egested a greater amount of P relative to C and N when they ate either diet. However, this pattern was particularly clear when larvae fed on the low-quality diet, even though P was in much lower concentration than in the high-quality diet. In addition, fecal N enrichment relative to C was detected in the low-quality but not in the high-quality diet. The greater fecal enrichment in P and the lower enrichment in N, could be related to bacteria and epithelial mucosa added to feces during the gut passage, which is particularly perceptible when nutrient assimilation is depleted. This enrichment was most apparent at 5°C, perhaps because of increasing gut retention time as temperature decreased (Welton et al. 1983). These results are consistent with the prediction that detritivorous insects have the lowest P requirements among aquatic consumers (Frost et al. 2006) as well as with results that show N is their main limiting nutrient (Balseiro and Albariño 2006, McManamay et al. 2011).

Reducing excretion of limiting nutrients to maintain homeostasis often results in an inverse relationship between the amount of excreted nutrients and the C:Nutrient ratio of food (McManamay et al. 2011, Fuller et al. 2015). However, this trend did not emerge in our results. Nitrogen excretion was relatively low and unaffected by diet at 5 and 10°C but increased sharply at 15°C in only the low-quality diet. This result suggests, as already reported elsewhere (Villanueva et al. 2011, Mas-Martí et al. 2015), that high temperatures strengthen deleterious effects of poor-quality food, at least in terms of the ability of A. mortoni to regulate the loss of N post assimilation. Excretion of P clearly increased with temperature, regardless of diet quality, which again points to less efficient mechanisms regulating P absorption, likely because of the lower limitation from this nutrient compared to N.

The evaluation of the effect of temperature on the kinetic parameters of the enzymes involved in food hydrolysis within the gut of the caddisfly must consider that these activities are the combination of digestion by the larvae and the intestinal microbiota. We measured activities as a whole without distinction of their origin. Thus, the observed variations in the kinetic parameters of a given enzyme represent the effects of temperature, but the possibility of thermal adaptation may depend on the relative contribution of either the microbiota or the invertebrate to enzyme production. Within the biological temperature range, K_m could be positively modulated, i.e., increasing (affinity decreases) with rising temperature, or kept low to ensure high enzyme-substrate affinity (Somero 1997). Thus, efficient digestion may place a premium on a low K_m value, to ensure that the enzyme remains saturated even at low substrate concentration, and on a high V_max to ensure rapid catabolism of ingested macromolecules.
Activity of β-glucosidase decreased significantly with temperature, which, in addition to its marked tendency to increase \( K_m \) with temperature, suggests a strong cancellation effect on this enzyme, i.e., positive thermal modulation (Somero 1997). This change would prevent a net increase in cellulolytic activity because of decreasing affinity of the enzyme for the substrate. Alkaline phosphatase showed a similar, but less marked, increase of \( K_m \) with increasing temperatures, which most likely resulted in the lack of a noticeable effect of temperature on total activity. In contrast, trypsin activity had a constant \( K_m \) over the temperature range of this experiment, ensuring an efficient enzyme conformation within the full temperature range tested (Somero 1997). This result could be interpreted as a useful adaptation for maintaining N-use efficiency under a warming context. Enzyme systems with lower temperature sensitivity could be beneficial in the face of global warming (Blagodatskaya et al. 2016).

The notable capability to balance N, together with a likely C regulation, likely enabled larvae to maintain a remarkably constant body C:N ratio across temperatures and diets and a highly similar ratio to the pre-experimental ratio. Under relatively non-N-limiting conditions (high-quality diet), larvae were likely able to accumulate energy reserves (lipids, glycogen) and growth (protein), which in turn may have led to elevated C:P and N:P ratios, respectively (Sterner and Elser 2002). Conversely, and somewhat unexpectedly considering the poor efficiency in balancing P, larvae maintained consistent body C:P and N:P ratios when they were fed the low-quality diet, regardless of temperature. This result might be related to losses of C and N that were equivalent to those of P because of increased respiration rates (Anderson et al. 2004) or because of the excretion of protein-breakdown products under stressful conditions (high temperature and low-quality food) (Jeong and Cho 2007, Cogo et al. 2018). A hierarchy of nutritional priorities determines which nutritional requirements are satisfied, and the related costs (allostatic load) associated with the unbalanced diet could result in an abnormal or inefficient regulation of nutrients (Raubenheimer et al. 2012). Therefore, associating an organism’s homeostasis with maintaining a constant given elemental ratio probably oversimplifies organismal responses (Persson et al. 2010) because different factors can modify body stoichiometry (Fink and Von Elert 2006, Halvorson and Small 2016). In this regard, it seems more appropriate to frame our results within a broader perspective of homeostatic regulation (rheostasis [Mrosovsky 1990] or allostasis [McEwen 1998]) that considers changes of metabolic variables as a necessary part of stability maintenance for organisms facing environmental changes.

As a corollary, the low-quality diet narrowed temperature tolerance of *A. mortoni* by shifting optimum range and critical temperatures: high temperature was tolerated when high-quality food was supplied, and low-quality food was tolerated under cold conditions (low metabolic rate). Following the energy-based stress classification of Pörtner and Farrell (2008), the transition from the pejus range (moderate stress, i.e., 5°C + low-quality food) into the pessimum range (high stress, i.e., 10°C + low-quality food) was determined by the critical value of temperature under low-quality food. The transition towards the lethal range is typically marked by a clear negative energy balance, which corresponds with 15°C + low-quality food in our experiment. The future global scenario characterized by increased temperature beyond seasonal variations—records between 15 and 17°C are already frequent during summer in Sierra Nevada streams—together with poorer nutritional quality of leaf litter could lead to a marked population decline of an endemic and locally-abundant shredder species in headwater streams from the southern Iberian Peninsula. Nutrient recycling can be compromised if these effects are shared by most of the shredding guild in headwater streams, likely with implications at the population and ecosystem functioning levels.

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