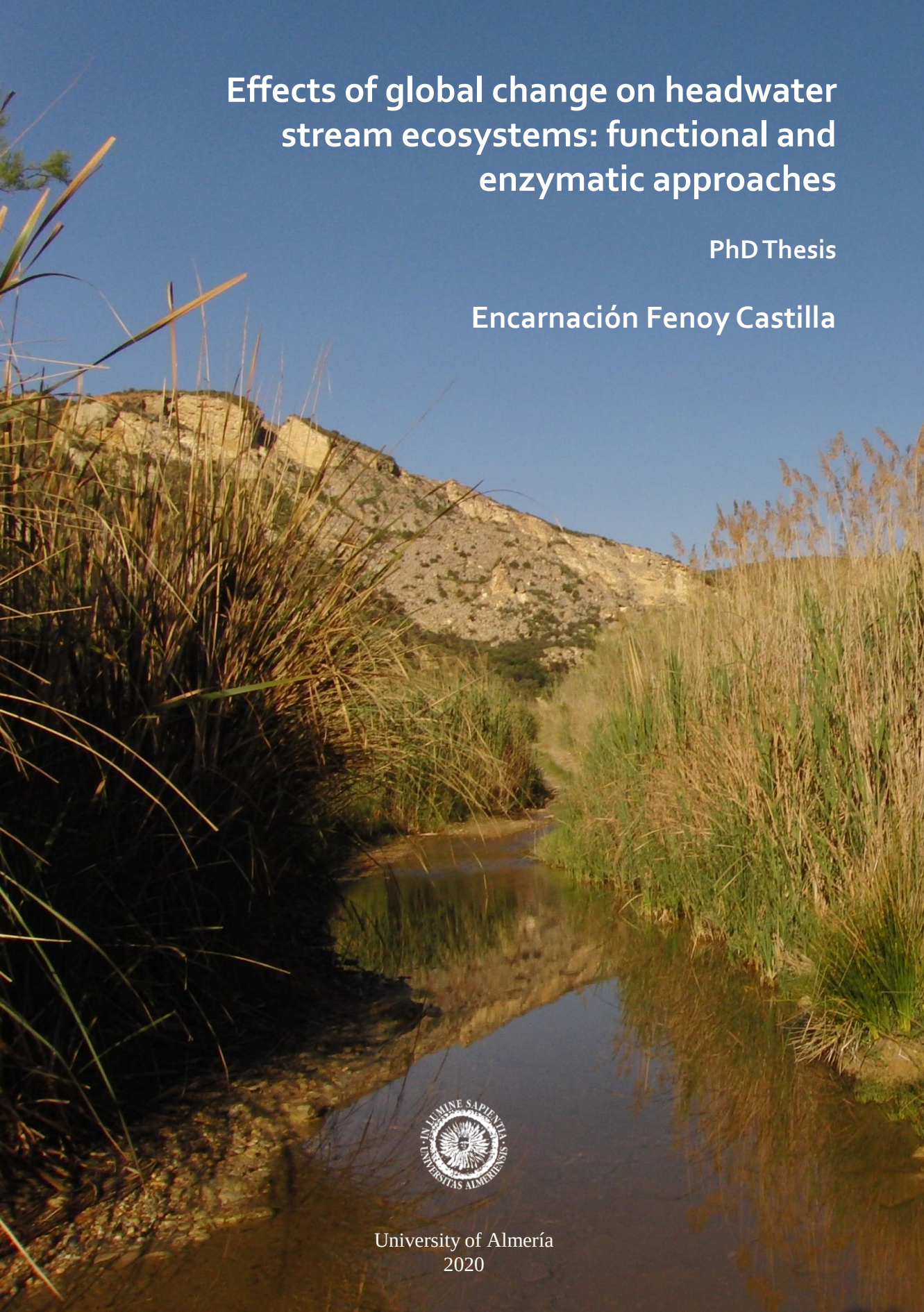
Effects of global change on headwater stream ecosystems: functional and enzymatic approaches

PhD Thesis

Encarnación Fenoy Castilla



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Encarnación Fenoy Castilla

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Effects of global change on headwater stream ecosystems: functional and enzymatic approaches

Efectos del cambio global sobre los ecosistemas fluviales de cabecera: aproximaciones funcional y enzimática

Encarnación Fenoy Castilla

Supervised by

Francisco J. Moyano López

J. Jesús Casas Jiménez

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A mi madre.

¡Y qué suerte si algún día logro parecerme a ti!

He who hears the rippling of rivers in these degenerate days will not utterly despair.

Henry David Thoreau

(A Week on the Concord and Merrimack Rivers)

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Summary

The concept of global change includes a variety of stressors (warming, species invasions and extinctions, changes in biogeochemical cycles, habitat degradation and loss, etc.) altering the whole of biodiversity and ecosystems functioning. Headwater streams, which are considered hotspots of biodiversity (especially those from Mediterranean regions), are particularly vulnerable to global change related stressors. These ecosystems are subsided by leaf-litter inputs from riparian vegetation, the major source of energy and nutrients into food webs. Thus, leaf-litter decomposition by aquatic fungi and macroinvertebrates is a key process within the nutrient recycling required for maintenance of such ecosystem.

The present dissertation aims to provide a better understanding on the potential alterations in stream ecosystem functioning due to the interaction of anthropogenic stressors. The main objective was to assess the effect of forecasted warming, increased dissolved nutrients and decreased leaf-litter quality on microbial diversity and on their decomposing activity, as well as on the performance of macroinvertebrates, both involved in leaf-litter decomposition. Additionally, microbial adaptations to decompose native leaf-litter, regardless of its quality (*home field advantage* hypothesis, HFA), were investigated to improve our knowledge on this scarcely addressed topic in aquatic systems. To achieve these goals, field experiments across subregions contrasting in abiotic factors, in conjunction with microcosms experiments, were performed to address this issue from a functional perspective.

Chapters 2 to 4 investigated the relative contribution of abiotic factors, leaf-litter traits and composition of decomposers community as primary drivers of leaf-litter decomposition. Chapter 2 shows that temperature has a key role on litter decomposition through its interaction with leaf-litter chemistry and that water pH affects the activity of some hydrolytic enzymes. Also, that although results suggest a potential HFA, this was not due to a particular enzyme profile.

Chapter 3 shows that leaf-litter quality is the major driver of decomposition since it modulates the size effect of abiotic factors and that the patterns and trends were analogous to those reported by studies carried out at larger scales. This field experiment also disclosed the existence of microbial adaptation to decompose more efficiently the recalcitrant but dominant litter species.

Results from Chapters 2 and 4 show the microbial ability to modulate functionality in relation to leaf-litter quality under different environmental conditions. Chapter 4 also evidenced that communities equally rich in species may present different

functional richness and diversity, highlighting the need of getting a better understanding on the functional structure of fungal communities.

The increased physiological rates of macroinvertebrates produced by the forecasted warming, combined to the increased recalcitrance of leaf-litter may reduce their ability to process leaf-litter unless they show adaptations to face it. However, little is known about potential intra-specific adaptations of shredders to process low-quality litters and conflicting results are obtained in experiments aimed to evaluate simultaneously the effects of both warming and depletion in food quality. To address these topics, two microcosms experiments were designed and detailed in Chapters 5 and 6. Results from Chapter 5 show that the response of shredders to leaf-litter quality is species-specific, with no particular intra-species adaptations of individuals from subregions where leaf-litter quality is already depleted.

The interaction of diet quality with temperature on growth, survival and strategies for homeostasis maintenance of shredders was studied in chapter 6, with results confirming the absence of compensatory feeding but reduced nutrient excretion. These results showed that the temperature tolerance was reduced when poor quality food is supplied, even though stoichiometric homeostasis and enzymatic rates related to limiting nutrient can be maintained with high efficiency. However, despite these compensatory mechanisms, high mortality was recorded when individuals faced high temperature and nutrient-poor diet simultaneously.

The overall findings from this Thesis underline the importance of decomposition as an indicator of the functional changes that may occur in headwater stream ecosystems affected by global change stressors. The loss of fungal functional diversity and the absence of metabolic adaptations in detritivores to face environmental and dietary alterations derived from global change might compromise nutrient recycling and ecosystem function in headwater streams ecosystems. This Thesis highlights the importance of exploring different functional aspects of such communities to assess their ability for adaptation and to maintain diversity.

Resumen

El cambio global incluye una variedad de factores de estrés (calentamiento, invasión y extinción de especies, cambio en los ciclos biogeoquímicos, degradación y pérdida de hábitats, etc.) que alteran la biodiversidad y el funcionamiento de los ecosistemas. Los ríos de cabecera, que son considerados puntos calientes de biodiversidad (especialmente aquellos de las regiones mediterráneas), son particularmente vulnerables al cambio global. Estos ecosistemas están subsidiados por las entradas de hojarasca desde la vegetación riparia, la mayor fuente de energía y nutrientes a las redes tróficas. Por lo tanto, la descomposición de la hojarasca por hongos acuáticos y macroinvertebrados es un proceso clave en el reciclado de nutrientes necesario para el mantenimiento de este ecosistema.

Esta tesis pretende proveer de una mayor comprensión de las alteraciones potenciales en el funcionamiento del ecosistema fluvial debido a la interacción de impactos antropogénicos. El principal objetivo fue evaluar el efecto del pronosticado calentamiento global, el incremento de los nutrientes disueltos y la disminución de la calidad de la hojarasca sobre la diversidad microbiana y su actividad descomponedora, así como en el desarrollo de los macroinvertebrados, ambos involucrados en la descomposición de la hojarasca. Además, se investigaron las adaptaciones microbianas a descomponer la hojarasca nativa, al margen de su calidad (la hipótesis del *home field advantage*, HFA), para mejorar nuestro conocimiento sobre este tema raramente abordado en ecosistemas acuáticos. Para lograr estos objetivos se llevaron a cabo experimentos de campo en subregiones que difirieron en los factores abióticos, en conjunción con experimentos de microcosmos, para abordar este tema desde una perspectiva funcional.

Los Capítulos 2 al 4 investigaron la contribución relativa de los factores abióticos, los rasgos foliares y la composición de la comunidad de microorganismos descomponedores como los principales impulsores de la descomposición de hojarasca. El Capítulo 2 muestra que la temperatura tiene un papel clave sobre la descomposición a través de su interacción con la composición química de la hojarasca y que el pH del agua afecta a la actividad de algunas enzimas hidrolíticas. También que, aunque los resultados sugieren una potencial HFA, esto no se debió a un perfil enzimático determinado.

El Capítulo 3 muestra que la calidad de la hojarasca es el mayor determinante de la descomposición, ya que ésta modula el tamaño del efecto de los factores abióticos y que los patrones y tendencias fueron análogas a las observadas en estudios llevados a cabo a escalas mayores. Este experimento de campo también reveló la existencia de adaptaciones

microbianas a descomponer más eficientemente las hojarascas de especies dominantes pero recalcitrantes.

Los resultados de los Capítulos 2 y 4 muestran la capacidad microbiana para modular la funcionalidad en relación con la calidad de la hojarasca bajo diferentes condiciones ambientales. El Capítulo 4 también evidenció que las comunidades con igual riqueza de especies pueden manifestar diferentes riquezas y diversidades funcionales, resaltando la necesidad de obtener una mejor comprensión de la estructura funcional de las comunidades fúngicas.

El incremento de las tasas fisiológicas de los macroinvertebrados producidas por el calentamiento, combinado con el incremento en la recalcitrancia de la hojarasca, podría reducir sus capacidades para procesar este recurso, a menos que éstos muestren adaptaciones para enfrentar este escenario. Sin embargo, poco se sabe sobre las potenciales adaptaciones intra-específicas de los fragmentadores para procesar la hojarasca de baja calidad, y de los experimentos que pretenden evaluar simultáneamente los efectos de las disminución de calidad de la hojarasca y el calentamiento se han obtenido resultados contradictorios. Para abordar estos temas, se diseñaron dos experimentos de microcosmos que se detallan en los Capítulos 5 y 6. Los resultados del Capítulo 5 muestran que la respuesta de los fragmentadores a la calidad de la hojarasca es especie-específica, sin adaptaciones intra-específicas particulares de los individuos provenientes de las subregiones donde la calidad de la hojarasca ya es menor.

La interacción de la calidad de la dieta con la temperatura sobre el crecimiento, la supervivencia y las estrategias para el mantenimiento de la homeostasis de los fragmentadores se estudió en el Capítulo 6, con resultados que confirmaron la ausencia de alimentación compensatoria, pero una reducida excreción de nutrientes. Estos resultados indicaron que la tolerancia a la temperatura se redujo cuando se ofreció alimento de baja calidad, aún incluso cuando pudo mantenerse con alta eficiencia la homeostasis estequiométrica y las tasas enzimáticas relacionadas con el nutriente limitante. Sin embargo, a pesar de estos mecanismos compensatorios, se registró una alta mortalidad con los individuos se enfrentaron simultáneamente a una alta temperatura y una dieta pobre en nutrientes.

Los resultados generales de esta tesis subrayan la importancia de la descomposición como un indicador de los cambios funcionales que pueden ocurrir en los ecosistemas fluviales afectados por el cambio global. La pérdida de diversidad fúngica funcional y la ausencia de adaptaciones metabólicas en los detritívoros para afrontar alteraciones ambientales y dietéticos derivados del cambio global podrían comprometer el reciclado de nutrientes y el funcionamiento de estos ecosistemas fluviales. Esta tesis resalta la importancia de explorar aspectos funcionales diferentes de estas comunidades para evaluar su capacidad de adaptación y de mantenimiento de la diversidad.

Chapter 1

General Introduction

Global change encompasses a variety of environmental alterations, including [CO_2] and temperature increments, changes in biogeochemical cycles, and habitat loss and degradation (Steffen et al. 2015). These alterations can trigger shifts in community composition and species distribution, species declines and ultimately extinctions (Pereira et al. 2010). Biodiversity loss, both at the interspecific and the intraspecific levels, may have strong effects on ecosystem functionality (Palkovacs et al. 2015, Raffard et al. 2019).

Freshwater ecosystems host around 9.5% of total biodiversity (Balian et al. 2008), despite their small surface at the global scale and the varied threats that impact them (Reid et al. 2019). Among them, headwater streams greatly contribute to whole-fluvial ecosystem biodiversity owing to their greater length (> 75% of total stream length in most basins) and, particularly, to their high habitat diversity which leads to high β-diversity (Finn et al. 2011). In this respect, streams in particular (e.g., Bonada et al. 2007, Tierno de Figueroa et al. 2013), and the Mediterranean region in general (Blondel et al. 2010), are considered global biodiversity hotspots.

Headwater streams are usually relatively cold water and light-limited ecosystems, in which allochthonous organic matter from the riparian plant community, i.e., leaf litter, constitutes the major basal source of energy and nutrients (Vannote et al. 1980, Wallace et al. 2015), being a pivotal component of food webs (Vannote et al. 1980, Moore et al. 2004). In these ecosystems aquatic hyphomycetes and shredding macroinvertebrates are the main agents of leaf-litter decomposition (Gessner and Chauvet 1994, Graça 2001, Pascoal and Cássio 2004, Wallace and Eggert 2009). This process remarkably contributes to CO_2 emissions, considering the small surface area of inland waters (Battin et al. 2009). Recent estimates indicate that about 80% (1.8 Pg C yr^{-1}) of the total C evaded from inland waters (Battin et al. 2009, Raymond et al. 2013) comes from rivers and streams (Raymond et al. 2013), although this figure has been lowered by a more recent estimate (Lauerwald et al. 2015). Furthermore, conservative estimates suggest that globally 36% of total CO_2 outgassing from rivers and streams originates from headwaters (Marx et al. 2017). However, this last estimate appears to have underestimated CO_2 evolved from biotic processing of leaf-litter in streams.

The increase of atmospheric [CO_2] and consequent global warming have the potential to alter the process of litter decomposition in streams, through inducing intra- and/or inter-specific changes in leaf-tissue chemistry of incoming plant detritus (Tuchman et al. 2002, Rier et al. 2005). This effect is likely to be more noticeable in subtropical and mid-latitude regions, where a marked aridity increase is predicted (Giorgi and Lionello 2008). Studies at global (Yuan and Chen 2009, Boyero et al. 2017) and regional (Chen et al. 2013, Salinas et al. 2018) scales indicate that warming could significantly reduce leaf nitrogen and/or phosphorus content. Shifts in other leaf traits—increasing toughness, and silicon or waxes content—may also occur due to changes in plant community composition, particularly if entail shifts in plant functional types or invasion of exotic species (Gritti et al. 2006, Salinas et al. 2018). In this context, Mediterranean ecosystems have been highlighted as highly vulnerable to the effects of

global change (Gritti et al. 2006, Filipe et al. 2013). In parallel, the Mediterranean Basin, due to its latitudinal location and mountainous relief, offers high environmental heterogeneity, particularly in certain regions such as Southern Spain (Andalusia) where a marked spatial gradient of aridity appears at present (Figure 1.1a) (Casas et al. 2006, 2011, Salinas et al. 2018). This makes this region quite suitable to infer past or future trajectories of ecosystems in warming scenarios from contemporary spatial patterns (space-for-time substitution sensu Pickett 1989).

Using the above-mentioned approach, a recent research regarding riparian communities in Mediterranean headwater streams, points that warming combined with increasing aridity are detrimental factors for deciduous plants, whereas favour the expansion of giant graminoids and evergreen (Salinas et al. 2018). This would imply that the forecasted warming and aridification of the Mediterranean region would trigger increasing recalcitrance of leaf litter inputs to headwater streams. This might limit the ability of decomposers to decompose more recalcitrant litter due, in accordance to the home-field advantage hypothesis (Gholz et al. 2000, Vivanco and Austin 2008), to miss-adaptations in the absence of previous co-evolution (Cross et al. 2013). Only a few studies have focused recently on this topic in aquatic environments.

In addition, extrinsic factors could interact with litter traits modifying microbial colonization and activity, and subsequently decomposition rates. The chemical composition of water (e.g. Suberkropp and Chauvet 1995), increasing concentration dissolved nutrients (Woodward et al. 2012, Biasi et al. 2017, Bastias et al. 2018), and temperature rise (Boyero et al. 2011, Fernandes et al. 2012, Ferreira et al. 2014), can increase microbial decomposition (e.g. Ferreira and Chauvet 2011), modify community structure (e.g. Jabiol et al. 2018) and potential species distribution (e.g. Duarte et al. 2013). Thus, it is critical to improve our knowledge on the relative importance of these factor, and how extrinsic and intrinsic (leaf-litter traits) factors interact affecting microbial decomposers and hence the flow of nutrients in headwater food webs (Canhoto et al. 2016).

The above mentioned factors can concomitantly affect macroinvertebrate shredder performance, since microbial conditioning of leaf-litter has the potential to reduce the large resource-consumer elemental imbalance that shredders face (Cross et al. 2005). Several strategies can be used by shredders to maintain elemental homeostasis when feeding on nutrient-poor resources: preferential selection of nutrient-rich resources, increased consumption of the nutrient-poor resource to compensate deficiencies, and/or mechanisms of post-ingestive regulation related with assimilation, egestion and excretion (Sterner and Elser 2002, Frost et al. 2005). Although these possible adaptations of shredders and the nutritional improvement of litter by microbial decomposers might enhance shredders performance, most studies generally conclude that increasing recalcitrance of leaf-litter cause detrimental effects on shredders (Graça 2001, Graça et al. 2015). However, the possible existence of intra-species adaptations in meta-populations of shredders inhabiting highly heterogeneous regions (varying

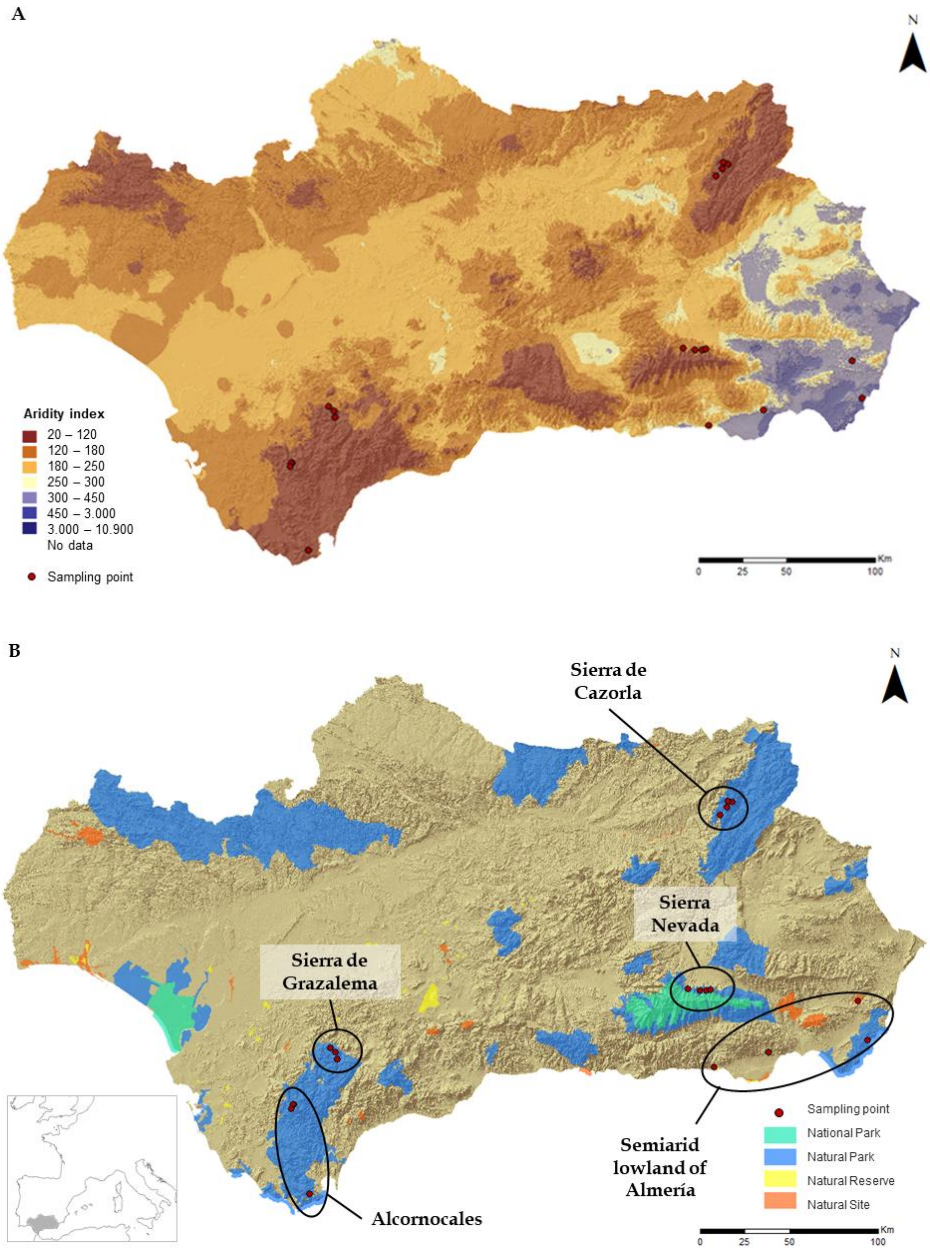

Figure 1.1. A) Bioclimatic classification of Andalusia based on aridity (aridity index: reference evapotranspiration/precipitation. Observed data from the period 1961-2000). Source: www.juntadeandalucia.es/medioambiente/site/rediam. B) Distribution low-order streams (sampling point) in selected sub-regions across Andalusia, most of them under some protection status and covering a wide environmental gradient.

in recalcitrance of leaf-litter inputs) to exploit more efficiently recalcitrant leaf-litter resources has been poorly studied.

Warming poses an additional drawback for shredders dealing with increasing leaf-litter recalcitrance, but studies on this respect show conflicting results. Rising temperature increases metabolic rates of ectotherms (Brown et al. 2004), and may also trigger other rates increment, such as consumption, but a mismatch between temperature scaling of metabolic and consumption rates has been reported (Lemoine and Burkepile 2012), probably due to a finite handling time (Suzuki-Ohno et al. 2012). This imply that, in order to survive under those altered conditions, shredders must have the ability to assimilate the limiting nutrients more efficiently, or to reduce their excretion. Previous studies have assessed strategies for homeostasis maintenance of different species of shredders by compensatory feeding and/or variations in excretion and egestion rates of key nutrients (Balseiro and Albariño 2006, Flores et al. 2014, Halvorson et al. 2018). Increases in water temperature within the range forecasted for headwater streams (2-4ºC) may deplete enzyme-substrate affinity, having pronounced effects on the enzymatic reactions (e.g. Hochachka and Somero, 1984). Thus, a high catalytic efficiency should be maintained under changing environmental conditions to ensure an efficient digestion (Hochachka and Somero 1984, Somero 1997). However, possible adaptations linked to the functionality of enzymes involved in nutrient digestion and metabolism are almost unknown in macroinvertebrate shredders.

Several Mediterranean sub-regions in southern Spain (Figure 1.1b), widely varying in bioclimatic conditions, were selected to study the direction and magnitude of the abovementioned changes on microbial decomposers and shredders involved in leaf-litter decomposition, a key process in the functioning of headwater ecosystems. Thus, the aim of the present thesis was to improve our current knowledge on how drivers of global change may affect leaf-litter processing in headwater streams, eventually altering ecosystem functioning. Different field and laboratory experiments were designed to assess the effects of increased temperature, changes in composition of riparian plant communities and adaptations of decomposers and shredders on leaf-litter processing and hence on nutrient inputs. The thesis was structured in five chapters with the following specific aims:

1. To evaluate the relative importance of abiotic factors (water temperature, pH and ionic content), interacting with a microbial community putatively adapted to exploit a basal resource of a given chemical composition, on litter decomposition and cellulolytic activity. (Field and laboratory experiment).

2. To assess how biotic and abiotic factors, and their interactions, affect the decomposition process of four leaf-litter species varying in recalcitrance, to represent potential alterations of incoming resources to headwater streams in the climate change context, and potential adaptations of decomposers to native litters. (Field experiment).

3. To examine the structural and functional response of decomposer communities (aquatic hyphomycetes) to the abiotic environment and litter quality. We explored the often-assumed functional redundancy of hyphomycetes. (Field and laboratory experiment).

4. To evaluate the intra-specific response of shredders from regions with contrasting recalcitrance of leaf-litter input to inter and intra-species differences in litter quality. We assessed the possible existence of local adaptations of shredders from relatively warm or warm/arid regions to cope with nutrient-depleted leaf-litter. (Laboratory experiment).

5. To assess the ability of an insect shredder to face simultaneously warming and decreasing food quality, through the commonly studied pre- and post-absorptive strategies, including the study of kinetic parameters of key digestive enzymes involved in nutrient processing (Laboratory experiment).

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Chapter 2

Temperature and substrate chemistry as major drivers of interregional variability of leaf microbial decomposition and cellulolytic activity in headwater streams

Encarnación Fenoy

J. Jesús Casas

Manuel Díaz-López

Juan Rubio-Ríos

José Luis Guil-Guerrero

Francisco J. Moyano-López

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2.1 Introduction

Organic matter decomposition is a key component of the global carbon cycle that is being altered by human activity (i.e. global warming and land-use changes) (Chapin et al. 2009). Low-order streams are ecosystems fundamentally subsidized by organic detritus (i.e. leaves) from the surrounding terrestrial habitats, notably in forested regions (e.g. Wallace et al. 2015). Thus, natural or human-induced changes affecting leaf litter decomposition have the potential to alter stream food webs (Graça et al. 2015). In terrestrial ecosystems, climate, substrate chemistry and decomposers community composition are the fundamental drivers of leaf litter decomposition (Gholz et al. 2000, Keiser et al. 2011). Temperature, moisture and substrate chemistry might together explain about 70% of variation of leaf litter decomposition in forest soils (Gholz et al. 2000, Parton et al. 2007), but factors contributing to the remaining variance and their relative contribution are yet uncertain (e.g. Freschet et al. 2012). Among these factors, the specialisation of decomposer communities on the litter type characteristic of its native ecosystem, commonly referred to as the 'home-field advantage' hypothesis (HFA; Hunt et al. 1988, Gholz et al. 2000), might contribute significantly to decomposition. For instance, in forest soils litter mass loss can be 4.2 % faster on average at the home environment (Wang et al. 2013), notably in undisturbed forest ecosystems (Austin et al. 2014).

In low-order streams, microbial decomposition of leaf litter is largely driven by temperature, through increased microbial biomass and/or enzymatic activity at elevated temperatures (Friberg et al. 2009, Boyero et al. 2011, Ferreira and Chauvet, 2011, Ferreira and Canhoto 2015, Ferreira et al. 2015). A global experiment in low-order streams (Boyero et al. 2011), reported that temperature explained up to 40% of the variation of microbial decomposition, but the authors concluded that factors other than temperature likely influenced litter breakdown rates, accounting for the considerable residual variability detected. This is to be expected given that low-order streams capture, as a whole, much of the catchment's environmental heterogeneity due to their intimate connection with the surrounding landscape (Lowe and Likens 2005). Such remarkable variability in many abiotic (i.e. water chemistry) and biotic (i.e. riparian and aquatic communities) factors, operating at regional and local scales, may significantly affect leaf litter decomposition in streams (Graça et al. 2015), interacting synergistically or antagonistically with temperature, potentially masking its effect on decomposition. This is particularly true for streams in the Mediterranean basin, where landscape heterogeneity is elevated at multiple scales (e.g. Casas et al. 2006, 2011), besides the consideration of this region as a global "hot-spot" in terms of high rates of climate change and land transformation (see reviews in García-Ruíz et al. 2011, Cooper et al. 2013).

The influence of direct effects of water chemistry on litter decomposition in streams has been widely tested: i.e. the concentration of dissolved nutrients (e.g Woodward et al. 2012), ionic concentration (e.g. Suberkropp and Chauvet 1995) and pH (e.g. Dangles et al. 2004). However, indirect effects of abiotic factors are less profusely studied in these ecosystems. Water chemistry and other abiotic factors may also exert indirect, historical, effects on decomposition by acting as ecological filters for community composition (e.g. Casas et al. 2011) and functional traits of species, likely affecting both the chemistry of terrestrial/riparian leaf-litter inputs (substratum) and decomposers capabilities.

As in terrestrial ecosystems (Aerts 1997, Makkonen et al. 2012), leaf litter chemistry is also a major determinant of decomposition rates in streams (Lecerf and Chauvet 2008, Schindler and Gessner 2009), depending to a great extent on its concentration in structural carbohydrates. Cellulose represents almost half of primary production and its decomposition is a key process in the C cycle (Sinsabaugh et al. 1991). Thus, the evaluation of its hydrolysis into glucose is considered a good tool to test possible functional differences in microbial assemblages (Gessner et al. 2007, Schneider et al. 2012), since they are much affected both by the aforementioned abiotic factors (Suberkropp and Chauvet 1995, Gulis and Suberkropp 2003) as well as by the chemistry of leaf litter inputs (Woodward 2009, Bärlocher et al. 2013). However, as temperature sensitivity of decomposition appears to increase with increasing molecular complexity of the substrate ("temperature–quality" hypothesis sensu Bosatta and Ågren (1999), Davidson and Jansen (2006)), determining how much decomposition rates respond to the interaction between substrate recalcitrance and temperature, in interplay with other abiotic and biotic factors, is, therefore, a critical issue to understand ecosystem responses to global changes (Conant et al. 2011, Gonçalves et al. 2013).

Regarding microbial communities, most studies, both in soil and streams, have concentrate on the effects of their diversity on decomposition (see reviews in Hättenschwiler et al. 2011a, Graça et al. 2015), but with controversial findings. A different approach to consider in this issue is the fact that microbial communities may adapt to exploit the prevalent litter inputs in the physic-chemical setting of their ecosystem, which will accelerate litter decomposition at home ('home-field advantage' [HFA]; Hunt et al. 1988, Gholz et al. 2000, Ayres et al. 2009, Strickland et al. 2009, Wang et al. 2013, but see Makkonen et al. 2012). If true, taxonomically and functionally dissimilar microbial communities are expected in ecosystems widely differing in physic-chemical features and prevalent chemical composition of litter inputs, which could lead to 'home-field advantage' effects, theoretically determining differential responses of decomposition to abiotic factors and substrate chemistry. However, while in some terrestrial ecosystems significant HFA effects on litter decomposition have been demonstrated (see above), to our knowledge this hypothesis has not been explicitly tested in stream ecosystems.

The present work was designed to evaluate the relative importance of abiotic factors (temperature, pH and ionic content), interacting with a microbial community putatively adapted to exploit a basal resource of a given chemical composition, on litter decomposition and cellulolytic activity. For this purpose, we performed a reciprocal litter transplant in a combined ‘‘ecosystem type’’ and ‘‘litter chemistry’’ experiment. The enzyme selected as indicator of functional adaptations (resource/environment) of the microbiota was β-glucosidase, since it plays a pivotal role in the final step of cellulose degradation (e.g. Seidle and Huber 2005) and has been widely used in ecological studies (Kourtev et al. 2002, Sinsabaugh and Shah 2011, Artigas et al. 2011, Ylla et al. 2012). In addition, the potential functional diversity of endoglucanases in the cellulase complex present in the leaf-degrading microbiota was evaluated using zymograms.

We hypothesized that decomposition rate and cellulolytic activity for each leaf species would be higher in its native ecosystem due to historical resource and abiotic environmental conditions to which microbial community has adapted. Differences between regions are expected to be more pronounced on the more recalcitrant leaf substrate (*Phragmites*) due to its putative higher intrinsic temperature sensitivity.

2.2 Material and methods

2.2.1. Study sites and experimental design

Six permanent streams from two adjacent regions —3 streams per region— with sharp biogeoclimatic contrast were selected in southern Spain. Streams from the Sierra Nevada mountains (hereafter mountain streams) are located at elevations ranging between 1300-1400 m asl, drain forested watersheds (i.e. pine afforestation) with siliceous lithology and have a flow regime influenced by snowmelt. Their riparian vegetation is dominated by alder (*Alnus glutinosa* (L.) Gaertner) and willow (*Salix* spp.). Streams of the semiarid lowland of Almería (hereafter lowland streams) are located at elevations ranging between 50-300 m asl and drain watersheds dominated by scrublands over calcareous-gypsum soils. The riparian vegetation of lowland streams is dominated by common reed (*Phragmites australis* (Cav.) Trin. ex Steud.) and oleander (*Nerium oleander* L.). Additional environmental information on these regions is given in Casas et al. (2011). We performed a reciprocal litter transplant in a combined ‘‘ecosystem type’’ and ‘‘litter chemistry’’ experiment, by using two species of leaf litter—*Alnus glutinosa* (hereafter *Alnus*) and *Phragmites australis* (hereafter *Phragmites*)—as representatives of mountain and lowland streams, respectively. A key trait differentiating litter chemistry of both species is that polymerized silicic acid strongly binds with cellulose in reed leaf litter (Ma and Yamaji 2006), which might confer higher recalcitrance and, thus, temperature sensitivity of decomposition to this substrate.

2.2.2. Environmental characterisation of streams

During the course of the field experiment we took triplicate measurements (2, 5 and 11 weeks) of electrical conductivity (EC), pH and dissolved oxygen in each stream with a multi-parametric probe (HACH® model HQ-30d, USA). With the same periodicity, stream discharge was measured as described in Casas et al. (2011), and we collected water samples: an aliquot of which was used to measure alkalinity by acid titration to an end point of pH 4.5, and the rest of the water sample was filtered through glass fibre filters (APFC, Millipore®) and frozen (-20 ºC) until analysed to determine, by standard methods (APHA 2005), the concentration of the following chemical parameters: Cl^-, argentometric titration method; SO_4^{2-}, turbidimetric method; NO_3^- -N, ion chromatography; soluble reactive phosphorus (SRP), colorimetric determinations involving the formation of the phosphomolybdic acid blue complex. Water temperature was recorded hourly with HOBO Pendant (Onset Computer Corporation, Bourne, MA, USA) loggers during the full incubation period.

2.2.3. Initial leaf litter characteristics

Senescent leaves were collected just after abscission using litterfall traps in the case of *Alnus* and directly from standing-dead shoots in *Phragmites.* The leaves were collected from the riparian zone of a single stream per region, transported to the laboratory and dried at room temperature for 2 weeks. The toughness of 25 senescent leaves per plant species was measured on moistened material using a calibrated texturometer (TA.XT2 Plus, Stable Micro Systems, London, UK). A constant needle tip surface area (0.38 mm^2) was used for all measurements, thus measures of toughness were expressed in units of mass (g).

Thereafter, leaf litter was oven-dried (70 ºC, 72 h) and grounded to less than 1 mm particle size to be used for triplicate chemical analyses. Nitrogen (N) and C were determined using a Perkin Elmer series II CHNS/O elemental analyser, with results expressed as % N and % C of leaf dry mass. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined using an ANKOM 200/220 fibre analyzer (ANKOM Technologies, Macedon, NY, USA). Percentages of hemicellulose, cellulose and lignin were calculated using the following equations: hemicellulose = (% NDF - % ADF) - % ash; cellulose = (% ADF - % ADL) - % ash; and lignin = % ADL - % ash. Ash concentration (% ash) was determined by incineration at 550 ºC for 5 h. The concentration of silica (Si) in leaf litter was determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES, Thermo ICAP 6500 duo, Thermo Fisher), after microwave sample digestion in nitric acid (65%) and hydrogen peroxide (30%).

2.2.4. Leaf litter bags and decomposition

Portions (5.0 ± 0.2 g dry mass) of leaf litter were spray moistened and introduced in bags (15 × 15 cm) of 1-mm mesh size, which allowed water circulation inside the bag but avoided the action of macroinvertebrates. At the end of December 2012, leaf bags —20 bags per species and stream—were incubated tied to iron stakes anchored to the stream bed in riffles along a 50-m stream reach. Four bags per stream and species were retrieved after 48 h—to estimate leaching loss—and after 2, 5 and 11 weeks. Leaf bags were transported to the laboratory in an icebox kept at 4 °C. The leaves were carefully removed from the bags, rinsed with filtered stream water to eliminate fine particles, oven-dried (70 ºC, 72 h) and weighed to determine % remaining dry mass to calculate decomposition rates. The four remaining leaf bags per species and stream were retrieved after 5 weeks of incubation, processed as above but, instead of being oven-dried, the material of the four bags was pooled, ground with a blender and used to measure ergosterol and prepare enzyme extracts (see below). Five weeks of incubation was considered enough for maximum colonization and partial degradation of the plant material by fungi in these streams (Casas et al. 2011).

2.2.5. Fungal biomass

Ergosterol was determined as a measure of fungal biomass in samples. Lipids were extracted from litter with alkaline methanol, the crude extract cleaned and concentrated by solid-phase extraction, and ergosterol finally purified and quantified by high-performance liquid chromatography (Gessner and Schmitt 1996). Ergosterol was detected at 282 nm and quantified against ergosterol standards (Fluka, Switzerland). The lipid concentration was converted into fungal biomass using a factor of 5.5 μg ergosterol mg^{-1} fungal dry mass (Gessner and Chauvet 1993) and the results were expressed as mg fungal biomass g^{-1} leaf dry mass.

2.2.6. Determination of activity and functional parameters of β-glucosidase

After incubation for 5 weeks, samples were grounded and homogenised in distilled water (1:3 w/v) with a blender. The extracts were centrifuged (12,000x g; 15 min; 4 °C) and the supernatant obtained was used as crude enzyme extract. The dry mass of samples was estimated by differential gravimetry of 1 g of each plant material after oven drying (70 °C, 72 h; n = 3). The activity of β-glucosidase was evaluated by determining the hydrolysis of p-nitrophenol-β-D-glucopyranoside (pNPG) 5 mM with readings at 405 nm. All assays were performed in triplicate.

We evaluated the effect of the main factors of divergence between streams from both regions—temperature, pH and ionic strength—on β-glucosidase activity. The effect of temperature was evaluated incubating extracts at temperatures ranging between 5 to

70°C. The enzyme efficiency under natural thermal conditions was calculated as the activity —measured at the average temperature registered in the stream of origin — expressed as a % respect to the maximum activity measured at optimal conditions. Values of activity were used to calculate enzyme activation energy (E_a) and temperature coefficients (Q_{10}). The E_a was calculated from the Arrhenius plots constructed with values of activity measured within the above mentioned temperature range. Temperature coefficients (Q_{10}), for the approximate temperature range (5-15 ºC) of divergence between the two regions, were obtained using the equation:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)}$$

where R_2 and R_1 are reaction rates observed at temperatures T_2 and T_1, respectively.

The effect of pH was evaluated within the range 4-9, using the following buffers: citrate (4 and 5), phosphate (6) and Tris-HCl (7, 8 and 9), 50 mM in all cases. Also, we measured enzyme activity at the average pH of the stream, which was expressed as a percentage of the maximum activity measured at optimal pH in each case. We evaluated the stability under different pH values by pre-incubating extracts in the above-mentioned buffers for different time periods (1, 2 and 4 h) and measuring residual activity in relation to the maximum.

We also calculated an adaptation of Q_{10} temperature coefficient to estimate the change in activity as pH increased one unit, for the pH range (7-8) in which both regions differ, approximately. For this purpose, we used reaction rates obtained from the pH stability test. The formula of "Q_1" pH coefficient would be as follows:

$$Q_1 = \left(\frac{R_2}{R_1}\right)^{\left(\frac{1}{pH_2 - pH_1}\right)}$$

where R_2 and R_1 are reaction rates observed at pH_2 and pH_1, respectively.

The effect of ionic strength (IS) on the activity of β-glucosidase was evaluated on extracts obtained from both plant species sampled incubated in the stream with higher EC (Río de Aguas, lowland). We used buffers with the same pH and total molar concentration, but differing in IS:

pH 6.0: IS 66 mM and 252 mM (citrate and phosphate, respectively)
pH 7.0: IS 46 mM and 108 mM (Tris-HCl and phosphate, respectively)
pH 8.0: IS 28 mM and 143 mM (Tris-HCl and phosphate, respectively)

Table 2.1 Environmental variables (mean ± SE; n = 3) measured in each stream, and the average for each region. Different superscript letters indicate significant ($P < 0.05$) differences among streams determined by post-hoc Tukey HSD test, following mixed-model nested ANOVA. Also shown are F values for the overall comparison between regions and among streams.

Location or comparison	O_2 (mg L^{-1})	O_2 (%)	Temperature (°C)	pH	EC (µS cm^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Cl^- (mg L^{-1})	SO_4^{2-} (mg L^{-1})	NO_3^--N (mg L^{-1})	SRP (µg L^{-1})	Discharge (L s^{-1})
Sierra Nevada											
Aldeire	10.8 ± 0.2^{a}	95.1 ± 0.7^{bc}	3.8 ± 1.0^{b}	6.8 ± 0.1^{d}	41 ± 1^{c}	18 ± 2^{c}	12 ± 2^{c}	$4 \pm <1^{c}$	0.38 ± 0.03^{b}	5 ± 2	48 ± 1^{a}
Jérez	10.7 ± 0.4^{ab}	93.9 ± 1.1^{bc}	3.8 ± 1.3^{b}	6.7 ± 0.1^{d}	48 ± 4^{c}	21 ± 1^{c}	10 ± 2^{c}	6 ± 1^{c}	0.17 ± 0.04^{c}	3 ± 2	52 ± 1^{a}
Lanteira	11.2 ± 0.3^{a}	94.9 ± 0.6^{bc}	3.1 ± 1.1^{b}	6.9 ± 0.1^{d}	42 ± 2^{c}	17 ± 1^{c}	16 ± 2^{c}	5 ± 1^{c}	0.44 ± 0.05^{b}	3 ± 1	43 ± 1^{a}
Region average	**11.0 ± 0.2**	**95.0 ± 0.4**	**3.5 ± 0.7**	**6.8 ± 0.1**	**43 ± 1**	**19 ± 1**	**13 ± 1**	**5 ± <1**	**0.33 ± 0.02**	**4 ± 1**	**48 ± 1**
Semiarid Lowland											
Aguas	9.5 ± 0.6^{b}	97.6 ± 5.3^{b}	$15.5 \pm <0.1^{a}$	8.1 ± 0.1^{c}	3150 ± 17^{a}	159 ± 7^{b}	240 ± 26^{a}	1629 ± 52^{a}	0.98 ± 0.08^{a}	4 ± 2	39 ± 1^{a}
Negras	$11.3 \pm 0.^{a}$	107.2 ± 0.8^{a}	13.1 ± 0.4^{a}	$8.5 \pm <0.1^{ab}$	2645 ± 9^{a}	263 ± 10^{a}	177 ± 33^{ab}	416 ± 54^{b}	0.14 ± 0.04^{c}	$6 \pm <1$	3 ± 1^{c}
Vícar	8.7 ± 0.1^{cb}	87.5 ± 0.7^{c}	$13.9 \pm <0.1^{a}$	$8.3 \pm <0.1^{bc}$	996 ± 10^{b}	253 ± 14^{a}	121 ± 7^{b}	196 ± 12^{b}	0.36 ± 0.01^{b}	4 ± 1	9 ± 1^{b}
Region average	**9.1 ± 0.12**	**92.6 ± 1.3**	**14.2 ± 0.1**	**8.3 ± 0.0**	**2073 ± 7**	**225 ± 6**	**172 ± 24**	**747 ± 223**	**0.73 ± 0.03**	**4 ± 1**	**18 ± 5**
Comparison											
Region	2.1^{ns}	0.6^{ns}	62.6***	90.1***	109.7***	185.1***	74.6***	50.5**	0.5^{ns}	1.7^{ns}	6.1^{ns}
Stream	11.9***	11.9***	14.5***	6.9**	198.0***	19.9***	5.5**	34.2***	127.0***	0.5^{ns}	95.3***

***$P < 0.001$; **$P < 0.01$; ns. not significant ($P > 0.05$).

Table 2.2 Variables (mean ± SE; n = 3) used to characterise litter quality (on leached material) of the two species assayed. Also shown are *t*-values and significance level of the comparisons.

Species or comparison	Toughness (g)	% C	% N	% Hemicellulose	% Cellulose	% Lignin	% Ash	% Si
Alnus glutinosa	69.8 ± 1.7	48.45 ± 0.40	1.64 ± 0.06	18.2 ± 1.9	10.9 ± 0.4	11.5 ± 2.4	6.30 ± 0.01	0.03 ± <0.01
Phragmites australis	238.1 ± 23.3	39.75 ± 0.87	1.50 ± 0.03	22.5 ± 1.7	27.6 ± 1.7	3.4 ± 0.9	12.45 ± 0.13	2.21 ± 0.06
Comparison	-13.7***	9.0***	2.0 ns	-6.4**	-12.6***	6.3**	-53.46***	-23.87**

***$P < 0.001$; **$P < 0.01$; ns not significant ($P > 0.05$).

Table 2.3 β-glucosidase activity (mean ± SE, n=3 measures per stream and species) as a function of temperature in decomposing litter of *Alnus* and *Phragmites* incubated for 5 weeks in streams from Sierra Nevada and the semiarid lowland of south-eastern Spain. All measurements were carried out at pH 7. Also shown are *F* values and significance level, following two-way ANOVA, to test the effect of region, species and the interaction region x species.

Factor or comparison	Max. Activity (μmol g^{-1} DM h^{-1})	Temperature of max. activity (ºC)	Activity at temperature of the stream (μmol g^{-1} DM h^{-1})	Efficiency (%)
Alnus glutinosa				
Sierra Nevada	**79.7 ± 14.5**	**60.0 ±10.0**	**4.4 ± 1.2**	**6.1 ± 1.9**
Aldeire	51.5 ± 1.3	40.0 ± <0.1	4.5 ± 0.1	8.7 ± 0.1
Jérez	87.8 ± 8.2	70.0 ± <0.1	6.5 ± 1.1	7.4 ± 1.2
Lanteira	99.9 ± <0.1	70.0 ± <0.1	2.3 ± 0.1	2.3 ± 0.1
Semiarid Lowland	**152.1 ±69.4**	**50.0 ± 5.8**	**100.0 ± 86.0**	**42.8 ± 25.6**
Aguas	92.8 ± <0.1	50.0 ± <0.1	13.5 ± 0.1	14.5 ± 0.2
Negras	290.3 ± 9.9	40.0 ± <0.1	272.1 ± 1.2	93.9 ± 3.5
Vícar	73.1 ± <0.1	60.0 ± <0.1	14.5 ± 0.2	19.9 ± 0.3
Phragmites australis				
Sierra Nevada	**92.1 ±49.4**	**43.3 ± 3.3**	**8.4 ± 2.3**	**11.9 ± 2.6**
Aldeire	49.1 ± 0.2	40.0 ± <0.1	6.7 ± 0.1	13.6 ± 0.1
Jérez	190.7 ± 6.2	50.0 ± <0.1	12.9 ± 0.1	6.8 ± 0.2
Lanteira	36.5 ± 0.25	40.0 ± <0.1	5.6 ± 0.1	15.3 ± 0.5
Semiarid Lowland	**63.0 ± 16.5**	**43.3 ± 3.3**	**18.0 ± 8.5**	**27.2 ± 7.1**
Aguas	64.5 ± <0.1	50.0 ± <0.1	9.1 ± 0.1	14.1 ± 0.1
Negras	90.8 ± 1.1	40.0 ± <0.1	34.9 ± 0.2	38.4 ± 0.3
Vícar	33.8 ± 0.9	40.0 ± <0.1	9.9 ± 0.4	29.2 ± 1.9
Comparison				
Region	0.2 ^{ns}	0.5 ^{ns}	5.3*	5.6*
Species	1.0 ^{ns}	3.2 ^{ns}	0.7 ^{ns}	0.1 ^{ns}
Region x Species	1.0 ^{ns}	0.5 ^{ns}	2.2 ^{ns}	0.8 ^{ns}

*$P < 0.05$; ns not significant ($P > 0.05$).

Table 2.4 β-glucosidase activity (mean ± SE, n=3 measures per stream and species) as a function of pH in decomposing litter of *Alnus* and *Phragmites* incubated for 5 weeks in streams from Sierra Nevada and the semiarid lowland of south-eastern Spain. All measurements were carried out at 22ºC. Also shown are *F* values and significance level, following two-way ANOVA, to test the effect of region, species and the interaction region x species.

Factor or comparison	Max. Activity (µmol g^{-1} DM h^{-1})	pH of max. activity	Activity at pH of the stream (µmol g^{-1} DM h^{-1})	Efficiency (%)
Alnus glutinosa				
Sierra Nevada	**10.32 ± 2.13**	**6.0 ± <0.1**	**2.79 ± 0.50**	**27.39 ± 2.64**
Aldeire	7.22 ± 0.06	6.0 ± <0.1	1.82 ± 0.16	25.20 ± 1.42
Jérez	14.40 ± 0.22	6.0 ± <0.1	3.50 ± 0.06	24.32 ± 0.59
Lanteira	9.33 ± 0.04	6.0 ± <0.1	3.05 ± 0.07	32.65 ± 0.83
Semiarid Lowland	**29.84 ± 11.61**	**5.5 ± 0.2**	**10.37 ± 2.54**	**38.46 ± 5.26**
Aguas	53.06 ± 1.22	5.7 ± 0.3	15.41 ± 0.27	29.04 ± 0.91
Negras	17.95 ± 0.45	5.7 ± 0.3	8.47 ± 0.21	47.21 ± 2.15
Vícar	18.50 ± 0.65	5.0 ± <0.1	7.24 ± 1.52	39.14 ± 8.15
Phragmites australis				
Sierra Nevada	**9.00 ± 1.04**	**5.0 ± 0.0**	**2.31 ± 0.35**	**25.84 ± 3.63**
Aldeire	7.79 ± 0.03	5.0 ± <0.1	2.54 ± 0.18	32.56 ± 2.24
Jérez	8.13 ± 0.27	5.0 ± <0.1	1.63 ± 0.06	20.08 ± 0.07
Lanteira	11.08 ± 0.18	5.0 ± <0.1	2.76 ± 0.04	24.87 ± 0.51
Semiarid Lowland	**11.28 ± 0.82**	**5.4 ± 0.3**	**3.28 ± 0.23**	**29.12 ± 0.78**
Aguas	12.81 ± 0.26	6.0 ± <0.1	3.73 ± 0.07	29.08 ± 1.07
Negras	9.99 ± 0.23	5.3 ± 0.3	3.04 ± 0.11	30.49 ± 0.89
Vícar	11.03 ± 0.19	5.0 ± <0.1	3.06 ± 0.13	27.79 ± 1.16
Comparison				
Region	4.5^{ns}	0.1^{ns}	12.0**	4.4^{ns}
Species	3.4^{ns}	7.4*	9.2*	2.4^{ns}
Region x Species	2.4^{ns}	7.4*	6.9*	1.1^{ns}

**$P < 0.01$; *$P < 0.05$; ns not significant ($P > 0.05$).

2.2.7. Zymograms for endocellulase activity

We used aliquots of extracts previously concentrated using Amicon® Ultra-15 (10.000 MWCO) for SDS-PAGE zymograms. These were prepared using 11 % polyacrylamide and 0.4 % carboxymethyl cellulose (CMC) as substrate for the activity of endocellulases. The gel was rinsed in sodium citrate buffer (50 mM, pH 5, containing 25 % isopropanol) for 30 min, and, thereafter, was incubated for 1 h in sodium citrate buffer (50 mM, pH 5) at 50 ºC to allow enzyme hydrolysis of the substrate. After rising pH with NaOH 0.1 N, gels were stained with Congo red (0.1 %) for 30 min and distained in 1M NaCl to reveal protein bands with cellulase activity. The soluble protein concentration in extracts was determined by the Bradford method (1976) using bovine serum albumin as standard.

2.2.8. Statistical analyses

We used mixed-model nested ANOVAs to test for differences in environmental variables among regions and streams ('region' fixed factor; 'stream' random factor nested within region), and two-sample t-tests to compare the physic-chemical characteristics of leaf litter between species.

Decomposition rates were calculated by fitting the % dry mass remaining over time (2, 5 and 11 weeks) to linear models ($M_t = M_0 - bt$) and exponential models ($M_t = M_0\, e^{-kt}$): where M_t is the remaining dry mass at time t, M_0 de initial dry mass (corrected for leaching), and b and k are the linear and exponential decomposition rates, respectively. Streams differed in temperature, thus coefficients were also calculated with degree-days as the independent variable, which were computed from records of daily mean water temperature. We used three-way mixed-model ANCOVAs to compare decomposition rates—'region' and 'species' as fixed factors and 'stream' as random factor—with time (days) or thermal sum (degree-days) as the covariate.

Two-way ANOVA was used to test the effects of region (stream as experimental unit) and species on fungal biomass, decomposition efficiency (ratio of decay rate to fungal biomass; Gonçalves *et al*. 2013), cellulolytic activity and its operational parameters as a function of temperature (efficiency and energy of activation) and pH, as well as on the number of isoenzymes. Three-way ANOVA was used to test for differences of enzymatic stability at different pH ('region', 'species' and 'pH' as factors; stream treated as experimental unit). We tested the effect of ionic strength, at different pH values, on the residual enzymatic activity, in leaf litter extracts from the stream with the highest ionic strength (Aguas), using repeated-measured ANOVA (three measures over time; 'ionic strength', 'pH' and 'species' as factors).

To assess the relative effect of temperature and pH on enzyme activity we compared paired (by stream) Q_{10} and Q_1 mean values for each species within a given region using

paired *t*-test. Furthermore, to evaluate the effect of region and species on differences in activity caused by temperature and pH (Q_{10}-Q_1) we used two-way ANOVA.

We used partial least squares regression (PLS) (Abdi 2003) to evaluate the relative importance of environmental variables as predictors of decomposition rates and cellulolytic activity: both activities, at the temperature and at the pH of the stream. Separate PLS models were developed for each dependent variable and leaf litter species to test the HFA hypothesis directly, by assuming that, given the large differences in environmental variables between regions, results would support the hypothesis if inverse relationship— among dependent variable and predictors— would be found between the two leaf litter species. All models were constructed with the autofit function in order to obtain the highest predictive (Q^2) value. A PLS model was considered significant when Q^2 exceeds a critical value of 0.097, and models with $Q^2 > 0.4$ were considered good (Eriksson et al. 2006). The relative influence of each predictor in a PLS model was expressed as the variable importance on projection (VIP). Predictors with a VIP >1 were considered as the most influential for the model (Eriksson et al. 2006).

All tests were performed on transformed variables, except pH, to make the variances homoscedastic, using log(x+1), or arcsine $\sqrt{x}$ for percentages. We used Tukey HSD tests for pair-wise post-hoc comparisons when factors had more than two levels. All tests were carried out using Statistica software (Statsoft, Tulsa, OK, USA), except PLS regressions that were performed with XLSTAT (Addinsoft, New York, NY, USA).

2.3. Results

2.3.1. Environmental setting

The sharp contrast in biogeoclimatic setting between regions was mirrored in stream's environmental conditions. Mountain streams had significantly lower temperature, pH, EC, alkalinity, Cl^- and SO_4^{2-} compared to lowland streams (Table 2.1). However, lowland streams formed a group more heterogeneous compared to mountain streams, primarily due to greater among-streams variability in EC, SO_4^{2-}, NO_3^--N and discharge in the lowland (Table 2.1).

2.3.2. Initial leaf litter characteristics

Nitrogen concentration did not significantly differ between species, while C concentration was significantly higher in *Alnus* leaf litter (Table 2.2). Significantly higher concentrations in cellulose, hemicellulose, Si and ash, but lower in lignin, were measured in leaves of *Phragmites* when compared to *Alnus*. The higher concentration of fibre and Si in leaf blades of *Phragmites* likely determined its significantly higher

toughness compared to *Alnus* (Table 2.2). From these data we also estimated the amount of non-structural carbohydrates that was higher in *Alnus* (42 %) than in *Phragmites* (25 %).

2.3.3. Breakdown rates

Leaf mass loss, corrected for leaching, fitted to negative exponential models slightly better than to linear models (average R^2 = 0.90 and R^2 = 0.88, respectively). Thus, exponential models instead of linear ones were used to compare decomposition rates (Figure 2.1). Decomposition rates calculated on a time basis were significantly affected by leaf litter species (F = 83.6, P < 0.001)—higher in *Alnus* than in *Phragmites*—region (F = 14.8, P < 0.05)—higher in lowland than in mountain streams—and stream (F = 9.2, P < 0.05). The significant interaction found between region and species (F = 31.3, P < 0.01) revealed that, while *Alnus* litter decomposed significantly faster than *Phragmites* in mountain streams, no significant differences between species were detected in lowland streams. On average, decomposition rate of *Alnus* was 1.5 times higher in lowland than in native mountain streams, while that of *Phragmites* was 3.4 times greater in its native region compared to the mountain one. Decomposition rates computed on a thermal sum (dd^{-1}) basis did not differ significantly between regions (F = 2.4, P > 0.05) or among streams (F = 5.1, P > 0.05), although differences between species (F = 94.1, P < 0.001) and the interaction region × species (F = 32.7, P < 0.01) still remained significant (Figure 2.1).

2.3.4. Fungal biomass and decomposition efficiency

Fungal biomass in leaf litter did not differ significantly between species or regions (F = 1.43, P = 0.26 and F = 0.05, P = 0.83; respectively). Noteworthy is, however, the tendency for both species of higher average fungal biomass in its native region (Figure 2.2). Decomposition efficiency was significantly higher in the lowland compared to the mountain region (F = 37.48, P < 0.001). No significant effect of species was detected (F = 0.02, P = 0.88) (Figure 2.2).

2.3.5. Activity and functional parameters of β-glucosidase

Maximum activity was measured at temperatures between 40-70 ºC, without significant differences between regions or species; however, activity at the stream temperature and enzymatic efficiency (% of the maximum activity measured at the average temperature in each stream) significantly differed between regions, but not between species (Table 2.3). The energy of activation (E_a) for β-glucosidase activity was not significantly affected by region (mountain: 37.70 ± 2.47 kJ mol^{-1}; lowland: 31.13 ± 3.92 kJ mol^{-1}; F = 1.55, P = 0.25) or species (*Alnus*: 37.94 ± 1.12 kJ mol^{-1}; *Phragmites*: 30.89 ± 4.41 kJ mol^{-1}; F = 2.02; P = 0.20).

Table 2.5 Model parameters of Partial Least Square (PLS) regression with model's predictive ability (Q^2), variance of dependent variable explained by predictor variables (R^2 [Y]), variable importance on projection (VIP) and standardized PLS partial coefficients. In all models only one PLS-component was selected by the autofit function. Models with significant predictive power ($Q^2 > 0.097$) are in bold.

Dependent variable	Q^2	R^2 [Y]	Predictor	VIP*	Standardized coefficient
Decomposition rate (k)					
Alnus	-0.22	0.54	Temperature	1.43 ± 0.23	0.22 ± 0.15
			$N\text{-}NO_3$	1.18 ± 1.04	0.18 ± 0.21
			Conductivity	1.02 ± 0.65	0.16 ± 0.15
			pH	1.02 ± 0.39	0.16 ± 0.11
Phragmites	**0.63**	**0.79**	**Temperature**	**1.34 ± 0.13**	**0.21 ± 0.04**
			pH	**1.19 ± 0.09**	**0.19 ± 0.04**
			Alkalinity	**1.18 ± 0.14**	**0.19 ± 0.03**
			Conductivity	**1.09 ± 0.28**	**0.17 ± 0.06**
β-glucosidase activity (stream temperature)					
Alnus	0.01	0.31	pH	1.15 ± 0.26	0.14 ± 0.25
			Alkalinity	1.15 ± 0.21	0.13 ± 0.24
			Temperature	1.10 ± 0.31	0.13 ± 0.24
			$P\text{-}PO_4$	1.06 ± 0.66	0.12 ± 0.30
Phragmites	**0.24**	**0.74**	**Conductivity**	**1.37 ± 0.31**	**0.23 ± 0.30**
			Temperature	**1.15 ± 0.36**	**0.19 ± 0.25**
			pH	**1.05 ± 0.24**	**0.17 ± 0.23**
β-glucosidase activity (stream pH)					
Alnus	**0.51**	**0.81**	**Conductivity**	**1.36 ± 0.29**	**0.23 ± 0.07**
			Temperature	**1.28 ± 0.18**	**0.21 ± 0.05**
			NO_3-N	**1.13 ± 0.48**	**0.20 ± 0.27**
			pH	**1.05 ± 0.08**	**0.18 ± 0.01**
Phragmites	**0.41**	**0.66**	**Conductivity**	**1.23 ± 0.29**	**0.18 ± 0.09**
			Temperature	**1.21 ± 0.22**	**0.18 ± 0.08**
			pH	**1.15 ± 0.17**	**0.17 ± 0.03**
			NO_3-N	**1.06 ± 0.78**	**0.16 ± 0.13**

*Only the predictors having a VIP score greater than 1 are shown

Maximum activity of β-glucosidase was recorded at pH between 5 and 6 in all cases, being not significantly affected by region or species, as occurred with the enzymatic efficiency (Table 2.4). In contrast, activity measured at the average pH of the stream was significantly higher in the lowland, and for *Alnus*, compared to the mountain region and *Phragmites*, respectively; also, a significant interaction region × species was detected, that was driven by significantly higher values in lowland *vs* mountain streams for *Alnus*, but a lack of significant differences between regions for *Phragmites* (Table 2.4).

Maximum enzyme stability against pH was obtained at pH=7, except for *Alnus* extracts from lowland streams (pH = 9) (Figure 2.3). Stability was significantly influenced by region (F = 43.5, P < 0.001; mountain streams > lowland streams), species (F = 38.8, P < 0.001; *Phragmites* > *Alnus*) and pH (F = 11.8, P < 0.001) (Figure 2.3). It is worth to highlight the significant effect of the interaction region × pH (F = 6.4, P < 0.001). Enzyme activity in extracts from lowland streams showed higher sensitivity to acidic—with decrements of activity between 60-80% after 4 h incubation—or circumneutral conditions. In contrast, a slightly higher sensitivity to alkaline pH was measured in extracts from the mountain streams (Figure 2.3).

For each species within both regions, Q_{10} coefficients were always significantly higher compared to Q_1 pH coefficients (all t-values between -7 and -4, P < 0.05 in the four comparisons) (Figure 2.4). Furthermore, while all mean Q_{10} temperature coefficients were clearly above 2, indicating that activity at least doubled with a 10 ºC temperature increase, all mean Q_1 pH coefficients resulted lower than 1, this indicating a reduction in the activity with increasing pH (between 7 to 8). Neither species nor region exerted significant effects on differences between Q_{10} and Q_1 (F = 3.69, P = 0.09; F = 1.19, P = 0.31; respectively).

High ionic strength produced a significant reduction in β-glucosidase activity in extracts from the stream with higher EC (F = 665.3, P < 0.001). This reduction was clearly detected in extracts of the two species for the three pH values tested, particularly after 2 h of incubation (Figure 2.5).

2.3.6. Cellulase zymograms

A higher number of endoglucanase isoenzyme forms was detected in extracts from *Phragmites* compared to *Alnus* (F = 22.84, P < 0.01), regardless of the region (F = 4.43, P = 0.07) (Figure 2.6). However, a significant effect of the interaction region × species was detected (F = 7.14, P < 0.05), which resulted from no differences between regions in the profiles measured in extracts from *Phragmites*, but a higher number of isoenzymes in *Alnus* from the lowland compared to mountain streams.

2.3.7. Effects of environmental factors on decomposition rates and enzyme activity

PLS regression models showed that decomposition rate and enzyme activity in *Phragmites* were significantly predicted by environmental factors, but that has not always the case in *Alnus* (Table 2.5). A high percentage of the variance of decomposition rates of *Phragmites* was explained by environmental factors, with temperature being the most important predictor (Table 2.5). Cellulolytic activity in *Phragmites*, both at the stream temperature (74% var. expl.) and the stream pH (66% var. expl.), were mainly predicted by water conductivity and temperature. For *Alnus*, only β-glucosidase activity

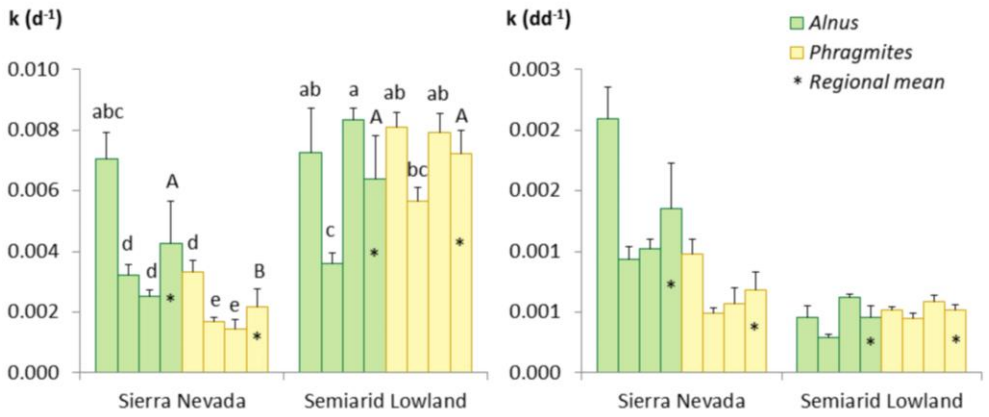

Figure 2.1 Decomposition rates (k ± SE) results for each of the three streams per region adjusted to negative exponential decay models for litter of *Alnus glutinosa* and *Phragmites australis*. The bars with an asterisk represent the mean value for each data series. Different letters indicate significant differences ($P \leq 0.05$) between streams (lowercase letters) or regions (capital letters).

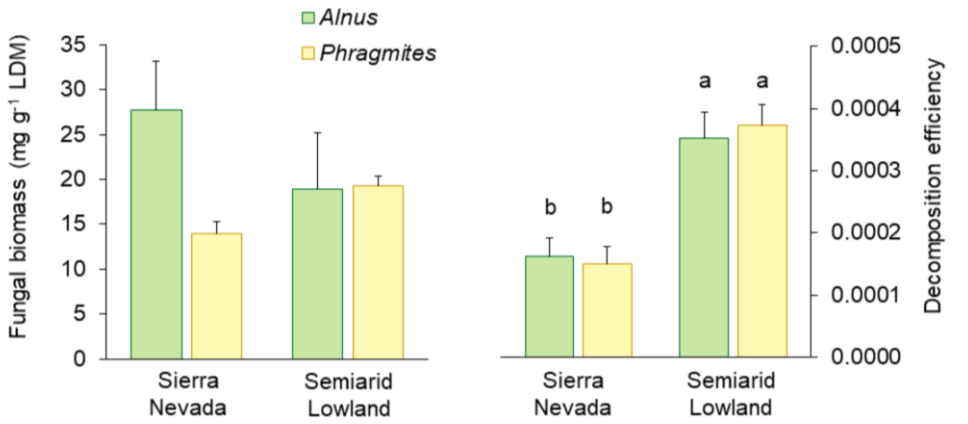

Figure 2.2 Fungal biomass and decomposition efficiency (mean ± SE) observed in the two leaf litters in both regions.

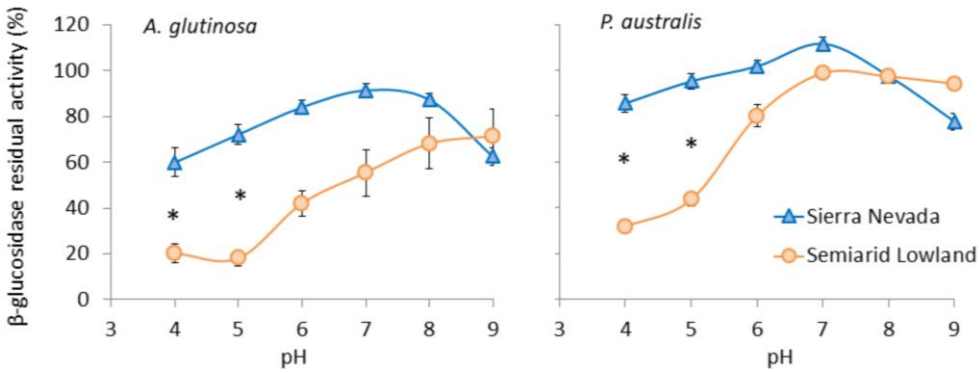

Figure 2.3 Enzyme stability measured as mid-term (4h incubation) pH sensitivity of β–glucosidase activity (n=3, mean ± SE). Statistically significant differences between regions for specific pH values are denoted with an asterisk (Tukey test, P<0.001).

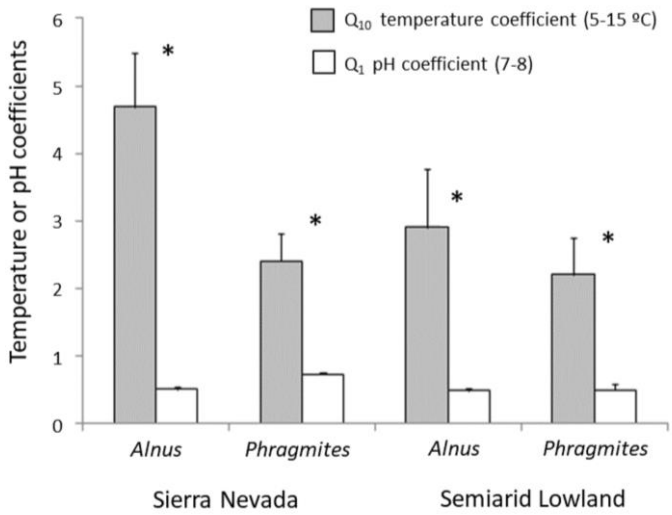

Figure 2.4 Apparent Q_{10} and Q_1 values for β–glucosidase activity from both leaf litters incubated in the two regions. An asterisk or different letter indicates significant differences ($P \leq 0.05$). Data are the mean of three replicates ± SE.

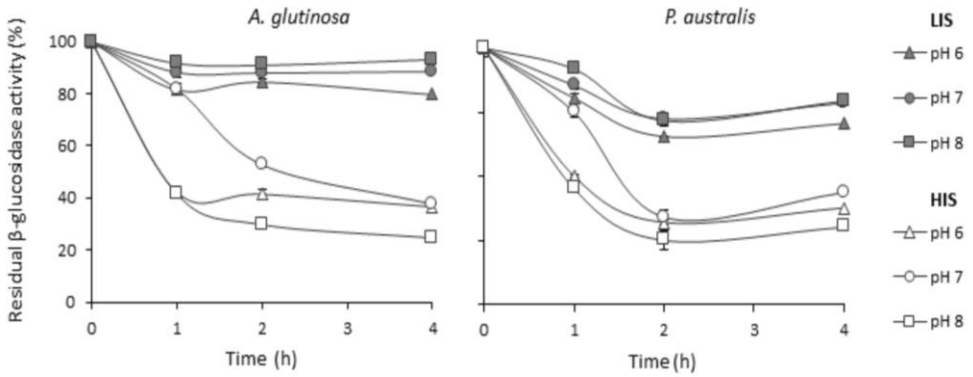

Figure 2.5 Effects of ionic strength on β-glucosidase activity in enzymatic extracts of *Alnus* and *Phragmites* leaf litter, from the stream with higher electric conductivity (Río de Aguas, Semiarid Lowland). Buffers with low ionic strength (LIS) and with high ionic strength (HIS) were evaluated at different pH values (n=3, mean ± SE).

at stream pH, was significantly predicted (81% var. expl.), mainly by water conductivity and temperature (Table 2.5). Overall, PLS regression models, when significant, revealed similar relationships between dependent variables and environmental factors for both species, which suggest limited support to the HFA hypothesis.

2.4. Discussion

Our results strongly support the leading role of temperature, interacting with substrate chemistry, on decomposition and cellulolytic activity, compared to water pH and ionic concentration, in accordance with the "temperature–quality" hypothesis (Bosatta and Ågren 1999). However, the part of our working hypothesis suggesting higher decomposition rates and cellulolytic activity in the native range of each leaf litter species—''home-field advantage'' hypothesis (Hunt et al. 1988, Gholz et al. 2000) — received much less support.

Acceleration of litter decomposition at higher water temperature has been frequently reported in correlative field studies (Irons et al. 1994, Fabre and Chauvet 1998, Friberg et al. 2009, Boyero et al. 2011) and laboratory experiments (Dang et al. 2009, Ferreira and Chauvet 2011, Martínez et al. 2014, Gonçalves et al. 2013, 2015), and may be due to a stimulation of fungal biomass and/or activity at higher temperatures (review in Ferreira and Chauvet 2011). The higher decomposition rates and β-glucosidase efficiencies in

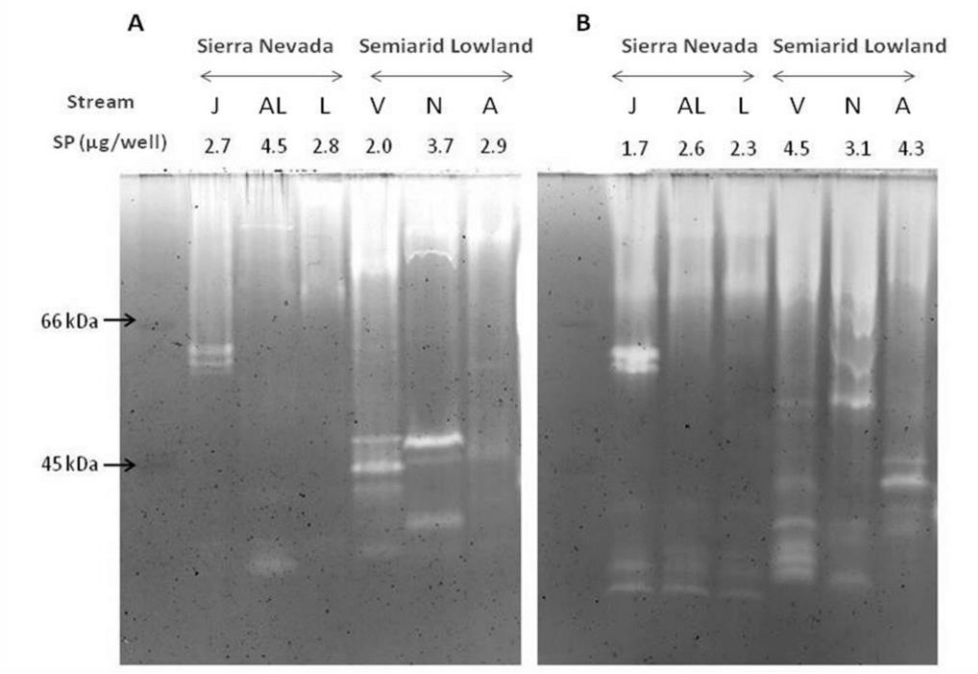

Figure 2.6 Zymograms for endo-β-1,4-glucanase activity in a concentrated enzyme solution of *Alnus* (A) and *Phragmites* (B). The first lane contains the molecular weight marker (MWM). Numbers under code in each lane indicate the soluble protein (SP) content in extracts (µg/well).

lowland than in mountain streams, the leading role of temperature as predictor of rates and enzyme activity, and the fact that differences between regions vanished when rates were expressed on a thermal sum basis, strongly suggest that the thermal step of around 10 °C between the two regions was the main driver of decomposition by stimulating fungal enzyme activity but not the increase in biomass. Certainly, β-glucosidase enzymes were closer to their thermal optimum in lowland than in mountain streams. This is not surprising since temperature greatly influences metabolic processes (Brown et al. 2004, Woodward et al. 2010) by driven enzyme kinetics and/or stimulating the rate of enzyme production (Wallenstein and Weintraub 2008), with the consequent increase in enzyme activity. Besides, accelerated degradation of recalcitrant substrates with temperature has been reported from soil (Trasar-Cepeda et al. 2007, Keiblinger et al. 2012) and aquatic (Ylla et al. 2012) environments.

An important effect of temperature on the degradation of complex crystalline polysaccharides consists in stimulating substrate accessibility to enzymes; marked increases with temperature of the relative adsorption of endoglucanases and cellobiohydrolases to cellulose have been, indeed, reported (Ooshima et al. 1983). A key

trait differentiating litter chemistry of both species assayed here, is the higher concentration of Si in *Phragmites*, which strongly binds with cellulose in the form of polymerized silicic acid (silica gel), determining a silico-cellulose cell membrane (Ma and Yamaji 2006). This could limit the accessibility of enzymes to cellulose fibres, thus making decomposition of this substrate highly sensitive to temperature. This is in agreement with the differences we detected in decomposition rates (d^{-1}) between regions for the two species: the rate of *Phragmites* was on average 3.4 times (3.0 in terms of Q_{10})—in *Alnus* only 1.5 times (1.4 in terms of Q_{10})—higher in lowland compared to mountain streams. Furthermore, the >2 times higher cellulose concentration of *Phragmites* than of *Alnus*, and its lower concentration of non-structural carbohydrates used as fuel by degrading microbiota, could have exacerbated temperature sensitivity of decomposition of the former substrate. In fact, both substrate accessibility and availability to exoenzymes have been proposed as major constrains of temperature sensitivity of organic matter decomposition in soils (Gershenson et al. 2009, Dungait et al. 2012).

Interestingly, although a high concentration in lignin, or lignin derived substances, has been linked to high sensitivity to temperature of decomposition (Davidson and Janssens 2006, Ylla et al. 2012), this was not the case observed for *Alnus*, which presented more than threefold the lignin concentration of *Phragmites*. As pointed out by several authors (Fierer et al. 2005, Kleber et al. 2011, Blagodatskaya et al. 2016) the inherent activation energy of the substrate is not the sole factor influencing the temperature sensitivity of decomposition, and in field conditions the relationship between such sensitivity and C quality may be obscured by complex interactions between temperature and a range of other abiotic and biotic factors. For instance, marked alkaline conditions in our lowland streams could have interfered with temperature sensitivity of lignin decomposition in *Alnus*, since enzymes involved in this process (e.g. laccases) often have maxima of activity and stability at acidic pH (3-4) (Bollag and Leonowicz 1984, Fukushima and Kirk 1995).

Our findings, however, strengthen the argument of a major role of temperature on differences in litter decomposition between the two regions, since they greatly agree with results obtained in laboratory experiments which reported similar values of temperature sensitivity for *Alnus* decomposition (Q_{10} *ca* 1.5) within the thermal range 5-15 °C and standard pH conditions (Ferreira and Chauvet 2011, Martínez et al. 2014). Furthermore, Donnelly et al. (1990) have reported higher decomposition sensitivity to temperature of cellulose compared to lignin in soil organic matter. These results point out to a fundamental dependence on substrate chemistry of temperature effects: the lower temperature sensitivity in *Alnus* compared to *Phragmites* perhaps due to a higher concentration of non-structural carbohydrates in the former that might attenuate its whole recalcitrance (Hättenschwiler et al. 2011b) and/or to the need of long-term experiments to

detect effects of environmental factors on lignin degradation (e.g. Berg et al. 1984, Di Nardo et al. 2004).

Our data on β-glucosidase activity as a function of pH also point out to a relatively weak effect of this factor, compared to temperature, on interregional differences in litter decomposition, at least regarding the cellulosic fraction. Overall, % efficiency of β-glucosidases as a function of pH was relatively low (<40%) in the two regions. Furthermore, Q_1 values (pH range 7-8) were below 1 in all cases, suggesting that the prevailing alkaline conditions in lowland streams could have depleted the final step of cellulose hydrolysis. This is in sharp contrast to values of Q_{10} determined within the temperature range 5-15 °C, indicating that this activity, at least doubled with a 10 ºC temperature increase regardless of the origin of the enzyme.

Activity at the stream pH was, nonetheless, significantly higher in lowland streams, despite their alkaline pH, and this factor emerges in PLS models with a significant VIP score to the enzyme's activity. This apparent conflict with results for optimum pH, might be driven by higher enzyme concentration (more than accelerated activity) in lowland compared to mountain streams, perhaps induced by higher enzyme production under the elevated temperatures in the former region (Connant et al. 2011, Blagodatskaya et al. 2016). Results for enzyme stability revealed some functional adaptation of enzymes to the pH regime of their environment of origin, but, overall, enzyme stability from mountain streams was slightly higher at pH 7, or very similar at pH 8, compared to lowland streams. This result adds some support to the notion that differences in pH between the two regions played a minor role on the final step of cellulose degradation compared with that of temperature.

Our results also suggest that β-glucosidase activity could have been hindered by the high ionic concentration of the water in lowland streams, since increasing ionic strength, regardless of pH conditions, reduced this activity even in extracts from the lowland stream with higher mineral concentration. In contrast, high litter decomposition has been detected in hardwater compared to softwater streams, which was related to the role of Ca^{2+} and Mg^{2+} as activating cations in hydrolytic enzyme reactions (Chróst 1991, Romaní and Sabater 2000), including β-glucosidase activity (King 1986). Nevertheless, high ionic concentration is not always ruled by alkalinity in inland waters, since ions others than bicarbonate, Ca^{2+} and Mg^{2+}, may be present, potentially affecting decomposers activity differently than alkalinity. Our lowland streams showed, indeed, higher concentrations of chloride and sulphate than the mountain ones, and leaf mass loss and activity of hydrolytic enzymes (Roache et al. 2006), cellulose decomposition (Mendelssohn et al. 1999), and β-glucosidase activity (Siabi et al. 2007) have been reported to be negatively affected by increasing NaCl concentration, salinity, or ionic strength, respectively.

Table 2.5 Model parameters of Partial Least Square (PLS) regression with model's predictive ability (Q^2), variance of dependent variable explained by predictor variables (R^2 [Y]), variable importance on projection (VIP) and standardized PLS partial coefficients. In all models only one PLS-component was selected by the autofit function. Models with significant predictive power ($Q^2 > 0.097$) are in bold.

Dependent variable	Q^2	R^2 [Y]	Predictor	VIP*	Standardized coefficient
Decomposition rate (k)					
Alnus	-0.22	0.54	Temperature	1.43 ± 0.23	0.22 ± 0.15
			$N\text{-}NO_3$	1.18 ± 1.04	0.18 ± 0.21
			Conductivity	1.02 ± 0.65	0.16 ± 0.15
			pH	1.02 ± 0.39	0.16 ± 0.11
Phragmites	**0.63**	**0.79**	**Temperature**	**1.34 ± 0.13**	**0.21 ± 0.04**
			pH	**1.19 ± 0.09**	**0.19 ± 0.04**
			Alkalinity	**1.18 ± 0.14**	**0.19 ± 0.03**
			Conductivity	**1.09 ± 0.28**	**0.17 ± 0.06**
β-glucosidase activity (stream temperature)					
Alnus	0.01	0.31	pH	1.15 ± 0.26	0.14 ± 0.25
			Alkalinity	1.15 ± 0.21	0.13 ± 0.24
			Temperature	1.10 ± 0.31	0.13 ± 0.24
			$P\text{-}PO_4$	1.06 ± 0.66	0.12 ± 0.30
Phragmites	**0.24**	**0.74**	**Conductivity**	**1.37 ± 0.31**	**0.23 ± 0.30**
			Temperature	**1.15 ± 0.36**	**0.19 ± 0.25**
			pH	**1.05 ± 0.24**	**0.17 ± 0.23**
β-glucosidase activity (stream pH)					
Alnus	**0.51**	**0.81**	**Conductivity**	**1.36 ± 0.29**	**0.23 ± 0.07**
			Temperature	**1.28 ± 0.18**	**0.21 ± 0.05**
			NO_3-N	**1.13 ± 0.48**	**0.20 ± 0.27**
			pH	**1.05 ± 0.08**	**0.18 ± 0.01**
Phragmites	**0.41**	**0.66**	**Conductivity**	**1.23 ± 0.29**	**0.18 ± 0.09**
			Temperature	**1.21 ± 0.22**	**0.18 ± 0.08**
			pH	**1.15 ± 0.17**	**0.17 ± 0.03**
			NO_3-N	**1.06 ± 0.78**	**0.16 ± 0.13**

*Only the predictors having a VIP score greater than 1 are shown

As litter decomposition is fundamentally driven by the interaction between resource chemistry and decomposers, both controlled by the environment, specific adaptations of microbial communities to be particularly capable of degrading the type of litter they encounter most often under the environmental conditions of their native range are to be expected. This ‘‘home-field advantage’’ (HFA) of litter decomposition (Hunt et al. 1988) did not find substantial support from our data, since decomposition rates of both species were faster in lowland than in mountain streams and both, rates and enzyme activity, showed similar patterns of variation with environmental factor in the two species. A recent meta-analysis of HFA quantification of litter decomposition in forest soils indicate that litter mass loss was 4.2 % faster on average at the home environment (Wang et al.

2013), notably in undisturbed forest soils (Austin et al. 2014). However, this percentage is relatively low compared to the about 70% of variation of leaf litter decomposition in forest soils explained by chemical characteristics of litter and climate (temperature and precipitation), particularly when studies embrace large regional scales (Gholz et al. 2000, Parton et al. 2007). Thus, given the ample differences in environmental conditions, certainly temperature, between the two regions studied here, it is possible that the hypothetical HFA of *Alnus* in mountain streams may have been overrides by the thermal step between regions.

Fungal assemblages, major drivers of leaf-litter decomposition, were species poor and compositionally different in lowland compared to mountain streams (Casas et al. 2011). Thus, differences in decomposition rates between regions cannot be attributed to fungal diversity, in agreement with studies suggesting considerable functional redundancy among aquatic hyphomycete species (Duarte et al. 2006, Gonçalves et al. 2015). Moreover, despite lower species richness, the number of endoglucanase isoenzymes detected in samples of *Alnus* was higher in lowland than in mountain streams and, overall, higher in *Phragmites* compared to *Alnus*. These results suggest a key control of environment and, particularly, substrate on gene expression of enzymes involved in cellulose degradation. Accordingly, results from forest soils suggest that microorganisms respond quickly to litter chemistry modifying gene expression of enzymes involved in decomposition (Kourtev et al. 2002). However, the observed variations in the number of isoenzymes were not correlated to inter-regional or between-substrates differences in decomposition rates. Functional enzyme parameters did not reveal major adaptations to perform better under the prevailing conditions (i.e. temperature and pH) of their environments of origin.

Overall, our results suggest that inter-regional differences in decomposition rates and cellulose degradation fundamentally depended on temperature that accelerated enzyme kinetics, and perhaps enzyme production, in lowland streams, largely overriding effects from other environmental factors or potential HFA. This temperature effect was highly dependent on substrate chemistry: the higher temperature sensitivity of decomposition in *Phragmites* was probably caused by its silico-cellulose cell membrane that might hinder accessibility of enzymes to cellulose fibres. The strong influence of leaf traits on temperature sensitivity of decomposition has been noted in recent studies from forest soils (Salinas et al. 2011) and headwater streams (Gonçalves et al. 2013, Ferreira et al. 2015). Nevertheless, our knowledge on how specific leaf traits respond under global warming scenarios remains still fragmentary.

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Chapter 3

Leaf traits and environmental controls on microbial litter decomposition in headwater streams: global patterns witnessed at a regional scale

Encarnación Fenoy

Jordi Moya-Laraño

José Luis Guil-Guerrero

Francisco J. Moyano-López

J. Jesús Casas

3.1. Introduction

Terrestrial-derived detritus constitutes a crucial functional component of many aquatic ecosystems, particularly in largely shaded headwater streams (Vannote et al. 1980, Moore et al. 2004). Leaf litter decomposition sustain most energy and nutrient flow through stream food webs, simultaneously releasing CO_2 back to the atmosphere, and substantially contributing to the global carbon cycle (Battin et al. 2009, Raymond et al. 2013).

Microbial decomposition of plant litter in streams is mainly driven by aquatic hyphomycetes, representing an essential link between litter resources and invertebrate consumers (Hieber and Gessner 2002, Pascoal and Cássio 2004, Canhoto et al. 2016). The decomposing activity of these fungi depends on many factors which can be grouped into two categories: the intrinsic characteristics of leaf litter (litter traits), to which a predominant control of decomposition have been attributed (Zhang et al. 2019), and the extrinsic in-stream abiotic factors (Gessner et al. 2007, Lecerf and Chauvet 2008). Thus, as global change is altering both types of factors, decomposer assemblages and the ecological processes in which they are involved may be deeply affected (Ferreira et al. 2014, Martínez et al. 2014).

On the one hand, rising atmospheric CO_2 concentration and associated global warming can trigger increasing recalcitrance of leaf litter inputs to streams, due to phenotypic intraspecific changes, altered relative abundance of species and/or species turnover. These changes involve mainly a reduced nutrient concentration (e.g., N), and increasing leaf toughness and/or Si content (e.g. Salinas et al. 2018). Some of these quality changes could slowdown leaf-litter decomposition by limiting enzyme diffusion and microbial access to the nutritional parts of the litter (e.g. Cornelissen et al. 1999), and/or by slowing down fungal growth or activity (Schaller et al. 2014).

On the other hand, it is well known that microbial decomposition of leaf litter is accelerated by global-change-related extrinsic factors, i.e. increasing temperature (Boyero et al. 2011, Ferreira et al. 2014, Amani et al. 2019) and moderate nutrient load in stream-water (Woodward et al. 2012, Biasi et al. 2017), with possible synergistic interactions among them (Ferreira and Chauvet 2011a, Fernandes et al. 2014). However, effects of the interaction between litter traits (intrinsic) and environmental (extrinsic) factors on leaf litter decomposition in streams are less studied, and published results are somehow conflicting (Canhoto et al. 2016). For instance, according to the "temperature–quality" hypothesis (Bosatta and Ågren 1999) the role of temperature accelerating decomposition becomes more prominent with increasing litter recalcitrance, thus counteracting to some extent the effect of a presumed reduced litter quality in warming scenarios (Fernandes et al. 2012, Gonçalves et al. 2013). The metabolic theory of ecology (MTE; Brown et al. 2004) provides a framework to quantitatively evaluate the temperature

sensitivity of decomposition of litters varying in quality, by calculating the activation energy (E_a) of chemical reactions. However, in field conditions other extrinsic factors can interact with temperature (e.g. water mineral content and nutrients, thermal adaptation of decomposers..), influencing the apparent temperature sensitivity of litter decomposition (Follstad Shah et al. 2017).

As global change proceeds, nutrient content in stream water increases, speeding up decomposition, in particular, of nutrient-poor litters (Ferreira et al. 2015). Nevertheless, the relative importance of stream-water dissolved nutrients and litter-quality, nu and their interactions, remains unresolved, perhaps due to the limited range of trait variability in the litter species and environmental settings studied so far (Canhoto et al. 2016).

Global change is modifying existing vegetation causing genetic changes in populations of currently dominant species, species losses, invasion of exotic species and/or land-use changes (Gritti et al. 2006, Kominoski et al. 2013). Thus, plant communities may spatially change faster than decomposer communities do, potentially decoupling production and decomposition processes (Bardgett et al. 2013). According to the home-field advantage hypothesis (HFA; Hunt et al. 1988, Gholz et al. 2000, Vivanco and Austin 2008), decomposition rates could be influenced by the particular adaptations of microbial assemblages to exploit more efficiently the type of resources historically dominant at home. Thus, alterations in energy flow and nutrient cycling are to be expected due to a lack of resource-consumer coevolution in a fast changing environment (Hobbs et al. 2006, Cross et al. 2013). In terrestrial ecosystems, the HFA hypothesis has been widely tested, but with conflicting results which hinder generalizations across scales and ecosystems (Palozzi and Lindo 2018). In aquatic ecosystems few studies have, implicitly or explicitly, evaluated HFA effects, yielding inconclusive results (Jackrel and Wootton 2014, Franzitta et al. 2015, Luai et al. 2019). The few studies that have explicitly tested this hypothesis for microbial decomposition in headwater streams, reported a limited support to it (Fenoy et al. 2016) or just significant HFA effects for recalcitrant litters (Yeung et al. 2019).

Given the simultaneous control of decomposition by numerous factors and their strong context dependence (Woodward et al. 2012, Boyero et al. 2016), determining the primary drivers of litter decomposition is challenging but of paramount importance in a changing world (Canhoto et al. 2016). However, logistical constraints of most studies limit the number of evaluated factors, and the different litter species and their associated functional traits used across studies have frequently hindered elucidation of general patterns and environmental controls of decomposition in streams (Canhoto et al. 2016, Tiegs et al. 2019, Zhang et al. 2019). This emphasizes the need for simultaneously examining decomposition rates of a number of leaf species differing in key functional traits across a wide range of abiotic factors, using experimental designs enabling reliable testing of potential HFA effects.

In this study, our aim was to evaluate the main intrinsic and extrinsic factors most often identified as drivers of the decomposition process in streams by adding a regional scale perspective. To this end, we studied the decomposition of four leaf litter species belonging to three functional groups (deciduous, broad-leaves evergreens and giant gramminoids) differing in leaf traits reflecting potential climate-driven alterations of riparian inputs to headwater streams (Salinas et al. 2018). We performed a reciprocal incubation experiment across four Mediterranean subregions with sharp contrasts in riparian vegetation and physico-chemical characteristics in stream waters. The study was conducted in an environmentally diverse but relatively small Mediterranean region (Casas et al. 2006, 2011), which provides a suitable framework to simultaneously evaluate target factors with smaller influences of historical biogeographic constraints compared to larger spatial scales (e.g. Leibold et al. 2010, Heberling and Fridley 2012). We hypothesize that 1) litter traits exert a preponderant control of the decomposition process, conditioning the size and direction of the effects of environmental variables; 2) the effect on decomposition of temperature and nutrient load in stream water are dependent on litter quality (i.e., a statistical interaction), particularly for recalcitrant substrates; and 3) we also predicted a significant effect of litter origin (native *vs* non-native) on decomposition, regardless of litter quality and its interaction with temperature and dissolved nutrients.

3.2. Material and methods

3.2.1. Study sites

We developed a decomposition experiment during winter 2017-2018 in four subregions with marked biogeoclimatic contrast located in Andalusia (southern Spain), selecting four low-order streams within each subregion. Streams located in Sierra Nevada (1148-1465 m a.s.l.) and Alcornocales (415-532 m a.s.l.) drain silica rocks, and those located in Sierra de Cazorla (686-1249 m a.s.l.) and Semiarid Lowland of Almería (47-300 m a.s.l.) drain limestone rocks. A typical Mediterranean-type climate dominates in this region, but with substantial variation among subregions in mean annual precipitation and mean annual air temperature: Sierra Nevada, 581 mm, 10.2 ºC; Alcornocales, 1227 mm, 15.1 ºC; Cazorla, 913 mm, 11.4 ºC; Semiarid Lowland, 350 mm, 16.4 ºC. Dominant riparian species were: alder (*Alnus glutinosa* [L.] Gaertn.) and grey willow (*Salix atrocinerea* Brot.) in Sierra Nevada; common rhododendron (*Rhododendron poncticum* subsp. *baeticum* [Boiss. & Reut.] Hand.-Mazz.), alder and Andalusian oak (*Quercus canariensis* Willd.) in Alcornocales; narrow-leaved ash (*Fraxinus angustifolia* Vahl), grey willow and black pine (*Pinus nigra* [Dunal]) in Cazorla; giant cane (*Arundo donax* L.) and common reed (*Phragmites australis* [Cav] Trin ex Steud.) in the Semiarid Lowland. Further bioclimatic information of the studied region is given in Salinas et al. (2018).

3.2.2. Stream water characteristics

Electrical conductivity (EC), pH and dissolved oxygen were measured in each stream with a multiparametric probe (HACH® model HQ-30d, Loveland, CO, USA). Water samples were collected and filtered (APFC, Millipore®, Darmstadt, Germany) in the field to measure alkalinity, total dissolved nitrogen (TN) and phosphorous (TP), nitrates (NO_3^-N) and soluble reactive phosphorus (SRP). Total alkalinity was measured by acid titration to a pH endpoint of 4.25 (Wetzel and Likens 2000). An aliquot of 100 mL of non-filtered water was wet mineralized for 30 min at 120ºC in an autoclave. After cooling to room temperature, an aliquot of 50 mL was acidified with concentrated sulphuric acid to determine TN (absorbance at 220 nm), whereas TP, mineralized to phosphate, was determined in the remaining 50 mL (Wetzel and Likens 2000). Dissolved inorganic nutrients were analyzed on filtered samples (0.45µm, Merk Millipore): NO_3-N, by the sodium salicylate method (APHA 2005), and SRP by the ascorbic acid method (Wetzel and Likens 2000). All measurements were performed twice in each stream, at the beginning and the end of the decomposition experiment. Water temperature was recorded hourly in each stream with HOBO Pendant (Onset Computer Corporation, Bourne, MA, USA) loggers during the full incubation period of leaf litter (40 days).

3.2.3. Leaf-litter selection and characterization

We selected one dominant species from each subregion, with contrasting leaf-litter quality: alder (hereafter *Alnus*) from Sierra Nevada, rhododendron (hereafter *Rhododendron*) from Alcornocales, ash (hereafter *Fraxinus*) from Cazorla and giant cane (hereafter *Arundo*) from the Semiarid Lowland of Almería. Senescent leaves were collected from the riparian zones in each subregion just after abscission (*Alnus* and *Fraxinus*) or from branches or standing-dead shoots (*Rhododendron* and *Arundo*). Leaf litter was air dried to constant weight (two weeks) at room temperature (ca. 23 ºC). Leaf toughness and SLA were measured on pre-incubated material and chemical characteristics were determined after in-stream leaching (see below). Toughness, SLA and percentages of hemicellulose, cellulose and lignin were determined as in Fenoy et al. (2016). Litter nitrogen (N) and carbon (C) concentrations were determined using a Perkin Elmer series II CHNS/O elemental analyser (EA-Thermo DELTA V Advantage, Fisher Scientific®), with results expressed as % N and % C of litter dry mass. Phosphorous (% P) was determined following the method described in Wetzel and Likens (2000), after sample incineration (500ºC, 5h).

3.2.4. Leaf-litter decomposition

Leaf litter portions (5.00 ± 0.05 g DM) of each species were spray moistened with distilled water and introduced in bags (15×20 cm; 1 mm mesh size). Leaf-litter bags—8 per species and stream—were incubated in 5 equidistant riffle sections along a 50-m stream reach. Three bags per stream and species were retrieved after 24 h in order to account for handling and leaching losses. The remaining five litter bags per species and stream were collected after 40 days to estimate decomposition. Upon retrieval, the litter bags were placed individually in zip-lock bags and transported to the laboratory in an icebox. Leaves were carefully rinsed with filtered stream water to eliminate fine particles, then were oven-dried (70ºC, 72 h) and weighed to the nearest 0.1 mg. Thereafter, litter was grinded to pass a 1 mm screen; a portion was ignited at 500ºC for 5 h to estimate mass loss (weighted to the nearest 0.1 mg) on an ash-free dry mass basis. Decomposition was expressed as ash-free leaf-mass loss per day (LML mg d^{-1}) and per degree-day (LML mg dd^{-1}).

3.2.5. Fungal biomass

An additional set of five litter bags per species and stream, incubated as above, was used to measure ergosterol content, to estimate fungal biomass. In the lab, a half portion of each litter bag was carefully rinsed with filtered stream water. These portions were pooled by species/stream to reduce the number of analyses to an affordable amount, frozen until needed, and then freeze-dried and grinded. Ergosterol was extracted from aliquots of 200 mg (< 500µm particle size) in 4 ml of methanol and treated with 1 ml of 2 M aqueous sodium hydroxide (100ºC, 30 min), partitioned by petroleum ether (2x5 ml) and evaporated to dryness in a rotatory evaporator (55ºC). Ergosterol was re-dissolved in 1 ml of methanol and quantified by high performance liquid chromatography (HPLC). Ergosterol was detected at 282 nm and quantified against ergosterol standards (Fluka, Buchs, Switzerland). Ergosterol content was converted into fungal biomass using a conversion factor of 5.5 mg ergosterol mg^{-1} fungal dry mass (Gessner and Chauvet 1993) and the results were expressed as mg fungal biomass g^{-1} litter DM.

3.2.6. Data analyses

All statistical tests were carried out using R software 3.5.2 (R Core Team 2018). We used one-way ANOVAs to compare environmental factors among subregions and leaf traits among species, and Tukey HSD tests for pair-wise post-hoc comparisons. We used the R 'factoextra' package to explore relationships among subregions and leaf species, respectively, by means of separate standardized principal component analyses (PCAs) of stream environmental characteristics and leaf traits, after varimax rotation. Because we were interested in extracting only a few dimensions, when a variable was highly correlated with others (Spearman rank correlation > 0.8, $P < 0.05$) it was removed from the final PCA. Except for pH, all analyses were performed on transformed variables, to make the

variances homoscedastic, using log(x+1) for most continuous variables or arcsine √(x/100) for percentages.

We first fitted linear mixed-effects models (LME; *lme4* package in R) to regress decomposition rate (in terms of time [d^{-1}] and thermal sum [dd^{-1}]) and fungal biomass with "region" and leaf litter "species" as fixed factors and "stream" as random factor, to look at the overall joint effects of extrinsic (region) and intrinsic (litter species) independent factors on each of the three dependent variables. The significance of independent factors was evaluated using type "III" sum of squares (*car* package in R). Pairwise comparisons of level means for a given factor within each level of the other factor were performed using the *multicomp* and *lsmeans* packages in R.

We then investigated which particular intrinsic or extrinsic factors, and which complex interactions among them, were driving decomposition. To this end, we documented the effect of the major environmental variables (extrinsic factors) and leaf traits (intrinsic factors) that jointly drive decomposition rate by analysing the data in two steps. First, the machine learning algorithm Random Forest (RF) (Breiman 2001), was applied to select influential drivers (Sandri and Zuccolotto 2006). Variables with Inc. Node Purity values before the inflection point were selected. RF performs well when facing multicollinearity, is relatively robust to over-fitting, automatically fits non-linear relationships and high-order interactions, and provides a measure of the importance of each variable in a model (Pitcher et al. 2012). This preliminary exploratory analysis was necessary given the large number of drivers and potential environment-trait interactions that could be at play, for most of which we lacked robust predictive hypotheses. Actually, one of the aims of this approach was to obtain decomposition estimates that are independent of litter trait and abiotic factors in order to test HFA (see below), for which the actual drivers are not of central relevance. Second, using the resulting set of selected variables, we applied linear mixed effects models to better understand the main interactions among the most important decomposition intrinsic and extrinsic factors as selected by RFs (LME). We included "stream" as random factor. Once the simplest fixed effects structure was found (that yielding the lowest AIC value using the ML option), the final mixed model was re-fitted with REML and standardized variables (*scale* function) to compare effect sizes, and diagnostic plots inspected again (Zuur et al. 2009). When necessary, we used Box-Cox transformation to make the data approximate to a normal distribution.

Relationships between temperature and decomposition rate was explored quantitatively based on the Metabolic Theory of Ecology (MTE) (Brown et al. 2004) for all species pooled and each one separately. The natural logarithm of the decay rate coefficient was regressed against the inverse of absolute temperature (T) in degrees Kelvin and the Boltzmann constant (k) (Brown et al. 2004).

We estimated Home Field Advantage (HFA) for each species in its native subregion, using the Additional Decomposition at Home of species i (ADH_i) of Ayres et al. (2009), which departed from a method originally developed to calculate HFA in sports. We adapted ADH_i for the four species used, calculated in each of the four streams per subregion. We calculated ADH_i using data of decomposition in terms of ash-free LML per day (d^{-1}) and per degree-day (dd^{-1}). We also computed ADH_i using the residuals (expressed in their original units) of the linear mixed regression model, assuming that the unexplained variance (residuals); i.e., that failing to be explained by extrinsic (environment) and intrinsic (litter traits) factors, could be due to the advantage of a microbial assemblage specialized on the litter species commonly exploited at home. A positive HFA effect, i.e. faster decomposition than expected at home, occurred if $ADH_i > 0$; if $ADH_i < 0$, litter decomposition at home would be slower than expected; and no HFA effect exist if $ADH_i = 0$. We used t-tests to determine whether mean ADH_i values (n = 4 streams per subregion) differed significantly from zero.

3.3. Results

3.3.1. Stream water characteristics

Environmental characteristics of streams showed wide ranges of variation across subregions (Table S3.1), the most prominent were those of mean water temperature (3.44-22.32 ºC), electric conductivity (62-3215 µS cm^{-1}) and TN (150-2096 µg L^{-1}). Water chemistry strongly reflected rock type. The limestone regions (Cazorla and Semiarid Lowland) showed higher values of pH, total alkalinity, electrical conductivity (EC), TN and NO_3-N, but lower content of phosphorus compared to subregions rich in silica rocks (Sierra Nevada and Alcornocales) (Table S3.1). The first two PCs of the PCA accounted for 74% of total variance. PC1 (38% expl. var.) showed positive loading of water temperature (0.92) and TN (0.86). Alkalinity (0.71) and NO_3^--N (0.61) showed the highest load on the positive RC2 (36% expl. var.) dimension, distinguishing between silica (below) and limestone (above) subregions (Figure 3.1). SRP showed the highest load on the negative dimension (-0.91) where streams from silica rocks subregions (Alcornocales and Sierra Nevada) were segregated (Figure 3.1).

3.3.2. Leaf-litter traits

Arundo showed significantly lower C, P and lignin, but higher cellulose, hemicellulose and Si contents compared to other species. N concentration significantly differed across all leaf litter species: *Alnus* >> *Fraxinus* > *Rhododendron* > *Arundo* (Table S3.2). The higher concentration of fibres and, particularly, of Si in leaf blades of *Arundo* likely determined its significantly higher toughness (Table S3.2). Total variance explained by the first two

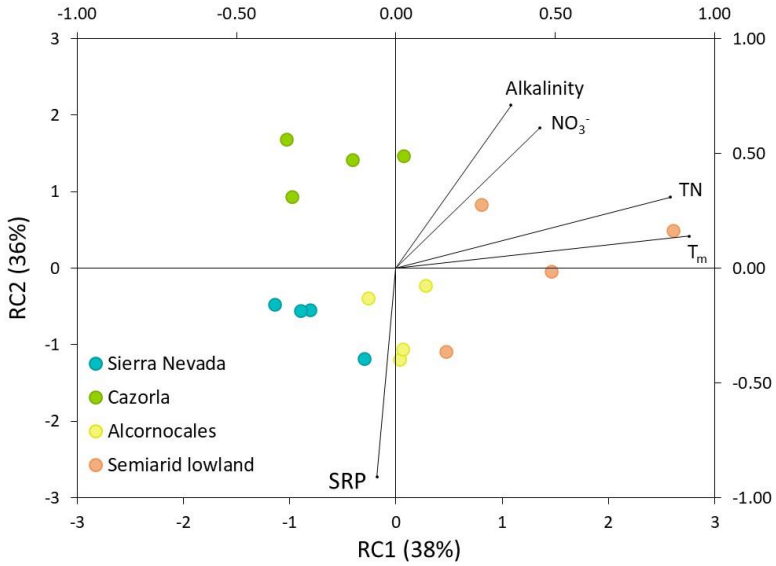

Figure 3.1. PCA ordination of environmental conditions in each region (4 streams per region), after varimax rotation. Variable loadings scale is indicated in right and upper axes.

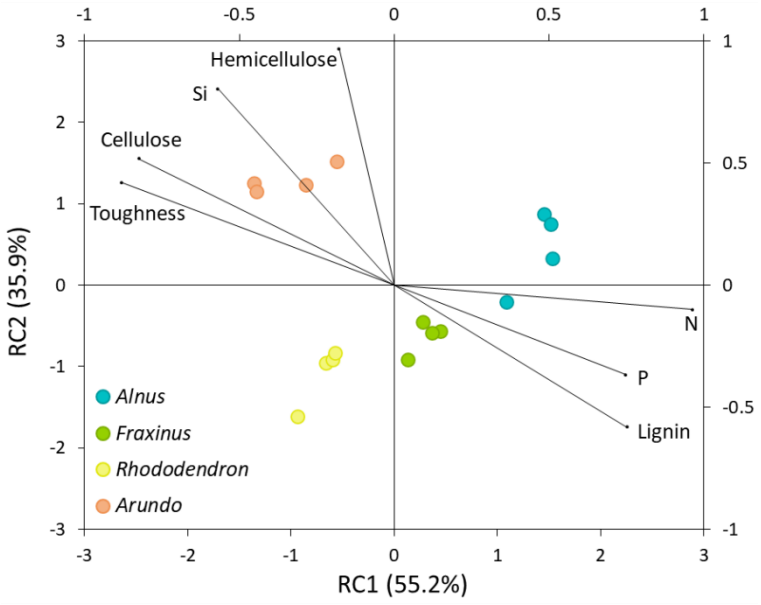

Figure 3.2. PCA ordination of each plant species selected as dominant in each region on the basis of leaf litter traits, after varimax rotation. Variable loadings scale is indicated in right and upper axes.

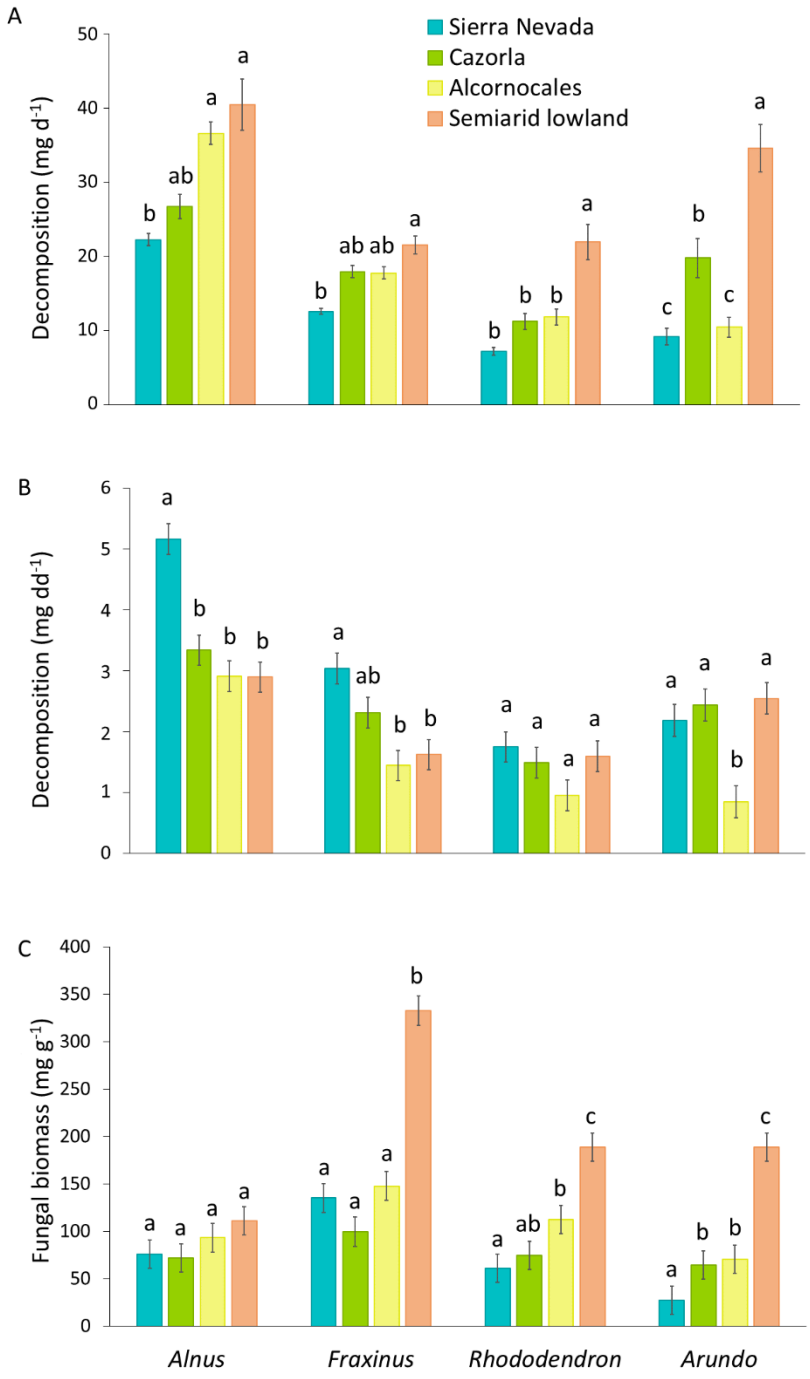

Figure 3.3. Decomposition rate (mean ± SE) computed on the basis of days (A) and degree days (B) of the four species incubated in the four regions. (C) Fungal biomass after incubation period (40 days). Different letters indicate significant differences among regions for each leaf litter (P<0.05).

components was 91.1%. RC1 (55.2% expl. var.) dimension showed high positive load of N (0.96), P (0.75) and lignin (0.75), and negative of toughness (-0.88) and cellulose (-0.83). This dimension segregated *Arundo* and *Rhododendron* (left) from *Alnus* and *Fraxinus* (right), much softer and richer in nutrients (Figure 3.2). RC2 (35.9% expl. var.) clearly segregated *Arundo* from other litter species due to its high content in hemicellulose (0.97) and silica (0.80) (Figure 3.2).

3.3.3. Effects of subregion and litter species on decomposition rates and fungal biomass

Leaf mass loss (g d^{-1}), corrected for leaching, was fundamentally affected by litter species ($\chi^2 = 278.4$, $P < 0.001$) and much less by region ($\chi^2 = 14.4$, $P < 0.01$), although both factors contributed significantly. Decomposition rates of *Alnus* were between 1.7 to 2.3 times higher than other species (all $P < 0.001$); no significant differences ($P > 0.05$) were detected between *Fraxinus* and *Arundo*; while *Rhododendron* was the species with the slowest decomposition rate (all comparisons $P < 0.001$). In general, decomposition was significantly higher ($P < 0.01$) in the Semiarid lowland (the warmest region) compared to Sierra Nevada (the coldest region). The highly significant interaction region × species ($\chi^2 = 118.1$, $P < 0.001$) revealed that decomposition of each species responded distinctively to the set of environmental characteristics in each subregion (Figure 3.3a, Table S3.3). Thus, while decomposition of *Alnus* showed a sustained increase from the coldest towards the warmest subregion, those of *Fraxinus* and *Rhododendron* were not significantly different between the subregions of intermediate temperature, and that of *Arundo* peaked in subregions with higher pH and water mineral content (Figure 3.3a, Table S3.3).

Decomposition rates computed on a thermal sum basis (g dd^{-1}) were also greatly affected by litter species ($\chi^2 = 147.5$, $P > 0.001$) and less by subregion ($\chi^2 = 54.4$, $P > 0.001$). Both *Alnus* and *Rhododendron* decomposed significantly ($P < 0.001$) faster and slower, respectively, than other species. Overall, significantly ($P < 0.01$) faster decomposition occurred in Sierra Nevada compared to Alcornocales. Interaction region × species ($\chi^2 = 137.8$, $P < 0.001$) remained highly significant as well (Figure 3.3b, Table S3). In this case a trend of increasing rates as regional temperature decreased was observed for the two litter species richer in nutrients (*Alnus* and *Fraxinus*), but greater evenness of rates among subregions were detected for more recalcitrant litters, although *Arundo* showed the lowest rate in Alcornocales.

As it was the case for decomposition rates, fungal biomass was much more affected by litter species ($\chi^2 = 169.8$, $P < 0.001$) than by subregion ($\chi^2 = 8.3$, $P = 0.041$). Significantly (all $P < 0.006$) higher biomass was measured in *Fraxinus*, followed by *Rhododendron*, *Alnus* and *Arundo*. Differences among subregions were mainly related with a trend of significantly higher biomass in the Semiarid lowland in all species, except in *Alnus* (Figure

3.3c), although not all species showed the same trend of biomass variation across subregions (species × region: $\chi^2 = 479.2$, $P < 0.001$) (Figure 3.3c, Table S3).

3.3.4. Intrinsic and extrinsic drivers of decomposition rates and temperature sensitivity

Random Forest (RF) regression selected litter N:P ratio as the most influential environmental variable on decomposition rate (g d^{-1}), followed by temperature, nitrate concentration in water, and leaf C:N ratio and toughness (Table S3.4). However, when the species with the highest N:P ratio (*Alnus*) was removed from RF regression, temperature was selected as the most important variable, followed by stream water N concentration (NO_3^-N and TN), and of secondary importance, litter hemicellulose, fungal biomass and stream elevation (Table S3.3). In the linear mixed-effects model, including the four litter species and the independent variables selected by the RF model, marginal R^2 (i.e. variance explained by fixed factors) equalled 0.66, representing 93% of conditional R^2 (0.71) (i.e. variance explained by fixed and random factors). Litter N:P had the highest positive effect on decomposition rate, followed by temperature and NO_3-N, with negative effects from litter C:N, although marginally significant (Table 3.1). The interaction between temperature and NO_3^-N concentration in water was negative but not statistically significant, indicating a lack of synergistic effects on decomposition (Table 3.1, Figure S3.1). However, the interactions of litter C:N ratio with both extrinsic factors (temperature and NO_3^-N) had positive and significant effects, suggesting accelerated decomposition of the more recalcitrant species (higher C:N ratio) as each of the other two environmental variable increases.

Table 3.1. Results of linear mixed-effects model to explain leaf mass loss (mg d^{-1}, n = 295) using selected (by RF regression) independent variables of litter quality (molar ratios C:N and N:P) and environment (stream water temperature and NO_3-N).

Factor	Estimate ± SE			χ^2	P-value
Intercept	0.084	±	0.089	0.87	0.35
Temperature	**0.347**	**±**	**0.093**	**14.02**	**<0.001**
NO_3-N	**0.427**	**±**	**0.186**	**5.30**	**0.021**
Litter C:N	-0.112	±	0.058	3.79	0.052
Litter N:P	**0.472**	**±**	**0.058**	**66.49**	**<0.001**
Temperature × NO_3-N	-0.110	±	0.087	1.60	0.206
Temperature × litter C:N	**0.103**	**±**	**0.042**	**5.82**	**0.016**
NO_3-N × litter C:N	**0.088**	**±**	**0.042**	**4.36**	**0.037**

Degrees of freedom of each independent variable = 1

Table 3.2. Summary of t-test results performed on results of additional decomposition at home (ADH) for each species. A positive HFA effect is detected if $ADH_i > 0$; no HFA effect exists if $ADH_i = 0$; and litter decomposition at home occurred slower than expected if $ADH_i<0$.Degrees of freedom: $n = 3$ in all cases.

	LML (mg d^{-1})		LML (mg dd^{-1})		Unexplained LML (mg d^{-1}) (residuals)	
Species	*t*-value	*P*-value	*t*-value	*P*-value	*t*-value	*P*-value
Alnus	-3.03	0.06	**5.01**	**0.02**	**-3.20**	**0.049**
Fraxinus	1.10	0.35	-0.58	0.60	0.047	0.67
Rhododendron	-1.17	0.33	0.38	0.73	-0.76	0.50
Arundo	2.87	0.06	2.66	0.08	**4.13**	**0.03**

The apparent activation energy (E_a) of litter decomposition of all species pooled was 0.47 ± 0.07 eV, representing 72% of the value (0.65 eV) predicted by metabolic theory (MTE) (Figure 3.4). Litter quality clearly affected E_a. Species with higher nutrient content showed E_a values about half of those predicted by MTE, while species of low litter quality showed E_a closer to the predicted values (*Rhododendron* 89%, *Arundo* 100%) (Figure 3.4).

3.3.5. Home Field Advantage

Additional decomposition at home (ADH) calculated using time-based decomposition rate (g d^{-1}) was not statistically significant for any of the four species, even though mean decomposition rate of *Arundo* at home (Semiarid lowland) was 3.7 times higher than elsewhere (Figure 3.5, Table 3.2). ADH computed for decomposition rate on a thermal-sum basis (mg dd^{-1}), showed that only *Alnus* decomposed significantly faster at home (Sierra Nevada); also *Arundo* showed higher decomposition at home, although marginally significant (Figure 3.5, Table 3.2). When ADH was calculated using residuals of the linear mixed effects model (i.e.; when the effects of influential environmental and litter trait variables had been removed), *Arundo* decomposed significantly faster in its native range (Figure 3.5, Table 3.2), but *Alnus* decomposed significantly slower in its subregion of origin (Sierra Nevada) (Figure 3.5, Table 3.2). Overall, *Arundo* was the only species that notably and consistently decomposed faster at home across approaches for estimating ADH.

3.4. Discussion

Leaf litter decomposition, a key ecosystem process in forested streams, is controlled by litter traits, the environment, and possible functional specialisations of decomposers, with complex scale-dependent interactions which make process rates greatly context-dependent (Woodward et al. 2012, Boyero et al. 2016, Bradford et al. 2016). In addition,

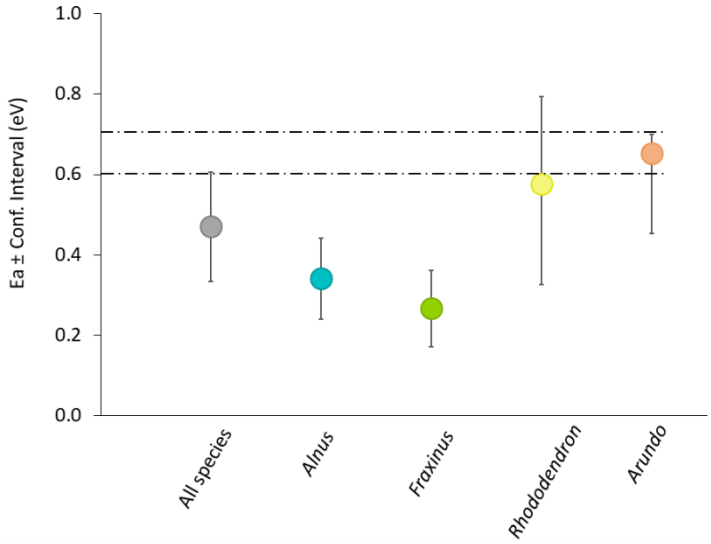

Figure 3.4. Temperature sensitivity (Ea ± CI, eV) for each leaf litter species estimated from mean decomposition in each stream (n=16).

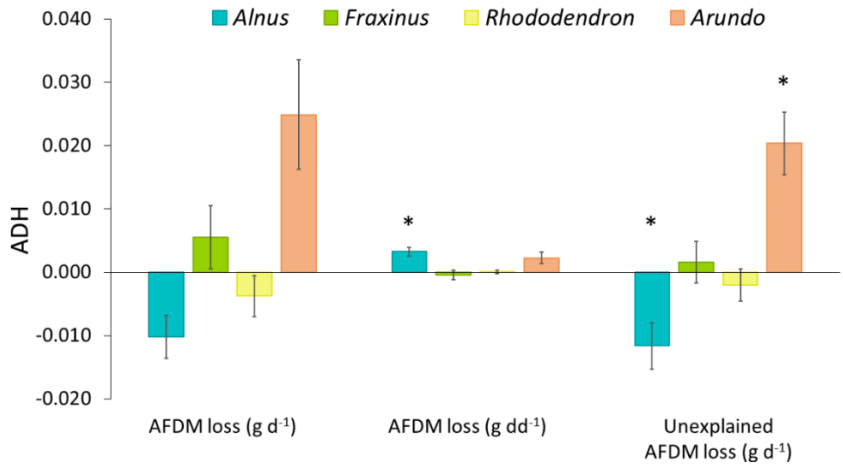

Figure 3.5. Additional decomposition at home (ADH) calculated for each species (mean ± SE) using decomposition rates computed by days, degree days or residuals from regression analysis (on their original units). Estimates that differ significantly from zero ($P < 0.05$) are indicated by an asterisk.

the contrasting and limited number of substrates and different methodologies used across studies, have often hindered the elucidation of key drivers of decomposition in streams (Canhoto et al. 2016), which are still under debate (Zhang et al. 2019). Thus, determining the dominant factors controlling litter decomposition, their relative influence and interactions, is challenging but critical in the face of ongoing global changes.

Our findings support the hypothesis of a leading role of litter traits on decomposition, in accordance with stream studies at regional (Casas et al. 2013, Four et al. 2019) and global (Zhang et al. 2019) scales, and soil ecosystems at global scale (Zhang et al. 2008, Makkonen et al. 2012). Overall, these studies conclude that high N (and related C:N and N:P ratios) and/or low lignin, also reported in local and laboratory studies (Schindler and Gessner 2009, Fernandes et al. 2012, Jabiol et al. 2019) are the main litter traits boosting decomposition. Our results highlight a major role for litter N, but not for lignin. This is likely related to the use in our study of deciduous and evergreen species differing much in N than in lignin, and to the inclusion of a slow-decomposing but lignin-poor and cellulose-rich giant gramminoid species, a functional group rarely considered in leaf litter decomposition studies in streams (see review by Zhang et al. 2019). Thus, litter N content and, particularly, its related C:N and N:P molar stoichiometric ratios, seem to be more suitable and widespread traits to predict decomposition of diverse functional types whose level of recalcitrance may depend on different structural (lignin, cellulose, Si…) compounds.

When *Alnus*, containing 2.6-6.4 times as much N as other species, was omitted from regression analyses, environmental variables—temperature and NO_3^- concentration—emerged as the most important drivers of decomposition. Given the key role of N stimulating microbial activity (Jabiol et al. 2018), when the intrinsic content of this nutrient is not limiting, decomposition is high regardless of extrinsic factors. Mean *Alnus* decomposition rate was indeed about twice that of other species. As pointed out by Canhoto et al. (2016), the use of "extreme" quality litters can bias results on drivers of decomposition, in our case changing the relative importance of environment and litter traits. Additionally, this result highlights the prime role that environmental drivers have on leaf litter decomposition of most plant species (i.e., those that are non N-fixers).

Our results point to significant effects on decomposition from two major global change driving factors—temperature and streamwater nitrate—but negligible effects from streamwater environmental factors—EC or alkalinity—dependent on basin rock lithology. Elevated water mineral content has previously been found to lower litter decomposition rates in streams (Reice and Herbst 1982). However, although some of our subregions showed levels 30 and 14-fold higher of EC and alkalinity, respectively, than others, this was insufficient to curb leaf-litter decomposition. Perhaps the levels of these two factors were not sufficiently elevated to counteract the strong and positive effect of temperature

on leaf-mass loss. As expected, elevated temperature significantly accelerated decomposition, likely due to increased metabolic rates of microbial decomposers (Dang et al. 2009), since a relationship between fungal biomass and leaf-mass loss was not observed, as reported in other studies (Bärlocher et al. 2013; but see Gessner and Chauvet 1994). This is consistent with results of a global study concluding that decomposition in streams is highly sensitive to temperature, more than in adjacent (riparian) terrestrial habitats, where moisture limitation may become a prime factor (Tiegs et al. 2019). Furthermore, in support of our second hypothesis, temperature sensitivity of decomposition depended on litter quality (Follstad Shah et al. 2017, Amani et al. 2019). The "temperature-quality" hypothesis suggest that temperature sensitivity of decomposition should be inversely related to litter quality (Bosatta and Ågren 1999, Davidson and Janssens 2006). The temperature sensitivity for all species pooled (E_a = 0.47 ± 0.07 eV; mean ± SE) was relatively low compared to that predicted by MTE (E_a between 0.6-0.7 eV; Brown et al., 2004). However, when activation energy (E_a) was calculated by species, the two less recalcitrant species —*Alnus* and *Fraxinus*— showed values approximately half of those measured for the two more recalcitrant species —*Rhododendron* and *Arundo*—, these last with E_a values rather close to the MTE prediction.

Studies have reported synergistic effects of temperature and dissolved nutrients (i.e. N) on leaf litter microbial decomposition (Ferreira and Chauvet 2011a, Martínez et al. 2014, Moghadam and Zimmer 2016), which have been attributed to increased nutrient-use efficiencies by decomposers at higher temperatures (Fernandes et al. 2014). However, we did not detect synergy (no significant interaction temperature × dissolved nutrients), despite our streamwater NO_3-N concentrations were similar to those in microcosms (Ferreira and Chauvet 2011a, Fernandes et al. 2014) or field studies (e.g., Woodward et al. 2012), and thus were sufficiently high to be able to elicit synergy with temperature on decomposition. What we observed, instead, was additive effects of temperature and NO_3-N, with a greater role of temperature stimulating decomposition compared to NO_3-N (compare the magnitude of the coefficients on Table 1). In support of our second hypothesis, both factors exerted more (cumulative) influence on low-quality litters (significant positive interactions temperature × litter C:N and NO_3-N × litter C:N). This is coincident with a recent meta-analysis reporting greater magnitude of nutrient-enrichment effects on decomposition of low-quality litters (Ferreira et al. 2015). As streamwater temperature and NO_3-N concentration are increasing at the global scale, it would be expected a particular acceleration of microbial decomposition in streams receiving litter inputs of relatively low quality, namely in warmer regions (Boyero et al. 2017, Salinas et al. 2018). On the other hand, if concomitant changes in litter quality occur in a particular location—i.e. decreasing litter quality as a consequence of increasing atmospheric CO_2 and temperature (Ainsworth and Long 2004, Way and Oren 2010)—the effect of extrinsic and intrinsic factors affecting microbial decomposition in streams might be counterbalanced to some extent (Fernandes et al. 2012). However, if atmospheric changes

lead to only slight variation in litter quality (Ferreira and Chauvet 2011b)—e.g. intra-specific changes instead of those associated with species replacement—litter decomposition will be fundamentally accelerated by rising streamwater temperature and NO_3-N concentration, likely with greater consequences in cold oligotrophic headwater reaches (Ferreira and Chauvet 2011a, Ferreira et al. 2015).

Our third hypothesis suggesting higher decomposition rates in the native range of each leaf litter species—'HFA' hypothesis (Hunt et al. 1988, Gholz et al. 2000)—was not supported for all species, regardless of the approach measuring additional decomposition at home (ADH). The exception was *Arundo*, the species of lowest quality, which showed faster decomposition at home (Semiarid lowland subregion), systematically across all measurement approaches, being statistically significant (LMM residuals) or marginally significant (k d^{-1} and k dd^{-1}). This could be attributed to a specialized microbial community decomposing more efficiently this low-quality species in the Semiarid Lowland, in accordance with many studies concluding that HFA is stronger for more recalcitrant litter because it requires more specialised decomposers (see review in Palozzi and Lindo 2018). The high Si content in Poaceae species strongly binds with cellulose in the form of polymerised silicic acid (silica gel), determining a silico-cellulose cell membrane (Ma and Yamaji 2006), that could restrict the accessibility of enzymes to cellulose fibres (Fenoy et al. 2016). However, recalcitrance should not be the only cause of the observed HFA in *Arundo*, since HFA was not observed in the recalcitrant *Rhododendron*. In addition to be recalcitrant, *Arundo* has sharp contrast in leaf traits (higher Si and cellulose contents) compared to the other three/shrub species assayed here that were much richer in lignin. One of the main tenets of the HFA hypothesis is that increasing dissimilarity in ecosystem properties and litter traits is a fundamental driver of HFA outcomes (Palozzi and Lindo 2018), suggesting a substrate quality-matrix quality interaction (SMI hypothesis) (Freschet et al. 2012). This hypothesis predicts an increasing positive interaction (facilitating microbial decomposition) between a given litter species and decomposer community as the concerned litter species and the ecosystem litter layer (the matrix driving local decomposer assemblages) become increasingly similar in quality. Following this argument, and considering that HFA results in relative estimates of strength, it seems logical having found HFA in *Arundo*, whose native range are streams with riparian vegetation overwhelmingly dominated by this invasive species along with other Poaceae species such as *Phragmites australis* (Cav.) Steud and *Tripidium ravennae* (L.) H. Scholz (Casas et al. 2011, Salinas et al. 2018). While, the native ranges of the other three species are more similar to each other, dominated by trees/shrub, whose level of litter recalcitrance greatly depend on lignin:N (weak litter quality-matrix quality contrast across them).

In addition to the differences/similarities in the litter matrix among native ranges, these also differed greatly in other environmental conditions. Some of the inconsistencies emerging in HFA studies have been linked to the strongly contrasting environmental

conditions, mostly associated with climate or nutrient availability (Austin et al. 2014 and references therein). That is why we have evaluated HFA using model residuals (unexplained variance of decomposition values) from the multiple regression analysis, which included environmental characteristics as independent factors. This estimate of decomposition could reflect patterns derived from a possible litter matrix-microbial community adaptation, once abiotic factors have been controlled for. This approach, indeed, gave the clearer and statistically significant HFA for *Arundo*, suggesting a true-positive HFA. Nonetheless, we must point out that interactions of environmental conditions with litter quality are not always considered in most HFA studies, and that these could contribute to a false, positive or negative, HFA (Palozzi and Lindo 2018), especially if the results are not confirmed reciprocally (e.g. all plants showing HFA in all allochthonous locations). One drawback of our study is that due to the large climatic and biogeographical differences among the four subregions we could not reciprocate the same plant species (or genera) among subregions. However, accounting in the models for most of the environmental and litter quality drivers explaining decomposition abroad is an alternative that we find valid to, at least tentatively, test for regional HFA effects.

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Chapter 4

Climate warming might impair functional but not taxonomic diversity of fungal assemblages decomposing leaf-litter in headwater streams

Encarnación Fenoy

Cláudia Pascoal
Fernanda Cássio
Arunava Pradhan
Daniela Batista

Juan Rubio-Ríos
Francisco J. Moyano-López
J. Jesús Casas

4.1 Introduction

Global change alterations of biogeochemical cycles and habitats (Steffen et al. 2015), are triggering shifts in community composition through changes in species distribution and/or extinctions (Pereira et al. 2010). Warming and species loss are predicted to increase simultaneously, exerting adverse effects on ecological processes; being the second particularly relevant, given the potential dependence of ecosystem functioning on biodiversity (biodiversity-ecosystem function hypothesis; hereafter B-EF) (Loreau et al. 2001, Cardinale et al. 2002, 2006, Hooper et al. 2005, 2012). Despite mounting evidence in support of the B-EF relationship, ecosystem functioning enhancement with species richness greatly depends on functional differences among the species and on the environmental context that may change species roles and interactions (Fetzer et al. 2015). Accordingly, functional diversity of an assemblage might be a more suitable predictor of ecosystem functioning than its taxonomic diversity (Reiss et al. 2009, Woodward 2009).

Headwater streams are particularly vulnerable to global change related stressors (Woodward et al. 2010, Reid et al. 2019). These ecosystems in forested areas greatly depend on terrestrial plant derived detritus, primarily leaf-litter (Webster et al. 1999), where aquatic hyphomycetes are key intermediaries of energy flow and nutrients to higher trophic levels (Gessner et al. 2010, Graça et al. 2015). Therefore, compositional alterations and/or species losses in hyphomycete assemblages as a consequence of global change stressors may have consequences on leaf-litter decomposition and nutrient cycling in stream food webs. Warming (Bärlocher et al. 2008, Ferreira and Chauvet 2011a, Fernandes et al. 2012) and increasing nutrient concentration in stream water (Duarte et al. 2009, Jabiol et al. 2018) can alter fungal assemblage composition and/or diversity in streams. Moderate increasing concentration of dissolved nutrients can reduce competition and allow the coexistence of more species (Gulis and Suberkropp, 2003, Pereira et al. 2016), but richness decreases at high levels of eutrophication (Duarte et al. 2015). Disagreement exist, however, regarding the effect of temperature on taxonomic diversity of decomposing fungi in streams (Bärlocher et al. 2008, Dang et al. 2009, Fernandes et al. 2012, Duarte et al. 2016a), and a recent study embracing a large latitudinal and thermal gradient have failed to establish clear effect of higher temperature on fungal richness at low latitudes (Seena et al. 2019). Furthermore, the B-EF relationship of hyphomycete diversity-litter decomposition remain fundamentally unsupported since often similar decomposition rates are performed by assemblages differing in composition and species richness (Bärlocher and Graça 2002, Dang et al. 2005, Pascoal et al. 2005, Ferreira and Chauvet 2012, Ferreira et al. 2016, 2019; but see Duarte et al. 2006, Pascoal et al. 2010), thus giving rise to the common perception of a high functional redundancy (Walker 1992) among hyphomycete species.

Despite the recognized importance of biodiversity in all its forms for the overall ecosystem functioning (Giller et al. 2004, Gessner et al. 2010), most studies on litter decomposition in streams have addressed this issue fundamentally by manipulating fungal assemblages—altered richness and composition in lab experiments (e.g. Geraldes et al. 2012, Gonçalves et al. 2015)—or by means of comparisons of reference *vs.* impacted sites with reduced taxonomic diversity (e.g. Pascoal et al. 2005, Duarte et al. 2008). A different approach, the *in vitro* physiological profiles, has been used in terrestrial environments (e.g. Pinzari et al. 2016, Rinnan et al. 2009) since Dobranic and Zak (1999) proposed a microarray method to study fungal functionality. This method directly measures fungal assemblage functionality and avoid the use of surrogate metrics to estimate functional diversity. This procedure uses carbon-sources utilization profiles as a mean of extracting fungal functionality data from an array of carbon substrates, representing a standardized measure that allows functional comparisons across a variety of ecosystems (Sobek and Zak 2003). This technique has been used in headwater streams to address bacterial functionality (Ylla et al. 2014, Gionchetta et al. 2020), but to our knowledge it has not yet been used to test the metabolic functionality of fungi decomposing leaf-litter in streams.

In this study we hypothesize that 1) increasing stream-water temperature and dissolved nutrients will lead to reduced diversity and 2) to altered species composition of fungal assemblage in decomposing leaf-litter. We also hypothesize that 3) functional diversity would be maintained across different environmental settings and litter substrates due to a presumed high functional redundancy among fungal species. To test these hypotheses, we performed a field decomposition experiment in four Mediterranean subregions differing in stream-water temperature and chemistry, and riparian plant composition. We used four leaf-litter species contrasting in traits, one species characteristic from each subregion, that were reciprocally incubated across subregions. Taxonomic and functional diversities were measured and analyzed looking for potential drivers of change in fungal decomposers biodiversity-decomposition relationship.

4.2. Material and methods

4.2.1. Study area

We studied streams (first to second order) from four subregions in Andalusia (Southern Spain): Sierra Nevada, Sierra de Cazorla, Alcornocales, and the Semiarid Lowland of Almería. These areas are under different nature protection status and show marked biogeoclimatic contrast. Riparian vegetation is dominated by deciduous tree species in the coldest subregions (Sierra Nevada and Sierra de Cazorla), by a mixture of deciduous trees and broad-leaf evergreen shrubs in Alcornocales, and by giant gramminoids in the Semiarid Lowland of Almería (Salinas et al. 2018) (see Chapter 3 for further information).

4.2.2. Environmental characterization of streams

Four permanent streams were selected in each one of the four subregions (total 16 streams). Electrical conductivity (EC), pH and dissolved oxygen were measured in each stream with a multiparametric probe (HACH® model HQ-30d, Loveland, CO, USA). Water samples were collected to measure alkalinity, total dissolved nitrogen and phosphorus, NO_3^--N and soluble reactive phosphorus (SRP) by standard methods (see methodology description in Chapter 3). All measures were performed at the beginning and the end of the field experiment (see below). Water temperature was recorded hourly with HOBO Pendant® loggers (Onset Computer Corporation, Bourne, MA, USA) during the full period of leaf-litter incubation.

4.2.3. Leaf-litter quality and field experiment

We selected one native and/or dominant species from each one of the four subregions to perform a decomposition experiment by reciprocal incubations across subregions. The four species widely differed in litter traits: two deciduous species—the nitrogen-fixer alder (*Alnus glutinosa* [L.] Gaertn.) from Sierra Nevada and ash (*Fraxinus angustifolia* Vahl) from Cazorla—the broadleaf evergreen shrub rhododendron (*Rhododendron poncticum* subsp. *baeticum* [Boiss. & Reut.] Hand.-Mazz.) from Alcornocales, and the graminoid giant cane (*Arundo donax* L.) from the Semiarid Lowland of Almería. Recently abscised leaves of each species were collected in autumn 2016 in the corresponding subregion. To characterize initial litter quality, we determined leaf toughness, specific leaf area (SLA), percentage of fiber (hemicellulose, cellulose and lignin), and nitrogen (N), phosphorus (P) and carbon (C) concentrations (see Chapter 3 for methods).

Portions (5.0 ± 0.05g dry mass) of each leaf-litter species were spray moistened and individually introduced in bags (15×20 cm;1-mm mesh size). Leaf bags—5 bags per species and stream—were incubated tied to iron stakes anchored to the stream bed in riffles along a 50-m stream reach. After 40 days, leaf bags were retrieved and placed individually in zip-lock bags, and transported to the laboratory in an icebox. There, leaves were removed from the bags and carefully rinsed with filtered stream water to eliminate fine particles. Then, leaves were oven-dried (70 ºC, 72 h) and weighed to the nearest 0.1 mg. Thereafter, litter was grinded to pass a 1 mm screen; a portion was ignited at 500ºC for 5 h to estimate ash-free mass loss (weighted to the nearest 0.1 mg). Decomposition was expressed as ash-free leaf-mass loss per day (LML mg d^{-1}) and per degree-day (LML mg dd^{-1}) (see chapter 3).

An additional set of 5 litter bags per species and stream, incubated and processed as above, was used to measure taxonomic and functional diversity. After retrieval from stream and elimination of fine particles, a portion of leaf-litter from each bag was frozen (-80 ºC)

until analyses of taxonomic diversity were performed, and other portion was immediately used for functional diversity analyses (see below).

4.2.4. Fungal taxonomic diversity

Taxonomic diversity of fungal assemblages was assessed as the number of operational taxonomic units (OTUs) from denaturing gradient gel electrophoresis (DGGE) according to Duarte et al. (2010). DNA was extracted from 25 leaf discs (1 cm Ø) per sample—5 discs from each one of the 5 bags per species and stream were pooled— using an Ultra Clean Soil DNA isolation kit (MoBio Laboratories, Solana Beach, CA). Fungal diversity was assessed using the primer pair ITS3GC/ITS4, which amplifies the ITS2 region of fungal rDNA. PCR reactions were carried out using the following program: initial denaturation (95°C, 2 min), followed by 36 cycles of denaturation (95°C, 30 s), primer annealing (55°C, 30 s) and extension (72°C, 1 min) in iCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). Final extension was done at 72°C for 5 min. DGGE analyses were performed using a DCode™ Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA). Amplified DNA (20 µL per sample) were loaded on 8% (w/v) polyacrylamide gel in 1x Tris-acetate-EDTA (TAE) with a denaturing gradient from 30 to 70% (100% denaturant corresponds to 40% formamide and 7 M urea). Gels were run at 55 V and 56°C for 16 h and stained with Midori Green (Grisp) for 10 min on a shaker at 40 rpm. Gel images were captured under UV light in a ChemiDoc XRS (BioRad Laboratories, Hercules, CA, USA). Digitized DGGE images were analysed using the BioNumerics software v5.0 (Applied Maths, Sint-Martens-Latem, Belgium). A matrix was constructed taking into account the relative contribution of each band (as a percentage) to the total intensity of the band line and the number of bands detected in each sample.

4.2.5. Community-level physiological profiles (CLPP)

We evaluated fungal functional diversity using the Soil FungiLog procedure described by Sobek and Zak (2003) with some modifications. Aliquot portions of litter from leaf bags (n = 5) of the same species incubated in each stream were pooled. Each pool was wet grinded with a blender, in filtered (0.45 µm pore size) water from the corresponding stream on an ice bath, and wet sieved through 500 and 250 µm mesh, collecting the material from the 250µm mesh sieve. We standardize inoculum by leaf-litter biomass (Preston-Mafham et al. 2002). We previously estimated (data not shown) the wet weigh of leaf-litter particles that resulted in initial optical density (OD) lower than 0.25 (Sobek and Zak 2003). Leaf-litter particles were added to a sterilized solution of agar (15 mL, 0.2% w/v), made with filtered stream water containing 200µL of an antibiotic stock solution (150 mg of streptomycin sulphate and 75 mg of chlortetracycline hydrochloride in 15 mL of sterile distilled water) to prevent bacterial activity. This was prepared, and gently agitated to obtain a homogenized suspension, immediately before microtiter plate inoculations. A volume of 100 µL of this mixture was transferred to each well in the Biolog FF microtiter

plates (Biolog, Hayward, California) using an eight-channel micropipette pump fitted with large orifice pipette tips.

The FF microtiter plates were incubated at the mean winter temperature of each subregion. OD measures (490 nm) of each plate were taken immediately after inoculation and every 24 h until an OD of 2 was reached in wells with higher colour development. Potential differences in the OD_{490} among individual wells were accounted for by subtracting the initial OD_{490} from values measured along the incubation period, allowing obtain net OD_{490} changes for each well. To account for the effect of temperature on colour development rates, data at a predefined average well colour development value of 0.15 (AWCD, i.e. the sum of differences between initial lecture and further lectures divided by 95) (Rinnan et al. 2009) was selected for the analysis of substrate utilization patterns.

4.2.6. Statistical analyses

Sample richness and Shannon's diversity were calculated for taxonomic (OTUs) and functional (CLPP) data and assessed for differences among subregions and leaf-litter species using two-way ANOVA with *Anova* function from 'car' package. Pairwise comparisons of level means for a given factor within each level of the other factor were performed using the *multicomp* and *lsmeans* packages in R.

We regressed the dependent variables, taxonomic and functional α-richness of samples (litter species/stream), against the independent environmental variables and leaf-litter traits using general linear models. A subset of environmental variables most related to global change drivers in stream-water (temperature, NO_3^-, and SRP) were used as predictors. Electric conductivity (EC) was also kept in the analysis because of its high inter-subregional (even intra-subregional) variability. Leaf traits were summarized using the first two rotated components of a PCA (RC 1 and RC 2 henceforth) performed in Chapter 3, where RC 1 was positively related with litter nutrient concentration (N and P) and negatively with toughness and cellulose, and RC 2 was positively related with hemicellulose and silica concentrations. Geographical distance and location of samples were included in exploratory initial models, but these variables were not retained. Models fit and parsimony were evaluated through the Akaike information criterion (AIC). We examined multicollinearity of the variables to be included in the models with the variance inflation factor (VIF) using the *vif* function in the R package 'car'. Normality of residuals was examined with Shapiro–Wilk's test. When necessary, we used Box-Cox transformation to achieve the normal distribution of residuals.

We conducted non-metric multidimensional scaling (nMDS) analyses to examine the dissimilarity, using Hellinger distance, among samples across subregions and leaf-litter species, using as criteria taxonomic composition (based on a sample-by-OTUs % abundance community matrix) and functionality (based on a sample-by-CLPP optical

densities community matrix). To test for significant differences among subregions and litter species we used the *adonis* function (with n= 999 permutations) in the 'vegan' package, which carries out a permutational MANOVA based on the dissimilarity matrix.

We calculated overall β-diversity (β_{Total}), separately for taxonomic and functional data, using the Hellinger distance as a measure of dissimilarity among pairs of samples (litter species/stream). To understand factors affecting environment-litter species related variation of fungal taxonomic or functional diversities, first we determined the local (sample) contribution to β_{Total} diversity (LCBD), for each type of data, following Legendre and De Cáceres (2013). In short, LCBD measures the relative ecological uniqueness of each sample based on the total variance in the community matrix, which is the total sum of squares divided by n-1. Then, we evaluated whether leaf traits (RC 1 and RC 2) and the environmental variables related to global changes stressors (the same used for α-diversity, see above) affected both taxonomic and functional LCBDs. To this end, we built a general linear model (*glm*), where LCBD was the response variable with leaf-litter traits and environmental variables were predictors. The model was assessed with regards to normally distributed errors and multicollinearity (as for α-diversity, see above). β_{Total} and LCBD were computed with function *beta.div*() from 'adespatial' package.

Finally, we tested the relationships of litter mass loss (AFDM loss, mg d^{-1} and mg dd^{-1}) with richness and Shannon's diversity, both taxonomic and functional, using linear models, after checking model assumptions (see above). All regressions were performed for the overall data set after log-transformation of dependent variables. All analysis were performed in R (R Core Team 2018), except NMDS that was performed in PRIMER 6 (Primer-E Ltd., Plymouth, UK; Clarke and Gorley, 2006).

4.3. Results

4.3.1. Stream-water characteristics

Subregions greatly differed in stream environmental characteristics, particularly in temperature, EC, alkalinity and nutrients (Table S3.1 in Annexes). Water chemistry was strongly related with lithology: calcareous subregions (Cazorla and Semiarid Lowland) showed higher pH, and particularly higher EC (18-fold) and total alkalinity (~10-fold) than siliceous ones (Alcornocales and Sierra Nevada). There was also 3- and 4-fold higher TN and NO_3^--N concentrations, respectively, in calcareous than in siliceous subregions, the last, on the contrary, generally showed 1.5- to 2-fold higher phosphorus concentrations (Table S3.1 in Annexes).

4.3.2. Leaf-litter traits

Litter species differed significantly in N concentration, being *Alnus* the species with higher concentration, doubling *Fraxinus* concentration, this latter with 1.5- and 2.5-fold higher N concentration than *Rhododendron* and *Arundo*, respectively (see Table S3.2 in Annexes). *Arundo* showed 1.8-fold more concentration of cellulose and >50-fold higher silicon concentration, which could explain its higher toughness, but lower C, P and lignin concentrations (1.3-, 1.6- and 4.6-fold, respectively) than the other litter species (see Table S3.2 in Annexes).

4.3.3. α-richness and Shannon's diversity variations across subregions and litter species

Taxonomic richness and Shannon's diversity did not differ among subregions or leaf-litter species, and their interaction was not significant either (all $P > 0.057$; Table S4.1; Figure 4.1). On the contrary, functional richness differed significantly among subregions ($F_{3,48} = 5.54$, $P = 0.002$), being on average 8% higher in the coldest (Sierra Nevada and Cazorla) compared to the warmest (Alcornocales and Semiarid Lowland) subregions (post-hoc, all $P < 0.001$) (Figure 4.2a, Table S4.2). Leaf-litter species had less effect, although significant, than subregions, with functional α-diversity just differing among *Fraxinus* and *Rhododendron* ($F_{3,48} = 3.16$, $P = 0.033$), and a significant interaction litter species × subregion ($F_{9,48} = 2.72$, $P = 0.028$). Similarly, functional Shannon's diversity differed among subregions ($F_{3,48} = 10.21$, $P < 0.001$), being on average 9 and 18% higher in the coldest—Sierra Nevada and Cazorla, respectively—than in the warmest subregions (post-hoc, all $P < 0.001$) (Figure 4.2b, Table S4.2). Lower functional Shannon's diversity was found in *Rhododendron* than in *Fraxinus* ($F_{3,48} = 5.59$, $P = 0.002$). Moreover, the significant interaction litter species × subregion ($F_{9,48} = 2.69$, $P = 0.013$), indicated different functional Shannon's diversity within litter species depending on subregion (Figure 4.2b, Table S4.2).

4.3.4. Taxonomic and functional compositional variation across subregions and litter species

Taxonomic composition (OTUs data) was significantly different among all subregions (*adonis*, $F = 4.39$, $R^2 = 0.19$, $P < 0.001$) (Figure 4.3a), but no significant effects of litter species (*adonis*, $F = 0.97$, $R^2 = 0.04$, $P = 0.51$) or interaction (*adonis*, $F = 0.59$, $R^2 = 0.08$, $P = 1.0$) were detected. Subregions (*adonis*, $F = 8.24$, $R^2 = 0.27$, $P < 0.001$) and litter species (*adonis*, $F = 1.63$, $R^2 = 0.05$, $P = 0.029$) were differentiated in terms of functionality (CLPP profiles), but the response varied depending on which subregions the litter species were incubated (*adonis*, $F = 1.56$, $R^2 = 0.15$, $P = 0.006$) (Figure 4.3b). Despite the above differences, taxonomic β_{Total} was much higher (0.81) than functional β_{Total} (0.20).

4.3.5. Predictors of α-richness and sample contribution to β-diversity

Taxonomic α-richness was not significantly affected by any of the predictors ($F_{49,63}$ = 0.92, P = 0.548,), but the overall model for functional richness was significant ($F_{49,63}$ = 8.71, P < 0.001, Table 4.1). Functional richness was negatively affected by temperature and SRP, and positively by NO_3^--N and EC (Table 4.1, Figure 4.4a,b), the last suggesting that high water's ion concentration favours functional richness. Moreover, a large and positive effect of the interaction of temperature with RC1 of leaf-litter traits (positively related to litter N and P concentrations) was significant, indicating that the negative effect of high temperature on functional richness decreases as litter quality increases (Table 4.1, Figure 4.4c). On the contrary, the significant negative interaction between stream-water NO_3^--N and RC 1, suggests that the positive effect of NO_3^--N on functional richness was counteracted in high quality litters (Table 4.1, Figure 4.4d).

The regression model for sample contribution to taxonomic β_{Total} diversity (taxonomic-LCBD) did not include RC 1 and RC 2 of litter traits as predictors (based on AIC criteria). Taxonomic-LCBD was positively affected by temperature (Table 4.2, Figure 4.5a) but negatively by EC and, particularly, by the interaction between NO_3^--N and EC (Table 4.2, Figure 4.5b). Functional-LCBD was significantly affected by temperature and NO_3^-N (strong positive and negative effects, respectively), and by SRP (negative effect) and EC (positive effect) (Table 4.2, Figure 4.6). However, the effects of temperature and NO_3^--N were modulated by litter quality. The negative interactions of temperature with leaf-litter traits, RC1 and RC2, suggest that as litter nutrient (N and P) and relatively labile C (hemicellulose) concentrations increase, the positive effect of temperature on functional LCBD decreases (Table 4.2, Figure 4.6c,d). The significant positive interaction of NO_3^--N with RC1, point to lower contribution of intermediate quality litters to functional LCBD at high NO_3^--N levels (Table 4.2, Figure 4.6e).

4.3.6. Relationship between α-diversity and leaf mass loss

Leaf-litter mass loss as a function of time (mg d^{-1}) did not show a significant relationship with taxonomic richness ($F_{1,62}$ = 0.002; P-value = 0.99) or Shannon's diversity ($F_{1,62}$ = 0.12; P-value = 0.73). Functional richness ($F_{1,62}$ = 0.74; P-value = 0.39) and Shannon's diversity ($F_{1,62}$ = 0.91; P-value = 0.34) neither showed an effect on leaf-litter mass loss measured on a time basis.

When leaf-litter mass loss was expressed in terms of accumulated heat (mg dd^{-1}) showed a significant and positive relationship with functional richness ($F_{1,62}$ = 19.32, P-value < 0.001, RMSE = 0.46, Adj. R^2 = 0.23) (Figure 4.7a) and with functional Shannon's diversity ($F_{1,62}$ = 25.81, P-value < 0.001, RMSE = 0.44, Adj. R^2 = 0.28) (Figure 4.7b). However, no significant relationship was found between litter-mass loss (mg dd^{-1}) and

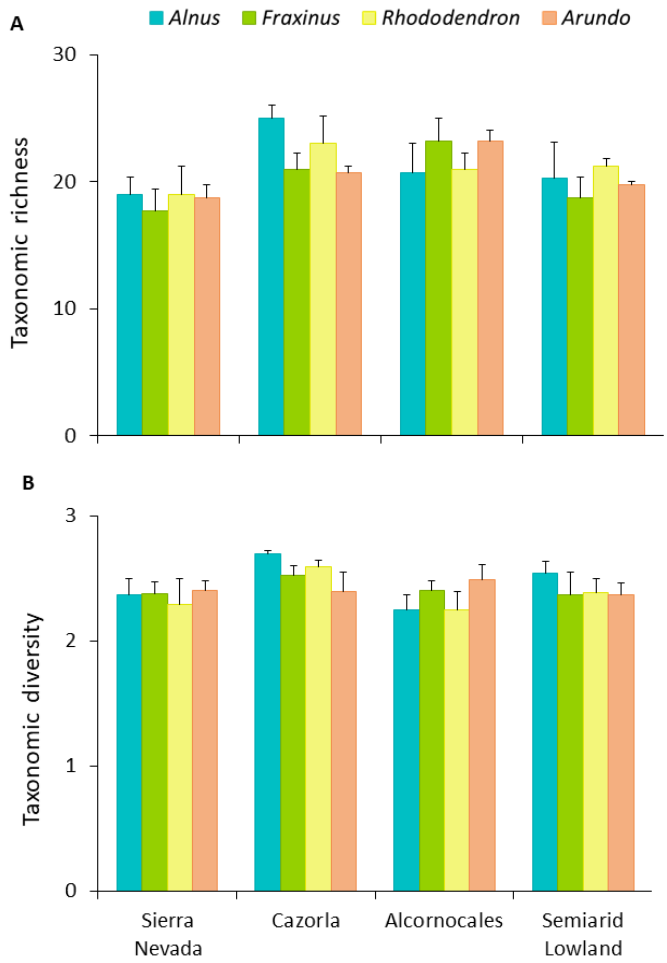

Figure 4.1. Taxonomic A) richness and B) Shannon's diversity index based on OTU data, for the four leaf litter species incubated in the four subregions.

taxonomic richness ($F_{1,62}$ = 2.97; *P*-value = 0.09) or Shannon's diversity ($F_{1,62}$ = 0.26; *P*-value = 0.61).

4.4. Discussion

Understanding how the link biodiversity-ecosystem functioning (B-EF) is affected by global change is a major concern, since species richness is rapidly decreasing as habitat degradation and global warming progress. In headwater streams, the magnitude of effects of putative functional changes in fungal community, caused by global change drivers, on

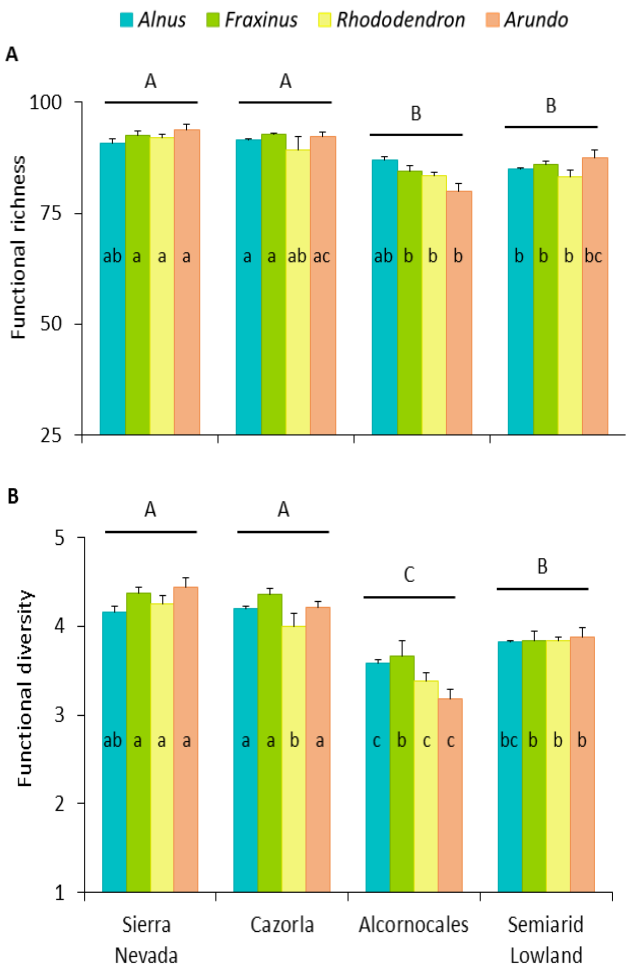

Figure 4.2. Functional A) richness and B) Shannon's diversity index based on CLPP data, for the four leaf litter species incubated in the four subregions.

litter decomposition has been scarcely studied despite the important consequences can trigger for ecosystem functioning.

Our regional-scale field study suggests substantial similarity in taxonomic richness or Shannon's diversity across subregions, even though their marked environmental contrast that could have acted as strong filters for some species. Thus, despite the considerable influence of environmental factors, mainly temperature, on diversity of fungal species, mainly reported in manipulative studies (Bärlocher et al. 2008, Fernandes et al. 2009, 2012, Ferreira and Chauvet 2011b), our first hypothesis was not supported, demonstrating

Table 4.1. Results of general linear model for environmental and litter determinants of functional α-richness. RC: Rotated components of a PCA on foliar traits.

Covariates	Estimate ± SE			*F*-value	*P*-value
Intercept	0.020	±	0.077		
Temperature	**-1.128**	**±**	**0.128**	**77.812**	**< 0.001**
NO_3^--N	**0.409**	**±**	**0.106**	**14.866**	**< 0.001**
SRP	**-0.255**	**±**	**0.083**	**9.345**	**0.004**
EC	**0.283**	**±**	**0.107**	**7.029**	**0.011**
Litter traits-RC1	0.102	±	0.080	1.610	0.210
Litter traits-RC2	0.090	±	0.081	1.244	0.270
Temperature × Litter traits-RC1	**0.409**	**±**	**0.133**	**9.392**	**0.004**
Temperature × Litter traits-RC2	-0.053	±	0.141	0.142	0.708
NO_3^--N × Litter traits-RC1	**-0.344**	**±**	**0.114**	**9.053**	**0.004**
NO_3^--N × Litter traits-RC2	0.090	±	0.107	0.713	0.403
SRP × Litter traits-RC1	0.034	±	0.081	0.182	0.672
SRP × Litter traits-RC2	-0.042	±	0.081	0.261	0.612
EC × Litter traits-RC1	-0.224	±	0.119	3.529	0.066
EC × Litter traits-RC2	0.055	±	0.113	0.241	0.626

AIC = 132.67; R^2 = 0.63; RMSE = 0.53; $F_{49,63}$ = 8.71; ***P*-value < 0.001**

that equally rich and diverse communities may occur in a wide range of temperature (3.4-22.3ºC) and other environmental conditions. These results suggest that streams located in contrasting ecoregions may harbour fungal communities similar in species richness and adapted to local conditions. The results of Seena et al. (2019) studying a large latitudinal gradient (also temperature: 3.1-26.2 ºC) pointed to a hump-shaped relationship between fungal taxonomic richness and absolute latitude, but decreasing richness towards warmer streams at lower latitudes could not be clearly attributed to temperature. Our regional results, which more likely circumvent historical biogeographic biases compared to global studies, highlight the notion that temperature or other stream-water characteristics have minor effects on fungal richness. However, we found that fungal assemblage composition was markedly different among subregions, giving support to our second hypothesis. Fungal compositional differences across subregions were not attributable to geographical distance or location, since these variables were not retained in regression analyses. These differences were indeed primarily attributable to stream-water characteristics, although a certain influence of the marked contrast in riparian vegetation cannot be ruled out (e.g. Bärlocher and Graça 2002, Ferreira et al. 2006a, Laitung and Chauvet 2005, Lecerf and Chauvet, 2008). Nonetheless, leaf-litter species did not significantly affect fungal assemblage composition, as previously reported in studies identifying fungal spores

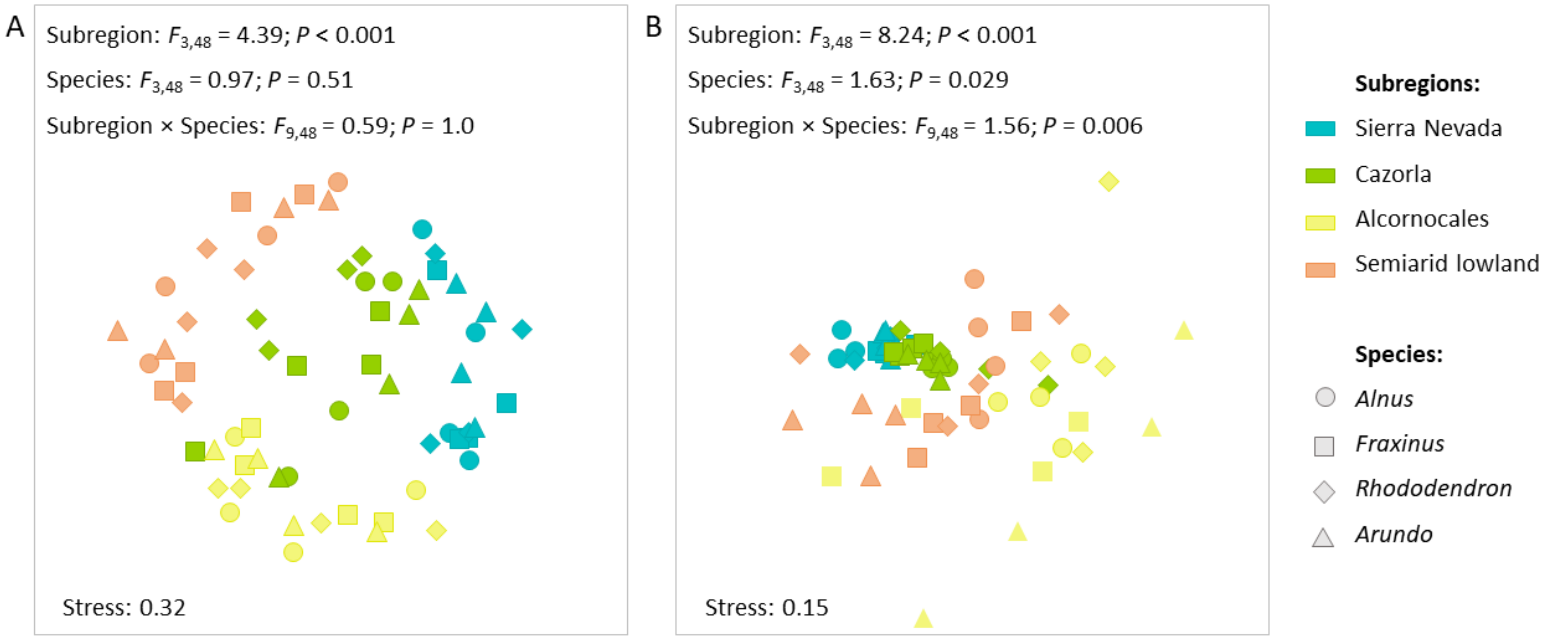

Figure 4.3. Non-metric multidimensional scaling (nMDS) of individual samples (n = 64) for A) OTUs and B) CLPP profiles with statistics from *adonis*. The stress for the 2-D nMDS shown in fungal species panel is 0.32, which is quite high. However, the stress for the 3-D nMDS of these samples is 0.23, which is more acceptable and has highly similar patterns to those shown in the 2-D solution.

Table 4.2. Results of general linear model for environmental and litter determinants of taxonomic and functional sample contribution to β-diversity (LCBD). RC: Rotated components of a PCA on litter traits.

Covariates	Estimate ± SE			*F*-value	*P*-value
Taxonomic β-diversity (taxonomic-LCBD)					
Intercept	-1.75	±	0.64		
Temperature	**1.43**	**±**	**0.61**	**5.46**	**0.023**
NO_3^--N	-0.95	±	0.49	3.80	0.057
SRP	-0.46	±	0.29	2.48	0.121
EC	**-2.58**	**±**	**1.28**	**4.10**	**0.048**
Temperature × NO_3^--N	0.86	±	0.60	2.07	0.156
Temperature × SRP	-0.37	±	0.34	1.20	0.278
Temperature × EC	2.39	**±**	1.27	3.53	0.066
NO_3^--N × SRP	-0.68	±	0.53	1.68	0.200
NO_3^--N × EC	**-1.81**	**±**	**0.57**	**10.11**	**0.002**
SRP × EC	0.75	**±**	0.45	2.73	0.105
AIC = 156.79; R^2 = 0.44; RMSE = 0.68; $F_{53,63}$ = 5.61; ***P*-value < 0.001**					
Functional β-diversity (functional-LCBD)					
Intercept	0.06	±	0.07		
Temperature	**1.33**	**±**	**0.12**	**120.94**	**< 0.001**
NO_3^--N	**-0.70**	**±**	**0.10**	**51.81**	**< 0.001**
SRP	**0.32**	**±**	**0.08**	**18.04**	**< 0.001**
EC	**-0.47**	**±**	**0.10**	**23.60**	**< 0.001**
Litter traits-RC1	-0.06	±	0.07	0.58	0.449
Litter traits-RC2	**-0.21**	**±**	**0.07**	**7.65**	**0.008**
Temperature × Litter traits-RC1	**-0.32**	**±**	**0.13**	**6.33**	**0.015**
Temperature × Litter traits-RC2	**-0.32**	**±**	**0.12**	**6.49**	**0.014**
NO_3^--N × Litter traits-RC1	**0.22**	**±**	**0.10**	**4.74**	**0.034**
NO_3^--N × Litter traits-RC2	0.16	±	0.10	2.32	0.135
SRP × Litter traits-RC1	0.01	±	0.07	0.03	0.856
SRP × Litter traits-RC2	-0.05	±	0.08	0.37	0.546
EC × Litter traits-RC1	0.05	±	0.10	0.21	0.649
EC × Litter traits-RC2	0.17	±	0.10	2.81	0.100
AIC = -479.99; R^2 = 0.67; RMSE = 0.004; $F_{49,63}$ = 9.99; ***P*-value < 0.001**					

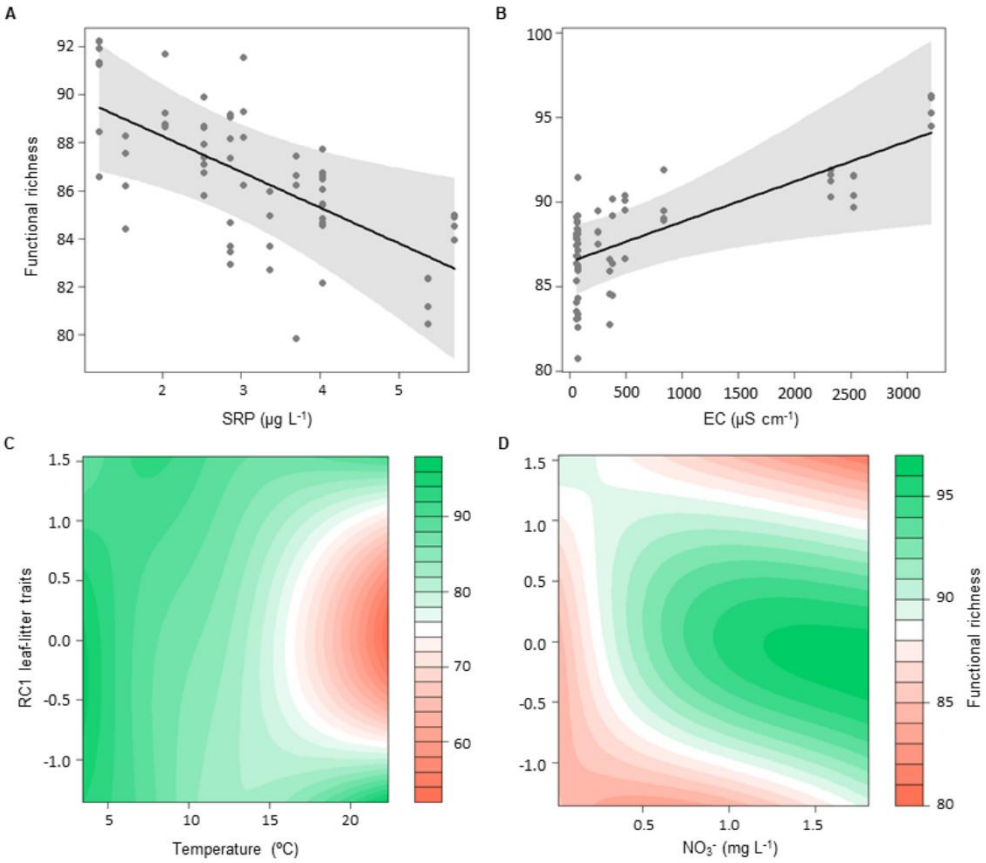

Figure 4.4. Significant main effects (A-B) of phosphorus (SRP) and electric conductivity (EC), and the interaction effects (C, D) of RC1 of leaf-litter traits with temperature and nitrates on functional richness. When a variable had a main effect and an interaction in the same model, only the interaction is shown.

released from different litter species (e.g. Bärlocher et al. 2011), which further strengthen the prime role of stream-water characteristics on species composition. This has been previously reported at regional scale (Solé et al. 2008, Duarte et al. 2009, Bärlocher et al. 2011), even high fungal community similarity have been found between geographically distant locations with similar environmental conditions (Duarte et al. 2016b, Seena et al. 2019). Our results also point to species replacement across subregions—net species turnover given the high similarity in α-richness—as the main component of β-diversity. This is in accordance with evidences indicating that microbial metacommunities are governed to a greater extend by environmental species sorting relative to other assembly rules (i.e. dispersal limitations), particularly when studying broad environmental gradients

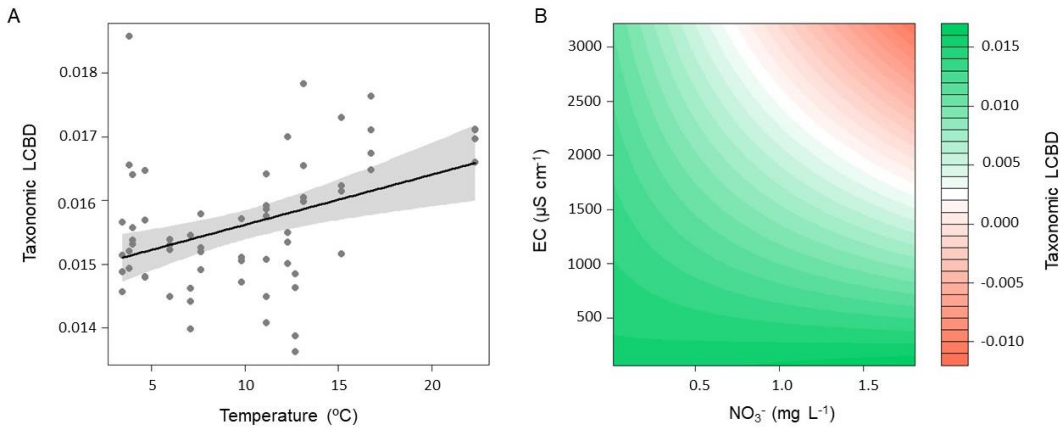

Figure 4.5. Significant (A) main and (B) interaction effects of temperature and NO_3^- interaction with electric conductivity (EC) on local contribution to taxonomic β-diversity (Taxonomic LCBD).

which tend to increase the importance of deterministic versus stochastic processes (Stegen et al. 2012, Zhou et al. 2014, Wu et al. 2018). Fungal taxonomic uniqueness of samples (sample contribution to β-diversity) was mainly driven by temperature with positive effect, but with negative effects from water mineral and NO_3^--N concentrations, particularly by the interaction of these last two variables. This suggest rather complex offsetting effects of global change drivers (temperature and NO_3^--N) on fungal community composition in stream ecosystems. Thus, while warming might boost sample uniqueness—increasing β-diversity—likely promoting the replacement of cold-stenotherm by relatively thermophilic species, by contrast, nutrient enrichment, particularly in streams with relatively high conductivity, tend to homogenize fungal assemblages. Increasing nutrient availability has been reported to produce stochasticity in community assembly (Chase 2010), but its consequences on β-diversity are conflicting and apparently depend on the taxonomic metacommunity studied (Chase 2010, Borer et al. 2014). Our results indeed agree with those of Daleo et al. (2018) studying a salt marsh fungal community. They concluded that increased nutrient inputs enhanced the relative importance of stochastic vs deterministic processes, driving to community homogenisation.

High functional redundancy—few species performing the same functional response than the whole community—has been attributed to aquatic hyphomycetes (Dang et al. 2005, Ferreira et al. 2006a, Ferreira and Chauvet 2012), suggesting that decomposers functionality would not be affected if species loss occurs within this functional group. Yet while all subregions were equally rich in species, fungal communities were functionally richer in colder than in warmer subregions. Temperature was the main predictor of sample

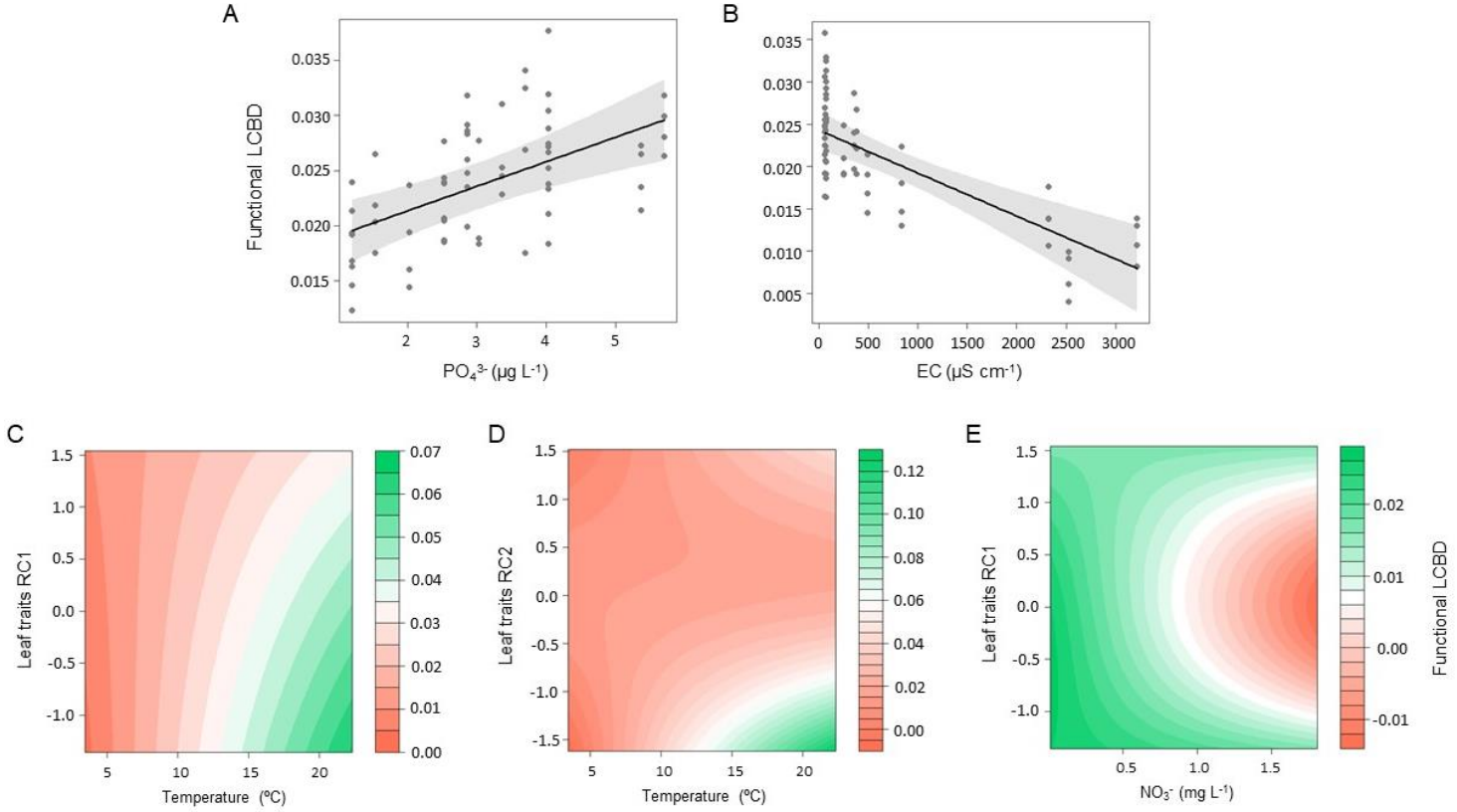

Figure 4.6. Significant (A,B) main effects of SRP and EC and (C-E) interactions effects of temperature with leaf-litter traits RC1 and 2, and NO_3^- with leaf-litter traits RC1, on local contribution to functional β-diversity (Functional LCBD).

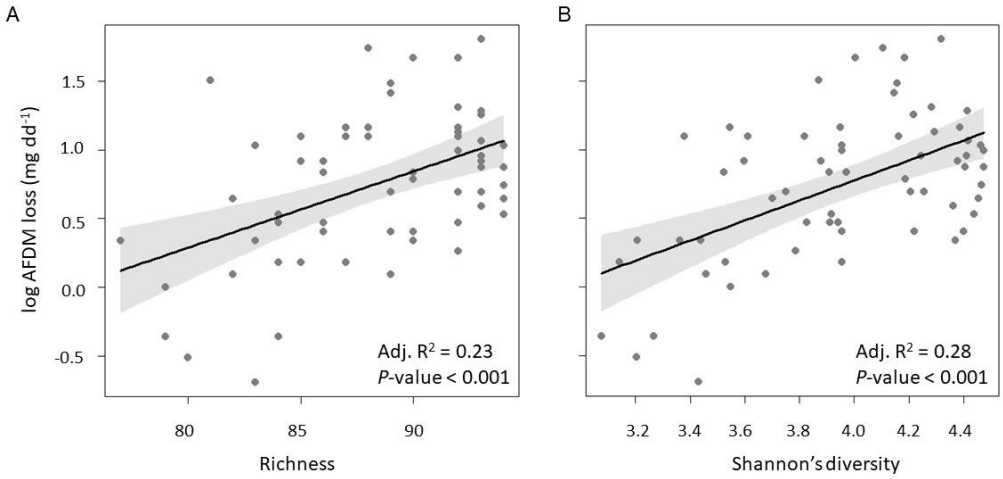

Figure 4.7. Relationship between log-transformed AFDM loss (mg dd^{-1}) and (A) functional richness and (B) Shannon's diversity across all four subregions (n = 64).

functional α-richness, although its negative effect was countered by litter nutrient and water mineral and NO_3^--N concentrations. Recent studies highlight the critical role that microbial dormancy and its environmental determinants can exert on the link B-EF (Kearns et al. 2016). Given the method we used to asses taxonomic diversity (OTUs), it could be hypothesized that as temperature rises could increase the proportion of dormant fungal taxa, thus rendering lower functional richness. This finds some support on culture data indicating that higher temperature increases the costs of sustaining life, thus fostering inactivation (Wörmer et al. 2019). Moreover, Jones and Lennon (2010) have reported that increasing nutrient availability in lakes can reduce rates of microbial dormancy, which might be somehow consistent with the trend detected here. Our data, however, cannot shed much light on these hypotheses, and some studies do not show clear support either, since while dormancy of bacteria strongly responded to subtle variations in environmental conditions that of eukaryotic microbial communities did not (Jones and Lennon 2010, Mueller et al. 2016). An alternative explanation for the positive effect of nutrients (in litter and water) on functional richness is that high nutrient availability supports higher number of fungal species (Gulis and Suberkropp 2003, Pereira et al. 2016, Ferreira et al. 2006b), which might lead to higher expression of functionality, but information regarding this issue is limited and our results do not suggest support for this explanation.

Our results also clearly show that, as for taxonomic composition, while temperature boosted functional heterogeneity among samples, water mineral and NO_3^--N concentrations determined a homogenizing effect. This strongly suggests that changes in functionality seems to be connected with environmental species sorting, and not just with

environmental effects on the functional expression of a given fungal taxon. Despite this connection, however, total functional β-diversity was much lower (4-fold) than the taxonomic one, which likely indicates strongest environmental filtering on species compare to functions. Most functions related to C-acquisition were indeed shared across samples. Our results also point to minor, but significant, effects of SRP and the interactions of temperature and NO_3^--N with litter quality on sample functional variability (LCBD-functional), proving the complex interplay of many factors on this variable.

Overall, our third hypothesis postulating that function may be more resilient than community structure, was supported as long as much low functional to taxonomic β_{TOTAL} was obtained. In stream ecosystems, fungal B-EF relationship has been mostly evaluated through the relationship between changes in fungal assemblages and those in litter decomposition, often concluding high functional redundancy of fungal species (Bärlocher and Graça 2002, Pascoal et al. 2005, Gonçalves et al. 2015). Similarly, in soils has been found a positive relationship between species richness and C cycling in most low-diversity experiments (≤ 10 taxa), but this relationship occurred less frequently in high-diversity experiments (> 10 taxa) (reviewed by Nielsen et al. 2011). Since taxonomic richness was very similar among samples in our study (over 10 OTUs in all samples) we were not able to test potential functional redundancy differences in terms of minimum number of species to reach saturation level of the response variable (functional richness). However, our study provides evidence that fungal communities very similar in species richness can differ in functionality, likely with all fungal communities reaching relatively high functional redundancy but with significant differences in functional richness. Thus, why do fungal communities invest more in functional richness in cold than in warm waters? When temperature rises, metabolic demand (protein turnover, cell repair, etc.) increases, thus incurring relatively greater respiratory costs that reduce carbon use efficiency (CUE). Then, if respiratory costs overcome the benefits of enzyme production, a reduction in enzyme investment might be expected (Allison 2014). As suggested by Allison (2014) warming could promote cooperating microbial communities (consortia) that reduce the number of resource acquisition genes needed by each taxon but being able to minimize the reduction of CUE under those conditions, to access nutrient resources. However, if this response is a consequence of environmental selection on genotypes or phenotypic plasticity of aquatic fungi remains unresolved.

Variation in functional richness observed in our study was positively related with leaf-litter mass loss, expressed in terms of accumulated heat (mg dd^{-1}). Higher microbial litter decomposition (dd^{-1}) under relatively cold conditions or at higher elevation have been previously reported (e.g. Chapter 3, Pérez et al. 2018, Taylor and Chauvet 2014), indicating greater CUE than under warm conditions likely due to lower metabolic stress which allows higher functional richness maintenance. While this study does not grant further exploration of these mechanisms, our findings highlight the need to gain more in-depth knowledge on

the effects of shifting environmental conditions on microbial functionality in stream ecosystems. This can likely help to improve our understanding of the consequences of global change on the B-EF relationships.

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Chapter 5

Feeding strategies of freshwater shredders for dealing with reduced leaf-litter quality in the climate change context: intra-species adaptations or fixed species traits?

Encarnación Fenoy

Juan Rubio-Ríos

José Manuel González

María J. Salinas

Francisco J. Moyano-López

J. Jesús Casas

5.1. Introduction

Forecasting ecosystem alterations triggered by climate change finds a great challenge in uncertainties caused by the inherent complexity of species interactions, along with the diversity of species-specific responses to climate variation (Winder and Schindler 2004, O'Connor et al. 2012). There is a relative lack of information on the effects of climate change at the species-interactions level compared to that on individual or species populations (Tylianakis et al. 2008, Woodward et al. 2010), although studies on resource-consumer interactions point to major impacts on the functioning of green and brown food webs (e.g. Stiling and Cornelissen 2007, Hoekman 2010, West and Post 2016).

In headwater streams limited by light, the most abundant watercourses in temperate regions, food webs rely greatly on terrestrial subsidies of organic matter, i.e. leaf-litter (Vannote et al. 1980, Collins et al. 2016). In these ecosystems, therefore, the trophic linkage between leaf-litter and macroinvertebrate shredders is pivotal, such that changes in resource-consumer interaction can modify the flux of energy and matter to higher trophic levels (Covich et al. 1999, Piscart et al. 2011). Two primary intrinsic factors drive this interaction, i) the quality of leaf-litter as a food, highly depending on the enzymatic hydrolysis performed by microbial decomposers, and ii) feeding behaviour and digestive performance of shredder species (Graça 2001, Graça et al. 2015).

Leaf traits of the riparian vegetation could change significantly in future global warming scenarios, mostly in subtropical and mid-latitude regions where a marked aridity increases and greater risk of heat waves are predicted (Giorgi and Lionello 2008). In such scenario, an increasing frequency of stream segments with intermittent flow is expected, this giving rise to canopy opening and major changes in the composition of riparian vegetation (Salinas and Casas 2007, Stromberg et al. 2013). Climate change might trigger significant shifts in leaf traits —i.e. increasing toughness and Si content but decreasing N content—due to changes in plant functional types of the riparian vegetation, even if the threshold between permanent and intermittent discharge is not crossed (Salinas et al. 2018). These traits are often reported to negatively affect palatability and digestibility of leaves (Schlief and Mutz 2006, Graça and Cressa 2010, Cooke et al. 2016). In addition, important intraspecific leaf trait changes could be prompted by climate change. The high phenotypic plasticity and elevated responsiveness of plants to climate variations at leaf-level (Sultan 2000) often lead high intraspecific variability of leaf traits, reaching even to level interspecific variation (Lecerf and Chauvet 2008, Albert et al. 2010). Nevertheless, few studies have rigorously accounted for intraspecific leaf-trait plasticity within a changing climate (Albert et al. 2011), even though growing evidence points to major consequences on resource-consumer interactions, with ripple effects on ecosystem functioning (Lecerf and Chauvet 2008, Graça and Poquet 2014, Jackrel and Wootton 2014).

Detritivores from headwater streams face much larger nutritional imbalances compared to consumers from living plant-based systems (Cross et al. 2005, Lauridsen et al. 2012). These large imbalances impose a crucial adaptive challenge, particularly in nutrient–poor headwaters, that might be exacerbated by climate change. The theory of ecological stoichiometry postulates that organisms can develop different strategies to maintain elemental homeostasis when feeding on nutrient-poor resources: i.e. preferential selection of nutrient-rich resources, increased consumption of the nutrient-poor resource to compensate deficiencies, and/or some mechanisms of post-ingestive regulation (Sterner and Elser 2002, Frost et al. 2005). Few studies have comprehensively tested these capabilities on streams shredders facing reduced nutritional quality of leaf-litter, and data available point to different limitations, conflicting results or species-specific idiosyncrasies (Fuller et al. 2015, Santonja et al. 2018). For example, it seems that successful search and finding of sparse food with a high nutritive value is a highly random process (Motyka et al. 1985) that requires a high investment of energy, this being deserved only to highly mobile species (Cruz-Rivera and Hay 2000). Yet, preferential feeding on high-quality resources appears to be the norm for shredders belonging to different phylogenetic groups when using single leaf-litter species treatments in laboratory experiments (e.g. Santonja et al. 2018). On the other hand, several studies have reported either that compensatory feeding on low-quality leaf-litter may be enough to balance, or even overcompensate, the growth obtained when fed on high-quality resources (e.g. Swan and Palmer 2006, Fuller et al. 2015), or not (Flores et al. 2014). These conflicting results have been described for the same shredder species; as an example, both preferential (Tuchman et al. 2002, Halvorson et al. 2015) and compensatory feeding (Anderson and Cummins 1979, Fuller et al. 2015) have been reported in *Tipula abdominalis*. This suggests that a significant degree of feeding/digestive phenotypic plasticity or genetic variation among populations might occurs in some shredder species. This trait plasticity of riverine macroinvertebrates may be greater than elsewhere in highly heterogeneous regions, like the Mediterranean Basin (Bonada et al. 2007). In the case of shredders, this could enable fine local adaptations aimed to exploit more efficiently leaf-litter resources dominant at home (Jackrel and Wootton 2014). However, to our knowledge this intra-species adaptation has been poorly studied in riverine shredders.

Here, we investigate whether different species of shredders present either fixed-species traits or intra-species adaptations to cope with variations in the nutritional value of leaf-litter, mostly related to toughness, Si and P concentration (interspecific changes) and N concentration (interspecific and intraspecific changes). We hypothesized that if adaptation to poorer diets exists, this should be perceptible in shredder's populations from streams present in warmer or warmer/arid zones, where we expected leaf-litter inputs of inferior nutritional quality compared to relatively mesic/cold regions. In line with stoichiometric theory, we expected that such presumed adaptation could be based on two strategies: i. an increased feeding rate on leaf-litter of lower quality to compensate deficient access to

limiting nutrients, and/or ii. an increased assimilation of the limiting nutrient relative to that of C. We also hypothesize that interspecific changes in the nutritional quality of leaf-litter would exert a greater influence on feeding performance than intraspecific ones, due to major interspecific variation in traits, other than elemental nutrients, affecting palatability/ digestibility. To this end, we performed feeding trials to evaluate the effect of variability in leaf-litter quality on feeding rates, survivorship, growth and energetic storage of three shredder species, each one from two regions with contrasting climate.

5.2. Material and methods

5.2.1. Regions of origin of shredders

We selected macroinvertebrate shredders from four subregions located in Andalusia (southern Spain). This Mediterranean region has remarkable lithological and climatic diversity (Casas et al. 2006), which auspicious the possibility to find a same species within contrasting climatic conditions. The shredder guild often exhibits notable differences in taxonomic composition depending on lithology of the drainage basin (Casas et al. 2011). Thus, for intra-species comparison of feeding performance we selected two subregions, widely differing in climate within each of the main lithological settings of Andalusia: siliceous *vs.* calcareous (Table 5.1). Within the siliceous setting, we chose Sierra Nevada and Alcornocales, which according to Emberger's pluviothermic quotient (Q_2) (Daget et al. 1988), have sub-humid and per-humid climates, respectively (Table 1). In the calcareous setting, we selected the two climatic extremes in the region: the semiarid lowlands of Almería and the per-humid subregion of Grazalema (Table 1). Sierra Nevada shows much lower temperatures, particularly minimum temperature, compared to its siliceous pair and the two calcareous subregions (Table 1). In the siliceous subregions, we chose two of the most frequent and abundant shredder taxa in mountain headwater streams in Andalusia: the caddisfly *Allogamus mortoni* (Navàs 1907) and the cranefly *Tipula leo* (Dufour 1991). In groundwater-fed low-order streams from the two calcareous subregions, we selected the snail *Melanopsis praemorsa* (Linnaeus 1758), which is an abundant generalist feeder but also behaves as a major leaf-litter shredder (Casas et al. 2011).

5.2.2. Quality of leaves from major riparian plants in each subregion, and of leaf-litter used in feeding tests

We characterised leaf quality of the major riparian plant species in three permanent low-order streams within each of the four subregions. In each stream, vegetation data were collected between June-July 2013 in six plots (36 m^2 each) randomly distributed in two strata (both stream sides) along a 100 m stream reach. We recorded percent coverage of all woody species and giant graminoids. For the four most abundant species in each stream,

102 leaves from six individuals (17 leaves per individual) were collected. We measured specific leaf area (SLA), toughness, %C, %N, %P, % lignin and Si as proxies of leaf nutritive value for detritivores (Graça and Cressa, 2010). We determined specific leaf area (SLA, $mm^2\ g^{-1}$) by measuring area (WinDIAS 3, Delta-T devices) and dry mass (60˚C, 78h), and toughness using a calibrated texturometer (TA.XT2 Plus, Stable Micro Systems). Concentrations of C and N were determined using IR mass spectrometry (DELTA V Advantage, Thermo Fisher Scientific®), and those of P and Si using ICP mass spectrometry (iCAP 6500 - ICP-OES, Thermo Scientific®). Percent lignin was measured using the acid-detergent method of Goering and van Soest (1970). In addition, we measured these parameters in 5 randomly selected portions per species and quality class of leaf litter used in feeding tests (see below), as well as hemicellulose and cellulose as described in Fenoy et al. (2016). The nutrient concentration (C, N and P) of leaf-litter was measured in pre-conditioned and post-conditioned material, as well as in faecal pellets produced by the experimental animals (see below).

5.2.3. Leaf-litter species for experimentation and microbial conditioning

To evaluate the effects of inter- and intraspecific variability of leaf-litter nutritional quality on feeding performance of shredders, we selected two riparian deciduous species, *Alnus glutinosa* (L.) Gaertn. and *Populus alba* L., with contrasting leaf traits that represent syndromes of alternative life history strategies within deciduous trees. The N fixer *Alnus glutinosa* (henceforward *Alnus*), common in temperate riparian corridors, is one of the species with higher leaf-N concentration and with relatively high lignin. *Populus alba* (henceforward *Populus*) is common in warm temperate and Mediterranean zones, being a species likely favoured by warming provided its thermophilic nature (Fussi et al. 2010). This species has relatively low leaf lignin and N concentrations, but high toughness and Si content. These traits seem to be favoured by climate warming, and the last three are often associated to low nutritional value for invertebrate shredders (Salinas et al. 2018). Senescent leaves of each species were collected in the two subregions from Andalusia where they showed the highest intraspecific differences in quality (primarily in N concentration), based on results of a previous extensive study (Salinas et al. 2018).

To standardize in-stream microbial conditioning of leaf litter offered to detritivores, litterbags (1 mm mesh-size, 5 bags per species and quality class) were incubated during winter in a stream of intermediate chemical and thermal characteristics to those in which the shredders were sampled (Table 1). Based on previous studies, three weeks of in-stream incubation was estimated enough for optimal microbial conditioning (Casas et al. 2011). After retrieval, litterbags transported to the lab in zip lock bags filled with stream water, in an icebox. Upon arrival, leaf-litter was immediately rinsed with filtered stream water to remove sediment slurry and leaf discs (1 cm Ø) were cut, frozen (-20 ºC), freeze-dried and preserved at -20 ºC until required.

5.2.4. Experimental set-up for feeding tests

Shredders were collected from three streams per subregion and acclimated during 7 days to laboratory conditions at the mean winter temperature of the streams of provenance (14°C for *Melanopsis*, and 8°C for *Allogamus* and *Tipula*) under a 12:12h LD photoperiod, in aquaria with forced aeration. During acclimation, shredders were fed *ad libitum* on leaf-litter from their original streams. After this period, only active individuals of similar size were selected for experiments. Mean size of *Melanopsis* from Grazalema and the Semiarid lowland was 34 ± 0.9 and 35 ± 1.1 mg DM respectively, without significant differences between regions (t = -3.4, *P* = 0.73). Mean size of individuals of *Allogamus* (19 ± 0.9 and 28 ± 1.1 mg DM) and *Tipula* (45 ± 2.3 and 82 ± 10.3 mg DM) were significantly smaller in Sierra Nevada compared to Alcornocales (t = 6.65, P < 0.001; t = 4.67, P < 0.001; respectively), probably due to lower temperature in the first subregion.

One hundred and twenty individuals per taxon (60 per subregion) were used in feeding tests. The initial body dry mass ($DM_{initial}$) of individuals of *Melanopsis* and *Allogamus* was estimated from measures on digital images of maximum shell length (SL) and head capsule width (HW), respectively, using the *SigmaScan® Pro v 5.0* image analyser. Size-body dry mass (DM: 60 ºC, 72 h; weighting to the nearest 0.1 mg) regressions were developed for *Melanopsis* (without shell) [$DM\ (g) = 5.9804 \times 10^{-5} \times SL(cm)^{2.6191}$, $R^2 = 0.90$, n = 78] and *Allogamus* (without case) [$DM\ (g) = 2.3391 \times 10^{-4}\ e^{3.1437 \times HW(cm)}$, $R^2 = 0.90$, n = 138] measuring different sets of individuals than those used for the experiment. Wet mass of *Tipula* was determined by transferring larvae to filter paper (10 s) and weighting to the nearest 0.1 mg. A different set of *Tipula* individuals was used to obtain wet mass (WM)-dry mass (DM) regression [$DM\ (g) = 0.1311 \times WM(g)^{1.1788}$, $R^2 = 0.96$, n = 66]. Experimental individuals were starved during 24 h before the experiment started to allow evacuation of their guts. Shredders were placed individually in cylindrical containers of 5 cm Ø, 7 cm height, with a 0.5 mm mesh screen at the bottom to allow faecal pellets to pass through. Each container was inserted in a slightly bigger container to collect faecal pellets, filled with filtered stream water, which was renewed every 5 days, and maintained with forced aeration (for more details see Rubio-Ríos et al. 2017). Fifteen individuals were randomly assigned to each one of the four dietary treatments. Two pairs of pre-weighed leaf-litter discs were either exposed to the feeding action of each shredder or immersed in the container in a 250 µm mesh bag to act as control to account for leaf-litter mass losses other than consumption. Along the 30-days period the experiment lasted, both sets of discs were renewed when approximately 50 % of discs exposed to the shredder were consumed. After retrieval, exposed and control discs were dried (60 ºC, 72 h) and weighed to estimate final dry mass (DM_t = dry mass of exposed discs – dry mass of control discs). Shredders were maintained under the same temperature and photoperiod regime as the acclimation period. At the end of the experiment, individuals were photographed (*Melanopsis* and *Allogamus*) or weighted (*Tipula*) to estimate final dry mass (DM_{final}) as above indicated,

and frozen (-20 ºC) until used for measurement of body composition (see below). Faeces were collected every day and preserved frozen (-20 ºC) until analysed for C, N and P concentrations (5 pools from 3 individuals per shredder species, diet and subregion). Differences in stoichiometry between food and faeces were used as an indicator of apparent assimilation efficiency.

5.2.5. Consumption, growth and energetic reserves

Relative feeding rate (RFR) was estimated as $RFR = (DM_i - DM_t) / (DM_{shredder} \times t)$: where DM_i and DM_t are initial and final dry mass (mg) of leaf discs, respectively, t is the exposure time (days) to consumption, and $DM_{shredder}$ is dry mass (g) of the shredder. Daily instantaneous growth rate (DIGR, d^{-1}) of individual shredders was calculated as the difference between ln DM_{final} and ln $DM_{initial}$, divided by the elapsed time (days).

Energetic reserves of shredders under the forms of lipids and glycogen were measured using the sulfo-phospho-vanillin and the anthrone reactions, respectively, following the methods described in Charron et al. (2014) with minor modifications. After removal of shells and cases, each individual was homogenized in 1 mL methanol using vortex and stainless steel balls. Each sample was aliquoted to measure total lipids and glycogen content. Optical density was measured at 525 nm for lipids and at 630 nm for glycogen. Calibration solutions were prepared using a commercial olive oil solution (5 g/L) solubilized in chloroform (for lipids) and glycogen (2.5 g L^{-1}) in distilled water.

5.2.6. Statistical analyses

We calculated the % cover-weighted mean per subregion (4 dominant riparian species) of each leaf trait as an integrative indicator of dietetic quality of riparian leaf inputs to streams. Then, we performed comparisons of weighted means for each leaf trait within lithological pairs of subregions differing in climate (Sierra Nevada vs. Alcornocales; Semiarid Lowland vs. Grazalema) using weighted t-tests (package *weights*, R Development Core Team 2018). We used factorial ANOVA and post-hoc Tukey's test to compare leaf-litter traits of species and quality classes. We used t-test to compare nutrient ratios (C:N, C:P and N:P) between faecal pellets egested by a shredder species and the corresponding post-incubated (in-stream) leaf-litter used to feed them. Factorial ANOVAs (subregion × leaf-litter species × quality class) were performed to assess effects of shredder provenance and changes in diet quality, on relative feeding rate, growth rate and energy reserves. Mantel-Cox Log-Rank tests were performed to determine whether independent factors significantly affected shredders survival. Log- or arcsin-transformed data were performed to improve homoscedasticity of variances. When these transformations were not possible due to negative values, 1/x transformation was used. All analysis, except t-tests (see above), were carried out in Statistica v7 (StatSoft, OK, USA).

5.3. Results

5.3.1. Leaf traits of major riparian species in the four subregions and experimental diets

Overall, a higher average foliar nutrient concentration (N and P) was detected for major riparian species in the coldest sub-humid subregion (Sierra Nevada) of the siliceous domain and in the per-humid subregion (Grazalema) of the calcareous domain, compared with their respective counterparts (Table S5.1). Moreover, leaves from Sierra Nevada showed lower C concentration and a slightly higher Si content compared to those from Alcornocales, but no significant differences in leaf toughness appeared between the two subregions. In the Semiarid Lowland Si content and toughness were significantly higher than in Grazalema (Table S5.1).

Leaf litter species used as experimental diets differed significantly in all traits measured (Table 5.2). *Alnus* showed significantly higher N concentration and lower molar C:N ratio compared to *Populus*. However, the opposite pattern was detected for P concentration and molar C:P and N:P ratios (Table 5.2). After in-stream incubation, a general increase in N and decrease in P concentration were measured, although interspecific differences in nutrient concentration remained. The much higher content of Si in leaf litter of *Populus* likely determined its significantly higher toughness compared to *Alnus*, which, on the other hand, was apparently independent of lignin concentration (higher in *Alnus*, Table 5.2).

Alnus showed significant intraspecific differences in a greater number of leaf-litter traits than *Populus* (Table 5.2). The "high quality" category of *Alnus* showed significantly higher N concentration (notably after in-stream incubation), higher hemicellulose, but lower cellulose and lignin concentrations and lower toughness but higher SLA when compared to the "low quality" category. The "high quality" category of *Populus* showed significantly higher C and N concentrations, but lower molar C:N ratio, and lower SLA than the "low quality" category within this species (Table 5.2).

5.3.2. Relative feeding rate

Leaf-litter species significantly affected relative feeding rate (RFR) in the three shredder species (Figures 5.1a, 5.2a and 5.3a, Table S5.2). Whereas *Melanopsis* consumed higher quantities of *Populus* than *Alnus*, *Allogamus* and *Tipula* showed the reverse interspecific pattern of food consumption. Subregion of origin only affected significantly RFR in *Allogamus*, with much higher consumption of *Populus* by individuals from Sierra Nevada compared to those from Alcornocales, which also determined significant interaction "subregion" × "litter-species" (Figure 5.2a, Table S5.2). Significant interaction "litter-species" × "quality-class" were detected in *Melanopsis* and *Tipula*, with greater consumption of the "high quality" class of *Populus* and the "low quality" class of *Alnus*, respectively (Figures 5.1a and 5.3a, Table S5.2).

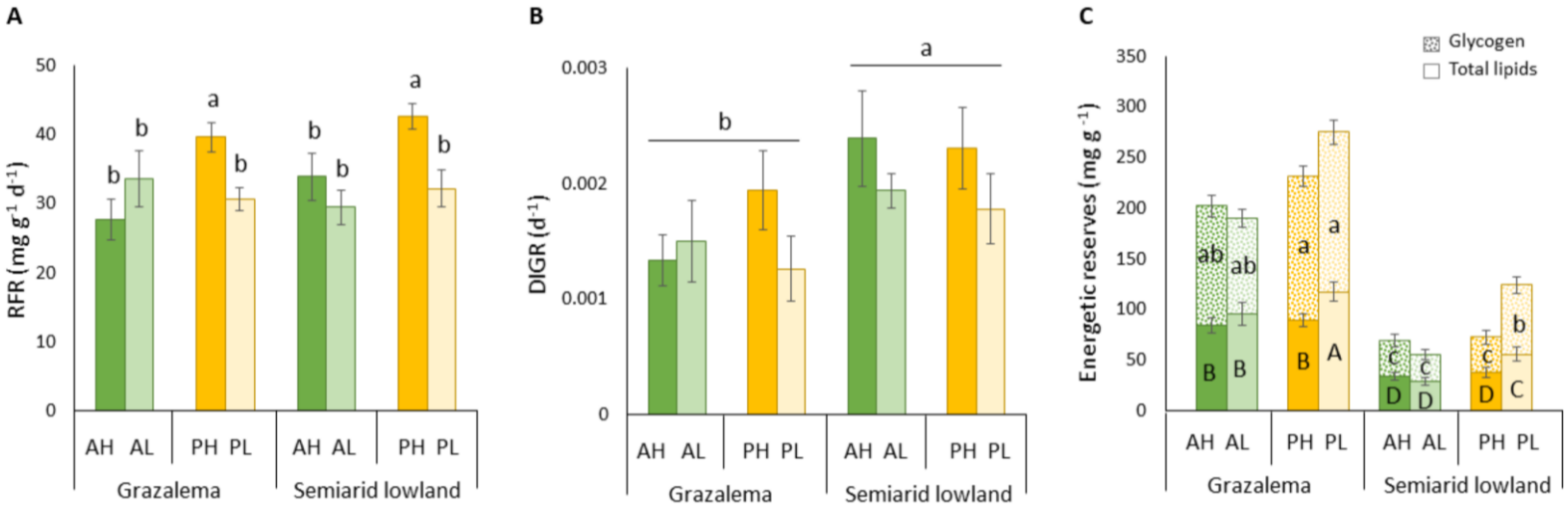

Figure 5.1. Mean ± SE of (A) relative feeding rate (RFR, $mg_{leaf\ litter}\ g_{shredder}^{-1}\ day^{-1}$), (B) daily instantaneous growth rate (DIGR, d^{-1}) and (C) energy reserves, i.e., total lipid and glycogen content (mg g^{-1}) for *Melanopsis praemorsa* fed on two qualities of *Alnus* (AH and AL: high and low, respectively) and *Populus* (PH and PL: high and low, respectively). Different letters indicate significant differences ($P < 0.05$).

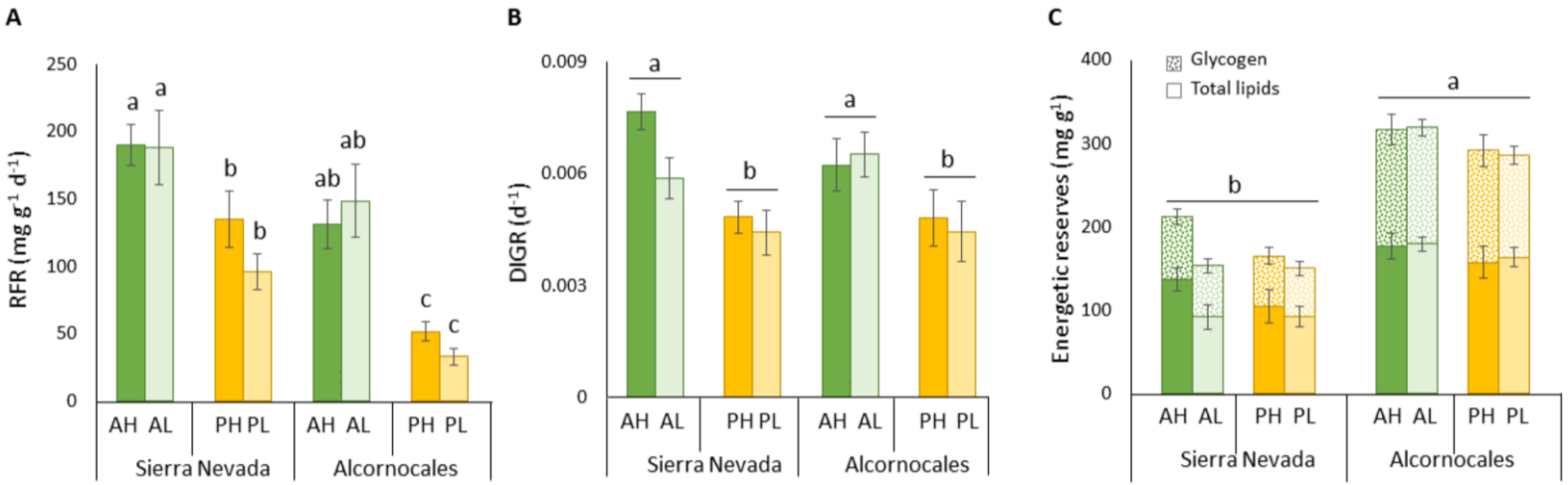

Figure 5.2. Mean ± SE of (A) relative feeding rate (RFR, $mg_{leaf\ litter}\ g_{shredder}^{-1}\ day^{-1}$), (B) daily instantaneous growth rate (DIGR, d^{-1}) and (C) energy reserves, i.e., total lipid and glycogen content (mg g^{-1}) for *Allogamus mortoni* fed on two qualities of *Alnus* (AH and AL: high and low, respectively) and *Populus* (PH and PL: high and low, respectively). Different letters indicate significant differences ($P < 0.05$).

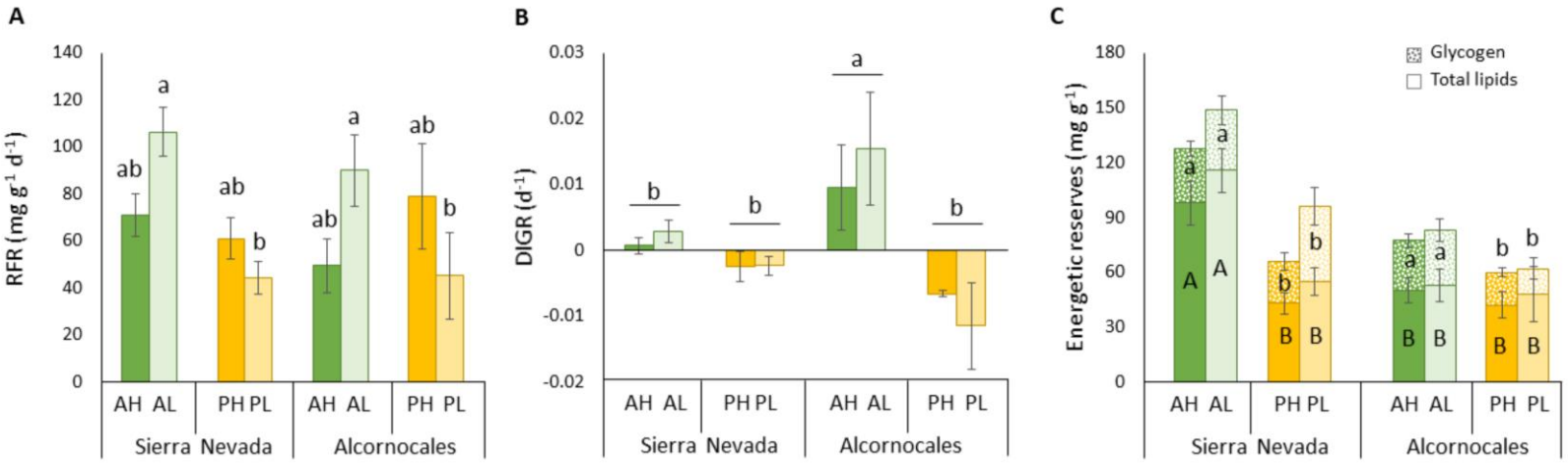

Figure 5.3. Mean ± SE of (A) relative feeding rate (RFR, $mg_{leaf\ litter}\ g_{shredder}^{-1}\ day^{-1}$), (B) daily instantaneous growth rate (DIGR, d^{-1}) and (C) energy reserves, i.e., total lipid and glycogen content (mg g^{-1}) for *Tipula leo* fed on two qualities of *Alnus* (AH and AL: high and low, respectively) and *Populus* (PH and PL: high and low, respectively). Different letters indicate significant differences ($P < 0.05$).

Table 5.1. Environmental characteristics (mean ± SE or range between parentheses; n = 3 streams) of the four subregions of origin of shredders. *Q_2: Emberger's bioclimatic coefficient; EC: electric conductivity. Annual temperature range: mean minimum temperature of the coldest month and mean maximum temperature of the warmest month.

Subregion	Lithology	Altitude (m a.s.l.)	Climate			Stream water		
			Annual rainfall (mm)	Annual temperature (ºC)	Q_2	Annual temperature (ºC)	pH	EC (μS cm^{-1})
Sierra Nevada	Siliceous	1438 (1419-1465)	554 (548-561)	10.7 (-1.1-27.7)	69 ± 4	9.5 (1.2-17.2)	7.25 ± 0.20	169 ± 66
Alcornocales	Siliceous	448 (388-532)	1240 (1116-1303)	15.2 (6.4-27.5)	215 ± 18	13.3 (8.2-20.1)	6.56 ± 0.14	71 ± 3
Semiarid lowland	Calcareous	377 (164-576)	348 (297-387)	17.2 (4.6-31.3)	45 ± 3	18.0 (10.4-28.6)	7.98 ± 0.30	2269 ± 710
Grazalema	Calcareous	460 (305-688)	1189 (1140-1414)	15.0 (4.5-31.3)	160 ± 12	14.8 (13.5-15.0)	7.68 ± 0.21	677 ± 160

*$Q_2 = 2000\ P / (M^2 - m^2)$: where P = mean annual precipitation; M = mean maximum temperature in the hottest month; m = mean minimum temperature in the coldest month.

Table 5.2. Mean values ± SE of leaf-litter traits for the two species, and the two quality classes within each species, used in feeding tests. Nutrients were measured before and after in-stream incubation for microbial conditioning. Different letters indicate significant differences following two-way ANOVA and post-hoc Tukey tests.

Leaf-litter traits	*Alnus glutinosa*		*Populus alba*		*F* – value and significance level		
	High quality	Low quality	High quality	Low quality	Species	Quality	Interaction
Pre-in stream incubation							
%C	47.76 ± 0.10^{b}	51.88 ± 0.17^{a}	45.02 ± 0.25^{c}	42.98 ± 0.22^{d}	918.9 ***	257.5 ***	29.9 ***
%N	2.45 ± 0.04^{a}	2.36 ± 0.07^{a}	1.14 ± 0.07^{b}	0.86 ± 0.01^{c}	652.3 ***	6.6 *	15.5 **
%P	0.044 ± 0.003^{b}	0.028 ± 0.004^{c}	0.056 ± 0.003^{ab}	0.061 ± 0.004^{a}	46.1 ***	11.1 **	4.5 *
C:N	22.8 ± 0.4^{c}	25.8 ± 0.7^{c}	46.6 ± 2.9^{b}	58.5 ± 0.9^{a}	366.3 ***	1.0 n.s.	22.5 ***
C:P	2835 ± 181^{b}	5043 ± 604^{a}	2085 ± 112^{b}	1842 ± 103^{b}	62.6 ***	17.4 ***	6.9 *
N:P	124.0 ± 6.7^{b}	193.6 ± 18.5^{a}	44.8 ± 0.7^{c}	31.5 ± 1.9^{c}	478.7 ***	37.8 ***	0.3 n.s.
Si (µg g^{-1})	144 ± 15^{b}	143 ± 21^{b}	1551 ± 45^{a}	2151 ± 216^{a}	702.1 ***	2.9 n.s.	2.2 n.s.
% Hemicellulose	25.8 ± 2.1^{a}	13.1 ± 1.5^{b}	13.2 ± 1.9^{b}	20.2 ± 3.0^{ab}	1.4 n.s.	18.2 **	1.5 n.s.
% Cellulose	18.9 ± 0.4^{b}	26.1 ± 0.6^{a}	16.7 ± 1.1^{b}	18.6 ± 1.0^{b}	30.6 ***	7.9 *	26.5***
% Lignin	13.0 ± 1.2^{b}	20.6 ± 0.6^{a}	6.3 ± 1.5^{c}	7.3 ± 0.5^{c}	77.8 ***	4.7 *	12.7 **
Toughness (g)	50.5 ± 1.7^{c}	60.5 ± 3.8^{b}	71.8 ± 1.5^{a}	68.0 ± 1.8^{ab}	38.1 ***	8.9 **	2.4 n.s.
SLA (cm^{2} g^{-1})	170.6 ± 4.9^{a}	131.9 ± 6.3^{b}	85.9 ± 1.3^{c}	142.5 ± 1.5^{b}	101.2 ***	163.2 ***	16.9 ***
Post-in stream incubation							
%C	49.156 ± 0.103^{ab}	50.504 ± 0.168^{a}	48.473 ± 0.247^{b}	48.200 ± 0.217^{b}	14.3 **	4.2 n.s.	1.9 n.s.
%N	3.168 ± 0.063^{a}	2.800 ± 0.037^{b}	1.835 ± 0.083^{c}	1.459 ± 0.062^{d}	384.1 ***	0.7 n.s.	30.7 ***
%P	0.032 ± 0.002^{b}	0.034 ± 0.002^{b}	0.047 ± 0.001^{a}	0.050 ± 0.001^{a}	69.4 ***	0.1 n.s.	1.7 n.s.
C:N	18.129 ± 0.341^{c}	21.065 ± 0.376^{c}	31.193 ± 2.055^{b}	38.802 ± 1.545^{a}	218.6 ***	0.9 n.s.	23.2 ***
C:P	4075.317 ± 286.240^{a}	3894.384 ± 279.519^{a}	2655.184 ± 63.389^{b}	2512.817 ± 82.701^{b}	62.2 ***	0.1 n.s.	0.9 n.s.
N:P	224.767 ± 15.013^{a}	184.233 ± 10.276^{a}	86.093 ± 4.150^{b}	65.206 ± 3.680^{c}	313.8 ***	0.5 n.s.	17.8 ***

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; n.s. = not significant ($P > 0.05$).

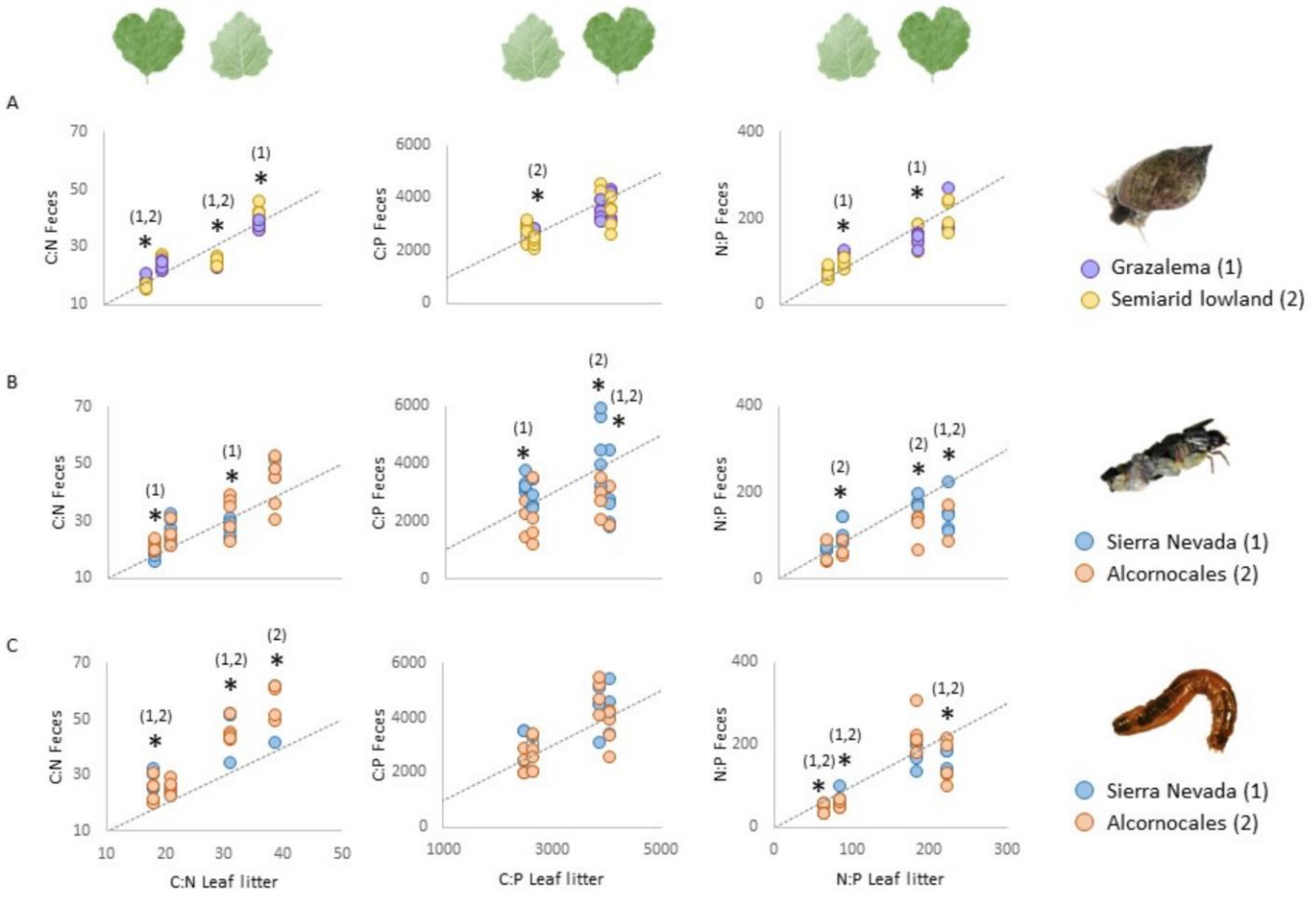

Figure 5.4. Relationship of molar C:N, C:P and N:P ratios between faeces and leaf-litter offered to *Melanopsis praemorsa* (A), *Allogamus mortoni* (B) and *Tipula leo* (C). The dotted line represents the ratio 1:1. The asterisks indicate a significant deviation from the 1:1 ratio ($P < 0.05$). The number in parentheses indicates the subregion for which differences were found.

5.3.3. Survival and growth

All individuals of *Melanopsis* survived throughout the course of the experiment in the four diets. Survival was slightly lower in *Allogamus* (97%) and notably lower in Tipula (71%). However, neither subregion of origin (log rank Mantel-Cox: $\chi^2_{Allogamus}$ = 0,402, P = 0.526; χ^2_{Tipula} = 0,272, P = 0.602) nor diet (log rank Mantel-Cox: $\chi^2_{Allogamus}$ = 1,072, P = 0,300; χ^2_{Tipula} = 3,012, P = 0.083) significantly affected survival of *Allogamus* and *Tipula*.

Melanopsis from the Semiarid Lowland showed significantly higher Daily Instantaneous Growth Rate (DIGR) than individuals from Grazalema, without significant effects of litter species, quality class or interactions (Figure 5.2b, Table S5.2). Individuals of *Allogamus* and *Tipula* fed on *Alnus* showed higher DIGR (Figures 5.2b and 5.3b, Table S5.2) than those fed on *Populus*. It is worth noting the negative growth rate obtained when *Tipula* fed on *Populus* (Figure 5.3b). Moreover, DIGR of *Tipula* was higher in individuals from Alcornocales compared to Sierra Nevada when fed with *Alnus*, but no significant differences between subregions appeared when fed *Populus* (Figure 5.3b, Table S5.2).

5.3.4. Energy reserves

Unlike growth rates, energetic reserves (lipids and glycogen) of *Melanopsis* were significantly higher in individuals from the per-humid region of Grazalema, these presenting a significantly higher accumulation when fed *Populus* litter (Figure 5.1c, Table S5.2). In *Allogamus*, significantly higher reserves were measured in individuals from Alcornocales compared to those from Sierra Nevada (Figure 5.2c, Table S5.2). In the case of *Tipula*, lipid reserves were significantly higher in individuals from Sierra Nevada when fed *Alnus* (significant interaction "subregion" × "litter-species"), but glycogen was significantly higher in individuals from both regions when fed *Alnus* (Figure 5.3c, Table S5.2).

5.3.5. Comparison of nutrient ratios between faeces and diets

Faecal nutrient ratios showed a general trend to increase with those of their corresponding diets. However, in many cases we found significant differences between faeces *vs.* diet in individuals of the two or at least one of the two subregions compared (Figure 5.4, Table S5.3). For *Melanopsis* fed on high quality of *Alnus* and *Populus*, appears a subtle trend, but still significant, of lower faecal C:N ratio than the corresponding diet, suggesting slight N-enrichment of faeces (Figure 5.4a, Table S5.3). In contrast, no significant differences in C:P or N:P ratios between faeces and diets were detected, with the exception of values measured for *Alnus* low quality. Results obtained with this diet point to a slightly higher assimilation efficiency of P in individuals from Grazalema compared to those from the Semiarid Lowland (Figure 5.4a, Table S5.3).

For *Allogamus*, the only statistically significant difference in faecal C:N ratio was measured in individuals from Sierra Nevada fed on the high quality class of *Alnus* and *Populus*, this suggesting a lower N assimilation efficiency relative to C, when compared to that measured in individuals from Alcornocales. Further, N:P ratio measured in faeces of individuals from Alcornocales fed on *Alnus* were significantly lower than those of diets, this suggesting a greater assimilation efficiency of N, relative to P, in the N-richer diets. No clear trend was detected for C:P ratios (Figure 5.4b, Table S5.3).

Tipula from both subregions showed a high capacity for assimilating N relative to that of C and P. In general, this pattern occurred in all diets except for *Alnus* low quality, in which no significant differences in ratios between faeces and diets appeared (Figure 5.4c, Table S5.3).

5.4. Discussion

The large resource-consumer elemental imbalance is one of the greatest challenges for detritivores in headwater streams (Cross et al. 2005). This imbalance is likely to be exacerbated by climate change if, as predicted, the nutritional value of leaf-litter inputs to streams will decrease (Salinas et al. 2018), either due to plant species turnover (interspecific variability) or phenotypic plasticity of species (intraspecific variability). Thus, considering the paramount trophic linkage exerted by shredders in stream food webs, significant effects on ecosystem functioning could arise (Lecerf and Chauvet 2008, García et al. 2012, Graça and Poquet 2014). However, little is known about the ability of riverine shredders to develop local adaptations to maintain performance when feeding on nutrient-depleted leaf-litter, which in highly heterogeneous regions, such as the Mediterranean, are more likely to occur compared to relatively homogeneous ones (Bonada et al. 2007). Pinpointing such putative adaptations might facilitate scaling-up predictions on the magnitude of climate change effects on stream ecosystems.

Our overarching hypothesis stated that if shredder's intra-species adaptations to cope with decreasing leaf-litter quality exists, these could be perceptible in populations already dealing with this scenario, i.e. streams located in warm or warm/arid zones presenting nutrient-depleted leaf-litter inputs. Our data revealed significant differences in the average nutritive value of the riparian foliage between subregions. However, results from feeding tests did not support our hypothesis; feeding strategy and its outcomes facing changes in resource quality were similar between the two populations compared of each species.

Adaptive phenotypic plasticity, or intra-species adaptations due to genetic diversity of populations, allows species to cope with environmental variability, decreasing their risk of extinction in a changing world (Gienapp et al. 2008). Nevertheless, despite the potential

benefits of these microevolutionary adaptations under a warming climate, adaptive phenotypic plasticity, for instance, is far from being widespread or maximal, due to genetics and environmental constraints (costs and limits) (e.g. Auld et al. 2009, Merilä and Hendry 2014), which could explain our results. Alternatively, it could be suggested that the average decrease in nutritional quality of leaf-litter inputs is not the prevailing selection pressure on shredders feeding traits. It may be thought that both, the relatively high diversity typical of riparian vegetation, together with microbial conditioning that enhances the nutritive value of recalcitrant leaf-litter, could dampen the strength as a selection pressure of dominant low-quality litter inputs.

Notwithstanding the above, in the case of *Melanopsis* we detected a significant effect of subregion regardless of diet quality: a higher growth but much lower (less than half) energy reserves were measured in individuals from the Semiarid Lowland when compared to the per-humid Grazalema. This suggests a physiological trade-off in resource allocation between these two traits—i.e. prioritization of growth in protein in the first subregion *vs.* accumulation of carbon reserves in the second one—indicative of a genetically-based differential metabolism of internal nutrients. The causes of such differential nutrient allocation between the two populations of *Melanopsis* are difficult to disentangle. It may be that the fast growth recorded for snails from the Semiarid Lowland could be simply a compensatory mechanism (Metcalfe and Monaghan 2001) produced when resource quality was improved (providing the experimental leaf-litter) after a period of retarded growth due to poor food quality (leaf-litter at home streams). However, compensatory growth is commonly driven by hyperphagia (Gurney and Nisbet 2004) and this was not observed in our experiment. Alternatively, it could be considered that under a situation of poor food-quality or scarcity, tissue protein can be the main contributor to meet imminent N demands for gametogenesis, as it has been shown in studies of marine bivalves exhibiting high phenotypic plasticity (Bayne 2004, and references therein). Thus, as no fundamental differences in assimilation efficiencies of C and N appeared between *Melanopsis* from both subregions, it seems that the excess of C acquired was mostly channelled toward energetic storage in individuals from Grazalema, while those from the Semiarid Lowland perhaps get rid the C excess increasing respiration rate (Hessen et al. 2013). This last response should be matched by reduced rates of N excretion to maintain fast growth. Extraordinary flexibility in rates as an adaptation to surplus dietary C seems to be a normal feature in freshwater snails (Fink and von Elert 2006), including the capacity to growth relatively fast on high C diets increasing its relative allocation to respiration (Rollo and Hawryluk 1988).

Significant differences were also detected between populations of *Allogamus* and *Tipula* but particularly in the first species, it could be more likely related to interregional differences in body size of individuals—smaller in Sierra Nevada compared to Alcornocales— than to intra-species adaptation. Higher relative feeding rates in smaller

individuals and greater energy storage as metamorphosis approaches in larger individuals, have been reported in other caddisfly species (e.g. Kiffer et al. 2016), and in most aquatic insects (e.g. Cavaletto and Gardner 1999), respectively.

Overall, our findings markedly highlight that feeding performance when facing decreasing nutritional value of leaf-litter is broadly a fixed-species trait of shredders, at least for the species studied here. These showed species-specific responses, but with a major distinction between the snail and the two insects. Furthermore, in line with our second hypothesis, interspecific changes in leaf-litter quality proved to have greater effects on feeding performance than intraspecific ones. Results on *Melanopsis* were in accord with our first prediction of increasing feeding rate on the poor-quality litter species, but not with the second one of increasing assimilation of the limiting nutrient relative to carbon. *Melanopsis* faced interspecific changes in food quality by increasing feeding rate on *Populus*. This litter was poorer in N but richer in P and Si compared to *Alnus*, and silicon appears to hinder N assimilation by preventing the leaf cell walls being broken apart and/or due to mid-gut damage (Massey et al. 2006, Massey and Hartley 2009, Hartley and De Gabriel 2016). Thus, compensatory feeding of *Melanopsis* on *Populus* likely offset reduced N concentration and possible difficulties for its assimilation, with respect to growth and energy reserves. In addition, the scraping feeding mode of the snail could allow individuals to perform a selective ingestion of the softer leaf matrix, leaving the vascular skeleton (Chergui and Pattée 1991, this study), overcoming adverse effects from high toughness of *Populus* as a pre-ingestive constraint. These and the above-mentioned putative post-assimilatory mechanisms to get rid of the excess carbon acquired, build evidence on the outstanding behavioural and physiological adjustments developed by freshwater snails to compensate poor-quality food (Rollo and Hawryluk 1988, Fink and von Elert 2006). Moreover, as freshwater molluscs appear to have greater P requirements than detritivorous insects (Frost et al. 2006), the higher P concentration of *Populus* might have provided value-added palatability of this leaf-litter to *Melanopsis* compared to insect species.

Decreasing interspecific leaf-litter quality had far-reaching consequences on the two insect species, particularly *Tipula*, compared to *Melanopsis*. In contrast with our predictions, both insect species showed preferential feeding: higher consumption and growth rates in the N-fixer *Alnus* compared to *Populus* and no fundamental differences in apparent assimilation efficiency of limiting nutrients relative to carbon were detected between litter species. This supports the common notion that nitrogen is a key driver of invertebrate consumption of litter, due to its limiting role for consumers (e.g. Evans-White et al. 2005, Frainer et al. 2016). However, an increase in the consumption of low-quality resources to maintain growth rates seems to be a more common response when offering single diets (this experiment) than in multiple-choice feeding experiments (Cruz-Rivera and Hay 2000, Swan and Palmer 2006, but see Santonja et al. 2018). Compensatory feeding

in low-quality resources has been indeed reported for caddisfly and *Tipula* species in single-leaf species experiments (Flores et al. 2014, Fuller et al. 2015), a strategy that in the case of our *Tipula* species, if this had been able to perform it, could have countered its negative growth in *Populus*.

Factors determining compensatory feeding in detritivore shredders are poorly known, but it has been suggested that it could depend on species idiosyncrasies interacting with certain leaf-litter traits (toughness, lignin…) other than limiting nutrients (Flores et al. 2014, Frainer et al. 2016). In this regard, our results obtained when the two insect species were faced to intraspecific qualities of *Alnus* litter appear particularly meaningful. Feeding rates of *Allogamus* were not affected by the quality of *Alnus*, this being in line with results obtained using artificial diets just differing in N and P concentrations (E. Fenoy, unpublished results). This suggests that the lack of compensatory feeding in this species is an idiosyncrasy independent of litter traits that might hinder its accomplishment. However, *Tipula* showed clear compensatory feeding between *Alnus* quality classes, a common adaptation to meet nutritional requirements in slow moving species, such as tipulids, when the quality of available food is low (Canhoto and Graça 2006). This point to the existence of a critical litter-quality threshold between *Alnus* and *Populus* from which *Tipula* could not perform this feeding strategy. These two litter species differed in several traits others than elemental nutrients, but the most prominent divergence was for Si, with between ten to fifteen-fold greater Si content in *Populus*. It is relatively well known, particularly in grasses, that silicification reduce herbivory (Massey et al. 2006), but little is known on the magnitude of this process in tree species and the subsequent effects on leaf-litter shredding by freshwater detritivores. Increasing silicon concentration in grasses reduces leaf consumption, digestion efficiency, and growth rates of folivorous insects (Massey et al. 2006, Massey and Hartley 2009). These effects have been firmly related to silicon' abrasiveness, which produces irreversible mandible wear and, perhaps, degradation of the mid gut of caterpillars, reducing N absorption (Massey and Hartley 2009). Our results on relative absorption of N in *Populus vs Alnus* diets, for both insect species does not support the presumed effects of abrasive silicon on digestion and assimilation. Thus, more likely feeding deterrence and/or mandible wear due to Si in *Populus* diets could have reduced consumption rates—by about half compared to *Alnus*—in *Allogamus*, and exerted critical constraints to putative compensatory feeding in *Tipula*, with detrimental post-ingestive consequences, particularly in the second species.

The ability of *Melanopsis* to perform compensatory feeding in *Populus* is in line with the general perception that effects of accumulated silicon in plant tissues seems to be less serious for snails. Several studies have reported no effects of increased Si content on consumption rates on herbivorous and detritivores snails (Schaller 2013, Horgan et al. 2017, but see Griffin et al. 2015), which has been attributed to the renewable teeth of their radula making it less susceptible to wear caused by silicon (Horgan et al. 2017).

Nevertheless, the significantly lower consumption rates of *Melanopsis* in the low-quality—with higher Si and lower N—compared to the high-quality class of *Populus*, is difficult to justify following the former rationale. It may be that the nearly 40% more Si in the low- than in the high-quality class, could have prevented compensatory feeding for having exceeded certain Si threshold of tolerance. This is, however, counter evidences on snail species of similar size to *Melanopsis* which do not exhibit feeding deterrence in common reed litter (Schaller 2013), which normally has much more Si content than poplar (Salinas et al. 2018). Alternatively, the threshold for compensatory feeding of *Melanopsis* between high and low quality classes of *Populus* could be related to litter C:N ratio. Given that this snail showed no particular ability to improve N assimilation relative to C, it appears that the excess of C gained eating low-quality *Populus* imposes metabolic constrains to increase feeding rate in this litter. This argument finds some support in the fact that feeding on this low-quality litter *Melanopsis* accumulated significantly higher reserves compared to high-quality *Populus*, as long as we may interpret this as a mechanism to get rid to the excess of carbon acquired (see above). Further research is needed to clarify this issue, but it seems that compensatory feeding could be conditioned by metabolic constrains in combination with thresholds of litter quality, as it has been suggested for herbivorous insects (Kerslake and Hartley 1997, Johnson et al. 2014).

No comprehensive conclusion can be drawn from results on a reduced number of shredder species. However, since the species studied here are widespread and locally abundant, our results may serve to outline possible trends of trophic interactions in Mediterranean low-order streams in the context of climate change. Elevated [CO_2], global warming, and increasing aridity can result in impoverished nutritional quality of leaf-litter inputs to stream detritivores: i.e. higher C:N ratio (Stiling and Cornelissen 2007, Ferreira et al. 2010) and Si content (Salinas et al. 2018). Recently, Rota et al. (2018) have encouraged studies to advance our knowledge on phenotypic determinants of resource use in shredders. Our results suggest that intra-species adaptations to improve feeding performance of shredders facing reduced litter quality are not common. Instead, this capability appears to be a fixed-species trait that varies among major phylogenetic groups: snails *vs*. insects. *Melanopsis*, though being a generalist feeder, has a key role for the incorporation of leaf-litter into detrital food webs in spring-fed lowland streams (Heller and Abotbol 1997, Casas et al. 2011). The remarkable capacity of this snail to cope with increasing C:N ratio and Si shown here, suggests that ecosystem processes are less likely to be affected by decreasing litter quality triggered by climate change. Water extraction and contamination that cause population decline or extinction of this snail (e.g. Bartolini et al. 2017), entail much more serious threats for the conservation of these lowland ecosystems. Conversely, headwater streams in mountainous regions are in general exempt of these anthropogenic impacts, for being in unpopulated watershed and/or under some nature conservation status. However, in these streams, where most shredders are insects, decreasing litter quality prompted by climate change, particularly if it is associated with

changes in riparian species composition, may cause major alterations in ecosystem processes.

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Chapter 6

Warming and nutrient-depleted food: two difficult challenges to face simultaneously by an aquatic shredder

Encarnación Fenoy

Francisco J. Moyano

J. Jesús Casas

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6.1. Introduction

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 ($[CO_2]$) is the main cause of global warming. Increases in both temperature and CO_2 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 $[CO_2]$ 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_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* Navàs (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 (β-glucosidase, trypsin, and alkaline phosphatase) responded to the combined effects of increasing temperature and decreasing food quality.

6.2. Material and 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.

6.2.1. 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 (±SE) annual temperature between 2013 and 2016 was 11.0 ± 1.6ºC, the winter mean was 3.5 ºC (range = 0.9–8.0ºC), the spring mean was 9.6ºC (range = 2.4–14.2), and the summer mean was 14.1ºC (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 concentration. 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 aquaria 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, 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 60ºC 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 60ºC 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 mm^2. 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 550ºC 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).

6.2.2. 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 15ºC), and we randomly assigned each of the 6 buckets in each incubator to 1 of 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 60ºC 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.

6.2.3. Consumption, survival, egestion, and excretion

We estimated the relative feeding rate (RFR) as follows:

$$\text{RFR } (mg_{disc}\ g_{larva}^{-1}\ d^{-1}) = (DM_t - DM_i)/(DM_{larva} \times t) \quad \text{(Eq. 1)},$$

where DM_t is dry mass of the disc at the end of the period of exposure to the larva, DM_i 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 DM_{larva} 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 DM_{larva}. We developed a head width–body DM (dried at 60ºC for 72 h,

weighed to the nearest 0.1 mg) regression for *A. mortoni* (without case): DM (g) = 2.3391 × 10^{-4} $e^{3.1437 \times \text{head width (cm)}}$ (R^2 = 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 (60ºC for 72 h) and then analyzed for C, N, and P concentration (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 Dunn'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 (NH_4^+) and phosphate (PO_4^{3-}) with the salicylate and the ascorbic acid methods, respectively (APHA 2005). Excretion rates were expressed as µg NH_4^+-N or PO_4^{3-}-P/mg 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:nutrient ratios of larvae. Changes in initial vs final (end of the experiment) body stoichiometry (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 of N and P as the difference between ingested and egested plus excreted nutrients following

Villanueva et al. (2011). Differences among treatments in net gain of N and P were tested with f-ANOVA-r.

6.2.4. Digestive enzyme activities

We measured enzyme activity at 5, 10, and 15ºC to assess the effect of temperature on the kinetic parameters (K_m and maximum enzyme activity [V_{max}]) of 3 digestive enzymes involved in the hydrolysis of C-, N-, and P-containing substrates (β-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 µmol/mL for β-glucosidase activity, 0–53 µmol/mL for trypsin activity, and 0–132 µmol/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 fluorogenic 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 β-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 µL of enzymatic extract, 120 µL borate buffer (50 mM, pH 7.5), and 10 µL of each substrate solution in a 96-well microplate. 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,

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

where V_{max} is the maximum enzyme activity, K_m is the half-saturation constant, which is the substrate concentration at which the reaction rate equals $V_{max}/2$, and S is the substrate amount. We estimated the enzymatic parameters V_{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_{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).

Table 6.1. 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.

	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

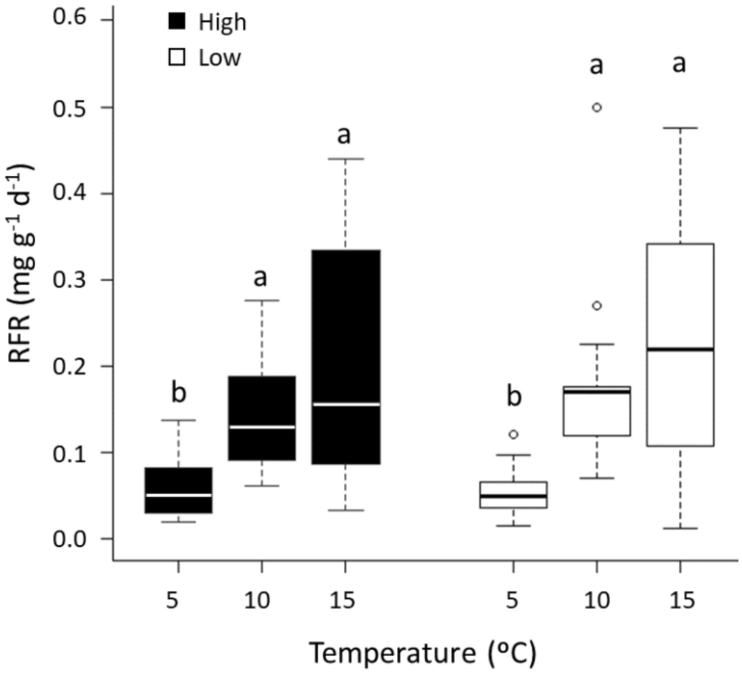

Figure 6.1. Mean (±SE) relative feeding rate (RFR; $mg_{diet}\ g_{larva}^{-1}\ d^{-1}$) of *Allogamus mortoni* fed on 2 diets differing in nutrient content (high and low) and exposed to temperatures of 5, 10, or 15ºC (n = 18 larvae/treatment). Box plots show median values (central line), 25th and 75th percentiles (box), and ranges (whiskers). Different letters indicate likely differences among temperatures ($F_{2,102}$ = 26.58, p < 0.001; post-hoc, p < 0.001). There were no diet or interaction effects.

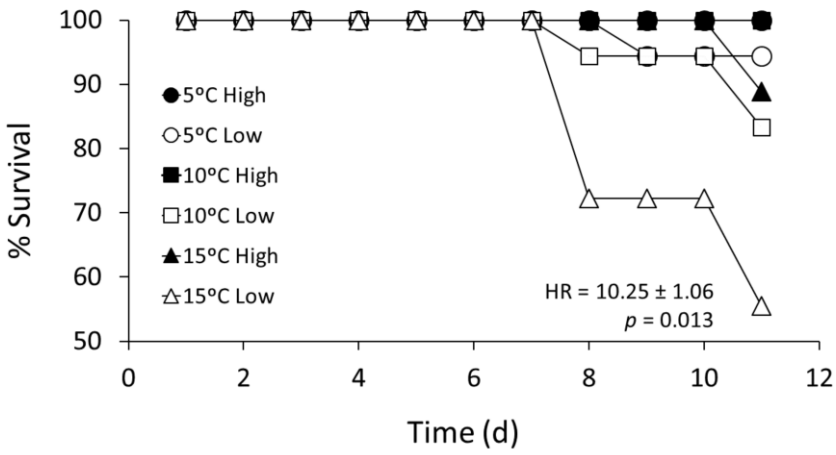

Figure 6.2. Survival (%) over 11 d of *Allogamus mortoni* fed on 2 diets differing in nutrient content (high and low) and exposed to 3 different temperatures (n = 18 larvae/treatment). Differences in survivorship among treatments were assessed with Cox proportional-hazards regression models. HR: Hazard's ratio.

6.3. Results

6.3.1. Artificial diets

Diets differed in toughness and stoichiometry except for N:P ratio (Table 6.1). The low-quality diet contained half of 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 S6.1). We used this result to set the exposure time of the diet to the larvae.

6.3.2. Feeding and survival

Feeding rates (Figure 6.1, Table S6.2) 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× higher at 15 than at 5ºC. 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 (logrank statistic = 20.79, p < 0.001; Figure 6.2). Individuals fed the low-quality diet had a higher probability of dying (Hazard's ratio, β = 10.25 ± 1.06) as temperatures increased (logrank statistic = 9.05, p = 0.013; Figure 6.2), but this trend was not as apparent for individuals fed the high-quality diet (logrank statistic = 4.12, p = 0.13).

6.3.3. 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 S6.3). With the high-quality diet, the C:N ratios of feces did not appear to differ from those of the food (ANOVA-r, $p = 0.329$). However, the C:N ratios in the low-quality food were higher than those in the corresponding feces (ANOVA-r, $p < 0.001$), especially at 5ºC at which food C:N was 1.5× higher than in feces (Figure 6.3A). Fecal C:P and N:P ratios were 1.8 and 1.6× lower on average, respectively, (ANOVA-r, both $p < 0.001$) than those in the corresponding diet in all temperature treatments (Figure 6.3B and 3C, respectively; Table S6.3). P-enrichment of feces was most obvious at 5ºC, whereas it tended to be lower for animals reared at 10 or 15ºC on either diet.

6.3.4. Excretion

The amount of excreted NH_4^+-N varied among temperatures (f-ANOVA-r, $p = 0.0012$) and between diets (f-ANOVA-r, $p = 0.011$). The effect of temperature on excretion depended on diet (f-ANOVA-r, $p = 0.002$; Figure 6.4A, Table S6.4). When larvae fed on the low-quality diet, NH_4^+-N excreta was 3 and 2× higher at 15ºC compared to 5 and 10ºC, 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 15ºC the excretion of N was 2.3× higher in the low- than in the high-quality diet (post-hoc test, $p = 0.016$; Figure 6.4A). Excreted PO_4^{3-}-P increased with increasing temperatures (f-ANOVA-r, $p < 0.001$), regardless of diet quality (Figure 6.4B, Table S6.4), but the only apparent difference among temperatures was between 5 and 15ºC, with excreted P 4× higher on average at 15ºC (post-hoc test, $p = 0.012$).

6.3.5. Enzyme assays

β-glucosidase activity decreased nearly 400 relative fluorescence units as temperatures increased from 5 to 15ºC (f-ANOVA-r, $p = 0.047$), regardless of diet quality (Figure 6.5A, Table S6.5). However, temperature did not influence the activity of trypsin or phosphatase (f-ANOVA-r, $p = 0.356$ and 0.082, respectively; Figure 6.5B, C, Table S6.5). Diet quality did not affect enzymatic activities (f-ANOVA-r, all $p > 0.063$; Figure 6.5A–C, Table S6.5).

The effect of temperature on K_m differed among enzymes. Apparent K_m (sensu Wallenstein et al. 2010) of β-glucosidase was doubled from 5 to 10ºC (ANOVA-r, $p < 0.001$; Figure 6.5D, Table S6.5). However, K_m could not be estimated accurately at 15ºC 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 (ANOVA-r, $p = 0.36$), although it appeared to decrease as temperatures rose (Figure 6.5E, Table S6.5). Apparent K_m of phosphatase activity increased as temperatures increased (ANOVA-r, $p = 0.013$), notably (1.7×) between 5 and 15ºC (ANOVA-r, $p = 0.013$; Dunn's test $p = 0.017$; Figure 6.5F, Table S6.5).

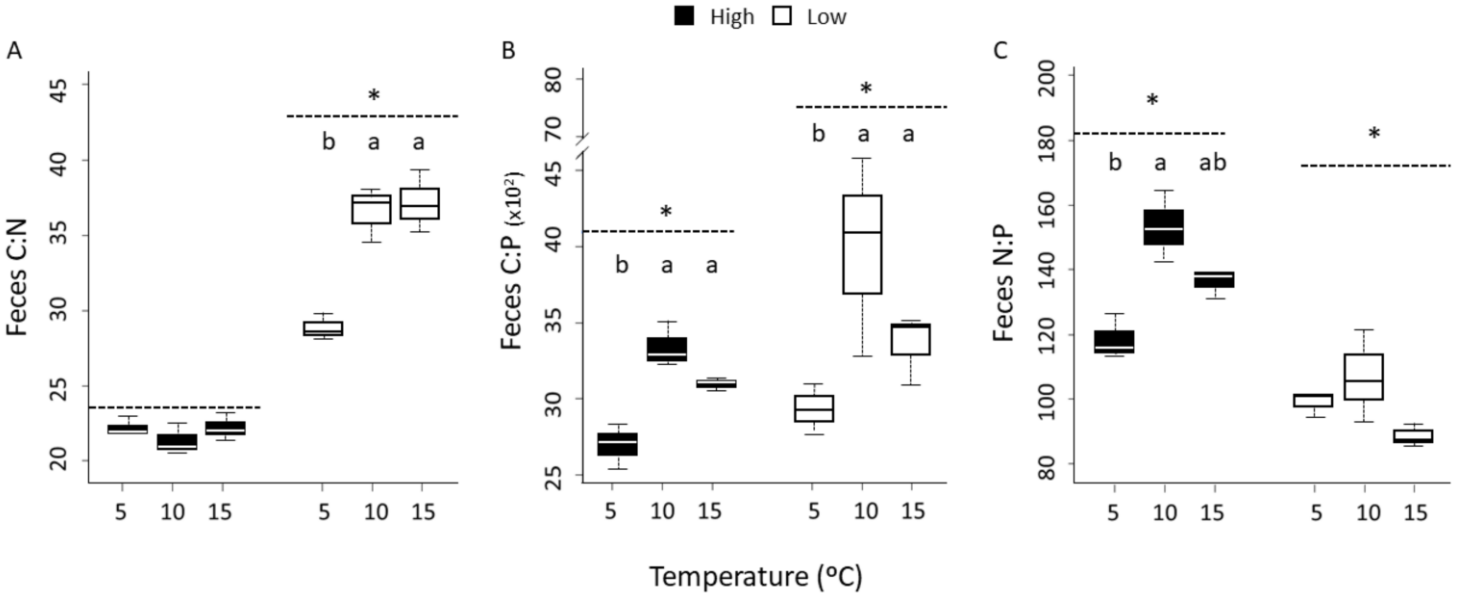

Figure 6.3. Mean (±SE) C:N (A), C:P (B), and N:P (C) molar ratios of feces egested by *Allogamus mortoni* larvae fed on 2 diets differing in nutrient content (high and low) and exposed to 3 different temperatures. Feces from each treatment were pooled into 3 replicates. Dashed lines show the ratios of the corresponding diets. Box plots show median values (central line), 25th and 75th percentiles (box), and the ranges (whiskers). * indicate that all feces stoichiometry differed between diets (ANOVA-r, $p < 0.001$). Different letters indicate significant differences (Dunn's post-hoc test, $p < 0.05$) among temperatures within each diet treatment.

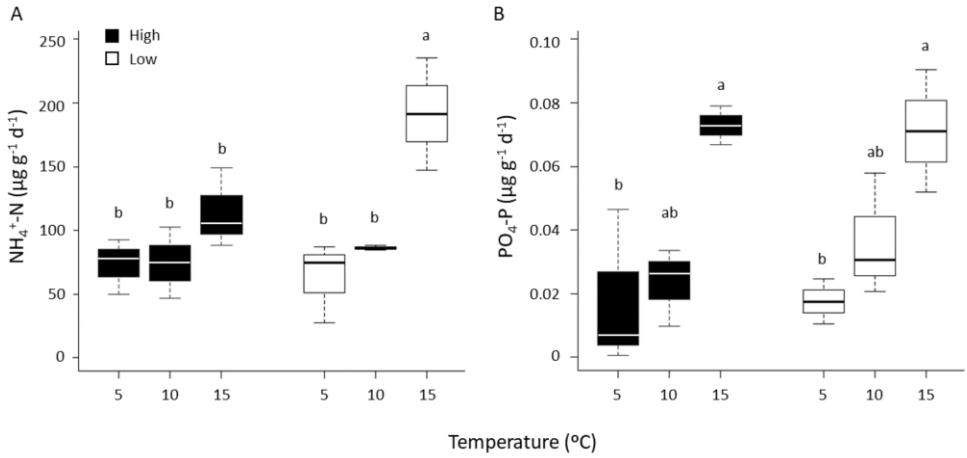

Figure 6.4. Mean (±SE) excretion rates ($\mu g_{nutrient}\ g_{larva}^{-1}\ d^{-1}$) of *Allogamus mortoni* fed on 2 diets differing in nutrient content (high and low) and exposed to 3 different temperatures. Larvae from each treatment were pooled into 3 replicates. Box plots show median values (central line), 25th and 75th percentiles (box), and the ranges (whiskers). Different letters indicate significant differences in ammonium (NH_4^+) 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 (PO_4^{3-}) excretion across temperatures (f-ANOVA-r, p < 0.001) (B).

6.3.6. 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; Figure 6.6A, Table S6.6). 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× higher C:P and N:P ratios at the end of the experiment relative to the beginning of the experiment at 10ºC (ANOVA-r, p = 0.008 and 0.038, respectively) and 2.3× higher at 15ºC (ANOVA-r, p = 0.025 and 0.016, respectively) (Figure 6.6B, C).

Individuals fed the high-quality diet assimilated 2.5× more N than those fed the low-quality diet (f-ANOVA-r, p < 0.001). 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 (Figure 6.7A, Table S6.7). 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 15ºC than at 5ºC (f-ANOVA-r, p < 0.001) (Figure 6.7B, Table S6.7).

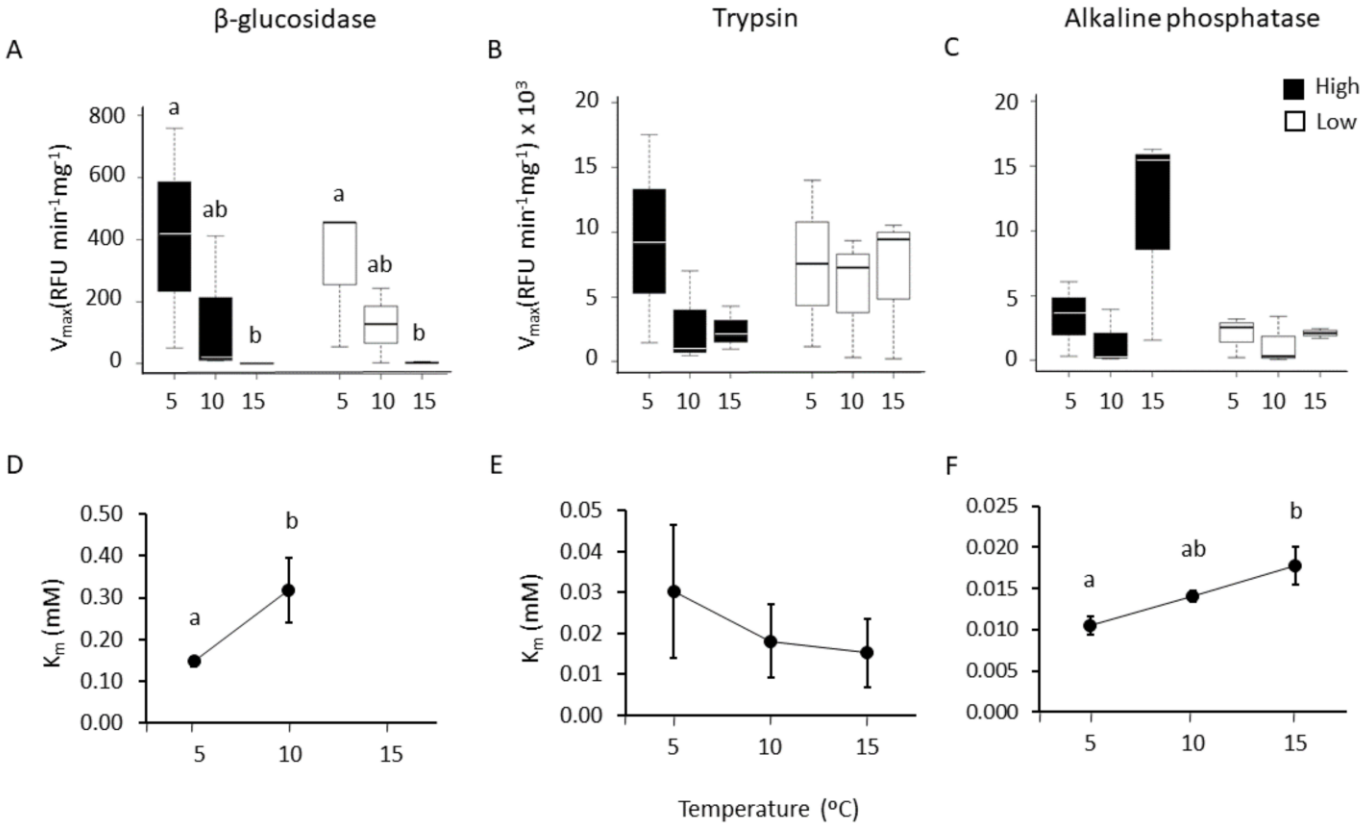

Figure 6.5. Mean (±SE) maximum enzyme activities (V_{max}) for β-glucosidase (A), trypsin (B), and alkaline phosphatase (C) of *Allogamus mortoni* acclimated to different temperatures when fed on 2 diets differing in nutrient content (high and low). Mean (±SE) effects of temperature on apparent Michaelis–Menten constant (K_m) for β-glucosidase (D), trypsin (E), and alkaline phosphatase (F). The K_m of β-glucosidase could not be estimated confidently at 15ºC. 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 V_{max} and K_m (f-ANOVA-r and ANOVA-r, respectively; all $p < 0.05$).

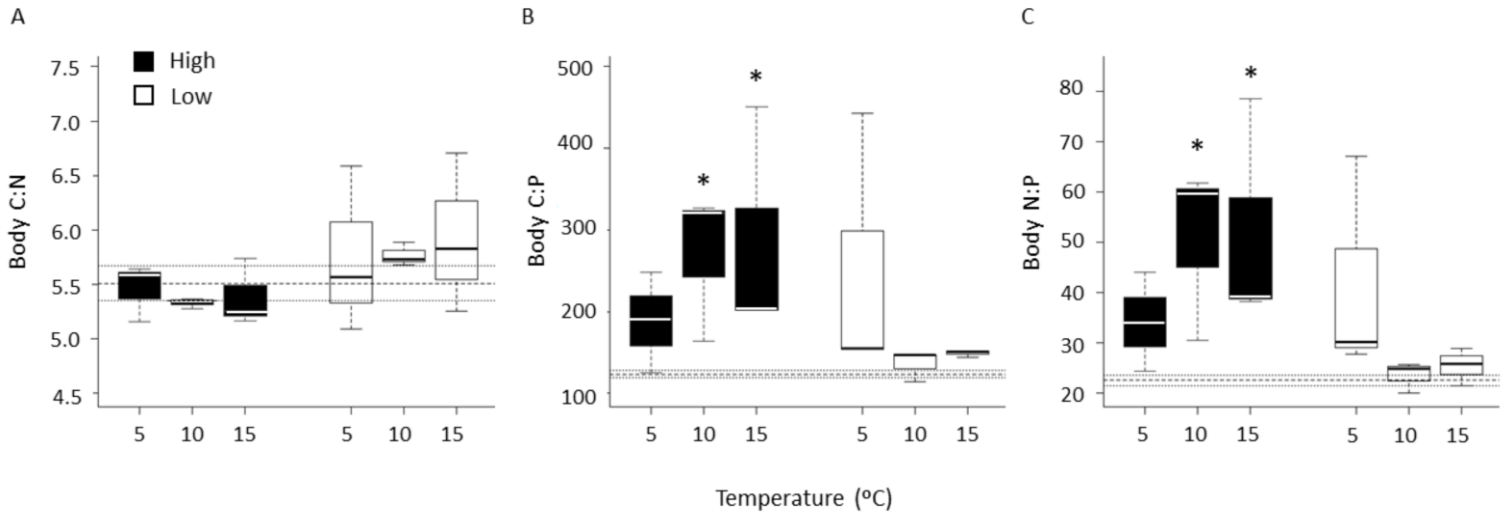

Figure 6.6. Mean (±SE) C:N (A), C:P (B) and N:P (C) molar ratios of body stoichiometry of *Allogamus mortoni* (n = 3) fed on 2 diets differing in nutrient content (high and low) at temperatures of 5, 10, and 15ºC. The solid lines that span the entire plot show the ratio of body stoichiometry before the experiment and the dashed lines show the standard error of the means (n = 9). Box plots show median values (central line), 25th and 75th percentiles (box), and ranges (whiskers). * indicate significant differences with initial body stoichiometry for a given temperature (ANOVA-r, $p < 0.05$). There were no differences in body stoichiometry across temperatures, diets, or interactions in any case.

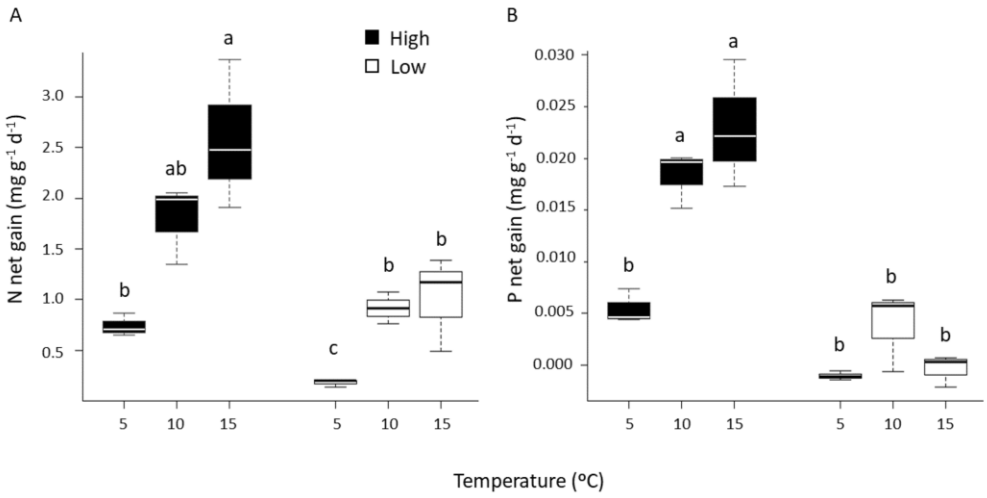

Figure 6.7. Net gain of N (A) and P (B) by *Allogamus mortoni* maintained at 5, 10, and 15°C and fed high- or low-quality diet (n = 3). Box plots show median values (central line), 25th and 75th percentiles (box), and ranges (whiskers). Effects of temperature, diet, and interaction were detected (f-ANOVA-r; all $p < 0.01$) for both N and P net gain. Different letters indicate significant differences (Holm-Sidack post-hoc test, $p < 0.05$) among treatments.

6.4. 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). Deleterious 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-Araneda 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 concentration, 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 5ºC, 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 10ºC but

increased sharply at 15ºC 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 (2002), the transition from the pejus range (moderate stress, i.e., 5ºC + low-quality food) into the pessimum range (high stress, i.e., 10ºC + 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 15ºC + low-quality food in our experiment. The future global scenario characterized by increased temperature beyond seasonal variations—records between 15 and 17ºC 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.

6.5. References

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Chapter 7

General discussion and conclusions

7.1. General discussion

The aim of the present PhD dissertation was to extend our current knowledge on how global change drivers may affect leaf-litter decomposition in headwater streams, a key ecosystem process in detritus-based stream food webs. The first part of this study was mainly focused on assessing effects of environmental factors on the functioning of microbial communities involved in leaf-litter degradation. In the second part, we aimed to evaluate the response of macroinvertebrate detritivores to changes in litter-quality potentially linked to global change.

Results presented in Chapter 2 demonstrate that the different factors affecting microbial decomposition of leaf-litter in streams can act in opposite directions: while temperature and dissolved nutrients had positive effect on breakdown rates, high stream-water pH and ion concentration (associated in the field experiment to warm and nutrient rich streams) depleted cellulolytic enzyme activity. However, the stimulatory effect of temperature overrides this negative effect by increasing metabolic rates (Brown et al. 2004), as well as total production and activity of cellulolytic enzymes (Wallenstein and Weintraub 2008). Results presented in Chapter 3 supported this finding in Chapter 2, since stream-water chemistry (total alkalinity, pH or electric conductivity) had negligible effect on litter mass loss of 4 plant species widely differing in leaf-traits, with temperature acting as the major abiotic driver of decomposition. The assessment of the activities and kinetic parameters of microbial hydrolytic enzymes can be useful to determine the consequences of warming within a given ecosystem, since warming might alter microbial stoichiometry modifying not only the demand of nutrients, but also the expression of enzymes (Sihi et al. 2019). As an example, it has been demonstrated that catalytic efficiency of enzymes in soil microbiota increases from low to intermediate temperatures, this resulting in the maintenance of high hydrolytic capacity as temperature rises, but also the existence of temperature thresholds that could trigger strong changes in catalytic efficiency and hence, on the decomposition process (Razavi et al. 2016).

Chapters 2 and 3 also evaluated the "home field advantage" (HFA) hypothesis. We assessed the HFA hypothesis in streams to know if microbial communities are adapted to decompose more efficiently the most abundant litter to which they are routinely exposed, regardless of its quality. Our results suggest that, once effects of the abovementioned extrinsic factors were accounted for, a positive HFA effect for low quality litter (i.e. giant graminoid species) emerges. This presumed community adaptation seems more likely to occur in the context of a region with low riparian plant diversity (taxonomically and functionally), producing inputs of low quality litter, when compared to other very different regions. However, this result contrasted with those obtained in Chapters 2 and 4. High richness of cellulase isoenzymes was measured on the low quality leaf-litter of the giant graminoid *Phragmites*, regardless this leaf-litter was incubated in its native or non-native range (Chapter 2), while fungal functional richness was lower in the native region of this

species, even in leaf-litter of the functionally similar *Arundo* (Semiarid Lowland, Chapter 4). Despite the lower functional richness, the number of cellulase isoenzymes on *Alnus* was higher in the Semiarid Lowland subregion (its non-native range). This might reveal a metabolic specialization on the use of the predominant form of C, since genes related to C and N acquisition are likely selected regarding dominant vegetation type and the biochemical traits of leaf-litter input (Paula et al. 2014). Additional analyses on potential changes in metabolic pathways due to the preferential use of some components of leaf-litter, should be performed to clarify this issue. However, results from Chapter 4 indicate that the ability of fungi to modulate their functionality, at least regarding C acquisition in terms of CLPP, is limited and depends on environmental factors, mainly temperature.

According to the biodiversity-ecosystem function (B-EF) hypothesis, species losses can alter ecosystem functioning as global change gain ground (Cardinale et al. 2002, 2006, Hooper et al. 2012). However, the fungal diversity-litter decomposition relationship is weakly supported from field and laboratory experiments which often attribute high level of functional redundancy to aquatic hyphomycete species (e.g. Bärlocher and Graça 2002, Dang et al. 2005, Pascoal et al. 2005). Results in Chapter 4 somehow support this statement, since overall taxonomic β-diversity was 4-fold higher than functional diversity, indicating a high rate of fungal species replacement with much lower changes in functionality. These small changes in functionality were related to the variation in functional richness and functions sorting across the environmental gradient studied, with temperature exerting a simplifying effect (lower functional richness) but introducing heterogeneity in the functional response of fungi. Thus, not species loss, but its replacement by other species likely better adapted to higher temperatures and to cooperate to reduce the energy cost of a high metabolic rate (Allison 2014). Thus, it seems that lower fungal functional richness resulted in lower decomposition efficiency (expressed in mg dd^{-1}, Chapter 3) in warmer streams, as reported in several other studies (Taylor and Chauvet 2014, Pérez et al. 2018).

As previously pointed by other authors (Bosatta and Ågren 1999, Fierer et al. 2005, Conant et al. 2011), our results support that decomposition of recalcitrant litters is more sensitive to warming. Despite the wide literature regarding temperature effect on the decomposition process and its interaction with litter quality, this key driver of the process has been not reliably established for a long time due to conflicting results (see review in Canhoto et al. 2016). Our findings suggest that litter quality determines the magnitude of effects of extrinsic factors, and therefore, it must be highlighted as the main driver of litter decomposition (Chapter 3, Zhang et al. 2019). The substantial quality differences in litter between N-fixing and non N-fixing plants likely determine which and how extrinsic factors affect the decomposition process. In Chapter 3, we aimed to shed some light on this conflicting issue by assessing the effect of both intrinsic and extrinsic factors on decomposition of a wide range of litter qualities. Our regional-scale study points to major

effects of global change drivers on leaf-litter decomposition in headwater streams, with patterns and trends analogous to those reported by studies carried out at larger scales (e.g. Zhang et al. 2019). Usually, when using N-fixing species, such as *Alnus*, the "identity" effect tends to predominate, overwhelming other extrinsic factors (Gonçalves et al. 2013, García-Palacios et al. 2016, Four et al. 2019). When such effect is not present or the differences between qualities are small (e.g. Ferreira and Chauvet 2011), extrinsic factors emerge as predominant. If climate change leads only to minor local changes in litter quality, microbial decomposition of leaf-litter would be additively accelerated by warming and nitrate enrichment of stream water, being these effects more pronounced for low-N litters. Thus, our results support predictions of no acceleration of litter decomposition if one or both of the above mentioned drivers fall out of the optimal range (Prescott 2010, Graça et al. 2015).

If microbial decomposition accelerates boosted by global change drivers, leaf-litter availability for shredders might decreases, thus altering trophic food web. In a less striking global change scenario, the time of in-stream permanence of the resource would not change significantly, but still being leaf-litter deficient in nutrients, and predictably accompanied by other deterrent factors— e.g. higher toughness due to increased siliceous or lignin content—which may affect consumption and assimilation rates of macroinvertebrates. The already high stoichiometric imbalance between aquatic shredders and their diet (Cross et al. 2005) will be exacerbated by the predicted reduction in nutritional value of leaf-litter inputs associated to global change scenarios. Both inter and intraspecific changes of plant litter, triggered by climate and land use changes, have the potential to affect substantially ecosystem functioning (Lecerf and Chauvet 2008, Graça and Poquet 2014). The ability of consumer's populations to face changes in resource stoichiometry should encompass individual's physiological and metabolic strategies (enhancing nutrient assimilation, reducing excretion, maintaining catalytic efficiency of enzymes, …). Overall, species of aquatic insects could be potentially well adapted, since they often show high assimilation or reduced excretion rates of limiting nutrients, i.e. nitrogen. However, as evidenced in Chapters 5 and 6, a high intake rate cannot be maintained with diets rich in structural components that hinder their processing or under high metabolic rates. Thus, insects might experience some decline due to these inter-specific quality changes of the trophic resource.

Potential intra-specific adaptations of shredders to succeed facing decreasing food quality have been scarcely tested. When compared individuals of the same species but from very distant populations in regions with sharp contrast in overall quality of leaf-litter inputs to streams, the results barely suggested the existence of intra-specific strategies (i.e. related to feeding behaviour or assimilation) to improve digestive efficiency of nutritionally poorer diets (Chapter 5). These findings suggest that *true* shredders (e.g. insects) can be particularly vulnerable to changes in riparian vegetation (Santonja et al. 2018). Conversely, "shredders" showing a more generalist feeding behaviour (i.e. snails)

can develop a differential use of food, depending on its nutritional value, even adopting different nutrient allocation strategies. Thus, these generalist consumers could play a key role in the incorporation of leaf-litter into detrital food webs, given their high capacity to cope with inter or intra-specific decreases in resource quality. The way interactions between warming and food stoichiometry may affect consumer homeostasis in aquatic environments remains unclear. Studies performed to date show conflicting results, perhaps due to the use of litters differing in nutrients besides other various components (e.g. Landeira-Dabarca et al. 2019), that add complexity to the interpretation of results. In Chapter 6 we tried to build-up knowledge in this area developing an alternative approach based on the use of artificial diets just differing in nutrient concentration, to minimize diet complexity. The results showed that tolerance to rising temperature was reduced when poor nutrients food was supplied, even though stoichiometric homeostasis and enzymatic rates can be maintained with high efficiency. To cope with nutrient imbalance linked to an increase of the metabolic rate, particularly in absence of compensatory feeding, some degree of flexibility in the stoichiometric niche might be necessary. In this line, assessment of the adaptation of enzymes involved in litter digestion under a warming scenario, mediated by variations in their functional parameters, should be considered in future studies of the nutritional ecology of shredders.

Little is known about the continuum between high homeostasis/low plasticity and low homeostasis/high plasticity of elemental body composition. The maintenance of strict homeostasis (low plasticity) should imply adaptations for a high efficiency in the acquisition of nutrients (compensatory or selective feeding, high assimilation, high efficiency of digestive enzymes, low excretion...). Thus, feeding behaviour and digestive physiology adaptations in order to achieve some degree of stoichiometric homeostasis, must necessarily be studied to evaluate nutrient flows from the individual to the ecosystem levels (Sperfeld et al. 2017). This represents an interesting line of research since changes in elemental availability, in combination with other abiotic factors (warming, eutrophication, salinization, etc.), could trigger phenotypic (intra-population) or genotypic (inter-population) selection, as well as modify interspecific relationships (competition, predation) and hence ecosystem function and organization. Some key questions arising could be: what species are more likely to be functionally displaced and what could be the consequences for the decomposition process, the whole food web and stream ecosystem functioning? Further studies focused on assessing inter and intra-specific trait adaptations regarding environmental change tolerance in a nutrient-depleted food scenario are needed to understand the whole ecosystem functioning and to predict its response to environmental changes. The geometric analysis of feeding and nutrition (Simpson and Raubenheimer 1993, Raubenheimer et al. 2012) of shredders, in combination with enzymatic or metabolomic approaches, can be a useful tool to understand, in short, how the Hutchinsonian hypervolume will be altered under this forecasted scenario. The overall findings from this Thesis underline the importance of decomposition as an indicator of the

functional changes that may occur in these ecosystems affected by anthropogenic stressors (Chauvet et al. 2016, von Schiller et al. 2017). The overall results from this work highlight the importance of riparian community changes and stresses derived from global change on structural and functional diversity in streams, suggesting the need to deepen in functional aspects of communities' response to preserve biodiversity and ecosystem functioning.

7.2. Conclusions

1. Leaf-litter traits have a leading role on decomposition process in headwater streams, with patterns and trends analogous to those reported by studies at larger scales. However, our knowledge on how specific leaf-traits and their interactions favour or constrain decomposition under global warming scenarios remains incomplete.

2. If climate change leads only to minor local changes in litter quality, microbial decomposition of leaf-litter would be additively accelerated by warming and streamwater nitrate enrichment, with more pronounced effects on low N litters.

3. Temperature is the abiotic factor with a major effect on microbial decomposition process, especially for the most recalcitrant species. Its effect largely overrides other environmental factors or the potential home-field advantage.

4. Home-field advantage hypothesis is supported for overwhelmingly dominant and low-quality leaf-litter species when abiotic factors are controlled but seems not attributable to enzyme adaptations to perform better under the prevailing conditions of their environments of origin.

5. Different fungal assemblages occurring in a wide range of environmental conditions are equally rich and diverse but differ in functionality. The reduction of functional richness by warming may cause lower decomposition efficiency in warm regions.

6. Intra-species adaptation of shredders to cope with depleted litter quality is not common, being essentially a fixed-species trait that varies among major phylogenetic groups. This suggests that ecosystem processes in lowland streams, where generalist shredder's snails are prevalent, are less likely to be affected by reduced litter quality.

7. Regulatory mechanisms present in shredders (insects) to reduce elemental constraints associated to poor food quality are less effective at higher temperatures. If similar responses occur in other detritivores, nutrient recycling and ecosystem function in headwater streams might be compromised as climate warms.

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Annexes

Table S3.1. Environmental variables (mean ± SE) measured in each stream, and the average for each region. Different superscript letters indicate significant differences determined by post-hoc Tukey HSD test ($P < 0.05$), following ANOVA. Also shown are *F*-values and significance level for the overall comparison among regions.

Regions and streams	O_2 (mg L^{-1})	Mean winter Temperature (ºC)	pH	E.C. (µS cm^{-1})	Total alkalinity (mg $CaCO_3$ L^{-1})
Sierra Nevada	**9.85 ± 0.04^{A}**	**3.97 ± 0.26^{A}**	**7.50 ± 0.04^{A}**	**68.28 ± 3.03^{A}**	**28.50 ± 3.88^{A}**
Lanteira	9.80 ± 0.27	3.80 ± 0.06	7.50 ± 2.25	63.65 ± 0.02	23.00 ± 1.00
Aldeire	9.93 ± 0.15	4.67 ± 0.06	7.42 ± 1.25	62.45 ± 0.05	26.00 ± 2.00
Ferreira	9.90 ± 0.39	3.44 ± 0.06	7.48 ± 1.65	73.20 ± 0.22	25.00 ± 1.00
Dólar	9.77 ± 0.21	3.99 ± 0.06	7.60 ± 1.40	73.80 ± 0.13	40.00 ± 12.00
Cazorla	**9.82 ± 0.31^{A}**	**7.65 ± 0.99^{B}**	**8.38 ± 0.06^{B}**	**374.25 ± 50.10^{B}**	**220.13 ± 20.86^{B}**
Chorrogil	10.51 ± 0.24	6.00 ± 0.05	8.51 ± 0.45	254.00 ± 0.01	199.50 ± 24.50
Gil Cobo	10.07 ± 0.28	7.10 ± 0.03	8.46 ± 0.60	385.50 ± 0.04	203.50 ± 28.50
Aguascebas	9.65 ± 0.09	6.55 ± 0.05	8.28 ± 0.05	359.50 ± 0.06	195.00 ± 15.00
Parra	9.04 ± 0.23	9.84 ± 0.04	8.28 ± 0.30	498.00 ± 0.03	282.50 ± 7.50
Alcornocales	**9.63 ± 0.02^{A}**	**11.81 ± 0.40^{C}**	**7.33 ± 0.12^{A}**	**72.68 ± 2.36^{A}**	**15.00 ± 1.73^{C}**
Tesorillo	9.63 ± 0.04	12.28 ± 0.02	7.16 ± 0.15	75.75 ± 0.14	14.00 ± 4.00
Aljibe	9.66 ± 0.26	11.14 ± 0.03	7.51 ± 0.70	74.85 ± 0.06	20.00 ± 10.00
Medio	9.58 ± 0.17	12.69 ± 0.02	7.10 ± 0.85	65.65 ± 0.20	14.00 ± <0.01
Cierva	9.65 ± 0.42	11.15 ± 0.03	7.55 ± 1.20	74.45 ± 0.05	12.00 ± 2.00
Semiarid lowland	**8.28 ± 0.41^{B}**	**16.85 ± 1.97^{D}**	**8.05 ± 0.13^{C}**	**2227.00 ± 500.57^{C}**	**205.63 ± 15.82^{B}**
Adra	7.58 ± 0.17	22.32 ± 0.01	7.78 ± 0.31	2325.00 ± 0.07	225.00 ± 5.00
Vícar	8.19 ± 0.19	16.77 ± 0.01	8.14 ± 0.22	838.00 ± 0.10	177.50 ± 32.50
Aguas	7.91 ± 0.40	15.17 ± 0.02	7.90 ± 0.52	3215.00 ± 0.12	180.00 ± 25.00
Negras	9.45 ± 1.01	13.15 ± 0.06	8.38 ± 0.58	2530.00 ± 0.16	240.00 ± 5.00
Comparison					
Region	20.30^{***}	186.87^{***}	79.16^{***}	631.04^{***}	201.34^{***}

$***P < 0.001$; $**P < 0.01$; $*P < 0.05$; n.s. $P > 0.05$.

Table S3.1. Continued.

Regions and streams	TN ($\mu g\ L^{-1}$)	$N\text{-}NO_3$ ($\mu g\ L^{-1}$)	TP ($\mu g\ L^{-1}$)	SRP ($\mu g\ L^{-1}$)
Sierra Nevada	**220.81 ± 60.68^{A}**	**85.60 ± 39.38^{A}**	**15.98 ± 1.81^{A}**	**4.28 ± 0.50^{A}**
Lanteira	157.14 ± 134.55	15.01 ± 7.15	14.69 ± 0.78	3.37 ± 0.83
Aldeire	149.93 ± 50.46	77.56 ± 8.93	11.56 ± 2.03	4.03 ± 0.17
Ferreira	402.21 ± 4.81	197.28 ± 28.59	17.81 ± 2.66	5.70 ± 3.50
Dólar	173.95 ± 40.85	52.54 ± 23.23	19.84 ± 0.31	4.03 ± 0.17
Cazorla	**569.20 ± 271.45^{AB}**	**237.94 ± 82.16^{B}**	**3.87 ± 1.13^{B}**	**1.62 ± 0.32^{B}**
Chorrogil	166.75 ± 19.22	138.31 ± 44.67	4.53 ± 2.81	2.53 ± 1.00
Gil Cobo	286.88 ± 81.69	268.76 ± 110.79	1.56 ± 0.78	1.20 ± 0.33
Aguascebas	459.88 ± 24.03	456.40 ± 87.56	2.66 ± 0.31	1.53 ± <0.01
Parra	1363.29 ± 941.85	88.28 ± 16.08	6.72 ± <0.01	1.20 ± <0.01
Alcornocales	**379.38 ± 112.13^{AB}**	**113.12 ± 73.23^{A}**	**9.84 ± 1.80^{AC}**	**3.41 ± 0.28^{A}**
Tesorillo	599.23 ± 172.99	330.59 ± 266.98	4.53 ± <0.01	3.03 ± 0.83
Aljibe	531.96 ± 120.13	11.44 ± 7.15	11.25 ± 0.16	3.70 ± 0.50
Medio	116.29 ± 60.07	52.54 ± 5.36	11.09 ± 0.31	4.03 ± 0.50
Cierva	270.06 ± 88.90	57.90 ± 35.74	12.50 ± 3.91	2.87 ± 1.33
Semiarid lowland	**1256.97 ± 422.97^{B}**	**673.52 ± 412.09^{B}**	**9.34 ± 4.02^{BC}**	**3.20 ± 0.74^{AB}**
Adra	2096.11 ± 16.82	1809.15 ± 96.50	5.94 ± 4.84	2.53 ± 1.33
Vícar	856.32 ± 12.01	753.04 ± 19.66	0.47 ± 0.31	2.03 ± 0.50
Aguas	1807.78 ± 1549.74	107.93 ± 14.30	11.72 ± 8.44	2.87 ± 1.00
Negras	267.66 ± 43.25	23.95 ± 5.36	19.22 ± 0.63	5.37 ± 2.17
Comparison				
Region	5.65^{**}	13.93^{***}	9.92^{***}	5.83^{**}

$***P < 0.001$; $**P < 0.01$; $*P < 0.05$; n.s. $P > 0.05$.

Table S3.2. Leaf litter traits (mean ± SE; n = 12 litter bags per region) of leached material of the four species assayed. Also shown are the ANOVA results for the comparisons.

	Alnus	*Fraxinus*	*Rhododendron*	*Arundo*	*F*- value
%C	50.077 ± 0.087^{a}	48.220 ± 0.145^{a}	49.384 ± 0.072^{a}	37.053 ± 2.155^{b}	93.3
%N	2.614 ± 0.049^{a}	1.005 ± 0.004^{b}	0.647 ± 0.033^{c}	0.410 ± 0.014^{d}	1141.1***
%P	0.032 ± 0.003^{a}	0.036 ± 0.004^{a}	0.024 ± 0.001^{ab}	0.019 ± 0.003^{b}	10.9***
C:N	22.440 ± 0.409^{a}	56.137 ± 0.279^{b}	90.403 ± 5.446^{c}	106.244 ± 5.206^{d}	628.9***
C:P	4285.20 ± 403.44^{ab}	3716.50 ± 218.28^{b}	5380.56 ± 125.01^{a}	5578.10 ± 659.33^{a}	5.1**
N:P	190.366 ± 15.16^{a}	66.119 ± 3.77^{b}	59.776 ± 2.41^{b}	53.063 ± 5.099^{b}	52.3***
%Hemicellulose	18.790 ± 1.51^{a}	14.770 ± 0.54^{b}	14.347 ± 0.94^{b}	26.386 ± 1.15^{c}	34.6***
%Cellulose	16.704 ± 0.56^{a}	19.771 ± 0.72^{b}	22.054 ± 0.69^{b}	35.178 ± 1.04^{c}	125.4***
%Lignin	17.121 ± 1.82^{a}	10.734 ± 0.55^{b}	11.282 ± 1.28^{b}	2.844 ± 0.34^{c}	75.5***
Si (µg g^{-1})	298.32 ± 34.25^{a}	213.47 ± 12.98^{a}	249.68 ± 69.62^{a}	13168.36 ± 3301.50^{b}	22.39***
Toughness	62.16 ± 2.48^{a}	65.49 ± 0.55^{a}	174.26 ± 9.14^{b}	393.64 ± 50.90^{c}	48.67***

$***P < 0.001$; $**P < 0.01$; n.s. $P > 0.05$.

Table S3.3. ANOVA results of mixed-effects models performed on leaf AFDM loss (mg d^{-1} and mg dd^{-1}) and fungal biomass (mg g^{-1}) on each species at each of the four subregions.

	AFDM loss (mg d^{-1})			AFDM loss (mg dd^{-1})			Fungal biomass (mg g^{-1})		
	n = 295			*n* = 295			*n* = 64		
	χ^2	df	*P*-value	χ^2	df	*P*-value	χ^2	df	*P*-value
(Intercept)	299.3	1	< 0.001	136.4	1	< 0.001	1450.8	1	< 0.001
Region	14.4	3	0.002	54.4	3	< 0.001	8.3	3	0.041
Species	278.4	3	< 0.001	147.5	3	< 0.001	169.8	3	< 0.001
Region × Species	118.1	9	< 0.001	137.8	9	< 0.001	479.2	9	< 0.001
Random-effect significance level	< 0.001			< 0.001			< 0.001		
Marginal R^2	0.65			0.66			0.80		
Conditional R^2	0.79			0.77			0.92		

Table S3.4. Detailed results of RF models on leaf mass loss as response variables (including or not *Alnus*). The variables used as predictors are environmental characteristics and leaf traits. Inc. node purity (the average decrease in node impurity measured as the residual sum of squares) is used to assess the importance of predictors in an RF model. The higher the value, the more important the variable.

Dependent variables	% Variance explained	Predictor variables	Inc. Node Purity
AFDM loss (mg d^{-1})	72.4	N:P	0.0063
		T_{mean}	0.0048
		NO_3	0.0045
		C:N	0.0039
		Toughness	0.0038
AFDM loss (mg d^{-1}) (without *Alnus*)	65.6	T_{mean}	0.0037
		NO_3	0.0025
		N_t	0.0018
		Hemicellulose	0.0014
		Fungal biomass	0.0013
		Elevation	0.0012

Table S4.1. Results of two-way ANOVA on taxonomic richness and Shannon's diversity (n = 64).

	Sum Sq	df	*F*-value	*P*-value
Richness				
(Intercept)	1722.25	1	170.274	< 0.001
Region	81.50	3	2.686	0.057
Species	22.69	3	0.748	0.529
Region × Species	76.31	9	0.838	0.585
Residuals	485.50	48		
Shannon's diversity				
(Intercept)	20.19	1	349.264	< 0.001
Region	0.46	3	2.671	0.058
Species	0.18	3	1.018	0.393
Region × Species	0.43	9	0.821	0.600
Residuals	2.77	48		

Table S4.2. Results of two-way ANOVA on functional richness and Shannon's diversity (n = 64).

	Sum Sq	df	*F*-value	*P*-value
Richness				
(Intercept)	30468.6	1	**4264.62**	**< 0.001**
Region	118.8	3	**5.54**	**0.002**
Species	67.6	3	**3.16**	**0.033**
Region × Species	174.9	9	**2.72**	**0.012**
Residuals	342.9	48		
Shannon's diversity				
(Intercept)	51.5	1	**1581.11**	**< 0.001**
Region	1.0	3	**10.21**	**< 0.001**
Species	0.5	3	**5.59**	**0.002**
Region × Species	0.8	9	**2.69**	**0.013**
Residuals	342.9	48		

Table S5.1. Percentage cover-weighted mean (± SE) of selected leaf traits of the four most abundant species in the riparian vegetation of the studied subregions (n=3 streams per subregion). % Abundance: mean % cover of each plant species per subregion. Total riparian canopy %cover and mean (± SE) leaf traits in each subregion are in bold. Also shown results of weighted t-tests for comparison of leaf traits between pairs of subregions within each lithological setting: siliceous (Sierra Nevada *vs.* Alcornocales) and calcareous (Grazalema *vs.* Semiarid Lowland). All ratios are molar.

REGIONS / Species	% Abundance	% C	% N	%P	C:N	C:P
SIERRA NEVADA	**55.22 ± 1.70**	**46.68 ± 0.73**	**2.580 ± 0.287**	**0.130 ± 0.011**	**19.75 ± 5.13**	**375.16 ± 35.06**
Alnus glutinosa	28.26 ± 9.53	47.09 ± 0.95	2.967 ± 0.167	0.117 ± 0.006	16.02 ± 0.67	407.91 ± 27.05
Pinus halepensis	2.66 ± 2.04	51.23 ± 0.50	0.881 ± 0.058	0.107 ± 0.036	58.34 ± 3.26	534.55 ± 173.02
Rubus ulmifolius	7.72 ± 2.77	45.05 ± 0.32	2.248 ± 0.073	0.122 ± 0.004	20.16 ± 0.77	371.84 ± 11.28
Salix atrocinerea	16.58 ± 4.08	46.03 ± 1.32	2.348 ± 0.089	0.160 ± 0.009	19.72 ± 0.77	295.32 ± 21.62
ALCORNOCALES	**52.26 ± 0.65**	**47.88 ± 0.71**	**1.984 ± 0.433**	**0.065 ± 0.004**	**29.66 ± 8.22**	**802.42 ± 51.45**
Alnus glutinosa	25.76 ± 4.58	48.47 ± 0.41	2.672 ± 0.211	0.070 ± 0.009	18.61 ± 1.56	753.34 ± 122.30
Nerium oleander	3.18 ± 1.57	47.08 ± 1.09	1.028 ± 0.024	0.069 ± 0.018	45.79 ± 0.00	738.33 ± 208.26
Rhododendron poncticum	13.19 ± 3.51	48.74 ± 0.39	0.948 ± 0.019	0.053 ± 0.005	51.54 ± 1.31	955.66 ± 90.58
Rubus ulmifolius	10.13 ± 3.90	45.50 ± 0.17	1.884 ± 0.044	0.068 ± 0.011	24.22 ± 0.63	747.81 ± 103.90
Comparison		4.836***	-4.853***	-22.996***	4.229***	30.703***
GRAZALEMA	**70.02 ± 2.07**	**45.19 ± 1.92**	**2.009 ± 0.174**	**0.108 ± 0.007**	**23.62 ± 3.41**	**441.27 ± 41.18**
Ficus caryca	13.50 ± 1.93	38.75 ± 0.29	2.375 ± 0.298	0.129 ± 0.014	16.78 ± 1.87	306.83 ± 29.35
Nerium oleander	7.94 ± 3.39	47.40 ± 0.07	1.269 ± 0.230	0.087 ± 0.010	38.65 ± 7.07	549.75 ± 64.47
Rubus ulmifolius	18.14 ± 9.31	44.95 ± 0.41	1.950 ± 0.065	0.099 ± 0.006	23.14 ± 0.60	459.03 ± 23.75
Salix pedicellata	30.44 ± 10.16	47.60 ± 0.20	2.074 ± 0.085	0.109 ± 0.017	23.02 ± 0.89	462.02 ± 84.35
SEMIARID LOWLAND	**55.46 ± 1.19**	**45.00 ± 1.51**	**1.449 ± 0.308**	**0.069 ± 0.010**	**39.17 ± 8.07**	**917.08 ± 144.66**
Arundo donax	14.22 ± 6.21	41.50 ± 0.98	2.063 ± 0.533	0.091 ± 0.028	24.38 ± 8.31	568.39 ± 188.29
Nerium oleander	12.69 ± 5.89	47.53 ± 1.06	1.045 ± 0.104	0.073 ± 0.034	46.27 ± 4.05	963.54 ± 357.64
Phragmites australis	12.44 ± 10.59	43.35 ± 17.33	1.921 ± 0.770	0.071 ± 0.033	26.12 ± 9.79	851.16 ± 414.16
Saccharum ravennae	16.11 ± 9.66	47.36 ± 0.53	0.861 ± 0.156	0.046 ± 0.020	56.72 ± 9.62	1239.16 ± 515.41
Comparison		-0.358 n.s.	-7.280***	-14.437***	8.006***	13.302***

*** P < 0.001; ** P < 0.01; * P < 0.05; n.s. = not significant (P > 0.05).

Table S5.1. Continued.

REGIONS / Species	Si (µg g^{-1})	% Lignin	Toughness (g)
SIERRA NEVADA	**271.19 ± 110.22**	**14.37 ± 4.20**	**79.98 ± 39.41**
Alnus glutinosa	300.37 ± 37.48	10.12 ± 1.09	62.16 ± 2.48
Pinus halepensis	1055.16 ± 803.16	16.19 ± 0.47	383.17 ± 124.25
Rubus ulmifolius	172.81 ± 22.96	6.32 ± 0.95	69.26 ± 3.82
Salix atrocinerea	141.49 ± 19.41	25.08 ± 0.84	66.70 ± 3.83
ALCORNOCALES	**202.193 ± 14.95**	**15.44 ± 2.48**	**111.01 ± 40.79**
Alnus glutinosa	190.52 ± 49.80	18.98 ± 0.27	66.12 ± 4.32
Nerium oleander	222.22 ± 14.35	11.21 ± 0.30	323.64 ± 11.55
Rhododendron poncticum	242.64 ± 54.10	15.35 ± 1.37	174.26 ± 9.13
Rubus ulmifolius	172.93 ± 32.60	7.87 ± 0.52	76.06 ± 2.23
Comparison	-2.618***	0.959 n.s.	2.302*
GRAZALEMA	**556.14 ± 397.46**	**13.88 ± 5.69**	**95.81 ± 39.54**
Ficus caryca	1959.94 ± 200.55	1.20 ± 0.12	83.64 ± 0.41
Nerium oleander	377.08 ± 137.95	12.34 ± 0.19	286.27 ± 6.62
Rubus ulmifolius	200.17 ± 61.76	6.02 ± 0.34	73.79 ± 2.01
Salix pedicellata	192.40 ± 64.12	24.60 ± 0.91	64.66 ± 3.64
SEMIARID LOWLAND	**9238.30 ± 3551.21**	**4.72 ± 1.95**	**311.15 ± 31.68**
Arundo donax	13168.36 ± 3301.50	2.40 ± 0.09	393.64 ± 50.90
Nerium oleander	388.06 ± 18.64	10.88 ± 1.95	326.90 ± 17.65
Phragmites australis	17136.47 ± 6451.82	2.76 ± 1.40	270.13 ± 91.74
Saccharum ravennae	6641.82 ± 1776.81	3.43 ± 0.10	257.62 ± 9.89
Comparison	10.716***	-7.173***	19.219***

*** P < 0.001; ** P < 0.01; * P < 0.05; n.s. = not significant (P > 0.05).

Table S5.2. Results of factorial ANOVAs (*F* statistic and significance level), comparing effects of subregion of shredder's provenance, leaf-litter species, and quality class on Relative Feeding Rate (RFR), Daily Instantaneous Growth Rate (DIGR), and energetic reserves (total lipids and glycogen contents) for the three shredders species used in feeding tests. Variables shown in figures 1 (*Melanopsis*), 2 (*Allogamus*) and 3 (*Tipula*).

Taxa/comparison	RFR ($mg\ g^{-1}\ d^{-1}$)		DIGR (d^{-1})		Total lipids ($mg\ g^{-1}$)		Glycogen ($mg\ g^{-1}$)	
Melanopsis praemorsa								
Subregion (Subr)	0.71	n.s.	7.3	**	156.74	***	156.29	***
Leaf-litter species (LLS)	8.8	**	0.02	n.s.	10.1	**	17.24	***
Quality class (QC)	3.6	n.s.	2.86	n.s.	3.73	n.s.	0.65	n.s.
Subr × LLS	0	n.s.	0.47	n.s.	1.47	n.s.	0.62	n.s.
Subr × QC	1.72	n.s.	0.28	n.s.	0.08	n.s.	1.96	n.s.
LLS × QC	5.61	*	1.09	n.s.	4.58	*	14.01	***
Subr × LLS × Q	0.78	n.s.	0.74	n.s.	1.59	n.s.	4.85	*
Allogamus mortoni								
Subregion (Subr)	34.04	***	0.2	n.s.	36.18	***	64.23	***
Leaf-litter species (LLS)	47.79	***	19.58	***	2.69	n.s.	2.07	n.s.
Quality class (QC)	3.29	n.s.	1.66	n.s.	0.67	n.s.	0.02	n.s.
Subr × LLS	5.95	*	0.2	n.s.	0.04	n.s.	0.18	n.s.
Subr × QC	0.01	n.s.	1.44	n.s.	2.69	n.s.	0.33	n.s.
LLS × QC	4.28	n.s.	0.16	n.s.	2.06	n.s.	0.15	n.s.
Subr × LLS × Q	0.93	n.s.	1.33	n.s.	1.34	n.s.	0.6	n.s.
Tipula leo								
Subregion (Subr)	1	n.s.	0.64	n.s.	9.18	**	1.18	n.s.
Leaf-litter species (LLS)	6.45	*	29.53	***	13.83	***	4.01	*
Quality class (QC)	0.01	n.s.	0.11	n.s.	0.94	n.s.	0.16	n.s.
Subr × LLS	0.79	n.s.	13.33	***	5.22	*	1.67	n.s.
Subr × QC	0.27	n.s.	0.02	n.s.	0.2	n.s.	0.62	n.s.
LLS × QC	13.99	***	1.88	n.s.	0.01	n.s.	0.18	n.s.
Subr × LLS × Q	1.43	n.s.	0.87	n.s.	0.01	n.s.	1.23	n.s.

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; n.s. = not significant ($P > 0.05$).

Table S5.3. Results of t-tests (*t*-value and significance level) comparing molar ratios of nutrients (C:N, C:P, N:P) between faeces egested by shredders species and the corresponding diet used to feed them.

Leaf-litter species and quality class	Molar ratio	*Melanopsis praemorsa*		*Allogamus mortoni*		*Tipula leo*	
		Grazalema	Semiarid Lowland	Sierra Nevada	Alcornocales	Sierra Nevada	Alcornocales
Alnus glutinosa							
High quality	C:N	-3.87 **	-10.49 ***	-3.84 **	-0.64 n.s.	2.33 *	3.43 **
	C:P	-1.20 n.s.	-1.9 n.s.	-2.63 *	-3.60 **	-0.06 n.s.	-0.64 n.s.
	N:P	-0.80 n.s.	-0.63 n.s.	-3.02 *	-3.63 **	-3.03 *	-2.78 *
Low quality	C:N	0.21 n.s.	-1.89 n.s.	1.29 n.s.	-0.29 n.s.	0.51 n.s.	-0.09 n.s.
	C:P	-1.47 n.s.	-0.27 n.s.	1.12 n.s.	-2.99 *	1.15 n.s.	2.28 n.s.
	N:P	-2.78 *	-1.92 n.s.	-1.07 n.s.	-2.95 *	-0.04 n.s.	1.81 n.s.
Populus alba							
High quality	C:N	-7.35 ***	-6.14 ***	-3.89 **	-1.18 n.s.	2.23 n.s.	3.32 *
	C:P	0.38 n.s.	-3.16 *	1.27 n.s.	-1.70 n.s.	0.77 n.s.	0.04 n.s.
	N:P	5.09 ***	1.03 n.s.	1.94 n.s.	-2.91 *	-2.36 *	-4.60 **
Low quality	C:N	-4.39 **	-1.72 n.s.	1.20 n.s.	-0.82 n.s.	1.71 n.s.	3.43 **
	C:P	1.80 n.s.	1.17 n.s.	5.30 ***	-1.72 n.s.	1.52 n.s.	-1.65 n.s.
	N:P	2.03 n.s.	0.24 n.s.	0.81 n.s.	-1.17 n.s.	-3.15 *	-2.77 *

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; n.s. = not significant ($P > 0.05$).

Annexe 6.1. Stability of artificial diets

We assessed stability of discs using distilled water at experimental temperatures (5, 10, and 15ºC) during different periods of submersion (6, 12, 24, 48, 72, and 120 h). We used 3 replicates per submersion time and temperature. It was done for each diet. Thereafter, discs were dried (60ºC, 72h) and weighed again, being remaining mass expressed as a percentage of the initial dry mass. Their stability (arcsin-transformed data) was tested using three-way ANCOVA, with diet quality and temperature as factors, and time as covariate.

Trials of stability in water produced very similar results between diets ($F_{1,101}$ = 2.76, P = 0.09) and temperatures ($F_{2,101}$ = 1.10, P = 0.33), with only a significant time effect ($F_{1,101}$ = 57.69, P < 0.001) on dry mass loss. Stability in water for the two diets at the three temperatures was significantly reduced when submersion time exceeded 100 h (post-hoc test, P < 0.001) compared to shorter submersion periods. Remaining mass until 72 h was on average 95.1 ± 0.3 %, whereas after 120 h decreased to 87.8 ± 1.7 %. Thus, we exposed disks of the diets to the action of the larvae for 72 h.

Table S6.1. Results of ANCOVA on stability in water of the artificial diets.

	SS	df	MS	F	p
Intercept	87.142	1	87.142	**17031.71**	**< 0.001**
Time	0.295	1	0.295	**57.69**	**< 0.001**
Temperature	0.011	2	0.006	1.11	0.334
Diet quality	0.014	1	0.014	2.76	0.100
Temperature × Diet quality	0.002	2	0.001	0.16	0.850
Error	0.517	101	0.005		

Table S6.2. Results of 2-way ANOVA of relative feeding rates of larvae feeding 2 diets at 3 temperatures.

	SS	df	MS	*F*	*p*-value
Intercept	1.807	1	1.807	**298.73**	**< 0.001**
Temperature	0.322	2	0.161	**26.58**	**< 0.001**
Diet quality	0.002	1	0.002	0.40	0.529
Temperature × Diet quality	0.004	2	0.002	0.33	0.721
Error	0.617	102	0.006		

Table S6.3. Results of the permutation test for analysis of variance (ANOVA-r) to compare nutrient stoichiometry between each diet and feces egested at a given temperature.

	High-quality diet		Low-quality diet	
	F_{obs}	*p*-value	F_{obs}	*p*-value
C:N	1.30	0.329	**53.55**	**< 0.001**
C:P	**134.73**	**< 0.001**	**147.80**	**< 0.001**
N:P	**52.35**	**< 0.001**	**110.85**	**< 0.001**

F_{obs}: observed *F*-statistic for each effect; *p*-value: proportion of permutations giving an *F*-value equal to or greater than that observed.

Table S6.4. Results of the permutation test for factorial analysis of variance (f-ANOVA-r) of nutrients excretion.

	NH_4^+		PO_4^{3-}	
	F_{obs}	*p*-value	F_{obs}	*p*-value
Temperature	**8.569**	**0.0012**	**18.054**	**< 0.001**
Diet quality	**0.913**	**0.011**	0.228	0.64
Temperature × Diet quality	**1.63**	**0.002**	0.383	0.69

F_{obs}: observed *F*-statistic for each effect; *p*-value: proportion of permutations giving an *F*-value equal to or greater than that observed.

Table S6.5. Results of the permutation test for factorial analysis of variance (f-ANOVA-r) of enzyme activity (V_{max}) and permutation test for analysis of variance (ANOVA-r) of enzyme affinity by the substrate (K_m).

	β-glucosidase		Trypsin		Alkaline phosphatase	
	F_{obs}	*p*-value	F_{obs}	*p*-value	F_{obs}	*p*-value
V_{max}						
Temperature	4.176	**0.047**	1.129	0.356	3.077	0.082
Diet quality	0.125	0.063	0.479	0.301	3.798	0.122
Temperature × Diet quality	0.065	0.059	0.513	0.350	2.375	0.072
K_m						
Temperature	**8.802**	**< 0.001**	6.167	0.360	**5.248**	**0.013**

F_{obs}: observed *F*-statistic for each effect; *p*-value: proportion of permutations giving an *F*-value equal to or greater than that observed.

Table S6.6. Results of the permutation test for factorial analysis of variance (f-ANOVA-r) of body stoichiometry of larvae fed high and low-quality diets and exposed to 3 temperature treatments; and permutation test for analysis of variance (ANOVA-r) of body stoichiometry changes of body larvae after experiment.

	C:N		C:P		N:P	
	F_{obs}	*p*-value	F_{obs}	*p*-value	F_{obs}	*p*-value
Differences in post-experiment body stoichiometry						
Temperature	0.081	0.926	0.045	0.960	0.016	0.988
Diet quality	3.722	0.837	2.112	0.077	4.422	0.057
Temperature × Diet quality	0.112	0.918	1.908	0.958	2.460	0.986
Differences in pre- and post-experimental body stoichiometry						
5ºC	0.346	0.894	1.283	0.331	1.634	0.254
10ºC	1.431	0.247	**6.855**	**0.008**	**7.082**	**0.038**
15ºC	0.437	0.719	1.473	0.025	2.158	0.016

F_{obs}: observed *F*-statistic for each effect; *p*-value: proportion of permutations giving an *F*-value equal to or greater than that observed.

Table S6.7. Results of the permutation test for factorial analysis of variance (f-ANOVA-r) of nutrients net gain.

	N net gain		P net gain	
	F_{obs}	*p*-value	F_{obs}	*p*-value
Temperature	**17.655**	**< 0.001**	**14.658**	**0.0013**
Diet quality	**28.632**	**< 0.001**	**89.79**	**0.003**
Temperature × Diet quality	**2.495**	**< 0.001**	**9.756**	**< 0.001**

F_{obs}: observed *F*-statistic for each effect; *p*-value: proportion of permutations giving an *F*-value equal to or greater than that observed.