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Interactive effects of UVR and nutrients on the primary producer-consumer interaction: An ecological-evolutionary perspective

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I. General introduction

The primary producer-consumer interaction in aquatic ecosystems

The study of structure and dynamics of networks pervades all of science (Strogatz, 2001; Clauset *et al.*, 2008). Ecological networks, unlike other networks, follow different rules since ecological interactions govern and constrain them in unique ways. Knowing how these operate is essential to understand the current response of species and ecosystems to perturbations in our global change world (Montoya *et al.*, 2006).

Within food-webs, each interaction contributes to the energy flow and biomass partitioning among different trophic levels, but some are especially relevant for the shape and regulation of the food-web. In addition, these are not the same across ecosystem types, being herbivory for aquatic and detritivory for terrestrial ecosystems (Fig. 1). The preferential fate of carbon (C) to the herbivorous pathway in aquatic ecosystems or detrital pathway in terrestrial ecosystems is strongly related with the structural, metabolic and stoichiometric properties of primary producers in each system as Lindeman firstly pointed out in 1942. There are strong evidences that variable selective pressures have driven differences between aquatic and terrestrial primary producers that propagate upward to shape food-web, what yields observed differences between both types of ecosystems (Shurin *et al.*, 2006). In Table 1, we summarize main characteristics of aquatic and terrestrial primary producers with major influence on the food-web. Different to the terrestrial, aquatic autotrophs are more productive per unit standing biomass (Nielsen *et al.*, 1996), and offer higher quality of food because of lack of structural tissues (Lindeman, 1942), limited chemical and structural defence strategies (Shurin *et al.*, 2006) and high content in

nutrients (N, P) (Elser *et al.*, 2000*a*). In addition, the higher metabolic and growth rate of consumer herbivores in aquatic systems compared with their terrestrial counterparts (Nielsen *et al.*, 1996; Niklas & Enquist, 2001), together with the apparent dominance of generalist consumers in water (Shurin *et al.*, 2006), make trophic efficiency at the primary producer-herbivorous consumer interface much higher in aquatic pelagic than terrestrial ecosystems. Thus, the primary producer-herbivorous consumer interaction (*i.e.* herbivory) plays an essential role for the food-web architecture in aquatic pelagic ecosystems (Shurin *et al.*, 2006). For instance, herbivorous zooplankton in lakes remove a three to four times greater proportion of primary productivity than grazers in terrestrial ecosystems, and aquatic consumers can be from six to sixty times more abundant on an areal basis within similar body size classes, although rates of net primary production are similar across ecosystem types (Cyr & Pace, 1993; Hairston & Hairston, 1993; Cyr *et al.*, 1997; Cebrian, 1999). Therefore, any perturbation that impacts on primary producers or their interaction with consumers has large consequences for the structure and functioning of aquatic food-webs.

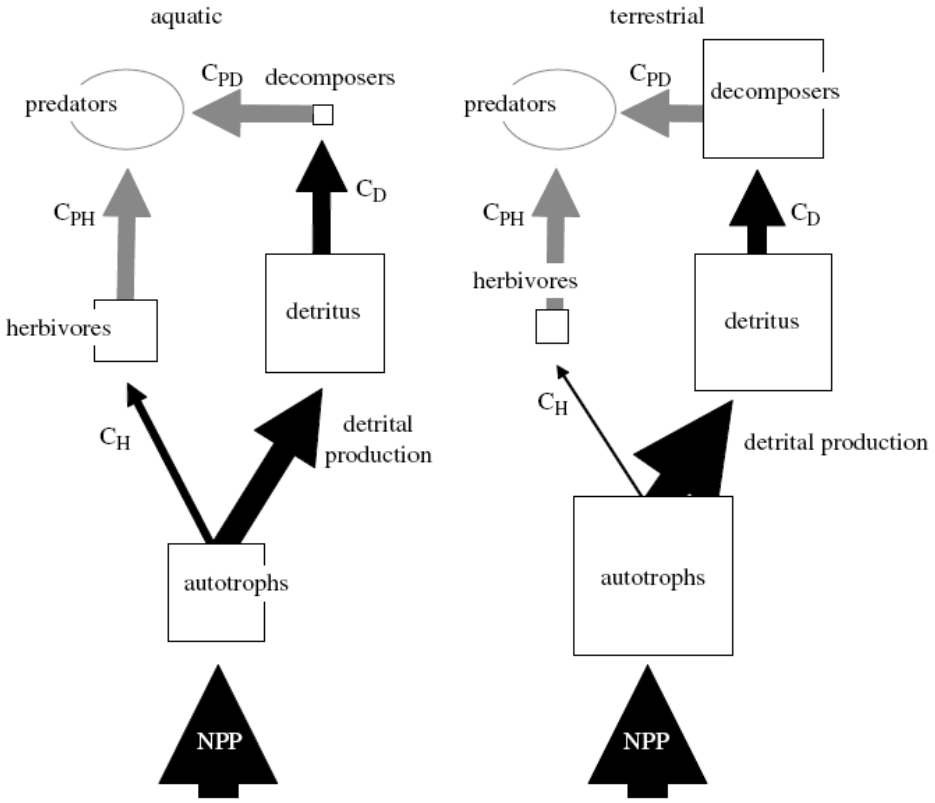


Figure 1. Differences in pathways of carbon flow and pools between aquatic and terrestrial ecosystems. The figure summarizes the patterns demonstrated in Cebrian (1999, 2004) and Cebrian & Latrigue (2004). The thickness of the arrows (flows) and the area of the boxes (pools) correspond to the magnitude. The size of the pools are scaled as log units since the differences cover four orders of magnitude. The C's indicate consumption terms (*i.e.* C_H is consumption by herbivores). Ovals and arrows in grey indicate unknown quantities. Source: Shurin *et al.,* 2006. *Diferencias en el flujo y contenido en carbono de los distintos compartimentos entre ecosistemas acuáticos y terrestres. La figura resume los patrones mostrados en Cebrian (1999, 2004) y Cebrian & Latrigue (2004). El grosor de las flechas (flujos) y el área de las cajas (compartimentos) se corresponde con la magnitud. El tamaño de los compartimentos se representa en escala logarítmica ya que las diferencias abarcan cuatro órdenes de magnitud. Los términos C's indican el consumo de un determinado nivel trófico* (i.e. C_H *es el consumo por los herbívoros). Los óvalos y las flechas en gris hacen referencia a cantidades desconocidas.*

Characteristic of PP	Aquatic pelagic PP	Terrestrial PP	Consequences for the food-web
Number of cells	(mainly) Unicellular	Multicellular	APE: Size-structured food-webs: $Size_{PP} < Size_{HC}$ *vs.* TE: Non-size-structured food-webs: $Size_{PP} > Size_{HC}$
Size	Small	Large	
Structural tissues	Absence	Developed system of phloem and xylem	APE: High food edibility for HC: High trophic efficiency *vs.* TE: Low food edibility for HC: Low trophic efficiency
Physical and/or chemical defences	Limited	Abundant	
Elemental content (stoichiometric C:N:P ratios)	Nutrient (N, P)-rich, C-low (Low C:N:P)	Nutrient (N, P)–low, C-rich (High C:N:P)	APE: Low elemental imbalance at the PP-HC interface: High trophic efficiency *vs.* TE: High elemental imbalance at the PP-HC interface: Low trophic efficiency
Productivity per unit of biomass	High	Low	APE: High food availability for HC: High trophic efficiency *vs.* TE: Low food availability for HC: Low trophic efficiency
Storage of photosynthetic material	Low	High	
Rate of tissues replacement	High	Low	
Metabolic rate	High	Low	APE: $MR_{PP} > MR_{HC}$: High trophic efficiency *vs.* TE: $MR_{PP} < MR_{HC}$: Low trophic efficiency
Growth rate	High	Low	APE: $GR_{PP} > GR_{HC}$: High trophic efficiency *vs.* TE: $GR_{PP} < GR_{HC}$: Low trophic efficiency

Table 1. Main characteristics of primary producers in aquatic pelagic and terrestrial ecosystems with major influence for the food web. Primary producers in aquatic benthic ecosystems share characteristics of both aquatic pelagic and terrestrial ecosystems. Abbreviations are: APE, aquatic pelagic ecosystems; GR, growth rate; HC, herbivorous consumers; MR, metabolic rate; PP, primary producers; and TE, terrestrial ecosystems. Source: Information has been obtained from the review by Shurin *et al.* (2006). *Principales características de los productores primarios de ecosistemas acuáticos pelágicos y terrestres, con especial relevancia para la red trófica. Los productores primarios de ecosistemas acuáticos bentónicos comparten características de tanto los de ecosistemas acuáticos pelágicos como terrestres. Abreviaturas: APE, ecosistemas acuáticos pelágicos; GR, tasa de crecimiento; HC, consumidores herbívoros; MR, tasa metabólica; PP, productores primarios; y TE, ecosistemas terrestres.*

Food quantity

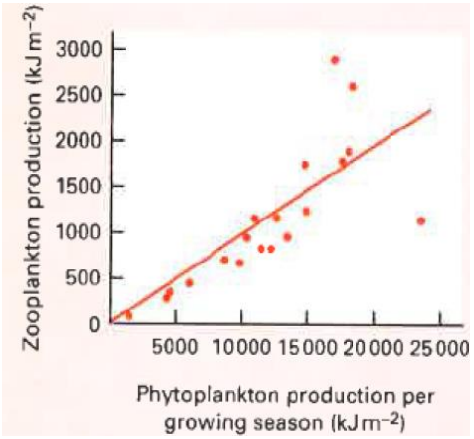
Herbivory in aquatic pelagic ecosystems is characterized because microscopic algal resources are entirely ingested by herbivorous zooplankton, which favours a strong connection between primary producer and herbivorous consumer dynamics. This implies more similarities to the prey-predator system based on Lotka-Volterra models, than to the primary producer-consumer interaction in terrestrial ecosystems, in which autotrophs do not necessarily die when are attacked by herbivores, and consequently, no compulsory link is observed between both trophic levels (Morris, 2009). This particularity made early studies to focus on how the availability of food resources predicted consumer abundance. These were based on the match-mismatch hypothesis, which predicts that consumer fitness depends on its temporal and spatial synchrony with the production of its resource (Cushing, 1974). This hypothesis, focused on the spatial-temporal dimension of this relationship, settled the framework for a high number of subsequent studies, although most of these also included the level of food quantity for consumers as a relevant factor for the interaction (*e.g.* Burns & Dodds, 1999; Guisande *et al.*, 2000). For instance, Durant *et al.* (2005) quantified the combined effect of abundance and timing of resource on consumer dynamics in order to better predict how environmental variability may affect ecological systems for different cases in both aquatic and terrestrial ecosystems. Several proxies of food quantity have been used for aquatic ecosystems, but among them algal biomass, chlorophyll *a* (Chl *a*) concentration, and overall seston C content have traditionally been considered from early studies (Lampert, 1977*a*). Studies using these predictors have successfully demonstrated the strong correlation between primary producers and consumers in planktonic food-webs, indicating that food quantity is the main factor controlling biomass and growth of herbivores (Brylinsk & Mann, 1973; Sterner & Schulz, 1998) (Fig. 2A, B). For instance, Fig. 2A shows the positive linear relationship between phytoplankton and zooplankton in the study carried out by Brylinsk & Mann (1973), indicating the predictability of secondary production from primary production. However, two important observations stand out from this relationship. First, that secondary production is about an order of magnitude below primary

production, indicating the major loss of energy as energy flows from autotrophs to herbivorous consumers. Second, the relatively wide scattered around this relationship, suggesting that variation in the rate of conversion from primary production to secondary production is quite large. Two questions arise from these observations:

1. Which is the ultimate reason for this loss of energy and matter?

2. Is there anything else beyond food quantity controlling the primary producer-herbivorous consumer interaction?

A



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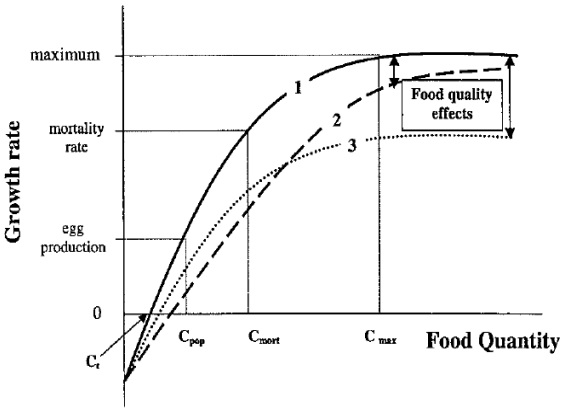


Figure 2. (A) The relationship between primary and secondary productivity for zooplankton in lakes (Brylinski & Mann, 1973). Source: Begon *et al.* 1997. (B) Hypothetical relationship between food quantity (carbon units) and specific growth rate of zooplankton (units of carbon gained per individual per time) on three food types. Type 1 (solid line) is 'ideal' or standard food, while types 2 (dashed line) and 3 (dotted line) are suboptimal foods. The zooplankter is able to compensate for the low nutritional value of food type 2, and can achieve maximal growth rate at high food concentrations. Food type 3, however, can never support maximal growth. Food quality is measured as the difference between growth on the standard food and growth on another food type at any given food quantity. Note that the shapes of the actual curves may vary. For example, in *Daphnia*, the growth curves may never truly level off, but may continue to increase slowly with increasing food levels (Lampert, 1977*b*). C_t is the threshold food concentration at which growth rate is exactly zero; C_{pop} is the population threshold food concentration at which egg production becomes possible; C_{mort} is the concentration of food necessary to make growth gains exactly equal mortality losses; and C_{max} is the lowest food concentration that supports maximal possible growth. If C_{mort} exceeds C_{max}, then no amount of available food will allow the population to persist. Source: Sterner & Schulz, 1998. *(A) Relación entre la producción primaria y la producción secundaria por el zooplancton en lagos (Brylinski & Mann, 1973). (B) Relación hipotética entre la cantidad de alimento (unidades de carbono) y tasa de crecimiento específica del zooplancton (unidades de carbono asimiladas por individuo y por unidad de tiempo) para tres tipos de alimento. El tipo 1 (línea continua) es el "ideal" o el alimento estándar, mientras que los tipos 2 (línea discontinua) y 3 (línea punteada) son alimentos subóptimos. El zooplancton es capaz de compensar el bajo valor nutricional del alimento tipo 2, y puede conseguir tasas de crecimiento máximas para niveles elevados de cantidad de alimento. El alimento tipo 3, no obstante, no puede dar lugar al crecimiento máximo. La calidad de alimento se mide como la diferencia entre el crecimiento para el alimento estándar y el crecimiento correspondiente a otro tipo para una determinada cantidad. Fíjese en que la forma de las curvas de crecimiento puede variar. Por ejemplo, para* Daphnia*, las curvas de crecimiento realmente nunca se estabilizan, dado que pueden continuar creciendo levemente para niveles altos de cantidad de alimento (Lampert, 1977*b*). C_t es el umbral de cantidad de alimento para el cual la tasa de crecimiento es exactamente cero; C_{pop} es el umbral poblacional de cantidad de alimento para el cual la producción de huevos es viable; C_{mort} es la concentración de alimento necesaria para que el aumento poblacional por reproducción se equipare con la reducción poblacional por mortalidad; y C_{max} es la cantidad de alimento más baja para la cual es viable el crecimiento máximo. Si C_{mort} excede C_{max}, no hay cantidad de alimento que permita la persistencia de la población.*

Food quality

Loss of energy and matter between links of the food chain has been generally explained in ecology textbooks as inefficient energy transfer, in accordance with the second law of thermodynamics. However, in words of Sterner & Elser (page 188 in 'Ecological stoichiometry: The biology of elements from molecules to biosphere', 2002), 'this paints a misleading picture of how food-webs work. Not all the losses in C or energy in going from one trophic level to another are due to unavoidable thermodynamics inefficiencies described by the second law'. Therefore, variation in C transfer should be also attributed to variation in food quality, which reduces the efficiency of energy and matter transfer up the food web and shapes the biological structure of ecosystems (Sterner & Elser, 2002).

Although zooplankton ecologists are in agreement with the essential role of algal food quality for consumer survivorship, growth and reproduction (Gulati & DeMott, 1997), there is no consensus on which characteristics of food determines its quality (Brett & Müller-Navarra, 1997). Thus, resource quality has been investigated in terms of algal shape (*e.g.* De Bernardi & Giussanig, 1990), ingestion defences and production of toxins (*e.g.* Ianora *et al.*, 2004), algal digestibility (*e.g.* Van Donk *et al.*, 1997) or community composition of primary producers (*e.g.* DeMott & Tessier, 2002). However, since biomass components are associated with C (energy), losses in C transfer between trophic levels have also been attributed to food quality imbalances in elements or biochemicals, with the development of distinct approaches advocating elemental (stochiometric approach) (*e.g.* Sterner, 1997; Sterner & Schulz, 1998; Sterner & Elser, 2002; Dickman *et al.*, 2008) or biochemical (biochemical approach) (*e.g.* Brett, 1993; Müller-Navarra, 1995*a*,*b*; Brett & Müller-Navarra, 1997; Gulati & De Mott, 1997; Müller-Navarra *et al.* 2000; Becker & Boersma, 2003; Müller-Navarra *et al.*, 2004; Leu *et al.* 2006*a*,*b*; Persson *et al.*, 2007) determinants of food quality (Fig. 3).

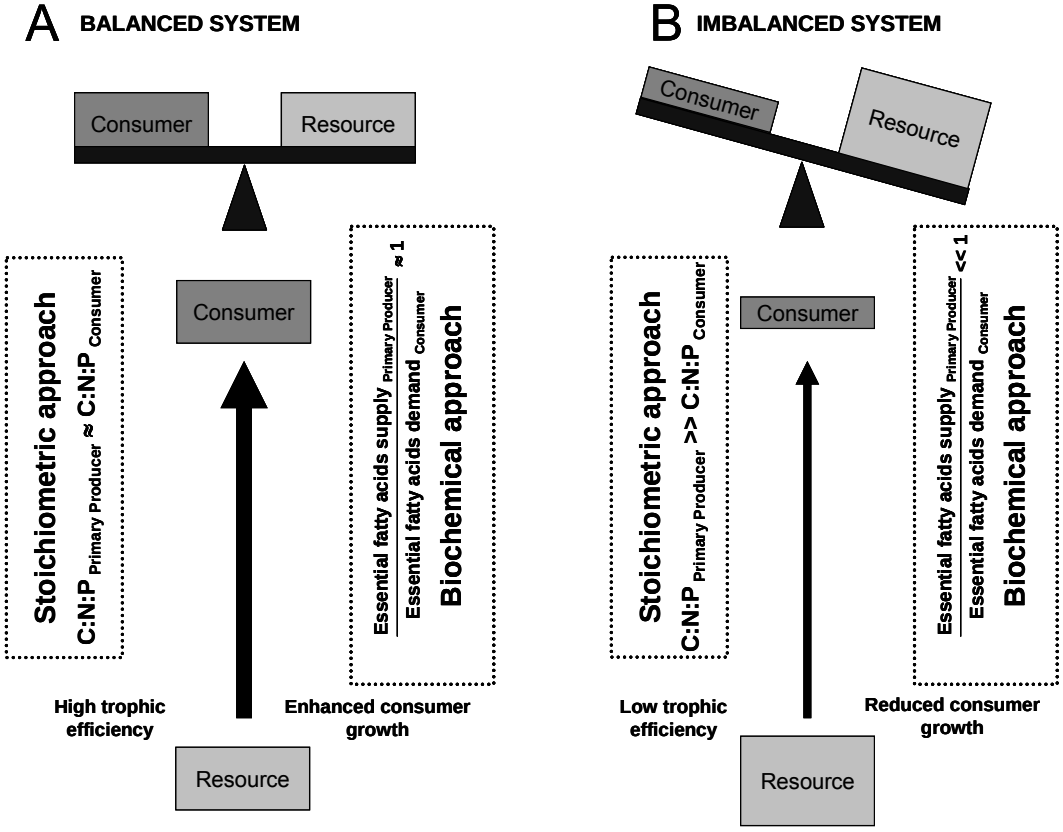


Figure 3. Differences between balanced and imbalanced resource-consumer systems. (A) In a balanced system, high trophic efficiency allows enhanced consumer growth. For the herbivory interaction, this is explained because of similar C:N:P ratios of both primary producer and consumer (stoichiometric approach) and/or consumer demands of essential fatty acids are fulfilled by primary producer supply (biochemical approach). (B) In an imbalanced system, low trophic efficiency inhibits consumer growth. For the herbivory interaction, this is explained because of higher C:N:P ratios of primary producer than those of consumer (stoichiometric approach) and/or consumer demands of essential fatty acids are not fulfilled by primary producers supply (biochemical approach). Thickness of arrows and box area corresponds to the magnitude of energy flow and size of the compartment, respectively. *Diferencias entre sistemas recurso-consumidor balanceados y desbalanceados. (A) En un sistema balanceado, la alta eficiencia trófica favorece el crecimiento del consumidor. En la herbivoría, esto se debe a razones C:N:P similares del productor primario y del consumidor (aproximación estequiométrica) y/o que las demandas del consumidor en ácidos grasos esenciales quede cubierta por el aporte de éstos por los productores primarios (aproximación bioquímica). (B) En un sistema desbalanceado, la baja eficiencia trófica inhibe el crecimiento del consumidor. En la herbivoría, esto se debe a razones C:N:P más altas de los productores primarios que de los consumidores (aproximación estequiométrica) y/o que las demandas de los consumidores en ácidos grasos esenciales no quede cubierta por el aporte de éstos por los productores primarios (aproximación bioquímica). El grosor de las flechas y el área de las cajas se corresponden con la magnitud del flujo de energía y el tamaño del compartimento, respectivamente.*

Stoichiometric approach

The conceptual framework known as ecological stoichiometry, and defined as the study of the balance of energy and multiple chemical elements in ecological interactions, argues that quality of food resources for consumers is directly determined by the differences between carbon:nitrogen:phosphorus (C:N:P) ratios of resource and consumer. This is particularly interesting at the primary producer-herbivorous consumer interface, since although all organisms are made of different elements, the proportions of C relative to N (C:N) and P (C:P) are generally high and variable in primary producers, and low and relatively fixed in consumers (Sterner & Hessen, 1994; Elser *et al.*, 2000*b*; Falkowski & Davis, 2004). This makes this interaction to be one of the most marked nutrient imbalances in food-webs (Brett & Müller-Navarra, 1997; Sterner & Elser, 2002). Therefore, according to this approach, food quality is directly determined by the relative content in essential elements like N or P in algal food (Sterner & Elser, 2002). Data supporting this hypothesis have been supplied by either laboratory experiments using cultured algae (for N limitation *see* Checkley, 1980; Kiørboe, 1985; and for P limitation *see* Sommer, 1992; Urabe & Watanabe, 1992; Sterner, 1993; Sterner & Hessen, 1994) and field studies (*e.g.* Elser & Hasset, 1994; Urabe *et al.* 2002*b*; Ferrao-Filho *et al.* 2005). To assess the impact of food quality and the strength of the herbivory interaction, apart from calculating arithmetic differences between primary producer and consumer elemental composition, ecological stoichiometry has also developed the meaningful index of threshold elemental ratio (TER; Sterner & Elser, 2002). TER, which functionally depends on animal physiological attributes and ambient food quantity (Sterner, 1997; Frost *et al.*, 2004; Logan *et al.*, 2004*a*,*b*; Anderson *et al.*, 2005), is defined as the dietary mixture where growth limitation switches from one element to another (Sterner & Hessen, 1994; Sterner, 1997). For example, for the well characterized *Daphnia*, $TER_{C:P}$ is approximately 300 (Urabe *et al.*, 1997; Sterner, 1997), meaning that *Daphnia* growth and reproduction should be strongly P-limited when food C:P ratios are above the $TER_{C:P}$, but C-limited below this threshold. By contrast, little is known about $TER_{C:P}$ and $TER_{C:N}$ of other crustacean species

such as calanoid copepods, or intraspecific differences within the same species. Further research on this topic will be carried out in chapter VI.

It should be noted that the wide range of variation of C:N:P ratios in algae, and also displayed by other photoautotrophic organisms (cyanobacteria, plants), reflects variable conditions in light, nutrient availability and growth rate. For example, under high light irradiance favouring high C fixation by photosynthesis or severe nutrient limitation, autotrophs produce biomass with extremely low nutrient content (high C:nutrient ratio), which directly limits consumers growth. Contrary to autotrophs, heterotrophic consumers have developed physiological mechanisms to strictly regulate their internal elemental composition and avoid any changes induced by extrinsic factors such as food. This resistance to change in elemental ratios is defined as stoichiometric homeostasis, and varies among species (Cowgill & Burns, 1975; Andersen & Hessen, 1991; Hessen & Lyche, 1991) and intraspecifically (Villar-Argaiz *et al.*, 2002; Ventura & Catalan, 2005). The degree of homeostasis is estimated by the slope of the linear trend that relates C:nutrient ratios of resource and consumer, as illustrated in Fig. 4. Further research is required to determine whether other factors, apart from food, may impinge on the homeostasis of consumers, inducing changes in their elemental composition. Chapter V will examine the potential role of abiotic factors (UVR, nutrients) as driving mechanisms behind the homeostatic composition of herbivorous consumers in nature.

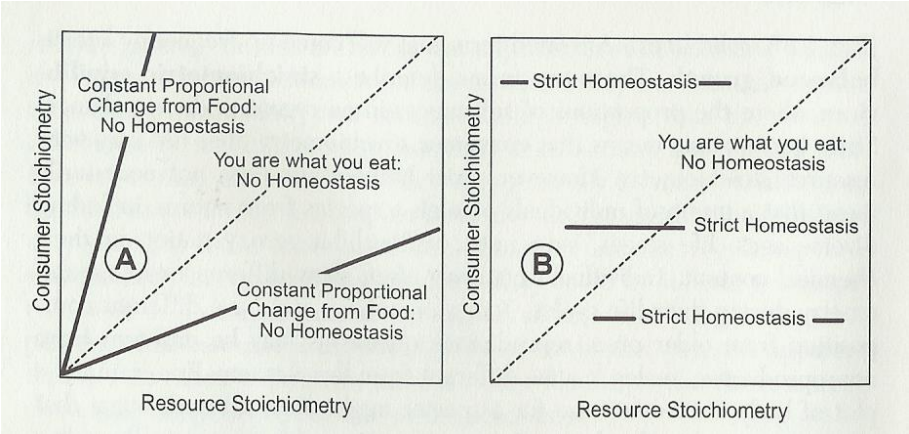


Figure 4. Relationships between consumer, and resource stoichiometry. Horizontal and vertical axes are any single stoichiometric measure, such as nitrogen content or carbon:phosphorus ratio. (A) Points on the 1:1 line (slope 1, intercept 0) represent identical stoichiometry in consumers and resources. This dashed line represents the stoichiometry of a consumer that always matches that of the resource ('you are what you eat' model). The solid lines represent consumers that perform constant differential nutrient retention ('constant proportional' model). (B) Strict homeostasis is defined as any horizontal line segment (slope 0, intercept > 0). Source: Sterner & Elser, 2002. *Relación entre la estequiometría del consumidor, y del recurso. Los ejes horizontal y vertical son cualquier variable estequiométrica, tales como el contenido en nitrógeno o la razón carbono:fósforo. (A) Los puntos en la línea 1:1 (pendiente 1, intercepto 0) representan idéntica estequiometría de consumidores y recursos. Esta línea discontinua indica que la estequiometría del consumidor siempre está ligada a la del recurso (modelo "eres lo que comes"). Las líneas continuas representan a los consumidores que siempre ofrecen un patrón invariable de retención de nutrientes (modelo "proporcional constante"). (B) La homeostasis estricta queda definida por cualquier recta horizontal (pendiente 0, intercepto > 0).*

Biochemical approach

The other scientific approach is based on biochemistry and attributes limitations in consumer growth to the deficiency of essential fatty acids in food, due to the inability of consumers to synthesize essential polyunsaturated fatty acids (PUFA) *de novo* (Müller-Navarra, 1995*b*; Brett & Müller-Navarra, 1997; Weers & Gulati, 1997). Among these, highly unsaturated fatty acids have been demonstrated to be especially relevant for growth and reproduction in zooplankton (Brett & Müller-Navarra, 1997). Correlative (Müller-Navarra *et al.*, 2000) and direct supplementation analyses (Ravet & Brett, 2006) have provided increasing evidence of PUFA limitation in nature.

However, there is growing evidence that algal PUFA content is specific for the different phytoplankton groups (Müller-Navarra *et al.*, 2004). For example, while diatoms can have high eicosapentaenoic acid [EPA (20:5ω3)] and docosahexaenoic acid [DHA (22:6ω3)] but little or none of α-linolenic acid [(ALA) 18:3ω3], other algal assemblages dominated by green algae, have only traces of EPA and DHA but high ALA content (Brett & Müller-Navarra, 1997). Such an ample diversity in the fatty acid spectrum of natural algal assemblages make necessary studies to consider the high profusion of biochemicals, as accurately as possible, in order to adequately infer consumer growth.

An urgent need for a 'stoichiometric-biochemical synthesis' and the integration of food quantity and quality studies

The high variety of studies determining the effects of a given qualitative characteristic of food on consumer performance emphasizes the need for theories that encompass distinct features of food quality to predict real limitation of herbivorous zooplankton in nature (DeMott & Tessier, 2002). Probably, the first step in doing so should be the synthesis of both stoichiometric and biochemical approaches. To date, most studies have investigated the prediction of consumer growth according to variations in PUFA content or algal elemental composition

(Müller-Navarra *et al.*, 2000; Sterner & Elser, 2002; Ravet & Brett, 2006) and considerable progress has been achieved using either parameter. However, questions have been raised about the general applicability of the P and *ω*3-PUFA limitation hypotheses (Gulati & DeMott, 1997; Hall *et al.*, 2004; DeMott & Pape, 2005; Ferrao-Filho *et al.*, 2007), and there is a pressing need to merge them into an integrated framework of consumer physiological ecology (Gulati & DeMott, 1997). Although impacts of algal *ω*3-PUFA and mineral contents appear to be independent of each other (Park *et al.*, 2002), some studies have elucidated the joint importance of both. Furthermore, there is a relative consensus that *ω*3-PUFA and P seem both to be *sine qua non components*, since *ω*3-PUFAs are fundamental when seston C:P ratios are below 350, *i.e.*, P-enriched seston relative to C (Becker & Boersma, 2003). Nonetheless, food quality indices which truly combine biochemicals and minerals in a single variable are still lacking. Because of the complex interrelationship between the biochemical and elemental composition of autotrophs (Ahlgren *et al.*, 1992; Weers & Gulati, 1997; Breteler *et al.*, 2005), we suggest that both must be considered if zooplankton limitation is to be successfully predicted.

However, beyond proxies used for the food quantity or the features of food quality considered, several ultimate questions that arise are: What is primarily limiting zooplankton populations: Food quantity or food quality? How do both interact in affecting consumer performance?

The distinction between constraints imposed by food quantity and quality is not an easy task, and this is partly because the effects of food quantity and quality interact. This means that the role of food quality is not the same across a food quantity gradient. Food quality should not matter much at very low food quantities, but have a prominent role as food quantity increases (Sterner & Schulz, 1998) (Fig. 2A). The widely assumed convention that food quality had no or minor relevance at low food quantities was later challenged by Boersma & Kreutzer (2002), who demonstrated, using laboratory experiments, that increased elemental food quality was also relevant for *Daphnia* growth at low quantities of food. However, there are no evidences for food quality effects at low levels of

food in nature. Therefore, because food quality and quantity interact, it is crucial to i) segregate the influence of each using laboratory and field experiments (Sterner & Schulz, 1998); and ii) demonstrate food quality effects on consumer performance at low food levels in ultraoligotrophic ecosystems. The goals of chapter III will specifically address these objectives for several species of zooplankton representing the major pelagic taxonomical groups of cladocerans, copepods and rotifers.

The primary producer-consumer interaction in a global change world

Half a century ago Lindeman (1942) identified the chief role of light and nutrients for ecosystems functioning (*see* his original scheme in Fig. 5). It is now widely recognized that ecosystem productivity is governed primarily by the inputs of nutrients and light (Urabe & Sterner, 1996). Solar energy or light is required by primary producers to fix C dioxide and produce organic matter *via* photosynthesis, and consequently, food-webs are built up from this energy substrate *via* trophic interactions.

However, metabolism also requires essential nutrients such as N and P for protein and nucleic acids biosynthesis. Because energy (C) and nutrients (mainly N, P) acquisition and losses are often not coupled in primary producers, the limiting role of nutrients affects the C:N:P ratios in algae, imposing restrictions to efficiently transfer energy from primary producers to upper trophic levels. Therefore, maximum efficiency of energy transfer in the primary producer-consumer interface should occur at the proper balance of light and nutrients. This was first experimentally supported by Urabe & Sterner (1996), who found that short-term *Daphnia* production was maximum for intermediate light intensities providing algal resource C:P ratio close to the $TER_{C:P}$. These results were soon assembled into the light-nutrient hypothesis (LNH), which states that seston C:nutrient ratios are functions of the ratio of available light and nutrients (Sterner *et al.*, 1997). Additional experiments in indoor plankton towers (Sterner *et al.*,

1998) and field enclosures (Urabe *et al.*, 2002) over long-temporal scales confirmed predictions of the LNH (Fig. 6).

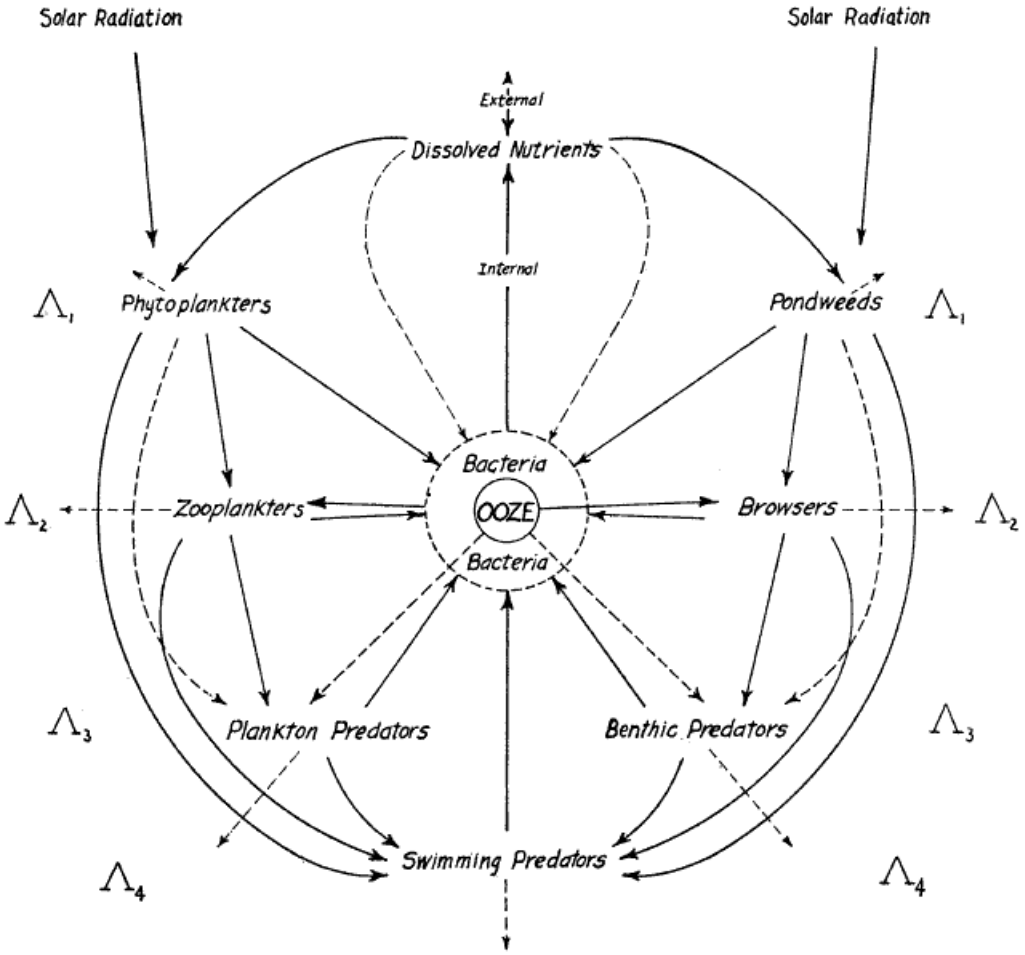


Figure 5. Generalized lacustrine food-cycle relationships. Source: Lindeman, 1942. *Relaciones genéricas en las redes tróficas de los lagos.*

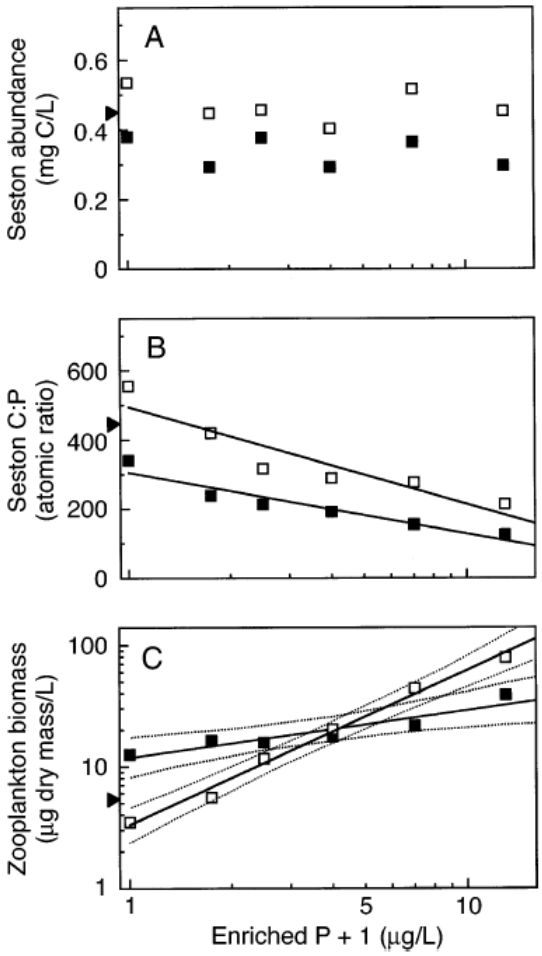

A
Seston abundance (mg C/L)
0
0.2
0.4
0.6
B
Seston C:P (atomic ratio)
0
200
400
600
C
Zooplankton biomass (µg dry mass/L)
1
10
100
1
5
10
Enriched P + 1 (µg/L)

Figure 6. Effects of light and nutrient balance on (A) mean seston carbon, and (B) mean carbon:phosphorus ratio during the 4.5-week experimental run, and (C) mean zooplankton biomass for the final three sampling dates in the experiments carried out by Urabe *et al.* 2002*b*. Closed squares denote shaded enclosures; open squares denote unshaded (control) enclosures. Black triangles indicate mean values in Lake 239 during the experiment. Regression lines are inserted when relationship is significant at *p*-value < 0.05. For zooplankton biomass (panel C), 95% confidence intervals are denoted by dotted lines to evaluate significant differences between control and shaded enclosures. Source: Urabe *et al.* 2002*b*. *Efectos del balance de luz y nutrientes sobre (A) el contenido promedio de carbono sestónico, y (B) promedio de la razón carbono:fósforo de las cuatro semanas y media del período experimental, y (C) promedio de la biomasa zooplanctónica para los tres últimos días de muestreo del experimento llevado a cabo por Urabe* et al.*, 2002*b*. Los cuadrados rellenos se refieren a los encerramientos sombreados; los cuadrados vacíos se refieren a los encerramientos sin sombrear (control). Los triángulos negros hacen referencia a los valores promedios del Lago 239 durante el período experimental. Se insertan las rectas de regresión cuando la relación es significativa para* p*-valores < 0.05. Para la biomasa zooplanctónica (panel C), los intervalos de confianza del 95% se representan por líneas punteadas para evaluar las diferencias significativas entre los encerramientos control y sombreados.*

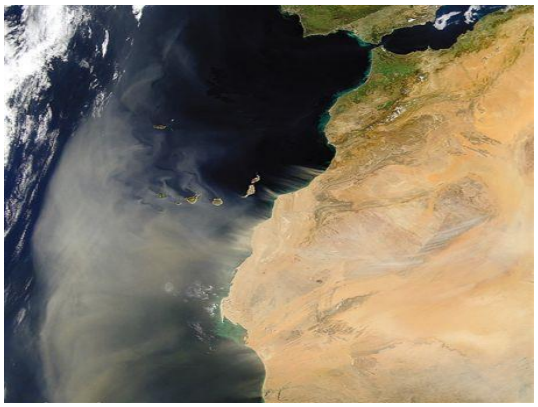
Ultraviolet radiation (UVR)

Solar energy not only includes photosynthetic active radiation (PAR) responsible for C dioxide fixation in organic matter, but also UVR with a major impact in the biota and ecosystems (Sinha & Häder, 2002; Häder *et al.*, 2003*a*,*b*; Helbling & Zagarese, 2003). UVR has considerably increased due to stratospheric ozone depletion over Antarctica, Arctic and high to mid latitudes during past 30 years. Among aquatic ecosystems, clear lakes in alpine regions, and oceans in polar regions may be particularly vulnerable due to deep UVR penetration into the water column. UVR deleterious effects on growth, development, reproduction and productivity have been demonstrated for a variety of taxa (Häder *et al*., 2007), although impact on trophic interactions, ecosystem structure and function needs to be investigated further. It is, therefore, important to assess the effects of UVR on the primary producer-herbivorous consumer interaction if we are interested in predicting the effect of higher UVR doses on ecosystems.

Several studies have demonstrated the role of UVR in modifying both food quantity and quality for consumers. The effects of UVR in reducing the amount of available food for consumers were early investigated by a number of researchers who demonstrated the deleterious role of UVR on C fixation by photosynthesis, gross and net primary production (Steeman-Nielsen, 1964; Worrest *et al.*, 1978; Lorenzen, 1979; Calkins & Thordarrdottir, 1980; El Sayed, 1988; El Sayed, 1990; Smith *et al.*, 1992; Moeller, 1994; Gala & Giessy, 1991; Boucher *et al.*, 1995). But these quantitative effects could be overridden by a variety of changes in food quality (Hessen *et al.*, 1997). Clearly, there is good evidence to support UVR-induced alterations on nutrient uptake for both N (Döhler & Alt, 1989; Döhler & Kugel, 1994; Braune & Döhler, 1994) and P (Hessen *et al.*, 1995), due to reduced enzyme activity, damage of cell membranes and wall, DNA mutations or altered protein synthesis. These and other UVR related processes may impinge on C:N:P stoichiometry of phytoplankton in multiple ways. Recent work has demonstrated a general reduction in algal C:nutrient ratio due to UVR, although there is no apparent consensus in the mechanisms behind this effect (Xenopoulos *et al.*, 2002; Hessen, 2006; Carrillo *et al.*, 2008; Hessen *et al.*, 2008*a*). Traditionally, a lower C:P ratio has been considered as an indicator of higher food quality for herbivores (Sterner & Elser, 2002). Hence, the observed reduction in sestonic C:P ratio by UVR might be expected to have an indirect beneficial effect by improving food quality (Xenopoulos *et al.*, 2002). However, recent studies have shown that low seston C:P ratios do not necessarily result in enhanced herbivorous growth (Leu *et al.*, 2006*a*) probably because, as Carrillo *et al.* (2008) demonstrate, a low seston C:P ratio is a consequence of the loss of C rather than the storage of P. In addition, UVR alters the biochemical composition of C in the food, increasing (Tank *et al.*, 2003; Leu *et al.*, 2007) or decreasing (Hessen *et al.*, 1997) the content of total lipids and essential PUFA. In summary, we now know that UVR has the potential to simultaneously alter the elemental and biochemical composition of autotrophs. *In situ* studies that address how these effects in turn translate to higher trophic levels are needed to better understand how natural communities will response to future global change. These issues are further investigated in chapters II and III.

Nutrients

Humans have dramatically altered the major biogeochemical cycles. In particular, human activities and industrialization have increased the amount of C, N, and P available by 12%, 112%, and 400%, respectively (Falkowski *et al.*, 2000). Atmospheric circulation is a major pathway for nutrient inputs into terrestrial and aquatic ecosystems *via* wet and dry deposition. Dust emissions are important regional components of climate with global consequences. Deserts are natural sources of dust, being the Sahara the major desert on Earth. It is widely known that dust plumes from deserts can provide essential nutrients that travel a great distance from their source areas to far sink regions (Neff *et al.*, 2008; Goudie, 2009). For example, dust from the Sahara desert can have major relevance for the nutrient budget of the Mediterranean basin (Ridame & Guieu, 2002), but can travel as far as the Amazon basin (Moulin *et al.*, 1997). While, dust contains relatively high amounts of key plant nutrients such as iron (Fe) and calcium (Ca), a recent study by Bristow *et al.* (2010) reported that dust can be 38 times richer in P than formerly thought. If, like some latest climate models agree, Africa will get dried than present conditions, more dust can be expected to reach the troposphere and transported with the predominant westerly winds. Ideal sites for the study of the effects of atmospheric depositions are the alpine lakes located in the National Park of Sierra Nevada because of (1) their location within the first 2000 km of the Sahara Desert where most dust is deposited (Jaenicke & Schütz, 1978), (2) their high altitude over 2000 m, within the range of the main jetstream of Saharan dust that travels between 1500 and 4000 meters above sea level (Talbot *et al.*, 1986), and (3) their extreme oligotrophy which makes primary production extremely sensitive to an allocthonous input of nutrients (Villar-Argaiz *et al.*, 2001; Morales-Baquero *et al.*, 2006) (Picture 1).



Picture 1. Satellite image of massive airborne plumes of dust from Sahara Desert. *Imagen satélite de plumas masivas de polvo del Desierto del Sahara aerotransportadas.*

Interactive effects of UVR and nutrients

To understand global change effects, work must necessarily encompass other important stressors that jointly affect ecosystems. It is increasingly recognized that multiple abiotic stressors, acting at different rates on local and global scales, affect the growth of organisms and the functioning of ecosystems (Carrillo *et al.*, 2008).

Testing interactive effects demand complex experimental designs in which more than one factor can be manipulated. The interaction among multiple factors results in effects on organisms and ecosystems not easily predicted from single-factor studies, due to the non-additive (synergistic and antagonistic) nature of their interactions. For instance, we have previously reported UVR reduces both primary production and nutrient uptake. Although one could expect higher availability of nutrients would compensate these UVR detrimental effects, Carrillo *et al.* (2008) reported synergistic effects in the interaction between UVR and nutrients on algal growth, *i.e.* decreased algal growth due to UVR at increased nutrient levels. However, we are still remarkably ignorant about how these effects could translate to the secondary production.

Future studies should pay particular attention to the combined effects of multiple stressors on the primary producer-consumer interface if accurate predictions for the trophic-web and ecosystem are to be made. The complexity of studying interactive effects on ecological interactions is a major challenge for ecologists. Broad integrative studies combining observational and experimental approaches using structural and functional variables would provide a more accurate assessment of how multiple factors interact to affect the primary producer-herbivorous consumer interface. Chapter II will analyze how the UVR, nutrients and their interaction affect the stoichiometric and biochemical composition of seston at the bottom of food webs. Chapter III will test how these effects transfer to the next trophic level of herbivorous consumers, by using coupled field and laboratory experiments and three zooplankton species as indicators.

In a previous study in Lake La Caldera, Villar-Argaiz *et al.* (2001) showed that atmospheric inputs into the lake, although favoured algal growth, did not result into higher zooplankton biomass (Fig. 7). This result was even more enigmatic after Carrillo *et al.* (2008) demonstrated that P inputs and UVR jointly decreased seston C:P ratio, thereby potentially enhancing food quality for herbivores. The counterintuitive result that enhanced food quantity and quality, due to nutrients and UVR, did not turn into higher zooplankton biomass, and therefore impinging the phytoplankton-zooplankton coupling, merits further investigation. This task will be covered in chapter IV.

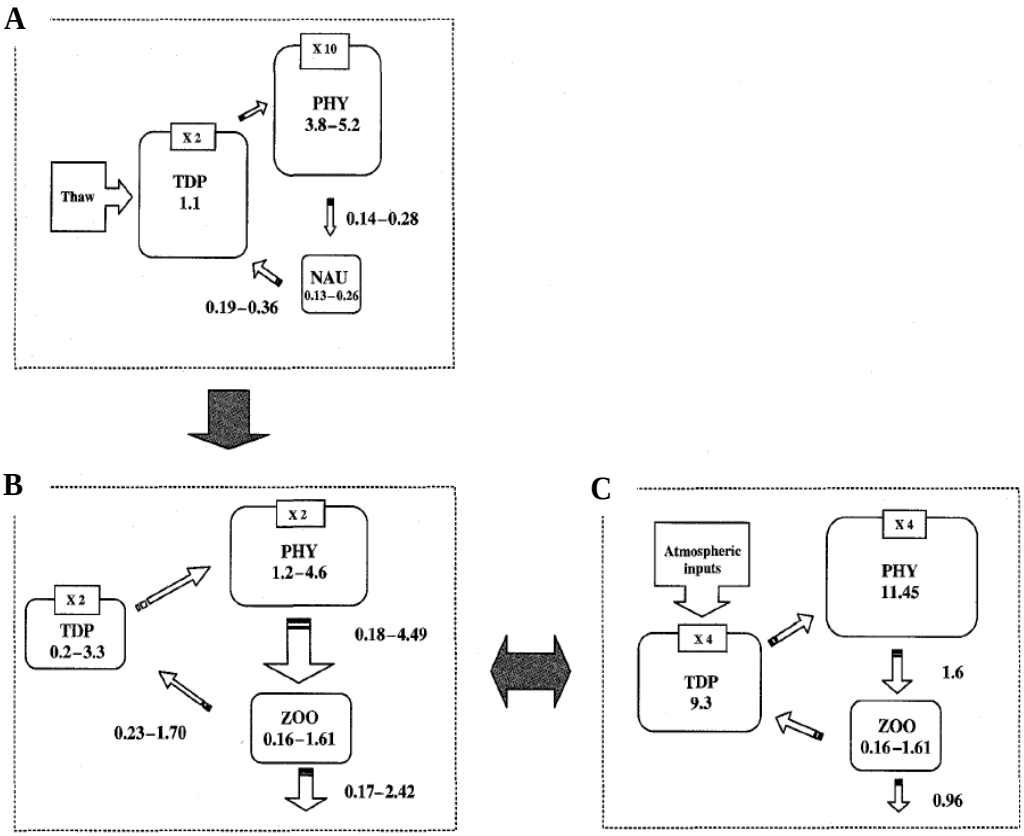


Figure 7. Simplified scheme of the major phosphorus (P) cycling routes in the pelagic zone of Lake La Caldera at (A) ice melting, (B) in a biologically driven community, and (C) after an atmospheric input. Boxes for total dissolved P (TDP) and phytoplankton are scaled to maximum zooplankton compartment sizes (μg P L^{-1}) and arrow thickness is proportional to flows (μg P L^{-1} day^{-1}). P ingestion rates were estimated from Reche (1995) for this system at similar conditions; P recycling rates from the model of Carrillo *et al.* (1996); and P release *via* faecal pellet from Villar-Argaiz *et al.* (2001). Note in panel (C), low P flux from phytoplankton to zooplankton when P atmospheric inputs take place. PHY, phytoplankton; ZOO, zooplankton (nauplii excluded); NAU, nauplii. Source: Villar-Argaiz *et al.* 2001. *Esquema simplificado de los principales flujos de fósforo (P) en la zona pelágica del lago de La Caldera durante (A) el deshielo, (B) en una comunidad gobernada biológicamente, y (C) tras una entrada atmosférica de nutrientes. Las cajas para el P disuelto total (*TDP*) y el fitoplancton están relativizadas al tamaño máximo del compartimento del zooplancton (*μg P L^{-1}*) y el grosor de las flechas es proporcional a los flujos (*μg P L^{-1}*). Las tasas de ingestión de P fueron estimadas por Reche (1995) para este sistema en condiciones similares; las tasas del reciclado de P a partir del modelo de Carrillo* et al. *(1996); y la liberación de P* via *excreción fecal por Villar-Argaiz* et al. *(2001). Fíjese en el panel (C), el bajo flujo de P de fitoplancton a zooplancton cuando tiene lugar una entrada de P atmosférico. PHY, fitoplancton; ZOO, zooplancton (nauplios excluidos); NAU, nauplios.*

Biological stoichiometry: A bridge between ecosystem ecology and evolutionary biology

In previous sections, ecological stoichiometry was defined as the study of the balance of energy and multiple chemical elements in ecological interactions (Sterner & Elser, 2002). Main contributions of this framework to the ecological study of the primary producer-consumer interaction are:

1. Close related zooplankton species of similar size, life stage, and taxonomical affiliations differ in their C:N:P stoichiometry (Andersen & Hessen, 1991; Hessen & Lyche, 1991; Elser *et al.*, 2000*a*), varying up to fivefold in body P content. These differences are strongly related with differences in specific growth rate, and life history strategies. For instance, while cladocerans with parthenogenetic reproductive cycles, short generation time, and high growth rates are characterized by N:P ratios between 12 and 18 (%P as dry weight ~1.2), calanoid copepods with sexual reproduction, long generation time, and low growth rates show N:P ratios exceeding 30 (%P as dry weight ~0.5) (Andersen & Hessen, 1991).

2. Stoichiometric homeostasis: Contrary to autotrophs, heterotrophic consumers regulate their internal elemental composition, showing relatively fixed C:N:P ratios (homeostatic organisms) in comparison to those of autotrophs (Sterner & Elser, 2002).

3. Bottom-up processes can affect elemental imbalance between primary producers and herbivores. An enhanced imbalance would result in reduced food quality for herbivorous consumers, therefore impairing their growth, reproduction, and disrupting the C flow in the food-web (Fig. 3B). On the contrary, a more elementally balanced interaction, would result in increased food quality for the growth of herbivorous consumers, consequently strengthening trophic interactions (Fig. 3A) (Hessen, 1992; Urabe & Watanabe, 1992; Sterner *et al.*, 1993; DeMott *et al.*, 1998; Elser & Foster, 1998; Boersma, 2000; Elser *et al.*, 2001; Hood *et al.*, 2005).

4. Top-down processes: Due to homeostatic nature of consumers and their interespecific differences in body elemental composition, shifts in the relative dominance of zooplankton species can alter internal nutrient cycling in pelagic food-webs (Elser & Urabe, 1999; Vanni, 2002). These impacts affect the nature of phytoplankton nutrient limitation, as reported by Elser *et al.* (1988) and Andersen & Hessen (1991). These authors found that while in communities dominated by rich-P *Daphnia* (low body N:P ratio) phytoplankton was driven to P limitation, others dominated by low-P copepods (high body N:P ratio) phytoplankton was driven to N limitation.

5. Environmental stressors (*e.g.* global change factors like increased UVR and atmospheric aerosol deposition) affecting energy and/or nutrients supply alter the nutritional balance of primary producer-herbivorous consumer interaction, and consequently ecosystem production (Urabe & Sterner, 1996; Sterner *et al.*, 1997, 1998; Diehl *et al.*, 2002; Hessen *et al.*, 2002; Urabe *et al.*, 2002*a*,*b*, 2003).

Although our interest has focussed on the role of ecological stoichiometry in explaining the primary producer-herbivorous consumer interaction in aquatic pelagic ecosystems, this framework has also been considered for other ecological interactions and for aquatic benthic and terrestrial ecosystems. In all cases, the link between variation in species and stage elemental composition and species interactions, food web dynamics, or nutrient cycling has proven valid (Sterner & Elser, 2002)… But, do stoichiometric patterns have further implications other than ecological processes?

A starting point to this question has been the exploration of, something as simple as, why species differ in elemental composition. Although, a ‘simple question’ might demand a ‘simple answer’, a great complexity of biological and evolutionary mechanisms might be behind this ‘simplicity’ (Elser, 2006). In the first attempt to explain interspecific differences in elemental composition, Reiners

(1986) proposed that these were driven by major interespecific differences in structural features for mechanical support. Such an argument was successfully applied to explain differences between terrestrial vascular plants with C-rich cellulose for support (high C:nutrient ratios) and non-vascular planktonic autotrophs (low C:nutrient ratios) (Reiners, 1986; Elser *et al.*, 2000*a*). However, strong evidences now support that variation in the elemental composition of organisms are not only related to structural features but also to the molecular biology of growth *via* production of ribosomal RNA. This idea is captured in the growth rate hypothesis (GRH), which states that variation in the P content (and thus C:P and N:P) of living things is driven by variation in allocation to P-rich ribosomal RNA that accompanies differences in growth rate, as elevated ribosome allocation is generally needed to meet the protein synthesis demands of rapid growth (Elser *et al.* 1996, 2000*b*, 2003) (Fig. 8). Therefore, considering:

1. Growth and fitness are intrinsically linked since organisms must necessarily grow before reproducing, and thus, growth rate is a central integrating parameter of overall life history strategy (Arendt, 1997).

2. Any selective pressure affecting organismal growth or developmental rates may be reflected on organismal C:N:P ratios, and consequently on organism´s sensitiveness to stoichiometric food quality constraints and its impacts on nutrient cycling (Sterner & Elser, 2002).

GRH links central concepts of cell and organism biochemistry and physiology [(elemental (C, N, P) and biochemical (ribosomal RNA, protein) composition, ribosome biogenesis)], evolutionary ecology of life histories [life history traits (growth rate), fitness] and ecosystems ecology (trophic interactions, nutrient cycling). Thus, GRH constitutes a central dogma in biological stoichiometry, defined as the study of the balance of energy and multiple chemical elements in living systems (Elser *et al.*, 2000*b*). This is the extension of ecological stoichiometry to the living systems, and therefore has applied similar principles from those of interactions between trophic levels to interactions between different levels of organization in biology (Elser *et al.*, 2000*b*).

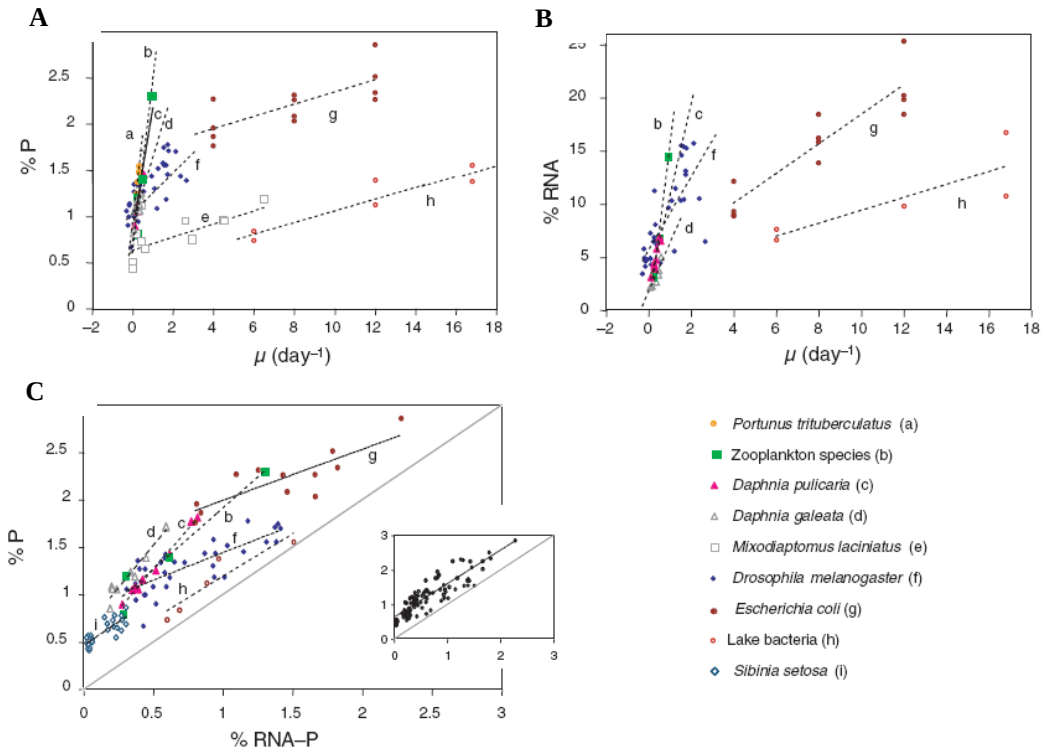

A
B
C
% P
% RNA
% RNA–P
μ (day⁻¹)
Portunus trituberculatus (a)
Zooplankton species (b)
Daphnia pulicaria (c)
Daphnia galeata (d)
Mixodiaptomus laciniatus (e)
Drosophila melanogaster (f)
Escherichia coli (g)
Lake bacteria (h)
Sibinia setosa (i)

Figure 8. Diverse species exhibit coupling of biomass phosphorus (P) content (% of dry mass) and biomass RNA content (% of dry mass) with specific growth rate (μ) and total P content with RNA-P content. In some cases (*Daphnia pulicaria*, *Daphnia galeata*) regression lines were extrapolated beyond the data to facilitate labelling. All relationships shown were statistically significant (one at a level of p-value < 0.10). (A) P content (*y*) *vs.* growth rate (*x*): line a, larvae of the swimming crab *Portunus tribulurculatus* during ontogeny; line b, different species of crustacean zooplankton; line c, the freshwater crustacean *Daphnia pulicaria* grown under P-limitation; line d, the freshwater crustacean *Daphnia galeata* grown under P-limitation; line e, the freshwater copepod *Mixodiaptomus laciniatus* during ontogeny; line f, the fruit fly *Drosophila melanogaster* during ontogeny; line g, the enteric bacterium *Escherichia coli* grown under P-limitation; line h, for mixed lake bacterial assemblages grown under P-limitation. (B) RNA content (*y*) *vs.* growth rate (*x*): line b, different species of crustacean zooplankton; line c, *Daphnia pulicaria*; line d, *Daphnia galeata*; line f, *Drosophila melanogaster*; line g, *Escherichia coli*; line h, mixed lake bacterial assemblages. (C) Biomass P content (*y*) *vs.* P content because of RNA (*x*): line b, different species of crustacean zooplankton; line c, *Daphnia pulicaria*; line d, *Daphnia galeata*; line f, *Drosophila melanogaster*; line g, *Escherichia coli*; line h, mixed lake bacterial assemblages; line j, field-sampled individuals of the mesquite-feeding weevil *Sibinia setosa*. The inset shows the fit to the aggregated data for %P and for %RNA-P. Source: Elser *et al.*, 2003. *Diversas especies exhiben un acople del contenido en fósforo (P) (porcentaje de peso seco) y en RNA (porcentaje de peso seco) con la tasa de crecimiento específica (*μ*), y del contenido total en P con el contenido en P en el RNA (RNA-P). En algunos casos (*Daphnia pulicaria, Daphnia galeata*), las rectas de regresión se extrapolan más allá de los datos para facilitar el etiquetado. Todas las relaciones mostradas son estadísticamente significativas (una al nivel de* p-*valor < 0.10). (A) Contenido en P (y)* vs. *tasa de crecimiento (x): línea a, larvas del cangrejo* Portunus tribulurculatus *durante la ontogenia; línea b, diferentes especies de zooplancton crustáceo; línea c, el crustáceo de aguas dulces* Daphnia pulicaria *alimentado bajo condiciones de limitación en P; línea d, el crustáceo de aguas dulces* Daphnia galeata *alimentado bajo condiciones de limitación en P; línea e, el copépodo de aguas dulces* Mixodiaptomus laciniatus *a lo largo de la ontogenia; línea f, la mosca de la fruta* Drosophila melanogaster *a lo largo de la ontogenia; línea g, la bacteria entérica* Escherichia coli *cultivada bajo condiciones de limitación en P; línea h, ensamblaje de bacterias de diversos lagos cultivadas bajo condiciones de limitación en P. (B) Contenido en RNA (y)* vs. *tasa de crecimiento (x): línea b, diferentes especies de zooplancton crustáceo; línea c,* Daphnia pulicaria*; línea d,* Daphnia galeata*; línea f,* Drosophila melanogaster*; línea g, la bacteria entérica* Escherichia coli*; línea h, ensamblaje de bacterias de diversos lagos. (C) Contenido en P (y)* vs. *contenido de P en el RNA (x): línea b, diferentes especies de zooplancton crustáceo; línea c,* Daphnia pulicaria*; línea d,* Daphnia galeata*; línea f,* Drosophila melanogaster*; línea g,* Escherichia coli*; línea h, ensamblaje de bacterias de diversos lagos; línea j, individuos muestreados en el campo del gorgojo* Sibinia setosa*. La figura interna muestra el ajuste para datos agregados de %P y %RNA-P.*

With the development of genomics during early twenty-first century, a great interest has emerged to explore the genetic basis of GRH, in an attempt to extend the biological stoichiometry at the genetic level. Major contributions of these studies include the finding that increased growth rate and associated increases in transcriptional capacity for ribosomal RNA production are positively associated with the length and content of the ribosomal DNA intergenic spacer (IGS) and/or in overall ribosomal DNA copy number (Weider *et al.*, 2005). However, this consistent pattern apparently contrasts with the pervasive relationships found between high growth rate and RNA content with small genome size in rapid-growth organisms. This phenomenon, initially observed for cladocerans, was explained by the P-allocation hypothesis, which states that differences in genome size between two major zooplankton groups of copepods and cladocerans (with overlapping niches and similar body size) could be due to differential P-allocation. Thus, the small genome size of cladocerans, characterized by high growth rates and rich P and RNA contents different to copepods may be a consequence of P allocation from DNA (mainly from non-coding DNA) to RNA under sustained selection for rapid growth in P-limited environments (Hessen *et al.*, 2008*b*). Although more experimental and observational support would be desirable, there are strong evidences to extend the validity of this hypothesis for the rest of eukaryotes under selective pressure for rapid growth in nutritionally limited environments (Fig. 9). This extension has been named as growth rate-genome size-nutrient limitation hypothesis (GRGSNLH) (Hessen *et al.*, 2009). Patterns in support of the GRH and P-allocation hypothesis will be tested in chapter VII.

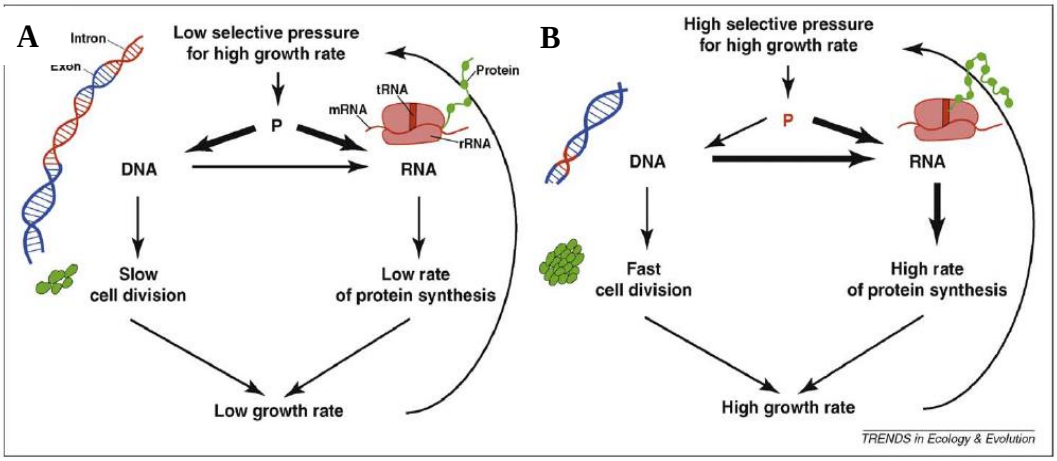


Figure 9. Two scenarios illustrating the effects of different evolutionary allocations of phosphorus (P) to DNA or RNA. (A) Under low selective pressure for high growth rate, a significant share of P is allocated to DNA (and implicit large genome size) potentially causing slow growth rate and low rate of protein synthesis. (B) Under strong selection for high growth rate, there will be selective pressure for reallocating P from non-coding DNA to RNA that promotes high growth rate. Selection favouring 'r-selected' life-history traits under nutrient scarcity might generate evolutionary pressure for this sequence of events. Thickness of arrows indicates relative importance of P allocation or causality. Scenario (A) indicates large genome, high intron:exon ratio, slow rate of protein synthesis and slow cell division as opposed to scenario (B). Source: Hessen *et al.*, 2009. *Dos escenarios que ilustran los efectos de la diferente asignación evolutiva de fósforo (P) al DNA o al RNA. (A) Bajo una presión selectiva relajada para alcanzar altas tasas de crecimiento, una fracción significativa del P es asignada al DNA (e implica tamaño de genoma grande) dando lugar potencialmente a bajas tasas de crecimiento y de síntesis proteica. (B) Bajo una presión selectiva intensa para alcanzar altas tasas de crecimiento, habrá una presión selectiva para reasignar P desde el DNA no codificante al RNA que de lugar a altas tasas de crecimiento. La selección que favorezca rasgos propios de estrategias adaptativas "r" bajo condiciones de escasez en nutrientes podría dar lugar a una presión evolutiva para esta secuencia de eventos. El grosor de las flechas indica la relativa importancia de la asignación en P o causalidad. El escenario (A) indica genomas grandes, una razón intron:exon alta, bajas tasas de síntesis proteica y división celular lenta en contraposición al escenario (B).*

Taken all these concepts together, biological stoichiometry mechanistically threads central concepts in biology that extends from streamlining of genome size and organization of major gene family (ribosomal DNA) through cellular allocation, and physiological nutritional demands, to trophic interactions and nutrient cycling in food webs. This implies that stoichiometric connections could provide a vertical integration across all levels of organization in biology, including the ecosystem level, which has traditionally being neglected in

evolutionary studies. Furthermore, although the above hypothesis have been enunciated for crustacean zooplankton (Elser *et al.*, 2003; Hessen *et al.*, 2008*b*), an increasing number of studies are corroborating them for other organisms in a variety of ecological conditions in aquatic as well as terrestrial ecosystems (for the GRH, *see* Sutcliffe, 1970; Elser *et al.*, 2000*a*; Elser *et al.*, 2000*b* and references therein; Frost & Elser, 2002; Jaenike & Markow, 2002; Makino *et al.*, 2003; Schade *et al.*, 2003; Makino & Cotner, 2004; Perkins *et al.*, 2004; Woods *et al.*, 2004; Elser *et al.*, 2005; Watts *et al.*, 2006; for GRGSNLH, *see* Smith & Holt, 1996; Gambi *et al.*, 1997; White & McLaren, 2000; Wyngaard *et al.*, 2005; Saliba *et al.*, 2006; Smith, 2007; Hessen *et al.*, 2008*b*; Gregory & Johnson, 2008; Hessen *et al.*, 2009 and references therein). Therefore, biological stoichiometry also provides horizontal integration across diverse taxonomic groups and type of ecosystems, strengthening the relationships between the evolutionary processes for the emergence of new species with the ecological processes that occur at the inhabited ecosystems. In words of Elser (2006), 'Biological stoichiometry, by providing an explicit conceptual framework to translate major evolutionary phenomena into currencies of interest to ecosystem ecologists (and viceversa), provides a clear means of integrating evolutionary and ecosystem perspectives'.

Objectives

The major goal of the present PhD Project is to study how single and interactive effects of UVR and (atmospheric) nutrient inputs affect the primary producer-consumer interface in high mountain lakes of the Iberian Peninsula (Sierra Nevada and The Pyrenees) (Fig. 10). The multiple approaches used in this work interrelates physiology, ecology and evolution knowledge, offering a unified view of the primary producer-herbivorous consumer interface in the actual global change scenario. Specific objectives were:

1. Examine the joint role of UVR and nutrients on food quality for consumers, in terms of elemental stoichiometry (C:N:P ratios) and biochemical composition (mainly fatty acids) of autotrophs (chapter II).

2. Assess how food quantity and quality (elemental and biochemical composition) affect consumers with contrasting life-history traits: Field and laboratory experiments (chapter III).

3. Determine how the interaction of UVR and nutrients *in situ* affects the strength of the coupling between primary producers and consumers (chapter IV).

4. Assess the effects of UVR and nutrients on consumer homeostasis (chapter V).

5. Model the growth response of herbivorous consumers to a gradient of food quality: Interannual observations and experimental test (chapter VI).

6. Test GRH and P-allocation hypothesis: Inter- and intra-specific variability in P-stoichiometry, nucleic acids and life-history strategies in crustacean zooplankton of clear high mountain lakes (chapter VII).

In order to fulfil these objectives, we conducted both experimental and observational studies. Experimental setups and observational long-data record were carried out in Lake La Caldera at the National Park of Sierra Nevada (chapters II-VI) (Picture 2). The observational inter-systemic study considered a set of 22 high mountain lakes, most of them located in the National Parks of Sierra Nevada and Aigüestortes i Estany de Sant Maurici (Southern and Northeastern Spain, respectively) (chapter VII).

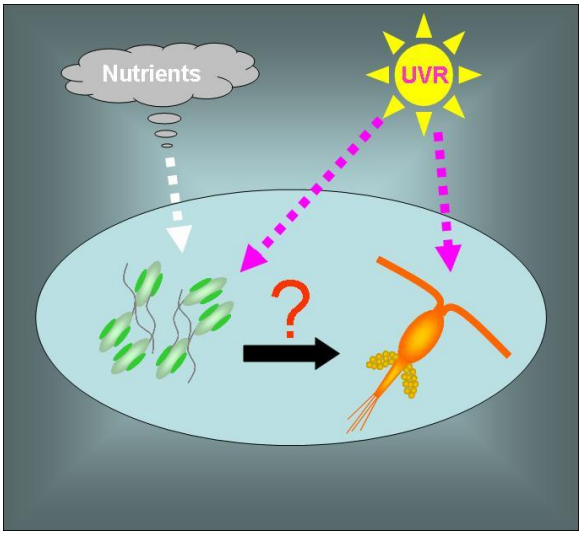


Figure 10. Scheme illustrating the main objective of this PhD project, *i.e.* the study of the interactive effects of UVR and nutrients on the primary producer-consumer interaction. *Esquema que ilustra el principal objetivo de este proyecto de tesis doctoral,* i.e., *el estudio de los efectos interactivos de la radiación ultravioleta y los nutrientes sobre la interacción productor primario-consumidor.*

Picture 2. Lake La Caldera. *Lago La*

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Efectos interactivos de la radiación ultravioleta y los nutrientes sobre la interacción productor primario-consumidor: Una perspectiva ecológico-evolutiva

I. Introducción general

La interacción productor primario-consumidor en ecosistemas acuáticos

El estudio de la estructura y dinámica de las redes incumbe a toda la ciencia (Strogatz, 2001; Clauset et al.*, 2008). Las redes ecológicas, a diferencia de otras, se rigen por otras reglas dado que las interacciones ecológicas las dirigen y restringen de manera única. Saber como éstas funcionan es esencial para comprender la respuesta de las especies y ecosistemas a las perturbaciones del cambio global (Montoya* et al.*, 2006).*

En las redes tróficas, cada interacción contribuye al flujo de energía y al fraccionamiento de la biomasa entre los diferentes niveles tróficos, aunque algunas son especialmente relevantes para su estructura y regulación. Además, estas no son las mismas para los diferentes tipos de ecosistemas, siendo la herbivoría la interacción fundamental para los ecosistemas acuáticos y la detritivoría para los terrestres (Fig. 1). El destino preferente del carbono (C) hacia los consumidores herbívoros en ecosistemas acuáticos o hacia los consumidores detritívoros en ecosistemas terrestres está estrechamente relacionado con las propiedades estructurales, metabólicas y estequiométricas de los productores primarios de cada tipo de ecosistema tal y como Lindeman ya señaló en 1942. Se ha evidenciado que las diferentes presiones selectivas en ambientes acuáticos y terrestres han dado lugar a las diferencias entre sus productores primarios, las cuales repercuten en la configuración de la red trófica, y por tanto contribuyen a las diferencias entre ambos tipos de ecosistemas (Shurin et al.*, 2006). En* Table 1*, se resumen las principales características de los productores primarios acuáticos y terrestres con especial relevancia para la red*

trófica. Diferentemente a los terrestres, los autótrofos acuáticos son más productivos por unidad de biomasa (Nielsen et al.*, 1996), y ofrecen una mejor calidad de alimento a los consumidores dada la ausencia de tejidos estructurales (Lindeman, 1942), la presencia limitada de defensas químicas y estructurales (Shurin* et al.*, 2006) y por su alto contenido en nutrientes (N, P) (Elser* et al.*, 2000*a*). Además, las mayores tasas metabólicas y de crecimiento de los productores primarios que las de los consumidores en ecosistemas acuáticos a diferencia de lo que ocurre en terrestres (Nielsen* et al.*, 1996; Niklas & Enquist, 2001), junto con la aparente dominancia de los consumidores generalistas en ambientes acuáticos (Shurin* et al.*, 2006), hacen que la eficiencia trófica de la interfase productor primario-consumidor herbívoro sea mucho mayor en ecosistemas acuáticos que en terrestres. Por tanto, la interacción productor primario-consumidor herbívoro (*i.e. *herbivoría) juega un papel esencial para la arquitectura de la red trófica en ecosistemas acuáticos pelágicos (Shurin* et al.*, 2006). Por ejemplo, el zooplancton herbívoro en los lagos moviliza una proporción de la producción primaria tres o cuatro veces mayor que la que movilizan los herbívoros de los ecosistemas terrestres, y los consumidores acuáticos son relativamente de seis a sesenta veces más abundantes, a pesar de que la producción primaria neta es igual para ambos tipos de ecosistemas (Cyr & Pace, 1993; Hairston & Hairston, 1993; Cyr* et al.*, 1997; Cebrian, 1999). Por lo tanto, cualquier perturbación que afecte a los productores primarios o a su interacción con los consumidores tiene enormes consecuencias para la estructura y funcionamiento de la red trófica acuática.*

Cantidad de alimento

La herbivoría en ecosistemas acuáticos pelágicos se caracteriza por el hecho de que las algas, de tamaño microscópico, son completamente ingeridas por el zooplancton herbívoro, lo que favorece un fuerte acople de las dinámicas de productores primarios y consumidores. Esto da lugar a que esta interacción tenga más similitudes con la interacción presa-depredador basada en los modelos de Lotka-Volterra, que con la interacción productor primario-consumidor de

*ecosistemas terrestres, en la que los autótrofos no tienen necesariamente por que morir cuando son consumidos por los herbívoros, y para la cual, consecuentemente, no existe una conexión estrecha entre ambos niveles tróficos (Morris, 2009). Esta particularidad hizo que los primeros estudios estuviesen enfocados en cómo la disponibilidad de los recursos podía predecir la abundancia del consumidor. Estos se basaron en la "*match-mismatch hypothesis"*, la cual argumenta que la eficacia del consumidor depende de la sincronía temporal y espacial con su recurso (Cushing, 1974). Esta hipótesis, basada en la dimensión espacio-temporal de esta relación, estableció las bases para una serie mayor de estudios posteriores, aunque la mayoría de estos también incluían la cantidad de alimento para el consumidor como un factor relevante para la interacción (*e.g. *Burns & Dodds, 1999; Guisande* et al.*, 2000). Por ejemplo, Durant* et al. *(2005) cuantificaron el efecto combinado de la presencia y abundancia de un recurso en la dinámica del consumidor con objeto de predecir mejor como la variabilidad ambiental puede afectar a los sistemas ecológicos para diferentes casos en ecosistemas acuáticos y terrestres. Diversos predictores de cantidad de alimento han sido utilizados en los ecosistemas acuáticos, pero entre ellos, biomasa algal, concentración de clorofila* a *(Chl* a*) y sobre todo contenido en C del seston han sido considerados desde los primeros estudios (Lampert, 1977*a*). Los trabajos que utilizan estos predictores han demostrado la fuerte relación entre los productores primarios y los consumidores en las redes tróficas planctónicas, apuntando a que la cantidad de alimento es el factor principal que controla la biomasa y el crecimiento de los herbívoros (Brylinsk & Mann, 1973; Sterner & Schulz, 1998) (Fig. 2A, B). Por ejemplo, la Fig. 2A muestra la relación lineal positiva entre fitoplancton y zooplancton en el estudio llevado a cabo por Brylinsk & Mann (1973), lo cual indica la predictibilidad de la producción secundaria a partir de la primaria. No obstante, dos observaciones importantes surgen de esta relación. Primero, que la producción secundaria es un orden de magnitud menor que la primaria, lo que indica una pérdida importante de energía cuando la energía fluye desde los autótrofos a los consumidores herbívoros. Segundo, la relativa gran dispersión a ambos lados de esta relación, lo que sugiere que la variabilidad en la tasa de*

conversión de producción primaria a secundaria es bastante grande. Dos preguntas surgen de estas observaciones:

1. *¿Cuál es la razón última para esta pérdida de energía y materia?*

2. *¿Hay algo más aparte de la cantidad de alimento controlando la interacción productor primario-consumidor herbívoro?*

Calidad de alimento

*La pérdida de energía y materia entre eslabones de la cadena trófica ha sido generalmente explicada en los libros de texto de Ecología como una transferencia ineficiente de la energía, de acuerdo con la segunda ley de la termodinámica. No obstante, en palabras de Sterner & Elser (página 188 de "*Ecological stoichiometry: The biology of elements from molecules to biosphere*", 2002), "esto pinta un cuadro engañoso sobre cómo funcionan las redes tróficas. No todas la pérdidas en C o energía al pasar de un nivel trófico a otro se deben a la inevitable ineficiencia termodinámica descrita por la segunda ley". Por lo tanto, la variabilidad en la transferencia de C debería ser atribuida a la variabilidad en la calidad de alimento, que reduce la eficiencia de la transferencia de energía y materia a través de la red trófica y por tanto, da forma a la estructura biológica de los ecosistemas (Sterner & Elser, 2002).*

*A pesar de que los ecólogos del zooplancton están de acuerdo con el papel esencial que juega la calidad del alimento algal para la supervivencia, el crecimiento y la reproducción de los consumidores (Gulati & DeMott, 1997), no hay consenso sobre que características del alimento determinan su calidad (Brett & Müller-Navarra, 1997). Así, la calidad del recurso ha sido estudiada en términos de morfología algal (*e.g. *De Bernardi & Giussanig, 1990), defensas frente a los consumidores y producción de toxinas (*e.g. *Ianora* et al.*, 2004), digestibilidad (*e.g. *Van Donk* et al.*, 1997) o composición de la comunidad de productores primarios (*e.g. *DeMott & Tessier, 2002). No obstante, dado que los*

*componentes de la biomasa están asociados con el C (energía), las pérdidas en la transferencia de C entre niveles tróficos han sido también atribuidas a los desequilibrios de la calidad del alimento debidos a desajustes elementales o bioquímicos, dando lugar por tanto al desarrollo de diversas aproximaciones que hacen referencia a las características elementales (aproximación estequiométrica) (*e.g. *Sterner, 1997; Sterner & Schulz, 1998; Sterner & Elser, 2002; Dickman* et al.*, 2008) y bioquímicas (*e.g. *Brett, 1993; Müller-Navarra, 1995*a,b*; Brett & Müller-Navarra, 1997; Gulati & De Mott, 1997; Müller-Navarra* et al. *2000; Becker & Boersma, 2003; Müller-Navarra* et al.*, 2004; Leu* et al. *2006*a,b*; Persson* et al.*, 2007) del alimento (Fig. 3).*

Aproximación estequiométrica

*El marco conceptual conocido como estequiometría ecológica (*ecological stoichiometry*), y definido como el estudio del balance de la energía y múltiples elementos químicos en las interacciones ecológicas, argumenta que la calidad de los recursos para los consumidores está directamente determinada por las diferencias entre las razones carbono:nitrógeno:fósforo (C:N:P) del recurso y del consumidor. Esto es particularmente interesante para la interfase productor primario-consumidor herbívoro, ya que aunque los organismos están constituidos por diferentes elementos, las proporciones del C con respecto al N (C:N) y al P (C:P) son generalmente elevadas y variables para los productores primarios, y bajas y relativamente constantes para los consumidores (Sterner & Hessen, 1994; Elser* et al.*, 2000*b*; Falkowski & Davis, 2004). Esto hace que esta interacción sea uno de los desequilibrios nutricionales más marcados en las redes tróficas (Brett & Müller-Navarra, 1997; Sterner & Elser, 2002). Por lo tanto, de acuerdo con esta aproximación, la calidad del alimento está directamente determinada por el contenido relativo en elementos esenciales como N o P en el alimento algal (Sterner & Elser, 2002). Los datos que apoyan esta hipótesis han sido proporcionados tanto por experimentos de laboratorio que utilizan cultivos algales (para la limitación por N* ver *Checkley, 1980; Kiørboe, 1985; y para la limitación por P* ver *Sommer, 1992; Urabe & Watanabe, 1992; Sterner, 1993;*

*Sterner & Hessen, 1994) como por estudios de campo (*e.g. *Elser & Hasset, 1994; Urabe* et al. *2002*b*; Ferrao-Filho* et al. *2005). Para evaluar el impacto de la calidad de alimento y la intensidad de la interacción de la herbivoría, además de calcular las diferencias aritméticas entre la composición elemental del productor primario y del consumidor, la estequiometría ecológica ha desarrollado también el índice* threshold elemental ratio *(TER; Sterner & Elser, 2002). TER, que funcionalmente depende de los atributos fisiológicos y de la cantidad de alimento disponible en el ambiente (Sterner, 1997; Frost* et al.*, 2004; Logan* et al.*, 2004a,b; Anderson* et al.*, 2005), se define como la dieta para la cual la limitación del crecimiento del consumidor pasa de estar determinada por un elemento a estar por otro (Sterner & Hessen, 1994; Sterner, 1997). Por ejemplo, para la bien caracterizada* Daphnia*, $TER_{C:P}$ es aproximadamente 300 (Urabe* et al.*, 1997; Sterner, 1997), lo que significa que el crecimiento y la reproducción de* Daphnia *deberían de estar fuertemente limitados por P cuando la razón C:P del alimento es más elevada que $TER_{C:P}$, pero limitados en C por debajo de este umbral. Por el contrario, se sabe poco acerca de $TER_{C:P}$ y $TER_{C:N}$ de otras especies de crustáceos tales como los copépodos calanoides, o sobre las diferencias intraspecíficas para una misma especie. Este tema será abordado con mayor profundidad en el capítulo VI.*

Debe de señalarse que el amplio rango de variabilidad de las razones C:N:P en algas, y también mostrado por otros organismos fotoautotróficos (cianobacterias, plantas), refleja condiciones variables de luz, disponibilidad de nutrientes, y tasas de crecimiento. Por ejemplo, bajo condiciones de alta irradiancia lumínica que favorezca la fijación de C por fotosíntesis o bajo severa limitación por nutrientes, los autótrofos producen biomasa con un contenido extremadamente pobre en nutrientes (razones C:nutrientes altas), lo que directamente limita el crecimiento de los consumidores. Contrariamente a los autótrofos, los consumidores heterótrofos han desarrollado mecanismos fisiológicos para regular estrictamente su composición elemental interna y evitar cualquier cambio inducido por factores externos como el alimento. Esta resistencia al cambio en las razones elementales se define como homeostasis estequiométrica, y varía entre especies (Cowgill & Burns, 1975; Andersen &

Hessen, 1991; Hessen & Lyche, 1991) e intraspecíficamente (Villar-Argaiz et al., *2002; Ventura & Catalan, 2005). El grado de homeostasis se estima por la pendiente del modelo lineal que relaciona las razones C:nutrientes del recurso y consumidor, tal y como se ilustra la Fig. 4. Se requiere un mayor esfuerzo para determinar si otros factores, además del alimento, pueden afectar a la homeostasis de los consumidores, induciendo cambios en su composición elemental. En el capítulo V se examinará el papel potencial de factores abióticos [radiación ultravioleta (*ultravioleta radiation, *UVR), nutrientes] como agentes que afectan la composición homeostática de los consumidores herbívoros en la naturaleza.*

Aproximación bioquímica

La otra aproximación científica se basa en la bioquímica y atribuye las limitaciones del crecimiento de los herbívoros a la deficiencia de ácidos grasos esenciales en el alimento, debido a la incapacidad de los consumidores para sintetizar de novo *ácidos grasos poliinsaturados (PUFA) (Müller-Navarra, 1995b; Brett & Müller-Navarra, 1997; Weers & Gulati, 1997). Entre estos, se ha demostrado que los ácidos grasos altamente insaturados son especialmente relevantes para el crecimiento y reproducción del zooplancton (Brett & Müller-Navarra, 1997). Los análisis de correlación (Müller-Navarra* et al., *2000) y los ensayos de suplementación directa (Ravet & Brett, 2006) han proporcionado firmes evidencias de la existencia de limitación por PUFA en la naturaleza.*

No obstante, hay cada vez un mayor número de evidencias de que el contenido algal en PUFA es específico para los diferentes grupos de fitoplancton (Müller-Navarra et al., *2004). Por ejemplo, mientras que las diatomeas pueden tener un alto contenido en ácido eicosapentaenoico [EPA (20:5ω3)] y docosahexaenoico [DHA (22:6ω3)] y un bajo o nulo contenido de ácido α-linolénico [(ALA) 18:3ω3], otras asociaciones algales dominadas por algas verdes tienen sólo trazas de EPA y DHA, pero sí un alto contenido en ALA (Brett & Müller-Navarra, 1997). Tan amplia diversidad en ácidos grasos de las*

comunidades naturales de algas hace necesario que los diversos estudios consideren la gran abundancia de estos componentes bioquímicos, tan preciso como sea posible, con objeto de inferir adecuadamente el crecimiento del zooplancton.

Necesidad urgente de la "síntesis estequiométrico-bioquímica" y de la integración de estudios de cantidad y calidad de alimento

La alta variedad de estudios evaluando los efectos de una determinada característica cualitativa del alimento sobre el consumidor enfatiza la necesidad de teorías que combinen las diferentes características de la calidad de alimento para inferir qué limita realmente al zooplancton herbívoro en la naturaleza (DeMott & Tessier, 2002). Probablemente, el primer paso para esto debería ser la síntesis de las aproximaciones estequiométrica y bioquímica. Hasta la fecha, la mayoría de los estudios han investigado cómo predecir el crecimiento del consumidor para diferentes calidades del alimento algal, en términos de PUFA o composición elemental (Müller-Navarra et al.*, 2000; Sterner & Elser, 2002; Ravet & Brett, 2006), dando lugar a un avance considerable mediante el uso de alguno de los predictores. No obstante, han surgido preguntas acerca de la aplicabilidad general de las hipótesis de limitación por P y ω3-PUFA (Gulati & DeMott, 1997; Hall* et al.*, 2004; DeMott & Pape, 2005; Ferrao-Filho* et al.*, 2007), estableciéndose así la urgente necesidad de vincular ambas aproximaciones en un marco conceptual integrado de ecología fisiológica de los consumidores (Gulati & DeMott, 1997). Aunque los impactos de ω3-PUFA y los contenidos minerales de las algas parezcan independientes unos de otros (Park* et al.*, 2002), algunos estudios han tenido indicios de la importancia conjunta de ambos. Es más, relativamente hay cierto consenso de que ω3-PUFA y el P parecen ser ambos componentes* sine qua non, *ya que los ω3-PUFAs son fundamentales cuando las razones C:P del seston están por debajo de 350,* i.e.*, seston enriquecido en P con respecto al C (Becker & Boersma, 2003). No obstante, no existen aun índices de calidad de alimento que realmente combinen componentes minerales y bioquímicos en una única variable. Debido a la*

compleja relación entre la composición elemental y bioquímica de los autótrofos (Ahlgren et al.*, 1992; Weers & Gulati, 1997; Breteler* et al.*, 2005), sugerimos que ambas deben ser consideradas para inferir correctamente qué limita al zooplancton.*

No obstante, más allá de los predictores de cantidad o las características de calidad de alimento consideradas, surgen varias preguntas: ¿Qué limita fundamentalmente a las poblaciones de zooplancton: Cantidad o calidad de alimento? ¿Cómo ambas interaccionan cuando afectan al consumidor?

La distinción entre las restricciones impuestas por cantidad y calidad de alimento no es una tarea fácil, y esto se debe en parte a que los efectos de cantidad y calidad de alimento interaccionan. Esto significa que el papel de la calidad no es el mismo a lo largo de un gradiente de cantidad. La calidad del alimento no debe de ser relevante a muy bajas cantidades, pero si ejercer un papel importante a medida que la cantidad de alimento es mayor (Sterner & Schulz, 1998) (Fig. 2A). La ampliamente aceptada asunción de que la calidad tiene poca o ninguna importancia para bajas cantidades de alimento fue rechazada por Boersma & Kreutzer (2002), quienes demostraron, por medio de experimentos de laboratorio, que una mayor calidad del alimento es también relevante para el crecimiento de Daphnia *a bajas cantidades. No obstante, no hay evidencias de efectos de calidad para bajas cantidades de alimento en la naturaleza. Por lo tanto, debido a que cantidad y calidad de alimento interaccionan, es crucial i) separar la influencia de cada una mediante el uso de experimentos de campo y laboratorio (Sterner & Schulz, 1998); y ii) demostrar los efectos de calidad sobre el consumidor para bajas cantidades de alimento, propias de ecosistemas ultraoligotróficos. Los objetivos del capítulo III específicamente abordarán estos aspectos para diversas especies de zooplancton que representen los principales grupos taxonómicos de cladóceros, copépodos, y rotíferos.*

La interacción productor primario-consumidor y el cambio global

*Hace medio siglo Lindeman (1942) identificó el papel clave de la luz y los nutrientes para el funcionamiento de los ecosistemas (*ver *su esquema original en la Fig. 5). Ahora se admite ampliamente que la productividad del ecosistema está gobernada por la disponibilidad de nutrientes y por la luz (Urabe & Sterner, 1996). La energía o luz solar es requerida por los productores primarios para fijar el dióxido de C y producir materia orgánica* via *fotosíntesis, y como consecuencia, construir las redes tróficas a partir de este sustrato energético* via *interacciones tróficas.*

No obstante, el metabolismo también requiere nutrientes esenciales como el N y el P para la biosíntesis de proteínas y ácidos nucleicos. Debido a que la adquisición y pérdida de energía (C) y nutrientes (principalmente N, P) están a menudo acopladas en los productores primarios, el papel limitante de los nutrientes afecta a las razones C:N:P de las algas, lo que impone restricciones para transferir la energía eficientemente de los productores primarios a niveles tróficos superiores. Por lo tanto, la eficiencia máxima de transferencia de energía en la interfase productor primario-consumidor tiene lugar para el balance óptimo de luz y nutrientes. Esto fue demostrado experimentalmente por Urabe & Sterner (1996), quienes observaron que el crecimiento de Daphnia *a corto-plazo es máximo para intensidades intermedias de luz que proporcionan una razón C:P del recurso algal próxima a* $TER_{C:P}$*. Estos resultados fueron pronto integrados en la hipótesis luz-nutrientes (*light-nutrient hypothesis, *LNH), la cual predice que las razones C:nutrientes del seston son dependientes de la razón entre la cantidad de luz y los nutrientes disponibles (Sterner* et al.*, 1997). Experimentos adicionales en torres de plancton (Sterner* et al.*, 1998) y en encerramientos de campo (Urabe* et al.*, 2002) confirmaron las predicciones de LNH para escalas temporales largas (Fig. 6).*

Radiación ultravioleta (*ultraviolet radiation*, UVR)

*La energía solar no sólo incluye la radiación fotosintéticamente activa (*photsynthetic active radiation*, PAR) responsable de la fijación del dióxido de C en la materia orgánica, sino también UVR que ejerce un gran impacto en la biota y en los ecosistemas (Sinha & Häder, 2002; Häder* et al.*, 2003*a,b*; Helbling & Zagarese, 2003). La UVR ha aumentado considerablemente debido al agotamiento del ozono estratosférico sobre la Antártida, Ártico, y en latitudes medias-elevadas durante los últimos 30 años. Entre los ecosistemas acuáticos, los lagos de aguas transparentes en los sistemas montañosos, y los océanos en las regiones polares pueden ser particularmente vulnerables debido a la alta penetración de UVR en la columna de agua. Se han observado efectos deletéreos de UVR en el crecimiento, desarrollo, reproducción y productividad para una gran variedad de taxones (Häder* et al.*, 2007), aunque su impacto en las interacciones tróficas, estructura y función de los ecosistemas requiere ser estudiado con mayor profundidad. Por lo tanto, es muy importante evaluar los efectos de UVR en la interacción productor primario-consumidor herbívoro si estamos interesados en predecir los efectos de una mayor dosis de UVR sobre los ecosistemas.*

Varios estudios han puesto de manifiesto el papel de UVR en modificar tanto la cantidad como la calidad de alimento para los consumidores. Los efectos de UVR en reducir la cantidad de alimento disponible para los consumidores fue pronto investigado por una serie de investigadores, quienes demostraron el efecto deletéreo de UVR sobre la fijación de C por fotosíntesis, la producción primaria bruta y neta (Steeman-Nielsen, 1964; Worrest et al.*, 1978; Lorenzen, 1979; Calkins & Thordarrdottir, 1980; El Sayed, 1988; El Sayed, 1990; Smith* et al.*, 1992; Moeller, 1994; Gala & Giessy, 1991; Boucher* et al.*, 1995). Pero estos efectos cuantitativos podrían ser rebasados por las modificaciones en la calidad del alimento (Hessen* et al.*, 1997). Claramente, hay firmes evidencias para justificar que UVR altera la adquisición de tanto N (Döhler & Alt, 1989; Döhler & Kugel, 1994; Braune & Döhler, 1994) como de P (Hessen* et al.*, 1995) mediante la inducción de una actividad enzimática reducida, daños en las*

membranas y paredes celulares, mutaciones en el DNA, o alteraciones de la síntesis proteica. Estos y otros procesos relacionados con la UVR podrían afectar a la estequiometría C:N:P del fitoplancton de diversas maneras. Trabajos recientes han puesto de manifiesto una reducción general de las razones C:nutrientes algales debida a UVR, aunque aparentemente no hay consenso sobre qué mecanismos son responsables de este efecto (Xenopoulos et al.*, 2002; Hessen, 2006; Carrillo* et al.*, 2008; Hessen* et al.*, 2008*a*). Normalmente, una razón C:P baja ha sido considerada como un indicador de mayor calidad de alimento para los herbívoros (Sterner & Elser, 2002). Por lo tanto, la observada reducción en la razón C:P del seston por UVR parece que podría tender efectos indirectos beneficiosos para los consumidores dado que significa una mejora en la calidad del alimento para ellos (Xenopoulos* et al.*, 2002). No obstante, estudios recientes señalan que razones C:P bajas del seston no implican necesariamente mayor crecimiento de los herbívoros (Leu* et al.*, 2006*a*) probablemente debido a que, tal y como Carrillo* et al. *(2008) demuestran, una baja razón C:P del seston es más consecuencia de la pérdida de C que de la acumulación de P. Además, UVR altera la composición bioquímica del C en el alimento, aumentando (Tank* et al.*, 2003; Leu* et al.*, 2007) o disminuyendo (Hessen* et al.*, 1997) el contenido total de lípidos y PUFA esenciales. En resumen, ahora sabemos que UVR tiene el potencial de alterar simultáneamente la composición elemental y bioquímica de los autótrofos. Se requieren estudios* in situ *diseñados para evaluar cómo estos efectos se trasladan a niveles tróficos superiores y comprender mejor cómo las comunidades naturales responderán al cambio global. Estos aspectos serán estudiados en profundidad en los capítulos II y III.*

Nutrientes

El ser humano ha alterado de una manera dramática los principales ciclos biogeoquímicos. En particular, las actividades humanas y la industrialización han aumentado la cantidad de C, N y P disponibles en un 12%, 112%, y 400%, respectivamente (Falkowski et al.*, 2000). La circulación atmosférica es el*

principal medio para el transporte y entrada de nutrientes a los ecosistemas terrestres y acuáticos gracias a la deposición húmeda y seca.

La emisión de polvo es un importante componente regional del clima con consecuencias a nivel global. Los desiertos son las fuentes naturales de polvo, siendo el Sahara el principal desierto de la Tierra. Se sabe ampliamente que las plumas de polvo desértico pueden proporcionar nutrientes esenciales que se trasladan a través de grandes distancias desde las áreas fuente hasta las regiones sumidero (Neff et al.*, 2008; Goudie, 2009). Por ejemplo, el polvo del Desierto del Sahara puede tener una gran relevancia para el contenido en nutrientes de la cuenca mediterránea (Ridame & Guieu, 2002), pero puede también llegar tan lejos como a la cuenca amazónica (Moulin* et al.*, 1997). El polvo contiene relativamente grandes cantidades de nutrientes clave para las plantas como hierro (Fe) y calcio (Ca), sin embargo un estudio reciente de Bristow* et al. *(2010) ha evidenciado que el polvo puede ser hasta 38 veces más rico en P de lo que antes se había pensado. Si tal y como apuntan los últimos modelos climáticos, África tiene condiciones más áridas en el futuro que las del presente, se espera que tendrá lugar una mayor emisión de aerosoles a la troposfera que accederán a áreas remotas por los vientos del oeste (*Westerly winds*). Ambientes ideales para el estudio de los efectos de la deposiciones atmosféricas son los lagos de alta montaña del Parque Nacional de Sierra Nevada debido a que (1) su localización dentro de los primeros 2000 km circundantes al Desierto del Sahara, en los cuales se deposita la mayor parte de su polvo emitido (Jaenicke & Schütz, 1978), (2) su elevada altitud por encima de los 2000 m, dentro del rango entre 1500 y 4000 m por encima del nivel del mar, por el cual transcurren las principales corrientes transportadoras de polvo sahariano (Talbot* et al.*, 1986), y (3) su extrema oligotrofia que hace que la producción primaria sea muy sensible a la entrada alóctona de nutrientes (Villar-Argaiz* et al.*, 2001; Morales-Baquero* et al.*, 2006) (*Picture 1*).*

Efectos interactivos de la UVR y los nutrientes

Para comprender los efectos a nivel global, el trabajo debe necesariamente ir enfocado a estudiar otros estresores importantes a nivel global que afecten conjuntamente a los ecosistemas. Está siendo cada vez más reconocido que los diversos estresores abióticos, que actúan a diferentes tasas a escalas local y global, afectan al crecimiento de los organismos y al funcionamiento de los ecosistemas (Carrillo et al.*, 2008).*

Testar los efectos interactivos requiere diseños experimentales complejos en los que más de un factor pueda ser manipulado. La interacción entre los diversos factores da lugar a efectos sobre los organismos y ecosistemas no fácilmente predecibles a partir de estudios unifactoriales, debido a la naturaleza no aditiva (sinérgica y antagónica) de sus interacciones. Por ejemplo, previamente se ha mencionado que UVR reduce tanto la producción primaria como la absorción de nutrientes. Aunque se podría esperar que la mayor disponibilidad de nutrientes compensara los efectos negativos de UVR, Carrillo et al. *(2008) observaron efectos sinérgicos de la interacción de UVR y los nutrientes sobre el crecimiento algal,* i.e.*, crecimiento algal aún más afectado por UVR a medida que aumentan los niveles de nutrientes en el medio. No obstante, aun carecemos de conocimiento de cómo estos efectos podrían trasladarse a la producción secundaria.*

La investigación del futuro debería prestar atención en particular a cómo los efectos combinados de diversos estresores afectan a la interfase productor primario-consumidor si queremos inferir más precisamente las consecuencias para la red trófica y el ecosistema. La complejidad de estudiar los efectos interactivos sobre las interacciones ecológicas es un gran desafío para los ecólogos. Estudios integrados que combinen aproximaciones experimentales con observacionales y variables estructurales con funcionales deberían proporcionar una evaluación precisa de cómo los diversos factores interaccionan cuando afectan a la interfase productor primario-consumidor herbívoro. En el capítulo II se analizará como UVR, nutrientes y su interacción afectan a la composición

estequiométrica y bioquímica del seston, en la base de la red trófica. En el capítulo III se testará cómo estos efectos se transfieren al siguiente nivel trófico de los consumidores herbívoros, mediante experimentos acoplados de campo y laboratorio, y tres especies de zooplancton como indicadoras.

En un estudio previo en el lago de La Caldera, Villar-Argaiz et al. *(2001) demostraron que los aportes atmosféricos al lago, a pesar de favorecer el crecimiento algal, no dieron lugar a una mayor biomasa zooplanctónica (Fig. 7). Este resultado fue aun más llamativo después de que Carrillo* et al. *(2008) demostraran que las entradas de P y UVR conjuntamente reducen la razón C:P del seston, mejorando por tanto potencialmente la calidad del alimento para los herbívoros. Este resultado contraintuitivo de que una mayor cantidad de alimento de mejor calidad, debido a los nutrientes y a la UVR, no dio lugar a una mayor biomasa de la comunidad de zooplancton, repercutiendo negativamente por tanto en el acople fitoplancton-zooplancton, merece investigarse con especial dedicación. Este aspecto será cubierto en el capítulo IV.*

Estequiometría biológica: Un puente entre la ecología de ecosistemas y la biología evolutiva

En secciones previas, la estequiometría ecológica fue definida como el estudio del balance de la energía y múltiples elementos químicos en las interacciones ecológicas (Sterner & Elser, 2002). Las principales contribuciones de esta disciplina al estudio ecológico de la interacción productor primario-consumidor son:

1. *Las especies de zooplancton de similar tamaño y estadío ontogenético, y parentesco filogenético cercano son distintas en sus razones estequiométricas C:N:P (Andersen & Hessen, 1991; Hessen & Lyche, 1991; Elser* et al.*, 2000*a*), difiriendo hasta cinco veces en su contenido en P. Estas diferencias se relacionan en gran medida con la tasa de crecimiento específica, y con la estrategia de vida. Por ejemplo, mientras*

que los cladóceros con ciclos reproductivos partenogenéticos, tiempo de generación corto, y elevadas tasas de crecimiento se caracterizan por razones N:P entre 12 y 18 (%P en peso seco ~1.2), los copépodos calanoides con reproducción sexual, tiempo de generación largo, y bajas tasas de crecimiento muestran valores de la razón N:P que exceden a 30 (%P en peso seco ~0.5) (Andersen & Hessen, 1991).

2. *Homeostasis estequiométrica: Contrariamente a los autótrofos, los consumidores heterótrofos regulan su composición interna, mostrando valores relativamente fijos en sus razones C:N:P (organismos homeostáticos) en comparación con aquellas de los organismos autótrofos (Sterner & Elser, 2002).*

3. *Los procesos* bottom-up *pueden afectar al desequilibrio elemental entre productores primarios y herbívoros. Un mayor desequilibrio consistiría en que una peor calidad de alimento impacta negativamente a los consumidores herbívoros, afectando por lo tanto a su crecimiento, reproducción, y flujo de C en la red trófica (Fig. 3B). Por el contrario, una interacción más equilibrada, consistiría en que una calidad mejorada favorece el crecimiento de los consumidores herbívoros, y consecuentemente fortalece las interacciones tróficas (Fig. 3A) (Hessen, 1992; Urabe & Watanabe, 1992; Sterner* et al.*, 1993; DeMott* et al.*, 1998; Elser & Foster, 1998; Boersma, 2000; Elser* et al.*, 2001; Hood* et al.*, 2005).*

4. *Procesos* top-down*: Debido a la naturaleza homeostática de los consumidores y a sus diferencias interespecíficas en la composición elemental somática, los cambios en la dominancia relativa de zooplancton pueden alterar el ciclo de los nutrientes en las redes tróficas pelágicas (Elser & Urabe, 1999; Vanni, 2002). Su impacto afecta a la naturaleza de la limitación por nutrientes del fitoplancton, tal y como observaron Elser* et al. *(1988) y Andersen & Hessen (1991). Estos autores vieron que mientras que en las comunidades dominadas por* Daphnia*, rica en P (baja*

razón N:P somática), el fitoplancton tiende a estar limitado por P, otras dominadas por copépodos, pobres en P (alta razón N:P somática), el fitoplancton tiende a estar limitado por N.

5. *Estresores ambientales (*e.g. *factores de cambio global como una mayor intensidad de UVR y de deposición de aerosoles atmosféricos) afectando a los aportes de energía y/o nutrientes alteran el equilibrio nutricional de la interacción productor primario-consumidor herbívoro, y consecuentemente a la producción del ecosistema (Urabe & Sterner, 1996; Sterner* et al.*, 1997, 1998; Diehl* et al.*, 2002; Hessen* et al.*, 2002; Urabe* et al.*, 2002*a,b*, 2003).*

Aunque nuestro interés ha estado dirigido a cómo la estequiometría ecológica explica la interacción productor primario-consumidor herbívoro en ecosistemas acuáticos pelágicos, esta disciplina ha sido también considerada para otras interacciones ecológicas y para ecosistemas terrestres y acuáticos bentónicos. En todos los casos, el vínculo entre la variación en la composición elemental específica y/o de estadío y la interacción entre especies, dinámica de red trófica, y/o flujo de nutrientes ha sido válida. (Sterner & Elser, 2002)… Pero, ¿tienen los patrones estequiométricos más implicaciones aparte de las correspondientes en los procesos ecológicos?

Un punto de partida para contestar a esta pregunta ha sido la búsqueda de una explicación para algo tan simple como, por qué las especies difieren en su composición elemental. Aunque, una "pregunta simple" podría requerir una "respuesta simple", existe una gran complejidad en los mecanismos biológicos y evolutivos que hay tras esta "simplicidad" (Elser, 2006). En un primer intento para explicar las diferencias interespecíficas en la composición elemental, Reiners (1986) propuso que éstas estaban originadas por las principales diferencias en los rasgos estructurales relacionados con el soporte mecánico. Tal argumento ha sido aplicado con éxito para explicar las diferencias entre plantas vasculares terrestres con estructuras de celulosa para el soporte ricas en C (razones C:nutrientes altas) y organismos autótrofos planctónicos no vasculares

(razones C:nutrientes bajas) (Elser et al.*, 2000*a*). No obstante, evidencias firmes apoyan ahora que la variación en la composición elemental de los organismos no sólo se relaciona con las características estructurales sino también con la biología molecular del crecimiento* via *producción de RNA ribosómico. Esta idea es recogida por la hipótesis de la tasa de crecimiento (*growth rate hypothesis*, GRH), que establece que la variación en el contenido en P (y por tanto en C:P y N:P) de los seres vivos está determinada por la variación en la asignación de P al RNA ribosómico, estrechamente vinculada con las diferencias en la tasa de crecimiento entre organismos, ya que se requiere P para la biosíntesis de ribosomas encargados de la síntesis proteica y por tanto para el crecimiento (Elser* et al. *1996, 2000*b*, 2003) (Fig. 8). Por lo tanto, considerando que:*

1. *El crecimiento y la eficacia biológica están íntimamente ligados dado que los organismos tienen que necesariamente crecer antes de reproducirse, y que por tanto, se puede considerar que la tasa de crecimiento es un parámetro fundamental que integra toda la estrategia de vida (Arendt, 1997).*

2. *Cualquier presión selectiva que afecte al crecimiento del organismo o a las tasas de desarrollo puede reflejarse en las razones C:N:P del organismo, y consecuentemente, en la sensibilidad del organismo a las restricciones impuestas por la calidad de alimento y en su influencia sobre el flujo de nutrientes (Sterner & Elser, 2002).*

*La GRH vincula conceptos fundamentales de la bioquímica y fisiología celular y del individuo [composición elemental (C, N, P) y bioquímica (RNA ribosómico, proteínas), biogénesis ribosómica], ecología evolutiva de los ciclos vitales [rasgos del ciclo de vida (tasa de crecimiento), eficacia biológica] y ecología de ecosistemas (interacciones tróficas, ciclo de nutrientes). Por lo tanto, GRH constituye un dogma central para la estequiometría biológica (*biological stoichiometry*), definida como el estudio del balance de la energía y múltiples elementos químicos en los seres vivos (Elser* et al.*, 2000*b*). Esto es la extensión de la estequiometría ecológica a los seres vivos, y por tanto, ha aplicado principios*

similares a aquellos de las interacciones entre niveles tróficos a las interacciones entre los diferentes niveles de organización biológica (Elser et al.*, 2000*b*).*

Con el desarrollo de la genómica durante el inicio del siglo XXI, ha emergido un gran interés por estudiar la base genética de la GRH, en un intento de extender la estequiometría biológica al nivel genético. Las principales contribuciones de estos estudios incluyen el descubrimiento de que una mayor tasa de crecimiento y el aumento asociado de la tasa de transcripción para la producción de RNA ribosómico están positivamente asociados con el tamaño y el contenido de los espaciadores intergénicos en el DNA ribosómico y/o con el número total de copias de DNA ribosómico (Weider et al.*, 2005). No obstante, este patrón consistente aparentemente contrasta con las observadas relaciones genéricas entre elevada tasa de crecimiento y alto contenido de RNA con tamaños de genoma pequeños en organismos de crecimiento rápido. Este fenómeno, inicialmente observado para cladóceros, fue explicado por* P-allocation hypothesis*, que establece que las diferencias entre tamaños del genoma entre los dos principales grupos de zooplancton, copépodos y cladóceros (que comparten el mismo nicho y son de similar tamaño) podría deberse a la asignación diferencial del P. Por tanto, el pequeño tamaño del genoma en cladóceros, caracterizados por tasas de crecimiento elevadas y un alto contenido en P y RNA, diferente a los copépodos, podría ser consecuencia de la reasignación del P desde el DNA (principalmente DNA no codificante) al RNA bajo una continua presión selectiva para crecimiento rápido en ambientes limitados por P (Hessen* et al.*, 2008*b*). Aunque más apoyo experimental y observacional sería deseable, hay firmes evidencias para extender la validez de esta hipótesis al resto de los eucariotas sometidos a selección para tener crecimiento rápido en ambientes limitados por nutrientes (Fig. 9). Esta ampliación ha sido llamada la hipótesis tasa de crecimiento-tamaño del genoma-limitación por nutrientes (*growth rate-genome size-nutrient limitation hypothesis*, GRGSNLH) (Hessen* et al.*, 2009). GRH y* P-allocation hypothesis *serán probadas en el capítulo VII.*

Tomados todos estos conceptos en conjunto, la estequiometría biológica vincula conceptos fundamentales de la biología que abarcan desde el diseño del

tamaño del genoma y la organización de la principal familia de genes (DNA ribosómico) pasando por la asignación intracelular y demanda fisiológica de nutrientes, a las interacciones tróficas y a los ciclos de los nutrientes en las redes tróficas. Esto implica que las conexiones estequiométricas podrían proporcionar una integración vertical entre todos los niveles de organización biológica, incluyendo al nivel ecosistémico, que ha sido tradicionalmente desatendido por los estudios evolutivos. Es más, a pesar de que las hipótesis mencionadas más arriba han sido enunciadas para el zooplancton crustáceo (Elser et al.*, 2003; Hessen* et al.*, 2008*b*), cada vez una serie mayor de estudios están corroborándolas para otros organismos bajo una amplia variedad de condiciones ecológicas tanto en ecosistemas acuáticos como terrestres (para la GRH,* ver *Sutcliffe, 1970; Elser* et al.*, 2000*a*; Elser* et al.*, 2000*b *y referencias incluidas; Frost & Elser, 2002; Jaenike & Markow, 2002; Makino* et al.*, 2003; Schade* et al.*, 2003; Makino & Cotner, 2004; Perkins* et al.*, 2004; Woods* et al.*, 2004; Elser* et al.*, 2005; Watts* et al.*, 2006; para GRGSNLH,* ver *Smith & Holt, 1996; Gambi* et al.*, 1997; White & McLaren, 2000; Wyngaard* et al.*, 2005; Saliba* et al.*, 2006; Smith, 2007; Hessen* et al.*, 2008*b*; Gregory & Johnson, 2008; Hessen* et al.*, 2009 y referencias incluidas). Por lo tanto, la estequiometría biológica también proporciona una integración horizontal entre los diversos grupos taxonómicos y los diversos tipos de ecosistemas, fortaleciendo las relaciones entre los procesos evolutivos responsables de la emergencia de nuevas especies con los procesos ecológicos que tienen lugar en los ecosistemas que habitan. En palabras de Elser (2006), "la estequiometría biológica, facilitando un marco conceptual explícito para trasladar los principales fenómenos evolutivos al interés de los ecólogos de ecosistemas (y viceversa), proporciona un medio de integración de las perspectivas evolutiva y ecosistémica".*

Objetivos

El principal objetivo del presente proyecto de tesis doctoral es estudiar cómo los efectos individuales e interactivos de UVR y entradas (atmosféricas) de nutrientes afectan a la interfase productor primario-consumidor en lagos de alta

montaña de la Península Ibérica (Sierra Nevada y Pirineos) (Fig. 10). Las múltiples aproximaciones de este trabajo relacionan fisiología, ecología, y conceptos evolutivos, ofreciendo así una visión integrada de la interfase productor primario-consumidor herbívoro en el actual escenario de cambio global. Los objetivos específicos fueron:

1. Examinar el efecto conjunto de UVR y nutrientes sobre la calidad de alimento para los consumidores, en términos de estequiometría elemental (C:N:P ratios) y composición bioquímica (principalmente ácidos grasos) de los autótrofos (capítulo II).

2. Evaluar cómo la cantidad y calidad (composición elemental y bioquímica) del alimento afectan a consumidores con diferentes rasgos del ciclo de vida: Experimentos de campo y laboratorio (capítulo III).

3. Determinar como la interacción de UVR y nutrientes afecta in situ *a la intensidad del acople entre productores primarios y consumidores (capítulo IV).*

4. Evaluar los efectos de UVR y nutrientes sobre la homeostasis de los consumidores (capítulo V).

5. Modelar la respuesta del crecimiento de los consumidores herbívoros a un gradiente de calidad de alimento: Observaciones interanuales y test experimental (capítulo VI).

6. Evaluar GRH y P-allocation hypothesis*: Variabilidad inter- e intraespecífica de la estequiometría del P, ácidos nucleicos y estrategias de vida en zooplancton crustáceo de lagos de alta montaña (capítulo VII).*

Con objeto de abordar estos objetivos, llevamos a cabo estudios tanto experimentales como observacionales. Los diseños experimentales y el registro observacional de series de datos fue llevado a cabo en el lago de La Caldera del Parque Nacional de Sierra Nevada (capítulos II-VI) (Picture 2). El estudio

observacional intersistémico consideró un conjunto de 22 lagos de alta montaña, la mayoría de ellos situados en los Parques Nacionales de Sierra Nevada y Aigüestortes i Estany de Sant Maurici (sur y noreste de España, respectivamente) (capítulo VII).

II

Effects of ultraviolet radiation and nutrients on elemental and biochemical food quality for consumers

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Abstract

Numerous laboratory studies have shown that food quality is suboptimal for zooplankton growth. However, little is known about how food quality is affected by the interaction of potential global change factors in natural conditions. Using field enclosures in a high altitude Spanish lake, seston was exposed to increasing phosphorus (P) concentrations in the absence and presence of UV radiation (UVR) to test the hypothesis that interactions between these factors affected the biochemical and stoichiometric composition of seston in ways not easily predicted from studies of single factors. P-enrichment increased the content of total fatty acids (TFA), ω3-polyunsaturated fatty acids (ω3-PUFA) and Chlorophyll *a*:carbon (Chl *a*:C) and C:N ratios in seston. The pronounced increase in ω3-PUFA was largely explained by the enhancement of 18:3n-3 (α-linolenic acid, ALA). In contrast, P-enrichment lowered the content of highly unsaturated fatty acids (HUFA), the HUFA:PUFA ratio and, at high P loads, seston C:P ratio. Although phytoplankton assemblages dominated by Chlorophytes were not rich in HUFA, seston in the control had substantially higher 20:4n-6 (arachidonic acid, ARA) content (79% of HUFA) than did P-enriched enclosures. UVR increased the content of ω3-PUFA and TFA in seston at the two ends of the trophic gradient generated at ambient and high concentrations of P, but decreased seston C:P and HUFA at all points on this gradient. ARA was not detected in the presence of

UVR. The interaction between P and UVR was significant for seston HUFA and C:P ratios, indicating that the effect of UVR in reducing HUFA (decreased food quality) and C:P ratios (enhanced food quality) was most pronounced at the low nutrient concentrations characteristic of oligotrophic conditions and disappeared as P increased. Therefore, any future increase in UVR fluxes will probably affect most strongly the food quality of algae inhabiting oligotrophic pristine waters although, at least in the Mediterranean region, these effects could be offset by greater deposition of P from the atmosphere.

Resumen

Numerosos estudios de laboratorio han puesto de manifiesto que la calidad de alimento es subóptima para el crecimiento del zooplancton. No obstante, se sabe poco sobre cómo la calidad de alimento está afectada por la interacción de factores potenciales de cambio global en condiciones naturales. Mediante el uso de encerramientos de campo en un lago de alta montaña español, se expuso al seston a concentraciones crecientes de fósforo (P) en presencia y ausencia de radiación UV (UVR) para testar la hipótesis de que las interacciones entre estos factores afectan la composición estequiométrica y bioquímica del seston de manera no fácilmente predecible a partir de estudios unifactoriales. El enriquecimiento en P incrementó el contenido total de ácidos grasos (TFA), ácidos grasos poliinsaturados ω3 (ω3-PUFA) y las razones clorofila a*:carbono (Chl* a*:C) y C:N del seston. El pronunciado aumento de ω3-PUFA fue explicado en gran medida por el aumento de 18:3n-3 (ácido α-linolénico). Por el contrario, el enriquecimiento en P disminuyó el contenido en ácidos grasos altamente insaturados (HUFA), la razón HUFA:PUFA y, para altos niveles de P, la razón C:P. Aunque las comunidades fitoplanctónicas dominadas por clorofitas no son ricas en HUFA, el seston en el tratamiento control tuvo sustancialmente mayor contenido en 20:4n-6 (ácido araquidónico, ARA) (79% de HUFA) que en los encerramientos enriquecidos con P. UVR aumentó el contenido de ω3-PUFA y TFA del seston en los dos extremos del gradiente trófico generado correspondientes a las concentraciones de P del medio y de mayor*

enriquecimiento, pero disminuyó la razón C:P del seston y el contenido en HUFA para el resto del gradiente. ARA no fue detectado en presencia de UVR. La interacción entre P y UVR fue significativa para el contenido en HUFA y la razón C:P del seston, lo que indica que mientras que el efecto de UVR disminuyendo HUFA (calidad de alimento reducida) y C:P (calidad de alimento incrementada) fue más pronunciado para concentraciones de nutrientes bajas, características de ambientes oligotróficos, desaparecía conforme P aumentaba. Por tanto, cualquier aumento futuro en la incidencia de UVR probablemente afecte en mucha mayor medida a la calidad de alimento algal en aguas claras oligotróficas aunque, al menos para la región mediterránea, estos efectos podrían estar compensados por una mayor deposición atmosférica de P.

Introduction

Light and nutrients are among the most important variables regulating the composition of autotrophs, which in turn affects that of herbivores *via* the quality of their food (Urabe & Sterner, 1996; Paul & Gwynn-Jones, 2003) and, hence, the efficiency at which energy is transferred through the food web (Sterner & Elser, 2002). Though the role of photosynthetic active radiation (PAR) on autotroph biochemical composition has received considerable attention, the indirect role of UVR is unclear. While there are reports that UVR enhances the food quality of herbivores, many data support the opposite conclusion. For example, decreased carbon:phosphorus (C:P) ratios (enhanced nutritional quality) have been found after exposing autotrophs to UVR (Xenopoulos *et al.*, 2002; Carrillo *et al.*, 2008), but also in epilithon in the absence of ambient UVR (Watkins *et al.*, 2001). Similarly, the effect of UVR on the quality of algae in terms of fatty acid composition remains unclear. Thus, some studies have reported a decrease in certain polyunsaturated fatty acids (PUFAs) under UVR (Goes *et al.*, 1994; Skerratt *et al.*, 1998), whereas others show no influence or an increase in certain types of PUFA (Skerratt *et al.*, 1998; Leu *et al.*, 2006*a*).

Perhaps, the species-specific response of biochemical composition to the stress of UVR or the relatively short-term nature of most of these studies underlies these conflicting results. However, other stressors such as nutrient availability (either in shortage or in excess) need also to be considered when studying the impact of UVR on the elemental and biochemical composition of autotrophs (Davison *et al.*, 2007). This is crucial given that the effects of UVR on the growth and somatic content of organisms are related to the availability of limiting mineral nutrients (Medina-Sánchez *et al.*, 2006). In the context of global change, understanding and predicting the simultaneous effects of multiple stressors, such as increasing UVR and nutrient concentrations, on species and food web interactions is a current challenge for ecosystem research and management. To achieve this goal, experiments that combine multiple stressors in the field are important, as they may have interdependent effects (not easily assessed from single-factor experiments) on food quality at the base of food webs.

Our aim was to examine the joint role of UV radiation and P-enrichment in the cellular stoichiometry and biochemical composition (fatty acids and Chl *a*:C ratio) of autotrophs. Our task was thus, firstly to elucidate the single role of each factor (P or UVR) on the biochemical composition of seston and, secondly, to study their joint effect (*i.e.* whether their single effects on seston interact). We used relatively long term experiments (over many generations of algae) which may have enabled the beneficial photorepair and/or photoprotective UVR mechanisms that ultimately determined the biochemical composition of algae.

Methods

Experimental set-up

The experiment was performed using field mesocosms to manipulate the supply of P and light quality (presence and absence of UVR) in La Caldera, an ultraoligotrophic fishless mountain lake in the National Park of Sierra Nevada (Southern Spain, 36º55′–37º15′N, 2º31′–3º40′W; 3050 m a.s.l.). The strong incidence of UV radiation at this latitude and altitude (Carrillo *et al.*, 2002), and the scarcity of nutrients (Villar-Argaiz *et al.*, 2001), make of La Caldera lake an ideal site at which to test the effects of nutrients and sunlight on seston. The experiment started on 1 August 2003. Unscreened lake water was pumped from 5 m depth into ten polyethylene cylindrical enclosures, closed at their lower end, and each with diameter of 1 m, depth of 7 m and volume of 2.7 m^3. Five of the enclosures, with polyethylene lid, received the full spectrum of solar radiation (+UVR treatment) and the other five (-UVR treatment) were covered with Plexiglas UF3 (Atohaas North America) sheets that blocked direct and refractory exposure to UV radiation. The optical properties of the filters used in the light treatments were verified before the experiments using a double-beam spectrophotometer (Perkin Elmer Lambda 40, Perkin Elmer Corporation, Norwalk, USA). The polyethylene plastic used in the +UVR treatment transmitted 90% of PAR, 60% of UVB and 75% of UVA, while the long-wave-pass Plexiglass transmitted 90% of PAR but completely blocked UV radiation (<390

nm). In both series of enclosures, phosphate (NaH_2PO_4) was added at the start to create an increasing P gradient of four concentrations (20, 30, 40 and 60 μg P L^{-1}), and inorganic nitrogen (N) (NH_4NO_3) was added to give a final N:P molar ratio of 30 according to concentrations of inorganic nutrients (NO_2^-, NO_3^-, NH_4^+ and total dissolved P, TDP) measured in the lake the day before the experiment. For each light treatment, one enclosure received no phosphate and served as a control to give a two (light) × five (P) factorial design with one replicate of each. Enclosures were sampled periodically (days 1, 3, 10 and 20 of incubations) for soluble reactive P (SRP) determinations. After SRP became depleted on day 20 of incubations, enclosures were sampled three times every two days (days 30, 32 and 34 of incubations) for phytoplankton abundance and seston (suspended particulate matter >1.0 μm) elemental and biochemical determinations, using a plastic bucket and after gently mixing the entire length of the enclosure (total number of samples per enclosure = 3).

Biological and biochemical analyses

Phytoplankton samples were fixed with Lugol´s solution. A 50-mL aliquot from the phytoplankton was counted at ×1000 magnification using an inverted microscope (Leitz Fluovert FS, Leica, Wetzlar, Germany) to estimate cell abundance.

Seston (pre-screened through a 40 μm mesh to remove macrozooplankton) was collected onto pre-combusted (24 h at 500ºC) 1-μm glass-fibre filters (Whatman GF/B) at low pressure (100 mm of Hg). Samples were analysed for C and N using a CNH analyser (Perkin-Elmer Model 2400) or for P content by colorimetric means after persulphate oxidation (APHA, 1992). All C:P ratios were calculated on a molar basis. Filters for Chl *a* were extracted in acetone and concentrations determined by fluorimetry (APHA, 1992). A Chl *a* standard (Fluka Chl *a* from algae) was used to transform the fluorescence data into Chl *a* concentrations.

Fatty acids in seston were analysed after extraction and transmethylation (Christie, 1982) using a gas chromatograph (Fisons Instruments GC 8000 Series, Thermo Electron Co., Rodano, Italy) equipped with a fused silica open tubular column (Tracer, TR-WAX, Tecknokroma, Spain) and a cold on-column injection system. We defined PUFA as polyunsaturated fatty acids with a chain length of 18 or more C atoms, and HUFA as a subset of PUFA molecules with 20 or more atoms of C. Units for fatty acids in this study are μg per mg of C in the seston.

Statistical analysis

The effects of P-enrichment for each light treatment on seston elemental and biochemical variables were assessed by regression analysis of mean values for the three sampling dates against P-enrichment level. It was checked that data fulfilled criteria for regression analysis. When no linearity was observed, differences between each level of P-enrichment and the control with no P added were assessed by dependent paired *t*-tests. For the effects of UVR, differences in biochemical and elemental composition at each P level were assessed by dependent paired *t*-tests.

For the analysis of the interactive UVR × P effects on seston composition ANOVA could not be performed, due to the unreplicated design of the experiment. Alternatively, interactive UVR × P effects on food quality variables were specifically tested depending on whether variables adjusted or not to linear trends when regressed against P-enrichment. When regressions were significant for the two light treatments, effects of UVR were tested by analysis of covariance. Statistically different slopes were indicative of an interaction between UVR and P, whereas homogeneous slopes indicated no interaction between UVR and P. In contrast, when no linearity was observed, analysis of covariance was precluded, and seston variables were relativized to the control (dividing the observation for each P level in +UVR by the respective control treatment replicate in –UVR) and regressed against P-enrichment. The existence of a linear trend between the UVR-relativized variable across the P gradient would indicate a significant UVR × P

effect on the biochemical constituent. To aid in the visualization of these results, and regardless of the method used to test for interactive effects, variables were relativized to the control before being represented graphically. The statistical analyses were performed using Statistica 7.0 for Windows software (StatSoft, 1997).

Results

The abundance of phytoplankton in the control enclosures without added P (Fig. 1) was within the range of those previously reported for Lake La Caldera (Villar-Argaiz *et al.*, 2001). Algal growth is strongly limited by P in this lake, as reflected by the low concentration of SRP in lake water (0.34-2.86 μg P L^{-1}) during the experiment and the dissolved inorganic N:total P ratio (DIN:TP) between 61 of 12 (Villar-Argaiz *et al.*, 2002), largely higher than the threshold of 12 proposed for P limitation by Morris & Lewis (1988). P-enrichment enhanced phytoplankton abundance by two orders of magnitude (Fig. 1) and the Chlorophyta *Dyctiosphaerium chlorelloides* (Nauman; Komárek & Perman) dominated in all experimental conditions. Variations in algal biochemical or stoichiometric content should, therefore, not be attributed to differences in algal communities among enclosures. Sampling for seston biochemical and elemental determinations was initiated after P (measured as SRP) became depleted after thirty days of incubation in the most enriched enclosures (Fig. 2) and clear differences in seston C:P ratio were detected.

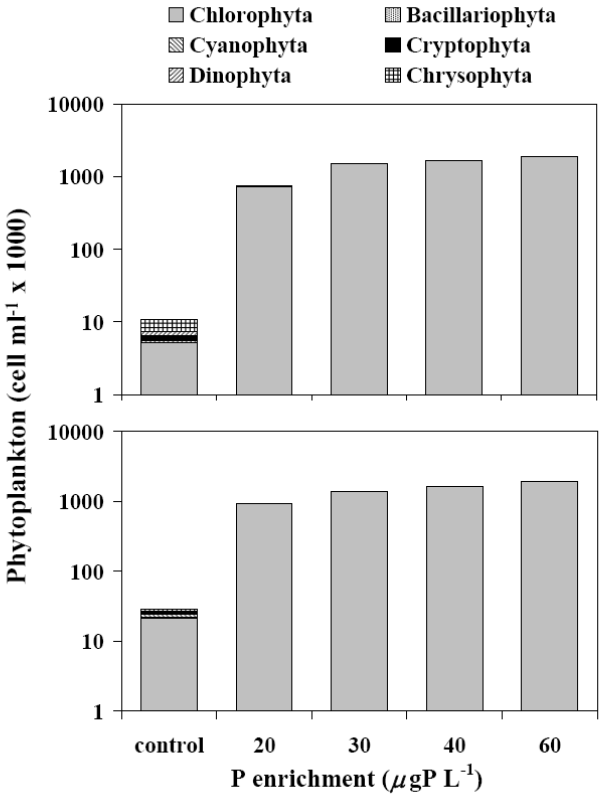

Figure 1. Taxonomic phytoplankton abundance in unenriched (control) and P-enriched enclosures under –UVR and +UVR after 30 days of incubation. *Abundancia fitoplanctónica por taxones en los encerramientos no enriquecidos (control) y enriquecidos en P para –UVR y +UVR después de 30 días de incubación.*

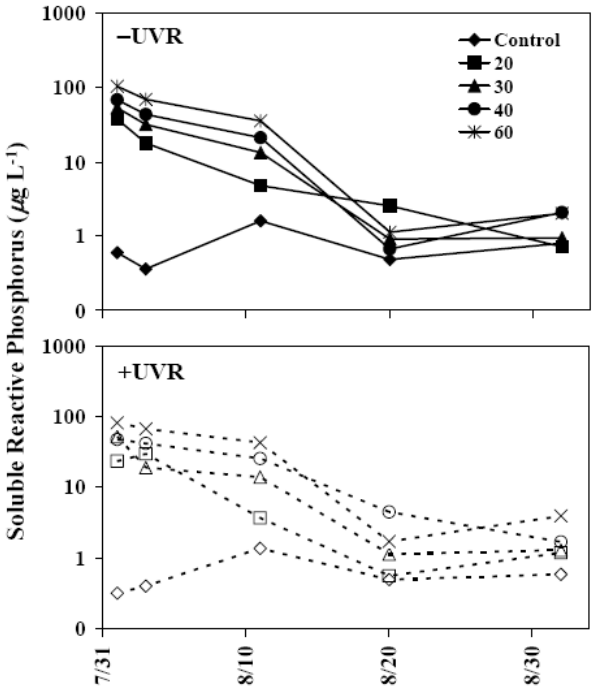

Figure 2. Changes in soluble reactive phosphorus as a function of the P-enrichment in the two UVR treatments. Solid symbols represent –UVR enclosures and open symbols represent +UVR enclosures. Data show means of three laboratory measurements. *Cambios en el fósforo reactivo soluble en función del enriquecimiento en P para ambos tratamientos de UVR. Los símbolos rellenos representan los encerramientos –UVR y los símbolos vacíos representan los encerramientos +UVR. Los datos muestran los promedios para cada tres medidas analíticas.*

Algal fatty acid content was more sensitive to P manipulation than to UVR (Fig. 3). Thus, P increased the fatty acid content linearly in terms of TFA and ω3-PUFA (Fig. 3a, c). The strength of this stimulation, however, varied between constituents, being much more pronounced for the ω3-PUFA [as shown by the over eight fold increase relative to the control (Fig. 3c) or the high slope of the regression between ω3-PUFA and P-enrichment (Table 1)]. For example, the absolute content of ω3-PUFA increased from 14 to 124 μg FA (mg C)$^{-1}$ in +UVR enclosures and from 7 to 81 μg FA (mg C)$^{-1}$ in the –UVR enclosures (data not shown). The strongest increases in FAs as a consequence of the enrichment with P were found in the concentrations of ω3-PUFA 18:3n-3 (α-linolenic acid, ALA) and 18:2n-6 (linoleic acid, LIN) which were 249-809% and 37-123% higher in the enriched treatments, respectively (Table 2; Fig. 4). The most abundant FA, however, was the saturated fatty acid (SFA) 16:0 that accounted for >25% of TFA in all enclosures or up to 123 μg FA (mg C)$^{-1}$ in the 60 μg P L^{-1}enclosure (Fig. 4). The SFA 18:0 was found in high concentrations in the control treatments, although it decreased after the addition of P and particularly in the 20 μg P L^{-1} and 30 μg P L^{-1} enclosures.

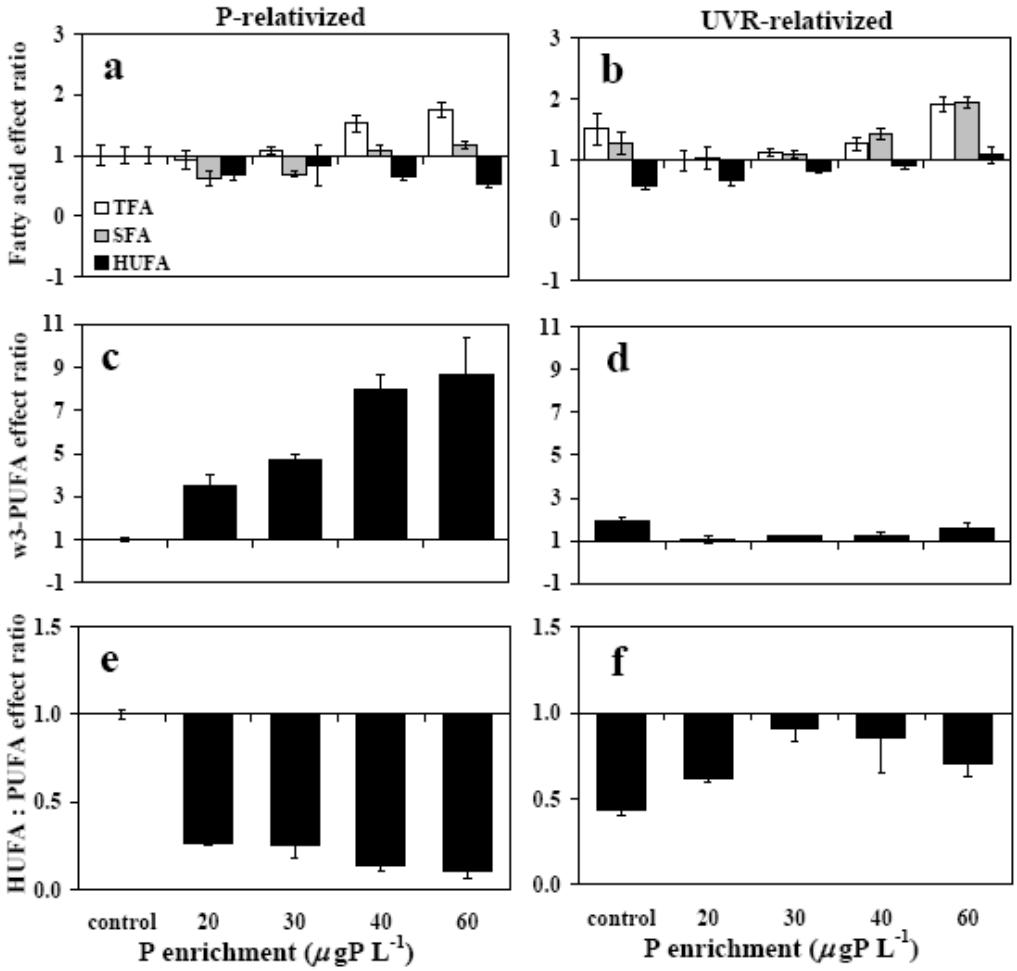
P-relativized
UVR-relativized
a
b
c
d
e
f
Fatty acid effect ratio
w3-PUFA effect ratio
HUFA : PUFA effect ratio
TFA
SFA
HUFA
control
20
30
40
60
P enrichment (μgP L-1)

Figure 3. Effects of P (left) and UVR × P (right) manipulations on seston in terms of (a, b) total, saturated and highly unsaturated fatty acids, (c, d) ω3-PUFA, and (e, f) HUFA:PUFA ratio in experimental enclosures. Response variables were specifically relativized to show P and UVR × P effects. P-relativized variables were calculated by dividing the replicate for each P treatment by the control replica (no P added) in +UVR enclosures. For the UVR × P effect, variables were first UVR-relativized by dividing the observation for each P level in +UVR by the respective control treatment replicate in –UVR and then regressed against P-enrichment (*see* Table 4 for linear regression parameters and Methods for further detail). A positive response was interpreted as stimulating the constituent production or ratio and a negative response as inhibiting the constituent production or ratio. Values represent the mean and standard deviation of three sampling dates. *Efectos del P (lado izquierdo) y de UVR × P (lado derecho) en el seston en términos de (a, b) ácidos grasos totales, saturados y altamente insaturados, (c, d) ω3-PUFA, y (e, f) la razón HUFA:PUFA en los encerramientos experimentales. Las variables respuesta fueron específicamente relativizadas para mostrar los efectos del P y de UVR × P. Las variables relativizadas en función del P fueron calculadas dividiendo para cada nivel de P en +UVR por la correspondiente réplica del tratamiento control en –UVR y luego regresionadas con respecto al enriquecimiento en P (*ver Table 4 *para conocer los parámetros de la regresión lineal y* Methods *para mayor detalle). Una respuesta positiva o negativa fue interpretada cómo estimuladora o inhibidora, respectivamente, de la producción del correspondiente componente o de la razón en cuestión. Los valores representan el promedio y la desviación estándar de los tres días de muestreo.*

Dependent variable (y)	Light treament	Linear regression			
		Slope	y-intercept	R^2	p-value
TFA [μg FA (mg C)$^{-1}$]	+UVR	**3.630**	**204.210**	**0.775**	**0.046**
	-UVR	1.290	196.910	0.335	0.306
SFA [μg FA (mg C)$^{-1}$]	+UVR	0.743	112.710	0.212	0.435
	-UVR	-0.300	110.310	0.269	0.370
ω3-PUFA [μg FA (mg C)$^{-1}$]	+UVR	**2.000**	**13.810**	**0.932**	**0.008**
	-UVR	**1.351**	**16.120**	**0.791**	**0.044**
HUFA [μg FA (mg C)$^{-1}$]	+UVR	**-0.050**	**6.847**	**0.792**	**0.043**
	-UVR	**-0.150**	**11.260**	**0.888**	**0.016**
HUFA:PUFA	+UVR	-0.318	17.476	0.719	0.069
	-UVR	-0.754	37.543	0.653	0.098
C:P	+UVR	-1.822	338.030	0.363	0.283
	-UVR	**-3.929**	**452.180**	**0.886**	**0.017**
pg P per cell	+UVR	-0.003	0.166	0.499	0.182
	-UVR	-0.002	0.158	0.489	0.189
C:N	+UVR	0.007	7.551	0.023	0.809
	-UVR	0.012	7.752	0.033	0.769
Chl *a*:C [μg Chl *a* (mg C)$^{-1}$]	+UVR	**0.679**	**32.130**	**0.956**	**0.004**
	-UVR	**0.767**	**28.684**	**0.863**	**0.022**

Table 1. Effects of P-enrichment, tested *via* regression analysis, on the biochemical and elemental composition of seston for each light treatment. Significant predictors are shown in bold. TFA, total fatty acids; SFA, saturated fatty acids; ω3-PUFA, ω3-polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids. *Efectos del enriquecimiento en P, testados* via *análisis de regresión, sobre la composición elemental y bioquímica del seston para cada tratamiento de luz. Los predictores significativos se muestran en negrita. TFA, ácidos grasos totales; SFA, ácidos grasos saturados;* ω*3-PUFA, ácidos grasos insaturados* ω*3; HUFA, ácidos grasos altamente insaturados.*

P-enrichment treatment (μg P L^{-1})	20		30		40		60	
Light treatment	+UVR	-UVR	+UVR	-UVR	+UVR	-UVR	+UVR	-UVR
SFA [μg FA (mg C^{-1})]	-37 (ns)	-22 (ns)	-31 (ns)	-19 *	7 (ns)	-4 (ns)	17 (ns)	-23 *
HUFA:PUFA	-74 **	-82 **	-74 **	-88 **	-86 **	-93 **	-90 **	-94 **
16:00	-23 (ns)	14 (ns)	-10 (ns)	17 (ns)	17 (ns)	43 (ns)	48 *	6 (ns)
16:4	234 **	(na)	322 ***	(na)	253 **	(na)	624 **	(na)
18:0	-52 *	-36 (ns)	-53 *	-50 *	-21 (ns)	-41 *	-25 *	-52 *
18:1n-9	1 (ns)	219 ***	-0.2 (ns)	168 **	-5 (ns)	135 *	22 (ns)	17 (ns)
18:2n-6	30 (ns)	156 ***	37 *	162 **	126 *	143 *	123 **	17 (ns)
18:3n-3	249 **	537 **	378 ***	671 **	487 **	1302 **	809 *	1050 **
20:4n-6	(na)	(na)	(na)	(na)	(na)	(na)	(na)	-94 *
C:P	51 *	18 (ns)	13 (ns)	-10 (ns)	4 (ns)	-26 (ns)	-31 **	-49 ***
pg P per cell	-91 **	-89 ***	-92 ***	-90 ***	-91 **	-89 ***	-87 ***	-84 ***
C:N	38 *	63 ***	38 *	47 **	28 *	33 **	11 (ns)	24 *

Table 2. Effects of the enrichment with P on seston biochemical and elemental composition for each light treatment. Numbers give the magnitude (%) and sign (-, inhibition; +, stimulation) the effect of each P concentration relative to the control (no P added). Only food quality variables that did not show a linear response to the enrichment with P (*see* Table 1) are shown here. Asterisks indicate the results of paired dependent *t*-test between the enriched treatments and the controls with no P added (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; na, not applicable). Abbreviations as in Table 1. *Efectos del enriquecimiento con P en la composición elemental y bioquímica del seston para cada tratamiento de luz. Los números proporcionan la magnitud (%) y el signo (-, inhibición; +, estimulación) el efecto de cada concentración de P en relación al control (sin P añadido). Sólo se muestran las variables de calidad de alimento que no mostraron una respuesta lineal con el enriquecimiento con P (*ver Table 1*). Los asteriscos indican los resultados de los* t*-test de muestras apareadas entre los tratamientos enriquecidos y los controles sin P añadido (ns, no significativo; *,* p < *0.05; **,* p < *0.01; ***,* p < *0.001; na, no aplicable). Las abreviaturas son como en* Table 1.

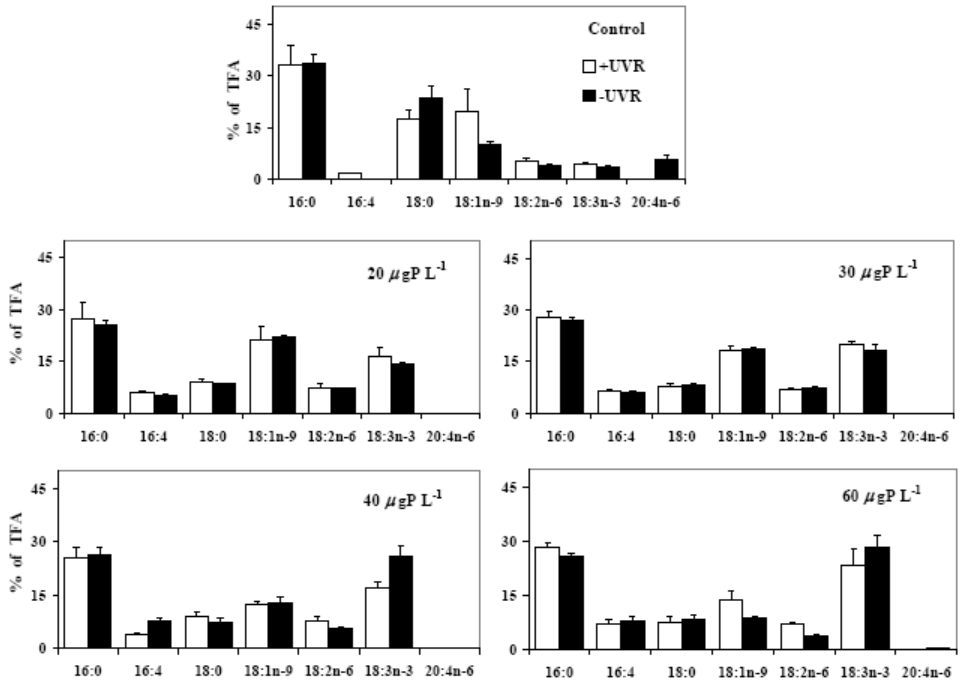

Figure 4. Effects of UV radiation and P manipulations on seston fatty acid composition. Values represent means from the three sampling dates. Fatty acids accounting for less than 5% of TFA in all treatments were not included. *Efectos de la manipulación con P y radiación UV sobre la composición en ácidos grasos del seston. Los valores representan promedios de los tres días de muestreo. Los ácidos grasos que se encuentran por debajo del 5% de TFA en todos los tratamientos no fueron incluidos.*

Most seston FAs (16:0, 16:4, 18:1n-9, 18:2n-6 and 18:3n-3) were also significantly enhanced by UVR although to a lesser extent than by P, particularly at the ends of the P gradient, *i.e.* in the controls with no P added and the most enriched enclosures (Fig. 4; Table 3). The strongest differences between UVR treatments were found in the concentrations of 18:1n-9 and 18:2n-6, which were over 200% higher in +UVR relative to –UVR in the 60 μg P L^{-1} enclosures (Table 3; Fig. 4). In contrast, HUFA was negatively affected by UVR and the addition of P. Thus, arachidonic acid [ARA (20:4n6)] reached 79% of HUFA and >5% of TFA only in the control in –UVR, but was not detected in the presence of UVR or after the addition of P (Fig. 4). Such a decline, together with the general increase in ω3-PUFA (Fig. 3c), was responsible for the strong decline in the HUFA:PUFA ratios with P (all *t*-test, $p < 0.01$; Table 2; Fig. 3e). Significant UVR × P effects were found on HUFA content (slope of the regression line: $F_{1, 6} = 7.87$, $p = 0.031$; Table 4). These effects were antagonistic, with a diminished effect of UVR as the enrichment with P increased (Fig. 3b).

P-enrichment treatment (μg P L^{-1})	Control	20	30	40	60
TFA [μg FA (mg C)$^{-1}$]	50 *	3 (ns)	11 (ns)	25 (ns)	90 **
SFA [μg FA (mg C)$^{-1}$]	26 *	8 (ns)	8 (ns)	41 *	93 **
ω3-PUFA [(μg FA mg C)$^{-1}$]	96 *	9 (ns)	19 *	22 *	54 *
HUFA [μg FA mg C)$^{-1}$]	-42 *	-35 **	-15 (ns)	-11 (ns)	12 (ns)
HUFA:PUFA	-57 **	-36 **	-8 (ns)	-20 (ns)	-28 *
16:00	48 *	3 (ns)	14 (ns)	22 (ns)	108 **
16:04	(na)	4 (ns)	23 *	-37 (ns)	67 **
18:0	13 (ns)	-15 (ns)	6 (ns)	53 (ns)	77 (ns)
18:1n-9	196 *	-6 (ns)	10 (ns)	19 (ns)	208 *
18:2n-6	94 *	-2 (ns)	2 (ns)	81 (ns)	269 **
18:3n-3	97 **	8 (ns)	22 **	-18 (ns)	55 (ns)
20:4n-6	(na)	(na)	(na)	(na)	(na)
C:P	-34 *	-12 (ns)	-7 (ns)	0.4 (ns)	-8 (ns)
pg P per cell	27 *	4 (ns)	3 (ns)	-2 (ns)	0.4 (ns)
C:N	4 (ns)	-12 *	-2 (ns)	0.3 (ns)	-7 (ns)
Chl *a*:C	27 **	4 *	-1 (ns)	-10 *	5 (ns)

Table 3. Effect of UVR on seston biochemical and elemental composition. Numbers give the magnitude (%) and sign (-, inhibition; +, stimulation) the UVR effects. Asterisks indicate the results of paired dependent *t*-test between +UVR and –UVR treatments for each P level (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Abbreviations as in Table 1. *Efecto de UVR en la composición elemental y bioquímica. Los números proporcionan la magnitud (%) y el símbolo (-, inhibición; +, estimulación) los efectos de UVR. Los asteriscos indican los resultados del* t-*test de muestras apareadas entre los tratamientos +UVR y –UVR para cada nivel de P (ns, no significativo; *,* $p < 0.05$*; **,* $p < 0.01$*; ***,* $p < 0.001$*). Las abreviaturas son como en* Table 1.

Variables	Interactive UVR × P effects					
	Analysis of covariance		Linear regression (UVR-relativized variables)			
	$F_{1,6}$	p-value	Slope	y-Intercept	R^2	p-value
TFA [μg FA (mg C)$^{-1}$]	–	–	0.007	1.130	0.202	0.447
SFA [μg FA (mg C)$^{-1}$]	–	–	0.012	0.980	0.541	0.157
ω3-PUFA [μg FA (mg C)$^{-1}$]	1.633	0.249	–	–	–	–
HUFA [μg FA mg C)$^{-1}$]	**7.867**	**0.031**	–	–	–	–
HUFA:PUFA	–	–	0.005	0.552	0.360	0.285
C:P	–	–	**0.005**	**0.732**	**0.870**	**0.050**
pg P per cell	–	–	**-0.004**	**1.173**	**0.678**	**0.045**
C:N	–	–	-0.001	0.992	0.103	0.600
Chl *a*:C [μg Chl *a* (mg C)$^{-1}$]	0.202	0.669	–	–	–	–

Table 4. Interactive UVR × P effects on seston biochemical and elemental composition. Interactive effects in variables showing linearity with P-enrichment for each light treatment were assessed by analysis of covariance, while variables not showing linearity were tested *via* regression analysis between UVR-relativized variables and P-enrichment (*see* Methods for further explanation). Significant predictors are shown in bold. *Efectos interactivos UVR × P en la composición elemental y bioquímica del seston. Los efectos interactivos en las variables que mostraron linealidad con el enriquecimiento en P para cada tratamiento de luz fueron evaluadas por análisis de la covarianza, mientras que las variables que no mostraron linealidad fueron testadas* via *análisis de regresión de las variables relativizadas por UVR con el enriquecimiento en P (*ver Methods *para mayor detalle). Los predictores significativos se muestran en negrita.*

P-enrichment did not cause a decrease in seston C:P at a linear rate in +UVR enclosures (Table 1; Fig. 5a). The relatively low C:P of 273 in the control and the post-bloom depletion of P in the least P-enriched enclosures were responsible for this result. Thus, seston C:P ratio was 360 in the 20 μg P L^{-1} (~30% higher relative to the control), decreased progressively with increasing P concentration, and achieved values below the control of 180 exclusively in the 60 μg P L^{-1} enclosure (Table 2; Fig. 5; note that only relativized response variables are shown). In contrast, UVR reduced mean seston C:P in all treatments and, particularly, in enclosures receiving ≤30 μg P L^{-1}, where seston C:P in the control was 34% lower in the +UVR enclosure than in the –UVR enclosure (Table 3; Fig. 5b). In addition, UVR and P showed an interactive effect on seston C:P (Table 4) which resulted in a reduced effect of UVR decreasing seston C:P at high P concentrations (Fig. 5b). P and UVR effects on P cell quota differed. While P-enrichment strongly decreased P cell quota (Table 3; Fig. 5c), the effect of UVR on P cell quota was significant and positive only in the control with no P added

(Table 3; Fig. 5d). As a consequence, a significant UVR × P effect was found on P cell quota indicating a stimulatory effect of UVR on P uptake that attenuated at high P-enrichment levels (Table 4).

Seston C:N ratios generally increased due to enrichment with P, and only remained unaltered relative to the control in the 60 μg P L^{-1} (Fig. 5e; Table 2). Also, seston C:N was not affected by UVR, except for the 20 μg P L^{-1} enclosure where this ratio was lower under UVR (from 9.9 ± 0.1 to 8.7 ± 0.3 in –UVR *vs.* +UVR enclosures; Table 3; Fig. 5f).

Chl *a*:C ratios strongly increased across the P gradient (Fig. 5g; Table 1), and was generally higher in the enclosures receiving UVR (Fig. 5h), and particularly in the control where UVR increased mean Chl *a*:C by >25% (Table 3).

Discussion

In this study we examined how a prolonged exposure to UVR affected phytoplankton biochemical and elemental composition with increasing pulses of P, the latter resembling natural inputs to this lake carried by winds from the Sahara (Morales-Baquero *et al.*, 2006). Our results contribute to our knowledge of the simultaneous effects of multiple stressors on the biochemical composition of primary producers. We found that P and UVR are important determinants of seston elemental and biochemical composition. While P was the main driver for most seston biochemical parameters, however, the precise effect of UVR depended strongly on the availability of P. Thus, consistently with an interactive UVR × P effect, the role of UVR in reducing HUFA and C:P ratios was most pronounced under oligotrophic conditions but vanished as P concentration increased.

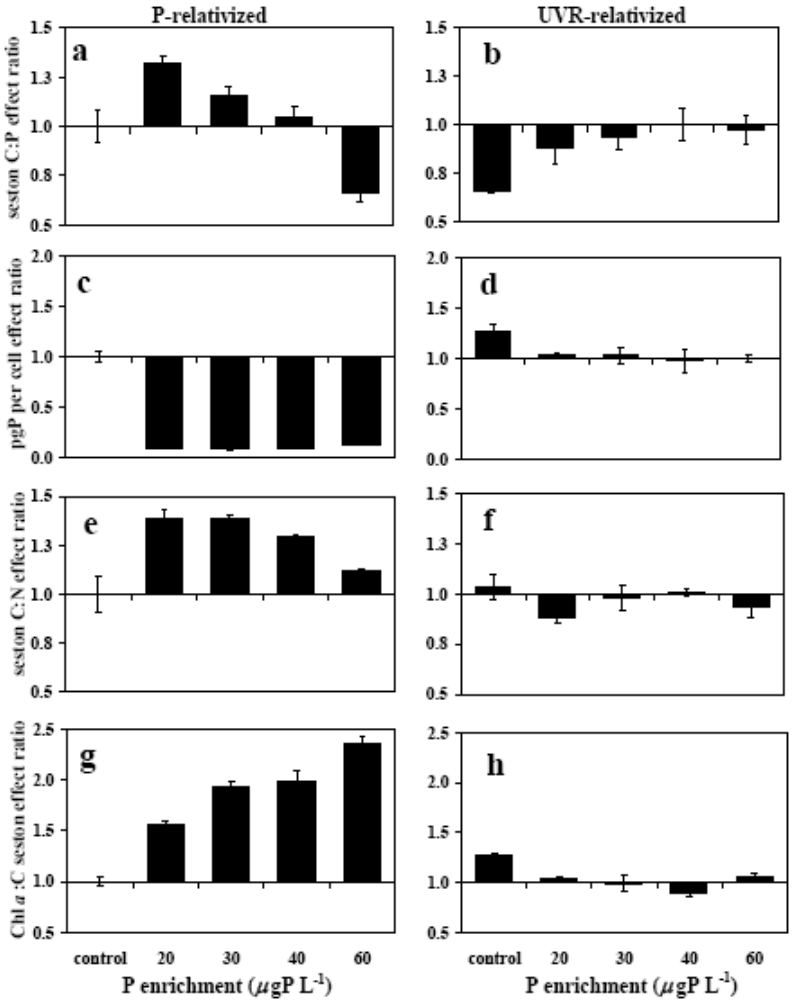

Figure 5. Effects of P (left) and UVR × P (right) manipulations on seston in terms of (a, b) seston C:P ratio, (c, d) P cell quota, (e, f) seston C:N ratio, and (g, h) Chl *a*:C in experimental enclosures. *See* Fig. 3 text for further details. *Efectos de la manipulación con P (lado izquierdo) y UVR × P (lado derecho) en el seston en términos de (a, b) razón C:P del seston, (c, d) cuota celular de P, (e, f) razón C:N del seston, y (g, h) razón Chl* a*:C en los encerramientos experimentales.* Ver *texto de Fig. 3 para mayor detalle.*

Single and interactive P × UVR effects on fatty acid composition

A seston fatty acid profile dominated by palmitic acid (16:0), as well as by the monounsaturated oleic acid (18:1n-9) and α-linolenic acid (18:3n-3), agrees well with expectations for a Chlorophyte dominated phytoplankton community (Brett *et al.*, 2006). However, seston fatty acid composition varied as a consequence of experimental manipulation. Thus, we found strong effects of P-enrichment on algal fatty acid composition, whereas the effects of UVR *per se* and its interaction with P were generally less pronounced. Notably, ω3-PUFA increased eight-fold in response to P-enrichment which corroborates observations for high arctic systems (Elser *et al.*, 2001; Leu *et al.*, 2006*b*). In particular, the higher concentration of ω3-PUFA was largely explained by the synthesis of C18 PUFAs and, particularly, of ALA. As discussed by Leu *et al.* 2006*a*, the pathway for the biosynthesis of C18 PUFAs starts at the SFA 18:0. Probably the enrichment with P caused the activation of this biosynthetic pathway towards the formation of 18C PUFAs (after the insertion of double bonds) at the expenses of the precursor 18:0.

Interestingly, the content of ω3-PUFA was also enhanced by UVR, although only at the ends of the experimental P gradient. Similar increments in ω3-PUFA due to UVR have been reported previously, but observations have not always being consistent among studies (Goes *et al*., 1994; Skerratt *et al.*, 1998). Although the proximate reasons for the increase in PUFA synthesis due to UVR are not obvious, it has been suggested that UVR could induce gene expression of desaturase enzymes responsible of the biosynthesis of C18 PUFAs (Leu *et al.*, 2006*a*). Ultimately, the increase in structural lipids could indicate enhanced cell growth and metabolism, benefiting the acclimation of UVR-stressed cells.

The fact that the HUFA ARA was found in substantial amounts in the control enclosure with no UVR, but disappeared when enclosures were P enriched, agrees with previous findings reporting an active accumulation of C in the form of ARA deposited in triacylglycerols under nutrient deprivation in unicellular chlorophytes (Merzlyak *et al.*, 2007). Further, it is generally accepted

that algae acclimate to stressful environments by alteration of their lipid composition and some species actively synthesize triacylglycerols (TGA) as an efficient C sink (Guschina & Harwood, 2006). The inhibition of ARA by UVR in the control enclosure can be explained by the detrimental effect of UVR in peroxidising HUFA rather than the more resistant short-chain PUFAs (Girotti, 2001). This view is supported by the finding of a higher content of 16:0, 16:4 or 18:1n-9 in the control in +UVR relative to –UVR enclosures.

As expected, the finding of an interactive antagonistic effect of UVR and P on the content of HUFA indicated that the detrimental effect of UVR in reducing HUFA and HUFA:PUFA was more intense at low P concentration. This is characteristic of oligotrophic conditions, under which UVR can penetrate deeper before extinction and less algae-shading effect is expected.

Single and interactive P × UVR effects on stoichiometric composition

The finding of higher seston C:P ratios in the low P-enrichment enclosures relative to the control after thirty days of incubations was not surprising given: (1) the intensive depletion of P in P-enriched enclosures after the algal bloom (as reflected by the mark reduction in P cell quota; Fig. 5c), and (2) the low seston C:P ratio in the unenriched +UVR treatment, which resembles those of lake La Caldera under natural conditions (181 and 115 are average seston C:P ratios for 30 samples dates during 1996 and 1997; data from Villar-Argaiz *et al.* (2002). The results of Xenopoulos *et al.* (2002) and, more recently, Carrillo *et al.* (2008) and Hessen *et al.* (2008) suggest that UVR has a key role in reducing seston C:P ratios. The finding in this study of a higher P cell quota in the control in +UVR relative to –UVR is consistent with previous work that relates the decrease in seston C:P in the presence of UVR with the increased P acquisition by algae (Leu *et al.*, 2006*a*; Carrillo *et al.*, 2008, Hessen *et al.*, 2008), although enhanced photosynthetic C release by algae can also have an important effect (Carrillo *et al.* 2008). The extra demands for P could be due to the high energetic cost (and consequently demand for ATP) associated with the bioconversion of ALA and

other PUFAs to HUFA through the elongation and desaturation processes (Kanawaza *et al.*, 1979; Brett & Müller-Navarra, 1997), or with other beneficial photo-mediated mechanisms such as nucleotide repair or enhanced protein biosynthesis (Hessen *et al.*, 2008). Together these observations are in line with previous work reporting that phytoplankton sensitivity to UVR is modulated by their P nutritional deficiency (Aubriot *et al.*, 2004).

As for HUFA, the fact that the effect of UVR in decreasing seston C:P was manifest at low P concentration and vanished as P load increased implied an interactive UVR × P effect, a result previously shown in three of five bioassays performed in two lakes at the Experimental Lake Area in Canada (Xenopoulos *et al.*, 2002). Therefore, P and UVR affected phytoplankton elemental composition by simultaneously decreasing C:P ratios (at least at the high P loads in this study). Thus, UVR might be of prime importance for phytoplankton elemental and biochemical composition in pristine waters where UVR penetrates more deeply (Fleischmann, 1989), and could help to explain deviations from the Redfield ratio towards lower C:P (Medina-Sánchez *et al.*, 2006).

Single and interactive P × UVR effects on Chl a*:C ratio*

Our observation that Chl *a*:C ratio strongly increased across the P gradient is supported by previous findings for green algae cultures (Hessen *et al.*, 2002). Further, UVR enhanced Chl *a*:C ratios, particularly in the controls, a result consistent with observations of strong C:Chl *a* disruptions caused by UVR (Xenopoulos *et al.*, 2002). Previous work has clearly shown that chlorophyll and C content depends strongly on the growth of the autotroph concerned (Healey & Hendzel, 1979), for which solar radiation and nutrients are essential factors. Therefore, responses to UVR and P could lead to multiple effects on photosynthetic C fixation/release (Carrillo *et al.*, 2008) and pigment synthesis/photolysis processes, by operating at different rates, could lead to variable Chl *a*:C ratios. It is logical to assume that a high Chl *a* per unit of C is characteristic of healthy algae and makes then a better food quality for consumers.

Although chlorophyll is chiefly used as an indicator of food quantity, the pronounced changes in Chl *a*:C observed in this study suggest that this factor may have pronounced consequences for zooplankton growth.

Implications for food quality

It is widely accepted that food quantity places the largest constraint on the growth of zooplankters in oligotrophic systems, and that food quality is more important as systems become more productive (Sterner, 1997; Persson *et al.*, 2007), but *see* Becker & Boersma (2003). This indeed might be the case in the present experiment, where food was well below the incipient limiting level (ILL) for most zooplankters in control enclosures, but well above these thresholds (Villar-Argaiz *et al.*, 2002) in the enriched enclosures.

With respect to the biochemical composition of seston, the strong synthesis of ALA due to P-enrichment may have implications for food quality, due to the known ability of herbivores to convert this fatty acid to long-chain polyunsaturated fatty acids (HUFAs) through elongation and desaturation processes (Brett & Müller-Navarra, 1997). Further, the relevance of ALA content in seston for zooplankton growth has been suggested based on field correlations between seston and zooplankton FA content (Sushchik *et al.*, 2003) or bioassays using natural seston or ALA-enriched algae as a food source for zooplankton growth (Wacker & Von Elert, 2001; Park *et al.*, 2003; Von Elert, 2002). However, simply because P and also UVR stimulated the content of ω3-PUFA, it may not be safe to conclude that these factors enhance food quality for zooplankton (Leu *et al.*, 2006*a*). In fact, our results indicate that the addition of P was detrimental to the content of HUFA, a well known essential group of biochemicals in animals (Müller-Navarra *et al.*, 2000). This is consistent with observations by Müller-Navarra *et al.* (2004) of more nutritious algae for zooplankton at low P concentration, due to their higher HUFA content per unit of C.

The question then rises as to which factor exerts the most relevant role determining food quality for herbivore consumers? Whether changes in the potential food quality of autotrophs, due to the simultaneous effect of P and UVR, add or offset each other is not a trivial question. For example, the beneficial effect of UVR or P in increasing ω3-PUFA and decreasing C:P could be offset by the detrimental effect of UVR peroxidising HUFA. Further, the diversity of potentially limiting substances to zooplankton is still highly debated (Sterner & Schulz, 1998; Ferrao *et al.*, 2007), and particularly since different zooplankton taxa might be limited by different characteristics of the food (Boersma & Stelzer, 2000). The strength of this food limitation may also vary greatly with zooplankton ontogeny (DeMott *et al.*, 2001) and lake trophic state (Persson *et al.*, 2007). Finally, there is covariation among seston food quality variables, making discrimination among the key food traits responsible for zooplankton nutritional limitation in nature far from straightforward. For example, a tight correlation between Chl *a*:C and ω3-PUFA ($r^2 = 0.82$, $p < 0.001$, df = 1, 8) was found in this study.

In summary, P addition had a positive effect on the nutritional quality of algae in terms of TFA, ω3-PUFA and Chl *a*:C ratios, negative effects on HUFA, HUFA:PUFA and C:N ratios, and effects on seston C:P that were specific to the concentration of P. Considering the magnitude of the responses and the ability of zooplankton to convert ω3-PUFA to HUFA, we suggest that the strong stimulation of ω3-PUFA may be the most relevant enrichment effect improving the nutritional quality of seston as food source for consumers in this study. The response to UVR depended, however, on the degree of P-enrichment. Overall, UVR had minor effects enhancing food quality in terms of PUFA at both ends of the P gradient applied, but adversely affected HUFA content by lowering, as a consequence, the HUFA:PUFA ratio. Nonetheless, this potentially detrimental effect of UVR could be counteracted by the simultaneously beneficial effect of decreasing seston C:P.

Global change has strong effects, not only on the amount of UV reaching the Earth (Shindell *et al.*, 1998; McKenzie *et al.*, 2007) including mid-latitudes

(Seckmeyer & McKenzie, 1992; KeilJakson & Hort, 2007), but also on the frequency and intensity of P inputs from the atmosphere reaching the Mediterranean region (Morales-Baquero *et al.*, 2006). Our results show interdependent and contradictory effects of UVR and P on the quality of algae for herbivore consumers. UVR has a predominant role in lowering seston C:P ratios (enhanced food quality) and HUFA content (decreased food quality) in low nutrient waters, but this effect vanishes as P load increases. This, in addition to food quantitative effects, could have consequences for the efficiency with which mass is transferred up the food web, particularly in highly oligotrophic systems.

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III

Effects of food quantity and quality, modified by ultraviolet radiation and nutrients, on consumers

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Abstract

In a 32-yr record in oligotrophic Lake La Caldera (Sierra Nevada, Spain) biomass of zooplankton was strongly correlated with precipitation, aerosol deposition intensity, and ultraviolet radiation (UVR). The food associated-effects of these factors for zooplankton growth were tested in field-laboratory experiments with the aim of separating the effects of food quantity from those of food quality at low food conditions, where there is good evidence to support the existence of food quality effects. Manipulation of nutrients generated a large food quantity gradient that exerted the strongest effect on zooplankton growth, with no significant role of UVR. Growth curves were fitted to a saturation function that reached a plateau at increasing seston levels of ca. 250, 500, and 1000 μg carbon (C) L^{-1} for the rotifer *Keratella cochlearis*, the copepod *Mixodiaptomus laciniatus*, and the cladoceran *Daphnia pulicaria*, respectively, and after which growth decreased in *Mixodiaptomus laciniatus*. Nutrients and to a lesser extent UVR also affected seston quality, which had a minor effect on zooplankton growth. *Keratella cochlearis* growth was strongly related to the phosphorus (P) content of seston, whereas *Mixodiaptomus laciniatus* and *Daphnia pulicaria* growth were positively correlated with a P-normalized ω3-polyunsaturated fatty acid index (ω3-PUFA:P). The increase in seston associated with more intense and frequent atmospheric depositions would adversely affect copepods, but improve growth of

C-limited cladocerans and P-limited rotifers in pristine ecosystems of the Mediterranean region.

Resumen

Para un registro de 32 años del lago oligotrófico La Caldera (Sierra Nevada, España), la biomasa del zooplancton se correlaciona fuertemente con la precipitación, la intensidad de deposición de aerosoles, y la radiación ultravioleta (UVR). Los efectos asociados al alimento de estos factores fueron evaluados mediante experimentos de campo y laboratorio con el objetivo de separar los efectos de cantidad de aquellos de calidad a bajas concentraciones de alimento, en donde hay evidencias consistentes que confirman la existencia de efectos de la calidad. La adición de nutrientes generó un amplio gradiente de cantidad de alimento, responsable de ejercer el mayor efecto sobre el crecimiento del zooplancton, mientras que no se observó ningún tipo de efecto debido a UVR. Las curvas de crecimiento se ajustaron a funciones de saturación que alcanzaban una meseta a niveles crecientes de seston a 250, 500, y 1000 μg *carbono (C)* L^{-1} *para el rotífero* Keratella cochlearis*, el copépodo* Mixodiaptomus laciniatus*, y el cladócero* Daphnia pulicaria*, respectivamente, tras la cual el crecimiento se redujo para* Mixodiaptomus laciniatus*. Los nutrientes y en menor grado UVR también afectaron a la calidad de alimento, que tuvo un menor efecto en el crecimiento del zooplancton. El crecimiento de* Keratella cochlearis *estuvo fuertemente relacionado con el contenido en fósforo (P) del seston, mientras que el crecimiento de* Mixodiaptomus laciniatus *y* Daphnia pulicaria *estuvo positivamente correlacionado con el índice de ácidos grasos poliinsaturados* ω*3 normalizado al P (*ω*3-PUFA:P). El aumento del seston asociado con la más intensa y frecuente deposición de aerosoles atmosféricos afectaría adversamente a los copépodos, pero mejoraría el crecimiento de los cladóceros limitados en C y el de rotíferos limitados en P en ecosistemas no perturbados de la región mediterránea.*

Introduction

A considerable amount of research has focused on characterizing the seasonal succession of plankton in aquatic ecosystems (Sommer *et al.*, 1986). Still, zooplankton dynamics is among the most poorly predicted component of planktonic systems (Zhao *et al.*, 2008). Typically the dominance of copepods is a clear established pattern in highly oligotrophic systems including oceans (Villar-Argaiz *et al.*, 2001; Nuwer *et al.*, 2008), while the relative importance of other functional groups (*e.g.*, cladocera, rotifera) increases in meso- and eutrophic systems (Carney & Elser, 1990). Although many attributes have been identified to qualitatively affect zooplankton succession (temperature, predation rates, etc.), food availability has been identified as a major regulatory factor of the zooplankton growth (Sterner & Elser, 2002). However, phytoplankton typically undergoes pronounced seasonal variations and often shows dramatic responses to external factors, which alter their quantity and quality as a food source for their herbivorous consumers.

With the recent surge of interest in global change effects, a crucial question is how induced changes in primary producers will affect herbivorous consumers *via* alteration in the quantity and quality of their food. Although both food quantity and quality have been shown to be important regulators of herbivore growth (Sterner & Elser, 2002), it is not yet clear which of these factors places a greater constraint on carbon (C) transfer efficiency across the primary producer-consumer interface (Hessen, 2008). Thus, most studies make no distinction between their effects, estimate them by using empirical models (Persson *et al.*, 2007) or indirectly by comparing the nutritional inadequacy of seston for zooplankton with a high-quality algal food (Müller-Navarra & Lampert, 1996). Although most studies frequently investigate dietary effect of a single stressor, it has long been recognized that more than just one factor can contribute to differences in zooplankton growth in nature (DeMott *et al.*, 2001). In addition, much of what is known about zooplankton nutrition comes from laboratory feeding experiments, where zooplankton species with high somatic growth rates (primarily belonging to the genus *Daphnia*) are fed cultures of phytoplankton.

There is, therefore, an urgent need for field studies to simultaneously deal with food quantity and quality constraints for different taxonomical groups that may add realisms to the contested debate on the mechanisms behind zooplankton nutrient limitation and succession in nature (Sterner & Schulz, 1998; Hessen, 2008).

Solar radiation and nutrient supply are two major ecological factors governing the amount of solar energy fixed as autotrophs and their quality as a food source for their grazers (Urabe *et al.*, 2002). Because solar radiation and nutrient supply are prone to the effects of global stressors, any alteration in either or both of these factors can strongly affect the efficiency with which energy is transferred across the producer-consumer interface. While there is ample evidence for food quantity alterations in terms of reduced primary production under ultraviolet radiation (UVR) exposure (Carrillo *et al.*, 2002), evidence for changes in the biochemical composition of food for herbivorous consumers is still sparse and frequently ambiguous. Thus, UV radiation has been shown to simultaneously decrease the C:phosphorus (P) (C:P) ratio and inhibit the synthesis of essential long-chained fatty acids in seston (suspended particulate matter), resulting in opposite effects on the quality of algae as a food source for zooplankton (Leu *et al.*, 2006; Villar-Argaiz *et al.*, 2009).

In addition, the interplay of multiple factors may act to amplify or reduce the effects of a given climate-forcing factor on ecosystems. For example, increased aerosol (particulate matter in the atmosphere) loading into many ecosystems can strongly interact with the effects that UVR may exert on species and organisms (Carrillo *et al.*, 2008). Although predictions for changes in overall atmospheric aerosols amounts are unreliable at global scales (IPCC, 2007), expectations for increased loading of aerosols seems to be a well established pattern in the Mediterranean region (Santese *et al.*, 2007). Because the composition of aerosol particles depends on their source, wind-blown aerosol from the Sahara desert, rich in phosphorus (P) (Morales-Baquero *et al.*, 2006), is considered to be a large contributor in the nutrient budget of neighboring aquatic

(Ridame & Guieu, 2002) and terrestrial Mediterranean ecosystems (Ávila & Peñuelas, 1999).

A recent study showed how the combination of higher atmospheric depositions and the extreme UVR levels characteristic of high altitudes can strongly affect the strength of phytoplankton-zooplankton coupling in a high mountain lake (Bullejos *et al.*, 2010). The mechanisms behind these effects are however difficult to discern, as both quantity and quality of food for herbivorous grazers are simultaneously altered by these global factors. The goals of this study are to examine the effects of climatic factors on the long-term dynamics of zooplankton in a high altitude lake, and to test the effects of nutrients mimicking atmospheric inputs and UVR on the growth of zooplankton *via* alteration in their food quantity and quality using coupled field and laboratory experiments. Our experiments comprised two steps. First, we exposed seston to the effects of UVR at increasing levels of nutrients to create a gradient in the quantity and quality of the seston, using field mesocosms in a high-mountain lake. Second, the nutritional suitability of the raised food was specifically tested in the laboratory to differentiate between the effects of food quantity and quality on the growth of three coexisting zooplankton species with contrastingly different life history traits: The calanoid copepod *Mixodiaptomus laciniatus*, the cladoceran *Daphnia pulicaria* and the rotifer *Keratella cochlearis*.

Methods

Study site

La Caldera is a small, relatively shallow (14 m maximum depth), alpine lake located above the tree line in Sierra Nevada Mountains of Southern Spain at an elevation of 3050 m. The lake is frequently ice-free from late June to mid November and water temperature during this time ranges from 4ºC after the thaw to a maximum of 15ºC in midsummer. The lake is fishless, lacks littoral vegetation and lake water is highly transparent (Secchi disk visibility reaching

maximum depth). Measurements of UVR and photosynthetic active radiation (PAR) on different sampling dates during the experimental field period in 2003 showed intense radiant energy at lake surface [PAR (400-700 nm) ~1000 W m^2, UVA (315-400 nm) ~100 W m^{-2}, and UVB (280-315 nm) ~2 W m^{-2}; LI-8000 spectroradiometer (LI-COR)], and high UVR penetration to several meters depth [*e.g.*, 50% UVB reaches 3.5 m, and 50% UVA reaches 6.0 m; values are means for six dates in 2003; details of spectral transmittance are provided in Carrillo *et al.* (2008)].

The dissolved organic C concentration is below 1 mg C L^{-1}. The quantity of available food for the zooplankton assemblage from bacteria and algae biomass conversions is frequently <100 μg C L^{-1} (Villar-Argaiz *et al.*, 2001) and the calanoid *Mixodiaptomus laciniatus* dominates the zooplankton community throughout the ice-free period (>90% in biomass).

Long-term zooplankton data series

Zooplankton was collected over different intervals (from two-day to month intervals) during the ice-free period in Lake La Caldera since 1975. Although the sampling program showed some disruptions, each decade included from two to six sampled-years. Samples were collected at a maximum depth station after sieving 18 or 24 L of water from different depths through a 40 μm mesh, and preserved in 4% formaldehyde. All taxa were identified to species level under an inverted microscope. Analysis protocols and original data are fully reported elsewhere (Cruz-Pizarro, 1981; Carrillo *et al.*, 1995; Villar-Argaiz *et al.*, 2001).

Remote sensing and climatic data series

Previous work in this as well as other high mountain lakes established that most nutrients enter the lake with snowmelt and *via* atmospheric processes (Psenner, 1999; Villar-Argaiz *et al.*, 2001). Both dry (aerosols) and wet

(precipitation) depositions have been recognized as major sources of nutrients particularly to low productivity ecosystems (Morales-Baquero *et al.*, 2006). We used the Total Ozone Mapping Spectrometer-Aerosol Index (TOMS-AI) developed by the Ozone Processing Team (National Aeronautics and Space Administration – Goddard Space Flight Center) as a proxy for dry deposition. It has been shown that TOMS-AI is well correlated to the amount of particulate matter and total P (TP) linked to dry atmospheric deposition collected at a station near the studied lake (Morales-Baquero *et al.*, 2006), and previous work in this lake emphasized that atmospheric depositions constitute readily available nutrient sources for plankton (Villar-Argaiz *et al.*, 2002). A TOMS-AI value >0.5 was considered to represent a deposition event, and the annual frequency of these events was calculated as the sum of all days for a given year showing a TOMS-AI value >0.5. We used annual averages of weekly TOMS-AI data given for 37.5ºN, 2.5-3.5ºW (coordinates integrating most high mountain lakes located in Sierra Nevada) as a measure of intensity of aerosol deposition (original data at http://ozoneaq.gsfc.nasa.gov). Total precipitation (rainfall and snowfall) was obtained from annual averages of weekly-collected samples at nearby meteorological station in Trevélez (Sierra Nevada) continuously measured by the *Agencia Estatal de Meteorología* (AEMET; original data at http://www.aemet.es). UV irradiances were obtained by using 325 nm wavelength data from the above NASA satellites and for the same geographic coordinates as for TOMS-AI data. This wavelength represented an intermediate value between the available UVA and UVB wavelengths (305, 310, 325, and 380 nm). UV irradiance data was restricted to the period corresponding to the annual average ice-free season duration (from 01 June to 15 November), and the resulting weekly time series was averaged over the years.

Field enclosures

The field experiment was conducted during the ice-free period in Lake La Caldera starting 01 August 2003. Lake water was pumped from 3 m depth into ten clear polyethylene enclosures (*Plásticos Andalucía*, Spain) that transmitted 90%

photosynthetic active radiation, 60% UVB and 75% UVA. Enclosures were closed at the bottom (1 m diameter, 7 m depth and 2.7 m^3 volume) and submerged with a top above the water to limit aerosol intrusions. Five of the enclosures, with polyethylene top, received natural solar radiation (+UVR treatment) and the other five (–UVR treatment) were covered with Plexiglas acrylic plastic sheets (Atohaas) that transmitted 90% PAR and completely blocked direct exposure to UV radiation (<390 nm). Additional Plexiglas sheets (1 m wide and 2 m long) were suspended below the water surrounding –UVR enclosures in order to avoid the incidence of oblique UV radiation. Optical properties of the polyethylene and Plexiglas cut-off filters used in the light treatments were verified before the experiment with a double-beam spectrophotometer (Perkin-Elmer Lambda 40, Perkin-Elmer). In both series of enclosures, phosphate (NaH_2PO_4) was added to create an increasing nutrient gradient of four concentrations (20, 30, 40, and 60 μg P L^{-1}) and inorganic nitrogen (N) (NH_4NO_3) was added to give a final N:P molar ratio of 30 according to concentrations of inorganic N:P ratios measured in the lake the day before the experiment. The addition of nutrients resembled natural P levels after large atmospheric depositions (Villar-Argaiz *et al.*, 2001) and the final N:P ratio of 30 was chosen to mimic the mean ratio found in atmospheric depositions (Morales-Baquero *et al.*, 2006). For each light treatment, an additional enclosure received no nutrients and served as a control to give a 2 (light) × 5 (nutrient) factorial design with one replicate. Each of the enclosures was used to raise the seston subsequently used as a food source to carry out fully replicated zooplankton growth assays in the laboratory (*see below*). After P addition and before taking samples, the water in mesocosms was vigorously mixed with a plastic bucket to avoid problems associated with the patchy distribution of organisms. Enclosures were sampled at the start of the experiment, day 3 and at 7-10 day intervals to measure changes in seston C and C:P ratios. After thirty days of incubations when significant differences in the quantity and quality of seston were detected, water from each of the ten enclosures was collected, filtered through a 40 μm net to remove zooplankton, and transported under cold and dark conditions to the laboratory.

Zooplankton growth bioassays

To discriminate between regulation of zooplankton growth *via* food quantity *vs.* quality we ran experiments in the laboratory using the *in situ* raised seston suspensions: Seston at increasing concentrations and identical qualities (hereafter food quantity bioassays) and seston of different food qualities at identical low quantities (hereafter food quality bioassays) (Fig. 1). For the food quantity bioassays we created two gradients of seston concentrations from the most enriched enclosures (60 μg P L^{-1} +UVR and –UVR enclosures) by mixtures of natural seston and 0.2 μm-prefiltered water from the same enclosure. For each UVR enclosure, four treatments were run simultaneously in which 6.25%, 12.5%, 25%, and 50% water with natural seston (filtered through 40 μm) was mixed with 0.2 μm-prefiltered water from the same enclosure (dilution treatments 1/16, 1/8, 1/4, and 1/2, respectively; Fig. 1). An additional treatment with undiluted seston was run (treatment 1). Experimental food thus consisted of a series of two food quantity gradients (from each UVR treatment), each with the same seston quality as it came from the same enclosure, but different quantity.

For the food quality bioassays, 40 μm filtered water from each of the P-enriched enclosures was diluted with 0.2 μm-prefiltered water from the same enclosure in the laboratory to achieve identical food C concentrations to those in the nutrient-free control enclosures. For this purpose, fluorimetric measurements of chlorophyll *a* (Chl *a*) *in vivo* (excitation wavelength of 430 nm and emission wavelength of 663 nm) were related to C content based on C-fluorescence calibration curves for each of the 10 enclosures (Perkin-Elmer Model LS 55) – the regression coefficients of all these regressions being > 0.95. No differences in seston abundance in terms of total C content were observed among treatments after dilution (all *t*-test, df = 4, $p > 0.05$). Experimental food thus consisted of seston with the same quantity, but different quality raised under the different enclosures. By monitoring zooplankton growth under standardized conditions in the laboratory, the well-established direct negative effects of UV radiation on zooplankton (Williamson *et al.*, 2001) were excluded.

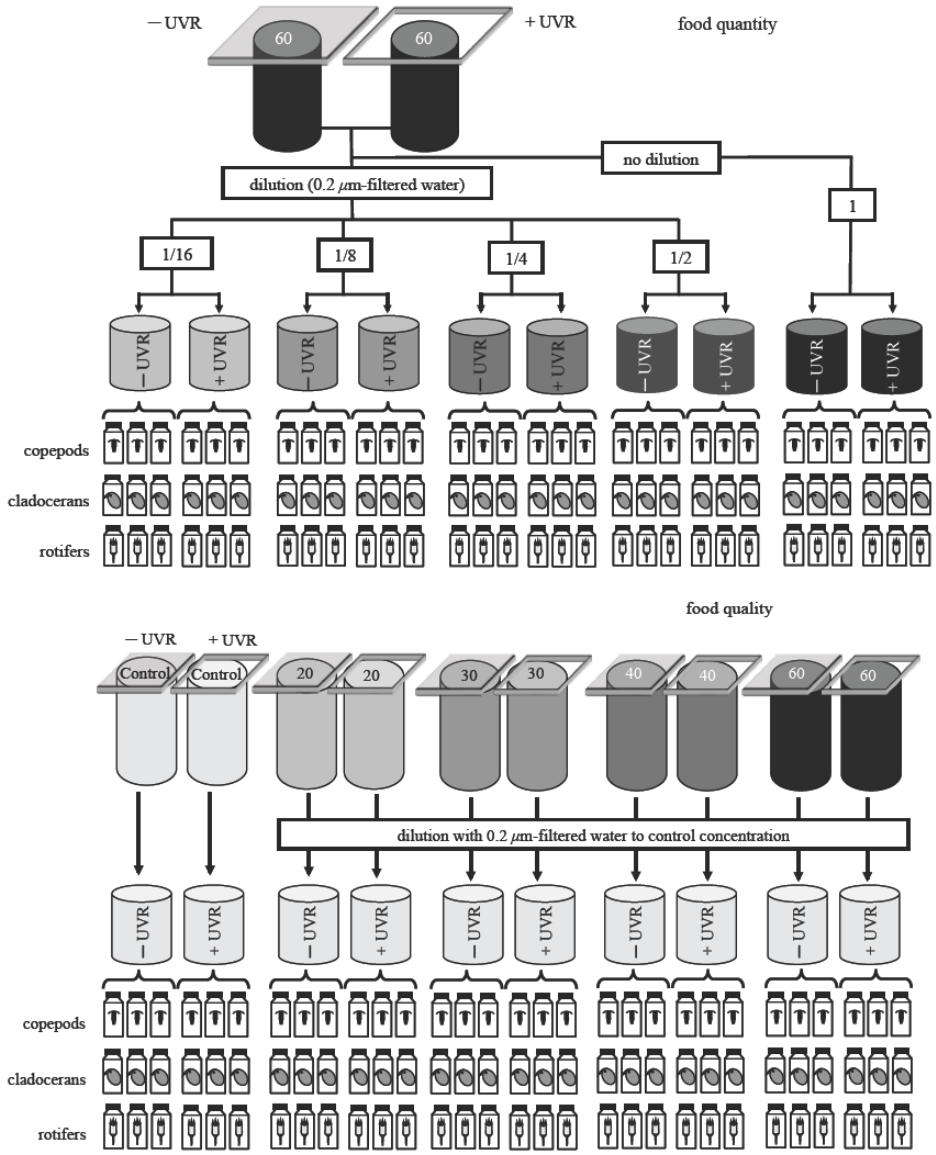
– UVR
+ UVR
food quantity
60
60
no dilution
dilution (0.2 μm-filtered water)
1
1/16
1/8
1/4
1/2
– UVR
+ UVR
copepods
cladocerans
rotifers
food quality
Control
Control
20
20
30
30
40
40
60
60
dilution with 0.2 μm-filtered water to control concentration

Figure 1. Scheme illustrating the (A) food quantity and (B) quality bioassay experiments. Field experimental enclosures are indicated by cylinders and the scale of grey colors define the concentration of seston due to nutrient enrichment after one month incubation from a pale grey (no nutrients added) to a dark grey (most nutrient enriched-treatment). Numbers on the cylinders indicate the amount of P added at the start of incubations (0 or no nutrients-added, 20, 30, 40, or 60 μg P L^{-1}). Arrows indicate how seston suspensions were prepared for the coupled-laboratory bioassays. Water with seston only proceeded from the most enriched enclosures in the food quantity bioassays and from each of the 10 enclosures in the food quality bioassays. Numbers in boxes represent the dilution factor, *i.e.*, the degree to which seston water was diluted with increasing amounts of 0.2 μm-prefiltered water from the mesocosms (1 denotes no dilution, 1/4 denotes 25% natural seston after mixing with 0.2 μm-prefiltered water, ...). Seston suspensions were then used in the laboratory to test for differences in the growth of each zooplankton species. This yielded 60 experimental vessels (30 for the food quantity and 30 for the food quality assays) for each zooplankter tested and a total of 180. *Esquema que ilustra los experimentos-bioensayo de (A) cantidad y (B) calidad de alimento. Los encerramientos experimentales de campo se esquematizan con cilindros, en los que la escala de grises define la concentración de seston debida al enriquecimiento en nutrientes tras un mes de incubación, que va desde el gris pálido (sin nutrientes añadidos) al gris oscuro (tratamiento de mayor enriquecimiento en nutrientes). Los números en los cilindros indican la cantidad de fósforo añadido al principio de la incubación (0 o sin nutrientes añadidos, 20, 30, 40, o 60 μg P L^{-1}). Las flechas indican como las suspensiones de seston fueron preparadas para los bioensayos apareados en el laboratorio. El agua con seston procedió solo del tratamiento más enriquecido para el bioensayo de cantidad de alimento y de cada uno de los 10 tratamientos en los bioensayos de calidad de alimento. Los números en las cajas representan el factor de dilución,* i.e., *el grado al que el seston se diluía con cantidades crecientes de agua prefiltrada a través de 0.2* μm *del correspondiente mesocosmos (1 denota no dilución, 1/4 denota 25% de seston natural tras mezclar con agua prefiltrada a través de 0.2* μm, *...). Las suspensiones de seston fueron luego utilizadas en el laboratorio para testar diferencias en el crecimiento de cada especie de zooplancton. Esto dio lugar a 60 vasos experimentales (30 para el ensayo de cantidad de alimento y otros 30 para el ensayo de calidad) para cada especie de zooplancton testada o bien un total de 180.*

The zooplankton *Daphnia pulicaria* and *Keratella cochlearis*, originated from single clones, were raised in the laboratory under standardized conditions. Ambient *Mixodiaptomus laciniatus* individuals were collected from Lake La Caldera the day before the growth bioassays and measured in the laboratory, isolating individuals belonging to stage III copepodites under an inverted microscope. At the start of the experiments, five *Daphnia pulicaria* neonates collected within 12 h of birth, or ten stage-III-copepodites of *Mixodiaptomus laciniatus* were placed into individual 170 mL glass tubes that each contained 150 mL of the experimental food suspension. For *Keratella cochlearis*, experiments began with 5 newly hatched females placed into 6-mL wells of 12-well, sterile, polystyrene tissue culture plates containing 5 mL of experimental food suspension.

Ten subsamples of the copepod and five of the daphnid were collected to determine initial body weight. The coefficient of variation [CV = (standard deviation / mean) × 100] among weighed samples never exceeded 2%. Zooplankton growth experiments for all treatments were run in triplicate in a growth chamber with a constant temperature (15ºC), and a photosynthetic active radiation of ~20 μmol photons m^{-2} s^{-1} from 25-W white fluorescent tubes (14 h light:10 h dark cycle). Twice a day, tubes and plates were gently shaken to prevent algal settlement. Every day, individuals were checked and transferred to clean tubes or new tissue culture plates containing fresh food suspension from the field enclosures, and rotifers counted under a dissecting microscope. After 5 days for *Daphnia pulicaria* and 12 days for *Mixodiaptomus laciniatus*, individuals were collected, dried (60ºC and 24 h) and weighed. Somatic growth rates (g) for these species were determined using the formula $g = [\ln(M_t) - \ln(M_0)] / t$, where M_t is the final body mass, M_0 is the initial body mass and t is the time (in days) at which animals were collected for their weight on a Mettler ultramicrobalance (±0.1 μg, Mettler).

The somatic growth rate measurement is somewhat difficult to apply to rotifers, as it requires sampling a large number of individuals. Therefore, intrinsic rate of population increase (r) was calculated for *Keratella cochlearis* by

following the techniques described in Stemberger & Gilbert (1985). Briefly, after day 13, the value ln (N_i :N_0) was regressed against time, where N_0 = initial rotifer abundance at day 3, N_i = population density at day i, and r the slope of the regression line. Both growth rates (g and r) were calculated as means for each treatment and both are often used as a measure of animal performance (Stearns, 1992; Sterner & Elser, 2002). Samples from the seston food treatments were collected at the beginning of the experiment and every two days for a total of three samples and prepared for food quality analyses (seston C, C:N:P ratios, Chl *a*, and fatty acids) as described below. The coefficient of variation for seston C, seston C:P or total polyunsaturated fatty acids (PUFAs) among samples was 4% on average and never exceeded 8%.

For the food quantity bioassays, the responses between seston concentration and the somatic growth rate (g) or intrinsic rate of population increase (r) were fit to a Monod model (Sterner & Elser, 2002):

$$g = \frac{g_{\max} C}{K_m + C} \qquad r = \frac{r_{\max} C}{K_m + C}$$

where g_{max} and r_{max} are the maximum growth rate and maximum intrinsic rate of population increase (d^{-1}), C is the seston concentration (μg C L^{-1}) and K_m is the seston concentration at which $g = g_{max} / 2$ or $r = r_{max} / 2$.

Sample analyses

Seston (pre-screened through 40 μm) was collected in triplicate onto pre-combusted glass-fiber filters (Whatman GF/B) for food quality determinations. Seston samples were analyzed for C and N using a CNH analyzer (Perkin-Elmer Model 2400) and for P content by colorimetric means after persulphate oxidation (APHA, 1992). All C:N:P ratios were calculated on a molar basis. Chl *a* was measured fluorimetrically after grinding of filters (Whatman GF/F) with pigments (concentrated by filtration of up to 300 mL at <100 mm Hg of pressure

differential) and extraction of the pigments in 90% acetone kept in the dark at 4ºC for 24 h. A Chl *a* standard (Fluka Chl *a* from algae) was used to transform the fluorescence data into Chl *a* concentrations. Total lipids were extracted using the method of Folch *et al.* (1957) and stored in chloroform:methanol (2:1, v:v) with 0.01% BHT as antioxidant. Lipid aliquots were transmethylated overnight (Christie, 1982) after the addition of nonadecaenoic fatty acid (19:0) as an internal standard. Fatty acid methyl esters were extracted with hexane:diethyl ether (1:1, v:v), and purified by thin layer chromatography using hexane:diethyl ether:acetic acid (85:15:1.5, v:v:v) as abluent system. The analyses of the methyl esters were performed with a Fisons Instruments GC 8000 Series gas chromatograph, equipped with a fused silica 30 m × 0.25 mm open tubular column and a cold on-column injection system, using N as carrier, and a 50ºC to 220ºC thermal gradient. Peaks were recorded and analyzed using Chrom-Card for Windows software and identified by comparison with known standards.

Statistical analysis

Forward stepwise regression analyses were carried out to evaluate the underlying mechanisms (temperature, precipitation, UVR irradiances, intensity, and frequency of TOMS-AI) controlling zooplankton long-term dynamics in Lake La Caldera. Linearity and orthogonality among independent variables were verified by previous correlation analysis and controlled by specifying 0.6 as the minimum acceptable tolerance (StatSoft, 1997). The *F*-values entering the multiple-regression model were established on the basis of the number of independent variables and cases. Regression models considered mean values for all variables.

Differences in food quality and quantity among field enclosures due to UVR and P manipulation were assessed by paired *t*-tests using samples collected during the growth assays (starting 01 September 2003 at the beginning of the zooplankton assays and every two days for a total of three samples per mesocosms). The effects of food quantity and food quality on zooplankton growth

were tested by two-way analysis of variance (ANOVA). The ANOVAs carried out used 'dilution treatment' and 'UVR' as the two factors for food quantity analysis, and 'P-enrichment' and 'UVR' as the two factors for food quality analysis. Differences in growth rate among the dilution factors and between UVR levels in the food quantity assays were assessed by *post-hoc* Tukey Honestly Significant Difference test (HSD), adjusting their probabilities using Bonferroni's test. The estimation of the food quantity level for maximum population growth was defined as the seston C content measured in the dilution factor at which zooplankton growth no longer increased. Zooplankton growth in the food quantity bioassays was also fit to saturation curves, and differences in g_{max} and K_m due to UVR treatment or taxonomical group were tested *via* one-way ANOVA.

To determine which food quality variable best predicted the growth of the zooplankton in the food quality bioassays, simple regression analysis were performed for each light treatment. To choose among candidate variables we used Akaike's Information Criterion (AIC) as a selection method (Burnham & Anderson, 1998; Park *et al.*, 2002). When regressions for each light treatment were significant, a homogeneity of slopes model [analysis of covariance (ANCOVA)] was used to test for differences between +UVR and –UVR treatments (categorical factor) across the food quality predictor (continuous predictor variable) that produced the highest fit on zooplankton growth (Quinn & Keough, 2002). When the slope of the regression line was different for +UVR and –UVR treatments, significant differences at each nutrient level were graphically determined by examining 95% confidence intervals of the regression lines (Urabe *et al.*, 2002). In this study we tested food quality predictors alone (*e.g.*, mineral or biochemical constituents) or in combination (*e.g.*, mineral and biochemical constituents), which we refer as to single or combined food quality indices, respectively. All data were checked for normal distribution by the Shapiro-Wilks' *W*-test. Homocedasticity was verified with the Levene's test, and the data were log transformed when these conditions were not met.

We conducted a meta-analysis of published data to examine the idea that the growth of zooplankton was affected by the ω3-PUFA and P content of its

food. For this purpose we performed a thorough literature search for studies that independently assessed the relevance of both of these constituents for zooplankton growth. We used Hedges' *d* as our metric of standardized effect size and calculated a weighted average to estimate cumulative effect size using fixed effects models. The size of a mean effect size was considered small when less than 0.2, moderate when 0.5, and large when greater than 0.8 (Gurevitch & Hedges, 1993). We used two electronic databases (Web of Sciences, Aquatic Sciences, and Fisheries Abstracts) to search for field and laboratory studies containing simultaneous data on the effects of seston P and fatty acid content on zooplankton growth as the response variable of interest. Although we included correlational and experimental supplementation studies, only eight articles met our criteria of simultaneous quantification of P and PUFA effects and all focused on *Daphnia* species. In studies where statistical significances were reported as probabilities, *p*-values were translated to correlation coefficients, and these used to calculate effect sizes (Rosenberg *et al.*, 2000). General statistical analyses were performed using Statistica 7.1 for Windows software (StatSoft, 1997), and MetaWin version 2.0 for meta-analysis calculations (Rosenberg *et al.*, 2000).

Results

Long-term biological and climatic data

Figure 2 shows the year-to-year variations in the abundance of zooplankton in Lake La Caldera between 1975 and 2007. The most striking feature of this time-series is the pronounced inter-annual variation but there does not appear to follow any long-term trend. Although there is a clear dominance of copepods, there are sporadic short-term appearances of cladocerans (mainly *Daphnia pulicaria*) and rotifers (Fig. 2).

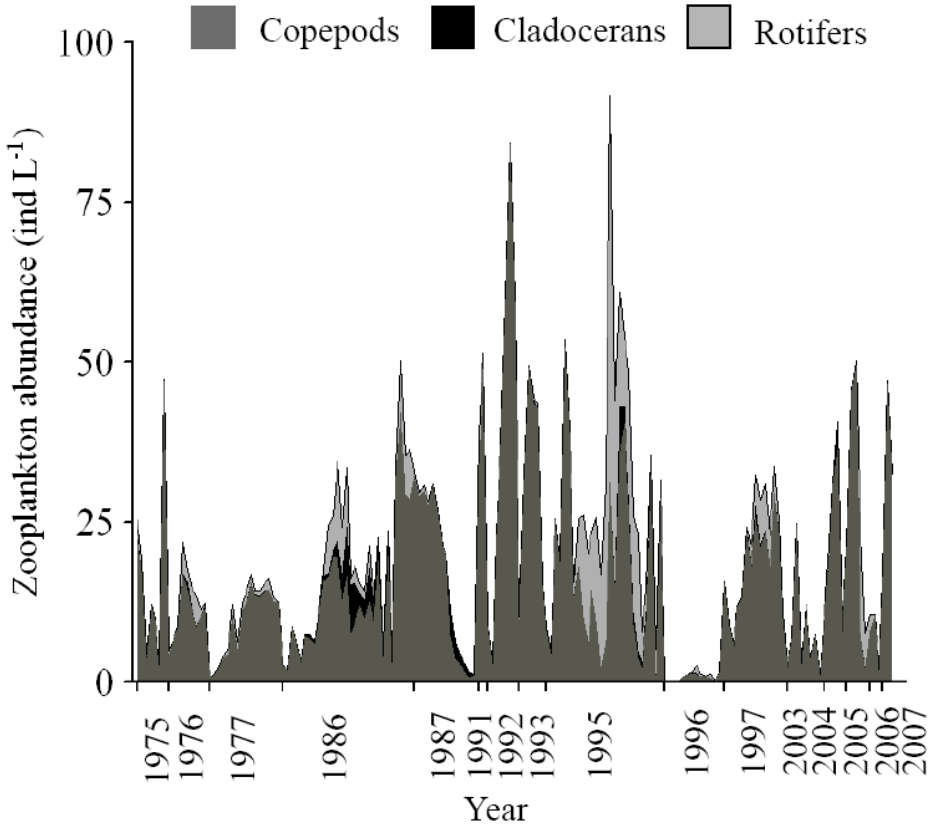

Figure 2. Long-term dynamics of the relative contribution of various zooplankton groups to total zooplankton abundance for the ice-free period between 1975 and 2007 in Lake La Caldera. *Dinámicas a largo plazo de la contribución relativa de varios grupos de zooplancton a la abundancia total de zooplancton durante el período libre de hielos entre 1975 y 2007 en el lago de La Caldera.*

Forward stepwise regressions showed that of all potential predictors, precipitation, TOMS-AI and UVR explained zooplankton biomass, although precipitation explained most (62%) of this variance, whereas TOMS-AI contributed 26% and UVR only with an additional 9% (Table 1). Because most zooplankton biomass was contributed by *Mixodiaptomus laciniatus*, copepod biomass correlated with the same set of predictor variables (precipitation, TOMS-AI, and UVR) as total zooplankton biomass (Table 1). In contrast, *Daphnia* did not show any long-term relationship with any of the predictor variables.

In situ *manipulation of UVR and nutrients: Seston quantity and quality gradient*

The incubations in the presence and absence of UVR at increasing nutrient regimes served our purpose of generating a wide gradient in the quantity and quality of seston. Seston C strongly increased in response to P-enrichment after incubation of 30 days (Table 2). UVR did not affect seston C except for the most P-enriched treatment, where seston C was 12% lower in the +UVR enclosure than in the –UVR enclosure (Table 2). As a result, seston C concentrations during zooplankton bioassays varied from 132 to 2001 μg C L^{-1} in +UVR enclosures and from 140 to 2333 μg C L^{-1} in the –UVR enclosures. By the time zooplankton bioassays were initiated, mean seston C:P ratios were lower in enclosures receiving no P, 20 and 30 μg P L^{-1} in +UVR than in their respective –UVR enclosures, with values well over 300. In contrast, UVR did not strongly affect seston C:P ratios in the enclosures receiving 40 μg P L^{-1} or more (Table 2). Therefore, enrichment with P and UVR generated a food quality gradient in terms of seston C:P ratio that ranged from 180 to 360 in +UVR and from 214 to 438 in –UVR enclosures (Table 2).

Table 1. Results of multiple stepwise regression analysis between zooplankton and potential predictors (precipitation, intensity and frequency of TOMS-AI, UVR irradiances, and temperature) for Lake La Caldera. *Resultados de los análisis de regresión múltiple por pasos entre el zooplancton y predictores potenciales (precipitación, intensidad y frecuencia de TOMS-AI, UVR, y temperatura) para el lago de La Caldera.*

Dependent variable	Independent variable	Beta	Multiple r^2	r^2 change	df1	df2	*F*	*p*
Copepod biomass (log)	Precipitation	-0.842	0.78	0.61	2	34	12.27	0.008
	TOMS AI	-0.515	0.92	0.24	2	25	10.75	0.014
	UVR	0.306	0.97	0.09	2	35	8.09	0.029
Total zooplankton biomass (log)	Precipitation	-0.853	0.78	0.62	2	34	12.84	0.007
	TOMS AI	-0.532	0.93	0.26	2	25	14.10	0.007
	UVR	0.309	0.98	0.09	2	35	15.26	0.008

Beta, standardized regression coefficient; Multiple r^2, coefficient of multiple determination; r^2 change, change in Multiple r^2 caused by entering a new variable in a single step (hierarchical analysis); df1, df2, degrees of freedom; $F_{df1,df2}$, *F*-test results of the relationship between the dependent variable and the set of independent variables entered in the analysis. Beta, *coeficiente de regresión estandarizado;* Multiple r^2, *coeficiente de determinación múltiple;* Change r^2, *cambio en* Multiple r^2 *debido a la adición de una nueva variable por cada paso (análisis jerárquico); df1, df2, grados de libertad;* $F_{df1,df2}$, *resultados del* F-*test de la relación entre la correspondiente variable dependiente y el conjunto de variables independientes introducidas en el análisis.*

Table 2. Effects of (A) UVR, (B) P-enrichment in +UVR, and (C) P enrichment in -UVR, tested by paired *t*-tests, on the C, C:P ratio, PUFA, HUFA, and HUFA:PUFA ratio of seston in the field enclosures. Values are the mean for the sampled dates (at the beginning of zooplankton assays and every two days for a total of three samples) ± 1 SD; *t*-values; significance levels (ns, not significant; * $p < 0.05$; ** $p < 0.001$; and *** $p < 0.001$). *Efectos de (A) UVR, (B) enriquecimiento en P en +UVR, y (C) enriquecimiento en P en –UVR, testados por* t-*test para muestras apareadas, sobre el contenido en C, razón C:P, PUFA, HUFA, y razón HUFA:PUFA del seston en los encerramientos de campo. Los valores son promedios de los días muestreados (al principio del bioensayo con zooplancton y cada dos días hasta obtener un total de tres muestras) ± 1 desviación estándar;* t-*valores; los niveles de significación (ns, no significativa;* * p < *0.05;* ** p < *0.001; y* *** p < *0.001).*

		C			C:P			PUFA			HUFA			HUFA:PUFA		
		Average ± SD (μg L^{-1})	*t*	*p*	Average ± SD (μg L^{-1})	*t*	*p*	Average ± SD (μg L^{-1})	*t*	*p*	Average ± SD (μg L^{-1})	*t*	*p*	Average ± SD (μg L^{-1})	*t*	*p*
A) UVR																
control	+UVR	132 ± 18	-4.40	ns	215 ± 35	-7.76	*	32 ± 02	29.42	**	7.1 ± 0.7	-5.67	*	22.0 ± 1.0	-9.97	**
	-UVR	140 ± 19			386 ± 42			24 ± 02			12.4 ± 2.3			51.3 ± 5.5		
20	+UVR	242 ± 12	-7.48	ns	376 ± 10	-21.73	*	83 ± 12	0.39	ns	4.9 ± 0.6	-4.00	ns	6.0 ± 0.0	-7.00	*
	-UVR	292 ± 32			420 ± 15			79 ± 02			7.6 ± 0.3			9.5 ± 0.7		
30	+UVR	485 ± 24	-7.77	ns	315 ± 13	-3.60	*	104 ± 04	13.30	**	5.9 ± 2.5	0.29	ns	5.7 ± 2.1	-0.76	ns
	-UVR	583 ± 52			386 ± 25			90 ± 06			5.6 ± 0.6			6.3 ± 0.6		
40	+UVR	969 ± 48	-7.59	ns	281 ± 38	-0.23	ns	140 ± 11	0.02	ns	4.6 ± 0.3	-1.60	ns	3.3 ± 0.6	-1.00	ns
	-UVR	1167 ± 95			286 ± 07			140 ± 16			5.1 ± 0.8			3.7 ± 0.6		
60	+UVR	2001 ± 95	-7.60	*	180 ± 12	-1.58	ns	184 ± 19	7.11	*	3.9 ± 0.4	0.99	ns	2.3 ± 0.6	-2.00	ns
	-UVR	2333 ± 36			214 ± 33			107 ± 06			3.6 ± 0.2			3.0 ± 0.3		
B) P-enrichment in +UVR																
control	20		-9.04	***		-5.41	*		-10.01	***		4.63	**		27.71	***
	30		-20.79	***		-4.59	*		-26.50	***		0.82	ns		12.25	***
	40		-28.65	***		-2.18	ns		-16.78	***		5.98	**		28.00	***
	60		-32.40	***		1.65	ns		-13.96	***		7.22	**		29.50	***
C) P-enrichment in –UVR																
control	20		-14.21	***		-1.60	ns		-32.57	***		2.80	*		10.15	**
	30		-40.77	***		-0.02	ns		-17.88	***		4.96	**		14.07	***
	40		-93.45	***		4.03	*		-12.71	***		5.20	**		14.91	***
	60		-194.33	***		5.36	**		-22.05	***		6.61	**		15.20	***

Response to experimental manipulation was also clear for fatty acids. Thus, PUFA increased from 32 to 184 μg mg C^{-1} with increasing P enrichment in +UVR enclosures and from 24 to 107 μg mg C^{-1} in –UVR enclosures (Table 2). In addition seston exposed to UVR had considerably higher mean PUFA content in the control and the most enriched treatment where PUFA was 25% and 42% higher in +UVR relative to –UVR, respectively (Table 2). α-linolenic acid (ALA, 18:3ω3) was the prevalent PUFA (57-85% of ω3-PUFAs and 24-61% of PUFAs), and other single ω3-PUFA [*e.g.*, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA)], for which experimental evidence of their importance for zooplankton exists, accounted together for 0-4% of ω3-PUFAs and less than 1% of total fatty acids in seston (data not shown). The seston fatty acid composition observed in this experiment was well explained by the dominance of the green algae *Dyctiosphaerium chlorelloides* (>95% biomass in all enclosures; Delgado-Molina *et al.*, 2009), a relatively poor algae in EPA and DHA content (Ahlgren *et al.*, 1997). Likewise, P-enrichment reduced highly unsaturated fatty acids (HUFA), although the effects varied between UVR treatments. Thus P-enrichment decreased HUFA between 39-71% in –UVR, and between 17-46% in +UVR treatments (Table 2). The increase in PUFA and the generalized decline in HUFA led to an overall decrease in the HUFA to PUFA ratio in the P enriched enclosures relative to the control enclosures (Table 2). In addition, the strongest differences in seston HUFA:PUFA ratio between UVR treatments were found in the control, which was 43% lower in the +UVR enclosure compared with –UVR (Table 2).

Effects of food quantity on zooplankton growth

The food quantity gradient explained between 89-94% of the total variation in zooplankton growth (Table 3). UVR did not significantly affect the growth response (Table 3). Neither was significant the interaction between P-enrichment and UVR for *Mixodiaptomus laciniatus* and *Daphnia pulicaria*, although it explained a minor percentage of the variance in *Keratella cochlearis* growth (Table 3; Fig. 3). Statistical differences in growth among experimental treatments indicated food quantity levels for maximum population growth (Table

4). Thus, *Mixodiaptomus laciniatus* in the food quantity gradient grew hyperbolically from 0.02 to 0.09 d^{-1} leveling off at 485 μg C L^{-1} (treatment 1/4; Fig. 4A), as indicated by the absence of differences in growth between treatments 1/4 and 1/2 (Table 4). A significant decline in growth rate was observed at the highest seston concentrations of ca. 2000 μg C L^{-1} (treatment 1/2 *vs.* 1; Table 4). The effect of P enrichment was strongest for *Daphnia pulicaria* that increased from 0.08 to 0.33 d^{-1} at a seston concentration ~1 mg C L^{-1} (treatment 1/2; Fig. 4C). A further food increase from ~1 mg C L^{-1} to ~2 mg C L^{-1} (treatment 1/2 to 1) no longer yielded a response in *Daphnia pulicaria* growth (Table 4). At the opposite end was *Keratella cochlearis* where P moderately stimulated growth from 0.11 to 0.23 d^{-1} (Fig. 4E) and no further differences in growth were observed after ~250 μg C L^{-1} (treatment 1/8; Table 4), indicating that maximum population growth was reached at much lower seston concentrations.

All zooplankters significantly fit a saturation curve (Fig. 4A, C, E; Table 5). UVR treatment had no effect on the shape of these curves (one way-ANOVA, $F_{1,1}$ = 0.106, p = 0.77). There were, however, marked differences in the g_{max} estimates due to taxonomical group (one way-ANOVA, $F_{1,2}$ = 259.82, p = 0.004) that resulted in different zooplankton shape responses. Highest g_{max} was found for *Daphnia pulicaria*, followed by *Keratella cochlearis* and *Mixodiaptomus laciniatus* (all *post-hoc* Tukey HSD tests, $p < 0.05$). Differences in K_m due to zooplankton taxonomical group or UVR treatment were not significant (one way-ANOVAs, $F_{1,2}$ = 8.23, p = 0.11 and $F_{1,1}$ = 4.56, p = 0.17, respectively) meaning that growth response with increasing seston concentrations was similar among species and between UVR treatments.

A) Food quantity						
Species	Effect	df1	df2	*F*	*p*	*PV*
Mixodiaptomus laciniatus	UVR	1	22	0.003	0.958	0.00
	dilution treatment	**4**	**22**	**50.927**	**<0.001**	**88.81**
	UVR × dilution treatment	4	22	1.664	0.199	2.90
	Error					8.28
Daphnia pulicaria	UVR	1	22	0.520	0.481	0.15
	dilution treatment	**4**	**22**	**76.629**	**<0.001**	**93.77**
	UVR × dilution treatment	4	22	0.908	0.482	1.07
	Error					5.00
Keratella cochlearis	UVR	1	22	0.001	0.975	0.00
	dilution treatment	**4**	**22**	**68.126**	**<0.001**	**88.60**
	UVR × dilution treatment	**4**	**22**	**5.018**	**0.009**	**6.53**
	Error					4.88
B) Food quality						
Mixodiaptomus laciniatus	UVR	**1**	**22**	**39.685**	**<0.001**	**9.93**
	P-enrichment	**4**	**22**	**73.904**	**<0.001**	**73.99**
	UVR × P-enrichment	**4**	**22**	**11.557**	**<0.001**	**11.57**
	Error					4.51
Daphnia pulicaria	UVR	1	22	2.010	0.173	2.37
	P-enrichment	**4**	**22**	**12.706**	**<0.001**	**59.88**
	UVR × P-enrichment	**4**	**22**	**3.511**	**0.028**	**16.55**
	Error					21.21
Keratella cochlearis	UVR	**1**	**22**	**34.274**	**<0.001**	**21.35**
	P-enrichment	**4**	**22**	**24.111**	**<0.001**	**60.07**
	UVR × P-enrichment	**4**	**22**	**2.965**	**0.048**	**7.38**
	Error					11.21

Table 3. Effects on zooplankton growth of (A) UVR, dilution treatment and their interaction in the food quantity bioassays, and (B) UVR, P-enrichment, and their interaction in the food quality bioassays, tested by two-way ANOVA. Reported are: Degrees of freedom (df1, df2), *F*-test results (*F*), significance level (*p*), and percentage of variance (*PV*) calculated as sums of squares of treatment:total sums of squares. Significant differences are highlighted as bold text. *Efectos sobre el crecimiento del zooplancton de (A) UVR, tratamiento de dilución y su interacción en los bioensayos de cantidad de alimento, y (B) UVR, enriquecimiento en P, y su interacción en los bioensayos de calidad de alimento, testados por ANOVA de doble vía. Se indican: Los grados de libertad (df1, df2), los resultados del* F*-test (*F*), el nivel de significación (*p*), el porcentaje de la varianza (*PV*) calculado como suma de cuadrados del tratamiento:suma de cuadrados total. Las diferencias significativas se señalan en negrita.*

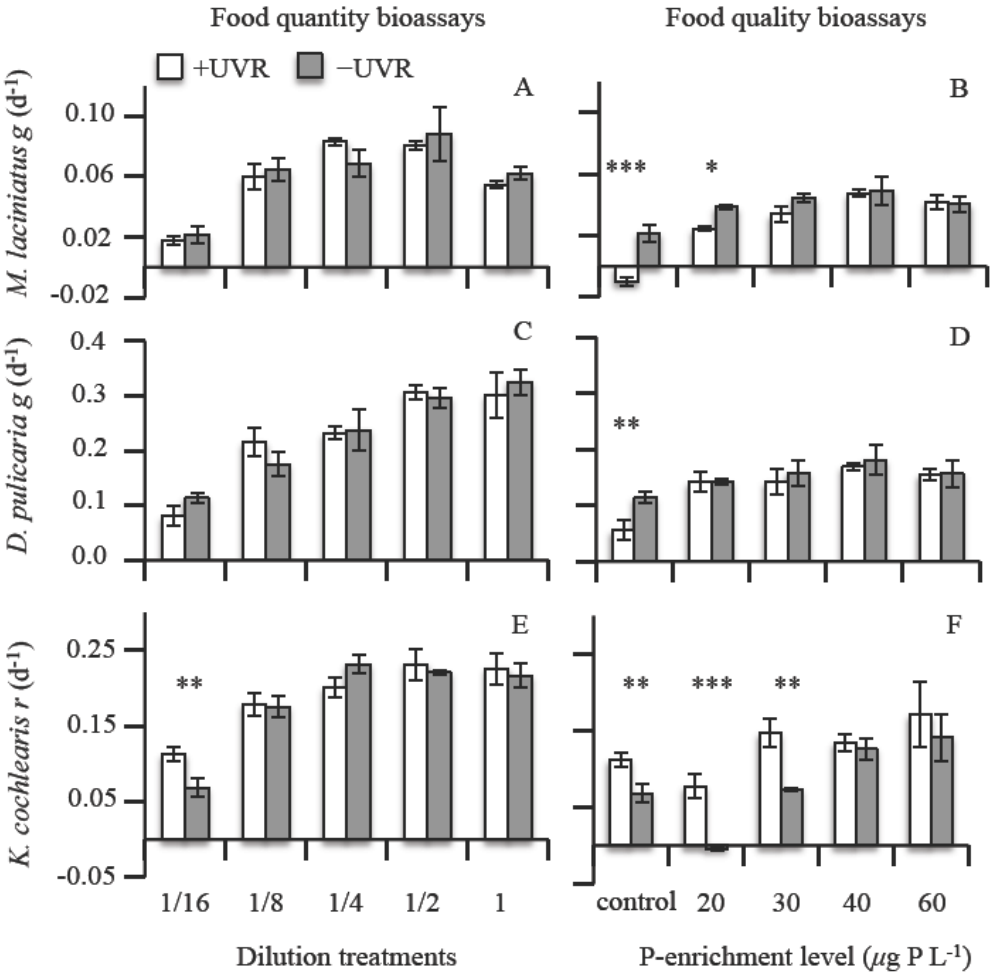

Figure 3. (A, C, E) Effects of UVR and dilution treatment on zooplankton growth in the food quantity assays, and (B, D, F) effects of UVR and P-enrichment on zooplankton growth in the food quantity assays. Error bars represent the mean ± 1SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ indicate the significance levels of the effect exerted by UVR on the response variable, tested by two-way ANOVA and *post hoc* Newman-Keuls' test. *See* Methods and Fig. 1 for a detailed description of the protocol used for preparation of food assays. *(A, C, E) Efectos de UVR y tratamiento de dilución sobre el crecimiento del zooplancton en los ensayos de cantidad de alimento, y (B, D, F) efectos de UVR y enriquecimiento en P sobre el crecimiento de zooplancton en los ensayos de calidad de alimento. Las barras de error representan la media ± 1 desviación estándar.* * p < *0.05;* ** p < *0.01;* *** p < *0.001 indican los grados de significación del efecto ejercido por UVR sobre la variable respuesta, testado por medio de un ANOVA de doble vía y un test* post hoc *de Newman-Keuls.* Ver Methods *y Fig. 1 para una descripción detallada de los protocolos usados para la preparación de los ensayos.*

	Dilution treatment	1/16	1/8	1/4	1/2	1
Mixodiaptomus laciniatus	1/16	–	↑ ***	↑ ***	↑ ***	↑ ***
	1/8	–	–	↑ **	↑ **	ns
	1/4	–	–	–	ns	↑ ***
	1/2	–	–	–	–	↓ ***
	1	–	–	–	–	–
Daphnia pulicaria	1/16	–	↑ **	↑ **	↑ **	↑ ***
	1/8	–	–	ns	↑ *	↑ **
	1/4	–	–	–	↑ *	↑ *
	1/2	–	–	–	–	ns
	1	–	–	–	–	–
Keratella cochlearis	1/16	–	↑ *	↑ **	↑ *	↑ **
	1/8	–	–	ns	ns	ns
	1/4	–	–	–	ns	ns
	1/2	–	–	–	–	ns
	1	–	–	–	–	–

Table 4. Differences in growth rate among food levels in the food quantity bioassays tested by one-way ANOVA and *post-hoc* Tukey HSD test, adjusting their probabilities using Bonferroni's test. Arrows express the increase or decrease in growth rate between treatments. Statistical significance level (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Because results were qualitatively similar for both UVR treatments, we report only those based on +UVR enclosures. *Diferencias en la tasa de crecimiento entre niveles de alimento en el bioensayo de cantidad de alimento, testadas por ANOVA de una vía y test* post-hoc *Tukey HSD, y ajustando sus probabilidades mediante un test de Bonferroni. Los niveles de significación estadística (ns, no significativo; *,* $p < 0.05$; **,* $p < 0.01$; ***,* $p < 0.001$). *Debido a que los resultados eran cualitativamente similares para ambos tratamientos de UVR, sólo presentamos aquellos de los encerramientos +UVR.*

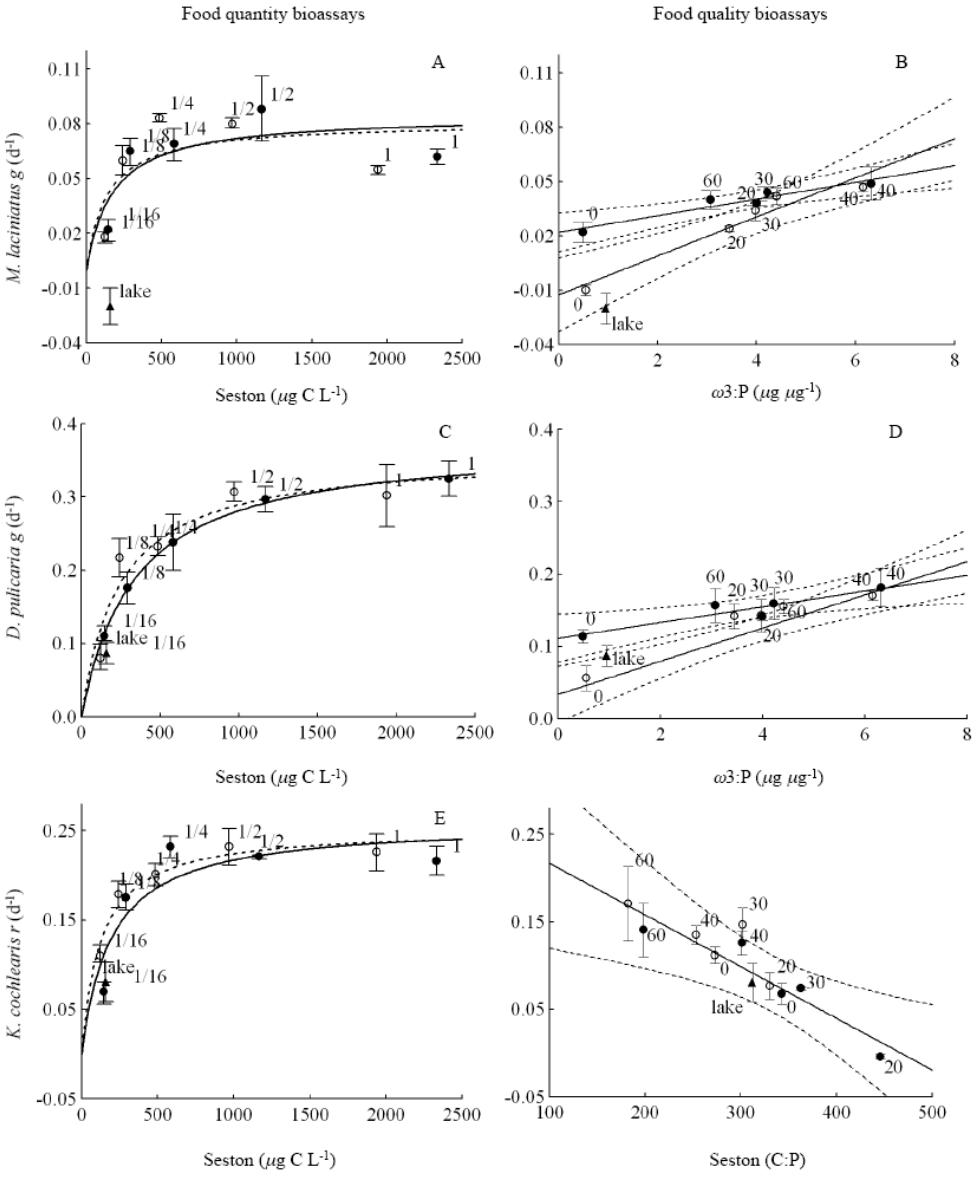
Food quantity bioassays
Food quality bioassays
A
B
C
D
E
M. laciniatus g (d-1)
D. pulicaria g (d-1)
K. cochlearis r (d-1)
Seston (μg C L-1)
ω3:P (μg μg-1)
Seston (C:P)
lake

Figure 4. Response of zooplankton growth to food quantity and quality assays. (A, C, E) Food quantity bioassay shows the relationships between seston concentration and zooplankton growth. (B, D, F) Food quality bioassay shows the relationship between best food quality predictors and zooplankton growth. The −UVR levels are represented by filled symbol, the +UVR levels by open symbols and a lake water control under the experimental conditions by a black triangle. Numbers next to the symbols indicate the dilution ratio from the most enriched enclosures (food quantity bioassays), the specific nutrient enclosure from which water was diluted to lake concentrations (food quality bioassays) or lake control (referred as to 'lake'). Dashed lines in food quality bioassays indicate 95% confidence intervals around the fitted models (solid lines). Lake control was not included in the regression analysis. Values are means for three replicates and error bars represent ± 1SD. *Respuesta del crecimiento del zooplancton en los ensayos de cantidad y calidad de alimento. (A, C, E) El bioensayo de cantidad de alimento muestra las relaciones entre la concentración del seston y el crecimiento del zooplancton. (B, D, F) El bioensayo de calidad de alimento muestra la relación entre los mejores predictores de calidad de alimento y el crecimiento del zooplancton. Los niveles de –UVR están representados por símbolos rellenos, los niveles de +UVR por símbolos vacíos y el control correspondiente al agua del lago bajo condiciones experimentales por un triángulo negro. Los números junto a los símbolos indican la razón de dilución del encerramiento más enriquecido (en el bioensayo de cantidad de alimento), el nivel específico de enriquecimiento en nutrientes del encerramiento del cual se diluyó su agua hasta alcanzar la concentración de la del lago (en el bioensayo de calidad de alimento), o control del lago (referido aquí como 'lago'). Las líneas discontinuas en los bioensayos de calidad de alimento indican el intervalo de confianza del 95% a ambos lados de los modelos ajustados (líneas continuas). El control del lago no fue incluido en los análisis de regresión. Los valores son promedios de tres réplicas y las barras de error representan ± 1 desviación estándar.*

Effects of food quality on zooplankton growth

There were substantial differences in the growth of zooplankton at the same seston concentrations in the food quality treatments (Fig. 4B, D, F). The ANOVA showed that P enrichment explained between 60% and 74% of total variance in zooplankton growth in the food quality bioassays (Table 3). The effect of UVR was significant for *Mixodiaptomus laciniatus* and *Keratella cochlearis* but not for *Daphnia pulicaria*. Interestingly, the interaction between P enrichment and UVR was significant for all zooplankton species and particularly strong for *Mixodiaptomus laciniatus* and *Daphnia pulicaria* explaining ~12% and 17% of the variance, respectively (Table 3). This analysis therefore showed that, as for food quantity bioassays, the growth of zooplankton was primarily affected by P-enrichment. Nonetheless, effects of food quality never reached those of the food quantity in magnitude. Thus, maximum zooplankton growth was 1.4 fold (*Keratella cochlearis*) and ~2 fold (*Mixodiaptomus laciniatus* and *Daphnia pulicaria*) lower in food quality than in food quantity bioassays (Fig. 4).

Species	Light treatment	g_{max} (SE)	K_m (SE)	df	r^2	p	
						g_{max}	K_m
Mixodiaptomus laciniatus	+UVR	0.081 (0.011)	138.20 (77.77)	13	0.481	<0.001	0.099
	-UVR	0.084 (0.011)	183.70 (89.67)	12	0.558	<0.001	0.063
	+UVR and -UVR	0.082 (0.007)	156.43 (58.81)	27	0.511	<0.001	0.010
Daphnia pulicaria	+UVR	0.376 (0.026)	252.81 (57.76)	11	0.874	<0.001	0.001
	-UVR	0.361 (0.017)	264.37 (45.59)	12	0.913	<0.001	<0.001
	+UVR and -UVR	0.366 (0.015)	253.03 (35.17)	25	0.887	<0.001	<0.001
Keratella cochlearis	+UVR	0.240 (0.013)	110.77 (26.21)	10	0.817	<0.001	0.002
	-UVR	0.257 (0.022)	202.46 (64.63)	11	0.761	<0.001	0.009
	+UVR and -UVR	0.248 (0.013)	156.76 (32.78)	23	0.748	<0.001	<0.001

Table 5. Parameters for the saturating curves for zooplankton growth from feeding at the different seston concentrations in the food quantity bioassays. Separate and combined models are given for UVR treatments. *Parámetros de las curvas de saturación para el crecimiento del zooplancton tras alimentarse con las diferentes concentraciones de seston en los bioensayos de cantidad de alimento. Se proporcionan los modelos en los que tanto se distinguen como no los tratamientos UVR.*

Of all predictors, seston ω3-PUFA was positively related to copepod and daphnid growth rates, although correlations were not strong and non significant in the case of *Daphnia pulicaria*, indicating that ω3-PUFA may not be a particularly useful food quality index in these experiments (Table 6). Neither was HUFA a good predictor of zooplankton growth because ALA comprised most of the fatty acid in seston (data not shown). In contrast, the P-normalized ω3-PUFA (ω3-PUFA:P) in seston yielded a considerably better prediction of the growth of calanoid copepods and daphnids with relatively lower AIC values than for single food quality indices. The slopes of these relationships were positive (Fig. 4B, D; Table 6). Also, the response of zooplankton growth rate to ω3-PUFA:P differed between UVR treatments in *Mixodiaptomus laciniatus* (analysis of covariance: slope of the regression line, $F_{1,6} = 26.54$, $p = 0.002$), and marginally in *Daphnia pulicaria* ($F_{1,6} = 5.61$, $p = 0.056$). In particular, differences between zooplankton growths were only significant for the controls, *i.e.*, treatments most similar to ambient lake conditions (Fig. 4B, D).

Lastly, seston C:P ratio was the sole factor predicting *Keratella cochlearis* growth (Table 6). The relationship was negative indicating that P-enriched seston favored rotifer growth (Fig. 4F). Although the response of rotifer growth to seston C:P did not differ between UVR treatments (analysis of covariance: Slope, $F_{1,6} = 0.44$ and $p = 0.84$), it is worth noting that higher growth rates for a given nutrient level were found under UV exposure due to the effect of this stressor decreasing seston C:P (Fig. 4F).

Table 6. Effects of food quality variables on the growth of zooplankton by means of simple linear regressions. Akaike's Information Criterion (AIC) is shown for significant regressions and best estimates shown in bold. *Efectos de las variables de calidad de alimento sobre el crecimiento del zooplancton evaluados por regresión lineal.* Akaike's Information Criterion (AIC) *se muestra para las distintas regresiones y las mejores estimas se muestran en negrita.*

Species	Food quality variable	Linear regression				
		Slope	*y* -intercept	r^2	*p*	AIC
Mixodiaptomus laciniatus	C:P	-0.00003	-0.036	0.01	0.889	-
	C:N	0.01400	-0.079	0.55	0.149	-
	P (μg L^{-1})	-0.01060	0.056	0.04	0.744	-
	ω3-PUFA (μg mg C^{-1})	-0.00050	-0.006	0.84	0.028	-14.94
	18:3 ω3 (μg mg C^{-1})	0.00580	-0.004	0.72	0.069	-
	ω3-PUFA:P (μg μg^{-1})	**0.01100**	**-0.013**	**0.95**	**0.006**	**-17.17**
	Carbon-specific Chl a (μg mg C^{-1})	0.00140	-0.045	0.86	0.022	-15.26
Daphnia pulicaria	C:P	-0.00005	-0.121	0.01	0.919	-
	C:N	-0.02900	-0.093	0.65	0.101	-
	P (μg L^{-1})	-0.03220	0.220	0.10	0.606	-
	ω3-PUFA (μg mg C^{-1})	-0.00080	-0.071	0.73	0.058	-
	18:3 ω3 (μg mg C^{-1})	0.00105	0.077	0.61	0.118	-
	ω3-PUFA:P (μg μg^{-1})	**0.02037**	**0.055**	**0.93**	**0.007**	**-13.89**
	Carbon-specific Chl a (μg mg C^{-1})	0.00254	-0.001	0.76	0.052	-
Keratella cochlearis	C:P	**-0.00060**	**0.286**	**0.82**	**<0.001**	**-27.22**
	C:N	-0.02110	0.267	0.22	0.172	-
	P (μg L^{-1})	0.07070	-0.063	0.44	0.035	-22.24
	ω3-PUFA (μg mg C^{-1})	0.00090	0.044	0.37	0.062	-
	18:3 ω3 (μg mg C^{-1})	0.00116	0.042	0.39	0.054	-
	ω3-PUFA:P (μg μg^{-1})	0.00707	0.074	0.07	0.473	-
	Carbon-specific Chl a (μg mg C^{-1})	0.00181	0.006	0.27	0.120	-

Notes: *n* = 5 for *Mixodiaptomus laciniatus* and *Daphnia pulicaria* when significant fits differed between UVR treatments (regressions shown are for +UVR treatment) and *n* = 10 for *Keratella cochlearis* when significant fits did not differ between UVR treatments (*see* ANCOVAs in Results section). Notas: *n = 5 para* Mixodiaptomus laciniatus *y* Daphnia pulicaria *cuando los ajustes significativos diferían entre tratamientos UVR (las regresiones no se muestran para los tratamientos +UVR) y* n *= 10 para* Keratella cochlearis *cuando los ajustes significativos no diferían entre los tratamientos UVR (*ver *ANCOVAs en la sección* Results*)*

Meta-analysis study

Meta-analysis was used to systematically review studies which have tested the single roles of ω3-PUFA and P on zooplankton growth. All studies showed significant positive influences of ω3-PUFA and P, and the mean effect size for either constituent was considered moderate as it was higher than 0.5 (Table 7). These results provide strong support for the effects of both ω3-PUFA and P content on zooplankton growth and were, therefore, congruent with the use of a combined food quality index merging the above single constituents (*i.e.*, ω3-PUFA:P).

Discussion

Copepods dominate the zooplankton in this lake and other highly oligotrophic systems including oceans (Carney & Elser, 1990; Nuwer *et al.*, 2008). However, the relationship found between copepod biomass and precipitation and aerosol intensity indicates that wet and dry depositions may have a negative effect on copepod populations. Several mechanisms might contribute to the link between precipitation and zooplankton community structure in the study lake. During wet years (snowfall in winter), zooplankton may be influenced by a relatively late thaw date, delayed warming of surface water and colder water temperatures, which might influence the extent of the delay of zooplankton increase (Romare *et al.*, 2005). During dry years, reduced precipitation contributes to early ice-melting processes, longer growth windows and warmer water temperatures in summer, which may have strong effect stimulating *Daphnia* emergence (Stross, 1966) and growth rate of herbivorous consumers. The negative effect of aerosol inputs on the long-term decline in copepod biomass is consistent with findings for this lake, where years of exceptionally high atmospheric loads caused reduced copepod populations but enhanced populations of cladocerans and rotifers (Villar-Argaiz *et al.*, 2001). Recent work has shown how atmospheric depositions affect nutrient limitation, which by impairing the trophic producer-consumer interaction extends into the food web (Elser *et al.*, 2010).

Table 7. Meta-analysis of the effects of PUFA and P on the somatic growth rate of zooplankton. *Meta-análisis de los efectos de PUFA y P sobre la tasa de crecimiento somática del zooplancton.*

Study	Species	Constituent	Size effect	Variance	Type of study	Observations
Boersma, 2000	*Daphnia magna*	P	0.85	0.06	Supplementation of P and FA to P-deficient algae	Effects of P addition on growth tested for the three first days of growth assays
		FA	0.64	0.04		
Boersma *et al.*, 2001	*Daphnia galeata × hyaline*	P	0.33	0.02	Supplementation of P and FA (HUFA) to natural lake seston	Correlation values used after supplementations
		FA (HUFA)	0.52	0.02		
Müller-Navarra, 1995	*Daphnia galeata*	P	0.29	0.05	Correlation of constituents *vs.* growth rate in natural lake seston	----
		FA (EPA)	1.95	0.05		
Müller-Navarra *et al.*, 2000	*Daphnia magna*	P	0.81	0.09	Correlation of constituents *vs.* growth rate in natural lake seston	Hypereutrophic pond
		FA (EPA)	2.09	0.09		
Park *et al.*, 2002	*Daphnia magna*	P	0.35	0.11	Correlation of constituent *vs.* growth rate in cultured algae	*Rhodomonas minuta*, *Scenedesmus acutus*, and *Synechococcus* sp.
		FA (ω3-PUFA)	1.19	0.11		
Plath & Boersma, 2001	*Daphnia magna*	P	0.20	0.11	Supplementation of FA emulsions to algae grown at various P levels	*Scenedesmus obliquus*
		FA (HUFAs)	0.26	0.11		
Ravet & Brett, 2006	*Daphnia pulex*	P	0.35	0.01	Supplementation of FA (Phosphatidylcholine liposome) to P-deficient algae	Cyanophytes, chlorophytes, and cryptophytes
		FA	0.35	0.01		
Wacker & Von Elert, 2001	*Daphnia. galeata*	P	1.66	0.07	Correlation of constituent *vs.* growth rate in natural lake seston	----
		FA (ALA)	2.09	0.07		
Cumulative effect size (fixed-effects models)			0.66	0.06	95% CI (0.56-0.76)	

Notes: Positive effect sizes values represent a positive effect of the constituent. The effect is considered to be significant at $p < 0.05$ when confidence limits do not bracket zero. CI stands for confident intervals. Notas: *Valores del efecto de tamaño positivos indican un efecto positivo del constituyente. El efecto se considera significativo para* $p < 0.05$ *cuando los límites de confianza no se equiparan a 0. CI significa intervalos de confianza.*

A major finding of our experimental work was that the effects of UVR and pulsed-nutrients mimicking aerosol inputs specifically affected the growth of distinct herbivorous consumer by influencing the quantity and quality of their food. We found that the response to experimental manipulation was largest for nutrient enrichment, which generated a wide food quantity gradient that most benefited the growth of the cladoceran *Daphnia* that reached a plateau at a higher seston concentration relative to the copepod and rotifer. Thus, the observation of constrained *Daphnia* growth below 1 mg C L^{-1} is coincident with the food level reported in previous classic research (Lampert & Muck, 1985). This study therefore, adds to the many predictions and observations to report that *Daphnia* growth is primarily food quantity limited (by means of seston C) in nutrient poor and arctic lakes (Persson *et al.*, 2007). Food quantity level for maximal population growth was observed at lower seston concentrations of around 500 μg C L^{-1} for *Mixodiaptomus laciniatus*. This is in agreement with the general consensus that copepods with complex life-histories, low growth rates and inferior filtering capacity than daphnids dominate the zooplankton communities in many oligotrophic lakes (Carney & Elser, 1990). In contrast high food levels resulted in slight decreased growth of the copepod *Mixodiaptomus laciniatus*, which indicates that future scenarios of increased nutrient enrichments in the Mediterranean region (Ávila & Peñuelas, 1999) could be particularly adverse to the dominance of these zooplankters, less capable of exploiting the bloom of phytoplankton associated with the P-rich allocthonous loads of aerosols of Saharan origin compared to *Daphnia*. With regards to *Keratella cochlearis* the low food level for maximum population growth of ca. 250 μg C L^{-1} indicated that this species is able to survive with extremely low amounts of food and is, therefore, often found in low food environments which cannot easily sustain larger zooplankton (Ramos-Rodriguez & Conde-Porcuna, 2003).

In the food quality bioassays we purposely concentrated on the effects of quality at low quantities by forcing algal concentration to levels that are usually found during most of the season in lakes of very low TP concentrations. In doing so, findings of this study improve our ability to predict effects of global stressors on the performance and fitness of the zooplankton in ultraoligotrophic systems

more vulnerable to global change (Marañón *et al.*, 2010). The potential for food quality constraints at low food levels was first investigated by Boersma & Kreutzer (2002). While our findings are consistent with this work, we also show that the strength of food quality limitation varied among species, was smaller than that imposed by food quantity, and was primarily induced by nutrients and to a lesser extend by UVR. A P-normalized ω3-PUFA index offered the best prediction for macrozooplankton nutrient limitation. This finding may be explained by the interconnected metabolisms of ω3-PUFA and P in autotrophs (Ahlgreen *et al.*, 1997) and their irreplaceable role for herbivorous consumers (Sterner & Elser, 2002), and is congruent with results of the meta-analysis which support the relevance of PUFA and P as single constituents for zooplankton growth (Table 7). The positive slope of the regressions between ω3-PUFA:P and macrozooplankton growth may have resulted from a greater influence of ω3-PUFA on algal food quality relative to P below the seston C:P threshold of 350 (Becker & Boersma, 2003), from an excessively P-rich seston akin to Elser's `stoichiometric knife edge´ hypothesis (Elser *et al.*, 2006), or from a combination of both. Likewise the slopes of the regression lines were different between UVR treatments (Fig. 4B, D). These results imply that UVR had an effect at low nutrient levels on the growth of the taxa by affecting a food quality component in addition to ω3-PUFA and P. We suggest that the beneficial effects of UVR (decreasing C:P ratio and increasing ω3-PUFA) might be overridden by its detrimental effect in peroxidating long-chain highly unsaturated fatty acids (HUFAs) *vs.* more resistant short-chain PUFAs (Girotti, 2001). In fact, differences in the HUFA between UVR treatments were particularly conspicuous in control enclosures receiving no nutrients where the HUFA:PUFA ratio was 43% lower in the +UVR treatment relative to the –UVR treatment.

Although knowledge of the effects that environmental stressors exert on rotifer ecology is still meager, one specific aspect that is being untangled is the determination that *Keratella* spp. is among the most UV-tolerant rotifer genus (Leech & Williamson, 2000). Indeed the inverse correlation found between *Keratella cochlearis* growth rate and seston C:P in this study indicates that UVR and nutrients may enhance the growth of this rotifer by the simultaneous

decreased in the C:P ratio of its food. We argue that cell number is fixed at birth in eutelic rotifers, and consequently may require less ω3-PUFA for membrane synthesis. Our study, consistent with previous reports of direct P limitation at low food levels (Ramos-Rodriguez & Conde-Porcuna, 2003), supports the conclusion that rotifer development is highly coupled to the availability of mineral nutrients, such as P linked to RNA necessary for protein synthesis and rapid growth.

Consistent with predictions (Santese *et al.*, 2007), the Mediterranean region is being increasingly affected by allocthonous nutrients owing to enhanced dust depositions (*see* Fig. 6A, B in Bullejos *et al.*, 2010). The negative relationship between copepod abundance and atmospheric inputs in concert with the higher capacity of *Daphnia* to exploit the high seston levels strongly suggest that changes in the intensity and frequency of aerosols could substantially affect zooplankton community structure facilitating the establishment of cladocerans and rotifers more susceptible than copepods to growth limitation by sestonic C and mineral P, respectively. Such a forecast is in agreement with the only paleolimnology study so far to report an increase in *Daphnia pulicaria* density for the last decades in a nearby Sierra Nevada lake (Conde-Porcuna *et al.*, 2009). Finally the effect of UVR on the growth of zooplankton would be species and nutrient-specific. Thus, at low nutrient conditions UVR would favor the rotifer by decreasing the C:P ratio of its food, but adversely affect growth of the copepod and *Daphnia*. These negative effects would be, however, offset under the scenario of increased nutrients associated to more frequent atmospheric loads. Clearly, reliable forecast of the effects of global change on the biological structure of aquatic ecosystems should take into account how dust deposition, UVR and precipitation may ultimately affect herbivorous consumers *via* alteration in the quantity and quality of their food.

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IV

Roles of ultraviolet radiation and nutrients in the strength of phytoplankton-zooplankton coupling

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Abstract

Ultraviolet solar radiation (UVR) and atmospheric nutrient inputs associated with aerosols are major world-wide stressors that simultaneously affect species and the interaction among them. A 2 × 5 field experimental design was used to determine how variations in light regimes [presence and absence of UVR (+UVR and –UVR)] and nutrients might influence the strength of phytoplankton-zooplankton coupling (PZC). We observed unimodal curves for zooplankton biomass in response to increased food supply from nutrient enrichment. These results challenge the 'more is better (or at least never worse)' concept, since high food levels resulted in weakened PZC. The effect of UVR on zooplankton was nutrient-dependent, significantly reducing zooplankton abundance at intermediate phosphorus (P) supplied-levels but not at the two ends of the trophic gradient generated (control and highest P level). Neither food quantity nor food quality explained observed differences in zooplankton biomass between light treatments, suggesting a deleterious direct effect of UVR on zooplankton at intermediate food ranges, resulting in a weakening of PZC. The location of this lake in the Mediterranean region has shown an increasing intensity and frequency of aerosol depositions over the past three decades (1973-2003), resulting in higher phytoplankton biomass. A combination of these higher atmospheric dust depositions with the high UVR levels characteristic of high mountain lakes might

underlie the interannual decoupling between phytoplankton and zooplankton dynamics observed in these oligotrophic ecosystems.

Resumen

La radiación ultravioleta (UVR) y las entradas atmosféricas de nutrientes asociados a los aerosoles son de los principales estresores globales que afectan simultáneamente a las especies y a la interacción entre éstas. Un diseño experimental 2 × 5 fue utilizado para determinar cómo variaciones en los tratamientos de luz [presencia y ausencia de UVR (+UVR y –UVR)] y nutrientes podrían afectar a la intensidad del acople fitoplancton-zooplancton (PZC). Observamos una respuesta unimodal de la biomasa zooplanctónica a una mayor disponibilidad de alimento debida al enriquecimiento en nutrientes. Este resultado pone en discusión el concepto "cuánto más mejor (o al menos nunca peor)", ya que una elevada cantidad de alimento dio lugar a un PZC reducido. El efecto de UVR sobre el zooplancton fue dependiente de la cantidad de nutrientes, reduciéndose así significativamente la abundancia zooplanctónica en los niveles intermedios de fósforo (P), pero no en los niveles extremos del gradiente trófico generado (correspondientes al control y al nivel más elevado de P). Ni la cantidad ni la calidad de alimento explicaron las diferencias observadas en la biomasa zooplanctónica entre ambos tratamientos de luz, lo que sugiere un efecto directo deletéreo de la UVR sobre el zooplancton para el rango intermedio de alimento, resultando así en un debilitamiento del PZC. La localización de este lago en la región mediterránea ha padecido una creciente deposición de aerosoles, tanto en intensidad como en frecuencia, a lo largo de las tres últimas décadas (1973-2003), lo que ha dado lugar a una mayor biomasa de fitoplancton. La combinación de una mayor deposición de polvo atmosférico junto a la elevada intensidad de UVR característica de los lagos de alta montaña podrían intensificar el desacople interanual entre las dinámicas de fito- y zooplancton observadas en estos ecosistemas oligotróficos.

Introduction

Global change factors, acting at different rates and spatial scales, promote changes in organisms that affect their interactions with other organisms and with the environment. Land use alterations, in combination with global warming, might generate or increase drought and dust emission in many areas and are a major driver of global change (Goudie, 2009). Higher dust activity in source areas like the Sahara might be responsible for more intensive nutrient depositions in sink areas (Neff *et al.*, 2008) such as the Mediterranean region (Santese *et al.*, 2007). These depositions are rich in phosphorus (P) (Morales-Baquero *et al.*, 2006) and might influence organisms and ecological interactions, especially in neighboring ecosystems with low nutrient availability. In addition, the interaction between global stressors adds a level of complexity to the study of global change effects. Ultraviolet radiation (UVR) is a major world-wide stressor with far-reaching implications for ecological interactions and an overall negative effect on the survival and growth of organisms (Bancroft *et al.*, 2007; Häder *et al.*, 2007). However, few studies have reported the effects of the interaction between global stressors, such as UVR and nutrient inputs, on trophic interactions (Medina-Sánchez *et al.*, 2006).

One crucial question is how global stressors affect the strength of the coupling between primary producers and herbivorous consumers, which has consequences for food web structure and the efficiency with which energy moves to higher trophic levels. The relevance in the study of the primary producer-consumer interface has increased since the recognition that it is at this level where nutrient imbalances are among the highest in nature (Sterner & Elser, 2002). Considerable research efforts have focused on aquatic systems due to the high turnover rates of the primary producers. To date, authors have investigated how the coupling between algae and zooplankton varies across trophic gradients (Elser *et al.*, 1990; Auer *et al.*, 2004; Hessen *et al.*, 2006) or in eutrophic ecosystems (Abrantes *et al.*, 2006). While there is empirical evidence of hump-shaped trends when zooplankton biomass is plotted against total phosphorus (TP) (Carney *et al.*, 1990; Persson *et al.*, 2007), others have reported logarithmic responses to the

increase in nutrients (Hessen *et al.*, 2006). Numerous studies have contributed new data on the mechanisms behind phytoplankton-zooplankton coupling (PZC). Durant *et al.* (2005) showed that food resource and consumer levels also determine the strength of PZC. Other characteristics that have been identified to qualitatively affect the strength of PZC include food quality (Dickman *et al.*, 2008), phytoplankton taxonomic composition (Auer *et al.*, 2004), zooplankton diversity (McCann *et al.*, 1998), alternative food resources such as microphytobenthos or microbial communities (Rautio & Vincent, 2006), and zooplankton predators (Hessen *et al.*, 2006; Dickman *et al.*, 2008). All of these might be prone to the effect of global stressors. While the role of global warming on PZC is well studied (Winder & Schindler, 2004; Domis *et al.*, 2007; Sommer *et al.*, 2007), additional work is required to elucidate the contribution of other joint global factors. There has been enormous research interest in developing our understanding of the response of organisms and ecosystems to the interaction between UVR and other global stressors (Williamson *et al.*, 2002; Vinebroke & Leavitt, 2005). However, the nature of this interaction is difficult to predict, and effects are not necessarily straightforward. For example, Carrillo *et al.*, (2008) demonstrated that P inputs from dust unmask deleterious UVR effects on algae instead of attenuating these effects, as expected. It was also reported that P-inputs increased algal biomass and that both P-inputs and UVR exposure reduced the seston carbon:phosphorus (C:P) ratio (Xenopoulos *et al.*, 2002; Carrillo *et al.*, 2008; Hessen *et al.*, 2008). As pointed out by these authors, these effects might positively enhance consumer growth by simultaneously improving food quantity and quality.

The aim of this study was to determine how the interaction between UVR and nutrient availability (mimicking atmospheric dust depositions) might affect the strength of PZC *via* changes in the quantity and quality of food for herbivore grazers. Our prediction was that plentiful food of high quality due to combined P-fertilization and UVR would promote high herbivore growth and, consequently, the strength of PZC. We tested this hypothesis in an experiment in which large mesocosms were incubated *in situ* in the presence and absence of UVR across an experimental P-gradient in a high-mountain lake. High-mountain lakes are

'sentinels of change' (Williamson *et al.*, 2009), offering ideal natural scenarios for testing the effects and mechanisms of climate change. These types of lakes are ultra-sensitive to atmospheric inputs, largely due to the oligotrophy associated with their remote location. Their high altitude exposes them to extreme UVR environments and favors the interception of dust-transporting atmospheric winds, especially when lakes are near major dust-emission sources (Morales-Baquero *et al.*, 2006; Di Ioiro *et al.*, 2009).

To assess whether changes in nutrients and UVR affect the long-term dynamics of phytoplankton-zooplankton populations in natural ecosystems, we analyzed phytoplankton and zooplankton biomass collected in Lake La Caldera from 1973 in relation to long-term aerosol index (AI) data as a proxy for P-deposition (Morales-Baquero *et al.*, 2006) and UV irradiances derived from original data of National Aeronautics and Spatial Administration (NASA).

Methods

Study site

The study was performed in Lake La Caldera in the National Park of Sierra Nevada (Spain, 36º55´-37º15´N, 2º31´-3º40´W) at an elevation of 3050 m above sea level (m.a.s.l.). La Caldera is a small lake with a surface area of ~0.02 km^2, maximum depth of <10 m and a mean depth of ~3 m immediately after ice-out in 2003. The ice-free period usually extends from the middle of June to the end of October, when temperature fluctuates between 5ºC and 15ºC. During this season, UVR of considerable intensity penetrates deeply in the lake (Table 1) due to the high transparency of the water (Secchi´s disk visibility reaching maximum depth) and low values of dissolved organic carbon (<1 mg L^{-1} as reported in references in Carrillo *et al.*, 2008). The pelagic community was strongly P limited during 2003, with a dissolved inorganic nitrogen:total phosphorus (DIN:TP) ratio of ~100 (by mass) (Carrillo *et al.*, 2008), which is characteristic of this lake (Villar-Argaiz *et al.*, 2001). Its altitude and geographical position very close to

Africa make it prone to high allocthonous P inputs from dust, since Saharan dust plumes are deposited within the first 2000 km, largely between 1500 and 4000 m.a.s.l. (Morales-Baquero *et al.*, 2006).

Date (N^{er} of days in period)	K_d UVB	K_d UVA	K_d PAR
01 August 2003 (1)	0.23	0.15	0.09
03 August 2003 (3)	0.11	0.07	0.05
20 August 2003 (20)	0.46	0.32	0.27
01 September 2003 (32)	0.36	0.23	0.17
11 September 2003 (42)	0.24	0.15	0.14
24 September 2003 (55)	0.13	0.06	0.07

Table 1. Diffuse attenuation coefficients for downward irradiance (K_d) in Lake La Caldera from different sampling days during experimental period in 2003 measured using a LI-8000 spectroradiometer (LI-COR). Days of the experimental period are given in brackets after dates. K_d were determined from the slope of the linear regression of the natural logarithm of downwelling irradiance *vs.* depth for each region of the solar radiation spectrum: UVB (300–319 nm), UVA (320–399 nm), and PAR (400–800 nm). *Coeficientes de atenuación difusa para la radiación incidente (K_d) del lago La Caldera para los diferentes días de muestreo durante el período experimental del 2003 medidos mediante un espectroradiómetro LI-8000 (LI-COR). Los días del período experimental vienen dados entre paréntesis a continuación de las fechas.* K_d *están determinados a partir de la pendiente de la regresión lineal del logaritmo natural de la radiación incidente* vs. *la profundidad para cada región del espectro de radiación solar: UVB (300-319 nm), UVA (320-399 nm), y PAR (400-800 nm).*

The phytoplankton community is mainly represented by *Chromulina nevadensis* (Chrysophyceae), *Dictyosphaerium chlorelloides* (Chlorophyceae) and *Cyclotella* sp. (Bacillariophyceae) (Delgado-Molina *et al.,* 2009). Herbivorous consumers are the highest trophic level in the pelagic community, and the calanoid copepod *Mixodiaptomus laciniatus* is the dominant zooplanktonic species (>90% in zooplankton biomass). Other zooplankton species such as *Daphnia pulicaria*, linked to littoral areas, or *Hexarthra bulgarica*, are scarce (Carrillo *et al.*, 1995).

In situ *experiment*

An *in situ* experiment was carried out with a 2 × 5 factorial design: Two light treatments [full-sunlight (+UVR) *vs.* photosynthetic active radiation (-UVR)] and five nutrient treatments. The unreplicated factorial design carried out to test interactive effects (UVR × P in this study) proved useful in previous studies (Carrillo *et al.*, 2008). Each treatment consisted of one mesocosm made of clear polyethylene tubes (0.7 m diameter × 7 m length), closed at the bottom, with a total volume of 2.7 m^3. A water pump was used to fill each mesocosm with unfiltered lake water collected from 3 m depth (photic layer affected by >5% of UVB). The ten mesocosms were set in two 3 × 3 m^2 racks made of 3 cm polyvinyl chloride pipe; each rack contained five enclosures for each light treatment. The two racks were separated by approximately 50 m to avoid shading effects. Both subsets were secured to a buoy attached to an anchored rope.

The +UVR treatment was obtained by using polyethylene plastic that transmits 90% of photosynthetic active radiation [PAR (400-700 nm)] and most of UVR [75% of UVA (320-399 nm) and 60% of UVB (295-319 nm)]. The –UVR treatment was obtained by using a cover of Plexiglass UF3, a long-wave-pass plastic that transmits 90% of PAR but blocks UVR (<390 nm). Optical properties of the cut-off filters used in light treatments were tested before experiments with a double-beam spectrophotometer (Perkin-Elmer Lambda 40). Further, the rack (subset) containing –UVR enclosures was surrounded by 2 m^2 layers of Plexiglass

UF3 below the lake surface to prevent incidence of refractory solar UVR. Comparisons between radiation water profiles (affected by >25% of UVB) within and outside the bags receiving no nutrients showed transmittances of 56% of UVB, 72% of UVA, and 73% of PAR in the +UVR treatments, mimicking natural conditions reasonably well. In the –UVR treatments, attenuations were 82% of UVB, 70% of UVA, and 17% of PAR, *i.e.*, considerably blocking UVR.

The five P-enrichment levels were set by adding a final concentration of 0, 20, 30, 40, and 60 μg P L^{-1} (as NaH_2PO_4). The enclosure with no added nutrient and +UVR served as control for nutrient-enriched enclosures and reproduced the closest conditions to those of the lake, with soluble reactive phosphorus (SRP) concentrations <1 μg P L^{-1}. The added nutrient concentrations generated the gradient produced by the natural atmospheric depositions of P. Although the 60 μg P L^{-1} treatment duplicated the maximum dissolved P concentration measured in this ecosystem after an allocthonous input (Villar-Argaiz *et al.*, 2001), it remained below the estimation of 81.4 μg P L^{-1} for a single event calculated from weekly collected atmospheric inputs in the lake area (Morales-Baquero *et al.*, 2006).

Previous investigations in this lake established clear connections between atmospheric deposition and lake nutrient concentrations (Morales-Baquero *et al.*, 2006). Highly similar concentrations in the P- and N-dissolved and total fractions were described immediately after an allocthonous input (Villar-Argaiz *et al.*, 2002*b*), implying that most of the nutrients adhered to dust particles are readily available for phytoplankton uptake. Hence, the nutrient pulses in our experiment represented an appropriate simulation of natural inputs. The amount of P to be added was calculated from the dissolved total phosphorus concentration found in the water column on the day before starting the experiment. To ensure that P remained as limiting nutrient, inorganic nitrogen (as NH_4NO_3) was added to reach a nitrogen:phosphorus (N:P) molar ratio of 30, mimicking the mean atmospheric dust TN:TP ratio (10-50, as reported by Morales-Baquero *et al.*, 2006). Nitrate and ammonium are major inorganic N compounds in the water-soluble fraction of aerosol particles (Chen *et al.*, 2007), supporting their use as N source in the

present experiment. After nutrient addition and before taking samples, the water in mesocosms was vigorously mixed with a plastic bucket to avoid problems associated with the patchy vertical and horizontal distribution of organisms. Sestonic and zooplankton samples were taken in triplicate from three randomly chosen mesocosms to determine the initial experimental conditions (*see* sampling methods below). The coefficient of variation [CV = (standard deviation / mean) ×100] for algal biomass and zooplankton abundance never exceeded 3%. Finally, the top of each enclosure was covered (polyethylene for +UVR and Plexiglass UF3 for –UVR) to avoid external nutrient inputs during the incubation period but allow air exchange.

The experiment lasted for 70 days, *i.e.*, most of the ice-free season (from 01 August to 10 October 2003). Sampling was performed on days 1, 3, 11, 20, 32, 42, 55, and 71, after mixing the entire length of the enclosure. The sampling frequency was appropriate for the study of phytoplankton dynamics, and the experiment was sufficiently long to allow calanoid copepods, with their low growth rates, to reach adulthood (Villar-Argaiz *et al.*, 2002*a*).

Chemical and biological analyses

Water samples for nutrients (DIN and SRP), sestonic elemental composition (C, N, and P), chlorophyll *a* (Chl *a*), and phytoplankton abundance were taken in triplicate with a plastic bucket after gently mixing the entire length of the enclosure before sampling and prefiltering with a 40 μm mesh to remove zooplankton. Zooplankton samples to determine abundance and biomass were taken using one vertical tow of a 40 μm mesh zooplankton net (12.5 cm diameter), which, covering the full depth of the enclosure, sampled 3% of the total enclosure volume. Immediately afterwards, samples were preserved in 4% formaldehyde. Additional zooplankton samples were also collected and brought in mesocosms water under dark and cold conditions for C-biomass determinations. The above variables were also monitored at three depths (0.5, 4, and 8 m) at a central station of the lake. We used a Van Dorn sampler for nutrients and seston and obtained

zooplankton samples for abundance and biomass determinations after sieving 12 L of water from each depth through a 40 μm mesh.

Samples for DIN and SRP were analyzed on the same day as their collection. DIN was considered the sum of NO_3^-, NO_2^-, and NH_4^+, which were determined by UV-spectrophotometric screening and sulphanilamide and phenol-hypochlorite techniques, respectively. SRP was analyzed by means of the acid molybdate technique (APHA, 1992). For sestonic C, N, and P determinations, samples were filtered through precombusted (1h at 550ºC) 1 μm glass fiber filters (Whatman GF/B) at low pressure (<100 mm Hg). Filters were then immediately analyzed for P, dried (24 h at 60ºC), and kept desiccated until C and N analysis. Particulate C and N were determined using a Perkin-Elmer model 2400 (Perkin-Elmer Corporation) elemental analyzer. For the analysis of particulate P, filters were introduced into acid-washed vials, digested with a mixture of potassium persulfate and boric acid at 120ºC for 30 min and determined as SRP in 10 cm quartz cuvettes using the acid molybdate technique (APHA, 1992). Blanks and standards were performed for all procedures. All C:N, C:P, algal C biomass:N, and algal C biomass:P ratios were calculated on a molar basis. Chl *a* was measured fluorimetrically after grinding filters (Whatman GF/F glass fiber filter, 25 mm diameter) with pigments (concentrated by filtration of up to 300 mL at <100 mm Hg of pressure) and extracting the pigments in 90% acetone kept in the dark at 4ºC for 24 h. A Chl *a* standard (Fluka Chl *a* from algae) was used to transform the fluorescence data into Chl *a* concentrations.

Phytoplankton was preserved by using Lugol´s reagent. Cells were counted in 100 randomly selected fields of view at ×2000 magnification under an inverted microscope (Leitz, Fluovert FS, Leica). Between 20 and 30 cells of each species were measured for each date, using image analysis (Quantimet 500, Leica) to estimate cell volume according to a corresponding geometrical shape. The biovolume density (mm^3 mL^{-1}) for each taxon was determined by multiplying mean cell volume by abundance. Cell volume was converted to C by using specific conversion factors (Carrillo *et al.*, 2008).

For zooplankton C-content, copepods from each mesocosm were identified to stage level, measured with the aid of a stereomicroscope, sorted alive into specific Petri dishes containing GF/F-filtered lake water, and then transferred to secondary Petri dishes with deionized water. Between 40 and 70 individuals of the most abundant stages were analyzed for C in triplicate following the methods described for seston. Zooplankton abundance was determined by counting under an inverted microscope at ×100 magnification. For each sample, 20 individuals of each species or copepod stage were measured by image analysis (Quantimet 500, Leica). Subsequently, zooplankton biomass was calculated by using the length-weight relationships specifically developed for the zooplankton species in this ecosystem (Carrillo *et al.*, 2001).

Long-term biological data series

Data on phytoplankton and zooplankton are available since 1973 and 1975, respectively. Samples were collected during the ice-free season of the lake at a maximum depth station. Standard collection protocols and original data are in Martinez (1977), Cruz-Pizarro (1981), Carrillo *et al.* (1995), Villar-Argaiz *et al.* (2001), Medina-Sánchez *et al.* (2004), Pulido-Villena (2004), and Delgado-Molina *et al.* (2009). Briefly, water for phytoplankton abundance and biomass determinations was taken from 3 or 4 depths (0.5 m below the surface, 0.5 m above the bottom and at one or two intermediate points) and, after filtration through a 40 μm mesh to remove zooplankton, preserved with Lugol´s reagent (1% vol/vol). Counts, measurements, and biovolume determinations were made as described above. Biomass (μg fresh weight L^{-1}) was obtained from biovolume-density conversions, assuming a specific weight of 1. Zooplankton samples to estimate abundance and biomass were obtained after sieving 18 or 24 L of water from different depths through a 40 μm mesh, and they were immediately fixed in 4% formaldehyde. Counts and size determinations were done as described above and biomass was estimated using standard length-biomass relationships (Bottrell *et al.*, 1976) or relationships specifically developed for the species in this system (Cruz-Pizarro, 1981; Carrillo *et al.*, 2001). A minimum of four measurements

were taken each sampled year, and the biomass values reported are annual averages.

Remote sensing

As a measure of aerosol content in the troposphere, we used the AI developed by the Ozone Processing Team [NASA – Goddard Space Flight Center (GSFC)] from measured irradiances by the Total Ozone Mapping Spectrometer (TOMS) on board the *Nimbus 7* (1978-1993) and *Earth Probe* (1996-2004) NASA satellites. Aerosol data were successfully used for the study of Saharan dust in previous studies of this ecosystem, due to the highly positive correlation of TOMS AI with total phosphorus (TP) and particulate matter (PM) linked to dry atmospheric deposition (Morales-Baquero *et al.*, 2006). We used annual averages of weekly TOMS AI data given for 37.5ºN, 3.075ºW (closest geographic coordinates to the lake) as a measure of the intensity of aerosol deposition. An AI value >0.5 was considered to represent a deposition event, and the annual frequency of these events was calculated as the percentage of days affected by aerosol deposition events.

Average annual UVR values were calculated by using 325 nm wavelength data given for 37.5ºN, 3.5ºW (closest geographic coordinates to the lake) and only considering the period corresponding to the ice-free season (approximately from 01 June to 15 November). This wavelength represented an intermediate value among the available wavelengths (305, 310, 325, and 380 nm). Original data are available at http://jwocky.gsfc.nasa.gov.

Statistical analyses

Simple linear regression analysis was used to test the effects of 1) experimental time on zooplankton biomass; 2) P-enrichment on TP; 3) TP on algal standing stock variables (algal biomass, Chl *a*, sestonic C, N, and P); 4) food

quality variables (sestonic C:N and C:P, algal C biomass:N, algal C biomass:P, Chl *a*:sestonic C) on zooplankton biomass; 5) time on TOMS AI, annual frequency of aerosol deposition events, and UV 325 nm irradiance; and 6) TOMS AI and annual frequency of aerosol deposition events on phytoplankton biomass.

When the regression was significant, a homogeneity of slopes model (ANCOVA) was used to test the effect of light treatment (categorical factor: +UVR, -UVR) across the continuous predictor variables (covariates: Time, P-enrichment and TP) on the response variables (zooplankton biomass, TP, and algal standing stock variables) (Quinn & Keough, 2002). When no significant differences were found in the *y*-axis intercepts, UVR effect was graphically checked along the covariate by examining 95% confidence intervals of the regression lines (*see* Urabe *et al.*, 2002 for a similar statistical analysis). When no significant regressions were observed, differences due to UVR were tested by a paired *t*-test. Paired *t*-tests were also used to examine differences in zooplankton abundance, size, and C-content due to UVR at each nutrient level.

Polynomical regression models were used to test the effects of TP and food quantity variables (algal biomass, Chl *a*, sestonic C, N, and P) on zooplankton biomass for each light treatment, determining the UVR effect by examining 95% confidence intervals of the regression lines, and the relationship between phytoplankton and zooplankton biomass (long-term biological data series). Statistical analysis of the experimental results, except for zooplankton temporal changes, considered mean values after the second week of the incubation (n = 5) to allow for the lag-response of zooplankton (dominated by a slow-growth copepod species) to experimental manipulation. Analyses of temporal variations in zooplankton biomass considered values for single days (n = 5). Normality was tested by Shapiro-Wilks' *W*-test and homoscedasticity by Levene's test. STATISTICA 7.0 for Windows software (Stat Soft, 2001) was used for the statistical analyses.

Results

UVR and P-enrichment effects on temporal dynamics

The temporal dynamics of DIN:SRP ratio and algal biomass are depicted in Fig. 1. The ratio followed a similar pattern in the two light treatments. The controls showed few changes in this ratio over the experimental period, with a mean value around 550, similar to the initial lake DIN:SRP ratio. The initial ratio in the nutrient-enriched enclosures was very similar to the experimental DIN:SRP ratio of 30, which increased up to day 20 and then progressively decreased until the end of the experimental period (Fig. 1A,B). No appreciable changes in algal biomass were detected in the control enclosures and the lake, with values remaining low throughout the experimental period. Algal biomass strongly augmented in response to nutrient enrichment up to day 32 and then steadily declined until the end of the experiment (Fig. 1C,D). Patterns were similar for other phytoplankton variables (Chl *a*, sestonic C, N, and P) due to their strong intercorrelations (all correlations $r \geq 0.90$ and $p < 0.05$). Phytoplankton was mainly composed of *Chromulina nevadensis* until day 20, but *Dictyosphaeium chlorelloides* dominated the algal community thereafter (Delgado-Molina *et al.*, 2009).

The calanoid copepod *Mixodiaptomus laciniatus* represented nearly 100% of the zooplankton biomass in the enclosures. The zooplankton assemblage was initially composed largely of copepodite stage III, with most reaching adulthood by the end of the experiment, *i.e.*, 70 days later. Fig. 2 shows temporal variations in zooplankton biomass for each UVR and nutrient treatment. Zooplankton biomass in the controls was low and did not show major temporal changes, reflecting the lake dynamics. Zooplankton biomass strongly increased in response to nutrient enrichment with the exception of the highest P-enriched treatment, in which the biomass barely changed and eventually decreased to a level lower than the control. Zooplankton biomass decreased after day 20 in enclosures 20 and 30 μg P L^{-1} and after day 42 in enclosures 40 and 60 μg P L^{-1}. These decreasing

trends were linear for +UVR in the 20 μg L^{-1} enclosure and for both light treatments in the 30 μg P L^{-1} enclosures (Table 2).

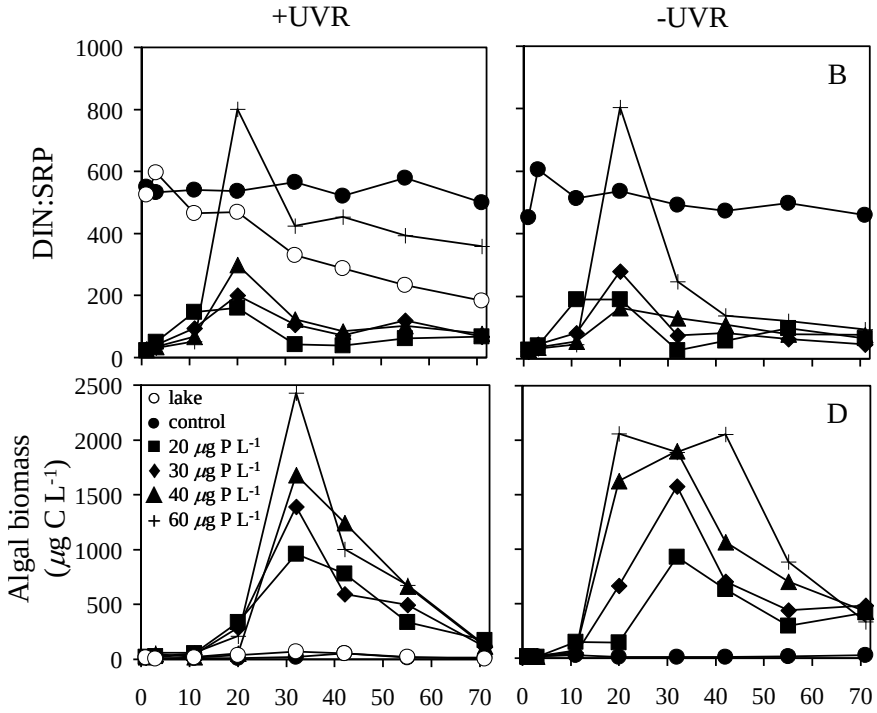

Figure 1. Temporal dynamics of dissolved inorganic nitrogen:soluble reactive phosphorus (DIN:SRP) and algal biomass under (A,C) +UVR and (B,D) –UVR in the lake, non-enriched (control) and P-enriched (20, 30, 40, and 60 μg P L^{-1}) treatments during the experimental period. Each point represents a single day. *Dinámicas temporales de nitrógeno inorgánico disuelto:fósforo reactivo soluble (DIN:SRP) y biomasa algal para (A,C) +UVR y (B,D) –UVR en el lago, tratamientos no enriquecidos (control) y enriquecidos en P (20, 30, 40, y 60* μg *P* L^{-1}*) durante el período experimental. Cada punto representa un día de muestreo.*

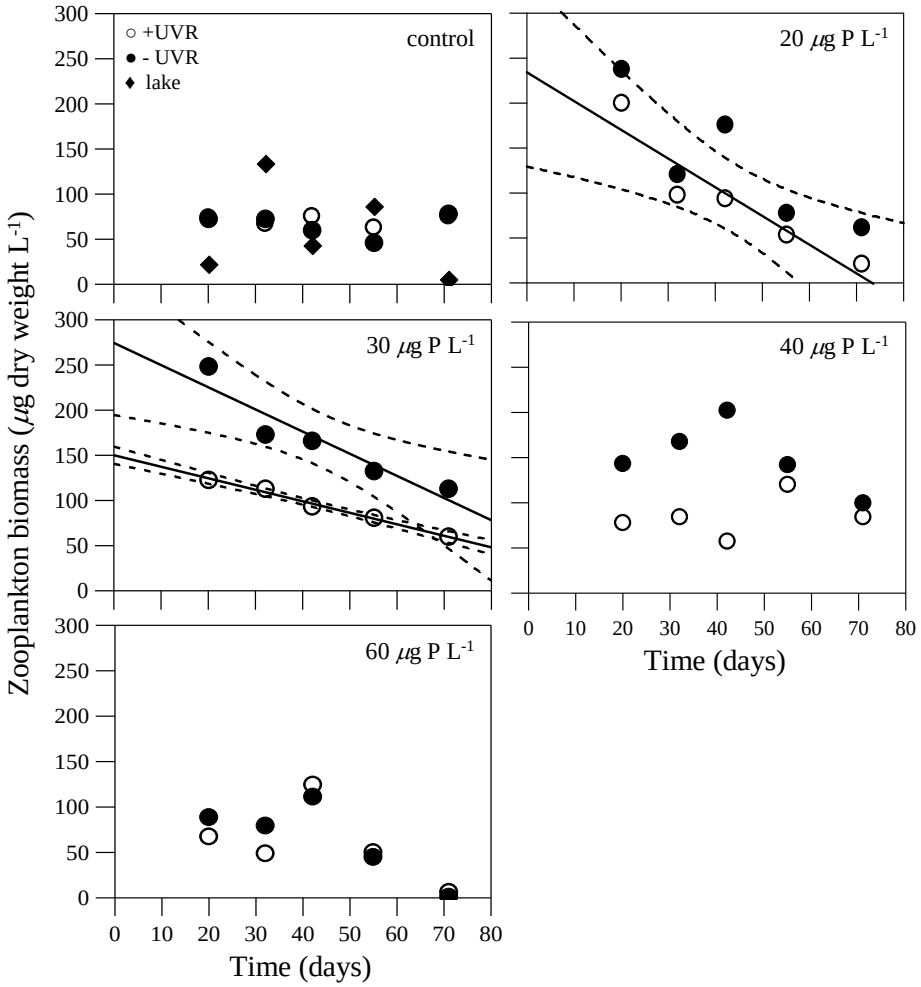

Figure 2. Relationships between experimental time and zooplankton biomass under +UVR and –UVR for non-enriched (control) and P-enriched (20, 30, 40, and 60 μg P L^{-1}) treatments. Dashed lines indicate 95% confidence intervals around the fitted regression lines (solid lines). Dots represent values for each of the last five sampling days for each light treatment. *Relaciones entre el tiempo experimental y biomasa zooplanctónica para +UVR y –UVR para los tratamientos no enriquecidos (control) y enriquecidos en P (20, 30, 40 y 60 μg P L^{-1}). Las líneas discontinuas indican los intervalos de confianza del 95% a ambos lados de las rectas de regresión (líneas continuas). Los puntos representan los valores para cada uno de los cinco últimos días de muestreo para cada tratamiento de luz.*

P-enrichment treatment (μg P L^{-1})	Light treatment	*b*	*a*	R^2	*p*-value
0	+UVR	-0.01	71.43	0.001	0.953
	–UVR	-0.06	68.53	0.009	0.880
20	+UVR	**-3.20**	**234.25**	**0.876**	**0.019**
	–UVR	-3.17	274.95	0.749	0.058
30	+UVR	**-1.27**	**150.11**	**0.992**	**<0.001**
	–UVR	**-2.45**	**274.46**	**0.877**	**0.019**
40	+UVR	0.38	68.40	0.114	0.579
	–UVR	-1.02	196.18	0.291	0.349
60	+UVR	-1.10	108.34	0.261	0.379
	–UVR	-1.77	143.46	0.663	0.093

Table 2. Results of regression analyses of the effect of experimental time (days) (*x*) on zooplankton biomass (μg dry weight L^{-1}) (*y*) for each light treatment at each P-enrichment level (μg P L^{-1}) for the last five sampling days. Regression model was: $y = bx+a$. Significant regressions are shown in bold. *Resultados de los análisis de regresión de los efectos del tiempo experimental (días) (*x*) sobre la biomasa zooplanctónica (*μg peso seco L^{-1}*) (*y*) para cada tratamiento de luz en cada nivel de enriquecimiento en P (*μg P L^{-1}*) durante los últimos cinco días de muestreo. El modelo de regresión fue:* y = bx+a. *Las regresiones significativas se muestran en negrita.*

At the intermediate P-enriched level (30 μg P L^{-1}), zooplankton biomass was significantly higher in the –UVR than in the +UVR enclosure (analysis of covariance: Intercept, $F_{1,6} = 282.45$, $p < 0.001$; slope, $F_{1,6} = 4.90$, $p = 0.068$). Treatments that did not show linear temporal trends were compared using *t*-tests, revealing that zooplankton biomass was also consistently higher in the 20 (*t*-test, $t = -3.80$, df = 4, $p = 0.019$) and 40 (*t*-test, $t = -2.81$, df = 4, $p = 0.048$) μg P L^{-1} treatments in the absence *vs.* presence of UVR.

The effects of UVR on zooplankton abundance, individual size and C-content were analyzed in order to elucidate the mechanisms responsible for the higher zooplankton biomass accrual in –UVR enclosures at intermediate P-enriched treatments. Mean zooplankton abundance for the last five sampling dates was 27%, 41%, and 61% higher in –UVR *vs.* +UVR enclosures for the 20 (*t*-test, $t = -3.81$, df = 4, $p = 0.019$), 30 (*t*-test, $t = -4.08$, df = 4, $p = 0.015$), and 40 (*t*-test, $t = -3.25$, df = 4, $p = 0.031$) μg P L^{-1} treatments, respectively (Fig. 3A). However, no UVR-induced size differences were observed (all *t*-tests, $p > 0.05$). The C-content of zooplankton was 33% and 29% lower in the absence *vs.* presence of UVR for the 20 (*t*-test, $t = 2.89$, df = 4, $p = 0.04$) and 30 μg P L^{-1} (*t*-test, $t = 3.89$, df = 4, $p = 0.018$) enclosures, respectively (Fig. 3B).

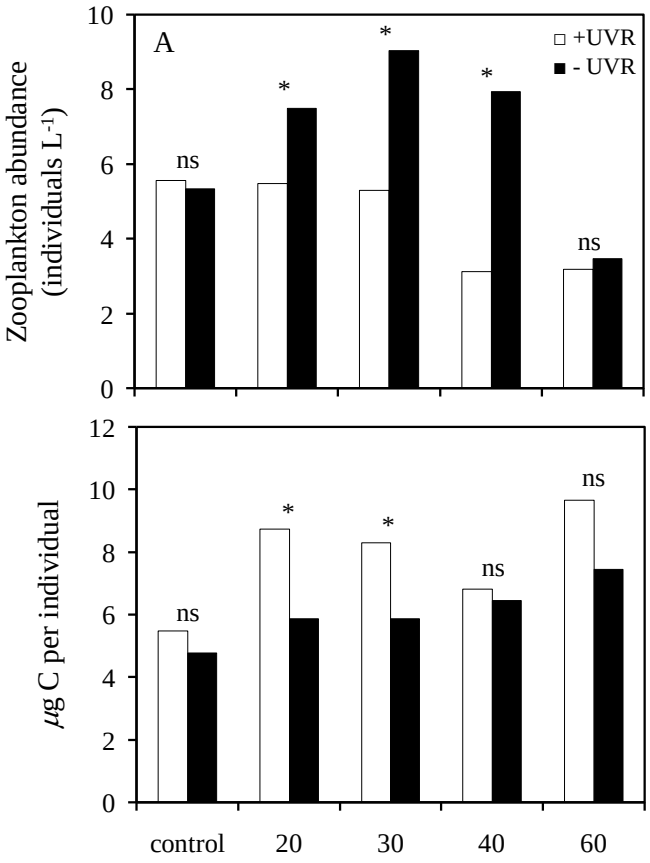

Figure 3. (A) Zooplankton abundance and (B) C-content per individual under +UVR and –UVR treatments in non-enriched (control) and P-enriched (20, 30, 40, and 60 μg P L^{-1}) treatments. Results of paired *t*-test comparisons between light treatments for each phosphorus level: * $p < 0.05$; ** $p < 0.01$; ns – not significant. Values represent the mean for the last five sampling dates. *(A) Abundancia zooplanctónica y (B) contenido en C por individuo para +UVR y –UVR para los tratamientos no enriquecidos (control) y enriquecidos en P (20, 30, 40, y 60* μg *P* L^{-1}*). Los resultados de las comparaciones por análisis* t-*test para muestras apareadas entre tratamientos de luz para cada nivel de fósforo:* * p < *0.05;* ** p < *0.01; ns – no significativo. Los valores representan la media para los últimos cinco días de muestreo.*

UVR and P-enrichment effects on phyto-zooplankton interface

A strong relationship was found between P-enrichment level and TP (Table 3), but no systematic differences were detected as a function of light or of the interaction between P-enrichment and light (Table 4). We therefore used TP as a predictor of algal and zooplankton biomass. Algal biomass, Chl *a*, and sestonic C, N, and P were positively correlated with TP in both +UVR and –UVR enclosures (Fig. 4A, Table 3). Although no differences were detected due to UVR, we observed significant UVR × P synergistic interactive effects on algal biomass and Chl *a*, but not on sestonic C, N, and P (Table 4). Examination of the UVR × P interaction effect in Fig. 4 showed no differences in algal biomass with the exception of the highest P-enriched level (Fig. 4A, Table 4).

Response of zooplankton biomass to the TP gradient was unimodal for both light treatments (Fig. 4B, Table 3). Differences due to UVR were significant for intermediate P-enriched levels receiving 20, 30, and 40 μg P L^{-1} (Fig. 4B). Mean zooplankton biomass in the 20, 30, and 40 μg P L^{-1} treatments was 31%, 44%, and 44% lower, respectively in +UVR *vs.* –UVR enclosures. The unimodal fits of zooplankton biomass were also significant for algal biomass, Chl *a*, and sestonic P (Fig. 5, Table 5).

We then examined whether food quality variables explained the differences observed in zooplankton biomass at intermediate P-enriched levels. Food quality in terms of the sestonic C:N molar ratio ranged from 6.0 to 8.5 in +UVR and from 6.1 to 9.7 in –UVR enclosures. Sestonic C:P ratios ranged from 159.4 to 289.5 in +UVR and from 184.6 to 339.6 in –UVR enclosures (Table 6). The only significant relationships between zooplankton biomass and seston food quality variables were for sestonic C:N in the –UVR treatment and sestonic C:P in the +UVR treatment, which showed positive regression slopes (Table 7).

Dependent variable (y)	Independent variable (x)	Light treatment	c	b	a	R^2	p-value
Total P	P-enrichment	+UVR	-	**0.65**	**0.18**	**0.988**	**<0.001**
		–UVR	-	**0.61**	**0.52**	**0.984**	**<0.001**
Algal biomass	Total P	+UVR	-	**20.95**	**141.69**	**0.856**	**0.024**
		–UVR	-	**40.11**	**17.20**	**0.978**	**0.001**
Chlorophyll *a*	Total P	+UVR	-	**2.21**	**8.73**	**0.932**	**0.008**
		–UVR	-	**3.70**	**5.88**	**0.979**	**0.001**
Sestonic C	Total P	+UVR	-	**33.79**	**388.76**	**0.770**	**0.050**
		–UVR	-	**45.20**	**387.84**	**0.821**	**0.034**
Sestonic N	Total P	+UVR	-	**5.18**	**57.55**	**0.834**	**0.003**
		–UVR	-	**6.85**	**43.20**	**0.941**	**0.006**
Sestonic P	Total P	+UVR	-	**0.55**	**1.48**	**0.991**	**<0.001**
		–UVR	-	**0.64**	**0.48**	**0.996**	**<0.001**
Zooplankton biomass	Total P	+UVR	**-0.07**	**2.71**	**68.03**	**0.950**	**<0.050**
		–UVR	**-0.32**	**12.28**	**46.10**	**0.990**	**<0.050**

Table 3. Results of regression analyses of the effect of P-enrichment on total P and of total P on algal biomass, chlorophyll *a*, sestonic C, N, P, and zooplankton biomass in both light treatments (+UVR, –UVR). Units are: μg P L^{-1} for P-enrichment and total P; μg C L^{-1} for algal biomass; μg L^{-1} for chlorophyll *a*, sestonic C, N, and P; and μg dry weight L^{-1} for zooplankton biomass. Regression model was: $y = cx^2+bx+a$. Significant regressions are shown in bold. *Resultados de los análisis de regresión del efecto del enriquecimiento en P sobre P total y de P total sobre la biomasa algal, clorofila* a, *C, N, P del seston, y la biomasa zooplanctónica para ambos tratamientos de luz. Las unidades son:* μg *P* L^{-1} *para el enriquecimiento en P y para P total;* μg *C* L^{-1} *para biomasa algal;* μg L^{-1} *para clorofila* a, *C, N, y P del seston; y* μg *peso seco* L^{-1} *para la biomasa zooplanctónica. El modelo de regresión fue:* $y = cx^2+bx+a$. *Las regresiones significativas se muestran en negrita.*

Response variable	Covariate	Intercept		Slope	
		$F_{1,6}$	p-value	$F_{1,6}$	p-value
Total P	P-enrichment	0.10	0.760	0.37	0.565
Algal biomass	Total P	1.28	0.301	**9.83**	**0.020**
Chlorophyll *a*	Total P	1.87	0.221	**10.24**	**0.018**
Sestonic C	Total P	4.37	0.081	0.50	0.507
Sestonic N	Total P	6.84	0.039	0.99	0.358
Sestonic P	Total P	5.18	0.063	5.24	0.062

Table 4. Results of analysis of covariance (ANCOVA) to test the effects of UVR and covariates (P-enrichment and total P) on response variables (total P, algal biomass, chlorophyll *a*, sestonic C, N, and P). Units are: μg P L^{-1} for P-enrichment and total P; μg C L^{-1} for algal biomass; and μg L^{-1} for chlorophyll *a*, sestonic C, N, and P. Significant results are shown in bold. *Resultados del análisis de covarianza (ANCOVA) para testar los efectos de UVR y las covariables (enriquecimiento en P y P total) sobre las variables respuesta (P total, biomasa algal, clorofila* a, *C, N, y P del seston). Las unidades son:* μg *P* L^{-1} *para el enriquecimiento en P y P total;* μg *C* L^{-1} *para la biomasa algal; y* μg L^{-1} *para clorofila* a, *C, N, y P del seston. Los resultados significativos se muestran en negrita.*

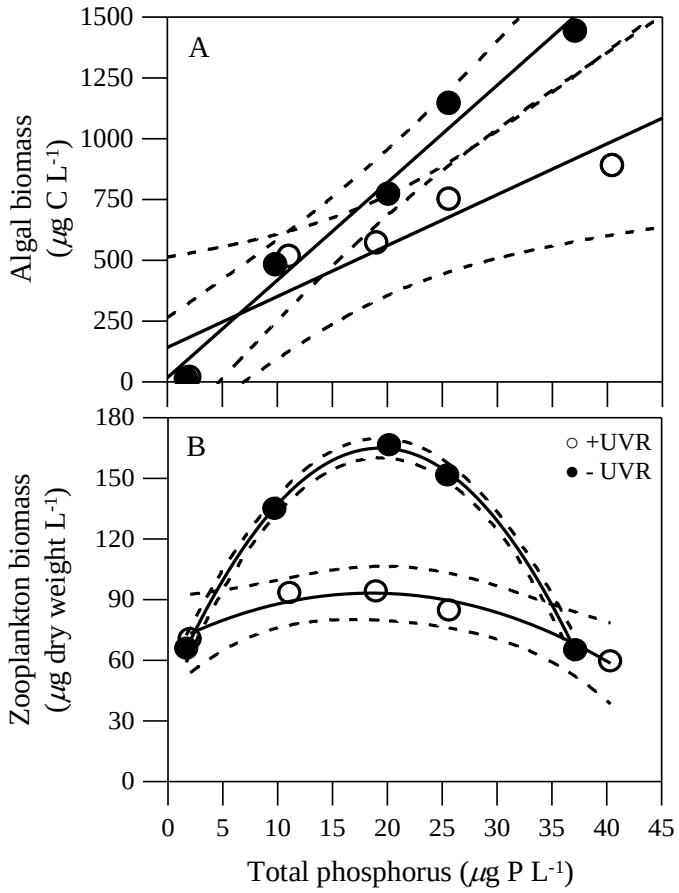

Figure 4. Response of (A) algal and (B) zooplankton biomass to the total phosphorus trophic gradient under +UVR and –UVR treatments. Dashed lines indicate 95% confidence intervals around the fitted models (solid lines). Values represent the mean for the last five sampling dates. *Respuesta de (A) biomasa algal y (B) zooplanctónica al gradiente de fósforo total para los tratamientos +UVR y –UVR. Las líneas discontinuas indican los intervalos de confianza del 95% a ambos lados de los modelos ajustados (líneas continuas). Los valores representan la media para los últimos cinco días de muestreo.*

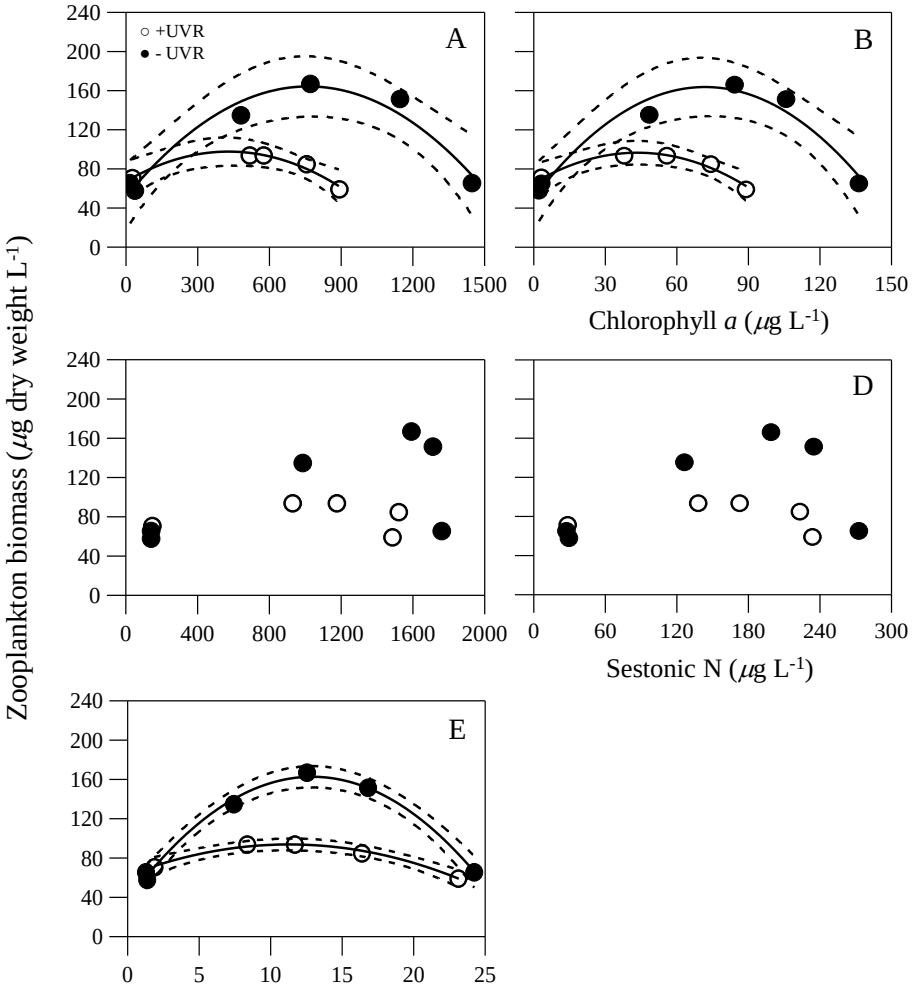

Figure 5. Response of zooplankton biomass to (A) algal biomass, (B) chlorophyll *a*, (C) sestonic C, (D) sestonic N, and (E) sestonic P under +UVR and –UVR treatments. Dashed lines indicate 95% confidence intervals around the fitted models (solid lines). Values represent the mean for the last five sampling dates. *La respuesta de la biomasa zooplanctónica a (A) biomasa algal, (B) clorofila* a, *(C) C sestónico, (D) N sestónico, y (E) P sestónico para los tratamientos +UVR y –UVR. Las líneas discontinuas indican los intervalos de confianza del 95% a ambos lados de los modelos ajustados (líneas continuas). Los valores representan la media para los últimos cinco días de muestreo.*

Dependent variable (y)	Independent variable (x)	Light treatment	c	b	a	R^2	p-value
Zooplankton biomass	Algal biomass	+UVR	**-0.00017**	**0.14**	**67.21**	**0.96**	**<0.05**
		–UVR	**-0.00019**	**0.29**	**55.52**	**0.94**	**<0.05**
	Chlorophyll *a*	+UVR	**-0.01643**	**1.43**	**65.56**	**0.96**	**<0.05**
		–UVR	**-0.02201**	**3.17**	**49.94**	**0.94**	**<0.05**
	Sestonic C	+UVR	-0.00005	0.09	59.65	0.54	ns
		–UVR	-0.00009	0.21	34.28	0.48	ns
	Sestonic N	+UVR	-0.00261	0.68	53.15	0.76	ns
		–UVR	-0.00617	1.95	9.77	0.83	ns
	Sestonic P	+UVR	**-0.24767**	**5.59**	**62.27**	**0.99**	**<0.05**
		–UVR	**-0.73859**	**18.94**	**41.02**	**0.99**	**<0.05**

Table 5. Effects of different food quantity predictors on zooplankton biomass under each light treatment. Variables: Zooplankton biomass (μg dry weight L^{-1}), algal biomass (μg C L^{-1}), chlorophyll *a* (μg L^{-1}), sestonic C (μg C L^{-1}), sestonic N (μg N L^{-1}), and sestonic P (μg P L^{-1}). Regression model: $y = cx^2+bx+a$. Significant regressions are shown in bold. ns – not significant. *Efectos de los diferentes predictores de cantidad de alimento sobre la biomasa zooplantónica para cada tratamiento de luz. Variables: Biomasa zooplanctónica (*µg *peso seco* L^{-1}*), biomasa algal (*µg C L^{-1}*), clorofila* a (µg L^{-1}*), C sestónico (*µg C L^{-1}*), N sestónico (*µg N L^{-1}*), y P sestónico (*µg P L^{-1}*). Modelo de regresión:* $y = cx^2+bx+a$. *Las regresiones significativas se muestran en negrita. ns – no significativo.*

P-enrichment treatment	Sestonic C:N +UVR	Sestonic C:N –UVR	Sestonic C:P +UVR	Sestonic C:P –UVR
0	5.96	6.07	211.66	305.87
20	7.88	9.05	289.48	339.61
30	8.51	9.66	262.02	329.31
40	7.92	8.76	234.05	264.06
60	7.44	7.57	159.38	184.63

Table 6. Mean values of sestonic C:N and C:P molar ratios for each light × P-enrichment treatment. *Valores medios de las razones molares C:N y C:P del seston para cada tratamiento de luz × enriquecimiento en P.*

Dependent variable (y)	Independent variable (x)	Light treatment	b	a	R^2	p-value
Zooplankton biomass	Sestonic C:N	+UVR	9.87	6.28	0.401	0.251
		–UVR	**30.72**	**-135.78**	**0.826**	**0.033**
	Sestonic C:P	+UVR	**0.29**	**13.51**	**0.928**	**0.008**
		–UVR	0.41	-1.26	0.295	0.344
	Algal C biomass:N	+UVR	3.34	69.11	0.092	0.620
		–UVR	9.59	76.31	0.165	0.500
	Algal C biomass:P	+UVR	0.22	56.75	0.462	0.207
		–UVR	0.58	37.71	0.493	0.186
	Chlorophyll *a* : sestonic C	+UVR	-258.56	90.62	0.027	0.791
		–UVR	380.38	97.57	0.021	0.817

Table 7. Effects of different food quality predictors on zooplankton biomass in both light treatments. Variables: sestonic C:N, sestonic C:P, algal C biomass:N, algal C biomass:P, and chlorophyll *a*: sestonic C. Regression model: $y = bx+a$. Significant regressions are shown in bold. *Efectos de los diferentes predictores de calidad de alimento sobre la biomasa zooplanctónica para ambos tratamientos de luz. Variables: C:N del seston, C:P del seston, biomasa de C algal:N, biomasa de C algal:P, y clorofila* a*:C sestónico. Modelo de regresión:* y = bx+a. *Las regresiones significativas se muestran en negrita.*

Long-term observational study

Data on aerosol depositions over the past three decades reveal a tendency for an increase in the magnitude and occurrence of these events, especially after 1990. Over this period, there was a >5-fold increase in TOMS AI ($r = 0.81$, $p < 0.001$; Fig. 6A) and a >3-fold increase in the annual frequency of aerosol deposition events ($r = 0.83$, $p < 0.001$; Fig. 6B), whereas the UV irradiance did not show significant temporal changes ($r = 0.10$, $p > 0.050$; Fig. 6C). The intensity and frequency of aerosol depositions during this time correlated positively with the increase in phytoplankton biomass ($r = 0.78$, $p < 0.05$, *see* Fig 6A inset; $r = 0.86$, $p < 0.01$, *see* Fig. 6B inset, respectively).

To assess whether our experimental results were representative of the natural plankton dynamics of the lake, we plotted phytoplankton and zooplankton biomass over the past three decades (Fig. 6D). The biomass of zooplankton was higher than that of phytoplankton until the beginning of the 1990s, when there was a clear trend to a lower accrual of zooplankton coinciding with the increase in phytoplankton biomass. As a result, zooplankton biomass was unimodally related to phytoplankton ($y = -0.009x^2 + 2.285x$, $p < 0.05$; *see* inset in Fig. 6D).

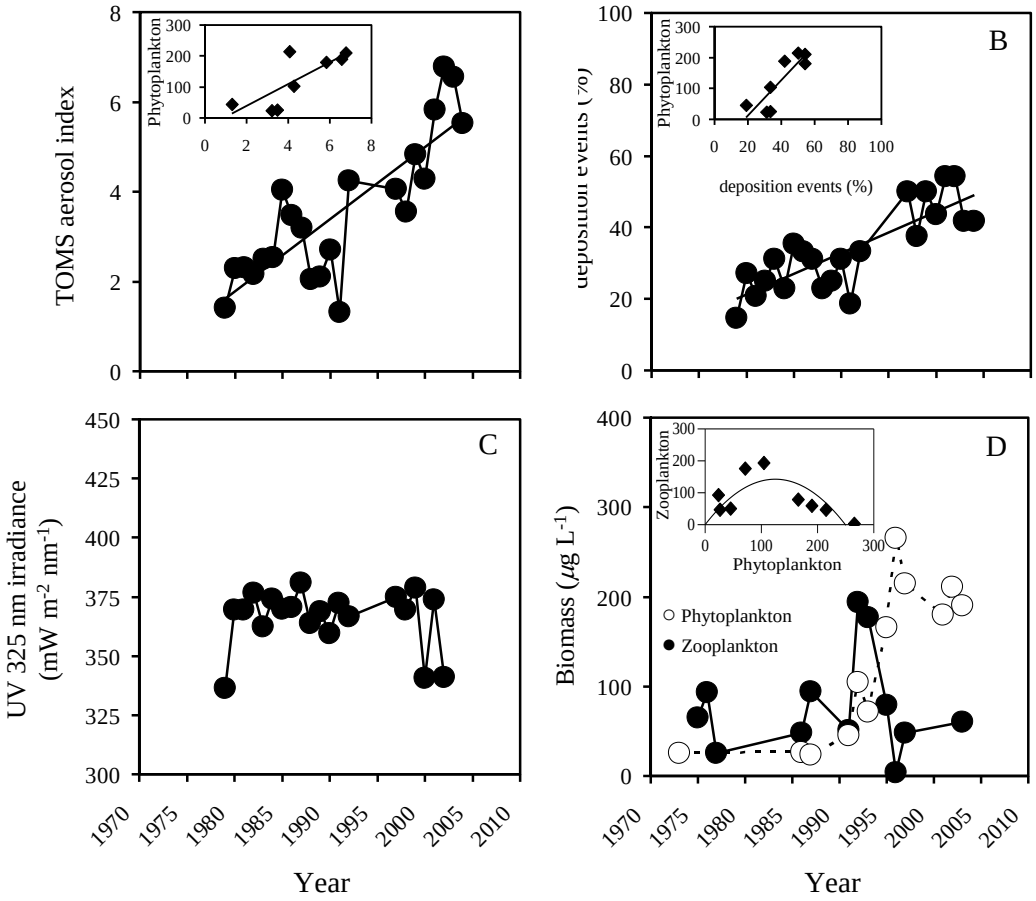

Figure 6. Interannual trends in (A) TOMS aerosol index, (B) annual frequency of aerosol deposition events, (C) UV 325 nm irradiance, and (D) phytoplankton and zooplankton biomass. Insets represent (A) the relationships of TOMS aerosol index and (B) annual frequency of aerosol deposition events to phytoplankton biomass, and (D) the relationship between phytoplankton and zooplankton biomass in years when mean values were available for both. Lines represent the best fits from least-square regressions. Biomass units are μg fresh weight L^{-1} for phytoplankton and μg dry weight L^{-1} for zooplankton. *Tendencias interanuales en (A) índice de aerosol TOMS, (B) frecuencia anual de eventos de deposición de aerosoles, (C) radiación UV 325 nm, y (D) biomasas fito- y zooplanctónica. Las figuras internas representan las relaciones de (A) índice de aerosol TOMS y (B) frecuencia anual de eventos de deposición atmosférica con la biomasa fitoplanctónica, y (D) la relación entre las biomasas fito- y zooplanctónica en los años para los cuales ambas están disponibles. Las líneas representan los mejores ajustes por regresión mínimo-cuadrática. Las unidades de biomasa son* μg *peso fresco* L^{-1} *para el fitoplancton y* μg *peso seco* L^{-1} *para el zooplancton.*

Discussion

Our experimental results show that the zooplankton response to increasing nutrients fits a UVR light-specific unimodal curve. Thus, zooplankton proved to be constrained by food at both ends of the nutrient gradient and by UVR at intermediate nutrient inputs. These findings do not support our hypothesis that zooplankton biomass accrual would be increased and PZC strengthened by the higher food quantity and quality from P-fertilization and UVR. However, this response of zooplankton is consistent with reports that zooplankton growth, and therefore transfer of energy and nutrients from primary producers to herbivore consumers, is highest at intermediate mesotrophic conditions and decreases towards both ends of the trophic gradient, resembling a unimodal function (Elser *et al.*, 1990; Persson *et al.*, 2007). This decoupling of the predator-prey relationship was recently described in long-term nutrient enrichments in stream ecosystems, reducing the overall food web efficiency (Davis *et al.*, 2010).

In the low seston range, the increase of zooplankton biomass up to an intermediate P-enriched level of 30 μg P L^{-1} indicates a strong effect of food quantity on zooplankton biomass, in full agreement with the observation that food availability is the dominant constraint on zooplankton growth in poor nutrient conditions (Persson *et al.*, 2007). Thus, the strength of PZC was maximal at intermediate trophic conditions (Fig. 5) but decreased with higher food quantity conditions. In other words, further increases in food quantity not only failed to yield higher zooplankton biomass but in fact had a detrimental effect. The cause of reduced zooplankton performance at high nutrient levels is a question of great interest. It has traditionally been associated with a wide range of negative conditions, including proliferation of inedible algae (Elser *et al.*, 1990; Auer *et al.*, 2004) or decreased food quality in terms of elemental content (Sterner & Elser, 2002) or biochemical composition (Brett *et al.*, 2006; Persson *et al.*, 2007). However, phytoplankton in this study was dominated by the edible algae *Dictyiosphaerium chlorelloides,* and no changes in species composition were observed during the study period (Delgado-Molina *et al.*, 2009). The concentration of other essential biochemicals in seston, including total fatty acids

and ω3-polyunsaturated fatty acids (ω3-PUFA), was also high in P-enriched enclosures (Villar-Argaiz *et al.*, 2009). The decrease in zooplankton biomass observed at the highest end of the trophic gradient may have various explanations. Thus, food 'in excess'may have a detrimental effect, since secretions of polysaccharides in high algal populations (Delgado-Molina *et al.*, 2009) may have a clogging effect on copepods by saturating their filtering capacity, as previously observed in large non-edible algae (Gliwicz, 2004). An alternative explanation derives from the 'stoichiometric knife-edge' hypothesis, which predicts a decline in herbivore performance and therefore biomass accrual from P-rich food with a low C:P ratio (Elser *et al.*, 2005). Accordingly, and contrary to long-held beliefs, seston values much lower than the C:N and C:P thresholds proposed by Urabe & Watanabe (1992) (22.5 and 300, respectively) could hamper the performance of zooplankters that have much lower mineral nutrient demands. Our results therefore question the classical hypothesis that 'more is better (or at least never worse)' (Boersma & Elser, 2006), since they reveal a strong mismatch situation, with high algal biomass and low zooplankton biomass accumulation. On the other hand, these findings are consistent with natural observations in the studied lake that a higher copepod biomass is not promoted by primary producer blooms from strong atmospheric nutrient loads (Villar-Argaiz *et al.*, 2001).

Few studies have considered the effects of increased nutrient availability and hence trophic status together with other potentially relevant environmental factors such as UVR. The present study shows that UVR is a major determinant of PZC, exerting the greatest constraint on zooplankton biomass accumulation at intermediate P-enriched levels. This result implies that UVR effects can prevail against the above-reported effects of food, reducing zooplankton biomass at intermediate food levels. Following this observation of nutrient level-specific UVR damage, a new intriguing question is whether UVR exerts a direct effect on consumer survival or rather an indirect effect on PZC *via* food quantity or quality regulation.

The absence of differences in food concentrations between UVR treatments, for a given intermediate P-enriched level, implies that food quantity

played no role in the UVR-related differences in the biomass of herbivores. We found no major changes in algal taxonomy, and the chlorophyte *Dictyiosphaerium chlorelloides* dominated all enclosures (Delgado-Molina *et al.*, 2009), therefore variations in food quality can not be attributed to differences in algal communities. With regard to the elemental content of the algae, sestonic ratios within the range reported for zooplankton in this experiment (C:N = 8-11, unpublished data from Bullejos *et al.*; C:P = 269-381, original data from Bullejos *et al.*, 2008) indicate high-quality food for this species. In addition, concomitant analysis of the experimental mesocosms revealed that the fatty acid content was equally high under both UVR regimes in the P-enriched treatments (Villar-Argaiz *et al.*, 2009). Therefore, our results indicate that the parameters of food quality measured had no influence on the negative effect of UVR at intermediate food levels, pointing out at some direct deleterious effect of UVR on zooplankton populations. Our results further support that the mechanism behind the detrimental UVR was organism death. However, the large C-content per individual under UVR suggests the storage of lipids associated with carotenoid pigmentation (Hylander *et al.*, 2009), which may be interpreted as a tolerance mechanism against UVR-induced stress.

Although copepods have traditionally been regarded as more resistant to UVR than cladocerans (Hylander *et al.*, 2009), they evidenced strong deleterious UVR effects in this study. Interestingly, the negative effects of UVR were only observed at intermediate food levels, when biomass accumulation was highest. These findings are surprising, since zooplankton can migrate at depth (Rhode *et al.*, 2001) and display antioxidant enzymes (Souza *et al.*, 2010). Moreover, reduced UVR would be expected in P-enriched enclosures due to the high algal biomass. Although these counterintuitive results warrant further investigation, they find support in previous studies by Carrillo *et al.* (2008) in which the deleterious effects of UVR on nutrient limited algae were only observed after moderate nutrient inputs. Regardless of the reasons for these effects of UVR, our results, taken together, imply that the potential benefits of enhanced nutrients linked to moderate aerosol inputs are largely offset by the deleterious effect of UVR.

Ecological implications

The long-term record reported in this study, in agreement with future projections for the Mediterranean region (Escudero *et al.*, 2005), indicates an increase in the magnitude and frequency of atmospheric dust aerosols, responsible for the increased phytoplankton biomass between 1978 and 2003. These patterns are consistent with previous reports that nutrients from Saharan atmospheric dust deposition are important sources of P, increasing the Chl *a* in these high mountain lakes (Morales-Baquero *et al.*, 2006) and enhancing the primary production of the mixed surface layer of the Western Mediterranean sea (Ridame & Guieu 2002).

However, the higher phytoplankton biomass was not followed by enhanced zooplankton biomass from the beginning of 1990s, resulting in a decoupled long-term dynamics. This pattern is analogous to our experimental observations of negligible zooplankton biomass augmentation with increasing seston after P-pulses applied in the presence of UVR. Our results also match observations of phytoplankton blooms after strong occurrences of atmospheric P inputs in these lakes, which did not translate into higher zooplankton accrual (*see* Fig. 6 in Villar-Argaiz *et al.*, 2001). Hence, the combination of greater atmospheric depositions (favoring phytoplankton development) and high UV irradiance (constraining zooplankton growth) in Mediterranean high-mountain lakes would not positively affect the development of herbivores, consequently weakening PZC.

High mountain lakes are climatically sensitive indicators to infer global change (Williamson *et al.*, 2009). Although extrapolation of our experimental results to the full-scale system is limited, since natural conditions are not completely reproduced by mesocosms closed at the bottom (*e.g.* by preventing nutrient sedimentation and constraining zooplankton migration), both experimental and long-term approaches can help to qualitatively explain the phytoplankton-zooplankton relationship in Lake La Caldera. Thus, the relationships reported here became uncoupled in the presence of UVR in both experimental and long-term observations. However, in the absence of UVR, from

low to moderate P-enrichments benefited zooplankton performance and reinforced PZC, while stronger P-enrichment impaired growth and disrupted PZC. These findings suggest that UVR makes a key contribution to the shape of the phytoplankton-zooplankton relationship. The intensity of the P- and UVR-induced decoupling effect may therefore depend on the magnitude and frequency of the atmospheric inputs, the exposure of lakes to UVR and atmospheric dust depositions, and the specific structure of the zooplankton communities in these high mountain lakes.

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V

Ultraviolet radiation and nutrient effects on zooplankton elemental composition

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Abstract

We conducted a 2 × 5 field experiment to test the effects of ultraviolet radiation (UVR) [presence (+UVR) *vs.* absence (-UVR)] and nutrients (0, 20, 30, 40 and 60 μg P L^{-1}) on zooplankton elemental composition [%carbon (C), %phosphorus (P), and C:P ratio] and assess zooplankton stoichiometric homeostasis against external stressors. We observed neither of %C and %P were affected by P-enrichment, but UVR did increase %C. When integrated both variables in C:P ratio, an inverse unimodal pattern for +UVR and a negative linear trend for –UVR emerged. Therefore, a noticeably increase in zooplankton C:P ratio due to UVR at both extremes of the P gradient was observed. This effect contrasts with the decreasing UVR effect on seston C:P ratio, and thus, not showing any relationship between both seston and zooplankton C:P ratios. Our results suggest that UVR is able to induce changes in zooplankton elemental composition not related to that of food, supporting the non-strict homeostatic nature of zooplankton.

Resumen

Llevamos a cabo un experimento de campo 2 × 5 para testar los efectos de la radiación ultravioleta (UVR) [presencia (+UVR) vs. ausencia (-UVR)] y de los nutrientes (0, 20, 30, 40 y 60 μg P L^{-1}) sobre la composición elemental del zooplancton [%carbono (C), %fósforo (P), y razón C:P] y evaluar la homeostasis estequiométrica del zooplancton frente a estresores externos. Observamos que ni %C ni %P fueron afectados por el enriquecimiento en P, aunque UVR si aumentó %C. Cuando se integran ambas variables en la razón C:P, emergen un patrón unimodal invertido para +UVR y una tendencia lineal negativa para –UVR. Por lo tanto, se aprecia un incremento notable en la razón C:P del zooplancton debido a UVR para ambos extremos del gradiente en P. Este efecto contrasta con el efecto reductor de UVR sobre la razón C:P del seston. Nuestros resultados sugieren que UVR es capaz de inducir cambios en la composición elemental del zooplancton que no se relacionan con el alimento, y que apoyan la naturaleza homeostática no estricta del zooplancton.

Introduction

Homeostatic elemental composition implies that the internal elemental composition of an organism resists changes despite external variability, and the process of regulation is known as stoichiometric homeostasis. While algae are a clear example of non-homeostatic organisms, zooplankton represents an intermediate degree of homeostasis (non-strict homeostasis) (Sterner & Elser, 2002). Previous studies showed that the degree of homeostasis in zooplankton is a function of different biotic features, including their ontogenetic development (Villar-Argaiz *et al.*, 2002), reproduction events (Ventura *et al.*, 2005) and the elemental composition of their food source (DeMott *et al.*, 1998).

However, other studies reported more subtle responses of zooplankton elemental composition to abiotic factors. Thus, Elser *et al.* (2000) described a higher P content in *Daphnia pulicaria* from Alaska than in *Daphnia pulex* from a temperate region, suggesting latitudinal effects on body elemental composition. While temperature has been considered a key abiotic factor explaining variations in the elemental composition of terrestrial organisms (Reich & Oleksyn, 2004), several studies have described changes in elemental composition produced by ultraviolet radiation (UVR) in algae (*e.g.* Xenopoulos *et al.*, 2002). Although the influence of food on the homeostatic nature of zooplankton has been examined (DeMott *et al.*, 1998), the relative importance of this factor with respect to other non-biotic factors such as UVR has been neglected.

The purpose of this work was to assess *in situ* the effects of UVR and food quality and quantity on biomass, carbon (C) and phosphorus (P) content and C:P ratio of the calanoid copepod *Mixodiaptomus laciniatus* across an experimentally generated trophic gradient.

Methods

This *in situ* experiment ran from August to October in 2003 in Lake La Caldera (Sierra Nevada, Spain) and had a 2 × 5 factorial design: Two light treatments and five nutrient treatments. Ten opened enclosures that allowed for air exchange were used, consisting of clear polyethylene tubes (1 m diameter × 7 m length; 2.7 m^3 total volume): Five enclosures were covered with polyethylene plastic (+UVR treatments), which transmits photosynthetic active radiation (PAR) and UVR, and the other five with plexiglass UF3, which transmits PAR but blocks UVR (-UVR treatments). Nutrient levels were set by adding a final concentration of 0, 20, 30, 40 and 60 μg P L^{-1} (NaH_2PO_4), after which water was vertically mixed using vertical tows of a plastic bucket. The enclosure with no added nutrient and +UVR served as control.

Incubations lasted for 70 days, and enclosures were monitored for seston and zooplankton biomass and chemical (C and P) determinations, after gently mixing, at the start of the experiment and on days 20, 32, 43, 56 and 70. Seston was sampled using a plastic bucket and zooplankton samples were taken using vertical tows of a 40-μm mesh zooplankton net.

Seston and zooplankton were then collected onto pre-combusted (24 h at 500 ºC) glass-fiber filters (Whatman GF/B) and analyzed for C by using a CNH analyzer or for P by colorimetry after persulfate oxidation. Algal and zooplankton abundances were counted using an inverted microscope. Algal biovolume was obtained by image analysis and converted to C units (biomass). Zooplankton biomass was calculated using length-weight relationships developed for *Mixodiaptomus laciniatus* in this ecosystem.

For each light treatment, effects of nutrient manipulation on algal and zooplankton biomass, C:P ratio, %C and %P of zooplankton were assessed by simple/polynomical regression of mean values for all sampling dates against P-enrichment level. UVR effect was determined by examining 95% confidence intervals of the regression lines. When no significant relationships between

zooplankton %C or %P and P-enrichment were found, effects of light manipulation were examined by independent *t*-test.

Results

Algal biomass ranged from 25 to 890 μg C L^{-1} under +UVR and from 17 to 1445 μg C L^{-1} under –UVR treatments. Seston C:P ranged from 159 to 288 under +UVR and from 183 to 338 under –UVR treatments. P-enrichment significantly increased algal biomass and decreased seston C:P ratio. UVR significantly decreased algal biomass at 40 and 60 μg P L^{-1} and seston C:P at every P-enrichment level (Fig. 1).

The zooplankton community was largely dominated by *Mixodiaptomus laciniatus*, which comprised >99% of total zooplankton biomass in the mesocosms. *Mixodiaptomus laciniatus* was in copepodite stage III at the start of the experiment, reaching maturity by the end of incubations. Mean zooplankton biomass increased from 60 to 94 μg dry weight L^{-1} in +UVR and from 65 to 166 μg dry weight L^{-1} in –UVR enclosures. Zooplankton biomass response was well explained by unimodal models, which accounted for 99% and 98% of the variance observed under +UVR and –UVR treatments, respectively (Table 1). These models differed at intermediate P-enrichment levels (20, 30, 40 μg L^{-1}), when presence of UVR significantly reduced zooplankton biomass (Fig. 2A).

Mean C content (%C) ranged from 36 to 60% in +UVR and from 29 to 38% in –UVR enclosures. Mean P content (%P) ranged from 0.34 to 0.43% under +UVR and from 0.31 to 0.37% under –UVR treatments. No significant relationships between %C or %P and P-enrichment were found under any light treatment (Table 1). In contrast to the effect of P-enrichment, UVR significantly increased %C ($p < 0.05$) but had no effect on %P (Fig. 2B, C).

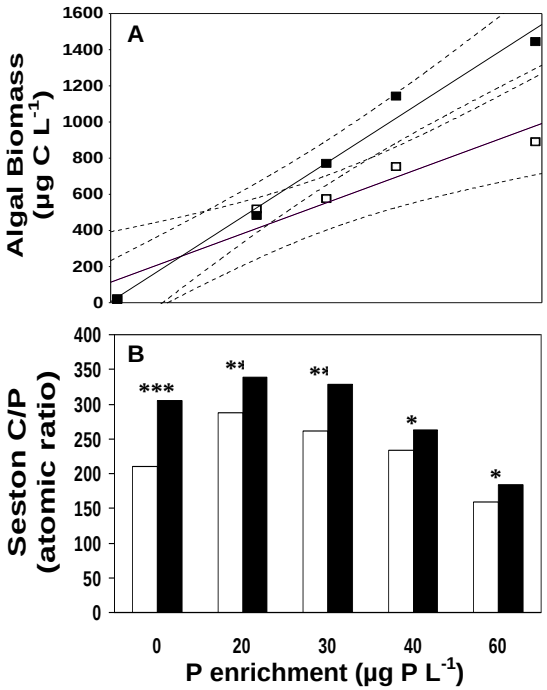

Figure 1. Linear relationships between (A) algal biomass and P-enrichment and (B) seston C:P ratio and P-enrichment under +UVR (white) and –UVR (black) treatments. In A, regression lines denote significant relationship ($p < 0.05$) for both light treatments. 95% confidence intervals are denoted by dotted lines to evaluate significant differences between +UVR and –UVR treatments. In B, asterisks indicate the significance (by paired *t*-test) of differences between light treatments for each P level: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. *Relaciones lineales entre (A) biomasa algal y enriquecimiento en P y (B) razón C:P del seston y enriquecimiento en P en los tratamientos +UVR (blanco) y –UVR (negro). En A, las líneas de regresión indican relaciones significativas (*p < *0.05) para ambos tratamientos de luz. Los intervalos del 95% de confianza representados por las líneas punteadas son para evaluar diferencias significativas entre los tratamientos +UVR y -UVR. En B, los asteriscos indican la significación (por* t*-test de muestras apareadas) de las diferencias entre los tratamientos de luz para cada nivel de P:* * p < *0.05;* ** p < *0.01;* *** p < *0.001.*

Variables		Light treatment	Fit model	*a*	*b*	*c*	*p* -value	R^2
y	*x*							
Algal biomass	P-enrichment	+UVR	1	**127.60**	**14.17**		**0.0094**	**0.92**
		-UVR	1	**30.55**	**24.74**		**0.0010**	**0.98**
Seston C:P ratio		+UVR	1	262.02	-1.06		0.4164	0.23
		-UVR	1	349.08	-2.19		0.1204	0.61
Zooplankton biomass		+UVR	2	**71.61**	**1.64**	**-0.03**	**<0.01**	**0.99**
		-UVR	2	**62.80**	**6.29**	**-0.10**	**<0.05**	**0.98**
Zooplankton %C		+UVR	1	44.89	0.09		0.6994	0.06
		-UVR	1	37.02	-0.13		0.1146	0.62
Zooplankton %P		+UVR	1	0.35	0.00		0.3439	0.30
		-UVR	1	0.33	0.00		0.5259	0.15
Zooplankton C:P ratio		+UVR	2	**385.56**	**-6.53**	**-0.10**	**<0.05**	**0.94**
		-UVR	1	**285.90**	**-1.16**		**0.0007**	**0.99**
Zooplankton biomass	Algal biomass	+UVR	1	82.19	-0.00		0.9258	0.00
		-UVR	1	109.72	0.01		0.8643	0.01
	Seston C:P ratio	+UVR	1	**13.51**	**0.23**		**0.0084**	**0.93**
		-UVR	1	-1.26	0.42		0.3440	0.30
Zooplankton C:P ratio	Seston C:P ratio	+UVR	1	463.05	-0.62		0.2571	0.40
		-UVR	1	160.59	0.32		0.1288	0.60

Table 1. Results of different regression analyses for both light treatments. Variables: Algal biomass (μg C L^{-1}), zooplankton biomass (μg dry weight L^{-1}), %C and %P of zooplankton (% of dry weight), seston and zooplankton C:P atomic ratios, and P-enrichment (μg P L^{-1}). Regression models were 1: $y = bx+a$ and 2: $y = cx^2+bx+a$. UVR: Ultraviolet radiation. Number in bold: Significant at $\alpha = 0.05$. *Resultados de los diferentes análisis de regresión para ambos tratamientos de luz. Variables: Biomasa algal (*μg C L^{-1}*), biomasa del zooplancton (*μg peso seco L^{-1}*), %C y %P del zooplancton (% del peso seco), razones atómicas C:P del seston y del zooplancton, y enriquecimiento en P (*μg P L^{-1}*). Los modelos de regresión fueron 1:* $y = bx+a$ *y 2:* $y = cx^2+bx+a$. *UVR: Radiación ultravioleta. Número en negrita: Significativo para* $\alpha = 0.05$.

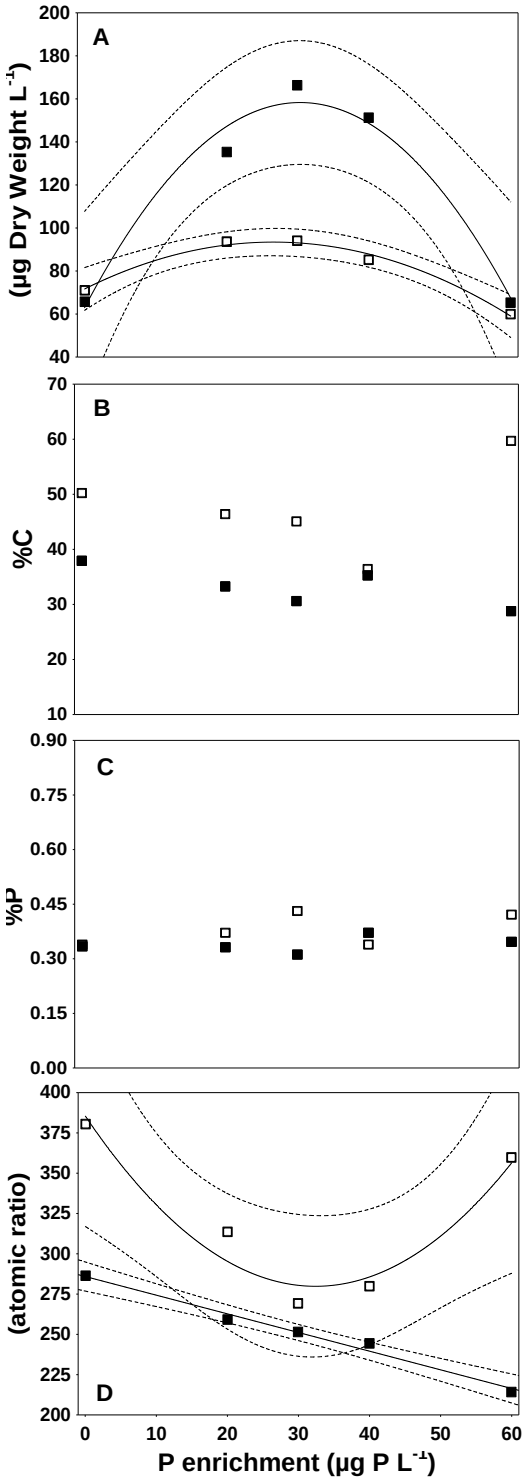
A
B
C
D
(µg Dry Weight L-1)
%C
%P
(atomic ratio)
P enrichment (µg P L-1)
200
180
160
140
120
100
80
60
40
70
60
50
40
30
20
10
0.90
0.75
0.60
0.45
0.30
0.15
0.00
400
375
350
325
300
275
250
225
200
0
10
20
30
40
50
60

Figure 2. Effects of UVR and P-enrichment on (A) zooplankton biomass, (B) %C, (C) %P, and (D) C:P ratio. White and black squares indicate +UVR and –UVR treatments. Regression lines are inserted when the relationship is significant ($p < 0.05$). In A and D, 95% confidence intervals are denoted by dotted lines to evaluate significant differences between +UVR and –UVR treatments. *Efectos de UVR y enriquecimiento en P para (A) biomasa, (B) %C, (C) %P, y (D) razón C:P del zooplancton. Los cuadrados blancos y negros indican los tratamientos +UVR y –UVR. Las líneas de regresión se insertan cuando la relación es significativa (*p *< 0.05). En A y D, los intervalos del 95% de confianza representados por líneas punteadas son para evaluar las diferencias significativas entre +UVR y –UVR.*

Mean zooplankton C:P ratio ranged from 269 to 381 in +UVR and from 214 to 286 in –UVR enclosures. In contrast to %C and %P, the model that best explained zooplankton C:P ratio differed between UVR treatments (Table 1). While a linear negative relationship explained 99% of the variance under –UVR treatment, an inverse unimodal accounted for 94% of the variance under +UVR treatment (Table 1 and Fig. 2D). Interestingly, the latter unimodal pattern was the opposite trend of that shown by zooplankton biomass. Analysis of confidence intervals of the two fits revealed significantly higher zooplankton C:P ratios at both ends of the P gradient [*i.e.*, no added nutrient (controls) and 60 μg P L^{-1}] but showed no significant differences at intermediate P levels.

No significant relationships were found between zooplankton biomass and algal biomass or seston C:P or between zooplankton C:P and seston C:P. Zooplankton biomass was only related to seston C:P ratio in +UVR enclosures, although their linear association showed a positive slope (Table 1).

Discussion

The P-induced increase in algal biomass and P- and UVR-induced decrease in seston C:P ratio were consistent with an improvement in the quantity and quality of seston as a food source for herbivore consumers (Sterner & Elser, 2002).

Because zooplankton biomass was not explained by the algal biomass (quantity) or seston C:P ratio (quality), differences in zooplankton biomass

between UVR treatments were likely due to a direct negative effect of UVR. The unimodal response of zooplankton to P-enrichment indicated an optimal trophic range at intermediate P levels, with maximum zooplankton development (Fig. 2A). The deleterious effects of UVR were unmasked in this optimal range. This was unexpected given the large enclosures used in the experiment (7 m), which allowed for vertical migration of organisms, and the high photoprotective pigment content in the zooplankton (data in prep.).

Because food (quantity and quality) and life history strategies (ontogenetic development differences or reproduction) had no impact on the zooplankton C:P ratio, the differences between light treatments suggest that UVR itself is mainly responsible for the higher zooplankton C:P ratios at each end of the trophic gradient generated. Differences in %C were responsible for this pattern, suggesting the storage of lipids. This is supported by the finding of higher contents of ω3-polyunsaturated fatty acids (ω3-PUFA) in seston at both ends of the P gradient (Villar-Argaiz *et al.*, 2007).

UVR-induced differences in zooplankton C:P ratio contributes evidence on the non-strict homeostatic nature of herbivore consumers. Previous studies demonstrated the role of UVR in modifying the elemental composition of autotrophs (*Xenopoulos et al.*, 2002) but, to our knowledge, this is the first to describe similar effects on herbivore consumers. However, in contrast to the seston C:P ratio, the zooplankton C:P ratio increased in response to UVR. The mechanisms behind these contradictory effects merit further attention.

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VI

Growth response of herbivorous consumers to a natural gradient of food quality: Interannual observations and experimental test

VI. Growth response of herbivorous consumers to a natural gradient of food quality: Interannual observations and experimental test

Close to submission as:

Bullejos FJ, Carrillo P, Gorokhova E, Medina-Sánchez JM, Villar-Argaiz M (2012) Nonlinear effects of food quality on herbivorous consumer growth: Surprising ontogenetic responses.

Abstract

We investigated the relationship between zooplankton growth, measured as %RNA and RNA:DNA ratio, and the nutrient content of seston in a high mountain lake during three years of intensive monitoring. Consumer growth response to the natural gradient of seston carbon:nutrient (C:nutrient) ratio was unimodal and stage-specific. Solution of the equation given by the first derivate function provided the optimum C:nutrient ratio for maximum stage-specific growth, which steadily increased ontogenetically. Solution of the equation given by the second derivative function, as a measure of function peakedness, indicated that animal vulnerability to suboptimal food quality decreased as nauplii and immature copepodites reached adulthood. Based on the tight correlation between light and nutrients and seston carbon:phosphorus (C:P) ratio, we performed an *in situ* light × nutrient experiment using replicated enclosures to test how their effects on primary producers transferred to consumers. The results, consistently with our interannual observations, demonstrated that consumer growth responded to variations in seston C:P ratio, and particularly for early developmental stages, more vulnerable to suboptimal food quality compared to adults.

Resumen

Investigamos la relación entre el crecimiento del zooplancton, medido como %RNA y la razón RNA:DNA, y el contenido en nutrientes del seston en un lago de alta montaña durante tres años de seguimiento intensivo. La respuesta del crecimiento del consumidor al gradiente natural de la razón carbono:nutriente (C:nutriente) del seston fue unimodal y específica de cada estadío. La solución de la ecuación dada por la primera función derivada proporcionó la razón C:nutriente óptima para el crecimiento máximo de cada estadío, que gradualmente aumentó a lo largo de la ontogenia. La solución de la ecuación dada por la segunda función derivada, como medida del grado de apuntamiento de la función, indicó que la sensibilidad animal a la calidad de alimento subóptima decreció a medida que nauplios y copepoditos inmaduros alcanzaban la madurez. Basándose en la estrecha correlación de la luz y los nutrientes con la razón carbono:fósforo (C:P) del seston, diseñamos un experimento luz × nutrientes in situ *utilizando encerramientos replicados para testar cómo sus efectos sobre los productores primarios se transferían a los consumidores. Los resultados, consistentemente con nuestras observaciones interanuales, demostraron que el crecimiento del consumidor responde a variaciones en la razón C:P del seston, y particularmente para los estadíos iniciales, más vulnerables que los adultos a la calidad de alimento subóptima.*

Introduction

Life history, shaped by natural selection to maximize organism fitness, is one of the most important concepts in evolutionary biology. As evolutionary optimization theory posits, both the wide diversity of life histories and the nature of the trade-offs among their traits are determined by resource availability, and especially, of those affecting growth, survivorship and reproduction (Stearns, 1992; Soler, 2002). Large contribution to the knowledge of this issue has been carried out by ecological stoichiometry (the study of the balance of energy and multiple chemical elements in ecological interactions, Sterner & Elser, 2002), which, among others, aims to study the role of limiting nutrients in constraining consumer performance. There are two basic fundamental principles on which ecological stoichiometry rely: (i) Autotrophs exhibit great flexibility in their elemental composition in response to the nutrient availability, whereas heterotrophs have physiological mechanisms to strictly regulate their elemental composition around taxon- or stage-specific values, what has been defined as stoichiometric homeostasis (Sterner & Elser, 2002); (ii) Differences in the elemental composition underlie the nutritional imbalance between autotrophs and their herbivorous consumers that can greatly affect consumer fitness and dynamics (Sterner *et al.*, 1993; Boersma, 2000) with consequences for the amount of energy and materials transferred up the food web (Urabe & Sterner, 1996; Urabe *et al.*, 2002) and the strength of trophic cascades (Elser *et al.*, 1998).

A key insight that emerges from this approach is that organism somatic composition and more specifically, the elemental mismatch between resources and consumers, is a fundamental trait as it provides an explicit mechanism that regulate nutritional demands for animal growth. A central concept for understanding nutrient deficiency in animals is the threshold elemental ratio (TER), *i.e.* the C:nutrient ratio at which growth limitation switches from energy (C) to mineral nutrient (Sterner & Hessen, 1994; Andersen *et al.*, 2004; Frost *et al.*, 2006). TER is calculated as a product of physiological nutrient efficiencies and somatic elemental composition. Although TER may change as a function of ambient food quantity (Sterner, 1997), quantitative estimates of TER have

assumed strict homeostasis and no variability in the chemical content of the consumer. However, while the distinctive elemental composition of consumers is not in dispute with large interspecific variations in consumers, several studies have explicitly described pronounced intraspecific variations in the somatic composition across the life cycle of a consumer. For example, DeMott *et al.* (1998) showed strong variations in the P content of *Daphnia* in response to the quality of food. Likewise, Villar-Argaiz *et al.* (2002) described large intraspecific changes in somatic C:N:P ratios throughout the entire ontogenetic development of a copepod species. In a coetaneous study, Villar-Argaiz & Sterner (2002) demonstrated that P limitation prevented transition between certain copepodite stages resulting in demographic bottlenecks. These data demonstrated that certain life stages may be more sensitive than others to variations in the stoichiometric content of its food resource, *i.e.* a given resource C:P ratio that can supply insufficient P for a given developmental stage might provide surplus P for another. In other words, these findings suggest that the degree of mismatch between the animal composition and that of its food resource may not be fix but can have multiple inherent variations depending on nutrient-requirements at each developmental stage. To add more complexity to the consumer-resource interaction, it has been reported that organism C:N:P ratios, not only varies ontogenetically, but also at developmental stage level (Villar-Argaiz *et al.*, 2002). For example, Carrillo *et al.* (2001) reported that intrastage-P content in the copepod *Mixodiaptomus laciniatus* can vary by, as much as, three fold.

Clearly, there is good evidence to support that the role of nutrients causing food quality limitation in organisms with complex life histories can not be estimated as hard cut-offs, since thresholds should take into consideration the intrinsic variability in elemental composition and nutrient requirements for growth associated with development. In order to investigate these issues, we first examined the relationship between seston C:nutrient ratio and the stage-specific growth of the calanoid copepod *Mixodiaptomus laciniatus* during three years of intensive monitoring in Lake La Caldera. Second, based on the significant relationship found between seston C:P ratio and nutrient and solar radiation in the field, we experimentally tested whether *in situ* manipulation of these two factors

affected the stage-specific growth of *Mixodiaptomus laciniatus*. As an ultimate goal we aim to characterize the optimum food quality at which the stage-specific growth is maximal. In doing so, we will emphasize the importance of considering the major unexplored feature of how stoichiometric constraints evolve across the animal life history. To fulfil these aims, we will use RNA derived indices as proxies of growth rate (*e.g.* Elser *et al.*, 2003). They have been demonstrated to be valid across biota (Chicharo & Chicharo, 2008), and particularly useful for both *in situ* and short-term experiments (*e.g.* Wagner *et al.*, 2001; Hessen *et al.*, 2002; Vrede *et al.*, 2002; Malzahn & Boersma, 2012).

Methods

Study system and organism

Our study was carried out in Lake La Caldera (36º55´-37º15´N, 2º31´-3º40´W), a natural permanent water body at 3050 m above sea level (asl) in the National Park of Sierra Nevada, Spain. The elevated UVR doses at this altitude [*see* Fig. 1 in Carrillo *et al.* (2002)], lake transparency (Secchi´s disk visibility reaching maximum depth) and low values of dissolved organic carbon (<1 mg L^{-1}) result in high UVR penetration in the water column [Table 1 in Appendix; *see* Fig. 1 in Carrillo *et al.* (2008)] during the ice-free season (from June to November). Strong P limitation [total P (TP): 1.38-11.30 μg P L^{-1}; dissolved inorganic nitrogen:total P (DIN:TP) molar ratio > 45] for lake production occurs, although it is partially alleviated by eventual atmospheric Saharan nutrient inputs (Villar-Argaiz *et al.*, 2001; Morales-Baquero *et al.*, 2006). The biological community is relatively simple with no fish and few planktonic species. Of particular interest for our study is the absolute dominance of the herbivorous zooplankter *Mixodiaptomus laciniatus*, representing >90% of total zooplankton biomass. This species was the focus of our study, as it strictly grazes on seston and due to the feasibility to study its ontogenetic development from nauplii to adult stages in its natural habitat. Its life-cycle includes an intensive hatching of

eggs as the thaw approaches, followed by the development of a single cohort during the ice-free season (Carrillo *et al.*, 2001; Villar-Argaiz *et al.*, 2002).

The low food availability (mostly <100 μg C L^{-1}) for herbivores in Lake La Caldera resembles the unproductive waters of extensive areas of the ocean as well as polar and other alpine lakes (Villar-Argaiz *et al.*, 2012). Hence, this ecosystem allow us to *in situ* assess the effects of elemental food quality, governed by light and (atmospheric) nutrient inputs (Villar-Argaiz *et al.*, 2001; Carrillo *et al.*, 2008), at low quantity on consumer performance. In addition, due to differential nutrient demands throughout ontogeny (Carrillo *et al.*, 2001; Villar-Argaiz *et al.*, 2002), this provides an unique opportunity to evaluate changing food quality restrictions on individual growth during organism development under natural conditions.

Field sampling and in situ *experiment*

Data used in this paper come from two sources: A 3-year (2005-2007) field monitoring program (hereafter, interannual study), and an *in situ* light × nutrient experiment carried out in July-August 2007 (hereafter, experimental study).

The interannual data were obtained by monthly monitoring during the ice-free seasons in 2005, 2006, and 2007. For each sampling day, we measured light ($UVR_{305,\ 320,\ 380\ nm}$ and PAR) and temperature profiles using a Biospherical Instruments Compact (BIC) radiometer (Biospherical Instruments Inc., San Diego, California, USA). For each UVR wavelength and PAR, the diffuse attenuation coefficient for downward irradiance (K_d) (Table 1 in Appendix) and the mean light in the mixed layer (I_m) were calculated. I_m, expressed as a fraction of surface irradiance, was calculated as:

$$I_m = \frac{1 - e^{-K_d Z_m}}{K_d Z_m}$$

(Riley, 1957; Sterner *et al.*, 1997), where Z_m is the depth of mixed layer. We define $I_{m\ UVR}$ as the mean UVR in the mixed layer and was calculated as the average of I_m at three UVR wavelengths (305, 320 an 380 nm). Water samples for chemical [total nitrogen (TN), and total phosphorus (TP)], and biological [chlorophyll *a* (Chl *a*), seston carbon (C), nitrogen (N) and phosphorus (P)] variables were taken in triplicate at two depths in 2005 (0.5 and 1 m) and four depths in 2006 and 2007 (0.5, 3, 5, and 8 m). Detailed collection protocols are further described in Villar-Argaiz *et al.* (2001). Samples of *Mixodiaptomus laciniatus* for analyses of nucleic acids (NAs) were collected by vertical hauls of a 64-μm mesh net and brought to the laboratory in lake water under dark and cold conditions. Between 10 and 20 individuals were isolated and transferred into 1.5 mL Eppendorf tubes containing 300 μL of RNA*Later* (Ambion Inc., Austin, Texas, USA) and storaged at -80 ºC until analyses [*see* recommendations in Gorokhova (2005)]. Primary production in terms of total organic carbon (TOC) and particulate organic carbon >1.0 μm (POC_1) was determined following the procedure described in Carrillo *et al.* (2002) (*see* Appendix for a detailed description of the method).

The experimental study comprised two steps. First, water collected at 3-m depth was filtered through 64 μm to remove zooplankton and incubated in six UVR-transparent polyethylene mesocosms (height 5 m, diameter 0.7 m, volume 2 m^3). Three enclosures were covered and surrounded with Plexiglass UF3 sheets to prevent UVR (PAR treatment). The other three enclosures received the full spectrum of solar radiation (UVR treatment), although they were covered with polyethylene to avoid atmospheric nutrient inputs during incubation. Second, after one month (from 19th July to 19th August), water from each mesocosm was used to fill two 20-L polyethylene microcosms (height 0.2 m, diameter 0.4 m) (total number of microcosms = 12). *Mixodiaptomus laciniatus* individuals collected from the lake were equally added to microcosms, reaching twice the lake population density. For each light treatment, three microcosms received P (as Na_2HPO_4) and N (as NH_4NO_3) to double TP concentration in the lake, and maintain a molar N:P ratio of 30, mimicking the mean value of the molar TN:TP ratio found in total atmospheric deposition (Bullejos *et al.*, 2010). This yielded a 2

(UVR *vs.* PAR) × 2 (no nutrient addition *vs.* nutrient addition) factorial design with 3 replicates per treatment: UVR, UVR+NP, PAR and PAR+NP. Microcosms were incubated for one week (from 19th August to 26th August) at the depth where UVR was 75% of that at the surface (0.1 m) for UVR treatments or under Plexiglass UF3 sheets for PAR treatments (*see* Fig. 1 in Appendix). After incubations, subsamples were taken in triplicate for Chl *a*, seston C, N, P, and zooplankton NAs, and treated as mentioned above. For further details of the experimental set-up and procedures *see* Souza *et al.* (2010).

Laboratory analyses

TN was analysed using the ultraviolet spectrophotometric screening method. TP was measured colorimetrically *via* the acid molybdate technique (APHA, 1992). Seston samples (obtained by GF/B filtration of 300 mL of water per replicate) were analyzed for P following the acid-molybdate technique (APHA, 1992), or dried (24 h at 60 ºC) and analyzed for C and N using a Perkin-Elmer model 2400 CHN elemental analyser (Perkin-Elmer Corporation, Waltham, Massachusetts, USA). Seston C:N and C:P ratios (hereafter, C:nutrient ratios) were calculated on a molar basis. Chl *a* was measured fluorimetrically after grinding filters with pigments (concentrated by GF/F filtration of 300 mL of water per replicate) and pigment extraction in 90% acetone (24 h under dark conditions at 4ºC).

A total of >500 and >120 individuals of *Mixodiaptomus laciniatus*, covering the full ontogenetic development, were measured and analyzed for NA content for the interannual and experimental studies respectively. RNA content was expressed relatively to dry weight (%RNA) after length-weight conversions (Carrillo *et al.*, 2001) or relatively to DNA content as RNA:DNA ratio [hereafter, NA indices (NAIs)]. NAs were individually measured using a microplate fluorimetric high-range assay with Ribogreen after N-laurylsarcosine extraction and RNase digestion, as described in Gorokhova & Kyle (2002). Fluorescence measurements were converted into RNA and DNA concentrations using curves

previously performed with RNA (16S and 23S from *Escherichia coli*; component C of the Ribogreen Kit) and DNA (calf thymus; Sigma-Aldrich) standards, and later expressed as NAIs (*see* Appendix for a detailed description of NA analysis).

Data and statistical analyses

In the interannual study, ontogenetic and gender differences in NAIs were tested using non-parametric Kruskal-Wallis ANOVA test. Polynomial regression models were used to test the effects of (i) temperature, (ii) algal standing stock (Chl *a*, seston C, TOC, and POC_1) and (iii) food quality (seston C:nutrient ratios) variables on NAIs for each developmental stage distinguishing between males and females in adults. Best polynomial fits adjusted to second-degree functions, expressed as:

$$f(x) = cx^2 + bx + a \quad (1)$$

and, as parameter *c* was always negative, graphically represented by an inverted parabola. Properties of these functions enable to assess how consumer growth, measured as NAIs, responds to food quality predictors. Thus, the maximum vertex point of the curve corresponds to the "optimum resource (seston) C:nutrient ratio" ($x_{opt\ C:nut}$) at which consumer growth is maximal [$f(x_{opt\ C:nut})$]. Since $f(x)$ was continuous and differentiable along the seston C:nutrient ratio interval, $x_{opt\ C:nut}$ that provided maximum growth [$f(x_{opt\ C:nut})$] was determined by the solution of the equation given by the first derivative function [$f\,'(x)$]:

$$f'(x) = 2cx + b = 0 \quad (2)$$

Therefore it follows that:

$$x_{opt\ C:nut} = -\frac{b}{2c} \quad (3)$$

By rearranging equation (1), maximal consumer growth (measured as either %RNA or RNA:DNA ratio) can be determined as:

$$f(x_{opt\ C:nut}) = c\left(-\frac{b}{2c}\right)^2 + b\left(-\frac{b}{2c}\right) + a = -\frac{\left(b^2 - 4ca\right)}{4c} \qquad (4)$$

(Fig. 1A). But also the absolute value of the second derivative function [$/f''(x)/$], as a measure of the degree of function´s peakedness, provides information concerning the sensitivity of the organism growth to the effects of food quality [hereafter, "growth sensitivity index" (GSI)].

$$GSI = /f''(x)/ = /2c/ \qquad (5)$$

Accordingly, a low GSI corresponded with a flat-topped peaked curve and was interpreted as low growth sensitivity to food quality variations around $x_{opt\ C:nut}$, whereas a high GSI corresponded with a highly peaked curve and indicated high sensitivity to food quality variations around $x_{opt\ C:nut}$ (Fig. 1B). Due to the more extended use of %RNA in the literature (*e.g.* Elser *et al.*, 2003) and because RNA:DNA ratio can be affected by factors modifying DNA content such as endopolyploidy (Gorokhova & Kyle, 2002) or egg production (Wagner *et al.*, 2001; Ikeda *et al.*, 2007), we calculated GSI from those mother functions where %RNA was used as proxy of growth.

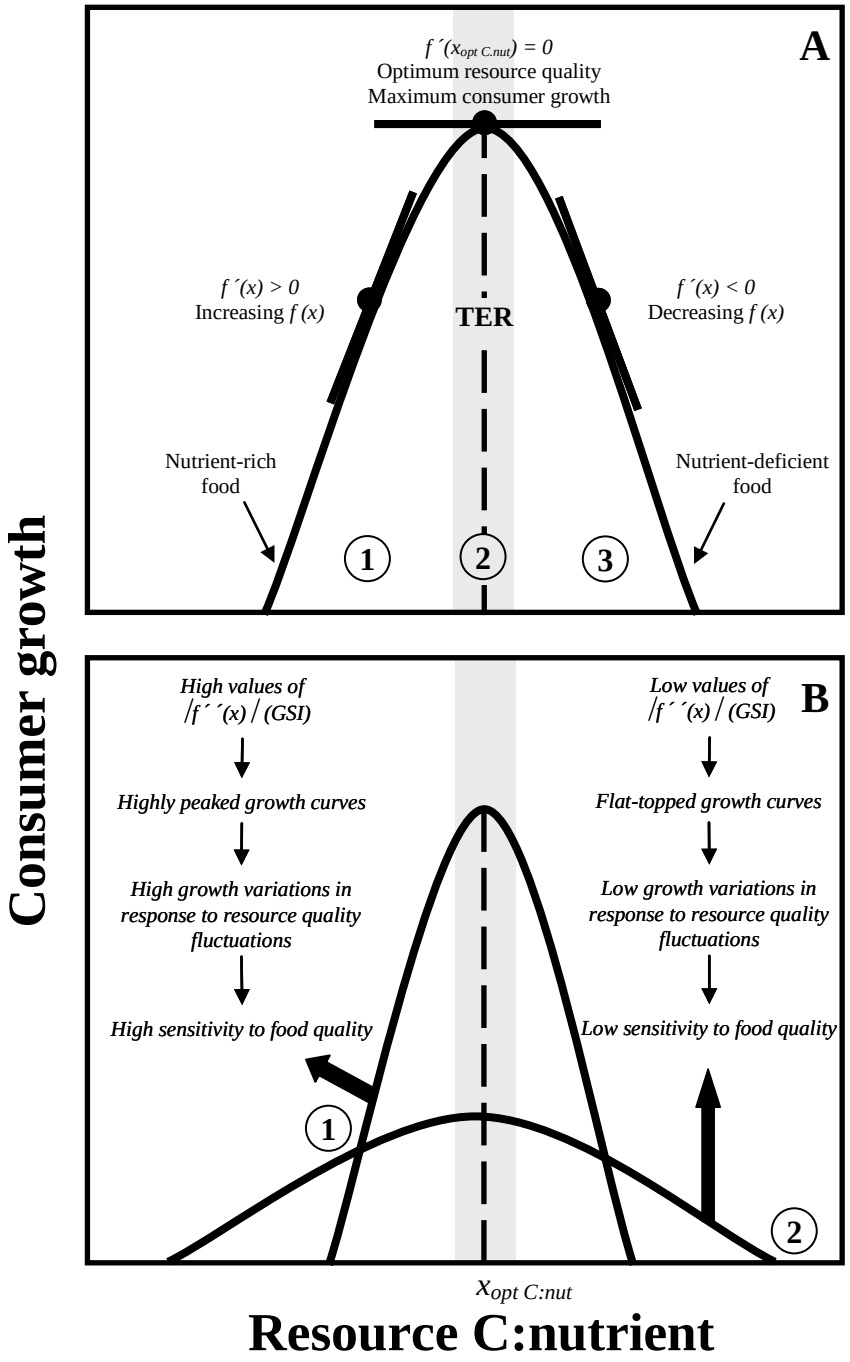
A
f ´(x opt C.nut) = 0
Optimum resource quality
Maximum consumer growth
f ´(x) > 0
Increasing f (x)
TER
f ´(x) < 0
Decreasing f (x)
Nutrient-rich food
Nutrient-deficient food
1
2
3
B
High values of /f ´´(x)/ (GSI)
Highly peaked growth curves
High growth variations in response to resource quality fluctuations
High sensitivity to food quality
Low values of /f ´´(x)/ (GSI)
Flat-topped growth curves
Low growth variations in response to resource quality fluctuations
Low sensitivity to food quality
1
2
Consumer growth
x opt C:nut
Resource C:nutrient

Figure 1. Conceptual diagram illustrating: (A) Unimodal response of consumer growth [*f(x)*] to resource C:nutrient ratio (*x*). Three regions characterize this curve: (1) Resource C:nutrient ratio that yields increasing *f(x)* with *f´(x) > 0*, indicating impaired growth because of nutrient-rich food; (2) Optimum resource C:nutrient ratio ($x_{opt\ C:nut}$, dashed line) that yields maximum consumer growth $f(x_{opt\ C:nut})$, close to the likely threshold elemental ratio (TER, grey shaded region), with $f´(x_{opt\ C:nut}) = 0$; (3) Resource C:nutrient ratio that yields decreasing *f(x)* with *f´(x) < 0*, indicating impaired growth because of nutrient-deficient food. (B) Two hypothetical scenarios of the unimodal response of consumer growth to resource C:nutrient ratio: (1) Highly peaked growth curves [high values of /*f´´(x)*/ ("growth sensitivity index", GSI) representing high consumer sensitivity to food quality fluctuations around $x_{opt\ C:nut}$, and (2) Flat-topped growth curves (low values of /*f´´(x)*/ (GSI)] representing low consumer sensitivity to food quality fluctuations. *See* Material and methods for further description. *Diagrama conceptual que ilustra: (A) Respuesta unimodal del crecimiento del consumidor [*f(x)*] a la razón C:nutriente del recurso (*x*). Tres regiones caracterizan a esta curva: (1) Razón C:nutriente del recurso que da lugar a* f(x) *creciente con* f´(x) > 0*, que indica crecimiento reducido debido a alimento enriquecido en nutrientes; (2) Razón C:nutriente óptima del recurso (*$x_{opt\ C:nut}$*, línea discontinua) que da lugar a crecimiento máximo del consumidor* $f(x_{opt\ C:nut})$*, próxima a la razón elemental umbral (TER, región sombreada de color gris), con* $f´(x_{opt\ C:nut}) = 0$*; (3) Razón C:nutriente del recurso que da lugar a* f(x) *decreciente con* f´(x) < 0*, que indica crecimiento reducido debido a alimento deficitario en nutrientes. (B) Dos escenarios hipotéticos de la respuesta unimodal del crecimiento del consumidor a la razón C:nutriente: (1) Curvas de crecimiento muy apuntadas [valores elevados de* /f´´(x)/ *("índice de sensibilidad del crecimiento", GSI) que representan una elevada sensibilidad del consumidor a las fluctuaciones de calidad de alimento alrededor de* $x_{opt\ C:nut}$*, y (2) curvas de crecimiento achatadas [bajos valores de* /f´´(x)/ *(GSI)] que representan una baja sensibilidad del consumidor a las fluctuaciones en la calidad de alimento. Ver* Material and methods *para una descripción en detalle.*

Simple linear regression was used to test the effects of (i) individual size on %RNA; (ii) $x_{opt\ C:nut}$ and GSI on mean stage-specific size; and (iii) TP, $I_{\mathrm{m\ UVR}}$, and $I_{\mathrm{m\ UVR}}$:TP ratio on seston C:P ratio. A homogeneity of slopes model [analysis of covariance (ANCOVA)] was used to test the effect of NAI (categorical factor) across the continuous predictor variables (covariates, $x_{opt\ C:P}$ and $x_{opt\ C:N}$) on mean stage-specific size (Quinn & Keough, 2002). To determine which variable best linearly predicted seston C:P ratio, we used Akaike´s Information Criterion (AIC) as selection method. The AIC was calculated as:

$$\mathrm{AIC} = n \log(\sigma^2) + 2K$$

where,

$$\sigma^2 = \frac{\sum \varepsilon_i^2}{n}$$

in which ε_i is an estimated residual for candidate regression model, n is the number of cases and K is the total number of estimated parameters plus 1 (for σ^2) (Burnham & Anderson, 1998).

In the experimental study, the light effects (UVR *vs.* PAR) on Chl *a*, and seston C:nutrient ratios in the mesocosms were tested by one-way ANOVA. The effects of light (UVR *vs.* PAR) and nutrients (no nutrient addition *vs.* nutrient addition) on seston C and C:nutrient ratios in the microcosms were tested by two-way ANOVA. Finally, a three-way ANOVA was used to test the effects of light, nutrients, ontogeny and gender on NAIs. A *post-hoc* Tukey hsd test was used to test differences between treatments or developmental stages. Normality was tested by Shapiro–Wilks *W*-test and homoscedasticity by Levene's test. Statistical analyses were performed using STATISTICA 7.1 for Windows software (StatSoft, 2005).

Results

Interannual study

The herbivore *Mixodiaptomus laciniatus* showed large intraspecific variability in growth rates, as reflected by the wide range in mean %RNA, with up to >12-fold higher values in nauplii than adults. There was a strong significant effect of ontogeny on both NAIs (Kruskal-Wallis ANOVA tests: %RNA, $H_{5,\ 34}$ = 23.43, *p*-value = <0.001; RNA:DNA ratio, $H_{5,34}$ = 11.54, *p*-value = 0.041). In particular, mean %RNA decreased from 8.25% in nauplii to 0.64% in adults. This reflected in a negative correlation between individual size and %RNA (%RNA = -0.007*size + 8.83, R^2 = 0.50, *p*-value <0.001). In contrast, no gender differences in NAIs were found (Kruskal-Wallis test: %RNA, $H_{1,\ 11}$ = 1.2, *p*-value = 0.27; RNA:DNA ratio, $H_{1,11}$ = 3.33, *p*-value >0.05) (Fig. 2 in Appendix). Much of the intraspecific variability in NAIs was due to differences observed within a given stage (*i.e.* intra-stage variability). Thus, the stage-specific coefficients of variation (CVs) ranged 21-37% for %RNA and 18-50% for RNA:DNA ratio. Variability within adults of a given gender was also considerable (CV = 21-38% for %RNA and 39-43% for RNA:DNA ratio). To investigate the mechanisms behind this high variability we analysed NAIs in response to factors potentially affecting animal growth (temperature, food quantity and quality variables). Only food quality variables were significant, and in all cases (copepodite and adult stages) the relationships were unimodal. Also NAIs showed pronounced variability in nauplii and copepodite CI, but the low number of samples precluded unimodal fits (Fig. 2, Table 1).

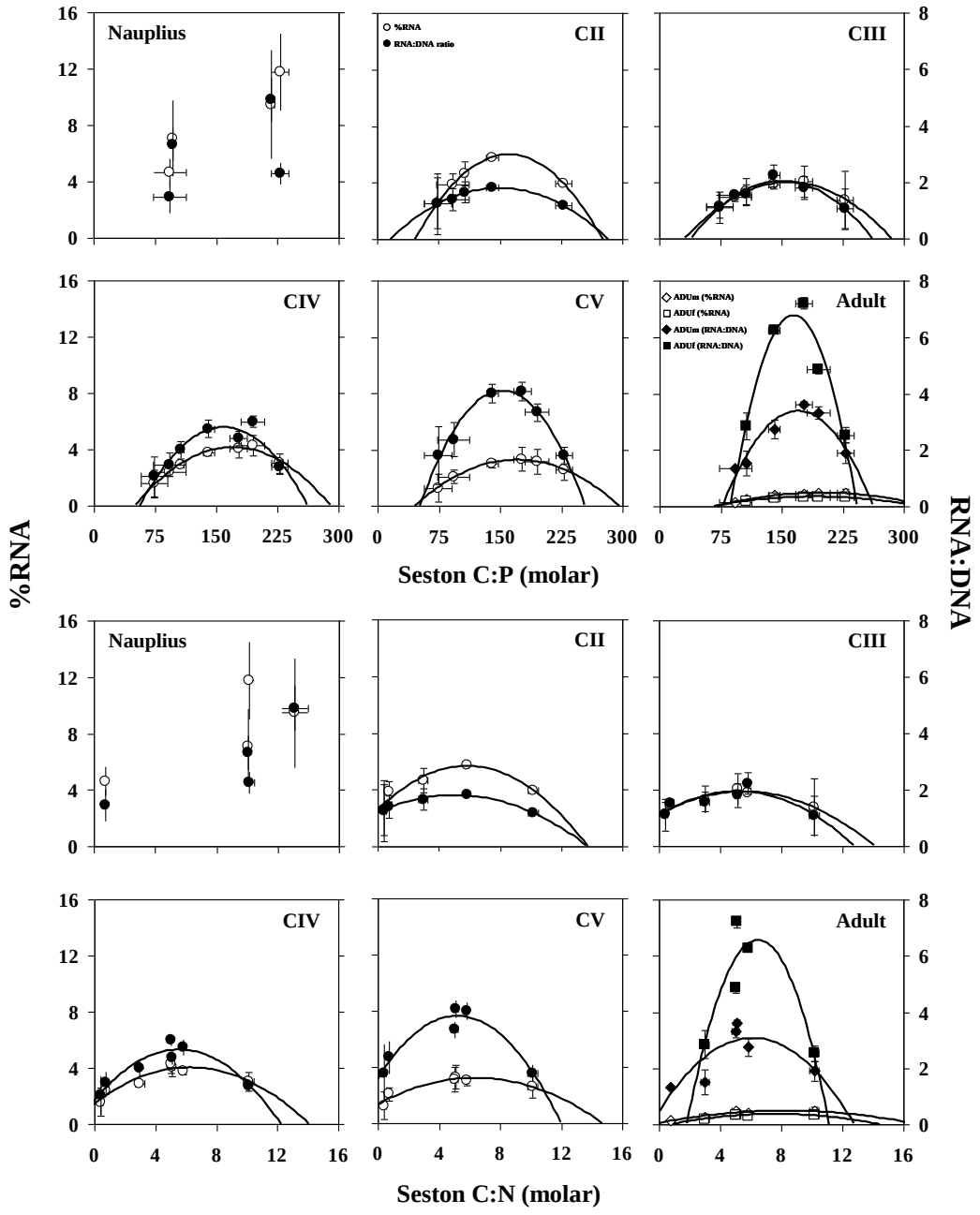
Nauplius
%RNA
RNA:DNA ratio
CII
CIII
CIV
CV
Adult
ADUm (%RNA)
ADUf (%RNA)
ADUm (RNA:DNA)
ADUf (RNA:DNA)
%RNA
RNA:DNA
Seston C:P (molar)
Seston C:N (molar)

Figure 2. Relationships between the seston C:P and C:N ratios and RNA content (% of dry weight, %RNA) and RNA:DNA ratio for each ontogenetic stage (nauplius; CII-CV, copepodites II-V) and adult gender (ADUm, adult male; ADUf, adult female) of *Mixodiaptomus laciniatus*. Circles and error bars represent mean values and standard deviations for each sampling day in the ice-free seasons of 2005, 2006, and 2007. Lines represent polynomial regression fits. *See* Table 1 for regression parameters and statistics. *Relaciones entre las razones C:P y C:N del seston y el contenido en RNA (% de peso seco, %RNA) y la razón RNA:DNA para cada estadío ontogenético (nauplius; CII-CV, copepoditos II-V) y género de adulto (ADUm, macho adulto; ADUf, hembra adulta) de* Mixodiaptomus laciniatus*. Los círculos y las barras de error representan los valores promedio y las desviaciones estándar para cada día de muestreo en los períodos libres de hielo en 2005, 2006, y 2007. Las líneas representan los ajustes por regresión polinómica.* Ver *en* Table 1 *los parámetros de regresión y los estadísticos.*

Dependent variable [*f(x)*]	Independent variable (*x*)	Stage-Sex	*c*	*b*	*a*	p_c	p_b	p_a	R^2
%RNA	Seston C:P	CII	**-0.00045**	**0.15**	**-5.78**	**<0.001**	**<0.001**	**<0.001**	**1.00**
		CIII	**-0.00025**	**0.08**	**-2.20**	**0.001**	**0.001**	**0.012**	**0.98**
		CIV	**-0.00029**	**0.10**	**-4.19**	**0.001**	**0.001**	**0.004**	**0.97**
		CV	**-0.00021**	**0.07**	**-2.80**	**<0.001**	**<0.001**	**0.001**	**1.00**
		Adult	**-0.00005**	**0.02**	**-1.12**	**<0.001**	**<0.001**	**<0.001**	**0.98**
		Adult male	**-0.00006**	**0.02**	**-1.28**	**0.043**	**0.024**	**0.047**	**0.96**
		Adult female	**-0.00004**	**0.02**	**-0.90**	**<0.001**	**<0.001**	**<0.001**	**1.00**
	Seston C:N	CII	-0.09102	1.06	2.55	0.074	0.063	0.037	0.88
		CIII	**-0.05320**	**0.59**	**2.23**	**0.013**	**0.012**	**0.002**	**0.91**
		CIV	**-0.06571**	**0.82**	**1.46**	**0.009**	**0.005**	**0.011**	**0.90**
		CV	**-0.04635**	**0.59**	**1.34**	**0.024**	**0.014**	**0.013**	**0.91**
		Adult	-0.01109	0.18	0.11	0.060	0.026	0.393	0.90
		Adult male	-0.01299	0.21	0.09	0.055	0.022	0.514	0.92
		Adult female	-0.01570	0.24	-0.17	0.157	0.135	0.627	0.78
RNA:DNA	Seston C:P	CII	-0.00010	0.03	-0.45	0.023	0.025	0.304	0.96
		CIII	-0.00016	0.05	-1.64	0.012	0.013	0.078	0.91
		CIV	**-0.00026**	**0.08**	**-3.86**	**0.012**	**0.010**	**0.036**	**0.85**
		CV	**-0.00040**	**0.12**	**-5.24**	**0.003**	**0.002**	**0.009**	**0.97**
		Adult	**-0.00067**	**0.22**	**-13.87**	**0.005**	**0.004**	**0.007**	**0.96**
		Adult male	**-0.00039**	**0.13**	**-7.98**	**0.017**	**0.014**	**0.025**	**0.92**
		Adult female	-0.00113	0.37	-23.87	0.039	0.040	0.060	0.92
	Seston C:N	CII	**-0.02394**	**0.24**	**1.20**	**0.007**	**0.008**	**0.001**	**0.99**
		CIII	-0.03307	0.34	1.08	0.053	0.058	0.019	0.76
		CIV	**-0.05906**	**0.65**	**0.84**	**0.004**	**0.004**	**0.024**	**0.90**
		CV	**-0.08222**	**0.85**	**1.60**	**0.009**	**0.009**	**0.014**	**0.93**
		Adult	-0.11639	1.41	-0.03	0.051	0.045	0.983	0.79
		Adult male	-0.06962	0.85	0.46	0.092	0.081	0.625	0.69
		Adult female	-0.30282	3.93	-6.17	0.087	0.097	0.268	0.85

Table 1. Regression analyses of the effect of food quality, measured as seston C:P and C:N molar ratios, on RNA content (% of dry weight, %RNA) and RNA:DNA ratio for each ontogenetic stage and gender in adults of *Mixodiaptomus laciniatus*. Adults have been analyzed with and without differentiation between genders. Regression model was given by the function $f(x) = cx^2 + bx + a$. Significant regressions are shown in bold. *Análisis de regresión del efecto de la calidad de alimento, medida por las razones molares C:P y C:N del seston, sobre el contenido en RNA (% de peso seco, %RNA) y la razón RNA:DNA para cada estadío ontogenético y género de los adultos de* Mixodiaptomus laciniatus*. Los adultos han sido analizados con y sin diferenciación entre géneros. El modelo de regresión vino dado por la función* $f(x) = cx^2 + bx + a$. *Las regresiones significativas se muestran en negrita.*

$x_{opt\ C:nut}$ were correlated to mean stage-specific size, indicating that seston food quality for maximum growth varied throughout ontogeny (Table 2, and Fig. 3 in the Appendix). The slopes of the relationships were positive and did not differ whether the covariate $x_{opt\ C:nut}$, obtained from %RNA or RNA:DNA ratio growth functions, was $x_{opt\ C:P}$ (ANCOVA: intercept, $F_{1,8}$ = 10.14, p-value = 0.012; slope, $F_{1,8}$ = 2.01, p-value = 0.194) or $x_{opt\ C:N}$ (ANCOVA: Intercept, $F_{1,8}$ = 2.35, p-value = 0.163; slope, $F_{1,8}$ = 1.34, p-value = 0.280). But also the sensitivity of consumers to food quality varied throughout organism development with a decreasing trend as ontogeny progressed as indicated by the negative linear relationships found between GSI and organismal size (Table 2; Fig. 3 in Appendix).

Independent variable (x)	b	a	p-value	R^2
$x_{opt\ C:P}$ (%RNA)	**10.32**	**-950.77**	**0.011**	**0.84**
$x_{opt\ C:P}$ (RNA:DNA ratio)	**18.69**	**-2090.96**	**0.034**	**0.71**
$x_{opt\ C:N}$ (%RNA)	**158.88**	**-200.24**	**0.015**	**0.81**
$x_{opt\ C:N}$ (RNA:DNA ratio)	**262.63**	**-610.57**	**0.038**	**0.70**
$GSI_{C:P}$ (%RNA)	**-573590.00**	**1105.41**	**0.005**	**0.89**
$GSI_{C:N}$ (%RNA)	**-2935.38**	**1136.15**	**0.005**	**0.88**

Table 2. Simple linear regressions of optimum seston C:P ($x_{opt\ C:P}$) and C:N ($x_{opt\ C:N}$) (molar) ratios and consumer growth sensitivity index for seston C:P ($GSI_{C:P}$) and C:N ($GSI_{C:N}$) ratios (independent variables, x) against mean stage-specific size (dependent variable, y). Nucleic acid index (%RNA or RNA:DNA ratio) used to obtain $x_{opt\ C:P}$, $x_{opt\ C:N}$, $GSI_{C:P}$, $GSI_{C:N}$ is given in brackets. Linear regression model was $y = bx + a$. Significant regressions are shown in bold. *Regresiones lineales simples de las razones (molares) óptimas C:P ($x_{opt\ C:P}$) y C:N ($x_{opt\ C:N}$) del seston y del índice de sensibilidad del crecimiento del consumidor a las razones C:P ($GSI_{C:P}$) y C:N ($GSI_{C:N}$) del seston (variables independientes,* x*) frente al tamaño promedio específico de cada estadío (variable dependiente,* y*). El índice de ácidos nucleicos (%RNA o razón RNA:DNA) utilizado para obtener* $x_{opt\ C:P}$, $x_{opt\ C:N}$, $GSI_{C:P}$, $GSI_{C:N}$ *viene dado en paréntesis. El modelo de regresión lineal fue* y = bx + a. *Las regresiones significativas se muestran en negrita.*

We measured a variety of physico-chemical characteristics (TP, $I_{m\ UVR}$ and $I_{m\ UVR}$:TP ratio) of the study lake, and regression analyses were performed to evaluate the underlying mechanisms controlling seston C:P ratio as food quality variable for herbivorous consumers. Thus, seston C:P ratio was positively correlated to TP (seston C:P ratio = 9.38*TP + 97.30, R^2 = 0.37, *p*-value = 0.027, AIC = 48.16). In contrast, seston C:P ratio was negatively correlated to $I_{m\ UVR}$ (seston C:P ratio = -231.13*$I_{m\ UVR}$ + 244.62, R^2 = 0.56, *p*-value = 0.005, AIC = 42.98), although based on the slightly lower AIC value, $I_{m\ UVR}$:TP ratio was a better predictor of seston C:P ratio (seston C:P ratio = -15.90*$I_{m\ UVR}$:TP ratio + 205.32, R^2 = 0.58, *p*-value = 0.004, AIC = 42.81).

Experimental study

Based on the results of the interannual study, we experimentally tested how food quality differences due to UVR and nutrient manipulation had an effect on consumer growth. Presence and absence of UVR affected seston food quality in terms of C:P (one-way ANOVA: $F_{1,\ 4}$ = 11.48, *p*-value = 0.028) and C:N (one-way ANOVA: $F_{1,\ 4}$ = 10.10, *p*-value = 0.034) ratios in the mesocosms. As a result, we found significantly lower values of both seston C:P and C:N ratios in the UVR *vs.* PAR ($C:P_{UVR}$ = 97.39 ± 31.23 *vs.* $C:P_{PAR}$ = 409.77 ± 156.77; $C:N_{UVR}$ = 3.01 ± 2.42 *vs.* $C:N_{PAR}$ = 9.81 ± 2.81) treatments. In contrast, UVR did not significantly affect Chl *a* (one-way ANOVA: $F_{1,\ 4}$ = 4.24, *p*-value = 0.11), which showed low values (Chl $a_{\ UVR}$: 1.44 ± 0.18 *vs.* Chl $a_{\ PAR}$: 2.52 ± 0.89) similar to those found in the system (mean Chl *a* < 2 μg L^{-1}, and 69% of observations < 0.7 μg L^{-1}). In the microcosms, joint manipulation of light and nutrients did not affect the quantity (seston C) and quality of food for zooplankton, except for seston C:P ratio which increased in the absence of UVR when no nutrients were added (Table 3; Fig. 5 in Appendix) (*post hoc* Tukey hsd tests: *p*-value = 0.001 for PAR *vs.* UVR; *p*-value < 0.001 for PAR *vs.* UVR+NP; *p*-value < 0.001 for PAR *vs.* PAR+NP).

NAIs values for the experimental study were within the range observed for the individuals collected from the lake. As for the interannual study, a clear

ontogenetic pattern was observed with decreasing %RNA from early copepodite stages to adults, and vice-versa for RNA:DNA ratios. The ANOVA showed that ontogeny explained most of the variance in %RNA (85%) and RNA:DNA ratio (69%), although light and nutrients contributed 1-5-2,8% in %RNA and 21-31% in RNA:DNA ratio (Fig. 3, Table 3). In particular, coinciding with the highest seston C:P ratio, the lowest %RNA was found in the PAR treatment for early copepodites stages (most *post-hoc* Tuckey hsd test resulted in p-value < 0.05 for inter-treatments comparisons for copepodites, and p-value > 0.05 for adults), which is consistent with the higher sensitivity to food quality found for these stages compared to adults in the interannual study. Differences in %RNA and RNA:DNA ratio between treatments in adults were only due to gender (three-way ANOVA: %RNA: Light, $F_{1,12}$ = 1.31, p-value = 0.274; nutrients, $F_{1,12}$ = 0.00, p-value = 0.977; gender, $F_{1,12}$ = 6.44, p-value = 0.026; RNA:DNA ratio: Light, $F_{1,12}$ = 3.34, p-value = 0.092; nutrients, $F_{1,12}$ = 4.06, p-value = 0.067; gender, $F_{1,12}$ = 4.83, p-value = 0.048) (*see* insets in Fig. 3).

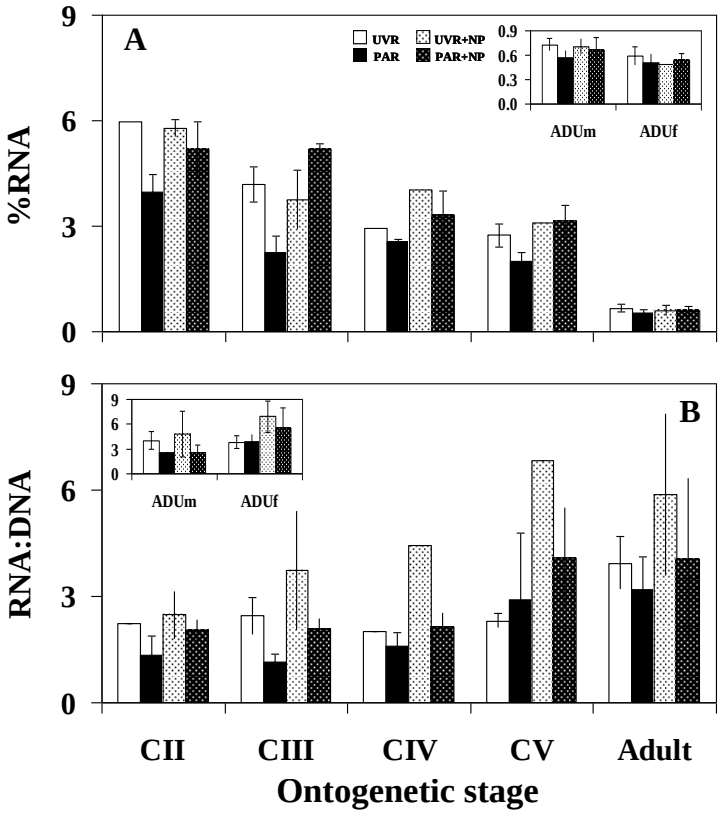

Figure 3. (A) RNA content (% of dry weight, %RNA) and (B) RNA:DNA ratio for each ontogenetic stage of *Mixodiaptomus laciniatus* in the field light × nutrient experiment after 1-week incubation. Insets represent (A) %RNA and (B) RNA:DNA ratio for each adult gender. UVR: Full sunlight; UVR+NP: Full sunlight + nutrient addition; PAR: Screened sunlight (>380 nm); PAR+NP: Screened sunlight (>380 nm) + nutrient addition. Columns are mean values and error bars are standard deviations. Stages are copepodites (CII-CV) and adults (ADUm, adult male; ADUf, adult female). *See* Table 3 and Results section for statistical results. *(A) Contenido en RNA (% de peso seco, %RNA) y (B) la razón RNA:DNA para cada estadío ontogenético de* Mixodiaptomus laciniatus *en el experimento de campo luz × nutrientes después de una semana de incubación. Las figuras internas representan (A) %RNA y (B) la razón RNA:DNA para cada género de los adultos. UVR: Luz total; UVR+NP: Luz total + nutrientes añadidos; PAR: Luz filtrada (>380 nm); PAR+NP: Luz filtrada + nutrientes añadidos. Las columnas son los valores promedio y las barras de error son las desviaciones estándar. Los estadíos son copepoditos (CII-CV) y adultos (ADUm, macho adulto; ADUf, hembra adulta).* Ver *en* Table 3 *y en la sección* Results *los resultados estadísticos.*

	Seston C				Seston C:P				Seston C:N				%RNA				RNA:DNA			
	df	*F*	*p* -value	*PV*	df	*F*	*p* -value	*PV*	df	*F*	*p* -value	*PV*	df	*F*	*p* -value	*PV*	df	*F*	*p* -value	*PV*
Light	1	1.38	0.284	0.93	**1**	**25.07**	**0.002**	**1.66**	1	0.05	0.838	0.00	**1**	**17.36**	**<0.001**	**1.54**	**1**	**7.36**	**0.010**	**21.51**
Nutrients	1	0.01	0.914	0.01	**1**	**48.52**	**<0.001**	**3.22**	1	2.77	0.147	0.30	**1**	**32.30**	**<0.001**	**2.86**	**1**	**11.50**	**0.002**	**33.62**
Ontogeny	-	-	-	-	-	-	-	-	-	-	-	-	**4**	**239.99**	**<0.001**	**85.08**	**4**	**5.88**	**0.001**	**68.72**
Light × nutrients	1	0.02	0.893	0.01	**1**	**34.24**	**0.001**	**2.27**	1	1.15	0.325	0.13	**1**	**19.96**	**<0.001**	**1.77**	1	2.05	0.161	5.99
Light × ontogeny	-	-	-	-	-	-	-	-	-	-	-	-	**4**	**3.59**	**0.015**	**1.27**	4	0.10	0.980	1.21
Nutrients × ontogeny	-	-	-	-	-	-	-	-	-	-	-	-	**4**	**4.69**	**0.004**	**1.66**	4	0.74	0.571	8.65
Light × nutrients × ontogeny	-	-	-	-	-	-	-	-	-	-	-	-	**4**	**7.91**	**<0.001**	**2.80**	4	0.52	0.722	6.07

Table 3. Results of the analyses of variance (ANOVAs) examining the effects of light and nutrients on seston C (μg C L^{-1}), C:P and C:N molar ratios, and light, nutrients, and ontogeny on RNA content (% of dry weight, %RNA) and RNA:DNA ratio of *Mixodiaptomus laciniatus*. Sample sizes were 3×2×2 (replicates × light × nutrients) = 12 for sestonic variables and 3×2×2×5 (replicates × light × nutrients × developmental stages) = 60 for %RNA and RNA:DNA ratio. Reported are: Degrees of freedom (df), *F*-test results (*F*), significance level (*p*-value) and % of variance (*PV*) calculated as sums of squares of treatment:total sums of squares. Significant results are shown in bold. *Resultados de los análisis de la varianza (ANOVAs) que examinan los efectos de la luz y los nutrientes sobre el C sestónico (*μg C L^{-1}*), y las razones molares C:P y C:N del seston, y los efectos de la luz, los nutrientes, y la ontogenia sobre el contenido en RNA (% de peso seco, %RNA) y la razón RNA:DNA de* Mixodiaptomus laciniatus*. Los tamaños de muestra son 3×2×2 (réplicas × luz × nutrientes) = 12 para las variables sestónicas y 3×2×2×5 (réplicas × luz × nutrientes × estadíos de desarrollo) = 60 para %RNA y la razón RNA:DNA. Se indican: Los grados de libertad (df), los resultados del* F-*test* (F*), el nivel de significación (*p-*valor) y % de varianza (PV) calculado como la suma de cuadrados:suma total de cuadrados. Los resultados significativos se muestran en negrita.*

Discussion

Our results indicate that consumer growth response to food quality was stage-dependent and, surprisingly, adjusted to unimodal fits where suboptimal food qualities were found due to the excess and deficient content of mineral nutrients. The thorough mathematical analysis of the unimodal curves discussed below, provided not only an optimum food quality, but also an indication of consumer sensitivity to mineral limitation, which characteristically decreased as ontogeny progressed, possibly attributable to differential energy and nutrient demands in the consumer life history.

Before making inferences about the importance of the unimodal responses observed in this study, it is necessary to highlight the sources of intraspecific variability in NAIs. First, both %RNA and RNA:DNA ratios showed strong significant changes during the animal life history from nauplii to late copepodite and adult stages, *i.e.* there was pronounced variation in NA content during ontogeny. Similar ontogenetic patterns in NAIs are observed for other crustacean species from both freshwater and marine systems (*e.g.* Wagner *et al.*, 2001; Gorokhova & Kyle, 2002; Bullejos *et al.*, 2012), and are consistent with ontogenetic variations described for other biochemical and mineral constituents (Carrillo *et al.*, 2001; Villar-Argaiz *et al.*, 2002; Ventura & Catalan, 2010). Second, this study shows that a given developmental copepod stage can exhibit pronounced differences in NA content, supporting previous observations that other factors, besides ontogeny, are responsible for NA variation in consumers (*e.g.* Wagner *et al.*, 2001; Van Geest *et al.*, 2010). The stage-specific relationship between NAIs and seston C:nutrient ratio highly reinforces that mineral availability is a chief component for consumer growth (*e.g.* Villar-Argaiz & Sterner, 2002; Vrede *et al.*, 2002; Elser *et al.*, 2005). Remarkably, the response of consumers to food quality was not linear but, instead, adjusted to unimodal curves. As a consequence, the performance of the organisms was highest at intermediate C:nutrient ratio, but weakened towards both ends of the food quality gradient.

The unimodal responses observed in this study have important implications. Because suboptimal food quality ensue from low and high concentrations of a potentially limiting nutrient it is reasonable to suggest that previously established C:nutrient ratio-thresholds should not be consider as hard cut-offs below which consumers do not respond to the nutrient content of food. Instead, thresholds should contemplate shifts associated with the varying demands of nutrients across consumer life history. In their relatively recent review, Frost *et al.* (2006) suggested that, as an average, $TER_{C:P}$ was 2.4 times higher than the average body C:P ratio. This means that when food is below the TER, one would expect organisms' limitation by C, and that P would not affect their growth. In a previous study, Villar-Argaiz *et al.* (2002) showed that molar C:N:P ratio for the copepod *Mixodiaptomus laciniatus* varied ontogenetically from 99:3:1 in nauplii to 165:13:1 in immature copepodites and 234:25:1 in adults. Because seston C:P and C:N ratios were always below Frost's hypothetical TER (C:P < 228, C:N < 18), copepods in this study were not expected to respond to the content of P and N in seston. Contrary, copepods showed strong changes in growth in response to food P-content at a low food quantity. This finding is consistent with recent observations for this ultraoligotrophic lake where *Mixodiaptomus laciniatus* growth was strongly related to the P content of seston (Villar-Argaiz *et al.*, 2012). If copepods with complex life histories and high C:N:P ratios suffer growth penalties due to P, one would expect a much stronger impact on other P-rich species such as *Daphnia*. Hitherto, considerable research of herbivore nutrition has focussed on establishing the boundary at which the mineral content of food can slow or limit the growth of consumers (Frost *et al.*, 2006). Our results show that thresholds might not only vary ontogenetically, but also suggest that consumer growth is sensitive to the excess in the nutrient content of the diet below TER.

Are unimodal responses common in nature? The unimodal fits that we document here are consistent with trends reported by Boersma & Elser (2006) at which consumer growth or overall performance was maximal at intermediate nutrient contents but decreased towards the lower and higher ends of the nutrient gradient. Therefore, nonlinear models may not only be a common phenomenon,

but could be the rule rather than the exception in the response of consumers to nutrient limitation. In a previous study, Elser *et al.* (2005) proposed the "stoichiometric knife-edge" hypothesis to argue the existence of an optimum resource C:nutrient ratio ("trophic or stoichiometric knife-edge"), close to the TER, that satisfies consumer´s requirements, and at which lower and higher values yield growth penalties. The unimodal growth responses shown here support the detrimental role of both nutrient shortage and excess for herbivorous growth, but for the first time, illustrates that the pronounced differences in consumer response to food quality not only occurred for a given species but also at the stage-level in natural conditions.

We further argue that examination of unimodal fits, and more specifically solving model derivate, have great potential to analyse nutritional ecology of consumers. Thus, the first derivate may be a powerful procedure when trying to infer consumer optimum demands for nutrients. In addition, the direct relationship between $x_{opt\ C:nut}$ and organismal size in this study reflects changes in the specific element requirements throughout the life history of the copepod. Thus, the increasing ontogenetic trend in their optimum $x_{opt\ C:nut}$ is consistent with previous published results for higher overall demands for C in adults which are able to divert their C sources to maintenance and reproduction (Villar-Argaiz *et al.*, 2002). Likewise, knowing the extent to which organisms are vulnerable to the effects of food quality is essential when trying to infer consumer nutrient limitation in nature. The second derivate of unimodal functions provides a powerful indication of consumer susceptibility to food quality. Thus, our results showing decreased growth sensitivity to food quality from early stages to late copepodite and adult stages are consistent with findings of a pronounced ontogenetic variation in copepod susceptibility to P-food quality, from high susceptibility in nauplii and immature copepodites to low susceptibility towards maturity (Villar-Argaiz & Sterner, 2002).

Natural selection operates on life history to maximize individual fitness. Therefore, one might expect that the selective pressure imposed by limiting nutrients in nature should determine organism adaptive strategy, coupling the life

history to the nutrient availability in order to minimize the elemental mismatch at the autotroph-herbivore interaction. Likely the ontogenetic variations described here might be sustained by fluctuating selection (Reznick *et al.*, 2000). This is supported by the fairly predictable seasonal variation in resource quality for herbivorous consumers. Thus, nutrient-rich algae (low seston C:N:P ratios) regularly grow early in the season (ice-out in high mountain lakes) coinciding with the development of high-growth nauplii with high nutrient requirements, whereas nutrient limitation in resources is more likely to occur towards the end of the growing season when copepodite and adults, with lower growth rates and nutrient demands, develop (Villar-Argaiz *et al.*, 2001). Søreide *et al.* (2010) observed that consumer life history varied predictably with the biochemical quality of its resource in Arctic marine ecosystems, suggesting that these types of selection constraints may also extend to other food quality parameters and environments.

Also, this study provides with evidence that, in addition to nutrients, UVR contributes to the qualitative yield of primary producers. While this is consistent with previous studies to report potential food quality alterations due to UVR (*e.g.* Xenopoulos *et al.*, 2002; Carrillo *et al.*, 2008; Hessen *et al.*, 2008), our experimental results showed that these effects transfer to consumers in nature, particularly early in the life history when nutrient demands are high. The fact that experimental suppression of UVR resulted in increased seston C:P ratios and dampened copepod growth suggests that UVR has a more complex effect than previously thought by affecting the nutritional imbalance at the primary producer-consumer interface. In contrast to the widely known detrimental effects of UVR (*e.g.* Helbling & Zagarese, 2003; Häder *et al.*, 2007), we showed that UVR may indirectly enhance copepod growth, possibly driving seston C:nutrient ratios towards values close to $x_{opt\ C:nut}$. Follow-up research should examine the generality of these results for other taxon with complex developmental life histories and analyse the impact of joint stressors at the plant-animal interface.

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Appendix:

I. Additional information for Methods section

This appendix offers an expanded description of some procedures described briefly in the main text.

Primary production measurements

Primary production was measured with the ^{14}C method proposed by Steeman-Nielsen (1952). Sets of four 50 mL quartz flasks (three clear and one dark) added with 0.37 MBq of $NaH^{14}CO_3$ [specific activity (SA): 310.8 MBq $mmol^{-1}$, NEN Dupont] were incubated *in situ*, at the depth where UVR was 75% of that at the surface, for 4 h symmetrically distributed around noon. All flask sets were horizontally held during the incubations. Primary production was measured as total organic carbon (TOC) by acidifying a 4-mL subsample in a 20-mL scintillation vial with 100 μL of 1 N HCl and allowing the vial to stand open in a hood for 24 h (no bubbling), as recommended by Lignell (1992). Particulate primary production >1.0 μm (particulate organic carbon >1.0 μm, POC_1) was determined by filtering an aliquot of 40 mL through 1.0 μm pore-size Nucleopore filters of 25-mm diameter. To minimize cell breakage, we applied low pressure (<100 mm of Hg). The filters were placed in scintillation vials and the dissolved inorganic ^{14}C was removed by adding 100 μL of 1 N HCl. We added 16 mL of liquid scintillation cocktail (Beckman Ready Safe) to the vials, and after 12 h the radioactivity was counted in a Beckman LS 6000 TA scintillation counter equipped with autocalibration (Beckman Instruments Inc., Fullerton, California, USA). The total CO_2 in the lake water was calculated from the alkalinity and pH measurements (APHA, 1992). In all calculations, dark values were subtracted from corresponding light values.

Nucleic acid analyses

RNA and DNA were measured on length-measured individuals using a microplate fluorimetric high-range assay with Ribogreen after N-laurylsarcosine extraction and RNase digestion, as described in Gorokhova & Kyle (2002). This method for nucleic acid quantification in zooplankton has been successfully applied, with some modifications, to both cladocerans (*e.g.* Gorokhova & Kyle, 2002) and copepods (*e.g.* Calliari *et al.*, 2006). The following working reagents were used: RiboGreen™ RNA Quantitation Kit (Invitrogen Corporation, Carlsbard, California, USA); RNase DNasefree (working solution: 5 μg mL^{-1}; Q-biogen, Weston, Masachussets, USA); N-lauroysarcosine (Sigma-Aldrich, St. Louis, Missouri, USA); Tris-EDTA buffer (Q-biogen). Fluorescence measurements were performed using a FLUOstar Optima fluorometer (microplate reader, filters: 485 nm for excitation and 520 nm for emission; BMG Labtechnologies, Ortenberg, Germany) and black solid flat-bottom microplates (Greiner Bio-One GmbH, Frickenhausen, Germany). The plate was scanned with a 0.2-s well measurement time and 10 measurements were made per well, before and after RNase digestion (30 min under dark conditions at 37 ºC). Fluorescence measurements were converted into RNA and DNA concentrations using curves previously performed with RNA (16S and 23S from *Escherichia coli*; component C of the Ribogreen Kit) and DNA (calf thymus; Sigma-Aldrich) standards. After obtained individual weights using specific length-weight relationships (Carrillo *et al.*, 2001), RNA content was expressed as percentage of dry weight (%RNA), and relatively to DNA content as RNA:DNA ratio.

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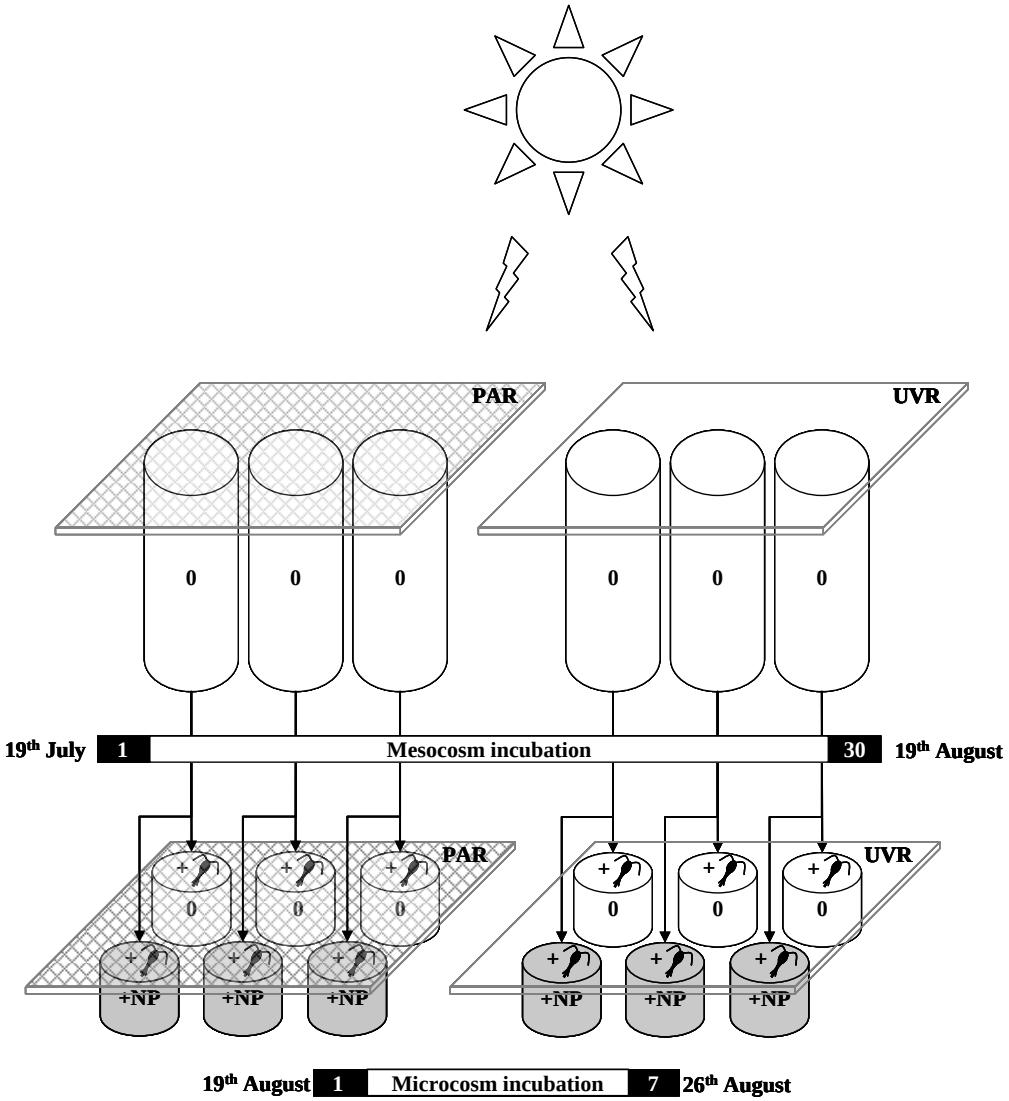
II. Additional tables

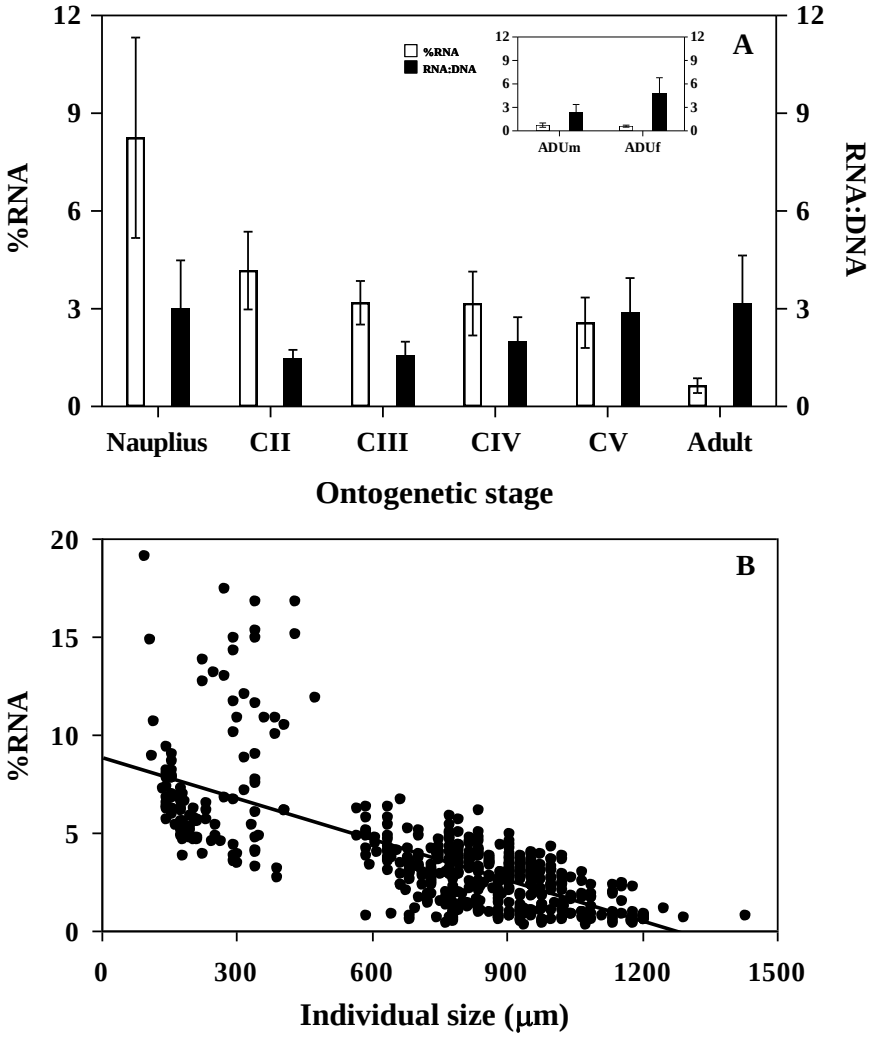
Date	$K_{d\ 305\ nm}$	$K_{d\ 320\ nm}$	$K_{d\ 380\ nm}$	$K_{d\ PAR}$
09 June 2005	1.29	1.14	0.72	0.36
01 July 2005	1.66	1.42	0.82	0.38
10 August 2005	3.18	2.75	1.55	0.54
15 September 2005	3.04	2.58	1.30	0.39
21 October 2005	3.26	2.55	1.57	0.46
12 June 2006	1.00	0.89	0.61	0.37
17 July 2006	2.57	2.35	2.05	0.56
31 August 2006	2.90	2.16	1.70	0.50
13 October 2006	1.87	1.62	0.91	0.48
13 July 2007	0.35	0.28	0.16	0.21
03 September 2007	0.77	0.64	0.37	0.30
27 September 2007	1.07	0.87	0.48	0.32

Appendix - Table 1. Diffuse attenuation coefficients for downward irradiance (K_d) in Lake La Caldera from different sampling days during ice-free seasons in 2005-2007. K_d were determined from the slope of the linear regression of the natural logarithm of downwelling irradiance *vs.* depth for three wavelengths of the UV region (305, 320 and 380 nm) and PAR (400-800 nm) of the solar radiation spectrum. *Coeficientes de atenuación difusa para la radiación incidente (K_d) en el lago La Caldera para los diferentes días de muestreo durante las estaciones libres de hielo de 2005-2007.* K_d *fue determinada a partir de la pendiente de la regresión lineal del logaritmo natural de la radiación incidente* vs. *la profundidad para las tres longitudes de onda de la región UV (305, 320 y 380 nm) y PAR (400-800 nm) del espectro de radiación solar.*

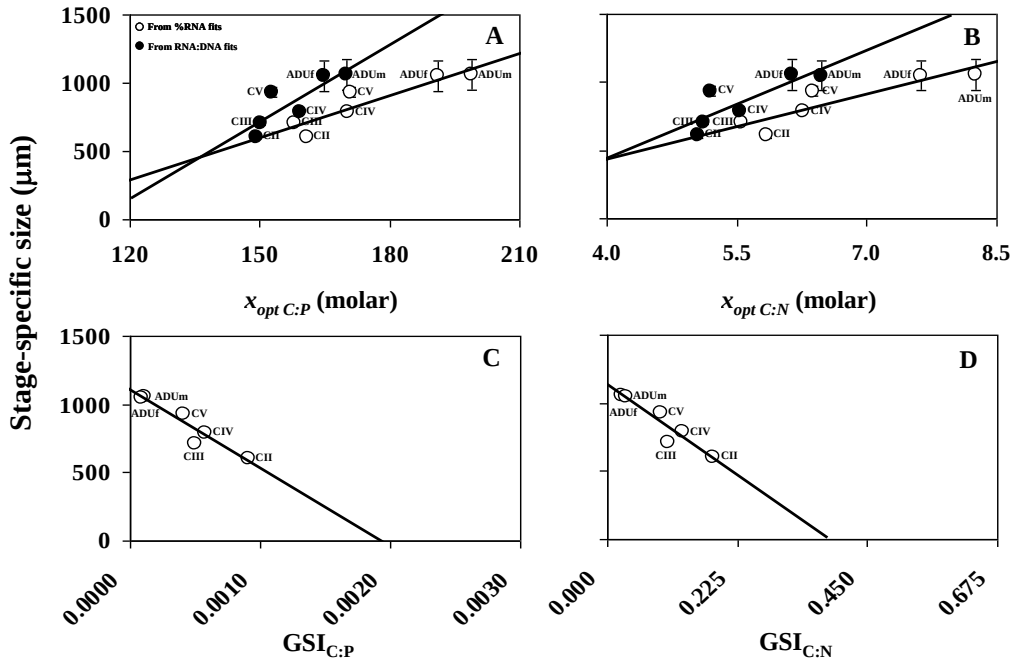
III. Additional figures



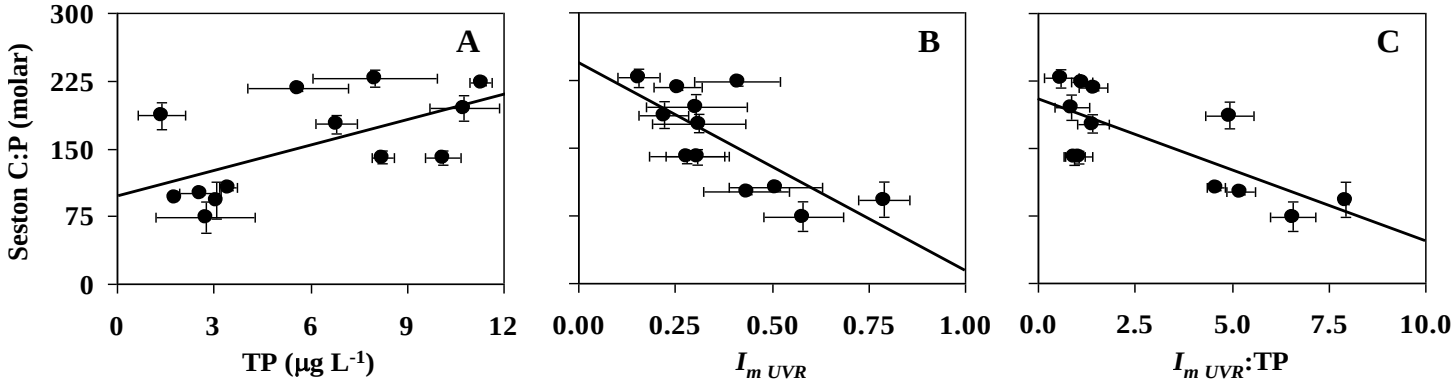
Appendix - Figure 1. Scheme illustrating the field 2×2 experimental design with two light treatments [full sunlight (UVR) *vs.* screened sunlight (>380 nm, PAR)] and two nutrient treatments [no nutrient addition (0) *vs.* nutrient addition (+NP)]. *See* Material and methods section for further details. *Esquema que ilustra el diseño experimental de campo 2×2 con dos tratamientos de luz [luz total (UVR)* vs. *luz filtrada (>380 nm, PAR)] y dos tratamientos de nutrientes [nutrientes no añadidos (0)* vs. *nutrientes añadidos (+NP)]*. Ver *la sección* Material and methods *para mayor detalle.*



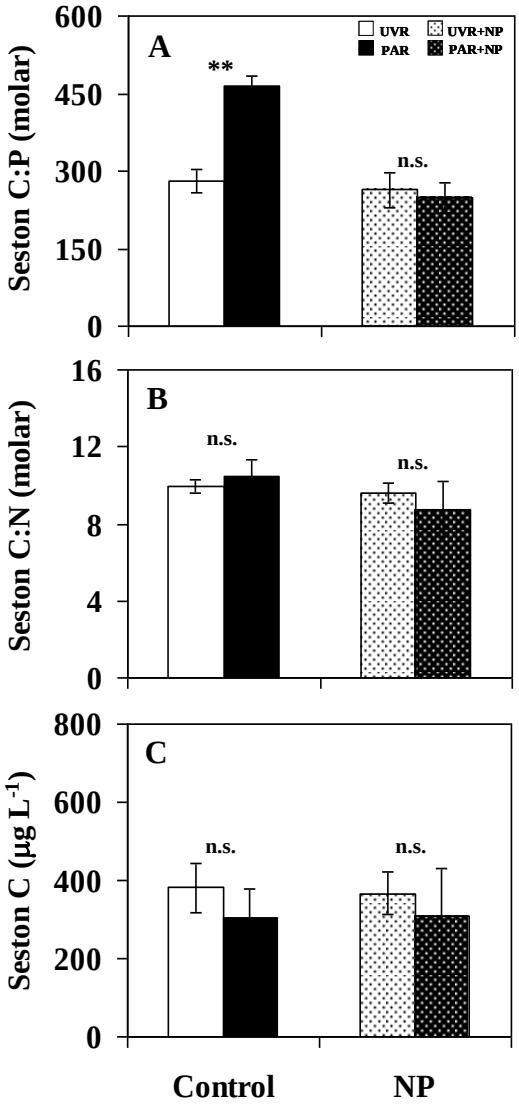
Appendix - Figure 2. (A) RNA content (% of dry weight, %RNA) and RNA:DNA ratio of *Mixodiaptomus laciniatus* developmental stages in the interannual study. Inset represents %RNA and RNA:DNA ratio for male and female adults. Columns are mean values and error bars represent standard deviations for nauplius, copepodite (CII-CV) and adults (ADUm, adult male; ADUf, adult female). (B) Relationship between individual size and %RNA. Points represent individual observations and solid line represents the linear regression fit. *See* Results section for regression parameters (slope, intercept) and statistical results. *(A) Contenido en RNA (% de peso seco, %RNA) y la razón RNA:DNA de los estadíos de desarrollo de* Mixodiaptomus laciniatus *para el estudio interanual. La figura interna representa %RNA y la razón RNA:DNA para machos y hembras adultas. Las columnas son los valores promedio y las barras de error representan las desviaciones estándar para nauplios, copepoditos (CII-CV) y adultos (ADUm, macho adulto; ADUf, hembra adulta). (B) Relación entre el tamaño individual y %RNA. Los puntos representan observaciones individuales y la línea continua representa el ajuste por regresión lineal.* Ver *en la sección* Results *los parámetros de regresión (pendiente, intercepto) y los resultados estadísticos.*



Appendix - Figure 3. Relationships between optimum seston (A) C:P ($x_{opt\ C:P}$) and (B) C:N ($x_{opt\ C:N}$) ratios, consumer growth sensitivity index for seston (C) C:P ($GSI_{C:P}$) and (D) C:N ($GSI_{C:N}$) ratios, and the stage-specific size in the interannual study. Circles are mean stage-specific sizes and vertical error bars indicate standard deviations. Solid lines represent linear regression fits. Stages are copepodites (CII-CV) and adults (ADUm, adult male; ADUf, adult female). *See* Material and methods section for definitions of "optimum seston C:P and C:N ratios", and "growth sensitivity index"; and Table 2 for regression parameters (slope, intercept) and statistical results. *Relaciones entre las razones (A) C:P ($x_{opt\ C:P}$) y (B) C:N ($x_{opt\ C:N}$) óptimas del seston, índice de sensibilidad del crecimiento del consumidor a las razones (C) C:P ($GSI_{C:P}$) y (D) C:N ($GSI_{C:N}$) del seston, y el tamaño específico de cada estadío en el estudio interanual. Los círculos son tamaños promedios específicos de cada estadío y las barras de error verticales indican las desviaciones estándar. Las líneas continuas representan ajustes por regresión lineal. Los estadíos son copepoditos (CII-CV) y adultos (ADUm, macho adulto; ADUf, hembra adulta).* Ver *en la sección* Material y métodos *las definiciones de "razones C:P y C:N óptimas del seston", e "índice de sensibilidad del crecimiento"; y en* Table 2 *los parámetros de la regresión (pendiente, intercepto) y los resultados estadísticos.*



Appendix - Figure 4. Relationships between (A) total P (TP), (B) mean UVR in the mixed layer ($I_{m\ UVR}$), and (C) $I_{m\ UVR}$:TP ratio, and seston C:P ratio. Error bars indicate standard deviations around mean values (closed circles). Solid lines are inserted for linear regression fits. *See* Results section for regression equations and statistics. *Relaciones entre (A) P total (TP), (B) UVR promedia en la capa de mezcla (*$I_{m\ UVR}$*), y (C) la razón* $I_{m\ UVR}$*:TP, y la razón C:P del seston. Las barras de error indican las desviaciones estándar alrededor de los valores promedio (círculos rellenos). Se insertan líneas continuas para los ajustes por regresión lineal.* Ver *en la sección* Results *las ecuaciones de las regresiones y los estadísticos.*


A
B
C
UVR
PAR
UVR+NP
PAR+NP
**
n.s.
Seston C:P (molar)
Seston C:N (molar)
Seston C (µg L-1)
600
450
300
150
0
16
12
8
4
800
200
Control
NP

Appendix - Figure 5. Seston (A) C:P and (B) C:N ratios, and (C) seston C in the experimental treatments after one week of incubation in Lake La Caldera. UVR: Full sunlight; UVR+NP: Full sunlight + nutrient addition; PAR: Screened sunlight (>380 nm); PAR+NP: Screened sunlight (>380 nm) + nutrient addition. Error bars indicate standard deviations. *See* Table 3 for statistical results of the effects of light and nutrients on seston C:P and C:N ratios, and seston C. Significance of *post hoc* Tukey hsd test comparisons between light treatments for each nutrient treatment: *, *p*-value < 0.05; **, *p*-value < 0.01; ***, *p*-value < 0.001; n.s., non-significant. *Razones (A) C:P y (B) C:N del seston, y (C) C sestónico en los tratamientos experimentales después de una semana de incubación en el lago La Caldera. UVR: Luz total; UVR+NP: Luz total + nutrientes añadidos; PAR: Luz filtrada (>380 nm); PAR+NP: Luz filtrada (>380 nm) + nutrientes añadidos. Las barras de error indican las desviaciones estándar.* Ver *en* Table 3 *los resultados estadísticos de los efectos de la luz y los nutrientes sobre las razones C:P y C:N del seston, y el C sestónico. La significación de las comparaciones entre tratamientos de luz para cada tratamiento de nutrientes por test Tukey hsd* post hoc*: *,* p-*valor < 0.05; **,* p-*valor < 0.01; ***,* p-*valor < 0.001; n.s., no significativo.*

VII

Life history strategies and inter- and intraspecific variability in P-stoichiometry and nucleic acids in crustacean zooplankton

VII. Life history strategies and inter- and intraspecific variability in P-stoichiometry and nucleic acids in crustacean zooplankton

Close to submission as:

Bullejos FJ, Carrillo P, Gorokhova E, Medina-Sánchez JM, Villar-Argaiz M (2012) Do copepods and cladocerans really differ in their life-history strategies? New insights from nucleic acids, and P-stoichiometry.

Abstract

We studied nucleic acids (NA) (RNA, DNA, RNA:DNA ratio) and phosphorus (P) stoichiometry for the dominant crustacean zooplankton in 22 high mountain lakes (Sierra Nevada and The Pyrenees, Spain) to assess their life-history strategies in relation to their ecological distribution, and test the validity of growth rate (GRH) and P-allocation hypotheses intra- and interspecifically. We believed that both molecular tools and stoichiometric approach will largely contribute to the understanding of the complex ongoing debate of copepods as *K*-strategists *vs*. cladocerans as *r*-strategists, and help to argue the ecological distribution of their species. Copepods had higher %RNA and %DNA than cladocerans, owing to higher values of both NA for *Cyclops abyssorum* and *Diaptomus cyaneus*, and remarkably low values of %DNA for *Daphnia* species. As a consequence, RNA:DNA ratio was much higher in cladocerans, but particularly in *Daphnia* species, than copepods. %body P resembled well patterns of NA, with higher P-investment in NA for copepods than cladocerans, although both drove more P to RNA than DNA. The analysis in detail carried out for *Mixodiaptomus laciniatus* revealed that both intra-stage and inter-stage differences in NA and P stoichiometry contributed to the observed intraspecific variability. Consequently, an ontogenetic pattern emerged, consisting of early high-growth naupliar stages with high P-investment in NA, especially in RNA (like *r*-strategists), that converted, during the ontogenetic development, into adults

with low P-investment in NA (like *K*-strategists). Our results from extensive data of NA and P from both *Mixodiaptomus laciniatus* and other crustacean species fulfilled predictions of GRH and P-allocation hypothesis, but in addition extend the taxonomically-restricted concepts of copepods as *K*-strategists and cladocerans as *r*-strategists. They point out that growth and life-history strategies are determined, apart from phylogenetic constraints, by selective pressures during ontogenetic development and those imposed by the environment.

Resumen

*Estudiamos los ácidos nucleicos (NA) (RNA, DNA, razón RNA:DNA) y la estequiometría del fósforo (P) de las especies dominantes de crustáceos del zooplancton de 22 lagos de alta montaña (en Sierra Nevada y en los Pirineos, España) para evaluar sus estrategias adaptativas con respecto a su distribución ecológica, y testar la validez de las hipótesis de la tasa crecimiento (*growth rate hypothesis, *GRH) y de* P-allocation *tanto intra- como interespecíficamente. Apostamos por que tanto las herramientas moleculares como la aproximación estequiométrica contribuirían en gran medida a la comprensión del debate, difícil de resolver, que considera a los copépodos como estrategas K frente a los cladóceros como estrategas r, y así ayudar a justificar la distribución de las especies de ambos grupos. Los copépodos mostraron mayores valores en %RNA y %DNA que los cladóceros, debido a mayores valores de ambos NA para* Cyclops abyssorum *y* Diaptomus cyaneus*, y valores marcadamente más bajos de %DNA para las especies de* Daphnia*. Como consecuencia, la razón RNA:DNA fue mucho mayor en cladóceros, particularmente en las especies de* Daphnia*, que en los copépodos. El %P somático reflejó bien los patrones de NA, con una mayor inversión de P en NA para copépodos que para cladóceros, aunque ambos grupos invirtieron más P en RNA que en DNA. El análisis en profundidad para* Mixodiaptomus laciniatus *reveló que tanto la variabilidad intra-estadío como inter-estadío en NA y en la estequiometría del P contribuían a la variabilidad intraespecífica observada. Consecuentemente, se puso de manifiesto un patrón ontogenético, que consistía en el desarrollo de estadíos naupliares con altas tasas*

de crecimiento y una alta inversión de P en ácidos nucleicos, especialmente en RNA (similar a las especies estrategas r), que se convierten, a lo largo del desarrollo ontogenético, en adultos con una baja inversión de P en NA (similar a las especies estrategas K). Nuestros resultados a partir del extenso registro de datos de NA y P de tanto Mixodiaptomus laciniatus *como de las otras especies de crustáceos cumplieron las predicciones de tanto de GRH como de* P-allocation hypothesis*, pero además amplían los conceptos taxonómicamente restringidos de copépodos como estrategas K y cladóceros como estrategas r. Muestran que tanto el crecimiento como las estrategias adaptativas están determinadas, aparte de por las restricciones filogenéticas, por presiones selectivas durante el desarrollo ontogenético y aquellas impuestas por el ambiente.*

Introduction

Understanding the life history strategy of organisms as the result of ecological forces driving and constraining the evolution of species, and to elucidate how life history traits are relevant for ecological dynamics are among the most important objectives of evolutionary ecology (Partridge & Harvey, 1988). Growth rate, defined as the increase in size (biomass) or protein content per unit of time, is among the most relevant life-history traits for ecologists, since it is an integrating parameter of overall life history strategy (Arendt, 1997). It does not only impinge on other important life-history traits and ecological features like the age at the first reproduction or the ability to inhabit temporal habitats (Elser *et al.*, 2006), but it is also used as a measure of animal fitness since organisms must grow to reproduce (Arendt, 1997; Elser *et al.*, 2000). Growth rate hypothesis (GRH), as central concept of biological stoichiometry [the study of the balance of energy and multiple chemical elements in living systems (Elser *et al.*, 2000)], proposes for organisms lacking major mineral storage of P (as vacuoles or bones), that elevated demands for increased allocation to P-rich ribosomal RNA under rapid growth drives variation in P content (and thus C:P and N:P ratios) of many biota. This concept states the strong connection among elemental composition, RNA content and growth rate (Elser *et al.*, 2000), being the basis for the use of RNA-based biomarkers like RNA per dry weight (%RNA) or RNA:DNA ratio as proxies for growth rate in a large variety of studies (*e.g.* Buckley, 1999; Vrede *et al.*, 2002; Shin *et al.*, 2003; Chicharo & Chicharo, 2008; Holmborn *et al.*, 2009), and leaving behind old-fashioned tedious methods that quantified growth rate by means of increased biomass overtime. These new molecular tools are expected to yield new perspectives on classic concepts of evolutionary ecology of life-histories, like the long-term debate on *K*- *vs.* *r*-strategies. In general, while *K*-strategists have been considered long-lived and slow-growing specialist species that have relatively low reproductive output and inhabit non-fluctuating and stable environments, *r*-strategists are those opportunist and generalist species with fast-growth that produce multiple cohorts of large offsprings and domain ephemeral temporal habitats. In aquatic ecosystems, copepods, with low growth rate, obligate sexual reproduction, ontogenetic development structured in stages, and

multivoltine life cycles, are the best adjusted profile to *K*-strategists, whereas cladocerans, characterized by high growth rates and simple cycles (no larval stages, direct development, and facultative parthenogenesis) are a clear example of *r*-strategists. According to GRH, natural selection operating on growth rates also shapes elemental composition of organisms, due to the tight connection among individual growth, ribosomal metabolism, and elemental composition (Elser *et al.*, 2000). Thus, we find that different trends in the evolution of growth rate of copepods and cladocerans have driven to differences in body P content (Andersen & Hessen, 1991; Elser *et al.*, 1996). Fast-growing cladocerans have high body P content owing to high demands of P to allocate to RNA for ribosomes and protein biosynthesis (Elser *et al.*, 2003; Hessen *et al.*, 2008). In contrast, lower growth rates have driven to lower body P content in copepods (Andersen & Hessen, 1991; Elser *et al.*, 1996).

With the development of genomics during the early twenty-first century, a great interest has emerged to explore the genetic basis of GRH. This has not only allowed biological stoichiometry to also include the genetic level, but in addition has constituted one of the few attempts of integrating how natural selection operate simultaneously on genome size and growth rate, concepts traditionally studied separately by evolution researchers. Main contributions of these studies found that increased growth rate and associated increases in transcriptional capacity for ribosomal RNA production are positively associated with the length and content of the ribosomal DNA intergenic spacers (IGS) and/or in overall ribosomal DNA copy number (Weider *et al.*, 2005). However, this consistent pattern apparently contrasts with the pervasive relationships between high growth rate, P and RNA content with small genome size in rapid-growth organisms like cladocerans, and particularly in *Daphnia* species. To explain this paradox, P-allocation hypothesis proposes that small genomes in cladocerans may be the consequence of P allocation from DNA (mainly from non-coding DNA) to RNA under sustained selection for rapid growth in P-limited environments (Hessen *et al.*, 2008). In contrast, copepods, with lower P content (Andersen & Hessen, 1991), have larger genomes than cladocerans (Hessen *et al.*, 2008). Consistent with this observation, one could expect that evolutionary pressure to reallocate P

from DNA to RNA would not be especially important in low-P demanding copepods. However, the finding that larval stages of copepods (nauplii) do have broad demands for P to sustain high growth rates (Carrillo *et al.*, 2001; Villar-Argaiz *et al.*, 2002; Villar-Argaiz & Sterner, 2002) add important caveats to the general arguments raised above. Although the extension of P-allocation hypothesis to other biota, called as growth rate-genome size-nutrient limitation hypothesis, expand its validity for other organisms demanding high growth rates under conditions of low nutrient availability (Hessen *et al.*, 2009), the generality of these arguments waits further corroboration in a broader context that includes the varying physiological stages of the ontogenetic development of organisms.

Cladocerans are dominant crustaceans in the pelagic zone of most freshwater ecosystems (Hessen *et al.*, 2006), whereas copepods are most prevalent crustaceans in marine open waters (*e.g.* Mauchline, 1998; Calbet *et al.*, 2001; Blachowiak-Samolyk *et al.*, 2008; Søreide *et al.*, 2008). While the ultimate reasons for this general pattern are highly controversial, numerous observations diverged from the expected distribution. In particular, high mountain lakes, despite being among the ecosystems with larger similarities throughout the planet (Catalan *et al.*, 2006), do not reflect a clear pattern of dominance by copepods or cladocerans (Miracle, 1982). However, these extreme low-nutrient environments are ideal scenarios to test hypotheses mentioned above. Their oligotrophic to ultra-oligotrophic status implies food limitation for zooplankton, playing a relevant role in zooplankton evolutionary history (Guisande *et al.*, 2005). In fact, it has been demonstrated as one of the main factors responsible for the extinction of zooplankton species in alpine lakes (Gliwicz, 1985). In addition, the short term of ice-free period, affected by high harmful UVR (Carrillo *et al.*, 2008) and low temperatures, and other typical stressors such as fluctuating hydrology, constitute strong selective pressures for high growth rates in order to early completion of the life-cycle (Woods *et al.*, 2003; Van Geest *et al.*, 2010). Also previous studies in high mountain lakes have shown a clear connection among growth, elemental composition and life-history strategy for copepod and/or cladoceran species (Carrillo *et al.*, 2001; Villar-Argaiz *et al.*, 2002; Ventura & Catalan, 2005).

We believe that the simultaneous analyses of stoichiometric composition, nucleic acid content and the ecological characterization of the natural sites inhabited by species will provide important insights into the ultimate reasons for the evolutionary success and distribution of major crustacean taxonomical groups in nature. In doing so, we wish to extend the application of biological stoichiometry, and strength its role as a bridge between the evolutionary thinking and ecosystems ecology (Elser, 2006).

Our goals were: (i) to assess how elemental (P) and NA (RNA, DNA, RNA:DNA ratio) content varied for the dominant crustacean zooplankton species in 22 high mountain lakes (Sierra Nevada and The Pyrenees mountains, Spain); (ii) to test the validity of GRH and P-allocation hypothesis, exploring P, RNA, and growth rate relationships and DNA content-body size allometry; (iii) to relate these results with their life history strategies and ecological distribution. Due to the scarcity of nutrients and the limited temporal "window" for the development of species, restricted to the ice-out period, we expect to observe strong evidences for both GRH and P-allocation hypothesis. We also believe that extensive use of the proposed molecular tools will provide important insights on the complexity of life history strategies in natural systems, largely contributing to the understanding of the debate of copepods as *K*-strategists *vs*. cladocerans as *r*-strategists, and together with additional information, help us to argue ecological distribution of species of both.

Methods

Study site

The study was carried out in a set of 22 high mountain lakes (1600-3100 m a.s.l), located in the National Parks of Sierra Nevada and The Pyrenees mountains (Spain). All ecosystems were small, shallow, highly transparent, and littoral vegetation was absent or very scarce. Physical and biological data of each lake were collected once between the 6th of July and 25th of August in 2005. Moreover,

Lake La Caldera was sampled over 3-6 week intervals during the ice-free periods (June-October) of 2005, 2006, and 2007.

Temperature and light [ultraviolet radiation (UVR) at 305, 320, and 380 nm ($UVR_{305,\ 320,\ and\ 380\ nm}$) and photosynthetic active radiation (PAR)] profiles were measured along the water column using a BIC Compact 4-Channel Radiometer (Biospherical Instruments Inc.; San Diego, California, USA). Each lake was characterized by the mean temperature of the water column and the diffuse attenuation coefficient (K_d, m^{-1}) for each wavelength, calculated from the slope of the linear regression of the natural logarithm of downwelling irradiance *vs.* depth. To provide an integrated extinction coefficient of UVR, we define $K_{d\ UVR}$ as the mean extinction coefficient of the three UVR wavelengths (305, 320, and 380 nm) (m^{-1}). Main physical and chemical characteristics for the study sites are summarized in Table 1.

Field sampling

The chemical and biological samples were taken with a 6-L Van Dorn sampler at the deepest point of the lake. Where possible, water from up to four depths (0.5 m below surface and above the bottom, and two intermediate sampling depths) was mixed in a 5 L-bucket and subsamples were taken in triplicate for total phosphorus (TP). After removing zooplankton by sieving water through 40-μm mesh, another set of subsamples were taken in triplicate for chlorophyll *a* (Chl *a*), sestonic carbon (C), nitrogen (N), and phosphorus (P).

Zooplankton samples were obtained after sieving 24 L of water from different depths through 40-μm mesh and preserved in 4% formaldehyde. Zooplankton abundance was determined by counting under an inverted microscope at $\times$100 magnifications. For each sample, when possible, 20 individuals of each cladoceran species or developmental stage for copepod species were measured for length by image analysis (Quantimet 500, Leica). Biomass of crustacean zooplankton was calculated using length-weight regressions

specifically developed for *Acanthocyclops vernalis* [copepodites by Rosen (1981) and McCauley (1984); adults by Bottrell *et al.* (1976) and McCauley (1984)]; *Cyclops abyssorum* [nauplii by Rosen (1981) and McCauley (1984); copepodites, and adults by Ventura (2004)]; *Diaptomus cyaneus* [nauplii by Rosen (1981) and McCauley (1984); copepodites and adults by Ventura (2004)]; *Eudiaptomus vulgaris* [nauplii by Rosen (1981) and McCauley (1984); copepodites and adults by Persson & Ekbolm (1980) and McCauley (1984)]; *Mixodiaptomus laciniatus* [nauplii, copepodites, and adults by Carrillo *et al.* (2001)]; *Alona affinis* (Dumont *et al.*, 1975); *Chydorus sphaericus* (Rosen, 1981; McCauley, 1984), *Daphnia longispina* (Bottrell *et al.*, 1976; McCauley, 1984), and *Daphnia pulicaria* (Ventura, 2004). For rotifers and ciliates, we considered individual weights directly measured by Dumont *et al.* (1975), Bottrell *et al.* (1976), and WaltzWalz (1987).

Additional samples of zooplankton were also collected by vertical hauls of a 40-μm mesh net and brought in lake water under dark and cold conditions for P and nucleic acids (NA) determinations. In the laboratory, zooplankton was concentrated by sieving through a 40-μm mesh and diluted to 1 L with 0.7-μm filtered lake water. For the analysis of P content, live individuals were identified to the species level with the aid of an inverted microscope and sorted into precombusted (1 h at 550 ºC) 1.0-μm glass fiber filters (Whatman GF/B). We distinguished among major ontogenetic stages for copepods and gender for adult copepods and cladocerans (males, females, and females carrying eggs). When possible, three replicates, containing 30-50 individuals of *Cyclops abyssorum*, 10-20 of *Diaptomus cyaneus*, 5-15 of *Alona affinis*, 20-25 of *Daphnia longispina*, and 5-15 of *Daphnia pulicaria*, were obtained. Simultaneously, some samples were taken and fixed in 4% formaldehyde for later individual size measurements, and thus, after biomass conversions, to estimate body P content as percentage in dry weight (%body P). Only in the case of *Mixodiaptomus laciniatus*, P content data were obtained from Carrillo *et al.* 2001, where an accurate study of the elemental composition and growth rate for each naupliar, copepodite, and adult stages was carried out. For NA analysis, up to 20 individuals for each species were sorted into 1.5 mL Eppendorf tubes containing 300 μL of RNA*Later*

(Ambion cat. nr 7024, Ambion Inc.; Austin, Texas, USA), and storaged at -80 ºC until analysis [*see* recommendations by Gorokhova (2005)].

For primary production (PP) measurements, sets of four 50 mL quartz flasks (three clear and one dark) added with 0.37 MBq of $NaH^{14}CO3$ [specific activity (SA): 310.8 MBq $mmol^{-1}$, NEN Dupont] were incubated *in situ*, at the depth where 75% of surface solar UVR reached, for 4 h symmetrically distributed around noon. All flask sets were horizontally held during the incubations. The full laboratory procedure for the determination of PP has been described in elsewhere in Carrillo *et al.* (2002). Briefly, it consisted of the determination of total organic carbon (TOC) and particulate organic carbon on samples filtered through 1.0-μm pore-size Nucleopore filters (POC_1).

Chemical and biological analyses

To determine TP, 50-mL aliquots were analyzed, using the acid molybdate technique after digestion with a mixture of potassium persulfate, boric acid, and sodium hydroxide at 120 ºC for 30 min (APHA, 1992). Up to 300 mL for sestonic C and N, and 400 mL for sestonic P per replicate were filtered through precombusted (1 h at 550 ºC) 1.0-μm glass fiber filters (Whatman GF/B) at low pressure (<100 mm Hg). Filters containing sestonic C and N were dried (24 h at 60 ºC), and kept desiccated until C and N analysis by a Perkin-Elmer model 2400 (Perkin-Elmer Corporation) elemental analyzer. Seston and zooplankton P were analyzed following the method described for TP. Blanks and standards were performed for all procedures. Sestonic C:N:P ratios were calculated on a molar basis. Chl *a* was measured fluorimetrically after filtration of 300 mL per replicate through 0.7-μm glass fiber filters (Whatman GF/F) at low pressure (<100 mm Hg) and a 24-h pigment extraction in 90% acetone in the dark at 4 ºC. A Chl *a* standard (Fluka Chl *a* from algae) was used to transform the fluorescence data into Chl *a* concentrations.

NA analysis was carried out with a microplate fluorimetric high-range assay with Ribogreen in previously length-measured individual zooplankters after extraction with N-laurylsarcosine followed by RNase digestion as described by Gorokhova & Kyle (2002). The following working reagents were used: RiboGreen™ RNA Quantitation Kit (Molecular Probes, cat. #R11490, Invitrogen); RNase DNasefree (Q-biogene, cat. #RNAS0500; working solution 5 μg ml^{-1}); N-lauroysarcosine (sarcosyl, Sigma, cat. #L-5125); TE buffer (Q7 biogene, cat. #TE1X0001). Fluorescence measurements were performed using fluorometer FLUOstar Optima (BMG Labtechnologies, microplate reader, filters: 485 nm for excitation and 520 nm for emission) and black solid flat-bottom microplates (cat. nr 675077, Greiner Bio-One GmbH; Frickenhausen, Germany). The plate was scanned with a 0.2-s well measurement time and 10 measurements were made per well, before and after RNase digestion (30 min under dark conditions at 37 ºC). Fluorescence measurements were converted into RNA and DNA concentrations using curves previously performed with RNA (16S and 23S from *Escherichia coli*, component C of the Ribogreen Kit) and DNA (calf thymus, Sigma-Aldrich, cat. nr D-1501) standards. RNA and DNA contents were expressed as μg per individual and, after biomass conversions with length-weight regressions, as percentage of dry weight (%RNA and %DNA), and RNA:DNA ratio. The percentages of body P contributed by RNA (%P-RNA) and DNA (%P-DNA) were calculated by multiplying their concentration by their P fraction (0.085 for RNA and 0089 for DNA; Ventura, 2006) and the total P associated to NA (%P-NA) was calculated as the sum of P in both RNA and DNA.

Statistical analyses

Kruskal-Wallis analysis of variance (Kruskal-Wallis ANOVA) was used to test inter-specific and inter-group (copepods *vs.* cladocerans) differences on size, %RNA, %DNA, RNA:DNA ratio, %P-NA and %body P. This analysis was also used to test for differences in the above variables due to ontogeny and sex at adulthood for copepod species, and reproduction within adult females and lake origin for both copepod and cladoceran species.

Linear regression analysis was used to test the correlation of size with P-RNA:body P, P-DNA:body P, and P-NA:body P ratios for specifically collected data of *Mixodiaptomus laciniatus* from Lake La Caldera; and log-transformed data of DNA content *per* individual (μg) with body size (μm) for each species and taxonomic group (copepoda, cladocera). When the regression was significant, homogeneity of slopes model (ANCOVA) was used to test the effect of taxonomic group on the correlation. Linear regression analysis was also used to test correlations among %body P, %RNA, and growth rate for all species and specific data of *Mixodiaptomus laciniatus* from Lake La Caldera. STATISTICA 7.1 for Windows software (Stat Soft 2005) was used for the statistical analyses.

Results

This set of high mountain lakes represented a narrow trophic gradient (TP: 4-34.5 μg P L^{-1}, Table 1) characterized by their low productivity (TOC: 0.30-30.36 μg C L^{-1} h-1; POC_1: 0.01-16.73 μg C L^{-1} h^{-1}) and low Chl *a* (0.25-11.85 μg L^{-1}, with 77% of observations <5 μg L^{-1}) and sestonic C (126.28-1032.15 μg C L^{-1}, with 86% of observations <500 μg L^{-1}) as food source for herbivorous consumers. In contrast, we found that food quality for herbivorous consumers was high with sestonic C:P and C:N ratios below 375 (104-364) and 12 (6-12), respectively.

Lake	Mountain region	Latitude	Longitude	Altitude	Perimeter	Area	Maximum depth	Water residence character	$K_{d\,UVR}$	$K_{d\,PAR}$	Temp.	TP
Laguna del Caballo	Sierra Nevada	37º00´52.59´´N	3º26´14.81´´W	2843	309.13	0.53	1.97	Permanent	5.75	0.64	14.96	17.84
Laguna de las Yeguas	Sierra Nevada	37º03´22.16´´N	3º22´49.98´´W	2881	775.13	3.10	5.45	Permanent	1.79	0.54	15.20	7.15
Lagunillo Chico de la Virgen	Sierra Nevada	37º03´09.30´´N	3º22´43.48´´W	2948	155.73	0.09	0.29	Temporary	7.12	0.94	20.26	34.32
Lagunillo Grande de la Virgen	Sierra Nevada	37º03´05.34´´N	3º22´43.41´´W	2954	290.28	0.55	0.77	Temporary	1.19	0.45	11.83	9.40
Laguna de Aguas Verdes	Sierra Nevada	37º02´54.98´´N	3º22´06.12´´W	3064	273.36	0.36	1.22	Permanent	7.11	1.05	16.23	28.05
Laguna Alta de Río Seco	Sierra Nevada	37º03´07.55´´N	3º20´48.10´´W	3032	183.28	0.14	1.28	Permanent	4.35	1.82	17.10	13.98
Laguna Grande de Río Seco	Sierra Nevada	37º03´07.97´´N	3º20´44.13´´W	3028	535.84	0.95	1.42	Permanent	4.07	2.66	15.46	14.22
Laguna de La Gabata	Sierra Nevada	37º03´36.12´´N	3º20´12.85´´W	2782	228.58	0.19	1.93	Permanent	0.92	0.38	12.21	11.68
Laguna Larga	Sierra Nevada	37º03´35.11´´N	3º20´03.61´´W	2783	714.47	2.35	3.38	Permanent	0.37	0.20	16.06	4.02
La Caldera	Sierra Nevada	37º03´17.47´´N	3º19´45.30´´W	3022	499.81	1.81	1.48	Permanent	2.49	0.54	14.87	8.25
La Caldereta	Sierra Nevada	37º03´11.52´´N	3º19´26.29´´W	3035	230.31	0.35	0.92	Temporary	2.02	0.66	17.64	28.83
Laguna del Borreguil	Sierra Nevada	37º03´08.32´´N	3º17´53.76´´W	2974	180.85	0.22	1.09	Permanent	3.74	0.82	16.42	11.38
Laguna Hondera	Sierra Nevada	37º02´53.15´´N	3º17´39.82´´W	2895	1113.02	3.90	0.21	Permanent	3.49	1.60	14.50	12.19
Llevreta	The Pyrenees	42º32´58.26´´N	0º53´17.91´´E	1657	1664.66	7.22	4.63	Permanent	2.31	0.36	16.47	9.29
Montcasan Inferior	The Pyrenees	42º38´26.99´´N	0º54´06.01´´E	2032	688.82	1.96	4.95	Permanent	1.47	0.24	14.89	7.77
Montcasan Superior	The Pyrenees	42º38´20.62´´N	0º54´18.01´´E	2059	688.40	2.01	5.31	Permanent	1.60	0.30	14.01	7.65
Llong	The Pyrenees	42º34´23.85´´N	0º57´01.22´´E	2019	1979.11	7.59	5.79	Permanent	1.33	0.27	17.37	7.20
Redó	The Pyrenees	42º34´51.46´´N	0º57´29.62´´E	2109	1010.80	6.34	6.87	Permanent	0.67	0.20	16.02	6.57
Barbs	The Pyrenees	42º35´58.06´´N	0º58´49.35´´E	2374	899.76	2.47	12.75	Permanent	0.96	0.44	12.27	13.45
Munyidera	The Pyrenees	42º36´02.18´´N	0º58´55.79´´E	2390	746.75	0.91	5.62	Permanent	0.68	0.13	15.43	8.99
Coveta	The Pyrenees	42º32´36.73´´N	1º02´08.45´´E	2396	721.00	2.03	5.47	Permanent	1.21	0.29	17.70	13.44
Cabanes	The Pyrenees	42º32´53.27´´N	1º02´20.30´´E	2380	690.13	2.20	5.85	Permanent	2.03	0.34	18.67	9.73

Table 1. Characterization of lakes from Sierra Nevada and The Pyrenees mountains during the study period. *Caracterización de los lagos de Sierra Nevada y de los Pirineos durante el período de estudio.*

Variables: Latitude; Longitude; Altitude (m a.s.l.); Perimeter (m); Area (ha); Maximum depth (m): $K_{d\ UVR}$: Mean extinction coefficient for ultraviolet radiation (UVR) of 305, 320 and 380 nm (m^{-1}); $K_{d\ PAR}$: Extinction coefficient for photosynthetic active radiation (PAR) (m^{-1}); Temp.: Temperature (ºC); TP: Total phosphorus ($\mu g\ L^{-1}$). *Variables: Latitud; Longitud; Altitud (m a.s.l.); Perímetro (m); Área (ha); Profundidad máxima (m);* $K_{d\ UVR}$*: Coeficiente de extinción promedio para radiación ultravioleta (UVR) de 305, 320 y 380 nm* (m^{-1}); $K_{d\ PAR}$*: Coeficiente de extinción promedio para radiación fotosintéticamente activa (PAR)* (m^{-1}); *Temp.: Temperatura (ºC); TP: Fósforo total (*$\mu g\ L^{-1}$*).*

Zooplankton biomass varied from <1 to a maximum of 686 μg dry weight L^{-1} (Fig. 1A). Despite the extremely low zooplankton abundance in some lakes, vertical hauls revealed the sporadic appearance of *Diaptomus cyaneus* in Lake Gabata, *Chydorus sphaericus* in Lake Hondera, *Cyclops abyssorum*, *Alona affinis* and *Daphnia longispina* in Lakes Montcasan Inferior and Munyidera. Metazooplankton species were dominant in most lakes, whereas microzooplankton species, mainly rotifers, were relevant in three of the study lakes (Lagunillo Chico de la Virgen, Llong, and Barbs). Metazooplankton species were comprised by mainly copepods, specifically *Mixodiaptomus laciniatus*, in lakes of Sierra Nevada and cladocerans in lakes of The Pyrenees (Fig. 1B).

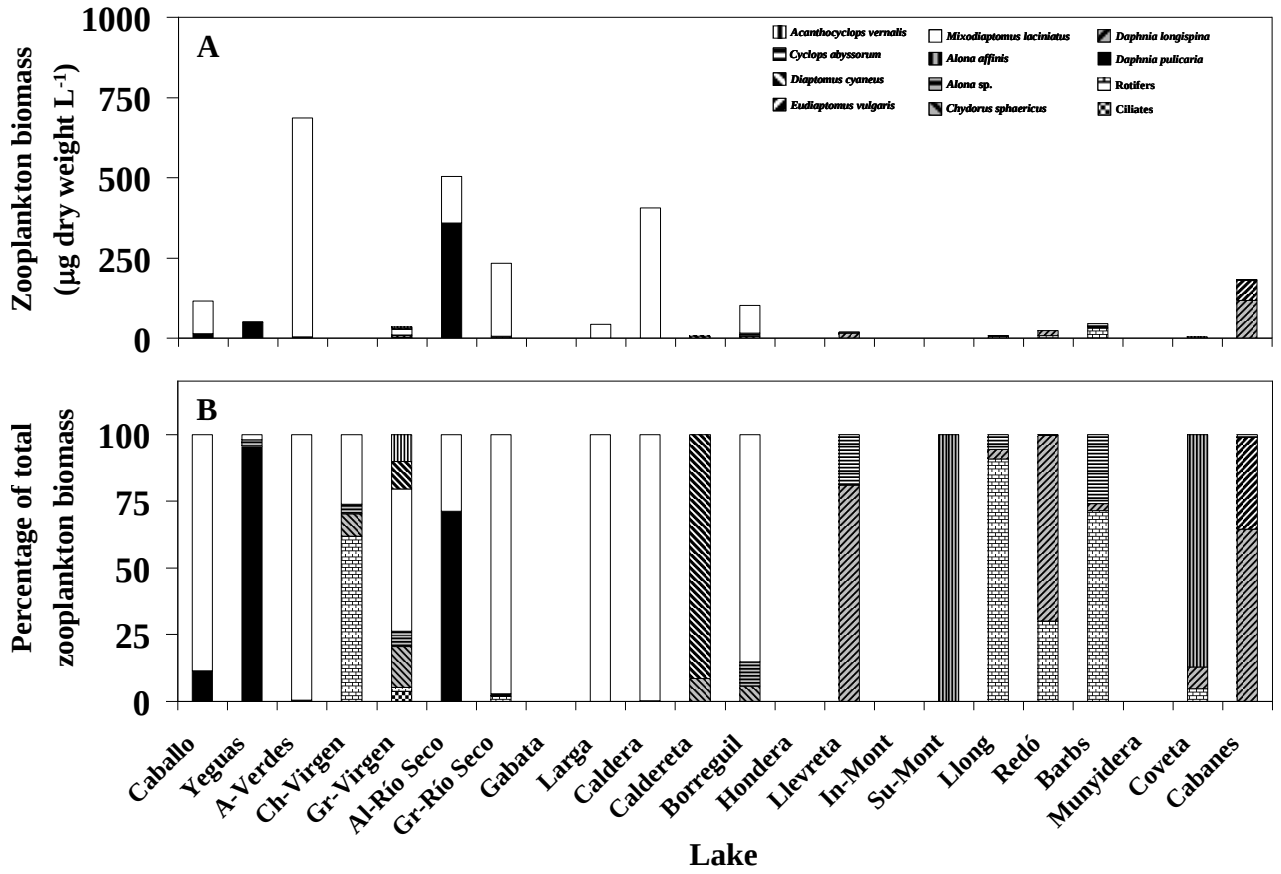

A
B
Zooplankton biomass (µg dry weight L-1)
Percentage of total zooplankton biomass
1000
750
500
250
0
100
75
50
25
0
Acanthocyclops vernalis
Cyclops abyssorum
Diaptomus cyaneus
Eudiaptomus vulgaris
Mixodiaptomus laciniatus
Alona affinis
Alona sp.
Chydorus sphaericus
Daphnia longispina
Daphnia pulicaria
Rotifers
Ciliates
Caballo
Yeguas
A-Verdes
Ch-Virgen
Gr-Virgen
Al-Río Seco
Gr-Río Seco
Gabata
Larga
Caldera
Caldereta
Borreguil
Hondera
Llevreta
In-Mont
Su-Mont
Llong
Redó
Barbs
Munyidera
Coveta
Cabanes
Lake

Figure 1. Biomass and taxonomic composition of the zooplankton (A) and percentage of total zooplankton biomass for each taxonomic group (B) of the study lakes. Lakes are Caballo, Laguna del Caballo; Yeguas, Laguna de las Yeguas; A-Verdes, Laguna de Aguas Verdes; Ch-Virgen, Lagunillo Chico de la Virgen; Gr-Virgen, Lagunillo Grande de la Virgen; Al-Río Seco, Laguna Alta de Río Seco; Gr-Río Seco, Laguna Grande de Río Seco; Gabata, Laguna de la Gabata; Larga, Laguna Larga; Caldera, La Caldera; Caldereta, La Caldereta; Borreguil, Laguna del Borreguil; Hondera, Laguna Hondera; Llevreta; In-Mont, Montcasan Inferior; Su-Mont, Montcasan Superior; Llong; Redó; Barbs; Munyidera; Coveta; and Cabanes. *Biomasa y composición taxonómica del zooplancton (A) y porcentaje de la biomasa total de zooplancton para cada grupo taxonómico (B) de los lagos de estudio. Los lagos son Caballo, Laguna del Caballo; Yeguas, Laguna de las Yeguas; A-Verdes, Laguna de Aguas Verdes; Ch-Virgen, Lagunillo Chico de la Virgen; Gr-Virgen, Lagunillo Grande de la Virgen; Al-Río Seco, Laguna Alta de Río Seco; Gr-Río Seco, Laguna Grande de Río Seco; Gabata, Laguna de la Gabata; Larga, Laguna Larga; Caldera, La Caldera; Caldereta, La Caldereta; Borreguil, Laguna del Borreguil; Hondera, Laguna Hondera; Llevreta; In-Mont, Montcasan Inferior; Su-Mont, Montcasan Superior; Llong; Redó; Barbs; Munyidera; Coveta; and Cabanes.*

Inter-specific variability in NA and P-stoichiometry

Despite the lower individual size of copepods compared to cladocerans (Kruskal-Wallis ANOVA test: $H_{1,\ 640} = 42.08$, $p < 0.001$) (*see* inset in Fig. 2A), copepods had higher %RNA and %DNA (Kruskal-Wallis ANOVA test: %RNA: $H_{1,\ 404} = 39.43$, $p < 0.001$; %DNA: $H_{1,\ 387} = 188.34$, $p < 0.001$) (*see* inset in Fig. 2B). These patterns mainly emerged due to higher values of both NA for *Cyclops abyssorum* and *Diaptomus cyaneus*, and remarkably lower values of %DNA for *Daphnia s*pecies (Fig. 2B). Consequently, RNA:DNA ratio was more than 8-fold higher in cladocerans than copepods (Kruskal-Wallis ANOVA: $H_{1,\ 335} = 147.18$, $p < 0.001$) (*see* inset in Fig. 2C), especially due to the considerable higher values reached by *Daphnia* species (Fig. 2C, Tables 2, 3).

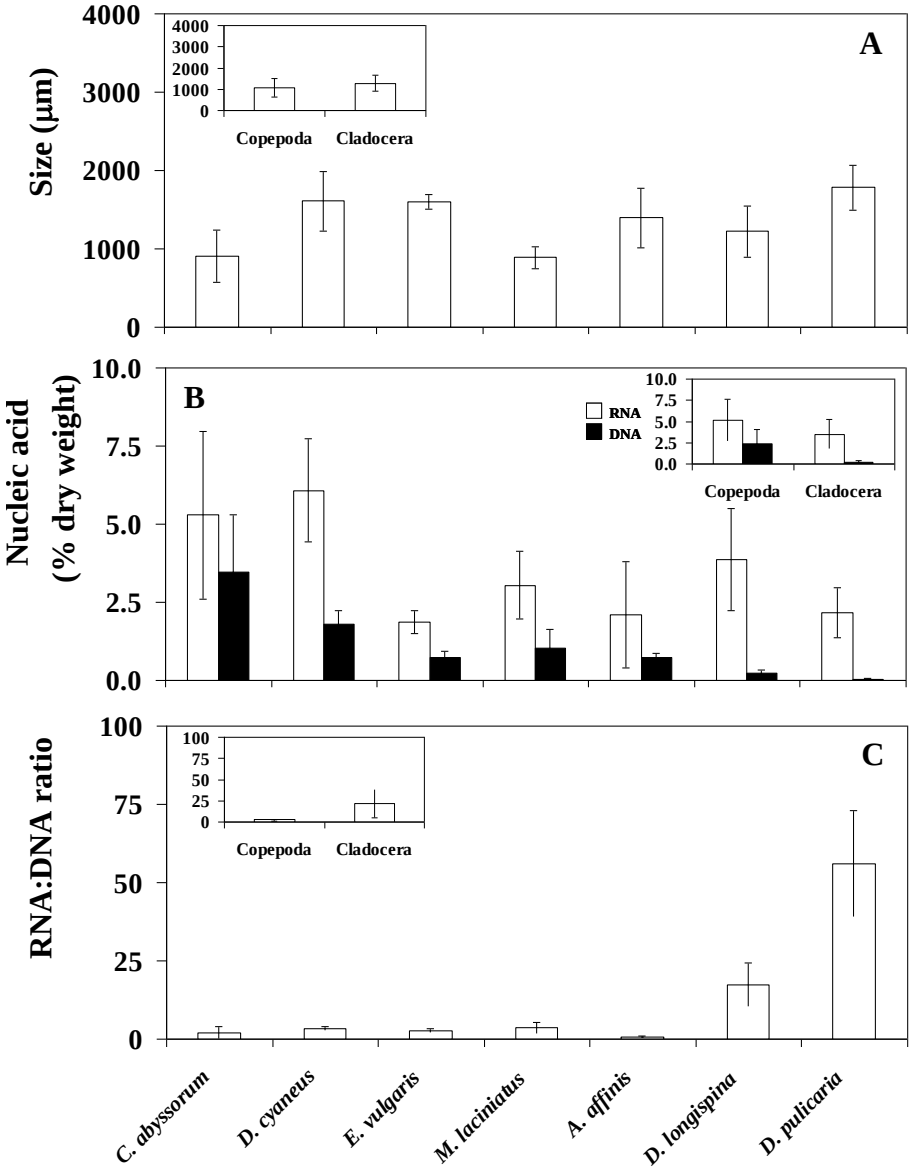

A
Size (µm)
4000
3000
2000
1000
0
Copepoda
Cladocera
B
Nucleic acid
(% dry weight)
10.0
7.5
5.0
2.5
0.0
RNA
DNA
C
RNA:DNA ratio
100
75
50
25
0
C. abyssorum
D. cyaneus
E. vulgaris
M. laciniatus
A. affinis
D. longispina
D. pulicaria

Figure 2. Size (A), nucleic acid content (RNA, DNA) (B), and RNA:DNA ratio (C) for each crustacean species: *C. abyssorum*, *Cyclops abyssorum*; *D. cyaneus*, *Diaptomus cyaneus*; *E. vulgaris*, *Eudiaptomus vulgaris*; *M. laciniatus*, *Mixodiaptomus laciniatus*; *A. affinis*, *Alona affinis*; *D. longispina*, *Daphnia longispina*; *D. pulicaria*, *Daphnia pulicaria*. Insets represent size (A), nucleic acid content (RNA, DNA) (B), and RNA:DNA ratio (C) for species grouped by copepods (copepoda) and cladocerans (cladocera). Closed squares are mean values, boxes are standard errors, and error barbs are standard deviations. *Tamaño (A), contenido en ácidos nucleicos (RNA, DNA) (B), y razón RNA:DNA (C) para cada especie de crustáceo:* C. abyssorum, Cyclops abyssorum*;* D. cyaneus, Diaptomus cyaneus*;* E. vulgaris, Eudiaptomus vulgaris*;* M. laciniatus, Mixodiaptomus laciniatus*;* A. affinis, Alona affinis*;* D. longispina, Daphnia longispina*;* D. pulicaria, Daphnia pulicaria. *Las figuras internas representan el tamaño (A), contenido en ácidos nucleicos (RNA, DNA) (B), y razón RNA:DNA (C) para las especies agrupadas por copépodos (copepoda) y cladóceros (cladocera). Los cuadrados rellenos representan los valores promedio, las cajas representan el error estándar, y las barras de error representan la desviación estándar.*

Response variable	*n*	*N*	*H*	*p-value*
Size	**6**	**640**	**271.75**	**<0.001**
RNA	**6**	**404**	**126.87**	**<0.001**
DNA	**6**	**387**	**281.60**	**<0.001**
RNA:DNA ratio	**6**	**335**	**257.52**	**<0.001**
P-Nucleic acids	**6**	**335**	**168.44**	**<0.001**
Body P	**5**	**36**	**26.82**	**<0.001**

Table 2. Results of Kruskal-Wallis analysis of variance (Kruskal-Wallis ANOVA) to test inter-specific differences on response variables: Size, RNA and DNA content, RNA:DNA ratio, phosphorus (P) allocated to total nucleic acids (P-Nucleic acids), and body P. Units are μm for size, and percentage (%) of dry weight for RNA, DNA, P-Nucleic acids and body P. Significant results are considered for *p*-values < 0.05, and are shown in bold. *Resultados de los análisis de la varianza Kruskal-Wallis (ANOVA Kruskal-Wallis) para testar diferencias interespecíficas en las variables respuesta: Tamaño, contenido en RNA y DNA, razón RNA:DNA, fósforo (P) invertido en el contenido total de ácidos nucleicos (P-Ácidos nucleicos), y P somático. Las unidades son* μm *para tamaño, y porcentaje (%) de peso seco para RNA, DNA, P-Ácidos nucleicos, y P somático. Se consideran resultados significativos para* p-*valores < 0.05, y se muestran en negrita.*

Species	Species	Size		RNA		DNA		RNA:DNA ratio		P-Nucleic acids		Body P	
		z	*p* -value	*z*	*p* -value	*z*	*p* -value	*z*	*p* -value	*z*	*p* -value	*z*	*p* -value
Cyclops abyssorum	*D. cyaneus*	**13.00**	**<0.001**	**3.51**	**0.010**	**4.44**	**<0.001**	**8.25**	**<0.001**	0.25	n.s.	1.40	.n.s.
	E. vulgaris	**6.30**	**<0.001**	**5.90**	**<0.001**	**5.65**	**<0.001**	2.72	n.s.	**6.75**	**<0.001**		
	M. laciniatus	0.75	n.s.	**4.15**	**0.001**	**8.35**	**<0.001**	**4.33**	**<0.001**	**5.17**	**<0.001**	**3.77**	**0.002**
	A. affinis	**4.23**	**<0.001**	**4.00**	**0.001**	2.95	n.s.	1.54	n.s.	**4.31**	**<0.001**	0.70	n.s.
	D. longispina	**8.04**	**<0.001**	**4.20**	**0.001**	**14.85**	**<0.001**	**14.29**	**<0.001**	**8.82**	**<0.001**	0.93	n.s.
	D. pulicaria	**7.23**	**<0.001**	**4.85**	**<0.001**	**8.15**	**<0.001**	**8.12**	**<0.001**	**6.20**	**<0.001**	1.91	n.s.
Diaptomus cyaneus	*E. vulgaris*	0.44	n.s.	**7.35**	**<0.001**	**3.31**	**0.020**	1.45	n.s.	**6.44**	**<0.001**		
	M. laciniatus	**10.98**	**<0.001**	**5.96**	**<0.001**	**4.04**	**0.001**	0.62	n.s.	**4.84**	**<0.001**	**4.70**	**<0.001**
	A. affinis	1.55	n.s.	**5.34**	**<0.001**	1.74	n.s.	**3.77**	**0.003**	**4.21**	**0.001**	1.32	n.s.
	D. longispina	**5.67**	**<0.001**	**6.70**	**<0.001**	**9.66**	**<0.001**	**6.14**	**<0.001**	**7.89**	**<0.001**	1.95	n.s.
	D. pulicaria	1.15	n.s.	**6.25**	**<0.001**	**6.06**	**<0.001**	**4.37**	**<0.001**	**5.96**	**<0.001**	2.86	n.s.
Eudiaptomus vulgaris	*M.laciniatus*	**6.30**	**<0.001**	2.14	n.s.	0.84	n.s.	0.77	n.s.	1.93	n.s.		
	A. affinis	1.51	n.s.	0.87	n.s.	0.07	n.s.	2.71	n.s.	0.62	n.s.		
	D. longispina	**3.17**	**0.032**	**3.70**	**0.004**	2.22	n.s.	**4.94**	**<0.001**	1.78	n.s.		
	D. pulicaria	0.53	n.s.	0.52	n.s.	2.56	n.s.	**4.55**	**<0.001**	0.27	n.s.		
Mixodiaptomus laciniatus	*A. affinis*	**4.34**	**<0.001**	0.98	n.s.	0.39	n.s.	**3.27**	**0.022**	1.83	n.s.	0.91	n.s.
	D. longispina	**6.69**	**<0.001**	1.48	n.s.	**4.95**	**<0.001**	**4.69**	**<0.001**	0.56	n.s.	1.91	n.s.
	D. pulicaria	**7.17**	**<0.001**	1.48	n.s.	**3.83**	**0.003**	**4.19**	**0.001**	2.04	n.s.	0.97	n.s.
Alona affinis	*D. longispina*	1.13	n.s.	2.15	n.s.	1.31	n.s.	**5.70**	**<0.001**	1.67	n.s.	0.17	n.s.
	D. pulicaria	2.06	n.s.	0.37	n.s.	1.81	n.s.	**5.70**	**<0.001**	0.42	n.s.	0.34	n.s.
Daphnia longispina	*D. pulicaria*	**3.99**	**0.001**	2.82	n.s.	1.18	n.s.	1.21	n.s.	1.89	n.s.	0.81	n.s.

Table 3. Multiple inter-species comparisons by Kruskal-Wallis analysis of variance (Kruskal-Wallis ANOVA) to test differences on response variables: Size, RNA and DNA content, RNA:DNA ratio, phosphorus (P) allocated to total nucleic acids (P-Nucleic acids), and body P. Units are μm for size, percentage (%) of dry weight for RNA, DNA, P-Nucleic acids, and body P. Significant results are considered for *p*-values < 0.05, and are shown in bold. n.s., not significant. *Comparaciones múltiples entre especies mediante análisis de la varianza Kruskal-Wallis (ANOVA Kruskal-Wallis) para testar diferencias en las variables respuesta: Tamaño, contenido en RNA y DNA, razón RNA:DNA, fósforo (P) invertido en el contenido total de ácidos nucleicos (P-Ácidos nucleicos), y P somático. Las unidades son* μm *para el tamaño, porcentaje (%) de peso seco para RNA, DNA, P-Ácidos nucleicos, y P somático. Se consideran resultados significativos para* p-*valores < 0.05, y se muestran en negrita. n.s., no significativo.*

Because of the high contribution of NA to the total P budget in the organisms (Elser *et al.* 1996), patterns in %body P resembled well those of NA, and particularly for %RNA. However, only *Cyclops abyssorum* and *Diaptomus cyaneus* showed significant higher values of %body P relative to *Mixodiaptomus laciniatus* (Fig. 3A, Tables 2, 3). In spite of these findings, we found non significant differences between copepods and cladocerans (Kruskal-Wallis ANOVA test: $H_{1,\ 36} = 1.24$, $p = 0.265$) (Fig. 3B). In order to determine specific differences in P allocation for each pool, we compared %P-RNA, %P-DNA and %P-NA among species. Thus, we found higher %P-NA in copepods, with 35-70% of body P allocated to NA, than in cladoceran species, with 12-30% of P-investment in NA (Kruskal-Wallis ANOVA test: $H_{1,\ 335} = 93.27$, $p < 0.001$) (Fig. 3B). Considering both NA, this enhanced P investment in copepods was even more pronounced for RNA (31-52%) than for DNA (8-20%). Such higher P-allocation in RNA than DNA was also shown by cladocerans (RNA: 20-30% *vs.* DNA: 0.4-7%) (Fig. 3B), and especially more pronounced in *Daphnia* species (RNA: 24-31% *vs.* DNA: 0.4-2%) due to their extremely low content in DNA (Fig. 3A, Tables 2, 3).

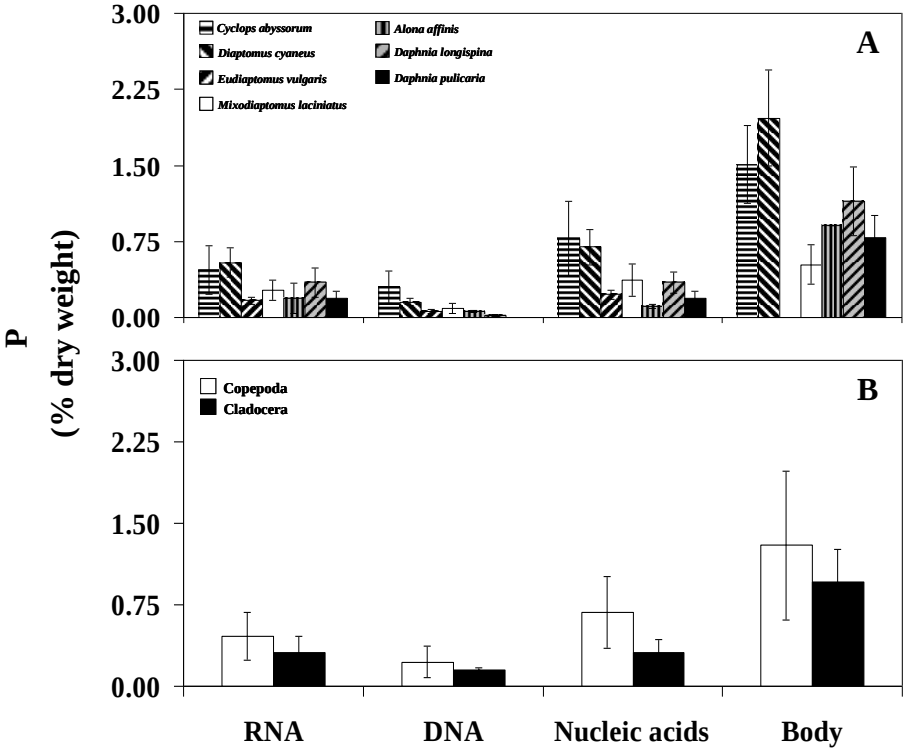


Figure 3. Phosphorus (P) allocated to RNA, DNA, and total nucleic acids, and total body P for each crustacean species (A) and species grouped by copepods (copepoda) and cladocerans (cladocera). Columns are mean values, and error barbs are standard deviations. *Fósforo (P) invertido en RNA, DNA, y contenido total de ácidos nucleicos, y P somático total para cada de especie de crustáceo (A) y para las especies agrupadas en copépodos (copepoda) y cladóceros (cladocera). Las columnas representan valores promedios, y las barras de error representan desviaciones estándar.*

Intraspecific variability in NA and P-stoichiometry.

We identified ontogeny, sex at adulthood, reproduction for adult females, and lake origin as possible sources of variation in NA and P variables for those species in which data were available. The effects of ontogeny varied among species. While a strong ontogenetic effect was detected for most variables in *Mixodiaptomus laciniatus*, no or minor effects were observed for *Cyclops abyssorum* and *Diaptomus cyaneus*. The opposite pattern was, however shown for sex at adulthood, where differences at least in %RNA, RNA:DNA ratio, and %P-NA were only observed in *Diaptomus cyaneus*. However, ovigerous females of *Diaptomus cyaneus* and *Daphnia longispina* yielded differences in most variables tested relative to non-ovigerous females. With respect to lake origin, effects on response variables varied widely depending on the species. Thus, although almost all variables were influenced by lake origin in the case of *Cyclops abyssorum*, only few were affected for all other species (Table 4).

		Cyclops abyssorum				*Diaptomus cyaneus*				*Mixodiaptomus laciniatus*			
Source of variation	Response variable	*n*	*N*	*H*	*p* -value	*n*	*N*	*H*	*p* -value	*n*	*N*	*H*	*p* -value
Ontogeny	Size	**2**	**260**	**80.38**	**<0.001**	2	96	0.00	n.s.	**1**	**83**	**8.31**	**0.004**
	RNA	2	168	1.27	n.s.	2	85	0.00	n.s.	**1**	**25**	**7.62**	**0.006**
	DNA	**2**	**143**	**6.38**	**0.041**	2	85	0.00	n.s.	**1**	**59**	**8.00**	**0.005**
	RNA:DNA	2	132	2.64	n.s.	2	85	0.00	n.s.	1	22	0.19	n.s.
	P-Nucleic acids	2	132	2.20	n.s.	2	85	0.00	n.s.	**1**	**22**	**7.43**	**0.006**
	Body P	**2**	**11**	**7.36**	**0.025**	2	7	0.00	n.s.	2	9	1.36	n.s.
Sex at adulthood	Size	1	5	0.33	n.s.	1	68	0.61	n.s.	1	3	1.50	n.s.
	RNA	1	5	0.00	n.s.	**1**	**64**	**33.06**	**<0.001**	1	3	1.50	n.s.
	DNA	1	5	0.33	n.s.	1	64	1.38	n.s.	1	3	1.50	n.s.
	RNA:DNA	1	5	3.00	n.s.	**1**	**64**	**32.57**	**<0.001**	1	3	1.50	n.s.
	P-Nucleic acids	1	5	0.00	n.s.	**1**	**64**	**27.04**	**<0.001**	1	3	1.50	n.s.
	Body P	1	2	0.00	n.s.	1	5	0.33	n.s.	1	2	0.00	n.s.
Reproduction for adult females	Size					**1**	**48**	**4.15**	**0.042**				
	RNA					**1**	**44**	**11.34**	**0.001**				
	DNA					**1**	**44**	**13.39**	**<0.001**				
	RNA:DNA					1	44	0.15	n.s.				
	P-Nucleic acids					**1**	**44**	**12.86**	**<0.001**				
	Body P					1	3	1.50	n.s.				
	Size	**6**	**260**	**36.63**	**<0.001**	**1**	**96**	**15.63**	**<0.001**				
	RNA	**6**	**168**	**121.75**	**<0.001**	1	85	0.72	n.s.				

		Alona affinis				*Daphnia longispina*				*Daphnia pulicaria*			
Source of variation	Response variable	*n*	*N*	*H*	*p* -value	*n*	*N*	*H*	*p* -value	*n*	*N*	*H*	*p* -value
Ontogeny	Size												
	RNA												
	DNA												
	RNA:DNA												
	P-Nucleic acids												
	Body P												
Sex at adulthood	Size												
	RNA												
	DNA												
	RNA:DNA												
	P-Nucleic acids												
	Body P												
Reproduction for adult females	Size					**1**	**155**	**11.22**	**0.001**				
	RNA					1	87	0.95	n.s.				
	DNA					**1**	**70**	**5.01**	**0.025**				
	RNA:DNA					**1**	**66**	**4.68**	**0.031**				
	P-Nucleic acids					1	66	1.72	n.s.				
	Body P					1	4	0.00	n.s.				
Lake	Size	**1**	**15**	**9.39**	**0.002**	**5**	**155**	**60.66**	**<0.001**				
	RNA	**1**	**11**	**7.50**	**0.006**	**5**	**87**	**54.21**	**<0.001**				
	DNA	1	4	0.00	n.s.	5	70	0.00	n.s.				
	RNA:DNA	1	4	0.00	n.s.	5	66	0.00	n.s.				
	P-Nucleic acids	1	4	0.00	n.s.	5	66	0.00	n.s.				
	Body P	1	1	0.00	n.s.	6	4	0.00	n.s.	3	4	0.00	n.s.

Table 4. Results of Kruskal-Wallis analysis of variance (Kruskal-Wallis ANOVA) to test the effects of ontogeny, sex at adulthood, reproduction for adult females and lake origin on response variables for *Cyclops abyssorum*, *Diaptomus cyaneus*, *Mixodiaptomus laciniatus*, *Alona affinis*, *Daphnia longispina*, and *Daphnia pulicaria*. Response variables are: Size, RNA and DNA content, RNA:DNA ratio, P allocated to total nucleic acids (P-Nucleic acids), and body P. Units are μm for size, and percentage of dry weight for RNA, DNA, P-Nucleic acids and body P. Significant results are considered for *p*-values < 0.05, and are shown in bold. n.s., not significant. *Resultados de los análisis de la varianza Kruskal-Wallis (ANOVA Kruskal-Wallis) para testar los efectos de ontogenia, sexo en la adultez, reproducción para las hembras adultas, y lago de origen en las variables respuesta para* Cyclops abyssorum, Diaptomus cyaneus, Mixodiaptomus laciniatus, Alona affinis, Daphnia longispina, *y* Daphnia pulicaria. *Las variables respuesta son: Tamaño, contenido en RNA y DNA, razón RNA:DNA, P invertido en el contenido total de ácidos nucleicos (P-Ácidos nucleicos), y P somático. Las unidades son* μm *para el tamaño, y porcentaje de peso seco para RNA, DNA, P-Ácidos nucleicos y P somático. Se consideran resultados significativos para* p-*valores < 0.05, y se muestran en negrita. n.s., no significativo.*

In order to disentangle the effects of intrinsic factors mentioned above, the intraspecific (ontogenetic) variability in NA for the copepod *Mixodiaptomus laciniatus* was examined from the broad comparison of a rather large number of samples (~500 individuals analyzed) collected over three years in Lake La Caldera (2005, 2006, and 2007). A high intra-stage variability was observed, and except for size, no differences were detected among single stages. However, when data were grouped into nauplii, copepodites and adults, a strong effect of ontogeny was observed for all study variables. Due to this ontogenetic effect, a decreasing trend through ontogenetic development was shown, except for size with an increasing trend and RNA:DNA with higher values for nauplii and adults than for copepodites (Fig. 4, Tables 5, 6). The decreasing trend of P investment in NA (%P-NA) was similar to that of %body P, although much more pronounced (Fig. 4C). Due to trends of both variables, P-NA:body P ratio strongly decreased through ontogenetic development (Fig. 4D), as it is indicated by the negative slope of the linear regression between size and P-NA:body P ratio (P-NA:body P ratio = -0.09*size + 118.53, $F_{1,7}$ = 96.25, r = -0.97, R^2=0.93, p-value < 0.001). P-investment in RNA was always higher than for DNA for each stage, although as %P-NA and %body P, both %P-RNA and %P-DNA decreased as organism reached maturity. However, the decreasing trend was much more strong for %P-RNA than for %P-DNA, as it is indicated by the 3-fold higher slope of the

relationship between size and P-RNA:body P ratio compared to that between size and P-DNA:body P ratio (P-RNA:body P ratio = -0.07*size + 87.92, $F_{1,7}$ = 33.55, r = -0.91, R^2 = 0.83; P-DNA:body P ratio = -0.02*size + 30.19, $F_{1,7}$ = 17.26, r = -0.84, R^2 = 0.71). No intersex differences were detected for any variable, but reproduction did affect body P content in adult females (*see* insets in Fig. 4A-C, Tables 5, 6).

Testing DNA content-body size allometry and growth rate hypothesis

To test potential allometric relationships between DNA content and body size, we regressed log-transformed data of DNA content per individual against log-transformed data of body size for each species, species grouped into copepods and cladocerans where both data were available, and for specifically collected data of *Mixodiaptomus laciniatus* from Lake La Caldera. All species showed positive linear relationships, except for *Eudiaptomus vulgaris*, *Mixodiaptomus laciniatus*, and *Alona affinis* with non-linear trends (Fig. 5A, Table 7). However, when considered the large interannual record of *Mixodiaptomus laciniatus*, we also observed a strong positive linear trend for this species (Fig. 5B, Table 7). Species grouped into copepods and cladocerans also showed strong positive correlations, although slopes were strikingly different (0.36 for copepods and 0.11 for cladocerans) (ANCOVA: intercept: $F_{1, 444}$ = 20612.44, $p < 0.001$; slope: $F_{1, 444}$ = 66.87, $p < 0.001$) (Fig. 5A, Table 7).

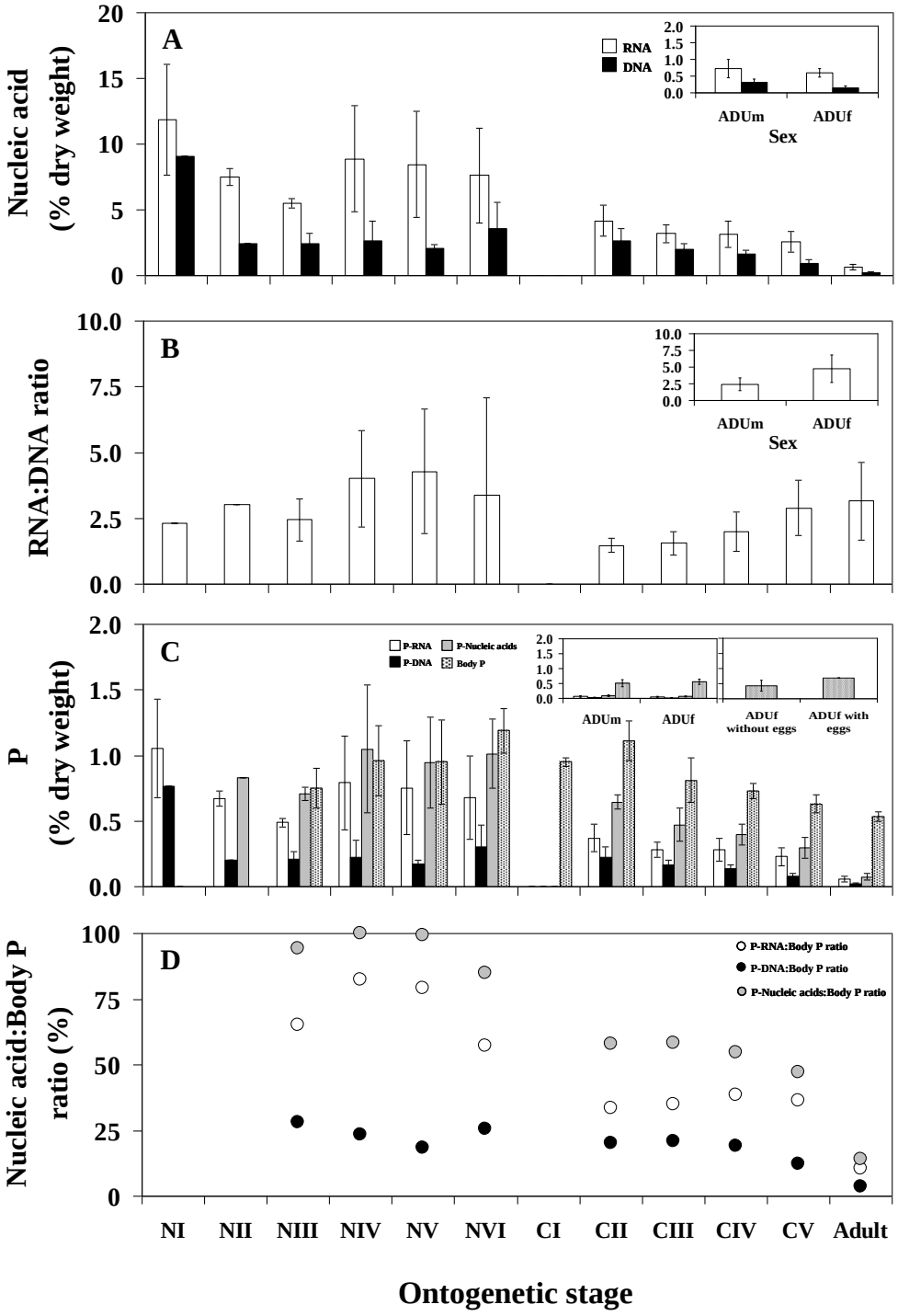

A
Nucleic acid (% dry weight)
RNA
DNA
ADUm
ADUf
Sex
B
RNA:DNA ratio
C
P (% dry weight)
P-RNA
P-DNA
P-Nucleic acids
Body P
ADUf without eggs
ADUf with eggs
D
Nucleic acid:Body P ratio (%)
P-RNA:Body P ratio
P-DNA:Body P ratio
P-Nucleic acids:Body P ratio
NI
NII
NIII
NIV
NV
NVI
CI
CII
CIII
CIV
CV
Adult
Ontogenetic stage

Figure 4. Nucleic acid content (RNA, DNA) (A), RNA:DNA ratio (B), P content allocated to RNA, DNA, total nucleic acids and body mass (C), and P-Nucleic acid:Body P ratio (D) of *Mixodiaptomus laciniatus* stages. Insets represent these variables for different sexes at adulthood. Columns in A-C and circles in D are mean values. Error barbs represent standard deviations for nauplius (NI-NVI), copepodite (CI-CV) and adult (ADUm, adult male; ADUf, adult female) stages. *Contenido en ácidos nucleicos (RNA, DNA) (A), razón RNA:DNA (B), P invertido en RNA, DNA, contenido total de ácidos nucleicos (RNA, DNA) y biomasa total (C), y razón P-Ácido nucleico:P somático (D) de los estadíos de* Mixodiaptomus laciniatus*. Las figuras internas representan estas variables para los diferentes sexos en la adultez. Las columnas en A-C y los círculos en D son valores promedios. Las barras de error representan las desviaciones estándar para nauplios (NI-NVI), copepoditos (CI-CV) y adultos (ADUm, macho adulto; ADUf, hembra adulta).*

	Ontogeny				Sex at adulthood				Reproduction for adult females			
Response variable	*n*	*N*	*H*	*p* -value	*n*	*N*	*H*	*p* -value	*n*	*N*	*H*	*p* -value
Size	**2**	**51**	**39.74**	**<0.001**	1	11	0.00	n.s.				
RNA	**2**	**47**	**34.27**	**<0.001**	1	11	1.20	n.s.				
DNA	**2**	**44**	**20.27**	**<0.001**	1	11	5.63	n.s.				
RNA:DNA ratio	**2**	**44**	**7.25**	**0.027**	1	11	3.33	n.s.				
P-Nucleic acids	**2**	**43**	**29.88**	**<0.001**	1	11	3.33	n.s.				
Body P	**2**	**47**	**19.76**	**<0.001**	1	12	0.11	n.s.	**1**	**6**	**4.35**	**0.037**

Table 5. Results of Kruskal-Wallis analysis of variance (Kruskal-Wallis ANOVA) to test the effect of ontogeny (nauplius *vs.* copepodite *vs.* adult), sex at adulthood (male *vs.* female), and reproduction for adult females (with *vs.* without eggs) on response variables of *Mixodiaptomus laciniatus*: Size, RNA and DNA content, RNA:DNA ratio, phosphorus (P) allocated to total nucleic acids (P-Nucleic acids), and body P. Units are μm for size, and percentage (%) of dry weight for RNA, DNA, P-Nucleic acids and body P. Significant results are considered for *p*-values < 0.05, and are shown in bold. *Resultados de los análisis de la varianza Kruskal-Wallis (ANOVA Kruskal-Wallis) para testar los efectos de ontogenia (nauplius* vs. *copepodito* vs. *adulto), sexo en la adultez (macho* vs. *hembra), y reproducción en las hembras adultas (con* vs. *sin huevos) en las variables respuesta de* Mixodiaptomus laciniatus*: Tamaño, contenido en RNA y DNA, razón RNA:DNA, fósforo (P) invertido en el contenido total de ácidos nucleicos (P-Ácidos nucleicos), y P somático. Las unidades son:* μm *para el tamaño, y porcentaje (%) de peso seco para RNA, DNA, P-Ácidos nucleicos y P somático. Se consideran resultados significativos para* p*-valores < 0.05, y se muestran en negrita.*

			Size		RNA		DNA		RNA:DNA ratio		P-Nucleic acids		Body P	
Source of variation	Stage	Stage	*z*	*p* -value	*z*	*p* -value	*z*	*p* -value	*z*	*p* -value	*z*	*p* -value	*z*	*p* -value
Ontogeny	Nauplius	Copepodite	**5.01**	**<0.001**	**4.35**	**<0.001**	2.25	n.s.	**2.49**	**0.038**	**3.84**	**<0.001**	1.39	n.s.
		Adult	**5.47**	**<0.001**	**5.31**	**<0.001**	**4.49**	**<0.001**	0.16	n.s.	**5.21**	**<0.001**	**4.35**	**<0.001**
	Copepodite	Adult	2.34	n.s.	**2.50**	**0.037**	**3.14**	**0.005**	1.66	n.s.	**2.73**	**0.019**	**3.31**	**<0.010**
Sex at adulthood	Male	Female	0.00	n.s.	1.10	n.s.	**2.37**	**0.018**	1.83	n.s.	1.83	n.s.	0.32	n.s.
Reproduction for adult females	With eggs	Without eggs											**1.96**	**0.050**

Table 6. Multiple comparisons by Kruskal-Wallis analysis of variance (Kruskal-Wallis ANOVA) to test differences due to ontogeny (nauplius *vs.* copepodite *vs.* adult), sex at adulthood (male *vs.* female), and reproduction for adult females (with *vs.* without eggs) on response variables of *Mixodiaptomus laciniatus*: Size, RNA and DNA content, RNA:DNA ratio, phosphorus (P) allocated to total nucleic acids (P-Nucleic acids), and body P. Units are μm for size, percentage (%) of dry weight for RNA, DNA, P-Nucleic acids, and body P. Significant results are considered for *p*-values < 0.05, and are shown in bold. n.s., not significant. *Comparaciones múltiples por análisis de la varianza Kruskal-Wallis (ANOVA Kruskal-Wallis) para testar diferencias debidas a ontogenia (nauplius* vs. *copepodito* vs. *adulto), sexo en la adultez (macho* vs. *hembra), y reproducción para las hembras adultas (con* vs. *sin huevos) en las variables respuesta de* Mixodiaptomus laciniatus*: Tamaño, contenido en RNA y DNA, razón RNA:DNA, fósforo (P) invertido en el contenido total de ácidos nucleicos (P-Ácidos nucleicos), y P somático. Las unidades son* μm *para tamaño, porcentaje (%) de peso seco para RNA, DNA, P-Ácidos nucleicos, y P somático. Se consideran resultados significativos para* p-*valores < 0.05, y se muestran en negrita. n.s., no significativo.*

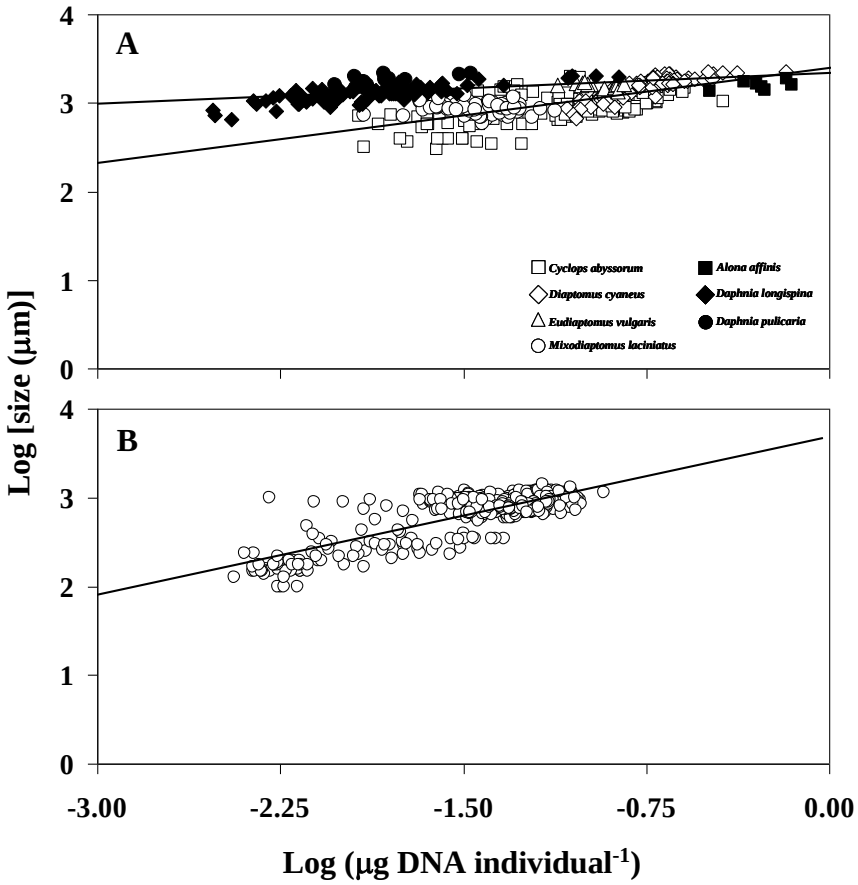


Figure 5. Relationships of log-transformed data of DNA content per individual and individual body size for each crustacean group [copepoda (open symbols), cladocera (solid symbols)] (A) and *Mixodiaptomus laciniatus* from Lake La Caldera (B). Solid lines are the fits of the linear regressions. *See* Table 7 for parameters of the regression analyses. *Relaciones de los datos transformados a logaritmos del contenido en DNA por individuo y el tamaño somático por individuo para cada grupo de crustáceos [copépodos (símbolos vacíos), cladóceros (símbolos rellenos)] (A) y* Mixodiaptomus laciniatus *del lago La Caldera (B). Las líneas continuas representan los ajustes por regresión lineal.* Ver *en* Table 7 *los parámetros de los análisis de regresión.*

	n	*N*	*F*-value	*p*-value	*r*	R^2	Slope	Intercept
Cyclops abyssorum	**1**	**166**	**102.99**	**<0.001**	**0.62**	**0.38**	**0.31**	**3.27**
Diaptomus cyaneus	**1**	**94**	**245.63**	**<0.001**	**0.85**	**0.72**	**0.60**	**3.64**
Eudiaptomus vulgaris	1	13	2.03	n.s.	-0.37	0.14	-0.11	3.10
Mixodiaptomus laciniatus	1	57	0.95	n.s.	-0.13	0.02	-0.06	2.86
Alona affinis	1	5	2.12	n.s.	0.55	0.30	0.24	3.27
Daphnia longispina	**1**	**89**	**146.96**	**<0.001**	**0.79**	**0.62**	**0.23**	**3.54**
Daphnia pulicaria	**1**	**10**	**9.21**	**0.012**	**0.69**	**0.48**	**0.21**	**3.65**
Copepoda	**1**	**336**	**312.85**	**<0.001**	**0.69**	**0.48**	**0.36**	**3.40**
Cladocera	**1**	**108**	**50.99**	**<0.001**	**0.57**	**0.32**	**0.11**	**3.34**
Mixodiaptomus laciniatus (ontogeny data)	**1**	**494**	**907.09**	**<0.001**	**0.80**	**0.65**	**0.59**	**3.69**

Table 7. Results of linear regression analyses between log-transformed data of DNA content *per* individual (μg) and body size (μm). Significant results are considered for *p*-values < 0.05, and are shown in bold. n.s., not significant. *Resultados de los análisis de regresión lineal entre los datos trasformados a logaritmos del contenido en DNA por individuo (*μ*g) y el tamaño corporal (*μ*m). Se consideran resultados significativos para* p-*valores < 0.05, y se muestran en negrita. n.s., no significativo.*

Our data for crustacean species from different high mountain lakes revealed close covariation of %body P and %RNA (Fig. 6A, Table 8). Data documenting these correlations are diverse, involving different ontogenetic development (copepods), sex condition for adult individuals (copepods), reproductive status for adult females and different lake origin (copepods and cladocerans). We also observed strong positive linear trends for the regressions relating %RNA, %body P, and growth rate using data of full ontogenetic development and both genders for adult individuals of *Mixodiaptomus laciniatus* from Lake La Caldera (Fig. 6B-D, Table 8).

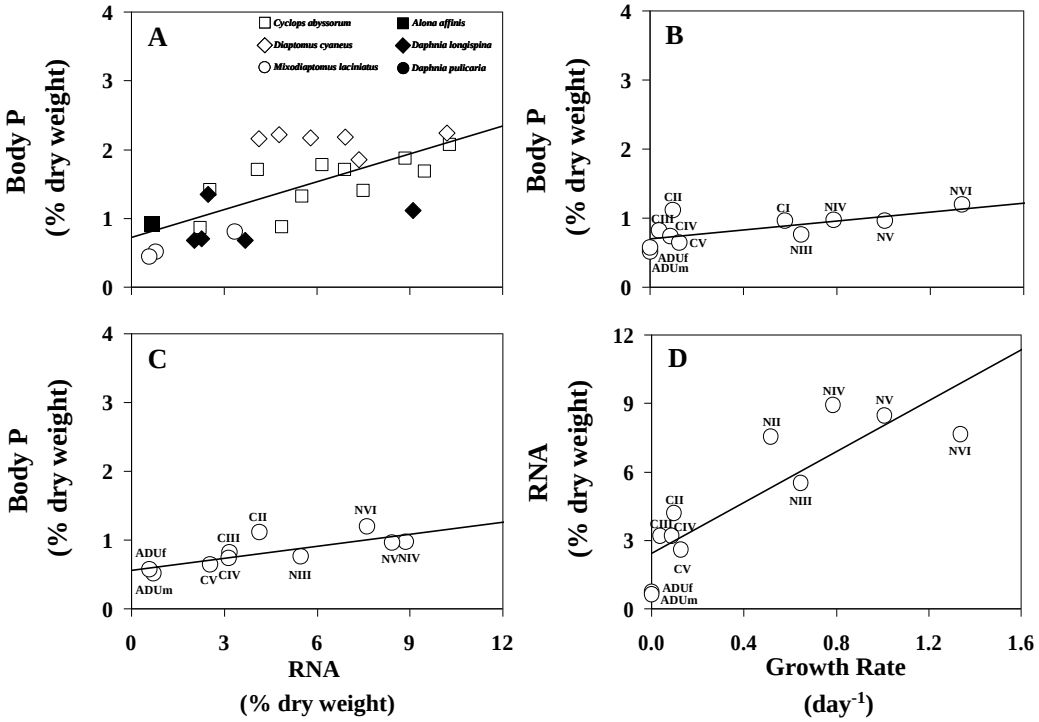


Figure 6. Relationships of RNA (A, C) with total body P content for all crustacean species in this study (A) and all ontogenetic stages of *Mixodiaptomus laciniatus* from Lake La Caldera (C); and relationships of growth rate with total body P (B) and RNA (D) for all ontogenetic stages of *Mixodiaptomus laciniatus* from Lake La Caldera. Each point represents mean values for each species × stage × lake combination in panel A, or ontogenetic stage in panels B-D, when all data were available. Stages are nauplii (NI-NVI), copepodites (CI-CV), and adults (ADUm, adult male; ADUf, adult female). Solid lines are the fits of the linear regressions. *See* Table 8 for parameters of the regression analyses. *Relaciones de RNA (A, C) con el contenido total de P somático para todas las especies de crustáceos en este estudio (A) y para todos los estadíos ontogenéticos de* Mixodiaptomus laciniatus *del lago La Caldera (C); y relaciones de la tasa de crecimiento con el contenido total de P somático (B) y RNA (D) para todos los estadíos ontogenéticos de* Mixodiaptomus laciniatus *del lago La Caldera. Cada punto representa un valor promedio para cada combinación especie × estadío × lago en el panel A, o estadío ontogenético en los paneles B-D, cuando todos los datos estaban disponibles. Los estadíos son nauplios (NI-NVI), copepoditos (CI-CV), y adultos (ADUm, macho adulto; ADUf, hembra adulta). Las líneas continuas representan los ajustes por regresión lineal.* Ver *en* Table 8 *los parámetros de los análisis de regresión.*

	Dependent variable	Independent variable	*n*	*N*	*F* -value	*p* -value	*r*	R^2	Slope	Intercept
All species	Body P	RNA	**1**	**24**	**20.34**	**<0.001**	**0.68**	**0.46**	**0.13**	**0.72**
	Body P	Growth rate	**1**	**9**	**7.88**	**0.020**	**0.68**	**0.47**	**0.32**	**0.69**
Mixodiaptomus laciniatus (ontogeny data)	Body P	RNA	**1**	**8**	**11.76**	**0.009**	**0.77**	**0.60**	**0.06**	**0.56**
	RNA	Growth rate	**1**	**9**	**26.36**	**0.001**	**0.86**	**0.75**	**5.57**	**2.39**

Table 8. Results of linear regression analyses to test correlations predicted by growth rate hypothesis (Elser *et al.*, 1996, 2000, 2003). Variables, in alphabetical order, are: Body phosphorus content (body P), growth rate, and RNA. Units are percentage of dry weight (%dry weight) for body P and RNA, and days^{-1} for the growth rate. Significant results are considered for *p*-values < 0.05, and are shown in bold. *Resultados de los análisis de regresión lineal para testar las correlaciones que predice la hipótesis de la tasa de crecimiento (Elser* et al., *1996, 2000, 2003). Las variables, por orden alfabético, son: Contenido en P somático (P somático), tasa de crecimiento, y RNA. Las unidades son porcentaje de peso seco (%peso seco) para P somático y RNA, días^{-1} para la tasa de crecimiento. Se consideran resultados significativos para* p-*valores < 0.05, y se muestran en negrita.*

Discussion

Interspecific variability in NA and P stoichiometry

Our results showing generally higher %RNA and %P for copepods than cladocerans, due to strikingly high values for *Cyclops abyssorum* and *Diaptomus cyaneus*, not only support the idea that copepods can exhibit high growth rates similar to those of *Daphnia* species, but contrast with the pre-settled concept that copepods, as *K*-strategists, are low-P, -RNA, and growth rate organisms (Elser *et al.*, 1996; Hessen *et al.*, 2008). We also observed contrasting patterns in NA and P allocation. Surprisingly, *Alona affinis*, in contrast to *Daphnia* species, showed similar patterns to those calanoid copepods *Eudiaptomus vulgaris* or *Mixodiaptomus laciniatus*, with characteristically low %P, %RNA, RNA:DNA ratio and higher %DNA. This also contrasts with the pre-settled concept that cladocerans, as *r*-strategists, are high-P, -RNA, and growth rate organisms (Elser *et al.*, 1996; Hessen *et al.*, 2008).

Results obtained for copepods might have been expected for *Cyclops abyssorum*, made up by naupliar and early copepodite stages with high growth rate, but not for *Diaptomus cyaneus*, made up by late copepodite and adult stages. This contrasting pattern is likely to be associated with the temporality of its habitat and its trophic diet. Contrary to, for example *Mixodiaptomus laciniatus* inhabiting permanent water bodies, *Diaptomus cyaneus* is typical in shallow and temporary lakes with short water residence time (Morales-Baquero, 1992). Thus, while pelagic *Mixodiaptomus laciniatus* completes its long ontogenetic development during the 4-5 months of ice-free season (Cruz-Pizarro, 1983; Villar-Argaiz, 1999, Carrillo *et al.*, 2001), strictly feeding on scarce high quality seston (Villar-Argaiz *et al.*, 2001; Villar-Argaiz *et al.*, 2012), *Diaptomus cyaneus* has adjusted its life cycle to shorter periods of 3-4 weeks (Ventura *et al.*, 2000; Ventura & Catalan, 2005), in which the selective pressure for fast growth implies high P-requirements. These are fulfilled by alternative P-enriched resources like bacteria (Sterner & Elser, 2002) obtained due to its omnivore behaviour (Ventura, 2004) and its ability of exploiting both littoral and benthic environments (Miracle,

1982). These P-enriched resources drive to P-unlimited conditions allowing fast growth, but also keeping large genomes. Moreover, polyploidy may have favoured maintenance of a high DNA content necessary for fast growth, as it has been shown for other zooplankton species under similar conditions (Dufresne *et al.*, 1991). Similarly, *Alona affinis*, inhabiting benthos and littoral with P-enriched resources (Miracle, 1978; Alonso, 1991; Alonso, 1998) and being omnivore like *Diaptomus cyaneus*, is presumably not limited by P, which has allowed to keep high DNA content, and presumably large genomes. Hence, reduction of genome size seems to be a recent phenomenon which has not been carried out by all cladoceran species, and only by those, as P-allocation hypothesis suggests, which inhabit P-limited environments and suffer selective pressure for rapid growth, like strictly pelagic *Daphnia* species (Alonso, 1991; Alonso, 1998; Sterner & Elser, 2002; Hessen *et al.*, 2008). Considering all these results together, growth does not seem to be strictly determined by genome size, and therefore by phylogenetic constraints (Hessen & Persson, 2009), but the role of other ecological factors such as resource availability and trophic interactions are also relevant. Species specialized in few low-P resources are prone to suffer P-limitation and be replaced by those with ability to exploit a wide range of alternative resources, with more possibilities to satisfy their demands for P in environments imposing rapid growth.

However, low DNA content, particularly in *Daphnia* species, does not implies reduced genome efficiency, since as P-allocation hypothesis argues, P reallocated to RNA to grow fast comes from non-functional DNA. In fact, genome efficiency seems to be maximized since much less DNA per individual is required by *Daphnia* species than copepods and *Alona affinis* to reach similar size. This is bound to be because reduced genomes promote increased growth rates since they reduce cell size and simplifies the cell division (Rasch & Wyngaard, 2006; Hessen *et al.*, 2009). DNA content per individual is a proper index of cell number especially used for aquatic metazoans, based on the amount of DNA per nucleus is quasi-constant, and each cell only contains one nucleus (Saiz *et al.*, 1998; Gorokhova & Kyle, 2002). Therefore, considering DNA content is function of the amount of DNA per nucleus or cell and the total number

of cells per individual, low DNA content observed for *Daphnia* species may be due to low DNA content per cell, *i.e.* small genome size, and/or low number of cells per individual. Although we lack C-values (the quantity of nuclear haploid DNA) data of our species, the low DNA content for *Daphnia* species reported in this study might support the P-allocation hypothesis pointing out reduced genomes for *Daphnia* species, and as it is suggested by low C-values of close related taxa (Table 9). Although, their more efficient genome apparently contrasts with the lower slope for *Daphnia* than copepod species when DNA content per individual is regressed against body size. However, this result must be cautiously interpreted since positive correlations only indicates that size increases with total DNA content, but nothing suggests about how varies with genome size or cell number. So, in order to make conclusions of genome efficiency, correlations with these later variables should be studied. Other evidences like high RNA:DNA ratios, and relatively higher P-investment in RNA relative to DNA especially in *Daphnia* species, and copepods with high DNA content (%DNA), low RNA:DNA ratios, and high C-values of other copepod species (Table 9) would provide additional support for P-allocation hypothesis.

Intraspecific variability in NA and P stoichiometry

The extensive data record in *Mixodiaptomus laciniatus* revealed a high intraspecific variability in NA and P stoichiometry. This variability was due to ontogenetic differences among stages, and especially to high intra-stage variability. This high intra-stage variability, especially observed for %RNA, RNA:DNA ratio, and %body P may be attributed to (i) intensive metabolic activity during the process of moulting in copepods, particularly accentuated before metamorphosis for the naupliar stages [(*see* Fig. 3 in Carrillo *et al.* (2001)], and (ii) high sensitivity of stage-specific growth to elemental food quality (Bullejos *et al.*, 2012).

Class	Order	Family	Species	C-value
Branchiopoda	Cladocera	Daphniidae	*Daphnia ambigua*	0.24
			Daphnia arenata	0.24-0.42
			Daphnia catawa	0.39
			Daphnia lacustris	0.37
			Daphnia melanica	0.48
			Daphnia minnihaha	0.28
			Daphnia neo-obtusa	0.42
			Daphnia nevadensis	0.39
			Daphnia obtusa	0.36
			Daphnia oregonensis	0.42
			Daphnia parvula	0.28
			Daphnia pilescens	0.31
			Daphnia pulex	0.23-0.37
			Daphnia pulicaria	0.24-0.49
			Daphnia retrocurva	0.29
			Daphnia tenebrosa	0.29-0.58
Copepoda	Cyclopoida	Cyclopidae	*Acanthocyclops robustus*	0.75
			Acanthocyclops vernalis	0.75-0.78
			Cyclops divulsus	1.80
			Cyclops furcifer	1.40
			Cyclops strennus	0.86-0.90
			Macrocyclops albidus	0.94
			Megacyclops latipes	2.01-2.67
			Mesocyclops edax	1.49
			Mesocyclops longisetus	0.89
	Calanoida	Clausocalanidae	*Diaptomus forbesii*	3.81
			Diaptomus insularis	1.91
			Diaptomus leptopus	2.77
			Diaptomus nudus	3.33
			Diaptomus sicilis	1.77
			Hesperodiaptomus articus	4.67
			Hesperodiaptomus nevadensis	5.71
			Hesperodiaptomus novemdecimus	3.23
			Hesperodiaptomus shoshone	3.11
			Hesperodiaptomus sp.	5.54
			Hesperodiaptomus victoriaensis	4.37
			Leptodiaptomus tyrrelli	1.33
			Leptodiaptomus wilsonae	3.25

Table 9. C-values of both cladoceran and copepod species. Source: Gregory (2008), www.genomesize.com. *Valores C de especies de cladóceros y copepodos. Fuente: Gregory (2008), www.genomesize.com.*

Although this intra-stage variability may be higher than inter-stage differences, an ontogenetic NA pattern emerged as observed for other species (Wagner *et al.*, 2001; Gorokhova & Kyle, 2002), and/or for other constituents such as elemental composition (Carrillo *et al.*, 2001; Villar-Argaiz *et al.*, 2002), lipids (Villar-Argaiz *et al.*, 2002), or aminoacids (Ventura & Catalan, 2010). The decreasing pattern from nauplii to adults was common for both %RNA and %DNA, although different trends were observed during naupliar development for %RNA. Thus, the decreasing trend during the early phase might be expected by the depletion of inherited maternal RNA for early proteins. Immediately after, when mature nauplii (after stage III) begin to feed on their own, RNA synthesis shoots up due to high protein demand for growth, cellular proliferation and differentiation before metamorphosis, as also observed for other species (Wagner *et al.*, 2001). For early copepodite stages, these requirements are also high due to both size growth and moulting processes. However, the decreasing trend for both variables when copepodites mature to adults is associated with the strong increase in weight because of lipids storage (Villar-Argaiz *et al.*, 2002). Increasing RNA:DNA ratio during naupliar and copepodite development reflects that growth due to protein synthesis is higher than growth due to cellular proliferation. However, the strong decrease when nauplii convert into copepodites is strongly related with cellular proliferation during metamorphosis. %body P resembled well pattern shown by %RNA as expected due to the strong correlation between both variables in agreement with GRH (Elser *et al.*, 1996, Elser *et al.*, 2000; Elser *et al.*, 2003), but in addition P-allocation to NA through ontogeny supports evidences for the life history strategy carried out for this species. High values of P-NA:body P and P-RNA:body P ratios during naupliar development reflect strong P-investment in NA, and particularly in RNA for protein synthesis during this phase. Later, the decrease in these ratios, especially at adulthood, points out that P is allocated to other biochemicals different to NA like phospholipids for membranes or ATP for energy storage (Elser *et al.*, 1996).

Taken together all the information given by NA and P-stoichiometric variables, a consistent ontogenetic pattern in the life history strategy emerges. This is characterized by a high-P demanding for RNA and protein synthesis and

fast-growing early period that changes into a low-P demanding and slow-growing period when organisms mature. This observed change in life-history strategy within the ontogenetic development provides a new insight of the ecological role of organisms and expands the classic concept of species as *K*- or *r*-strategists. Therefore, depending on the degree of development, the same organism may play different ecological strategy and vary the kind of interaction with other species and surrounding environment. However, lower slopes of regressions between growth rate and %body P or %RNA and %body P than those found by Elser *et al.* (2003) for *Daphnia* species (*Daphnia pulicaria*: 1.32 and 1.69; *Daphnia galeata*: 0.73 and 1.78), make us define *Mixodiaptomus laciniatus* as a typical low-P and slow-growing species, at least for most of its life-cycle. Although this may be a risky assumption since not early instars were included in the regressions for *Daphnia* species in the study carried by Elser *et al.* (2003), comparisons with other species like *Drosophila spp.* (Elser *et al.*, 2006) or *Portunus tribuberculatus* (Elser *et al.*, 2003), where all ontogenetic data were included, reinforces our appreciation.

Fig. 7 illustrates how the magnitude of intraspecific variability of NA and P stoichiometry may be as important as interspecific variability. In addition, although only restricted to *Mixodiaptomus laciniatus*, the degree of intra-stage variability is as relevant as inter-stage variability. These observed patterns of intraspecific variability in NA go one step beyond than that by Villar-Argaiz *et al.* (2002) for elemental composition, settling the biochemical mechanisms for the concept of rheostasis they propose in their paper, and supporting that species vary in its adaptive strategy through ontogeny, from a fast-growing short (like *r*-strategists) to a slow-growing long period (like *K*-strategists). Therefore, ecological forces (environmental fluctuations, trophic resources), reflected in the NA and elemental composition of organisms, together with phylogenetic constraints govern the growth of the organisms. This not only touches upon central concepts in evolutionary ecology such as *K*- *vs.* *r*-strategies, but contributes to extend our knowledge on species ecology and distribution in nature.

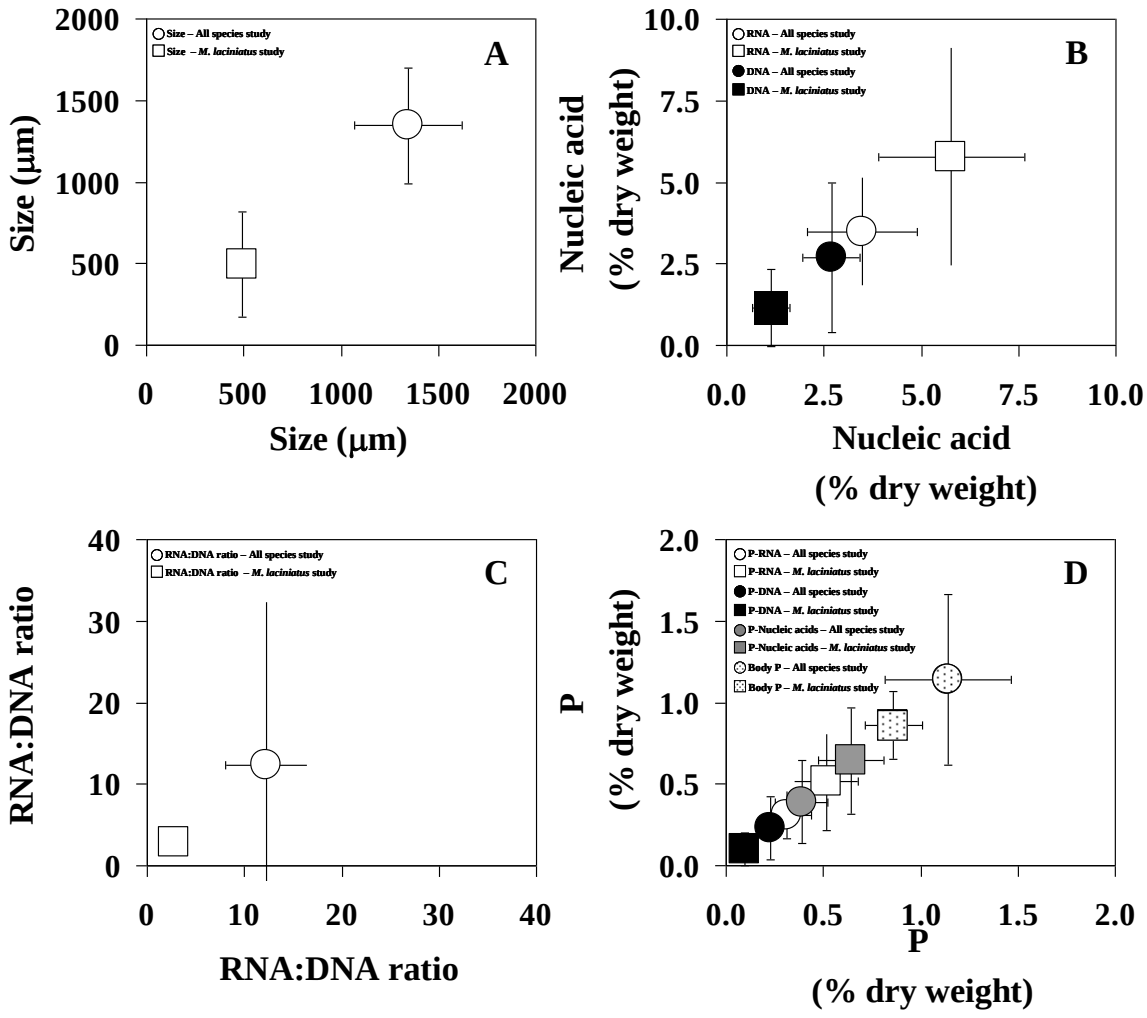


Figure 7. Diagram illustrating mean size (A), nucleic acid content (RNA, DNA) (B), RNA:DNA ratio (C), and P content allocated to RNA, DNA, total nucleic acids, and body mass (D) for all crustacean species in this study (circles) and all ontogenetic stages of *Mixodiaptomus laciniatus* from Lake La Caldera (squares). Vertical and horizontal bars are interspecific and intraspecific standard deviations for multispecies study, and inter-stage and mean intra-stage standard deviations for *Mixodiaptomus laciniatus* study. *Diagramas que ilustran los promedios de tamaño (A), contenido en ácidos nucleicos (RNA, DNA) (B), razón RNA:DNA (C), y contenido de P invertido en RNA, DNA, ácidos nucleicos totales, y biomasa total (D) para todas las especies de crustáceos de este estudio (circulos) y todos los estadíos ontogenéticos de* Mixodiaptomus laciniatus *del lago La Caldera (cuadrados). Las barras verticales y horizontales representan las desviaciones estándar interespecíficas e intraespecíficas para el estudio multiespecífico, y las desviaciones estándar interestadío e intraestadío para el estudio con* Mixodiaptomus laciniatus.

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VIII

Synthesis

Síntesis

Interactive effects of UVR and nutrients on the primary producer-consumer interaction: An ecological-evolutionary perspective

VIII. Synthesis

The major goal of this PhD project was to investigate the interactive effects of ultraviolet radiation (UVR) and nutrients on the primary producer-consumer interaction. For this purpose, it was first evaluated how the combination of UVR and nutrients altered the primary producers as food resource for herbivorous consumers using large field mesocosms in an oligotrophic high mountain lake (chapter II). Second, the nutritional suitability of the *in situ* raised food for consumers was assessed using a variety of experimental and observational approaches carried out at different spatial and temporal scales, from short bioassays to mid-term experiments and long-term field observations. From these heterogeneous studies, a single consistent picture of how the strength of the coupling might evolve in response to UVR and nutrient availability emerged (chapters III-VI).

Our experimental results for the effects of UVR and phosphorus (P) enrichment on the elemental and biochemical composition of seston showed that: (i) P-enrichment increased the content of total fatty acids (TFA), ω3-polyunsaturated fatty acids (ω3-PUFA) [mainly 18:3n-3 (α-linolenic acid)] and chlorophyll *a*:carbon ratio (Chl *a*:C) and carbon:nitrogen (C:N) ratio, but lowered the content of highly unsaturated fatty acids (HUFA) [mainly 20:4n-6 (arachidonic acid, ARA)], the HUFA:PUFA ratio and, at high P loads, C:P ratio in seston; (ii) UVR increased TFA and ω3-PUFA at control and highest P-enrichment, but decreased HUFA and C:P ratio of seston at all points of the trophic gradient. The interaction between UVR and P-enrichment was significant for seston HUFA and C:P ratio, indicating that the effect of UVR in reducing HUFA (decreased biochemical food quality) and C:P ratio (enhanced elemental

food quality) was most pronounced at low nutrient concentrations, and vanished as P-enrichment increased. These results suggest that any potential future increase in UVR fluxes might affect more strongly the algal food quality inhabiting oligotrophic pristine waters, although these effects could be offset by P-inputs from atmospheric dust depositions.

Food associated-effects of these factors on zooplankton growth were tested in coupled short-term laboratory experiments with the aim of separating food quantity from food quality effects at low food conditions, similar to those given in oligotrophic pristine waters (chapter III). This objective was carried out for three zooplankton species with contrasting life-history traits: The copepod *Mixodiaptomus laciniatus*, the cladoceran *Daphnia pulicaria*, and the rotifer *Keratella cochlearis*. Results showed that increased nutrient concentrations generated a large nutrient gradient that most affected the zooplankton growth, with no significant role of UVR. The growth of each zooplankter adjusted well to a saturation curve that reached a plateau at increasing seston levels of ca. 250 for *Keratella cochlearis*, 500 for *Mixodiaptomus laciniatus*, and 1000 μg C L^{-1} for *Daphnia pulicaria*, and after which growth decreased for *Mixodiaptomus laciniatus*. By contrast, nutrients and also UVR affected food quality for zooplankton growth, although to a lesser extent compared to food quantity. The food quality parameter that best explained zooplankton growth was species-specific. Thus, in comparison with previous findings for single food quality predictors, we found that P-normalized ω3-PUFA index (ω3-PUFA:P) of primary producers was a better predictor of the growth of *Mixodiaptomus laciniatus* and *Daphnia pulicaria*, two freshwater metazoans representing some of the major planktonic groups. A key to understand consumer growth appears to be offered by marrying hitherto opposed schools of thought in a joint consideration of essential fatty acids and mineral P, both indispensable for herbivorous consumers, which are linked together in autotroph metabolism. Of all predictors, growth rate of the rotifer *Keratella cochlearis* was strongly related to the P-content of seston. Altogether, food quantity and quality bioassays suggest that seston increase associated with more intense and frequent atmospheric depositions could result in

impaired growth for dominant copepods, but might favour C-limited cladocerans and P-limited rotifers in pristine ecosystems of the Mediterranean region.

Because the above laboratory assays isolated the effects of food quantity and quality and excluded the impact of UVR, mid-term incubations (70 days) were carried out *in situ* to examine how the joint effects of UVR and nutrients might affect the strength of the phytoplankton-zooplankton coupling (PZC) in nature (chapter IV). Our experimental results on *Mixodiaptomus laciniatus* showed that zooplankton biomass unimodally responded to food quantity, challenging 'the more is better (or at least never worse)' concept, since high levels of food resulted in weakened PZC. The effect of UVR on zooplankton was nutrient dependent, significantly reducing zooplankton abundance at intermediate nutrient concentrations (20, 30 and 40 μg P L^{-1} treatments), but not at both extremes of the trophic gradient generated at control and 60 μg P L^{-1} treatments. These observed differences were not due the role of UVR affecting food quantity or quality, suggesting direct deleterious effects of UVR on zooplankton at intermediate food ranges and, as a consequence, weakening PZC. These results contributed to explain the long-term decoupled dynamics of phyto- and zooplankton in Lake La Caldera as result of the increasing intensity and frequency of aerosol depositions over the past three decades (1973-2003) and the characteristic high UVR levels reaching high mountain lakes.

Detrimental effects of UVR resulted in decreased zooplankton abundance but not size, suggesting a direct lethal UVR effect. These detrimental effects of UVR were, however, not observed at both ends of the trophic gradient, where UVR exerted a more subtle effect by enhancing the somatic C content of zooplankton (increased body C:P ratio). Such an increase in %C was interpreted as a protective mechanism against UVR stress. UVR-induced effects in zooplankton elemental composition contribute evidence on the non-strict homeostatic nature of herbivorous consumers. Interestingly, the opposed impact of UVR decreasing phytoplankton C:P ratios but increasing zooplankton C:P ratios would contribute to enhance the nutritional imbalance at the primary producer-consumer interface (chapter V).

The use of nucleic acid indices (NAIs) (%RNA and RNA:DNA ratio) as proxies for growth during three years of intensive monitoring in Lake La Caldera allowed for the examination of nutrient conditions that favoured maximal growth during the ontogenetic development of zooplankton (chapter VI). A most intriguing result was that zooplankton growth, primarily limited by food quantity in Lake La Caldera, was strongly affected by food quality as seston C:nutrient ratio (C:P and C:N ratios). Furthermore, the relationship between NAIs and seston C:nutrient ratio was unimodal and stage-specific. These results also challenged 'the more nutrient is better (or at least never worse)' concept for the consumer, and indicate that food quality effects not only occurred at extremely low food quantities, but also affected zooplankton growth in ways not described before. Thus, the performance at each consumer's developmental stage decreased towards both ends of a food quality gradient and was maximal at an optimal resource C:nutrient ratio. While several mechanisms might account for this phenomenon, the extended assumption that below a given threshold elemental ratio organism performance is not affected by the nutrient content of its food is challenged here. These results are consistent with the knife-edge hypothesis and has strong bearings on the nutritional imbalance at the primary producer-herbivorous consumer interface as it suggests that food C:nutrient ratio can impair consumer growth, whether that C:nutrient ratio is higher or lower that consumer's requirements. The unimodal curves described here were supported by the results of the experimental set-ups, in which manipulation of UVR and nutrients altered food quality of seston, affecting the zooplankton growth that showed considerable developmental-stage variations.

Finally, we studied NAIs and P-stoichiometry in distinct planktonic crustacean species from 22 high mountain lakes (Sierra Nevada and The Pyrenees) in order to examine how variations in NAIs and P-stoichiometry between and within major taxonomical groups related to their life history strategy. Our results were consistent with predictions of growth rate and P-allocation hypotheses. In addition, these results successfully contributed to extent the knowledge of the life history strategy played by *r*-species that place emphasis on rapid growth and *K*-species that invest in competitive abilities at the expense of

growth. We found that both copepods and cladocerans can grow according to each strategy and that *r* and *K* strategies are likely to occur within the life history of any given species. For example, nauplius stages of copepods had high %RNA allowing for a rapid growth, but progressively decreased as copepods reached maturity. All together, the results presented in this chapter strongly suggest that growth strategies are determined, apart from phylogenetic constraints, by selective ontogenetic pressures and those imposed by the environment.

Efectos interactivos de la radiación ultravioleta y los nutrientes sobre la interacción productor primario-consumidor: Una perspectiva ecológico-evolutiva

VIII. Síntesis

El principal objetivo de este proyecto de tesis doctoral fue investigar los efectos interactivos de la radiación ultravioleta (UVR) y los nutrientes sobre la interacción productor primario-consumidor herbívoro. Para este objetivo, se evalúo primero cómo la combinación de la UVR y los nutrientes alteraron a los productores primarios como recurso alimenticio para los consumidores herbívoros usando grandes mesocosmos de campo en un lago oligotrófico de alta montaña (capítulo II). Segundo, la calidad nutricional del alimento generado in situ *para los consumidores fue evaluada mediante una variedad de aproximaciones experimentales y observaciones llevadas acabo en diferentes escalas espaciales y temporales. De toda esta variedad de estudios, emergió un patrón de cómo la intensidad del acople podría evolucionar en respuesta a la UVR y a la disponibilidad de nutrientes (capítulos III-VI).*

Nuestros resultados experimentales de los efectos de UVR y enriquecimiento en fósforo (P) sobre la composición elemental y bioquímica del seston mostraron que: (i) El enriquecimiento en P incrementó el contenido de ácidos grasos totales (TFA), ácidos grasos poliinsaturados ω3 (ω3-PUFA) [principalmente 18:3n-3 (ácido α-linolénico)] y la razón clorofila a*:carbon (Chl* a*:C) y la razón carbono:nitrógeno (C:N), pero disminuyó el contenido de ácidos grasos altamente insaturados (HUFA) [principalmente 20:4n-6 (ácido araquidónico, ARA)], la razón HUFA:PUFA y, para altos niveles de P, la razón C:P del seston; (ii) UVR incrementó TFA y ω3-PUFA en el control y en el tratamiento de mayor enriquecimiento en P, pero disminuyó HUFA y la razón C:P del seston para todos los puntos del gradiente trófico. La interacción entre UVR y el enriquecimiento en P fue significativa para HUFA y para la razón C:P del seston, lo que indica que el efecto de UVR en reducir HUFA (calidad*

bioquímica del alimento reducida) y la razón C:P (calidad elemental del alimento favorecida) fue más pronunciado para bajas concentraciones de nutrientes, y se amortiguó conforme el enriquecimiento en P se incrementó. Estos resultados sugieren que cualquier potencial aumento en los flujos de UVR podrían afectar más fuertemente a la calidad del alimento algal en aguas oligotróficas pristinas, aunque estos efectos podrían ser anulados por las entradas de P asociadas a las deposiciones de polvo atmosférico

Los efectos asociados al alimento de estos factores sobre el crecimiento del zooplancton fueron testados en experimentos acoplados de laboratorio a corto plazo con el objetivo de separar los efectos de cantidad de alimento de los efectos de calidad en condiciones de baja cantidad, similares a las dadas en aguas oligotróficas pristinas (capítulo III). Este objetivo fue llevado acabo para tres especies de zooplancton con diferentes rasgos del ciclo de vida: El copépodo Mixodiaptomus laciniatus*, el cladócero* Daphnia pulicaria*, y el rotífero* Keratella cochlearis*. Los resultados mostraron que concentraciones crecientes de nutrientes generaron un gran gradiente trófico que fue el que principalmente afectó al crecimiento del zooplancton, sin ningún papel significativo de UVR. El crecimiento de cada especie de zooplancton se ajustó bien a una curva de saturación que alcanzó una meseta para niveles crecientes de seston de 250 para* Keratella cochlearis*, 500 para* Mixodiaptomus laciniatus*, y 1000* $\mu g\ C\ L^{-1}$ *para* Daphnia pulicaria*, y tras la cual el crecimiento disminuyó para Mixodiaptomus laciniatus. En contraposición, los nutrientes y también UVR afectaron a la calidad del alimento para el crecimiento del zooplancton, aunque en menor grado en comparación con la cantidad de alimento. El parámetro de calidad de alimento que mejor explicó el crecimiento del zooplancton era específico para cada estadío. Por tanto, en comparación con previas observaciones que apuntanban a predictores simples de calidad de alimento, nosotros observamos que el índice ω3-PUFA normalizado por el P (ω3-PUFA:P) de los productores primarios fue el mejor predictor para el crecimiento de* Mixodiaptomus laciniatus *y* Daphnia pulicaria*, dos metazoos de aguas dulces que representan algunos de los principales grupos del plancton. Un concepto clave para comprender el crecimiento del consumidor aparece al proporcionar la posibilidad de unir dos*

escuelas de pensamiento tradicionalmente opuestas hasta ahora considerando conjuntamente ácidos grasos esenciales y P mineral, ambos indispensables para los consumidores herbívoros, que están vinculados en el metabolismo autótrofo. De todos los predictores, la tasa de crecimiento del rotífero Keratella cochlearis *estaba fuertemente relacionada con el contenido en P del seston. Todo junto, los bioensayos de cantidad y calidad de alimento sugieren que el aumento de seston asociado con las más intensas y frecuentes deposiciones atmosféricas podría dar lugar a un menor crecimiento de los copépodos dominantes, aunque podría favorecer a los cladóceros limitados en C y a los rotíferos limitados en P en ecosistemas pristinos de la región mediterránea.*

Debido a que los ensayos de laboratorio mencionados anteriormente separaban los efectos de cantidad y calidad de alimento y excluían el impacto de UVR, se llevaron acabo incubaciones in situ *a medio plazo (70 días) para evaluar cómo los efectos conjuntos de UVR y nutrientes podrían afectar a la fuerza de la interacción fitoplancton-zooplancton (PZC) en la naturaleza (capítulo IV). Nuestros resultados experimentales sobre* Mixodiaptomus laciniatus *mostraron que la biomasa de zooplancton respondía unimodalmente a la cantidad de alimento, discutiendo así el principio "cuanto más mejor (o al menos nunca peor)", ya que elevados niveles de alimento dieron lugar a PZC debilitado. El efecto de UVR sobre el zooplancton era dependiente de la cantidad de nutrientes, de tal modo que reducía la abundancia de zooplancton en las concentraciones intermedias de nutrientes (tratamientos 20, 30 y 40* $\mu g\ P\ L^{-1}$*), pero no en ambos extremos del gradiente trófico generado en los tratamientos control y 60* $\mu g\ P\ L^{-1}$*. Las diferencias observadas no se debían al papel de UVR afectando a la cantidad o calidad de alimento, lo que sugiere efectos deletéreos directos de UVR sobre el zooplancton para el rango intermedio de alimento y, como consecuencia, debilitando PZC. Estos resultados contribuyeron a explicar las dinámicas desacopladas a largo plazo del fito y del zooplancton en el lago de La Caldera como resultado de un aumento de la intensidad y frecuencia de deposiciones de aerosoles a lo largo de las tres últimos décadas (1973-2003) y de los niveles característicos de alta incidencia de UVR en los lagos de alta montaña.*

Los efectos negativos de UVR dieron lugar a una menor abundancia del zooplancton aunque no afectó al tamaño del mismo, lo que sugiere un efecto directo letal de UVR. Estos efectos negativos de UVR, no obstante, no fueron observados en ambos extremos del gradiente trófico, en los que UVR ejerció un efecto más sutil aumentando el contenido de C somático del zooplancton (aumento de la razón C:P somática). Tal incremento en %C fue interpretado como un mecanismo protector frente al estrés por UVR. Los efectos inducidos de UVR sobre la composición elemental del zooplancton aportan evidencia sobre la naturaleza homeostática no estricta de los consumidores herbívoros. Interesantemente, el impacto opuesto de UVR reduciendo las razones C:P del fitoplancton, pero aumentando las razones C:P del zooplancton contribuyen a favorecer el desequilibrio nutricional en la interfase productor primario-consumidor (capítulo V).

El uso de los índices de ácidos nucleicos (NAIs) (%RNA y razón RNA:DNA) como predictores del crecimiento durante tres años de seguimiento intensivo en el lago de La Caldera permitieron la evaluación de las condiciones nutricionales que favorecían el crecimiento máximo durante el desarrollo ontogenético del zooplancton (capítulo VI). Un resultado más que interesante fue que el crecimiento del zooplancton, primeramente limitado por cantidad de alimento en el lago de La Caldera, estaba fuertemente afectado por la calidad de alimento medida por la razón C:nutriente (razones C:P y C:N). Es más, la ralación entre NAIs y la razón C:nutriente del seston era unimodal y específica de estadío. Estos resultados también discuten el principio "cuanto más nutriente mejor (o al menos nunca peor)" para el consumidor, e indican que los efectos de calidad de alimento no sólo tenían lugar para cantidades de alimento extremadamente bajas, sino que también afectaban al zooplancton de maneras jamás descritas anteriormente. Por tanto, el crecimiento potencial de cada estadío de desarrollo del consumidor era menor hacia ambos extremos del gradiente de calidad de alimento y era máximo para la razón C:nutriente óptima del recurso. Mientras varios mecanismos podrían explicar este fenómeno, este resultado discute la asunción extendida de que por debajo de una determinada razón elemental umbral, el crecimiento potencial del organismo no se afecta por

*el contenido en nutrientes del alimento. Estos resultados son consistentes con la hipótesis del filo de la navaja (*knife-edge hypothesis*) y tiene fuertes repercusiones para el desequilibrio nutricional en la interfase productor primario-consumidor herbívoro ya que sugiere que la razón C:nutriente del alimento puede afectar al crecimiento del consumidor, tanto si la razón C:nutriente es mayor como si es menor de los requerimientos del consumidor. Las curvas unimodales descritas aquí estaban fuertemente apoyadas por los resultados de los diseños experimentales, en los que la manipulación de UVR y nutrientes afectaban a la calidad de alimento del seston, afectando al crecimiento del zooplancton que mostró variaciones considerables entre estadíos de desarrollo.*

*Finalmente, estudiamos NAIs y la estequiometría del P en diferentes especies de crustáceos del plancton procedentes de 22 lagos de alta montaña (Sierra Nevada y Pirineos) con objeto de evaluar cómo las variaciones en NAIs y en la estequiometría del P entre y dentro de los principales grupos taxonómicos se relaciona con sus estrategias del ciclo de vida. Nuestros resultados fueron consistentes con las predicciones de la hipótesis de la tasa de crecimiento (*growth rate hypothesis*) y* P-allocation hypothesis*. Además, estos resultados contribuyeron a extender el conocimiento de las estrategias del ciclo de vida desempeñadas por las especies* r *basadas en el crecimiento rápido y por las especies* K *que invierten en las capacidades competitivas a expensas del crecimiento. Observamos que tanto copépodos como cladóceros pueden crecer de acuerdo a cada estrategia y que es posible que las estrategias* r *y* K *tengan lugar dentro del ciclo de vida de cualquier especie. Por ejemplo, los estadíos naupliares de los copépodos tenían un alto %RNA que les permitía tener un rápido crecimiento, que progresivamente disminuyó a medida que los copépodos alcanzaban la madurez. En su conjunto, los resultados presentados en este capítulo fuertemente sugieren que las estrategias de crecimiento están determinadas, aparte de por las restricciones filogenéticas, por presiones ontogenéticas y por aquellas impuestas por el ambiente.*

IX

Conclusions

Conclusiones

IX. Conclusions

1.- Ultraviolet radiation (UVR) and phosphorus(P)-enrichment enhanced food quality for herbivorous consumers in terms of total fatty acids (TFA), polyunsaturated fatty acids (ω3-PUFA), chlorophyll *a*:carbon (Chl *a*:C) and C:P ratio, although both reduced the sestonic content in highly unsaturated fatty acids (HUFA). Interactive UVR × P-enrichment effects were significant and opposed in sign for HUFA and sestonic C:P ratio. Thus, UVR specifically reduced HUFA content (decreased food quality) and seston C:P ratio (enhanced food quality) at low nutrient concentrations, but these effects vanished as P-enrichment increased.

2. Food quantity limitation was the primary factor constraining herbivorous consumer growth (in order of importance that of cladocerans > copepods > rotifers) in high mountain lakes of Sierra Nevada. Food quality also limited zooplankton growth under low food quantity conditions, although effects were less pronounced compared to those of food quantity, and in addition, the factor determining food quality was species-specific. Thus, a P-normalized ω3-PUFA index offered the best prediction for copepod and cladoceran growth limitation, while seston C:P ratio was the sole factor predicting rotifer growth.

3. The response of zooplankton biomass to increased food quantity from *in situ* nutrient enrichment was unimodal. Thus, zooplankton proved to be constrained by food (too scarce or 'in excess') at both ends of the nutrient gradient, but grew optimally at intermediate food levels. Strong detrimental UVR effects were observed at these intermediate food levels, pointing out a nutrient-dependent role of UVR. Results of this experimental approach were consistent with long-term observations of decoupled interannual dynamics between primary producers and consumers over the last three decades in Lake La Caldera, where there is evidence

for a pronounced increase in the magnitude and frequency of atmospheric aerosol inputs.

4. UVR was responsible for the increase in zooplankton C:P ratio at each end of the trophic gradient generated after nutrient manipulation. Differences in somatic C content were responsible for this pattern, suggesting the storage of lipids, probably associated with carotenoid pigmentation. From these results, relevant ecological implications emerged. First, UVR-induced differences in zooplankton elemental composition contribute evidence on the non-strict homeostasis of herbivorous consumers. Second, UVR, by generating contradictory effects on the C:P ratios of primary producers and their consumer, might amplify the nutritional imbalance at the primary producer-consumer interface.

5. *In situ* response of stage-specific herbivore growth to food quality was unimodal, characterized by an optimum C:nutrient ratio that maximizes growth, and lower and higher ranges impairing growth because of nutrient-rich and nutrient-deficient food, respectively. This response demonstrated that: (i) Consumers are sensitive to nutrient content in food at low quantities and bellow their threshold elemental ratios; and (ii) organisms respond differently to food nutrient content in their ontogeny because of differential requirements in C (energy) and nutrients during their development.

6. The analysis in detail of nucleic acids and P-stoichiometry in the dominant zooplankton species in high mountain lakes of Sierra Nevada, and The Pyrenees mountains supported the validity of growth rate and P-allocation hypotheses for organisms in these ecosystems, but in addition provided a new insight of *K* and *r* ecological strategies. Contrary to the long-held believe that both strategies were taxonomically restricted, our results pointed out that both may be played within the ontogenetic development, and given the proper environmental selective pressures, either by copepods or cladocerans.

IX. Conclusiones

1. La radiación ultravioleta (UVR) y el enriquecimiento en fósforo (P) favorecieron la calidad de alimento para los consumidores herbívoros en términos de ácidos grasos totales (TFA), ácidos grasos poliinsaturados (ω3-PUFA), y las razones clorofila a:carbono (Chl a:C) y C:P del seston, aunque ambos redujeron el contenido en ácidos grasos altamente insaturados (HUFA). Los efectos interactivos UVR × P fueron significativos y opuestos en signo para HUFA y para la razón C:P del seston. Así, UVR particularmente redujo el contenido en HUFA (calidad de alimento disminuida) y la razón C:P del seston (calidad de alimento favorecida) para bajas concentraciones de nutrientes, aunque estos efectos desaparecían conforme aumentaba el enriquecimiento en P.

2. La limitación por cantidad de alimento fue el principal factor limitando el crecimiento de los consumidores herbívoros (por orden de importancia: cladóceros > copépodos > rotíferos) de los lagos de alta montaña de Sierra Nevada. La calidad de alimento también limitaba el crecimiento del zooplancton bajo condiciones de baja cantidad de alimento, aunque los efectos eran menos pronunciados en comparación con los de la cantidad de alimento, y además, el factor determinante de la calidad era específico de especie. Así, el índice ω3-PUFA normalizado por el P era el mejor predictor para el crecimiento de los copépodos y de los cladóceros, mientras que la razón C:P del seston era el único factor predictivo del crecimiento de los rotíferos.

3. La respuesta de la biomasa del zooplancton a una mayor cantidad de alimento a partir del enriquecimiento en nutrientes in situ *fue unimodal. Así, el zooplancton manifestó estar limitado por el alimento (demasiado escaso o "en exceso") en ambos extremos del gradiente trófico, pero creció óptimamente para*

los niveles intermedios de alimento. Se observaron efectos negativos intensos de UVR para estos niveles intermedios de alimento, sugiriendo que los efectos de UVR son dependientes de los nutrientes. Los resultados de esta aproximación experimental fueron consistentes con las observaciones a largo plazo de dinámicas interanuales desacopladas entre los productores primarios y los consumidores durante las tres últimas décadas en el lago La Caldera, en donde hay evidencia de un pronunciado incremento de tanto la magnitud como de la frecuencia de aportes atmosféricos de aerosoles.

4. UVR fue responsable de un incremento en la razón C:P del zooplancton en cada extremo del gradiente trófico generado tras la manipulación con nutrientes. Las diferencias en el contenido de C somático fueron responsables de este patrón, lo que sugiere el almacenamiento de lípidos, probablemente asociados con la pigmentación por carotenoides. De estos resultados, relevantes implicaciones ecológicas emergieron. Primero, las diferencias inducidas por UVR en la composición elemental del zooplancton, aportan evidencia sobre la homeostasis no estricta de los consumidores herbívoros. Segundo, UVR, por medio de la generación de efectos contradictorios sobre las razones C:P de productores primarios y de sus consumidores, podría amplificar el desequilibrio nutricional en la interfase productor primario-consumidor.

5. La respuesta in situ *del crecimiento específico de estadío de los herbívoros a la calidad del alimento fue unimodal, caracterizada por una razón C:nutriente óptima que maximiza el crecimiento, y rangos por encima y por debajo de ésta que afectan al crecimiento debido a un alimento enriquecido y deficiente en nutrientes, respectivamente. Esta respuesta demostró que: (i) Los consumidores son sensibles al contenido en nutrientes del alimento para bajas cantidades y por debajo de su razón elemental umbral; y que (ii) los organismos responden diferencialmente al contenido en nutrientes del alimento durante su ontogenia debido a los requerimientos diferenciales en C (energía) y nutrientes durante su desarrollo.*

*6. El análisis en detalle de los ácidos nucleicos y la estequiometría del P para las especies dominantes de zooplancton de los lagos de alta montaña de Sierra Nevada, y de los Pirineos apoyan la validez de la hipótesis de la tasa de crecimiento (*growth rate hypothesis*) y de la* P-allocation hypothesis *para los organismos de estos ecosistemas, pero además proporcionó una nueva perspectiva de las estrategias ecológicas* K *y* r. *Al contrario de la creencia establecida de que ambas estrategias están taxonómicamente restringidas, nuestros resultados señalan que ambas puedes ser desempeñadas durante el desarrollo ontogenético, y dada ciertas presiones selectivas ambientales, tanto por copépodos como por cladóceros.*