UNIVERSITAT DE BARCELONA

Fish as local stressors of Pyrenean high mountain lakes: Arrival process and impact on amphibians and other organisms

Alexandre Miró Pastó

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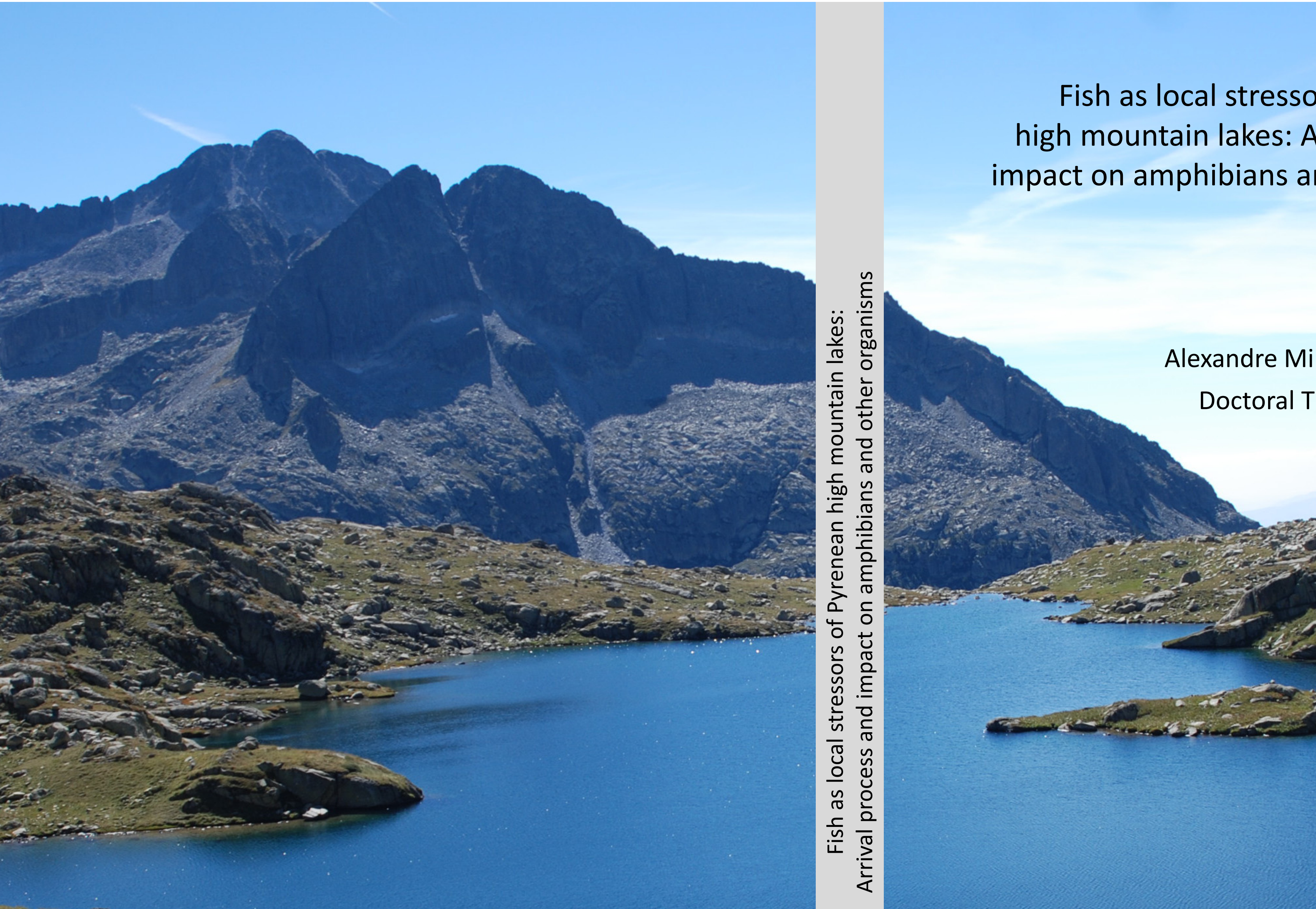

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Fish as local stressors of Pyrenean high mountain lakes: Arrival process and impact on amphibians and other organisms

Alexandre Miró Pastó

Doctoral Thesis

Tesi doctoral
Universitat de Barcelona
Facultat de Biologia – Departament d'Ecologia
Programa de doctorat en Ecologia Fonamental i Aplicada

Fish as local stressors of Pyrenean high mountain lakes: Arrival process and impact on amphibians and other organisms

Els peixos com a pertorbació local als estanys d'alta muntanya dels Pirineus: Procés d'introducció i impacte sobre els amfibis i altres organismes

Memòria presentada per Alexandre Miró Pastó per optar
al grau de doctor per la Universitat de Barcelona

Alexandre Miró Pastó
Centre d'Estudis Avançats de Blanes (CEAB)
Consejo Superior de Investigaciones Científicas (CSIC)
Pallars Sobirà, setembre de 2015

Vist i plau del director de la tesi
Dr. Marc Ventura Oller
Investigador del CEAB-CSIC

Vist i plau de la tutora de la tesi
Dra. Isabel Muñoz Gracia
Professora del Departament
d'Ecologia (UB)

«One last effort brings us, after a regular fifteen minute trek in the snow, to the last of the three lakes, known as The Island Lake. Though the most beautiful and twice as large as the others, it is devoid of the life that trout have given to the other two lakes...»[1]

Count of Carlet, 1905

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[1] Fragment translated from the Count of Carlet's recorded trek through the Pyrenean Valley of the river Madriu, Andorra, in 1905. The Count of Carlet, Salvador Armet Ricart (Barcelona, 1860-1928) was a cultured intellectual who was a university professor, an archaeologist and folklorist.

Reference: Comte de Carlet, 1905. Del Segre a l'Ariège a travès d'Andorra. Butlletí del Centre Excursionista de Catalunya: 127: 225-241; 128: 257-284; i 129: 300-317. http://ddd.uab.cat/record/27613

To my family

To the lakes

Thanks for being there

Acknowledgements

I met my first Pyrenean Lake in June 1988. It was the impressive Lake Besiberri, in the Barrabés Valley. The gorgeous scenery and the pure blue of the lake inspired in me a curiosity about the life in those waters.

Ten years later, in 1998, when I was doing my Biology degree, I was able to collaborate for some years with the research group of the University of Barcelona that had been studying Pyrenean high mountain lakes. They introduced me to the world of research and taught me field sampling methods. I want to thank: Jordi Catalan, Marc Ventura, Lluís Camarero, Sergi Pla, Marisol Feliu, Teresa Buchaca, Anna Clua, Jaume Piera, Frederic Bartumeus, Guillermo de Mendoza, Gemma Cots, Anna Centellas, Maria Vásquez and Esther Fanlo. Walkngi around the lakes and collecting samples next to you was very important to me.

In 2001, after finishing my Biology degree, Marc Ventura offered me a great suggestion. *«You could study the role of fish in the Pyrenean high mountain lakes. I'm sure they are very important, and there is almost nothing known about them»*. A few days later I began the job. In 2005, after learning about the fish, we started to look at amphibians and other organisms, and to study their ecology in relation to the fish in Pyrenean lakes. We have been working for years on these topics, always near our beloved lakes. Thank you very much Marc, for your suggestion and for your support during many years.

When I started to collect historical data about the presence and exploitation of fish in the Pyrenean high mountain lakes, some specialists in history, old documents and archives supplied us with very useful information. We want to thank Carme Maria Marugan and Jesús Sanchez Pellicer from the Arxiu Comarcal del Pallars Sobirà, Maria Pau Gómez from the Arxiu Generau d'Aran, Maria Àngels Sanllehy from the Biblioteca Nacional de Catalunya and Joan Ramon Segura from the Biblioteca del Centre Excursionista de Lleida. We also want to thank the staff of the Arxiu

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Since five years ago I haven't spoken a word of English, so for me to write this thesis in English was the hardest challenge. I was able to do it properly thanks to the help of my supervisor Marc Ventura and my friends from Scotland David O'Brien and Jeanette Hall who kindly assisted in editing the manuscripts. Mary Loughlin also improved my spoken and written English. Thank you very much!

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Thanks a lot to all of you!

Alexandre Miró Pastó
Pallars Sobirà, September 2015

Report of the thesis supervisor

Dr. Marc Ventura Oller, researcher from the Department of Continental Ecology, Centre for Advanced Studies of Blanes (CEAB-CSIC), supervisor of the Doctoral Thesis written by Mr. Alexandre Miró Pastó with the title "*Fish as local stressors of Pyrenean high mountain lakes: Arrival process and impact on amphibians and other organisms".*

INFORMS

That the research papers written by Mr. Alexandre Miró Pastó as part of his pre-doctoral training and included in his Doctoral Thesis have resulted in two published papers, and three more that are being prepared for submission in international journals. Following is a list of the papers published or in preparation and their impact factors according to SCI of ISI Web of Knowledge of the journals where the papers have been published:

Chapter 1. Miró, A., and M. Ventura. 2013. Historical use, fishing management and lake characteristics explain the presence of non-native trout in Pyrenean lakes: Implications for conservation. Biological Conservation 167:17-24. DOI: dx.doi.org/10.1016/j.biocon.2013.07.016. The journal had an IF at 2012 of 3.794 and is at the 1st quartile of the following areas: Biodiversity Conservation, Ecology and Environmental Sciences.

Chapter 2. Miró, A., and M. Ventura. 2015. Evidence of exotic trout mediated minnow invasion in Pyrenean high mountain lakes. Biological Invasions 17:791-803. DOI: 10.1007/s10530-014-0769-z. The journal had an IF at 2014 of 2.586 and is at the 2nd quartile of the following areas: Biodiversity Conservation, Ecology.

Chapter 3. Miró, A., Buñay, D. & Ventura, M. 2015. Multiple stressor impacts on native high mountain lake fauna: the role of trout, minnows and water-level fluctuation.

Chapter 4. Miró, A. & Ventura, M. 2015. Amphibian distribution in Pyrenean high mountain lakes and ponds is best explained by spatial patterns and fish introductions

Chapter 5. Miró, A., Buchaca, T., Pla, S., Buñay, D., Vila, M., Larsen, T., Sabas, I. & Ventura, M. 2015. Introduced fish mediate trophic cascade on the epilithon of high mountain lakes and ponds through tadpole eradication.

Also CERTIFIES

That Mr. Alexandre Miró Pastó has participated actively in the development of the research activity that has led to the writing of all the papers of the PhD. In particular he has participated in the study design, all the field work, has generated all the data with the exception of the zooplankton of chapter 3, temperature modelling of chapter 4 and diatoms, algal groups, bacteria, archaea, chemistry and physical modelling of chapter 5. He also has analysed the results and written all papers.

Finally, I also certify that none of these papers are being part of any other PhD theses completed or under development.

Sincerely,

Marc Ventura Oller

Blanes, September 18th 2015

Contents

Part I. Arrival, distribution and ecology of introduced fish

Part II. Impacts on amphibians and other organisms

General abstract

Pyrenean high mountain lakes are naturally fishless due to natural barriers that have prevented the colonisation of fish species from lower streams. However, there have been numerous introductions of trout to such ecosystems, both in historical and recent periods. Unlike other high mountain regions, some such lakes in the Pyrenees were exploited traditionally for trout cultivation. This activity started some centuries ago and lasted until the 1950s, affecting approximately 25% of the lakes. Since 1950, and similarly to other high mountain regions, a wave of modern introductions of exotic species has affected between 35% and 85% of the lakes, depending on the valley. In recent decades, minnows have also been introduced as a result of recreational fishing with live-bait and, by 2000, were present in 27% of Pyrenean high mountain lakes.

The first part of the thesis was intended to provide an objective description of the fish introduction process in the Pyrenees. For that purpose we collected data on trout and minnow occurrence from 520 high mountain lakes >0.5 ha in the southern Pyrenees. We then quantified, by generalized additive models, which particular environmental and anthropogenic factors best explained their present distribution, and as a result the conservation status of the lakes.

The distribution of *Salmo trutta* in the lakes of the southern slopes of the Pyrenees was best explained by both anthropogenic factors and lake characteristics, while only anthropogenic factors linked to recreational fishing were associated with the distribution of the exotic trout *Salvelinus fontinalis* and *Oncorhynchus mykiss*. In the case of minnow occurrence, previous presence of trout in the lake was the most explanative variable, confirming its association with recreational fishing using it as live-bait.

Trout and minnow introductions can cause large ecological problems and ecosystem change in high mountain lakes and ponds, since both trout and minnows occupy the top of a lake’s food chain. In the second part of the thesis, we sampled 1736 Pyrenean high mountain lakes and ponds at

different levels of intensity, and investigated the effects of introduced fish on indicator faunal groups such as amphibians and conspicuous macroinvertebrates in the littoral, and crustaceans in the plankton. We also considered whether there was a trophic cascade from fish to the littoral epilithic community of these ecosystems.

Our results showed that fish presence was linked with the disappearance of most amphibian species and of most conspicuous macroinvertebrates. Minnows also showed a sizeable impact on the pelagic habitat, reducing the abundance of some herbivorous species of zooplankton that appeared to be unaffected by trout. In the case of amphibian species, we found that, although introduced fish had a high local impact, western-eastern patterns of some environmental variables are the main drivers of amphibian species distribution at Pyrenean range scale. In addition, we confirmed the presence of a littoral trophic cascade that defines most of the characteristics of the littoral epilithon of Pyrenean high mountain lakes and ponds through fish predation of tadpoles and hence, by a drastic reduction of grazing activity.

General introduction

Introduced fish as local stressor

Several anthropogenic stressors have been identified worldwide as major conservation threats in aquatic and terrestrial ecosystems. These include habitat destruction and fragmentation, introduction of invasive species, land-use changes, pollution, human population growth, overharvesting and climate change (Brook et al. 2008, Stendera et al. 2012). Amongst them, the impact of multiple species invasions on native organisms and communities can be difficult to interpret, because invaders can interact among themselves and affect native species in several ways (Simberloff 2006).

In general, ecological factors don't function individually, and an ecosystem's integrity is often affected by complex interactions from multiple stressors and environmental variables (e.g. Davis et al. 2010, Matthaei et al. 2010, Ban et al. 2014). Therefore, multi-factor studies are a more adequate framework for investigating the relative weight of the different natural and anthropogenic factors (Davidson and Knapp 2007, Hof et al. 2011).

Some invasive species have been identified as a cause of large impacts on the receiving ecosystems by direct predation on native species (Lockwood et al. 2013, Simberloff et al. 2013). Previous studies of the effects of invasive species in freshwater ecosystems have shown strong negative ecological consequences (Vitule et al. 2009). Freshwater fishes are amongst the animal groups with the greatest number of invasive species, as has been shown in Europe (Hulme et al. 2009). At present, the introduction of freshwater fishes is closely related to human activities (Marchetti et al. 2004), and particularly with angling, in the case of salmonid fishes (Cambray 2003).

Introduced fish effects in high mountain lakes

High mountain lakes are found in remote headwaters, far from populated areas, and may support unique plant and animal communities and hence have tremendous ecological and environmental value (Cole and Landres 1996, Kernan et al. 2009a). Many of them are situated within protected areas with scenic, ethnographic, biodiversity and conservation interest, and varying levels of protection from the most basic to highly protected areas (Knapp 1996a, Wiley 2003).

Although high mountain lakes are often perceived to be pristine, they have been threatened by global stressors such as climate change and by long-distance atmospherically transported pollution including acid deposition, persistent organic pollutants and trace metals (Davidson and Knapp 2007, Catalan et al. 2013), which cannot be managed at regional scale (Brown et al. 2013). Nevertheless, their major direct anthropogenic stressors are likely to be the introduction of invasive fish stocks (Pister 2001, Wiley 2003) and, in some areas, artificial water-level fluctuations because of impoundment for hydroelectric power exploitation (Catalan et al. 1993) both of which can be managed directly by local or regional administration (Brown et al. 2013).

High mountain lakes are naturally fishless due to natural barriers that have prevented the colonisation of fish species from lower streams. However, there have been numerous trout introductions to such ecosystems around the world since the 19th century, mainly for angling. These introductions have been reported in mountain ranges such as the Rocky Mountains and Sierra Nevada in western North America, the Tatras, Cantabric range and Sistemas Central and Ibérico in Europe, or the Canterbury high elevation plains in the South Island of New Zealand (Terrero 1951, Brancelj 2000, Schindler 2000, Martinez-Solano et al. 2003, Wiley 2003, Toro et al. 2006, Wissinger et al. 2006). Moreover, since the 1900s, minnows have also been introduced to some mountain areas as live-bait for recreational fishing, for example in the north European lakes of Scotland and Norway (Maitland and Campbell 1992, Museth et al. 2007).

Several studies since the 1980s have investigated the effect of introduced trout on a range of autochthonous species or assemblages of high mountain lakes worldwide, using data from a large number of sites. They have found that Introduced trout becomes the top predator of high mountain lake ecosystems, leading to profound ecological changes. These include the drastic reduction or elimination of amphibian and reptile populations (Knapp 2005, Orizaola and Braña 2006, Pope et al. 2008, Pilliod et al. 2010, Tiberti and von Hardenberg 2012), changes in zooplankton and benthic macro-invertebrate species composition and size structure (Brancelj 2000, Knapp et al. 2001b, de Mendoza et al. 2012, Tiberti et al. 2014b), large-scale alteration of ecosystem processes such as planktonic food web or nutrient cycling (Sarnelle and Knapp 2005, Magnea et al. 2013) and indirect effects on surrounding habitats through resource depletion (Epanchin et al. 2010). Nevertheless, the indirect effect of introduced fish on the benthic community of high mountain lakes through removing grazers has not yet been studied.

Furthermore, the impact of invasive trout on the amphibians of high mountain lakes can be exacerbated by interaction with other stressors, such as airborne pesticides (Davidson and Knapp 2007). The negative effect of introduced fish on amphibians is particularly worrying, since they have decreased globally in recent decades, and are one of the most threatened animal groups worldwide (Beebee and Griffiths 2005).

Pyrenean high mountain lakes and ponds

The Pyrenees comprise a continuous high mountain biogeographic area situated in the south west of Europe, running from the Atlantic ocean to the Mediterranean sea (2°05’W–3°15’E, 43°18’–42°16’N). There are approximately 4500 high mountain lakes and ponds, all of which are potentially suitable habitats for freshwater organisms. These lakes have been well-studied since the late nineteenth century (e.g. Jeanbernat 1874), and especially during recent decades. Work has focused on describing their main environmental and ecological characteristics (e.g. Catalan et al. 2009a, Kernan et al. 2009a, Kernan et al. 2009b, Thompson et al. 2009) and studying their role as sentinels of global change (e.g. reviewed by Catalan et al. 2006, Bartrons et al. 2012b, Camarero and Catalan 2012, Catalan et al. 2013, Arellano et al. 2015). Contrasting with these extensive studies of lakes, the ecological characteristics of ponds have not been described.

There have been many studies of the faunal communities of Pyrenean high mountain lakes and ponds, for example planktonic crustaceans and macroinvertebrates are well studied at Pyrenean range scale (Miracle 1978, Kernan et al. 2009a, de Mendoza and Catalan 2010, de Mendoza et al. 2012, Ventura et al. 2014, de Mendoza et al. 2015). There are six widely distributed amphibians in Pyrenean lakes or ponds across most of the mountain range (e.g. Pleguezuelos 2002, Pottier 2008). These comprise three anurans: the common frog (*Rana temporaria* Linnaeus 1758) living in both lakes and ponds (Balcells 1956, Vences et al. 2003), the midwife toad (*Alytes obstetricans* (Laurenti, 1768)) with two subspecies, *A. o. obstetricans* more frequent in the western range and *A. o. almogavarii* in the east (Maia-Carvalho et al. 2014), the common toad (*Bufo spinosus* Daudin, 1803)(Recuero et al. 2012); and three caudatans: the Pyrenean newt (*Calotriton asper* (Dugès, 1852)), an endemic species of the Pyrenees (Carranza and Amat 2005), the palmate newt (*Lissotriton helveticus* (Razoumowsky, 1789)) and the fire salamander (*Salamandra* (*salamandra* L 1758)). In addition, the Pyrenean frog (*Rana pyrenaica* Serra-Cobo, 1993) is a recently discovered endemic found in some streams of the central Pyrenees (Serra-Cobo et al. 2000). Although Pyrenean amphibian distribution is generally well known, there have been no ecological studies of the assemblage performed at the whole biogeographical scale.

Conservation status of Pyrenean high mountain lakes

Most Pyrenean lakes are protected through inclusion within the Natura 2000 network, and different National or Natural Parks. The lakes as habitats are included in the Habitats Directive, as well as three of the six amphibian species present (*R. temporaria, A. obstetricans* and *C. asper*). The species' conservation status is classified as Near Threatened (*C. asper*) or Least Concern (the rest) by the IUCN and they are all protected by national and regional laws. Taking into account the strong ecological interest of Pyrenean lakes and their associated fauna and their conservation value, it is surprising that little or no studies have approached an evaluation of their conservation status at a Pyrenean scale.

Species distributions do not follow political borders but environmental features or gradients; therefore identification of Pyrenean scale problems can aid environmental managers in improving management policies and, therefore, the conservation of these ecosystems. At present there are six different countries or regions that have environmental responsibilities in the Pyrenean range: in general, the northern side is part of France and the southern is part of the Spanish regions of Basque Country, Navarre, Aragon and Catalonia, with Andorra in the south-east. Therefore, there is a strong heterogeneity of protection agencies and environmental management policies across the range. A regional overview of Pyrenean conservation status provides a sound basis for conservation policies and actions at mountain range scale, removing artificial borders and involving all countries and administrations present.

The two main types of threats to Pyrenean high mountain lakes are diffuse pollution, and local stressors such as fish introduction or water level alteration for hydroelectricity. While many studies have described the presence of different types of airborne pollutants in the Pyrenees (e.g. reviewed by Bartrons et al. 2012b, Camarero and Catalan 2012, Catalan et al. 2013, Arellano et al. 2015), there are few or no studies describing the effects of water level alteration or invasive fish. The former is known primarily to affect larger lakes (Catalan and Ventura 2003) that have different species composition to smaller lakes (Catalan et al. 2009a). In contrast, there are no accounts of the distribution of invasive fish across the whole range.

Previous studies have suggested that most fish introductions are relatively recent (Sostoa and Lobón-Cerviá 1989). Trout species introduced include *Salmo trutta* Linnaeus, 1758*, Oncorhynchus mykiss* (Walbaum, 1792) and *Salvelinus fontinalis* (Mitchill, 1814) in the southern (Spanish) Pyrenees (Sostoa and Lobón-Cerviá 1989) and these species together with *Salvelinus alpinus* (Linnaeus, 1758) and *Salvelinus namaycush* (Walbaum in Artedi, 1792) in the northern (French) Pyrenees (Delacoste et al. 1997). Moreover, the presence of minnows (*Phoxinus* sp.) in Pyrenean high mountain lakes has also been reported, probably associated with their use as live-bait for recreational fishing (Lobon-Cervia et al. 1989).

Despite the existing body of knowledge on Pyrenean high mountain lakes, there has been little work on fish introduction into these ecosystems, fish ecology, or the effects of introduced fish on lake ecosystems. This thesis presents an exhaustive investigation of the historical and ecological factors of fish introduction and their ecological consequences in Pyrenean high mountain lakes, and also considers the ecological consequences of a second anthropogenic stressor, artificial water level changes and the interaction of the two. The general aim of the thesis is to describe the magnitude and impact of fish introductions across the whole of the Pyrenees. For that reason, and because previous studies have shown that the most conspicuous animal groups (see above) are those most affected by fish introductions, we focussed on amphibians and conspicuous macroinvertebrates from the littoral zone, and planktonic crustaceans (the largest and major natural animal group of the pelagic habitat). Finally we have also studied the effect of the fish trophic cascade on the littoral epilithic community via removal of tadpole grazers. Such studies, analysing the effects of different stressors together with environmental factors on ecosystems, can be helpful to inform appropriate management and conservation policies (Hart and Calhoun 2010).

General objectives and hypotheses

Objectives

The general aim of the thesis was to reconstruct the introduction process of fish in Pyrenean high mountain lakes and ponds, and to investigate the ecological consequences of their introduction.

Specifically, we had the following five objectives, which match the five chapters of the thesis:

1. Reconstruct the trout arrival process into Pyrenean high mountain lakes and disentangle the environmental and anthropogenic factors that best explain their current distribution.

2. Reconstruct the minnow arrival process into Pyrenean high mountain lakes, and disentangle the environmental and anthropogenic factors that best explain their current distribution and, specifically, the relationship with previous trout introductions.

3. Investigate the impact of trout, minnows and artificial water level fluctuation, and their interactions, on assemblages of amphibians, conspicuous macroinvertebrates and planktonic crustaceans of Pyrenean high mountain lakes.

4. Disentangle the importance of environmental spatial patterns from the local effect of introduced fish, in explaining amphibian distribution in the Pyrenean high mountain lakes and ponds at mountain range scale.

5. Investigate whether there is a trophic cascade mediated by introduced fish in the littoral of Pyrenean high mountain lakes and ponds, and the indirect impact of fish on epilithon characteristics, through predation on grazing amphibian tadpoles.

Hypotheses

1. Arrival process and ecology of trout; we hypothesised that their arrival in the naturally fishless high mountain lakes was mainly human-mediated, and that anthropogenic factors should explain most of their distribution.

2. Arrival process and ecology of minnows; we hypothesised that their introduction resulted from their use as live-bait and was thus largely associated with the previous introduction of trout for angling in the lake.

3. Impact of introduced fish on Pyrenean lake fauna; we hypothesised that there would be a marked impact on amphibians and conspicuous macroinvertebrates, as well as on some planktonic crustaceans, as shown in previous research from other mountain ranges.

4. Spatial patterns of environmental variables; we hypothesised that these would exert a strong influence on the regional distribution of amphibian species at mountain range scale, in addition to the localised impact of introduced fish.

5. Impact of introduced fish on the littoral epilithon community; we hypothesised that there would be a demonstrable indirect effect of introduced fish on the characteristics of the littoral epilithon community in the Pyrenean high mountain lakes and ponds, through direct predation on grazing tadpoles.

Part I

Arrival, distribution and ecology of introduced fish

Chapter 1

Historical use, fishing management and lake characteristics explain the presence of non-native trout in Pyrenean lakes: Implications for conservation[2]

Abstract

High mountain lakes are naturally fishless, although many have had introductions of non-native fish species, predominantly trout. Predation on native fauna by introduced trout involves profound ecological changes. The objective of this study was to reconstruct the historical process of trout introduction in 520 high mountain lakes >0.5 ha of the southern Pyrenees and quantify which particular factors either environmental or anthropogenic, best explained their present distribution and lake conservation status. The first written evidence of trout introductions dated back to 1371 AD. By 1900, trout had been introduced to 26.5% of lakes during preceding centuries. A subsequent wave of introductions began in 1960 when stocking led to trout becoming established in 52.5% of lakes. From 1900 to 1950, walking distance from nearby urban centres was the dominant factor explaining 29-60% of the variation in trout distribution, indicating that trout introductions were in lakes closer to human settlements. In contrast, with the onset of modern fish management that took place during the period 1960–2000, the most significant factors were

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both the management practices and lake characteristics which are likely related with the probability of survival of the fish populations. After 2000, the remaining fishless lakes were those of highest altitudes, and the shallowest and those with the lowest surface area. The recent fishing ban in protected areas of National Parks has resulted in a stabilization of the number of lakes with fish, suggesting that this is the only reliable management policy to avoid new introductions and preserve the conservation status of high mountain lakes.

Keywords: invasive species, trout, high mountain lake, National Park, lake conservation, fish introductions

1. Introduction

Previous studies of the effects of invasive species in freshwater ecosystems have shown negative ecological consequences (Vitule et al. 2009). Freshwater fishes are one of the animal groups with higher number of invasive species, as has been shown in Europe (Hulme et al. 2009). At present, the introduction of freshwater fishes is closely related to human activities (Marchetti et al. 2004), and particularly with angling in the case of salmonid fishes (Cambray 2003). Within high mountain lakes, the introduction of non-native fish species (mainly salmonids), is a global threat with common origins, mainly related with recreational angling and often promoted by different relevant governmental agencies (Pister 2001, Schindler and Parker 2002). These ecosystems are originally fishless due to natural barriers that have prevented the natural colonisation of fish species from lower streams (Pechlaner 1984, Knapp et al. 2001a).

Introduced trout become the top predators of high mountain lake ecosystems leading to profound ecological changes. Predation on native fauna can lead to the elimination of amphibian and reptile populations (Knapp et al. 2001b, Martinez-Solano et al. 2003, Knapp 2005, Orizaola and Braña 2006, Pope et al. 2008), changes in zooplankton and benthic macro-invertebrate species composition and size structure (Brancelj 2000, Knapp et al. 2001b, Toro et al. 2006, de Mendoza et al. 2012), alteration of ecosystem process such as nutrient cycling (Schindler et al. 2001) and indirect effects through resource depletion (Epanchin et al. 2010). Trout introductions are therefore a threat for the conservation of high mountain lake biodiversity in general and in particular for the most conspicuous animal groups.

In order to implement effective conservation measures, it is necessary to have a precise knowledge of trout distribution and the factors that determine their presence. Detailed information on the causes of the spread of salmonid fishes exist for some high mountain areas such as western North America (Bahls 1992, Schindler 2000, Wiley 2003), where introductions took place between the end of the nineteenth and beginning of the twentieth century. Trout introductions were undertaken initially by individual fishermen and, a few decades later, by governmental agencies responsible for fisheries management (Schindler 2000). In contrast to the American continent, in European high mountain lakes the colonisation process has not been studied in detail (Gliwicz and Rowan 1984, Pechlaner 1984, Sostoa and Lobón-Cerviá 1989). The first introductions in the Alps were carried out at the end of the sixteenth century (Pechlaner 1984) and in the Tatra mountains at the end of the nineteenth century (Gliwicz and Rowan 1984, Brancelj 2000). In the Cantabric mountains (Iberian Peninsula) introductions also occurred at the end of the nineteenth century (Terrero 1951) and even more recently in the Sistema Central and Sistema Iberico, also in the Iberian Peninsula (Martinez-Solano et al. 2003, Toro et al. 2006).

In the Pyrenees it has been suggested that most fish introduction events are relatively recent (Sostoa and Lobón-Cerviá 1989). The different trout species introduced in the Pyrenees include *Salmo trutta, Oncorhynchus mykiss* and *Salvelinus fontinalis* in the southern (Spanish) Pyrenees (Sostoa and Lobón-Cerviá 1989) and these species together with *Salvelinus alpinus* and *Salvelinus namaycush* in the northern (French) Pyrenees (Delacoste et al. 1997). At a global scale, *S. trutta* and *O. mykiss* are considered among the 100 most invasive alien species of the world (Lowe et al. 2000). Within Europe, *O. mykiss, S. fontinalis* and *S. namaycush* have been introduced from North America, while *S. trutta* and *S. alpinus* which are native European species, have been widely introduced beyond their native range, mostly in high mountain areas. Therefore they are all classified as European alien species (Hulme et al. 2009).

Among the different approaches used to reconstruct the fish introduction processes in lakes, the review of historical information has been very useful (Le Cren et al. 1972, Pechlaner 1984). Studies with a historical perspective are necessary to understand the impacts of introduced species

(Kulhanek et al. 2011), which in turn are the basis for establishing more appropriate management policies. For example, the historical perspective has been used successfully in the North American Great Lakes, where it has had a significant potential for establishing restoration goals (Steedman et al. 1996). The combined analysis of historical information and the factors that might explain the distribution of high mountain lake fishes has not yet been conducted.

The objective of this study was to reconstruct the historical process of trout introduction events in high mountain lakes of the southern slope of the Pyrenees, to quantify which particular factors, either environmental or anthropogenic, best explain their present distribution and to evaluate which conservation measures have been most effective. In particular, we had the following specific hypotheses: (*i*) we expected that lakes with historical presence of trout populations would be those closer to towns and only hold the autochthonous species *S. trutta; (ii)* that modern introductions would be more likely in areas where active fish management has taken place and would involve the occurrence of other species than *S. trutta*; *(iii)* the absence of trout would be more likely in lakes with acid pH, higher altitudes and lower lake sizes; and finally *(iv)* prohibitions on fishing in National Parks would result in stopping fish introductions.

2. Materials and Methods

2.1. Study area

The studied lakes are spread along the Pyrenean mountain range (0°42'W–2°09'E, 42°52'–42°23'N; Fig. 1). From among 1062 lakes >0.5 ha we surveyed 520 water bodies which comprise all those lying within the Catalan–Aragoneese (Spanish or southern) Pyrenees. Pyrenean lakes originated from glacial processes or were modified by the activity of quaternary glaciations. A more detailed description of the lake characteristics is provided in Appendix 1.

2.2. Data collection and environmental variables

The occurrence data for the different fish species were obtained from various sources depending on the period. Historical information of fish introductions (before 1900) was obtained from historical documents from local, regional and national archives. For introductions between 1900 and 2000 we collected *ca.* 6000 citations of species presence from interviews with local elderly fishermen or nature reserve guards, from local reports of fishing or walking societies and from our own visual encounter surveys. In a few cases we used gill nets to validate the information from the interviews. On average, we collected 11.6 citations per lake. We chose this method instead of only using our own surveys, since our aim was to provide a historical perspective of fish introductions and to be able to provide an objective account of the main factors responsible for these introductions.

To quantify which factors were responsible for fish introductions we generated a set of environmental variables summarizing both physical lake characteristics that might have affected the distribution of fish species and anthropogenic factors. Physical attributes from each water body included altitude, surface area, temperature and pH. Anthropogenic factors were divided into two groups, those describing or indicators of the mode of fish introductions (walking distance from the nearest town, the size of the population of the nearest town and their number of hotel beds, records of helicopter stocking and the presence of forestry roads), and those describing the management practice where the lake is situated (Table 1). A detailed description of how the variables were obtained is described in Appendix 1.

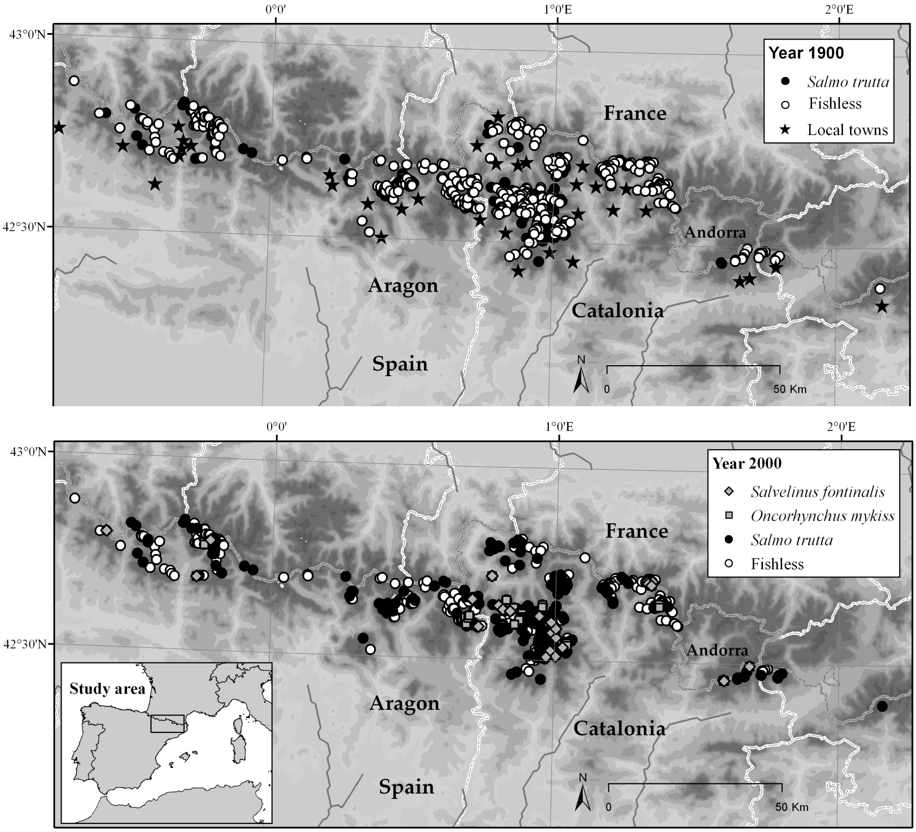

Figure 1. Fish species occurrence and distribution of studied lakes along the Pyrenean range at the year 1900 (upper panel) and at the year 2000 (lower panel). Note that in 43 lakes there are more than one trout species per lake that are not visible in the map. In particular we have found that 32 lakes have two trout species, mainly *S. trutta* with *S. fontinalis* or *O. mykiss*. In addition, 11 lakes have the three trout species. Asterisks in the upper panel are the local towns.

Table 1. Description of predictor variables used in the generalised additive models.

Variable type	**Variable name**	**Description**
Physical and chemical	Altitude (ALT)	Elevation of the lake (m)
	Surface area (SURF)	Surface of the lake (ha)
	Water body location (LOCATION)	Latitude and longitude of the lake, UTM reference system
	Accumulated degree days (ADD)	Thermal accumulation in degree days > 7.8°C (°CDay)
	pH<5.5 (pH)	Lakes with pH < 5.5
Mode of introduction	Walking distance (WALK)	Walking distance from the nearest town (minutes)
	Population (POP)	Inhabitants of the nearest town where the lake is situated.
	Hotel beds (HOT)	Number of hotel beds in the nearest town where the lake is situated.
	Helicopter stocking (HEL)	Helicopter stocking in the lake
	Forestry road (FROAD)	Lakes with forestry road access
Management practice	Fishing zone (FIZ)	Active fish management, at present or in the past
	National Park (NATP)	Part of the National Park where no fishing is allowed at present
	Hydroelectrical power (HEP)	Lakes with water level regulation
Biological parameters	*Salmo trutta* presence (STRU)	Presence of *S. trutta.* Only for *S. fontinalis* and *O. mykiss* models
	Salvelinus fontinalis presence (SFON)	Presence of *S. fontinalis.* Only for *S. trutta* and *O. mykiss* models
	Oncorhynchus mykiss presence (OMYK)	Presence of *O. mykiss.* Only for *S. fontinalis* and *S. trutta* models

2.3. Statistical analysis

We compared the data on the presence/absence of the different fish species in the Pyrenean lakes with the different environmental variables including lake characteristics, site location variables, mode of introduction variables and fish management practice descriptors. We used generalized additive models for the analyses because they are similar to generalized linear models, but relax the assumption that the relationships between the dependent variable (when transformed to a logit scale) and predictor variables are linear by estimating a nonparametric loess smooth function for each continuous predictor variables (Hastie and Tibshirani 1990, Knapp

2005). See Appendix 1 for details on the regression procedure. The analysis was run first to obtain a historical perspective every decade from 1900 until present (year 2000) with all the taxa together and secondly with the present day data for each species separately.

Finally, we compared the presence/absence of the different fish species with the categorical predictor variables by 2x2 contingency tables. With the different continuous predictor variables, we tested if there were significant differences between the lakes that are fishless, those stocked before the year 1900 and those after 1900 with one-way ANOVA and a Tukey post hoc test. Data had previously been normalized.

3. Results

3.1. Historical process

More than 98% of the 520 studied lakes had actually natural barriers left after the glaciers retreated that had prevented the natural colonisation of fishes. Therefore, most fish populations found in them at present are presumed to have come from non-natural sources. Research in historical archives confirmed that exploitation of lakes by local citizens was a common traditional activity in the Pyrenees. In this case, lakes were stocked with brown trout from nearby streams where it is native. The first explicit citations dated back to the 14 th and 15th centuries in Lake Evol (1371 AD and 1423 AD), in Lakes Naut and Major de Saboredo (1581 AD), in Piedrafita Lake (1624 AD) and Escrita and Peguera catchments (1674 AD) (Miró 2011). These old documents describe both the presence of trout in some lakes, and fishing for commercial exploitation of trout. After these initial historical documents, we found an increase in the number of citations, probably due to the increased amount of preserved written information. Our findings suggest that human exploitation of some of these lakes might have originated further back in time, possibly with the human colonisation of the Pyrenees.

We found sufficiently detailed information to be able to perform a detailed historical reconstruction of introductions in the 20th Century. As of 1900, there were 138 lakes (26.5% of the total) that had been stocked with trout during preceding centuries for traditional exploitation (Fig. 1). The first modern introductions using large stocks of juvenile trout grown in local fisheries occurred occasionally at the beginning of the 20th century, but it was not until 1960 that most fish stockings were recorded. During this period the number of lakes with introduced trout increased to 272 (52.5% of the sample). Prior to 1900, the trout species introduced was exclusively brown trout, which was also the species with the highest increase during the last decades of the 20th Century, increasing from 153 lakes in 1950 (29.4%) to 262 (50.4%) in 2000 (Fig. 2). The other species found, *O. mykiss* and *S. fontinalis,* originally from North America, were first recorded in 1950 being present in 32 (6.2%) and 33 (6.3%) lakes respectively (Fig. 2). These latter introductions were carried out mostly by governmental agencies but also by hydroelectric exploitation companies and local fishermen's societies.

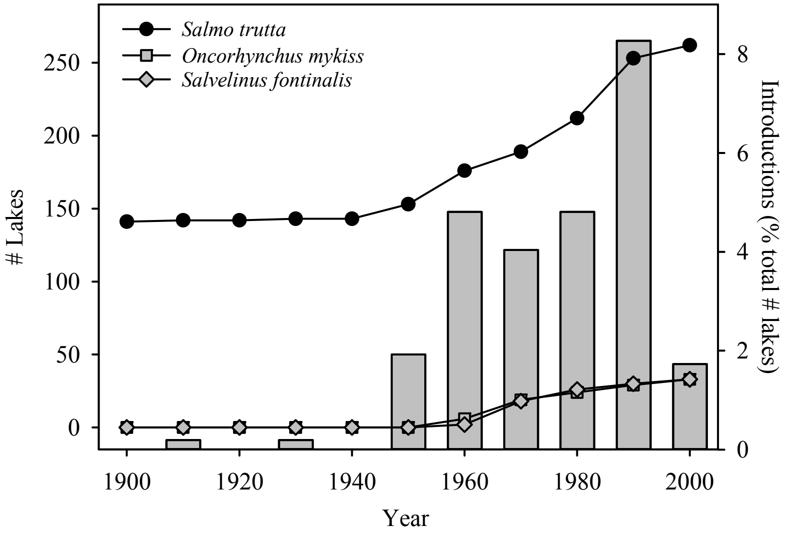

Figure 2. Introduction process of non-native trout species in the southern Pyrenees during the twentieth century. Vertical gray bars are the decadal total number of lakes that have suffered fish introductions in percentage of the total number of lakes > 0.5 ha of the study area (n=520).

Those lakes that had been stocked before 1900 had significantly higher surface area, shorter walking distance from the nearest town and higher catchment area compared to the lakes stocked after 1900. In contrast, fishless lakes had the highest altitudes, greatest walking distances and lowest temperatures (Fig. A1). Lakes stocked after 1900 had intermediate values for these variables.

Among the different factors explaining trout introductions during the 20th century, there was a clear difference between the first and second half of the 20th century. From 1900–1950, walking distance from the nearest settlement was clearly the dominant factor explaining from 29 to 60% of deviance: the lakes where fish were introduced were those closer to human settlements. This factor was followed in significance by lake surface area (13 – 30% of deviance increase) and lake location and altitude (Fig. A2). In contrast, with the onset of modern fish management, walking distance ceased to be the most explicative factor during the period 1960–2000. Management factors, such as fishing zone or helicopter stocking area (only during the last decade, 1990–2000), and lake characteristics such as lake surface area, altitude and temperature were the most relevant factors. These results indicated that both the management practices and lake characteristics were the most relevant factors for explaining the presence of the fish population in modern fish introductions.

3.2. Factors explaining the present distribution of the three introduced salmonids

Among the three trout species found in south Pyrenean lakes, *S. trutta* is clearly the most widely distributed. It was found in 258 (49.6%) lakes and had a significantly higher chance of being found in the lakes within active fish management areas (χ^2 = 138.7, $p < 0.0001$), in lakes with HEP (χ^2 = 44.7, $p < 0.0001$), with nearby forestry roads (χ^2 = 44.7, $p < 0.0001$) or in helicopter stocking areas (χ^2 = 77.65, $p < 0.0001$). In contrast, the probability of occurrence in lakes within National Parks was not statistically different than outside them (χ^2 = 0.55, $p = 0.457$).

The generalized additive model selected nine of the thirteen predictor variables (helicopter stocking, active fish management, surface area, altitude, location, pH < 5.5, walking distance from nearest settlement, forestry road and HEP) which were significantly associated with the probability of *S. trutta* occurrence (Table A1). The relationship between the probability of *S. trout* occurrence (on a logit scale) and the important continuously distributed predictor variables were all significantly nonlinear (Fig. 3). The response curve describing the estimated effect of lake surface area on the probability of *S. trutta* occurrence (p_i) indicated that p_i was low at the smallest lakes, but increased steadily until ca. 3 ha when it was constant. *S. trutta* had an increasing function of altitude until 2300 m, when it decreased progressively, and it had a constant, decreasing function with walking distance from the nearest town. Categorical variables representing different management practices contributed to approximately half of the deviance explained by the model (28.1%), with morphometric variables representing lake characteristics the other half (23.9%). The response surface for water body location is not provided in this or subsequent figures as it was often complex and offered no additional insights into the effects of the different predictor variables on species occurrence.

O. mykiss was found in 32 (6.2%) of the lakes and had a significantly higher chance of being found in lakes within active fish management areas (χ^2 = 196, $p < 0.0001$), in lakes with HEP (χ^2 = 5.2, p = 0.023), with nearby forestry roads (χ^2 = 43.2, $p < 0.0001$), and within National Parks (χ^2 = 17.9, $p < 0.0001$), but its occurrence was independent of the lake being within helicopter stocking areas (χ^2 = 0.05, p = 0.817). The generalized additive model selected two of the thirteen predictor variables (active fish management area and forestry roads) which were significantly correlated with the probability of *O. mykiss* occurrence (Fig. A3a). Both variables were equally important within the model.

S. fontinalis was found in 33 (6.3%) of the lakes and had a significantly higher chance to be found in lakes within active fish management areas (χ^2 = 9.62, p = 0.002), in lakes with HEP (χ^2 = 22.4, $p < 0.0001$), with nearby forestry roads (χ^2 = 51.3, $p < 0.0001$), and within National Parks (χ^2 = 4.66, p = 0.031), but its occurrence was independent of the lake being within helicopter stocking areas (χ^2 = 0.43, p = 0.518). The generalized additive model selected two of the thirteen predictor variables (to have a forestry road access and to be within an active fish management area) which were significantly correlated with the probability of *S. fontinalis* occurrence (Fig. A3b). Access to a forestry road had a stronger weight in the model explaining 71.3 % of deviance compared to the other significant variable (20.1%).

Eventually, for the three trout species, none of the biological variables (e.g., the occurrence of other trout species) were significant, suggesting that the probability of finding one particular trout species is not directly linked with the previous presence of another species.

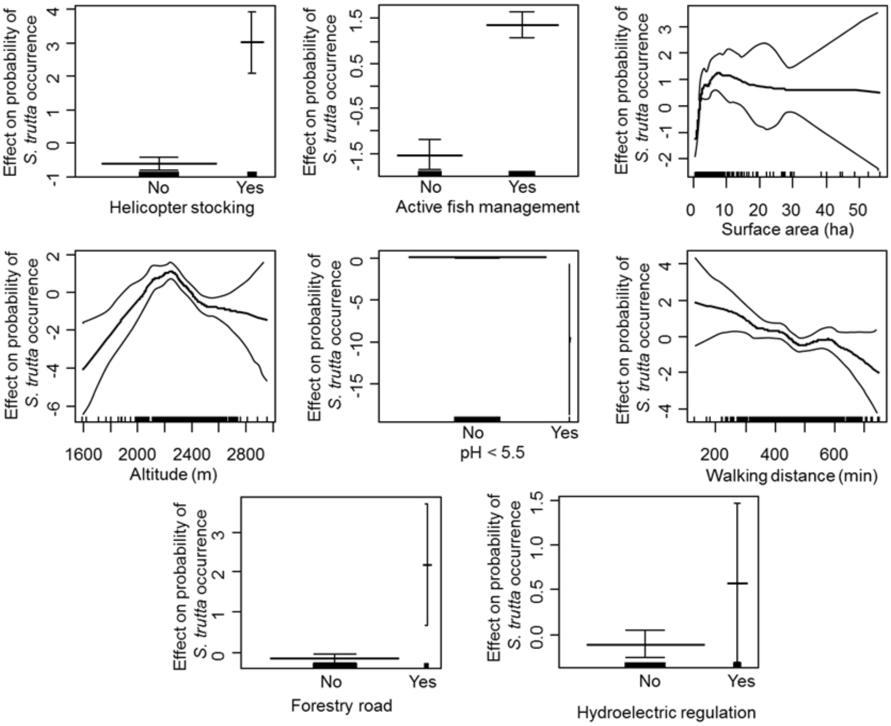

Figure 3. Estimated effect of each of the highly significant (P ≤ 0.01) predictor variables on the probability of occurrence by Salmo trutta, as determined from the generalized additive model (span = 0.5). Response curves are based on partial residuals and are standardized to have an average probability of zero. Thin lines are approximate 95% confidence intervals and hatch marks at the bottom are a descriptor of the frequency of data points along the gradient in continuous variables or within each category for categorical variables. The width of horizontal lines in categorical variables is proportional to the frequency of the data within each category. Numbers in parenthesis are the percentage of explained deviance of each variable. See Table A1 for model details.

3.3. The role of National Parks in the conservation of lakes

In the previous statistical models, National Parks did not appear as a significant variable in explaining the historical changes or the present trout distribution. Therefore, in order to evaluate the particular role of National Parks in lake conservation, we compared the changes in the number of lakes with fish during the twentieth century in Aigüestortes i Estany de Sant Maurici National Park with the other areas, distinguishing the two management areas of the National Park (Fig. 4a). We found that among the three areas, the number of lakes with trout only stopped increasing at the time of the ban (1988) in the area where fishing was prohibited, while at the other areas it continued to increase.

The increase in the number introductions could partially be masked if, at the same time, there were some lakes in which trout populations had gone extinct. Therefore we counted the number of lakes that had been stocked during the 20th century and the number that at present are fishless in the three areas. The percentage of lakes that lost salmonid populations was lowest in the fishing area of the National Park, while it was higher in the area of the National Park where fishing is not allowed (Fig. 4b), although, these differences were not significant in the chi square tests (p >0.1 in all cases). In contrast, the lakes where fish disappeared had a significantly higher altitude and only half of the surface area (Figs. 4c and d respectively), suggesting that the disappearances basically occurred in lakes where populations cannot persist and that had not been re-introduced, independently of the type of protection area. Within the National Park, the slightly higher rate of population disappearances in the fishing-prohibited area was likely due to re-stocking in the fishing-allowed area.

4. Discussion

4.1. First introductions: traditional exploitation of lakes

The first written documents describing the existence of fishing rights in high mountain lakes of the Pyrenees dated back to the 14th and 15th centuries. These initial introductions for traditional exploitation resulted in 26.5% of the lakes having introduced trout by 1900. Similarly, in the northern side of the Pyrenees, it has been described that ca. 25% of the lakes had fish prior to the onset of widespread introductions after 1936

(Delacoste et al. 1997), which might also be attributed to traditional fishing activities by local fishermen. Our findings suggest that human exploitation of some of these lakes might have originated further back in time, possibly back to Neolithic times, when primitive residents already used the high altitude pastures (Miró 2011). However, written evidence found suggest that the first major historical introductions occurred within the medieval warm period (1000–1300 AD) when the human population in the Pyrenees was highest (Miró 2011).

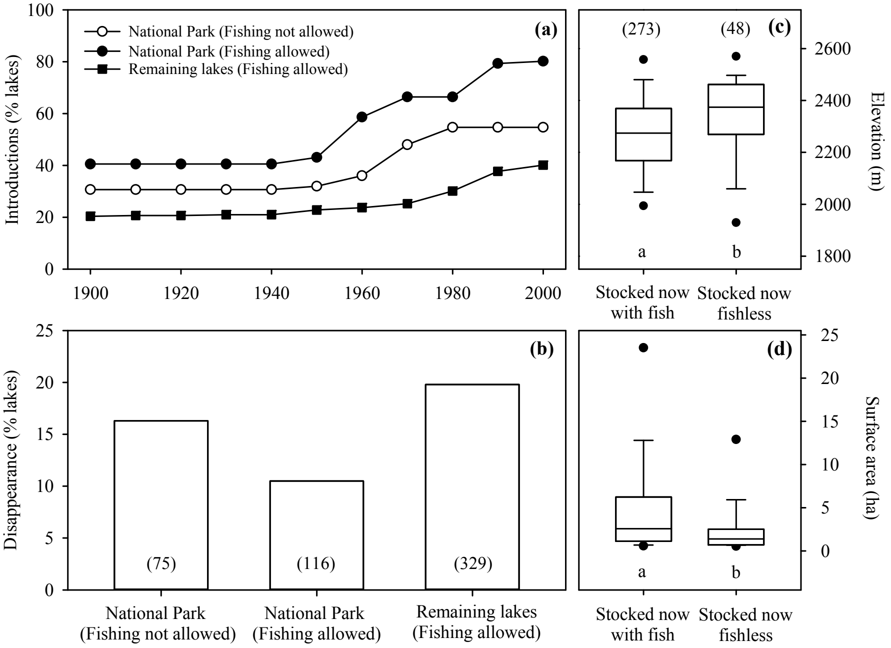

Figure 4. (a) Effect of Aigüestortes i estany de Sant Maurici National Park on the introduction of nonnative trout species in high mountain lakes of the Pyrenees. Circles are the lakes within the National Park, and squares are lakes outside the National Park. White circles are the area of the National Park where fishing is not allowed and black circles are the lakes in fishing allowed areas. (b) Percentage of lakes (respect to the total number of each area, in brackets) in the two management areas of the National Park compared to the rest of the study area. (c and d) Box plots of the altitude and surface area of the lakes that have been stocked with trout after 1900 distinguishing those that are still with fish from those that are now fishless. The lines within each box follow Figure 3. Sample sizes are given in parentheses in (c). Categories with different letters are significantly different at the P = 0.05 level (Mann Whitney U test).

Our results contrast with what has been described from other alpine areas of the world where trout introductions are a relatively recent process. In North America, introductions started in the 19th century with the arrival of European colonists (Schindler 2000, Wiley 2003) similar to most areas of Europe where it was not until the end of the 19th and 20th centuries that most alpine areas were populated with trout (Gliwicz and Rowan 1984, Sostoa and Lobón-Cerviá 1989, Martinez-Solano et al. 2003, Toro et al. 2006). Similarly, in northern European regions with many alpine lakes, such as Scotland or Scandinavia, trout introductions have occurred since the Middle Ages but were mainly at low altitudes, and it was not until the late nineteenth century that introductions to remote high mountain lakes began, mainly related with angling activities (Nilsson 1972, Maitland and Campbell 1992). The only exception is in Tyrol, in the Alps, where there are records of introduction of Arctic char (*Salvelinus alpinus*) at the end of the 15th and beginning of the 16th centuries (Pechlaner 1984). The main difference between the introductions in the Alps and the Pyrenees was that in the Alps the introductions and fishing were performed by the nobility (e.g., King Maximillian I; Pechlaner 1984), while in the Pyrenees the rights of exploiting the lakes were given to local towns in order to keep the population in the area. As a result of this historical process those lakes that had been traditionally exploited for fish were typically those closer to towns, and were also those of higher surface area, lower altitude and bigger catchment size (Figs. 1 and A2).

4.2. Modern introductions and the role of management practices

With the onset of modern fish management that took place during the period 1960–2000, the most significant factors explaining trout distribution were related with management practices. However, different factors were important for the different species. For *S. trutta*, the probability of occurrence was highest in helicopter stocking areas, and in active fish management areas. The repeated use of helicopter stocking in recent decades has resulted in extensive occurrence of trout in these areas and, as a result, in a high percentage of lakes with fish (52.5%). In these areas the inverse relationship between trout presence and altitude is lost. This result is similar to that found in other areas of the world where helicopters and aeroplanes have been used for stocking trout, such as several mountain areas of West USA, where trout is present in ca. 60% of lakes (Bahls 1992).

In contrast to *S. trutta*, *S. fontinalis* was preferentially found in lakes with forestry road access, while for *O. mykiss* it was preferentially found in lakes that had a forestry road access but in areas that active fish management has been carried out. These two last species are present in a much lower number of lakes, and also were introduced mainly between 1960 and 1980 (Fig. 2). Their presence is closely linked with the majority of construction of hydroelectric schemes at high altitudes in the Pyrenees (Catalan et al. 1997). Hydroelectric companies compensated local citizens by developing local fisheries that were used to introduce these two species to the lakes around the area where hydroelectric schemes were developed. The same hydroelectric development was responsible for the construction of forestry roads that were in turn, the main routes serving for introducing these two species. Amongst the species, *S. fontinalis* was mainly introduced in lakes with vehicle access. In the 1980s administrative changes shifted stocking responsibilities to local fishermen's societies together with governmental agencies. This favoured the closure of *S. fontinalis* and *O. mykiss* hatcheries and the consolidation of North and Central European strains of *S. trutta* in official fish hatcheries. As a result, there has been a shift from using local stocks to using races from different parts of central and northern Europe (Araguas et al. 2009).

Modern management practices have therefore resulted in high fish introductions during the last few decades with a maximum during 1990s (Fig. 2). As a result, trout have been introduced in most lakes with higher fishing interest (e.g., lower altitude or bigger surface area). This also explains that fish introductions dropped considerably during 2000.

4.3. Limits to trout survival: the role of lake characteristics

Lake characteristics were the second most important group of variables (altogether explaining 23.9% of deviance; Table A1) after those related with management. Amongst them lake surface area was the most important variable. Similarly, in the Sierra Nevada (USA) Knapp (1996b) also found that those lakes with fish were significantly bigger than those without. The possible role of lake surface area on trout occurrence is likely related with the probability of population persistence. We found that those lakes that lost their fish populations had significantly smaller surface area and higher altitude. Lake surface area and lake depth are highly correlated in alpine lakes due to their common glacial origin (Catalan et al. 2009b). Smaller, and therefore shallower, lakes are those where ice-cover thickness (e.g., 2–4 m, typical of these lakes; Ventura et al. 2000) can reach lake bottom or close to it, and therefore kill all fish either directly due to disappearance of water or indirectly through disappearance of oxygenated layers below the ice-cover. The second most important factor is lake altitude, strongly correlated with lake temperature in alpine areas (Thompson et al. 2009). However, temperature itself does not seem to be the main factor under altitude, since we explicitly incorporated the accumulated summer temperature (Table 1) in the model and this variable was not selected in the analysis. It is possible that the fraction of deviance explained by altitude is more related with lake characteristics, such as benthic substrate composition, or the probability of finding inlet streams, both needed for trout reproduction and therefore population persistence (Wiley 2003).

The presence of lakes with pH <5.5 also explained a significant proportion of deviance of *S. trutta*, being the probability of occurrence positively associated with pH. This species cannot survive below this pH (Rosseland et al. 1999/2000). The Pyrenees has some lakes with such a naturally low pH occurring due to substrate geology (Casals-Carrasco et al. 2009), where although they have been stocked in the past, we do not find the presence of trout in any of them. Other trout species such as *S. fontinalis* potentially would be able to survive in some of these lower pH lakes (Korsu et al. 2007, Westley and Fleming 2011). However, the history of *S. fontinalis* introduction was closely linked with hydroelectric exploitations (see Section 4.2), and therefore the species has never been introduced in these lakes.

4.4. The role of National Parks

In this study we have found no significant effect of National Parks in the occurrence of *S. trutta*, and surprisingly the probability of finding *O. mykiss* and *S. fontinalis* was higher inside than outside National Parks. This finding contrasts with previous studies where the probability of finding trout is lower in National Parks where fishing is forbidden (Knapp 1996b, Wiley 2003). For the two species with higher occurrences inside the National Park, this apparent contradiction is likely due to the higher abundance of hydroelectric exploitation inside the Park. For these two species the presence of forestry roads (build almost exclusively during lake impoundments) is one of the two most important factors explaining their distribution. In addition, before prohibition of fishing in the National Park, these areas were included within normal fish management areas, and therefore National Parks before the 1980s, had the coincidence of the two most relevant variables for explaining the occurrence of these two species.

A closer examination of the number of lakes with fish in Aigüesteortes i Estany de Sant Maurici National Park compared to the rest of the area (Fig. 4a) showed that in the area of the National Park where fishing is forbidden, the number of lakes with trout stopped increasing just at the time of the prohibition, while at the other areas, it continued to increase. This finding illustrates that the only management practice that had an effect in the protection of lakes is the prohibition of fishing. Although the most obvious route to avoid introductions would be for governmental agencies to stop stocking trout, this is complicated by the fact that through the history of trout introductions in the Pyrenees there have been several other agents involved in these introductions in addition to governmental agencies (e.g., local citizens in the past or local fishermen's societies in the last decades). As a result we have found some lakes that have been stocked recently without the collaboration of the administration.

The social implications of prohibiting fishing would not be relevant if the prohibition is implemented in those lakes that are at present fishless (at the moment there is the paradox that some lakes have fishing rights while never being stocked with fish). It could also be implemented in some particular lakes or groups of lakes that are especially important for the conservation of some animal groups (e.g., Pyrenean newt *Calotriton asper*). Finally, prohibition on fishing has already been performed in the Aigüestortes i estany de Sant Maurici National Park, with no documented adverse social impact: basically local citizens realized that the social benefits of nature preservation (through higher tourism) have been greater than those that were obtained via fishing.

Although the purpose of this paper was not to explicitly quantify the relative proportion of established (self-sustainable) populations, our results suggest that there is a high proportion of the populations that are established. This is obvious with the historical introductions prior to 1900 (the lakes still have trout at present) but less clear with the introductions that occurred along the twentieth century. By comparing the data of the recorded introductions with the persistence of populations at the year 2000, we were able to quantify the number of lakes where trout populations have not been able to survive. We found a small percentage of stocked lakes where trout disappeared (Fig. 4b). As we discussed in Section

4.3. the disappearance was not related with any of the protection figures, but with physical lake characteristics, such as higher altitudes and smaller surface areas (Fig. 4c and d). Moreover, previous studies have suggested that fish naturalisation is relatively infrequent, due to the requirement of stream inlets connected with the lakes (Lek et al. 1996). It is also possible that we did not find a higher reduction of trout disappearances in the non-fishing area of the National Park due to the relatively recent date of fishing prohibition. Previous studies have described that it takes several decades (between 20 and 30 years, but in some circumstances even 40) before populations disappear from the lakes where they have been introduced and that have unsuitable characteristics for trout populations to persist (Knapp et al. 2005). We would expect, therefore, that in the next few decades there will be a higher increase of fish disappearances in the protected area of the National Park.

5. Conclusion

The results found in this study, together with those of other areas (Knapp 1996b, Wiley 2003), show that when fishing or fish stocking is forbidden, the number of lakes with trout stop increasing and tend to decrease. In contrast, in National Parks where fishing and stocking are allowed, the number of lakes with trout increase until nearly all have fish (fishing allowed area of Aiguestortes i estany de Sant Maurici National Park, Fig. 4; and National Park of Pyrénées; Delacoste et al. 1997). In this latter case the conservation status of those animal groups affected by fish introductions will be affected.

Acknowledgments

The authors would like to acknowledge all those people we interviewed or who provided information on the history of trout introductions in the Pyrenees, but especially Carme Maria Marugan, Miguel Alonso, Òscar Arribas, Joseph Jourdane, Agustí Esteban, Josep Maria Bringué, Maria Àngels Sanllehy, Maria Pau Gómez, Àngel Ferrer, Josep Antoni Luque, Rosendo Vila and Xuan Vila. David O'Brien assisted in editing the manuscript. Three anonymous referees are acknowledged by their helpful comments. Economic support was provided by the Spanish Government projects Fundalzoo (CGL2010–14841) and Invasivefish (427/2011).

Appendix Chapter 1

Supplementary methods, tables and figures

1. Materials and Methods

1.1. Additional description of the study area

The studied lakes range in altitude between 1600 and 2960 m with the highest frequency found at ca. 2400 m and are relatively small and deep (average surface area of 2 ha and average maximum depth of 17 m, with the largest of 54 ha and deepest 105 m). Due to their common origin, they have a close positive relationship between surface area and maximum depth (Catalan et al. 2009b). Most of the lakes are above the tree line with catchments partially covered by meadows, although some of them are within or below the tree line. Approximately half of the lakes have catchments on granodiorite bedrocks, the remaining being located in catchments with metamorphic (25%), detrital (15%) or carbonate (10%) bedrock with a minority with Silurian slate (Casals-Carrasco et al. 2009). One part of the latter bedrocks, due to their high sulphate content, give natural acidity to the training waters (pH < 5.5). The ionic content is generally low, being the chemical variability mostly related with bedrock composition (Catalan et al. 1993). The lakes have low phosphorous and chlorophyll content being of oligotrophic nature (Buchaca and Catalan 2007). A general description of the climate, physic-chemical and biological characteristics of Pyrenean lakes and their comparison with other European high mountain lakes can be found elsewhere (Camarero et al. 2009, Catalan et al. 2009a, Kernan et al. 2009a, Thompson et al. 2009).

1.2. Detailed description of the data collection and environmental variables

Altitude and surface area were obtained from a GIS generated from 1:25000 maps from the various national agencies (Casals-Carrasco et al. 2009). Water temperature was the accumulated degree days (ADD) during the ice-free period calculated from daily max-min temperature data using the sine-wave method, assuming the trigonometric sine curve as an approximation of the diurnal temperature curve and adding the area under the curve and above lower threshold of each day (Baskerville and Emin

1969). We used the lower developmental temperature of 7.8°C as the lower threshold below which salmonid reproduction is not feasible (Elliott et al. 1995). Daily water temperatures were obtained from 45 automatic thermometers (Vemco Minilog-T) deployed at a depth of ca. 1m in lakes covering a wide range of altitudes, surface areas and catchment sizes. This ice-free accumulated temperature was assumed to be representative of the epilimnion (fish mainly feed and spend their time in this upper layer of lakes during the ice-free period) following previous limnological knowledge of these lakes and calibration measurements performed seasonally at different depths. The calculated ADDs from the lakes with automatic thermometers were extrapolated to all other lakes using a regression model between ADD and various morphometric parameters. This was possible since the temperature variability in alpine areas follows a close relationship with altitude and also with lake size and water renewal time among other variables (Thompson et al. 2009). We chose this approach rather than assuming that altitude was the parameter mostly explaining differences in water temperature for getting a closer estimation of the differences in water temperature among this big subset of lakes covering a wide range of altitudes and morphometric variables. We introduced the measured ADD from the 45 lakes as the dependent variable in a multiple linear regression model and chose the minimum combination of morphometric variables that described most of the variance with stepwise forward selection procedure obtaining the following model:

$$\mathrm{DegreeDays}(>7.8°\mathrm{C}) = 4410.4874 - 1.39\ \mathrm{ALT} - 21.73\ \mathrm{SURF} - 117.72\ \ln(\mathrm{CS}/\mathrm{SURF}) - 0.08\ \mathrm{CS} - 53.71\ \ln(\mathrm{DC}/\mathrm{CS}) \qquad (1)$$

$R^2 = 0.833$; $F_{5,\,20} = 19.9$, $P < 0.0001$

Where ALT and SURF are lake altitude and surface area (Table 1), CS is the lake total catchment size and DC is the lake direct catchment size (i.e. the proportion of catchment not shared with other lakes that are upstream of the lake). This latter parameter equals CS when there are no lakes upstream and the ratio DC:CS is relevant since the presence of other lakes in the catchment changes the temperature of the streams flowing out of them. The ratio CS:SURF is indicative of the lake renewal time.

When using pH as an environmental variable, we used a binary variable which grouped lakes with pH<5.5, as below this pH fish are known not to be able to survive (Rosseland et al. 1999/2000). In the Pyrenees, lakes with such low pH are naturally found in the areas underlain with Silurian slate. These rocks contain a high proportion of sulphates that give this natural acidity. The occurrence of these lakes was found by combining a lake lithological classification (Casals-Carrasco et al. 2009) with the validation of field measurements of pH.

Among the factors indicating the mode of fish introductions we estimated the walking distance from the nearest town (WALK; in minutes) which could be a good predictor of the likelihood of a lake to have fish introduced, especially for the period prior to 1900, when all introductions are believed to have been undertaken by local citizens on foot. The walking distance is largely a function of two variables, the difference in height from nearest town and the lake, and the distance. To estimate it, we chose a representative subset of lakes (ca. 70 lakes from different areas) for which we quantified the walking time from the nearest town by climbing ourselves to each of these lakes. Then we regressed the walking time with two predictor variables: the altitudinal difference between the lake and the town (ALTDIFF; in meters) and the linear distance between them (DIST; in meters), both obtained from a GIS and 1:25.000 cartographic maps. Since climbing uphill takes more time than downhill, we used two regressions, one for uphill (UPWALK) and the other for downhill (DOWNWALK):

$$\text{UPWALK} = 0.157\text{ALTDIFF} + 0.008\text{DIST} - 4.279 \tag{2}$$

$R^2=0.942$; $F_{2,72}=563.6$; $P<0.001$

$$\text{DOWNWALK} = 0.078\text{ALTDIFF} + 0.013\ \text{DIST} - 5.732 \tag{3}$$

$R^2=0.885$; $F_{2,69}=258.9$; $P<0.001$

The total walking distance was then obtained by adding UPWALK with DOWNWALK. We also used the size of the nearest town and the number of hotel beds at the time of the fish introduction obtained from national historical inventory surveys with the hypotheses that the bigger the town, the higher the probability of having people interested in introducing fish or that those towns with higher number of beds would have more tourists and that this therefore might increase the pressure for introducing fish. We also recoded if there was helicopter fish stocking in the area nearby to the lake during a certain period or if the lake had a forestry road giving car access to the lake. The management practice variables considered were whether lakes belonged to National Parks, whether the location was within an area with active fish management or if the lake had water fluctuations for hydroelectric production (HEP). Within the study area there were two National Parks: Valle de Ordesa y Monte Perdido National Park, that due to its carbonate lithology has very few lakes (2% of the total) where fishing is allowed. In addition, Aigüestortes i Estany de Sant Maurici National Park has 37% of the total lakes under two different management regimes: a core area with strict regulations where fishing has been banned since 1988 and a peripheral area where fishing is allowed. For evaluating the effectiveness of the two management strategies within National Parks, we only used the data from Aigüestortes i Estany de Sant Maurici National Park (NATP) and did not take into account the other National Park. Hydroelectric production (HEP) is not only relevant for the potential direct effect of changing water levels on fish breeding, but also because HEP companies performed fish introductions in order to compensate local towns (owners of the historical rights of exploitation of lakes) from the potential negative effects of the water level fluctuations. Finally we also used the presence of other trout species as a categorical variable to explore potential interferences and relationships among fish species.

1.3. Statistical analyses

Prior to analyses with the generalised additive models, we tested for collinearity among predictor variables by Pearson correlation coefficients (r) for all pair-wise combinations of continuous predictor variables. Correlation coefficients ranged between –0.71 and 0.60 (Table A2) and were well below the suggested cut-off of $|r| \geq 0.85$ that would indicate collinearity for the sample size used in these analyses (Berry and Felman, 1985 in Knapp 2005). Therefore, all predictor variables were included initially in the regression models. In the regression models, p_i is the probability of finding the species at location *i*, and is defined as

$$p_i = \frac{e^{\theta_i}}{1+e^{\theta_i}},$$

Where the linear predictor (i.e., logit line) ϑ_i is a function of the independent variables. For all fish species, the specific relationship we used for ϑ was the following function of covariates:

$$\vartheta = lo(ALT) + lo(SURF) + lo(LOCATION) + lo(ADD) + pH + lo(WALK) + lo(POP) + lo(HOT) + HEL + FROAD + FIZ + NATP + HEP + STRU + SFON + OMYK. \quad (4)$$

Where lo(·) represents a nonparametric loess smoothing function that characterizes the effect of each continuous independent variable on p_i. The location covariate lo(LOCATION) was a smooth surface of UTM easting and northing (see Table 1 for variable abbreviations).

From Eq. (4) we selected a subset of significant variables explaining the greatest proportion of deviance. The best combination of independent variables was selected by stepwise forward selection using AIC criteria. The use of this procedure ensured that at each selection step only those variables explaining a significant proportion of previously non-explained variance would be selected. In other words, while altitude and temperature (ADD) and surface area share a significant amount of variance, their inclusion in the model would only take place if each variable would explain a fraction of variance not explained by the others. The proportion of variance explained by each variable was determined by evaluating the change in deviance resulting from dropping each variable from the model in the presence of all other variables. Analysis of deviance and likelihood ratio tests (based on the binomial distribution) were used to test the significance of the effect of each predictor variable on the probability of occurrence by each fish species. Because the large sample sizes used in the regression models could result in predictor variables being statistically significant despite very weak associations with species presence/absence, predictor variables were considered to have significant effects only when $P \leq 0.01$.

For all regression models, the relationship between the significant predictor variables and the probability of species occurrence is shown graphically in separate plots separating the unshared fraction of variance that each factor explains. Each plot depicts a response curve that describes the contribution of the predictor variable to the logit line. More generally, the response curve shows the relative influence of the predictor variable on the probability of species occurrence. This response curve is based on partial residuals, is plotted on a log-scale, and is standardized to have an average value of 0. For example, a hump shaped response curve for the predictor variable elevation indicates that the fish species was, in a relative sense, most likely to be detected at sites at intermediate elevations and less likely to be detected at sites of both low and high elevations (Knapp et al. 2003). All regression-related calculations were conducted using *R* (R Development Core Team 2010) using the function library *gam*.

Table A1. Results of generalized additive models developed for each fish species introduced in the Pyrenean lakes. Deviance increase means the increase in deviance resulting from dropping the selected variable from the model. The percentage increase is given in parentheses, and was calculated as (deviance increase/(null deviance-model deviance[a]))x100 (Knapp 2005). Asterisks indicate the level of statistical significance associated with each variable: * P ≤ 0.01 and P > 0.001,.** P ≤ 0.001, *** P ≤ 0.0001 and NS not significant (P > 0.01). [a]Sometimes referred to as ''residual'' deviance.

Parameter	Species					
	S. trutta		*O. mykiss*		*S. fontinalis*	
Null deviance	721		240		246	
Degrees of freedom (null model)	519		519		519	
Model deviance[a]	345		197		210	
Degrees of freedom (full model)	495		517		517	
Explained deviance (% of total)	52		18		15	
Deviance increase						
Helicopter stocking	63.6	(16.9)***		NS		NS
Active fish management	42.0	(11.2)***	19.1	(43.8)***	7.2	(20.1)**
Surface area	38.8	(10.3)*		NS		NS
Altitude	32.3	(8.6)***		NS		NS
Location	19.9	(5.3)*		NS		NS
pH < 5.5	18.9	(5.0)**		NS		NS
Walking distance	11.2	(3.0)***		NS		NS
Forestry road	11.3	(3.0)***	20.8	(47.5)***	25.6	(71.3)***
Hydroelectrical Power	1.4	(0.4)***		NS		NS

Table A2. Pearson correlation among continuous variables. *denote correlations significantly different from zero (P < 0.05).

	SURF	ADD	WALK	POP	HOT
ALT	-0.125*	-0.715*	0.605*	-0.128*	-0.063
SURF		0.313*	-0.090*	-0.020*	-0.042
ADD			-0.407*	0.124*	0.001
WALK				0.118*	0.173*
POP					0.519*

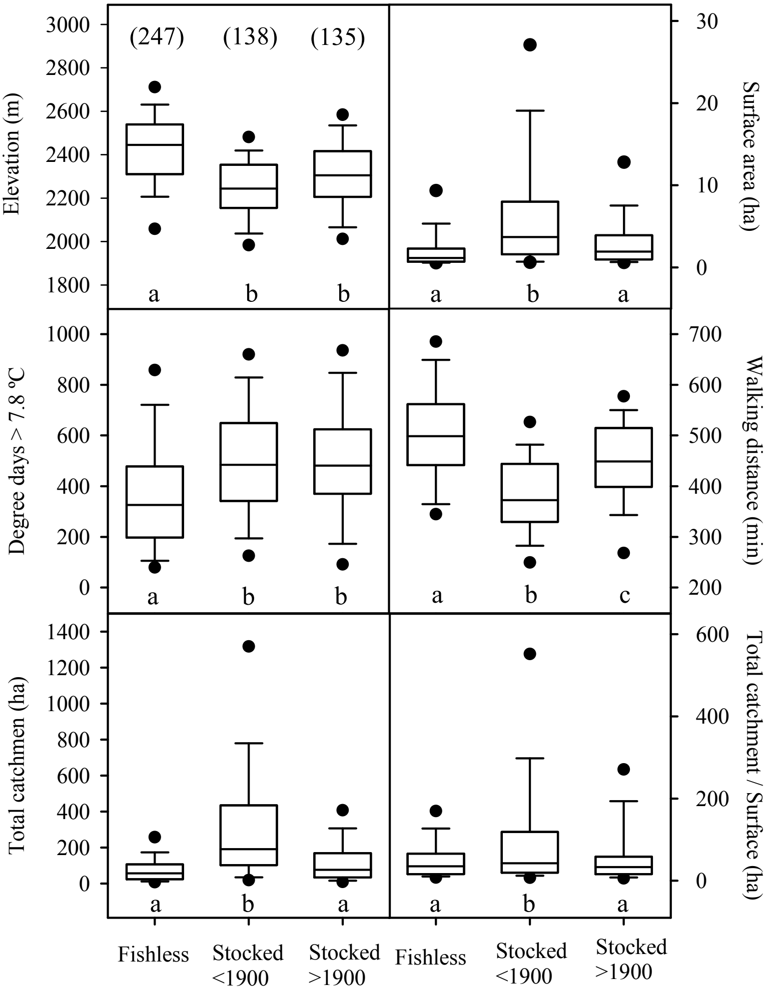

Figure A1. Box plots showing elevation, surface area, accumulated degree days above 7.8 °C, walking distance (in minutes), total catchment, the ratio of total catchment to lake surface area, and the walking distance from the nearest town to each lake, for fishless lakes, stocked <1900 and stocked >1900. The line within each box marks the median, the bottom and top of each box indicate the 25th and 75th percentiles, the whiskers below and above each box indicate the 10th and 90th percentiles, and the points above and below the whiskers indicate the 5th and 95th percentiles. Sample sizes for each category are given between brackets in the first panel. Categories with different letters are significantly different at the p = 0.05 level (One way ANOVA, Tukey Post-hoc).

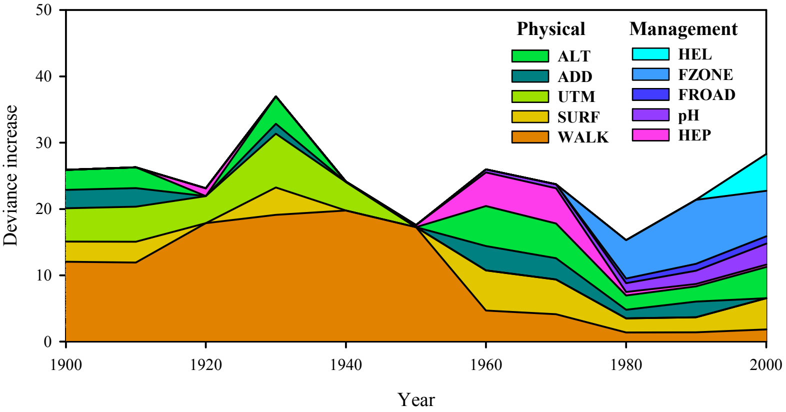

Figure A2. Relative importance of the variables affecting salmonid introductions in the southern Pyrenean lakes during the last century. Only the deviance increase explained by each variable and for each decade is plotted.

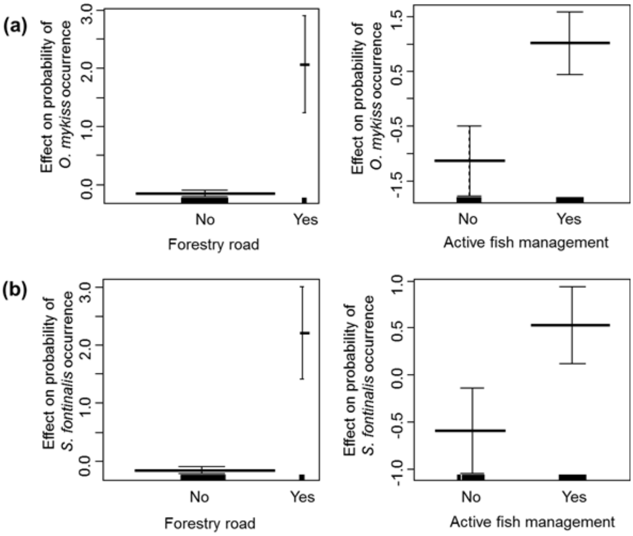

Figure A3. Estimated effect of each of the highly significant ($p \leq 0.01$) predictor variables on the probability of occurrence of (a) *Onchorynchus mykiss* and (b) *Salvelinus fontinalis*, as determined from the generalized additive model. Thin lines are approximate 95% confidence intervals and hatch marks at the bottom are a descriptor of the frequency of data points within each category. The width of horizontal lines is proportional to the frequency of the data within each category. Numbers in parenthesis are the percentage of explained deviance of each variable. See Table A1 for model details

Chapter 2

Evidence of exotic trout mediated minnow invasion in Pyrenean high mountain lakes[3]

Abstract

Although high mountain lakes are naturally fishless, there have been numerous trout introductions to such ecosystems in many areas of the world with negative ecological consequences. In recent decades other fishes, such as minnows, have been introduced to some mountain areas, including the Pyrenees. These introductions may cause further ecological problems, since minnows also occupy the top of the food chain, and are difficult to manage since such introductions occur without permission from the authorities. In this study we have analysed the process of minnow introductions in all high mountain lakes of the southern slope of the Pyrenees to find out which particular factors best explained their present distribution and to evaluate which management measures have been most effective for stopping introductions. We found 27 % of the lakes had minnows (*Phoxinus* sp.) present, 52 % had trout and 47 % were fishless. Trout presence was the most significant variable explaining 27 % of deviance of minnow presence data in a generalized additive model. Recreational fishing using minnows as live-bait is likely responsible for these introductions. Minnow introductions are therefore mediated by a preceding invasive species and facilitated by human activity. We also compared the number of minnow introductions in non-fishing areas of

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[3] Miró, A., and M. Ventura. 2015. Evidence of exotic trout mediated minnow invasion in Pyrenean high mountain lakes. Biological Invasions 17:791-803.

National Parks with other areas where managed fishing takes place. We found that the number of lakes with minnow introductions was increasing in all areas except those where fishing was prohibited, indicating that prohibiting fishing is an effective management practice for stopping minnow introductions.

Keywords: *Phoxinus phoxinus*, Minnow, Invasive species, National Park, Lake conservation, High mountain lakes.

1. Introduction

Invasions by human-introduced non-indigenous species are one of the main threats to biodiversity. They have been identified as the main cause of extinction of wildlife in many occasions, but also as a potential phenomenon generating evolutionary stress and moreover causing biotic community homogenization (Clavero and García-Berthou 2005, Lockwood et al. 2013, Marr et al. 2013, Simberloff et al. 2013). Freshwater fishes are one of the animal groups with a high number of invasive species (Hulme et al. 2009). Ecological impact of invasive species in aquatic environments has been described as severe when they become top predators (Vitule et al. 2009), since in addition to decreasing the relative abundances or completely eradicating their prey, they also alter the trophic relationships inducing trophic cascades to the base of the food web (e.g. Byström et al. 2007, Wahl et al. 2011). Furthermore, the introduction of multiple predator species can have cumulative effects on the receiving ecosystems (Nyström et al. 2001).

Angling practices with live-bait represents a worrying pathway for alien species introductions (Kerr et al. 2005, Webb 2007, DiStefano et al. 2009, Ward et al. 2012). The largest organisms used as live-bait are several species of small fishes, but other animals such as amphibians, earthworms, crayfishes, grubs and insects are also used (Lindgren 2006, Keller and Lodge 2007). Improper disposal of live-bait has been attributed as the source of introduction of at least 14 species of fishes in Ontario (Kerr et al. 2005). In the English Lake District, individuals of at least 12 native and non-native fish species have been brought to Windermere for the purpose of live-baiting (Winfield et al. 2011). Live-bait use was also responsible for the introduction of 47 known freshwater species in United States Mid-Atlantic slope drainage systems, among which are at least 5 non-native fishes, 4 non-native crayfishes and 9 non-native earthworm species (Kilian et al. 2012). Live-baits may arrive in the environment by accidental escape or more often, by being released into the water at the end of the fishing trip (Winfield et al. 2011, Kilian et al. 2012). In some cases, it has been shown that bait-related introductions have resulted in established populations of invasive species (e.g. Callaham et al. 2006, Migge-Kleian et al. 2006).

The use of small fishes as live-bait, mainly used to catch salmonids, has previously been reported in boreal and mountain lakes. For example, during the second half of the twentieth century, Scottish lochs have received, at least 7 non-native species used as live-bait, minnows among them (Maitland and Campbell 1992). Also, minnows have expanded outside their native distribution in Scandinavia since the beginning of the twentieth century, mainly in mountain areas and in association with angling practices (Museth et al. 2007). However, invasions linked with released live-bait have not been very well described in high mountain watersheds so far.

High mountain lakes are originally fishless ecosystems due to natural barriers that have prevented the natural colonization of fish species from lower streams or dispersal among interconnected lakes (Knapp et al. 2001a, Pister 2001). However, the Pyrenees like many other high mountain areas of the world nowadays have introduced trout in more than half of the lakes (Miró and Ventura 2013). These introductions first took place in lower altitude lakes (*ca*. 25% of the lakes) historically associated with traditional fish exploitation dating back at least to the 14th century. More recently, during the second half of the twentieth century, introductions have taken place in another 25% of the lakes, mainly for recreational fishing purposes and promoted by local fishermen associations, environmental administrative offices and hydroelectrical power companies (as compensatory actions) (Miró and Ventura 2013).

Complementary to the spread of trout, minnows have also been detected in many Pyrenean lakes during the last few decades (Miró 2011). Although minnows apparently are used as live-bait for trout angling, this type of angling is forbidden in the southern part of the Pyrenees since 1966. The illegal introduction of minnows, therefore, occurs without the control of public authorities and generates a challenge for resource managers tasked with the conservation of high mountain lake ecosystems. Within the Pyrenees, there are areas with different degrees of protection (*e.g.*

National Parks where fishing is prohibited and other areas where fish management takes place) that offer an interesting opportunity to explore how different fish management practices (authorized or prohibited trout angling) have affected the spread of minnows.

The objective of this study was to understand the distribution and spread of minnows in high mountain lakes of the southern slope of the Pyrenees, to find out which particular factors, either environmental or anthropogenic, best explained their present distribution and to evaluate which conservation measures have been most effective. We expected to confirm that successful minnow introductions were closely related with their use as live-bait for trout fishing. In particular, we had the following specific hypotheses: (*i*) we would only find minnows in lakes where trout had been previously introduced; *(ii)* minnow introductions would be more likely in areas where active fish management has taken place; *(iii)* the probability of finding minnows would be higher in lower altitude lakes and in those with larger sizes; and finally *(iv)* fishing prohibition (for trout) in National Parks should result in stopping minnow introductions.

2. Material and methods

2.1. Description of the study area and species

The studied lakes are spread along the Pyrenean mountain range (0°42’W–2°09’E, 42°52’–42°23’N; Fig. 1). From among 1062 lakes > 0.5 ha, we surveyed 520 water bodies which comprise all those lying within the Catalan-Aragoneese (Spanish or southern) Pyrenees. Pyrenean lakes originated from glacial processes or were modified by the activity of quaternary glaciations. The surveyed lakes range in altitude between 1600 and 2960 m with the highest frequency found at ca. 2400 m and are relatively small and deep (average surface area of 2 ha and average maximum depth of 17 m, with the largest of 54 ha and deepest 105 m). Due to their common origin, there is a close positive relationship between surface area and maximum depth (Catalan et al. 2009b). Most of the lakes are above the tree line with catchments partially covered by meadows, although some of them are within or below the tree line. Approximately half of the lakes have catchments on granodiorite bedrock, the remaining being located in catchments with metamorphic (25%), detrital (15%) or carbonate (10%) bedrock with a minority with Silurian slate (Casals-

Carrasco et al. 2009). One part of the latter bedrocks, due to their high sulphate content, give natural acidity to the waters (pH < 5.5).

Within the study area there is a National Park, Aigüestortes i Estany de Sant Maurici National Park which has 37% of the total studied lakes. These lakes are under two different management regimes: a core area with strict regulations where fishing has been prohibited since 1988 (with 75 lakes) and a peripheral area where fishing is allowed (with 116 lakes).

The study species is the European minnow (*Phoxinus phoxinus* (L. 1758)), that was considered a single species until recently, and therefore most citations were referred as *P. phoxinus.* A recent taxonomical revision has distinguished various species: *P. phoxinus* sensu stricto in the northern (French) Pyrenean slope, *Phoxinus bigerri* Kottelat, 2007 native in the north and south-western Pyrenean streams, and *Phoxinus septimaniae* Kottelat, 2007 native in the north-eastern Pyrenean streams (Kottelat 2007). During summer of 2013 we were able to examine the species present in fifteen lakes of both north and south slopes of Pyrenees, and only found *P. phoxinus* sensu stricto. To avoid taxonomical confusion, in this paper we use the term minnow to refer to *Phoxinus* sp. found in the Pyrenean lakes.

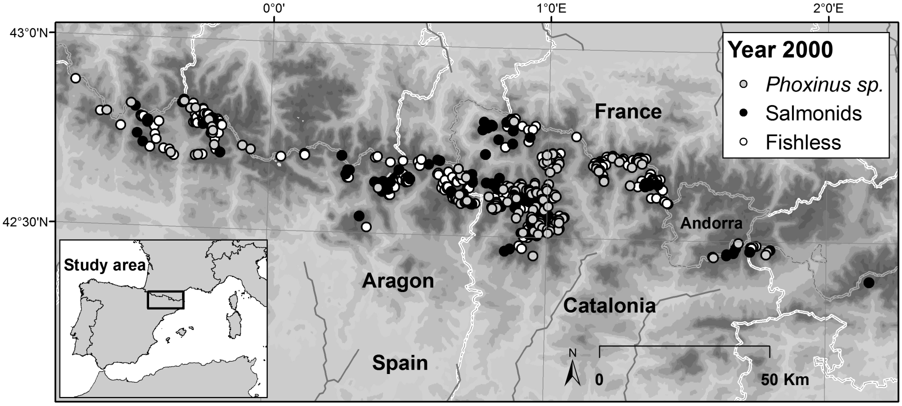

Figure 1. Distribution of the 520 studied lakes along the southern Pyrenean range and fish presence at year 2000. Minnows (*Phoxinus* sp.) usually share the lake with trout or are alone in some cases; see Results section to more details.

2.2 Data collection and environmental variables

We collected 435 citations of minnow presence and 379 of minnow absence in the study area from interviews with local elderly fishermen or nature reserve wardens, from local reports of fishing or walking societies and from our own littoral's visual encounter surveys. In fifteen lakes, we also used minnow traps to validate the information from the interviews, confirming all cases. Historical information of minnow absence was also obtained from historical documents from local, regional and national archives. The collected data from the different complementary sources covered the whole twentieth century and allowed us to reconstruct the decade when minnows arrived to each lake. We chose this method instead of only using our own surveys, since our aim was to provide a historical perspective of minnow introductions and to be able to provide an objective account of the main factors responsible for these introductions.

To detect which factors were associated for minnow introductions, we generated a set of environmental variables summarizing both physical lake characteristics and anthropogenic factors that might have affected the distribution of minnows (Table 1). Altitude and surface area were obtained from a GIS generated from 1:25000 maps from the Spanish, Aragonese an Catalan geographical agencies (Casals-Carrasco et al. 2009). A binary factor of connectivity (CONN) was included to assess the possible natural dispersion of minnows through streams or their secondary spread after introduction in one lake, coding the presence/absence for a given lake of a stream connection with another lake with an established minnow population. Water temperature was the accumulated degree days (ADD) during the ice-free period calculated from daily max-min temperature data using the sine-wave method, assuming the trigonometric sine curve as an approximation of the diurnal temperature curve and adding the area under the curve and above the lower threshold for each day (Baskerville and Emin 1969). As a lower threshold we used 7.8°C, the lower developmental temperature below which *Salmo trutta* reproduction is not feasible (Elliott et al. 1995). Daily water temperatures were obtained from 27 automatic thermometers (Vemco Minilog-T) deployed at a depth of *ca*. 1m in lakes covering a wide range of altitudes, surface areas >0.5 ha and catchment sizes from 2008 to 2009. The calculated ADDs from those lakes with automatic thermometers were extrapolated to all other lakes using a multiple linear regression model between ADD and various morphometric parameters and choosing the minimum combination of morphometric variables that described most of the variance with stepwise forward selection procedure. The following model obtained was:

$$\mathrm{DegreeDays}(>7.8°\mathrm{C}) = 4410.4874 - 1.39\ \mathrm{ALT} - 21.73\ \mathrm{SURF} - 117.72\ \ln(\mathrm{CS}/\mathrm{SURF}) - 0.08\ \mathrm{CS} - 53.71\ \ln(\mathrm{DC}/\mathrm{CS}) \quad (1)$$

$R^2 = 0.833$; $F_{5,\ 20} = 19.9$, $P < 0.0001$

Where ALT and SURF are lake altitude and surface area (Table 1), CS is the lake total catchment size and DC is the lake direct catchment size (i.e. the proportion of catchment not shared with other lakes that are upstream of the lake). This latter parameter equals CS when there are no lakes upstream and the ratio DC:CS is relevant since the presence of other lakes in the catchment changes the temperature of the streams flowing out of them. The ratio CS:SURF is indicative of the rate of water renewal.

When using pH as an environmental variable, we used a binary variable which separated out lakes with pH<5.5, as below this pH fish are known to be unable to survive (Rosseland et al. 1999/2000).

Among the factors indicating the mode of fish introductions, we estimated the walking effort from the nearest town (WALK; in minutes) which could be a good predictor of the likelihood of a lake having fish introduced. To estimate it, we chose a representative subset of 72 lakes from different areas, for which we quantified the walking time from the nearest town by ourselves climbing to each of these lakes. Then we regressed the walking time with two predictor variables: the altitudinal difference between the lake and the town (ALTDIFF; in meters) and the linear distance between them (DIST; in meters), both obtained from a GIS and 1:25000 cartographic maps. Since climbing uphill takes more time than downhill, we used two regressions, one for uphill (UPWALK) and the other for downhill (DOWNWALK):

$$\mathrm{UPWALK} = -4.279 + 0.157\mathrm{ALTDIFF} + 0.008\mathrm{DIST} \quad (2)$$

$R^2 = 0.942$; $F_{2,72} = 563.6$; $P < 0.001$

$$\mathrm{DOWNWALK} = -5.732 + 0.078\mathrm{ALTDIFF} + 0.013\ \mathrm{DIST} \quad (3)$$

$R^2 = 0.885$; $F_{2,69} = 258.9$; $P < 0.001$

Table 1 Description of predictor variables used in the generalized additive models

Variable type	Variable name	Description
Physical and chemical	Altitude (ALT)	Elevation of the lake (m)
	Surface area (SURF)	Surface of the lake (ha)
	Water body location (LOCATION)	Latitude and longitude of the lake, UTM reference system
	Connectivity (CONN)	Binary factor that has a value of 1 in each lake that has a direct stream connection without any natural barrier with another lake with an established minnow population.
	Accumulated degree days (ADD)	Thermal accumulation in degree days > 7.8°C (°C day)
	pH<5.5 (pH)	Binary factor indicating lakes with pH < 5.5
Mode of introduction	Walking Effort (WALK)	Walking effort from the nearest town (minutes)
	Population (POP)	Inhabitants of the nearest town to the lake
	Hotel beds (HOT)	Number of hotel beds in the nearest town to the lake
	Helicopter stocking (HEL)	Binary factor determined by the existence of helicopter stocking in the lake
	Forestry road (FROAD)	Binary factor marking the lakes with forestry road access
Management practice	Fishing zone (FIZ)	Binary factor determined by the existence of active fish management, at present or in the past
	National Park (NATP)	Binary factor indicating the lakes which belonging to the part of the National Park where fishing is prohibited at present
	Hydroelectrical power (HEP)	Binary factor marking the lakes with water level regulation
Trout facilitation	Trout presence (TROUT)	Binary factor determined by trout presence in the lake

The total walking effort was then obtained by adding UPWALK with DOWNWALK. We also used the size of the nearest town (POP) and the number of hotel beds (HOT) at the time of the minnow introduction, both obtained from national twentieth century historical inventory surveys. We also recoded if there was helicopter stocking of trout (HEL) in the area nearby to the lake during a certain period (1970s – 2000s) or if the lake had a forestry road giving car access to the lake (FROAD). The management practice variables considered were whether the location was within an area with active fish management (FIZ), whether lakes belonged to fishing prohibited areas of National Park of Aigüestortes i Estany de Sant Maurici (NATP) or if the lake had water fluctuations for hydroelectric production (HEP). Hydroelectric production (HEP) is not only relevant for the potential direct effect of changing water levels on minnow breeding, but also because HEP companies performed trout introductions in order to compensate local towns from the potential negative effects of the impounding. Finally we also used the presence of trout species (TROUT) as a categorical variable to explore potential interferences and relationships between minnow and trout species.

2.3. Statistical analyses

First of all we calculated the introduction rate of minnow and trout for the twentieth century by dividing the number of lakes where they have been introduced by the period in years that the introductions lasted. In the case of trout we only included the modern trout introductions that took place during the second half of the twentieth century to compare the rate with those of minnows. Then, we used generalized additive models (GAMs) as statistical analyses to compare the data on the presence/absence of minnows in the Pyrenean lakes with the different environmental variables described above. We used GAMs for the analyses because they are similar to generalized linear models, but distend the assumption that the relationships between the dependent variable (when transformed to a logit scale) and predictor variables are linear by estimating a nonparametric loess smooth function for each continuous predictor variable (Hastie and Tibshirani 1990, Knapp 2005). Prior to analyses with the GAMs, we tested for collinearity among the predictor variables by Pearson correlation coefficients (*r*) for all pair-wise combinations of continuous predictor variables. The strongest correlation coefficient was |0.72|, below the suggested cut-off of $|r| \geq 0.85$ that would indicate collinearity for the sample size used in these analyses (Berry and Felman 1985). Therefore, all predictor variables were included initially in the regression models. In the regression models, p_i is the probability of finding the species at location *i*, and is defined as:

$$p_i = \frac{e^{\theta_i}}{1 + e^{\theta_i}},$$

Where the linear predictor (i.e., logit line) ϑ_i is a function of the independent variables. For minnow, the specific relationship we used for ϑ was the following function of covariates:

$$\vartheta = \mathrm{lo(ALT)} + \mathrm{lo(SURF)} + \mathrm{lo(LOCATION)} + \mathrm{CONN} + \mathrm{lo(ADD)} + \mathrm{pH} + \mathrm{lo(WALK)} + \mathrm{lo(POP)} + \mathrm{lo(HOT)} + \mathrm{HEL} + \mathrm{FROAD} + \mathrm{FIZ} + \mathrm{NATP} + \mathrm{HEP} + \mathrm{SALM} \tag{4}$$

Where lo(·) represents a nonparametric loess smoothing function that characterizes the effect of each continuous independent variable on p_i. The location covariate lo(LOCATION) was a smooth surface of UTM easting and northing (see Table 1 for variable abbreviations).

From Eq. (4) we selected a subset of significant variables explaining the greatest proportion of deviance. The best combination of independent variables was selected by stepwise forward selection using AIC criteria. The use of this procedure ensured that at each selection step only those variables explaining a significant proportion of previously non-explained variance would be selected. In other words, while altitude, temperature (ADD) and surface area share a significant amount of variance, their inclusion in the model would only take place if each variable would explain a fraction of variance not explained by the others. The proportion of variance explained by each variable was determined by evaluating the change in deviance resulting from dropping each variable from the model in the presence of all other variables. Analyses of deviance and likelihood ratio tests (based on the binomial distribution) were used to test the significance of the effect of each predictor variable on the probability of occurrence by minnow. Because the large sample sizes used in the regression models could result in predictor variables being statistically significant despite very weak associations with species presence/absence, predictor variables were considered to have significant effects only when $P \leq 0.01$.

The relationship between the significant predictor variables and the probability of minnow occurrence is shown graphically in separate plots separating the unshared fraction of variance that each factor explains (Fig. 3). Each plot depicts a response curve that describes the contribution of the predictor variable to the logit line. More generally, the response curve shows the relative influence of the predictor variable on the probability of minnow occurrence. This response curve is based on partial residuals, is plotted on a log-scale, and is standardized to have an average value of 0. For example, a hump-shaped response curve for the predictor variable “elevation” indicates that minnow was, in a relative sense, most likely to be detected at sites at low elevations and less likely to be detected at sites at high elevations (Knapp et al. 2003).

We used the estimated effect of previous trout presence in the binomial equation to approximate the change in the likelihood (*i.e.* odds ratio) of finding minnow in the presence *vs.* absence of trout after having controlled for the effects of habitat and spatial variables (Hastie and Tibshirani 1990, Welsh et al. 2006).

All regression-related calculations were conducted using R statistical software (R Development Core Team 2013) with the function library *gam* (Hastie 2013). The analyses were run with the data up to the year 2000.

To analyse in more detail the factor variables, we compared the presence/absence of minnows with the categorical predictor variables by 2x2 contingency tables. To test if the morphometric characteristics of the lakes where minnows were introduced were different from the other lakes, we compared the values of the continuous predictor variables with one-way ANOVA and a Tukey post-hoc test, for distinguishing among three groups: fishless lakes, lakes stocked with trout only and lakes stocked with trout and minnows. Data had previously been normalised.

Finally, in order to assess the effectiveness of banning fishing to prevent new introductions of minnows, we compared the changes in the number of lakes with minnows in Aigüestortes i Estany de Sant Maurici National Park during the twentieth century. Drawing line charts, we compared the number of lakes with new minnow introductions since the fishing ban (1988) in the 75 lakes where fishing was banned and the 116 lakes where fishing continued. We grouped the rest of the lakes, outside of the National Park, in a third group (with 329 lakes) and plotted the changes in each category during the twentieth century.

3. Results

We found minnows in 141 of 520 (27%) of the surveyed high mountain lakes (Fig. 1). In 133 of these lakes there were also at least one trout species. The remaining 8 lakes had trout in the past which had gone extinct but still had minnows present. In addition, we found 132 lakes (25.4 %) with only trout (mainly *Salmo trutta*, but also in some lakes *Salvelinus fontinalis* and *Onchorynchus mykiss*) and 247 lakes (47.5 %) that were fishless. All the minnow introductions registered up to the year 2000 took place during the last three decades of the twentieth century (Fig. 2a). Before 1970 there was no Pyrenean high mountain lake with minnow. Since then, the number of minnow introductions has been much higher than those of trout. The introduction rate for minnow was 4.7 *p.a.* (141 lakes introduced between 1970 and 2000) and 2.2 *p.a.* for trout (133 lakes introduced between 1940 and 2000). The documented causes of introduction of minnows were transport to the lake in water containers by fishermen to be used as live-bait. According to our interviews, minnows were then introduced to the lakes by throwing the remaining individuals into the lake in the belief that this species would be food for trout.

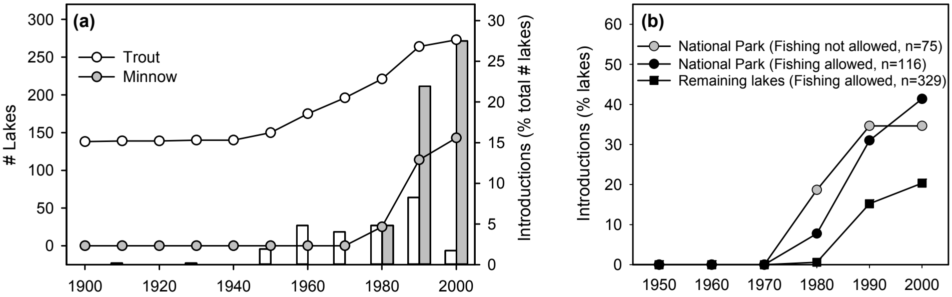

Figure 2. (a) Introduction process of the minnow compared to trout in the southern Pyrenees during the twentieth century. Vertical grey bars are the decadal total number of lakes that were stocked with trout in percentage of the total number of lakes > 0.5 ha of the study area (n=520). (b) Effect of Aigüestortes i Estany de Sant Maurici National Park on the introduction of minnows in high mountain lakes of the Pyrenees. Circles are the lakes within the National Park, and squares are lakes outside the National Park. Grey circles are the area of the National Park where fishing is not allowed and black circles and squares are the lakes in fishing allowed areas.

Table 2. Results of generalized additive models developed for minnows (*Phoxinus* sp.) introduced in the Pyrenean lakes. Only variables that were significant at the stepwise procedure are included in the table.

Parameter	*Phoxinus* sp.	
Null deviance	608	
Degrees of freedom (null model)	519	
Model deviance[a]	288	
Degrees of freedom (full model)	502	
Explained deviance (% of total)	53	
Deviance increase[b]		
Trout presence	86.8	(27.2)***
Surface area	38.8	(12.1)***
Location	37.1	(11.6)***
Altitude	34.3	(10.8)***
Helicopter stocking	15.5	(4.8)***
Forestry road	2.9	(0.9)***

[a] Sometimes referred to as ‘‘residual’’ deviance

[b] Deviance increase means the increase in deviance resulting from dropping the selected variable from the model. The percentage increase is given in parentheses, and was calculated as (deviance increase / (null deviance-model deviance))x100 (Knapp 2005)

Asterisks indicate the level of statistical significance associated with each variable: * $P \leq 0.01$ and $P > 0.001$, ** $P \leq 0.001$, *** $P \leq 0.0001$ and NS not significant ($P > 0.01$)

The forward selection procedure for the generalized additive model selected six of the fourteen predictor variables by order of importance which were significantly correlated with the probability of minnow occurrence: trout presence, surface area, location, altitude, helicopter stocking and forestry road (Table 2). The relationship between the probability of minnow occurrence (on a logit scale) and the important continuously distributed predictor variables were all significantly nonlinear (Fig. 3). The presence of trout explained 27.2 % of deviance of minnow occurrence (higher probability when trout were present either now or previously in the lake), more than twice than the second most important variable. The response curve describing the estimated effect of lake surface area on the probability of minnow occurrence (p_i) indicated that p_i was low at the smallest lakes, but increased steadily until ca. 3 ha when it increased slowly until *ca*. 25 ha and then decreased gradually. Minnow presence was a constant function of altitude until 2300 m, when it decreased progressively. Categorical variables representing different management practices (helicopter stocking and forestry road) contributed only a little part on the deviance explained by the model. The response surface for water body location is not provided in this figure as it was complex and offered no additional insights into the effects of the different predictor variables on species occurrence.

Minnow appears to be particularly linked to the previous trout presence in the lake. After controlling for the effects of habitat and spatial variables, minnows were 54 times more likely to be found in trout-containing water bodies than lakes without trout (odds ratio, with approximate 95% confidence limits 17-169).

The lakes with introduced minnows had significantly lower altitude, greater surface area, higher accumulated temperature, shorter walking effort from the nearest town and greater catchment area compared to the lakes stocked only with trout. In contrast, fishless lakes had the highest altitudes and walking effort and lowest temperatures and catchment areas (Fig. 4). Lakes stocked only with trout had intermediate values for these variables.

Minnows were significantly more likely to be found in the lakes with prior trout presence ($\chi^2 = 159.9$, $p < 0.0001$), within active fish management areas ($\chi^2 = 49.8$, $p < 0.0001$), in lakes with hydroelectrical power exploitation ($\chi^2 = 44.1$, $p < 0.0001$), or with nearby forestry roads ($\chi^2 = 44.8$, $p < 0.0001$). In contrast, the probability of occurrence in lakes within National Parks was not statistically different from the lakes outside them ($\chi^2 = 1.36$, $p = 0.244$), as well as in lakes with helicopter-based trout introductions compared to lakes where trout were introduced with other methods ($\chi^2 = 2.36$, $p = 0.124$).

In the previous statistical models, National Park does not appear as a significant variable in explaining minnow distribution. However, if we examine the accumulated percentage of lakes with minnow at the final part of the twentieth century (Fig. 2b), we can see that only in the non-fishing area, the number of lakes with minnows stopped increasing from the time of the prohibition, while in the other areas it continued to increase.

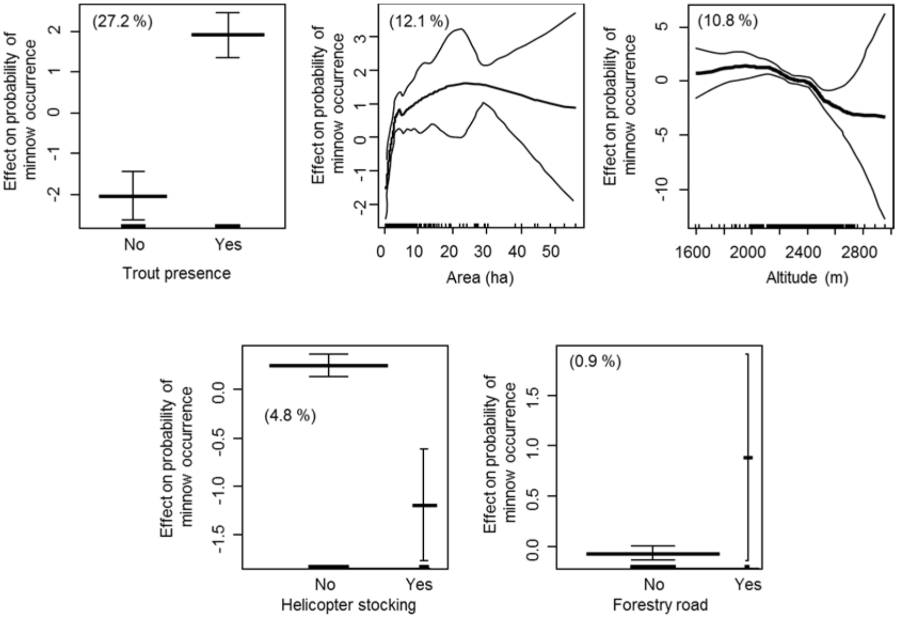

Figure 3. Estimated effect of each of the highly significant ($p \leq 0.01$) predictor variables on the probability of occurrence by minnow, as determined from the generalized additive model (span = 0.5). Response curves are based on partial residuals and are standardized to have an average probability of zero. Thin lines are approximate 95% confidence intervals and hatch marks at the bottom are a descriptor of the frequency of data points along the gradient in continuous variables or within each category for categorical variables. The width of horizontal lines in categorical variables is proportional to the frequency of the data within each category. Numbers in parenthesis are the percentage of explained deviance of each variable. See Table 2 for model details

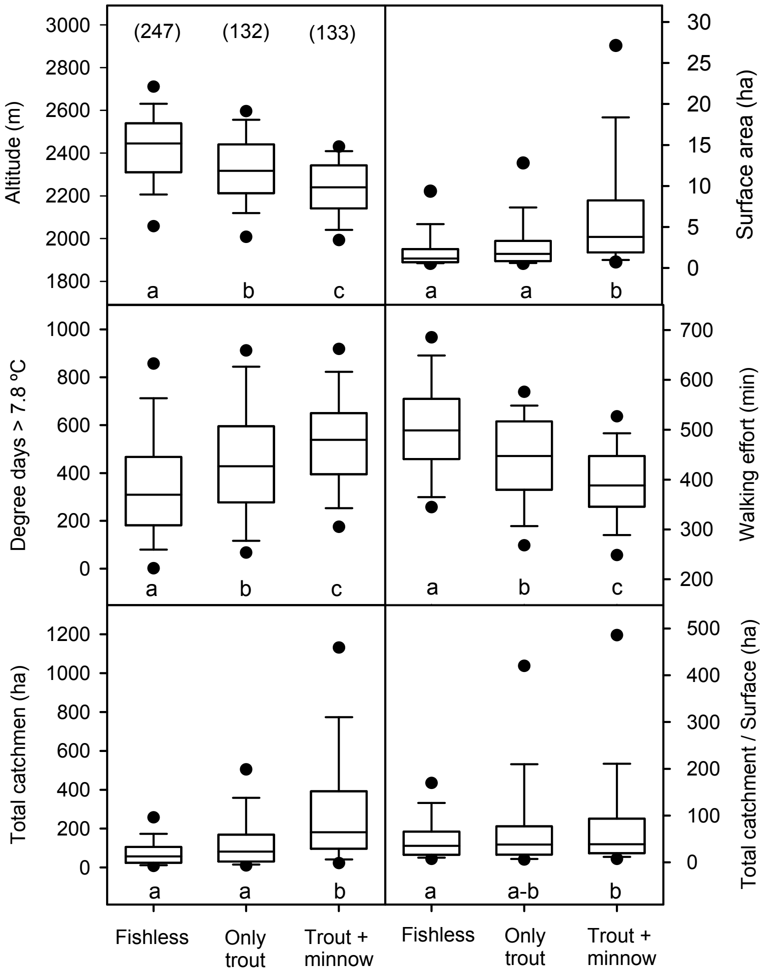

Figure 4. Box plots showing elevation, surface area, accumulated degree days above 7.8 °C, total catchment, the ratio of total catchment to lake surface area, and the walking effort from the nearest town to each lake, of fishless lakes and stocked lakes with trout and with trout+minnow. The line within each box marks the median, the bottom and top of each box indicate the 25th and 75th percentiles, the whiskers below and above each box indicate the 10th and 90th percentiles, and the points above and below the whiskers indicate the 5th and 95th percentiles. Sample sizes for each category are given between brackets in the first panel. Categories with different letters are significantly different at the $p < 0.05$ level (One way ANOVA, Tukey post-hoc).

4. Discussion

4.1. Introduction mediated by invasive trout

The results of this study strongly suggest that the invasion of minnows in the high mountain lakes of the Pyrenees is mediated by a preceding invasive species and facilitated by human activity. We found that the introduction of minnow is a more recent and faster process than those of salmonids (Fig. 2a). Since 1970, when the first introduction took place, it has now spread to 27% of the lakes of the southern Pyrenees with an introduction rate of 4.7 *p.a.*, compared to those of trout, at 2.2 *p.a.* for the period between 1940 and 2000. The results of GAM indicate that the presence of trout before minnow introduction is by far the most important variable explaining minnow's distribution. In fact, we have not found any case of minnow introduction in naturally fishless lakes, indicating that recreational fishing with live-bait is likely to be responsible for these introductions (*ca.* half of the lakes with salmonids; Miró and Ventura 2013). The higher probability of finding minnows at lower altitude lakes and closer to forestry roads also support this idea, since both are elements facilitating the access of fishermen to lakes.

In high mountain lakes, trout are the only species group authorised for introduction by governmental agencies worldwide (e.g. Sostoa and Lobón-Cerviá 1989, Wiley 2003), mainly associated with recreational fishing (Cambray 2003). Unlike trout, minnow introductions are in general not authorised by governmental authorities and their introduction is often an illegal angling practice. The end result in many cases is that fish unused as live-bait are released at the end of the fishing expedition (Maitland and Campbell 1992, Kerr et al. 2005, Winfield et al. 2011, Kilian et al. 2012). This has been quantified to be done by 36% of the fishermen in Michigan and Wisconsin, 41% in Ontario and 65% in Maryland (Litvak and Mandrak 1993, Kerr et al. 2005, Kilian et al. 2012). To prevent the widespread release of non-native species used as live-bait, many US states and Canadian territories have restricted the use, sale, or transport of bait (Kerr et al. 2005, Peters and Lodge 2009). A similar situation exists on the southern slope of Pyrenees, where the release of any organism to the environment without government authorization is also strictly prohibited (Miró 2011). Nevertheless, similar to our findings from the southern valleys of the Pyrenees, in some regions of North America a large proportion of anglers appear to be unaware of, or choose to ignore, the current regulation prohibiting the release of live organisms because they believe their actions are compassionate and that the released unused bait is suitable food for angling fishes (Kerr et al. 2005, Kilian et al. 2012). Our results strongly suggest the need to intensify preventive actions by giving accurate information of the potential negative effects for the local environment of the release of non-native organisms to fisheries boards and local communities. These actions are one of the best guiding principles to prevent the spread of invasive species together with regulation and legislation (Simberloff et al. 2013).

Live-bait related introductions of minnows have occurred in lower, boreal and arctic lakes where trout are present, but they have not been previously described in high mountain lakes. The distribution of minnow expanded considerably throughout the 1900s in the north European lakes of Scotland and Norway, especially in mountain areas, due mainly to the use of minnows as live-bait for angling (Maitland and Campbell 1992, Museth et al. 2007). When minnow is introduced in lakes with autochthonous trout, it reduces recruitment and annual growth rates of trout, causing a decrease of the trout abundance by 35 % on average; however, the effect on other native fauna takes place primarily in the shallow littoral areas (Museth et al. 2007).

In the particular case of extreme habitats such as high mountain lakes that are naturally fishless, such as those of the Pyrenees, the introduction of trout results in a substantial impact for native fauna, especially for the more conspicuous organisms such as amphibians and macroinvertebrates which may be extirpated (e.g. Knapp et al. 2001b). However, minnows, like other small cyprinids, have an omnivorous and opportunistic diet similar to trout (Oscoz et al. 2008, Museth et al. 2010). They predate on zooplankton, benthic macroinvertebrates (Vinebrooke et al. 2001, Naestad and Brittain 2010) and also feed on fish eggs and hatchlings (Kottelat 2007). Their small size allows them to access shallow areas which trout cannot reach, thus eliminating the possibility that other taxa such as amphibians can shelter in littoral areas of the lake. Consequently, the introduction of minnows, which behave like second top predator, will likely result in a stronger negative effect on the native fauna as has been found in other aquatic ecosystems (Nyström et al. 2001). In fact, preliminary results obtained in Pyrenean lakes seem to confirm the negative effects of this small fish (A. Miró, Pers. Comm.).

4.2. Minnow invasive potential

Differences in minnow life history characteristics compared to those of trout give them a higher acclimation success and therefore an extraordinary invasive potential. The minnow, like other small widely-distributed freshwater fish, displays a remarkable variability in its life history depending on the site temperature. For example, minnows have maximum age ranges between 3 and 13 years reaching maturity between 1–2 and 5–7 years in hot and cold places respectively, while show significantly lower growth increments in cold summers (Mills 1988). This plasticity in their life history is what has allowed the species to easily adapt to high mountain lakes, showing higher resistance to harsh conditions than trout. In our dataset, we have not found any lake where minnows have disappeared once established. In contrast, in lakes with trout, between 10 and 44 % of the populations go extinct after 20–30 years due to a lack of favourable conditions (Knapp et al. 2005, Miró and Ventura 2013). Moreover, in Pyrenees we have found that in eight lakes preexisting trout populations have disappeared after minnow introductions. Some of these lakes had brown trout introduced centuries ago while others were stocked recently.

Also, minnow presence was more likely at lakes with lower elevations, greater surface areas and higher temperatures. This could be a result of the pattern of introductions (*e.g.* fishermen using live-bait do not fish so often at the upper altitude lakes) or to acceptable habitat conditions for released minnows to become established. Taking into account the high adaptability of minnows, it seems more likely that our finding is a result of the pattern of introduction.

As well as minnows, other species might be used as live-bait, increasing the number of invasive species to lakes. This is the case for gudgeon (*Gobio* sp.), which is now found in some lakes of the northern slope of the Pyrenees (Miró 2011). This fish has similar size and flexible life-history features to those of minnows (Tang et al. 2011). Thus the ecological effects of fish introductions in high mountain lakes can result in stronger unpredicted consequences.

4.3. Management practices

In this study we have found that the only area where minnow introductions have stopped increasing is at that area of the Aigüestortes i estany de Sant Maurici National Park where fishing has been prohibited since 1988 (Fig. 2b). Similar results have also been found for trout (Miró and Ventura 2013) and this indicates that to date the only management practice that is effective in stopping minnow introductions in high mountain lakes is to ban fishing. This is especially the case for the southern Pyrenean lakes, where the use of live-bait is prohibited, and therefore minnow introductions occur out of the control of governmental agencies. It is necessary to improve the regulation of the activity, and in the cases where maximum protection are required, authorities might even consider the prohibition of fishing.

5. Conclusions

Minnows have been introduced in 27% of high mountain lakes of the Southern slope of the Pyrenees as a result of releases by fishermen using it as live-bait. This invasion took place in only the last three decades of the twentieth century. Although this fishing technique is forbidden in the Southern slope of the Pyrenees since 1966, this has not prevented their introduction in high mountain lakes and the spread of the species. In contrast, trout fishing prohibition since 1988 in the core area of Aigüestortes i Estany de Sant Maurici National Park has been an effective measure to stop minnow introductions. Our results show that there is almost no natural minnow spread beyond the lake of introduction. Therefore, if new introductions are prevented, spread may be contained to only those lakes where introductions already occurred. In order to prevent further minnow introductions we suggest, apart from to studying fishing prohibition in high protected areas, conducting intensive information campaigns to fishermen, local communities and administrative offices in the areas where trout fishing is allowed.

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Part II

Impact on amphibians and other organisms

Chapter 3

Multiple stressor impacts on native high mountain lake fauna: the role of trout, minnows and water-level fluctuation[4]

Abstract.

Introduced trout have been reported to be a major local stressor of high mountain lake fauna during recent decades. Two more human-mediated disturbances affecting these ecosystems, exotic minnows and habitat alteration by artificial water-level fluctuation, may co-occur with trout but remain poorly studied. Detailed knowledge of the local stressors that affect high mountain lake fauna can help to inform environmental policies and effective natural habitat management efforts in mountain areas. We tested the role and the relative importance of trout, minnows, level fluctuation and habitat characteristics on the amphibian, conspicuous macroinvertebrate and planktonic crustacean assemblages in the high mountain lakes of the Pyrenees. Through performing redundancy analyses, variation partitioning and log-linear models, we worked on faunal presence/absence data from 471 lakes >0.5ha: 245 were sampled for planktonic crustaceans and 338 for amphibians and conspicuous macroinvertebrates. Our results showed that trout, minnows and water-level fluctuation had a cumulative and antagonistic impact on high mountain lake fauna. At assemblage level, trout and minnows were the factors that most determined the presence or absence of amphibians and conspicuous macroinvertebrates as well as their taxonomic richness. Trout

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[4] Miró, A., Buñay, D. & Ventura, M. 2015. Multiple stressor impacts on native high mountain lake fauna: the role of trout, minnows and water-level fluctuation.

and minnows together as a stressor fraction explained approximately a third of the exclusive explained variance for amphibian and conspicuous macroinvertebrate assemblages. At taxon level, trout and minnows presence was linked to a lower occurrence of most amphibian species and conspicuous macroinvertebrates taxa, while minnows alone reduced the occurrence of some planktonic crustaceans. The largest impact of minnows on these crustaceans was seen when minnows were found as the only fish in the lake. Artificial water-level fluctuation also reduced the occurrence of some planktonic crustaceans that appeared unaffected by trout and minnows. There have been some successful trout eradication initiatives in high mountain lakes during the past decades, however generate knowledge on removing minnows or restoring natural water-level fluctuation in dammed high mountain lakes is becoming a great challenge for the future.

Keywords: human disturbances; high mountain lakes; amphibians; conspicuous macroinvertebrates; planktonic crustaceans; redundancy analysis; variation partitioning; log-linear models.

1. Introduction

Several anthropogenic stressors have been identified worldwide as major conservation threats in aquatic and terrestrial ecosystems. These include habitat destruction and fragmentation, introduction of invasive species, land-use changes, pollution, human population growth, overharvesting and climate change (Brook et al. 2008, Stendera et al. 2012). Usually, however, ecological factors don’t work individually and an ecosystem’s integrity is often affected by complex interactions from multiple stressors (e.g. Davis et al. 2010, Matthaei et al. 2010, Ban et al. 2014). Multiple stressors can have an additive impact if there is no interaction among them and their combined effect is equal to the sum of the individual stressor impacts, or can be involved in synergistic or antagonistic interactions when the combined effect is greater or lower respectively than the additive expectation (Didham et al. 2007). The impact of multiple invasions on native assemblages might be difficult to understand, because invaders can interact among themselves and affect native species in several ways (Simberloff 2006). However, the greatest opportunity to understanding the situation is by studying and comparing the impacts of the invasions from other co-occurring stressors (Strayer 2010, Ricciardi et al. 2013). In this context it is useful to analyze those interactions along with the relative weight of human disturbances that impact on ecosystems (Ormerod et al. 2010, Stendera et al. 2012). Such studies, analyzing the effects of different stressors together can be helpful for managers of protected areas, who can use the results to inform appropriate management and conservation policies (Hart and Calhoun 2010).

Freshwater ecosystems cover only 0.8% of the earth's surface, but they support more than 10% of all animal species and 35% of all vertebrate species (Stendera et al. 2012). However, ecosystem integrity and specifically biodiversity have decreased far more in standing waters than in other habitats (Revenga et al. 2005, Dudgeon et al. 2006, Stendera et al. 2012). Within freshwaters, high mountain lakes are found in remote headwaters, far from populated areas, may support unique plant and animal communities and hence have tremendous ecological and environmental value (Cole and Landres 1996, Kernan et al. 2009a). Many of them are situated within protected areas with differing degrees of protection and scenic, ethnographic, biodiversity and conservation interest, varying from basic to highly protected areas (Knapp 1996a, Wiley 2003). Although high mountain lakes are often perceived to be pristine, they have been threatened by climate change and by long-distance atmospherically transported pollution including acid deposition, persistent organic pollutants and trace metals (Davidson and Knapp 2007, Catalan et al. 2013). However, their major direct anthropogenic stressors are likely to be the introduction of invasive fish stocks (Pister 2001, Miró and Ventura 2013) and, in some areas, artificial water-level fluctuation by impoundment for hydroelectric power exploitation (Catalan et al. 1993).

Some invasive species cause large impacts on the receiving ecosystems by direct predation on native species (Lockwood et al. 2013, Simberloff et al. 2013). This is the case where trout are introduced into high mountain lakes which were originally fishless ecosystems, due to natural barriers that have prevented the natural colonization of fish species from lower streams or by dispersal between interconnected lakes (Knapp et al. 2001a, Pister 2001). Introduced trout have become the top predator, leading to profound ecological changes. The predation of trout on the native fauna of high mountain lakes can lead to the elimination of amphibian and reptile populations (Knapp 2005, Orizaola and Braña 2006, Pope et al. 2008, Pilliod et al. 2010, Tiberti and von Hardenberg 2012), changes in zooplankton and benthic macro-invertebrate species composition and size structure (Brancelj 2000, Knapp et al. 2001b, de Mendoza et al. 2012, Tiberti et al. 2014b), large-scale alteration of ecosystem processes such as nutrient cycling (Schindler et al. 2001, Magnea et al. 2013) and indirect effects on surrounding habitats through resource depletion (Epanchin et al. 2010). Furthermore, invasive trout can impact on high mountain lakes by interaction with other stressors, such as airborne pesticides (Davidson and Knapp 2007). Trout introductions are therefore a threat for the conservation of high mountain lake biodiversity in general, and in particular for the most conspicuous animal groups.

Another group also introduced to high mountain lakes are minnows. Their introduction was generally a result of being used as live-bait by anglers and their subsequent release to the lakes (Museth et al. 2007, Miró and Ventura 2015). The effect of minnows on lakes where trout are native, results in the reduction of the annual trout recruitment and growth rates, causing a decrease in trout abundance by 35 % on average (Museth et al. 2007). In the particular case of extreme habitats such as high mountain lakes that are naturally fishless, minnows, like other small cyprinids, have an omnivorous and opportunistic diet similar to that of trout (Oscoz et al. 2008, Museth et al. 2010). Their small size allows them to access shallow areas which trout cannot reach, thus eliminating the possibility that other organisms such as amphibians can shelter in littoral areas of the lake. Consequently, the introduction of minnows, which become a second top predator, could be expected to result in a stronger negative effect on the native fauna of high mountain lakes, as has been found in other aquatic ecosystems in similar situations (e.g. Nyström et al. 2001). To our knowledge, and in contrast to trout, the impact of introduced minnows, either individually or by interaction with other disturbances, has not extensively been examined in high mountain lakes (but see Schabetsberger et al. 2006).

Invasive species also can interact with other stressors which might increase the negative effects on standing waters (Strayer 2010). Impoundment is a habitat alteration that can become a large individual threat to aquatic ecosystems mainly through water level fluctuation (Sutela et al. 2013), besides facilitating new invasions (Johnson et al. 2008). The even strongest impact of lake exploitation as water storage has been found on macrophytes (Gacia and Ballesteros 1996, Krolova et al. 2013), but depending on the magnitude of the water-level changes it can also affect nutrient concentration through sediment-resuspension processes (Catalan et al. 1993), as well as altering phytoplankton composition by changes in hydraulic dynamics and water-renewal rates (Leon Lopez et al. 2012). However, artificial water-level fluctuation is also a poorly studied stressor in high mountain lakes.

The aim of this study was to evaluate the impact of multiple local anthropogenic stressors on high mountain lake fauna. We performed an extensive study on 471 Pyrenean high mountain lakes using both multivariate and univariate statistical approaches to assess the relative effect of two invasive fishes (trout and minnow) and one habitat perturbation (artificial water-level fluctuation), on three faunal taxonomic groups (amphibians, conspicuous macroinvertebrates and planktonic crustaceans). Furthermore, we aimed to assess the type of interaction that occurs among the three stressors. We expected to find a clear, cumulative but antagonistic impact caused by all three stressors on both littoral (amphibians and conspicuous macroinvertebrates) and pelagic organisms (planktonic crustaceans).

2. Methods

2.1 Description of the study Area

The studied lakes are spread along the Pyrenean mountain range (0°42'W - 2°09'E, 42°52' - 42°23'N; Fig. 1). There are 1 080 lakes > 0.5 ha either of glacial origin or modified by the activity of quaternary glaciations. They range in altitude between 1 500 and 2 960 m with the highest frequency found at *ca.* 2 300 m. The unmodified lakes are relatively small and deep (average surface area of 4.5 ha and average maximum depth of 15 m, with the largest 44 ha and the deepest 105 m). Approximately 15% of the total lakes are impounded, mostly the biggest. Among these semi-natural dammed lakes the largest has 160 ha of surface and the deepest 123 m of maximum depth. Due to their common glacial origin, there is a close positive relationship between surface area and maximum depth (Catalan et al. 2009b). Most of the lakes are above the tree line with catchments partially covered by meadows, although some of them are within or below the tree line. Approximately half of the lakes have catchments on granodiorite bedrock, the remaining being located in catchments with metamorphic (25%), detrital (15%) or carbonate (10%) bedrock with a minority on Silurian slate (Casals-Carrasco et al. 2009). The latter bedrocks, due to their high sulfate content, give natural acidity to the waters (pH < 5.5). The ionic content is generally low, being the chemical factor most strongly related to bedrock composition (Catalan et al. 1993). The lakes have low phosphorous and chlorophyll content, being of an oligotrophic nature (Buchaca and Catalan 2007). A general description of the climate, physico-chemical and biological characteristics of Pyrenean lakes and a comparison with other European high mountain lakes can be found elsewhere (Camarero et al. 2009, Catalan et al. 2009a, Kernan et al. 2009a, Thompson et al. 2009).

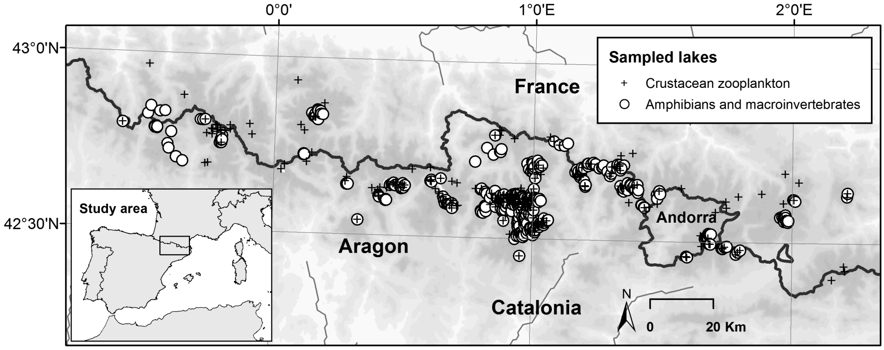


Figure 1. Distribution of studied lakes along the Pyrenean range

2.2. Studied animal assemblages

The studied amphibian assemblage contained up to five species: the anurans common frog (*Rana temporaria*), midwife toad (*Alytes obstetricans)* and common toad (*Bufo spinosus*) and the caudatans palmate newt (*Lissotriton helveticus*) and Pyrenean newt (*Calotriton asper*), the last one endemic to the region. We assessed the occurrence of *A. obstetricans* and *B. spinosus* based on the presence of egg masses or larvae that are indicative of breeding. In the same way, we based the occurrence of *L. helveticus* and *C. asper* on the presence of at least one of the different life stages (eggs, larvae, sub-adults or adults) when they were found in the lake. In the case of *R. temporaria*, we counted the sub-adults or adults separately (which we coded as *R. temporaria* frog) from the egg masses or larvae (which we coded as *R. temporaria* tadpole). *R. temporaria* is the most widespread amphibian of the Pyrenees. This species breeds in the

Pyrenean lakes in late spring or early summer, just when the ice/snow cover is melting. The adults leave the lake immediately after the breeding season ends, moving to other lakes and their surrounding meadows (Balcells 1975). .For this reason we distinguished between these two stages within *R. temporaria* within the amphibian assemblage.

The assemblage of conspicuous macroinvertebrates included those taxa of invertebrates that are easiest to see in the littoral of Pyrenean lakes: water striders (Family Gerridae), backswimmers (Family Notonectidae), dragonflies (Order Odonata), beetles (Order Coleoptera), caddisflies (Order Trichoptera) and mollusks (Phylum Mollusca).

The planktonic crustacean assemblage contained 10 species in total, four belonging to the Suborder Cladocera (*Daphnia longispina*, *Daphnia pulicaria*, *Holopedium gibberum* and *Scapholeberis mucronata*) and six belonging to the Subclass Copepoda, of which two were from the order Cyclopoida (*Acanthocyclops vernalis*, *Cyclops abyssorum*) and four from the order Calanoida (*Diaptomus cyaneus*, *Diaptomus castaneti*, *Eudiaptomus vulgaris* and *Mixodiaptomus laciniatus*). We found a further 8 species of planktonic crustaceans but we excluded them from the dataset as they occurred at fewer than 5% of sites.

2.3. Taxon data collection

We surveyed *ca.* half of the lakes >0.5 ha of the Pyrenees (471 lakes), with 162 sampled for littoral amphibians, macroinvertebrates and planktonic crustaceans, 83 sampled only for planktonic crustaceans and 226 only for littoral amphibians and conspicuous macroinvertebrates. Therefore, the amphibian and conspicuous macroinvertebrate datasets have 388 sites and the planktonic crustacean dataset had 245 sites (the main features of the sampled lakes are summarized in Table 1 and some photos of example can be viewed in Appendix B). The field surveys were conducted during the lakes' ice-free periods from 1974 to 2013 for the planktonic crustacean surveys (including data from Miracle 1978, Catalan et al. 2009b), and from 2008 to 2013 for the amphibian and conspicuous macroinvertebrate surveys. To avoid the seasonal variations in organism occurrence during the lake ice-free periods as found in some studies in high mountain and boreal lakes (e.g. Laugen et al. 2003, Knapp 2005), we performed the surveys during the season by following the altitudinal and insolation gradients, from warmer to colder sites. Moreover, when there were doubts about the data validity, we repeated the survey in the following years. In total we conducted 496 amphibian and conspicuous macroinvertebrate and 359 planktonic crustacean surveys.

Table 1. Main characteristics of the two datasets of studied lakes. In the continuous variables we show the mean and the range values. In the presence/absence variables we show the number of lakes with presence and the % on the total lakes in parentheses. Maximum depth was calculated on 207 lakes from the amphibian and conspicuous macroinvertebrate dataset and on 187 lakes from the planktonic crustacean dataset. Field pH was calculated on 80 lakes from the amphibian and conspicuous macroinvertebrate dataset and on 43 lakes for the planktonic crustacean dataset.

	Dataset	
Variable	Amphibians and conspicuous macroinvertebrates (n=388)	Planktonic crustaceans (n=245)
Altitude (m)	2307 (1598-2880)	2296 (1618-2960)
Surface area (ha)	4.27 (0.50-56.05)	6.75 (0.51-56.05)
Total catchment (ha)	192.95 (2.67-5437.93)	237.83 (2.85-3939.76)
Maximum depth (m)	10.5 (0.15-105)	17.8 (0.2-123)
Conductivity (µS/cm)	25.41 (3.1-176.3)	29.7 (3.2-190.8)
Field pH	8.03 (5.55-9.76)	7.94 (5.55-9.12)
Trout presence	194 (50%)	143 (58.4%)
Minnow presence	133 (34.3%)	101 (41.2%)
Impounding presence	52 (13.4%)	41 (16.7%)
Artificial water-level fluctuation presence	42 (10.8%)	35 (14.3%)

We evaluated the occurrence of amphibians and conspicuous macroinvertebrates by visual encounter surveys (Crump and Scott Jr. 1994) of sufficient shore segments (between 10 and 30 depending on the lake perimeter) to cover all the habitat variability along the perimeter of each lake. Shore segments were 10 meters long, parallel to the shoreline (2 m into the water and 2 m into the next terrestrial strip) and were proportionally distributed for the different substrates found in the water-bodies and their edges. When no amphibians were detected, the entire shoreline of the lake was resurveyed. Because high mountain lakes are extremely clear, large areas could be surveyed from shore and macroscopic animals could be easily detected, as has been shown by previous studies (e.g. Knapp 2005, Davidson and Knapp 2007, Pilliod et al. 2010). Samples to verify the occurrence of planktonic crustacean species were obtained by vertical net hauls from the deepest point of the lake to the surface with a 160 µm conic net in most of the lakes, with the exception of the early surveys of 1974-77 when part of the lakes were sampled from the shore by throwing a net towards the center of the lake (Miracle 1978). A recent comparison has shown little sampling method effect for the ten species considered in this study (D. Buñay, personal observation). Zooplanktonic samples were immediately preserved in 5% sucrose formalin solution (Ventura and Catalan 2005) and identified to species level later in the laboratory.

2.4. Explanatory variables

To detect which factors were responsible for assemblages' compositions we generated a set of explanatory variables summarizing the environment, stressors and spatial characteristics (see below) that might have affected the distribution of animal taxa (Table 2). All of them were used in the analyses of the amphibian-macroinvertebrate dataset, but six variables related to the littoral and shore zones were not used in the planktonic crustacean analyses (see caption of Table 2).

Altitude (ALT) and surface area (SURF) of the lakes were obtained from a GIS generated from 1:25000 maps from the various national agencies (Casals-Carrasco et al. 2009). Conductivity (COND), referred to 20ºC, was gathered during the field surveying with handheld meter WTW Cond 340i.

We used the accumulated degree days (ADD) during the ice-free period calculated from daily max-min temperature data using the sine-wave method, to describe the thermal characteristics of the lakes, assuming the trigonometric sine curve as an approximation of the diurnal temperature curve and adding the area under the curve and above the lower threshold for each day (Baskerville and Emin 1969). As the lower threshold of the method, we used the minimum developmental temperature of *Rana temporaria,* 7.64°C (Balcells 1975) below which growth is not feasible. Field data of the daily water temperatures were obtained during the years 2009, 2010 and 2011 from 30 automatic thermometers (Vemco Minilog-T) situated at a depth of *ca.* 1m in 30 Pyrenean lakes larger than 0.5ha, covering a wide range of altitudes, surface areas and catchment sizes. In total, we had 80 ADD field data: 28 from 2009, 27 from 2010 and 25 from 2011. This ice-free accumulated temperature was assumed to be representative of the epilimnion following previous limnological knowledge of these lakes and calibration measurements performed seasonally at different depths. The ADDs of all studied lakes were interpolated using a regression model between field ADD and various morphometric parameters. This was possible since the temperature variability of lakes in alpine areas follows a close relationship with lake altitude, but also with lake size among other morphometric variables (Thompson et al. 2009). We introduced the 80 measured ADD from the 30 lakes as the dependent variable in a multiple linear mixed model (Lindstrom and Bates 1988) with the sampling year as a random factor and chose the minimum combination of morphometric variables that described most of the variance (Burnham and Anderson 2002). The seven explanatory variables used were: lake altitude (ALT), lake surface (SURF), total catchment (TCAT), direct catchment or proportion of catchment not shared with other lakes that are upstream of the lake (DCAT), ratio between total catchment and lake surface (TCAT/SURF) which is indicative of the rate of water renewal, geographic longitude (UTMX), and finally, two binary insolation factors (NORTH and SOUTH) according to the orientation of the valley where the lake is. East and west orientations were not taken in account in these orientation factors. The quantitative variables SURF, TCAT, DCAT and TCAT/SURF were natural logarithm transformed to bring them closer to the normal distribution (Zuur et al. 2007) and ALT was centred around 0 to keep the intercept in low values (Zuur et al. 2009). We started to search for the best model with all explanatory variables, dropping the least significant at each step. We then compared the new and the previous model with a likelihood ratio test using the maximum likelihood estimation method (ML) until we found significant differences. Afterwards we rejected the last model and took the previous one. Eventually, with an additional step, we removed TCAT/SURF from the optimal model so as to not contradict previous knowledge. This variable had positive slope in our model, while the rate of water renewal, which is the inverse of water residence time, must be negatively related to the water temperature (e.g. George et al. 2007). When the final model was found, we refitted it to have more accurate slopes with the restricted maximum likelihood estimation (REML) procedure (Zuur et al. 2009). At the same time, we removed three outlier data so as not to influence the analysis incorrectly (Zuur et al. 2007). The final optimal model obtained was:

$$\mathrm{ADD}(>7.64^{\circ}\mathrm{C}) = 1425.01 + 124.75\ \mathrm{SOUTH[Yes]} - 1.37\ \mathrm{ALT} - 67.52\ \ln(\mathrm{TCAT}+1) - 92.55\ \ln(\mathrm{DCAT}+1)\ |\ \mathrm{RANDOM\ FACTOR\ YEAR}$$

The final model had an acceptable correlation structure (Venables and Ripley 2002) ranging from -0.61 to 0.31. The residuals based on the REML fit showed homogeneity of variances and residual normality (Zuur et al.

2009). All variables and slopes were significant and had *P*-values <0.0001. The marginal R^2 , which describes the proportion of variance explained by the fixed part, and the conditional R^2 , which describes the variance explained by both fixed and random parts (Nakagawa and Schielzeth 2013), were 0.673 and 0.855 respectively. The model was built with R statistical software (R Development Core Team 2013) using the package *nlme* (Pinheiro et al. 2013).

Littoral and shore habitats were characterized by visually estimating substrate features and composition of the same shore segments where we had looked for amphibians (see above, section Taxa data collection). Data were expressed as % coverage for each category of 3 characteristics: littoral slope, littoral substrate composition and shore substrate composition. Littoral slope was categorized as shallow (<10 cm deep, <15º slope), slight slope (15º–30º slope), sloping (30º–70º slope) or sub vertical (70º–90º slope). Littoral substrate composition was categorized as organic material, silt-sand (<0.5 cm), gravel (>0.5–2.5 cm), cobble (>2.5–25 cm), boulder (>25 cm–1 m), big boulder (>1 m) or bedrock. Shore substrate composition was categorized as cobble-boulder (2.5 cm–1 m), big boulder-bedrock (>1 m) or terrestrial vegetation. We adapted these classifications from previous studies about amphibians and macroinvertebrates in high mountain and boreal lakes (Knapp et al. 2001b, Johnson et al. 2004). To reduce the dimensionality of the substrate data, we subjected the littoral slope, littoral substrate and shore substrate data to separate principal component analyses (Knapp 2005). In all three analyses, axis 1 explained a substantial amount of the total variation in substrate characteristics and composition (littoral slope: 58.2%; littoral substrate: 50.0%; shore substrate: 82.6%), so the scores were used as the independent variable representing littoral zone slope (SLOPE), littoral zone substrate composition (LITTO) and shore substrate composition (SHORE). High values of SLOPE were associated with low littoral slope and vice versa; high values of LITTO were associated with fine grain substrate, while low values were associated with coarse grain substrate; high values of SHORE were associated with vegetated shores, while low values were associated with bare rocky shores (Table 3).

Table 2. Description of predictor variables used in the analysis. Variable name abbreviations are given in parentheses. The symbol * marks the six variables related to the littoral and shore zones, not used in the planktonic crustaceans analyses.

Variable type	Variable name	Description
Environment	Altitude (ALT)	Elevation of the lake (m)
	Surface area (SURF)	Surface of the lake (ha), natural logarithm transformed
	Conductivity (COND)	Water conductivity (µS/cm), natural logarithm transformed
	Accumulated temperature (ADD)	Estimated Accumulated Degree Days of the lake above 7.64ºC
	Littoral slope* (SLOPE)	First principal component for littoral zone slope. High values of the variable are associated with low littoral slope and vice versa
	Littoral substrate* (LITTO)	First principal component for littoral zone substrate types. High values of the variable are associated with fine grain substrate, while low values are associated with coarse grain substrate
	Shore substrate* (SHORE)	First principal component for shore zone substrate types. High values of the variable are associated with vegetated shores, while low values are associated with bare rocky shores
	Aquatic vegetation* (AQVEG)	% of littoral aquatic vegetation surface, natural logarithm transformed
	Predation refuge* (REFU)	Predation refuge areas presence/absence in the lake, dummy transformed
	Ponds around* (POND)	Number of ponds within 200m of the lake divided by the lake perimeter in 100 m units, square root transformed
Stressors	Trouts (TROUT)	Introduced Salmonid presence/absence in the lake, dummy transformed
	Minnows (MINNOW)	Introduced *Phoxinus sp.* presence/absence in the lake, dummy transformed
	Level fluctuation (LEVEL)	Artificial water-level variation (m), natural logarithm transformed
Spatial broad-scale	MEMx	Moran's Eigenvector Maps of lake spatial variance decomposition with positive Moran's Index
Spatial fine-scale	MEMx	Moran's Eigenvector Maps of lake spatial variance decomposition with negative Moran's Index

Aquatic vegetation (AQVEG) is the % of macrophyte coverage in the littoral zone, taken in the same segments where we characterized the littoral habitat. We created the binary variable predation refuge (REFU) to reflect the presence in the lake of littoral areas where fish were assumed to be unable to access: shallow areas either with or without macrophytes and stones. To evaluate the degree of isolation of the studied sites, we created the variable POND to include the existence of likely favorable aquatic ecosystems near the lake which allow amphibian breeding. We generated POND with GIS, counting the number of ponds present 200 m around the lake divided by the perimeter of the lake in 100 m units.

The occurrence data for salmonids (TROUT) and minnows (MINNOW) were obtained from historical documents, from interviews with local elderly fishermen or nature reserve wardens, from local reports of fishing or walking societies and from our own visual encounter and field surveys (Miró and Ventura 2013, 2015). The different trout species found in Pyrenean lakes are *Salmo trutta, Oncorhynchus mykiss* and *Salvelinus fontinalis* in the southern (Spanish) Pyrenees and these species together with *Salvelinus alpinus* and *Salvelinus namaycush* in the northern (French) Pyrenees (Miró and Ventura 2013). Currently the only minnow species found in Pyrenean lakes is *Phoxinus phoxinus* (Miró and Ventura 2015). Artificial level fluctuation (LEVEL FLUCTUATION) was obtained from several literature sources (e.g. MOPU 1988, Catalan et al. 1997) and defined as the maximum water-level fluctuation that can be reached in impounded lakes.

In order to assess the contribution of space to the assemblages' compositions, we used Moran's eigenvector maps (MEM; Dray et al. 2006) as spatial predictors, generated on basis of the geographic coordinates. We also used MEMs to account for some of the unrecorded or unavailable information as proxies for missing predictors related to spatially structured ecological processes (Leibold et al. 2010). We extracted MEMs from the spatial weighting matrix (SWM) which was computed through a neighbourhood defined by the relative neighbourhood criterion (Toussaint 1980) and spatial weights determined by linear function of distance (Dray et al. 2006). We made these decisions to emphasize in the SWM the highly heterogeneous geographical distances that exist in our datasets. MEM represents a spectral decomposition of the spatial relationships among the observation points, and results into a set of multiscale spatial explanatory variables called spatial eigenfunctions (Dray et al. 2012). In fact, the eigenvectors associated with high positive eigenvalues allow users to model global or broad-scale spatial patterns while the eigenvectors associated with negative eigenvalues allow to model local or fine scales (Dray et al. 2006, Dray et al. 2012). After removing the MEMs associated with no significant Moran's indices, we obtained 351 MEMs for the amphibian and conspicuous macroinvertebrate dataset (176 positive and 175 negative), and 222 MEMs for the planktonic crustacean dataset (113 positive and 109 negative).

Table 3. Pearson's correlations among the variables littoral slope, littoral substrate and shoreline substrate categories with the principal component axis 1. The variance contained in the first axis is given in parentheses and above the correlations (% explained variance).

Habitat characteristics	Axis 1	Sig.
Littoral slope (SLOPE)	*(58.2% explained variance)*	
Shallow (<10 cm deep, <15º slope)	0.93	<0.001
Slight slope (15º–30º slope)	0.23	<0.001
Sloping (30º–70º slope)	-0.88	<0.001
Vertical (70º–90º slope)	-0.54	<0.001
Littoral substrate (LITTO)	*(50.0% explained variance)*	
Organic material	0.25	<0.001
Silt-sand (<0.5 cm)	0.91	<0.001
Gravel (>0.5–2.5 cm)	-0.018	0.715
Cobble (>2.5–25 cm)	-0.28	<0.001
Boulder (>25 cm–1 m)	-0.83	<0.001
Big boulder (>1 m)	-0.1	0.048
Bedrock	-0.24	<0.001
Shoreline substrate (SHORE)	*(82.6% explained variance)*	
Terrestrial vegetation	0.99	<0.001
Cobble-boulder (2.5 cm–1 m)	-0.89	<0.001
Big boulder-bedrock (>1 m)	-0.59	<0.001

2.5. Statistical analysis

Redundancy analysis (RDA; Wollenberg 1977) was used to identify the main structures explained by the measured environmental and stressor variables on each fauna assemblage (Table 2). In the RDA analyses of amphibians and conspicuous macroinvertebrates, we included 10 environment variables (altitude, surface, conductivity, accumulated temperature, littoral slope, littoral substrate, shore substrate, aquatic vegetation, predation refuge and ponds within 200 m) and 3 stressor variables (trout, minnows and level fluctuation). In the planktonic crustacean analyses we included 4 environment variables (altitude, surface, conductivity and accumulated temperature) and 3 stressor variables (trout, minnows and level fluctuation), but we did not use the environmental variables related to the littoral habitat (littoral slope, littoral substrate, shore substrate, aquatic vegetation, predation refuge and ponds within 200 m). We accounted for space by repeating the three aforementioned RDA analyses including also the significant spatial MEMs variables. To search for parsimony and prevent the problem of inflation of the overall type I error, before all RDA analyses we performed an appropriate variable forward selection process using a double stopping criterion for each candidate variable: (a) traditional significance level $\alpha = 0.05$ and (b) global adjusted R^2 threshold of the RDA made with all potential explanatory variables, only if that test was significant (Blanchet et al. 2008). In addition, we examined for linear dependencies among the explanatory variables in the RDA which could render the regression coefficients of the explanatory variables unstable (Legendre and Legendre 1998) using Variance Inflation Factor (VIF, Borcard et al. 2011). After the forward selection processes, all VIF values obtained were below 4.96, clearly below 10, the VIF threshold value indicative of worrisome collinearity (Borcard et al. 2011). We tested the significance of all RDAs and their axes through permutation tests with 999 permutations.

To visualize RDA results we drew, for each assemblage, a correlation triplot diagram (scaling 2), which allows one to focus on the variables' ordination (Legendre and Legendre 1998). For each RDA, we plotted the two most explanatory canonical axes, RDA1 and RDA2. The site scores were drawn as linear combinations of explanatory variables (lc scores) to avoid excessive overlap that was otherwise found when we used weighted sums of taxa (wa scores) due to the same combination of taxa occurring at several sites. Spearman's correlation between each selected variable and RDA axes allowed us to know accurately which variables were more related to each canonical axis. To compare the constrained (RDA) and unconstrained (PCA) configuration of the variables, also we drew a PCA for each assemblage using the variables selected by the RDA forward selection process.

To know the relative influence of all stressors together on the assemblages structure, we also performed a variation partitioning analysis of each assemblage by partial RDAs (Borcard et al. 1992, Peres-Neto et al. 2006) taking into consideration the 4 following components: environment, stressors, spatial broad-scale, and spatial fine-scale (see above and Table 2 for the variables within each group). In each partial RDA we applied the same forward selection, VIF test and significance permutation test as described above. After applying the forward selection to the MEMs of spatial fine scale (negative Moran's indices), none of them was selected in any assemblage. Consequently, we computed the variation partitioning analysis taking into consideration only 3 components: environmental, stressor and spatial broad-scale. In each partial RDA, we also computed the Spearman's correlation between each environmental and stressor selected variable and the partial RDA1 and RDA2 axes.

The dependent variable used in all multivariate analyses was the presence/absence of any life stage identified of the amphibian, conspicuous macroinvertebrate or planktonic crustacean taxa of interest. We decided to make lineal RDAs instead of db-RDAs with binary similitude index, because the results that we obtained were similar in both analyses and db-RDAs always had one decimal lower in adjustment due to the particularities of the statistical procedure. Before running the analyses, we transformed continuous variables to bring them closer to the normal distribution when necessary (Table 2) and all variables were standardized to zero mean and unit variance to correct their heterogeneous dimensions (Borcard et al. 2011). Binary variables of presence/absence were used directly as dummy (1/0) variables (Legendre and Legendre 1998).

Finally, to investigate the specific impact of the anthropogenic stressors on the individual taxa, we generated a four-way contingency table for each taxon and analysed them by building log-linear models (Legendre and Legendre 1998) with the presence/absence of each taxon as a factor together with the presence/absence of the three stressors: trout, minnows and artificial water-level fluctuation . We choose to build a log-linear model from a four-way contingency table for each taxon instead of analysing the three stressors separately because our three stressors have a high degree of coincidence: 95.2% of the lakes with artificial water-level fluctuation have also trout, 88.1% of the lakes with level fluctuation have also minnows and 92.5% of the lakes with minnows have also trout. Therefore, the effects may appear confused and masked in simple 2x2 contingency tables. Log-linear analyses were performed through GLM models with Poisson distribution, which allowed us to test the significance of each interaction term that involved the taxon (Crawley 2007), which were [T*tx*], [M*tx*], [L*tx*], [TM*tx*], [TL*tx*], [ML*tx*] and [TML*tx*], where T indicates the factor trout, M indicates the factor minnows, L indicates the factor water-level fluctuation and *tx* indicates the different taxa also as a presence/absence factor. We began each analysis with the saturated model containing all main effects and interaction terms between the factor presence/absence of the taxon and the stressor factors trout, minnows and level fluctuation. The dependent variable was the frequency of each contingency. Afterwards, by successive steps, we dropped the interaction terms that contained the taxon from the highest to the lowest-order and computed the χ^2 test to check the significance of the dropped term between the models before and after dropping the term (Crawley 2007). To determine the effect of the factors involved in each significant interaction term and visualize the relationship among them, we drew interaction plots showing the mean % of occurrence of the taxon on the Y axes.

All analysis were computed with R statistical software (R Development Core Team 2013) using the basic functions and the packages *spacemakeR* (Dray 2013), *packfor* (Dray et al. 2013), *vegan* (Oksanen et al. 2013b) and *vcd* (Meyer et al. 2013).

3. Results

3.1. Amphibians

We obtained a significant RDA (F = 14.02, P = 0.001) which explained 25% of the variance of the amphibian assemblage (adjusted R^2 = 0.23) with 9 variables identified by the forward selection process (in the selection order and followed by the cumulative percentage of fit based on the adjusted R^2 value): trout (15.4%), shore substrate (17.7%), altitude (19.1%), ponds within 200 m (20%), predation refuge (20.9%), minnows (21.8%), conductivity (22.4%), littoral slope (22.8%) and aquatic vegetation (23.2%).

We found two significant canonical axes RDA1 and RDA2 (F = 107.88, P = 0.001 and F = 10.86, P = 0.001 respectively) which explained 21.2% and 2.1% of the total variance respectively. Trout and minnows had a high and negative correlation with RDA1 (Spearman coefficient -0.84, $P < 0.001$ and -0.71, $P < 0.001$ respectively) while the other selected variables were mainly positively correlated with RDA1 (Fig. 2A and Table A1). Trout and minnows moved from nearer the second axis in the unconstrained configuration (PCA) to closer to the first axis in the constrained form (RDA) (see Fig. 2A in comparison with insert). In the RDA diagram all amphibian taxa, except *B. spinosus* and *L. helveticus*, were situated in the opposite direction from trout and minnows, showing a negative correlation between fish and amphibian presence. Particularly, tadpoles of *R. temporaria* were highly negatively correlated with fish, but also closely associated with lower littoral slope. Adults of *R. temporaria* were correlated with high altitudes, and *A. obstetricans* and *C. asper* were more related to the existence of predation refuges and high conductivities. *B. spinosus* and *L. helveticus* appeared in the central, undefined part of the chart. Amphibian richness was clearly lower in the RDA region closer to trout and minnow presence and higher in the opposite area (see symbol size at Fig. 2A). Level fluctuation was not related to the amphibian assemblage structure and it was excluded in the forward selection step. The RDA analysis including significant MEMs to account for spatial patterns showed roughly the same pattern and importance for the selected environmental and stressor variables, but the overall explained variance increased to 35% (adjusted R^2 = 0.31, Fig. A1).

The forward selections made before the variation partitioning resulted in 6 significant environmental variables: surface (7.1%), ponds within 200 m (8.7%), shore substrate (9.9%), altitude (11.9%), littoral slope (12.5%) and conductivity (13.1%); 2 variables in the stressor fraction: trout (15.4%) and minnows (16.5%); and 42 MEMs in the spatial broad-scale fraction. Level fluctuation wasn’t selected in the stressor fraction, and no MEMs were selected in the spatial fine-scale fraction. Variation partitioning (Table 4 and Fig. A4A) identified a significant pure broad-scale spatial fraction (adjusted R^2 = 0.116, P = 0.001), a significant pure stressor component (adjusted R^2 = 0.054, P = 0.001) that was twice those of the pure significant environmental fraction (adjusted R^2 = 0.028, P = 0.001). Trout and minnows together as a stressor fraction, explained approximately 27% of the exclusive explained variance for the amphibian assemblage.

Between 67% and 89% of the amphibian occurrences were placed in the contingency where there were no stressors present, except by *B. spinosus* which also occurred if there was presence of trout or minnows (Table A3; Fig. A5A). In the log-linear models (Table 5), the factor 'trout' showed significant negative effect on the occurrence of *R. temporaria* (both tadpoles and frogs), *A. obstetricans* and *C. asper* (Fig. 3A1, 3B1, 3C and 3E); but not on the occurrence of *B. spinosus* and *L. helveticus*. The factor 'minnows' had significant negative effect on *R. temporaria* tadpoles (Table 5; Fig. 3A2). The factor 'level fluctuation' had significant and also negative effect only on *R. temporaria* frogs (Table 5; Fig. 3B2). We also found 3 three-way significant interactions, 2 of them involved *R. temporaria* tadpoles and the third *B. spinosus* (Table 5). In the significant interaction among *R. temporaria* tadpoles, trout and level fluctuation (Fig. 3A3), trout showed a huge negative effect on the species occurrence, irrespective of whether there was presence or absence of level fluctuation. However, when trout was absent, the presence of level fluctuation caused a large negative effect on *R. temporaria* tadpole occurrence. We found exactly the same pattern in the significant interaction among *R. temporaria* tadpoles with minnows and level fluctuation (Fig. 3A4). In the significant interaction among *B. spinosus* with trout and minnows (Fig. 3D), trout did not exhibit any negative effect on the species occurrence, irrespective of whether there was presence or absence of minnows. In contrast, when trout was absent, there was a slight negative effect related to minnows.

3.2. Conspicuous macroinvertebrates

We performed a significant RDA ($F = 16.41$, $P = 0.001$) which explained 26% of the variance of the conspicuous macroinvertebrates assemblage (adjusted $R^2 = 0.24$) with 8 variables identified by the forward selection process: trout (12.2%), aquatic vegetation (17%), minnows (19.4%), accumulated temperature (21.6%), shore substrate (22.4%), littoral slope (23.1%), conductivity (23.7%) and predation refuge (24.1%). We found two significant canonical axes RDA1 and RDA2 ($F = 108.88$, $P = 0.001$ and $F = 15.7$, $P = 0.001$ respectively) which explained 21.2% and 3.1% of the total variance respectively. Trout and minnows were highly positively correlated with RDA1 (Spearman coefficient 0.75, $P < 0.001$ and 0.69, $P < 0.001$ respectively) while the other selected variables were all negatively correlated with RDA1 (Fig. 2B and Table A1). Trout and minnows moved from nearer the second axis in the unconstrained configuration (PCA) to closer to the first axis in the constrained diagram (RDA) (see Fig. 2B in comparison with insert). In the RDA diagram all conspicuous macroinvertebrate taxa, except Mollusca, were situated in the opposite direction of trout and minnows, showing negative correlation among them. Particularly, Notonectidae, Coleoptera and Trichoptera, were highly negatively correlated with fish. Gerridae and Odonata were correlated with low littoral slopes and vegetated shores, but also appeared quite associated with high accumulated temperatures and conductivities, and slightly connected to the presence of macrophytes and predation refuges. Mollusca appeared in the central undefined part of the figure. Conspicuous macroinvertebrate richness was clearly lower in the RDA region closer to trout and minnows presence and higher in the opposite area (Fig. 2B). Level fluctuation was not significant in explaining the conspicuous macroinvertebrate assemblage structure and it was excluded in the forward selection procedure. The RDA analysis including significant MEMs to account for space showed almost the same pattern and importance of the selected environmental and stressor variables, but explained variance rose to 34% (adjusted $R^2 = 0.31$, Fig. A2).

Figure 2 (next page). RDA correlation triplot of (A) amphibian, (B) conspicuous macroinvertebrates and (C) planktonic crustacean assemblages. Variables are written with capital letter and indicated by solid arrows. Taxa are written with low case italic font and indicated by dashed arrows. Lakes are drawn with circles as site scores (linear constraint scores). Circle size indicates the richness of taxa, between 0 and 5 (smaller and larger circles respectively). Frogs of *R. temporaria* were not considered as a taxon to compute amphibian richness. The small charts show the unconstrained ordination (PCA) of the selected variables by the RDA forward selection. Variable name abbreviations are given in Table 2. Taxa name abbreviations are; Amphibian assemblage (A): *R. temporaria* tadpole (*rtem.t*), *R. temporaria* frog (*rtem.f*), *A. obstetricans* (*aobs*), *B. spinosus* (*bspi*), *L. helveticus* (*lhelv*) and *Calotriton asper* (*casp*); Conspicuous macroinvertebrates (B): Gerridae (*ger*), Notonectidae (*not*), Odonata (*odo*), Coleoptera (*col*), Trichoptera (*tri*) and Mollusca (*mol*); Planktonic crustaceans assemblage (C): *Daphnia longispina* (*dlon*), *Daphnia pulicaria* (*dpul*), *Holopedium gibberum* (*hgib*), *Scapholeberis mucronata* (*smuc*), *Acanthocyclops vernalis* (*aver*), *Cyclops abyssorum* (*caby*), *Diaptomus cyaneus* (*dcya*), *Diaptomus castaneti* (*dcas*), *Eudiaptomus vulgaris* (e*vul*) and *Mixodiaptomus laciniatus* (*mlac*). Note that high values of principal component SLOPE are related to low littoral slope and vice versa. Also, high values of principal component SHORE are related to vegetated shore and low values are related to bare rocky shore (Table 3).

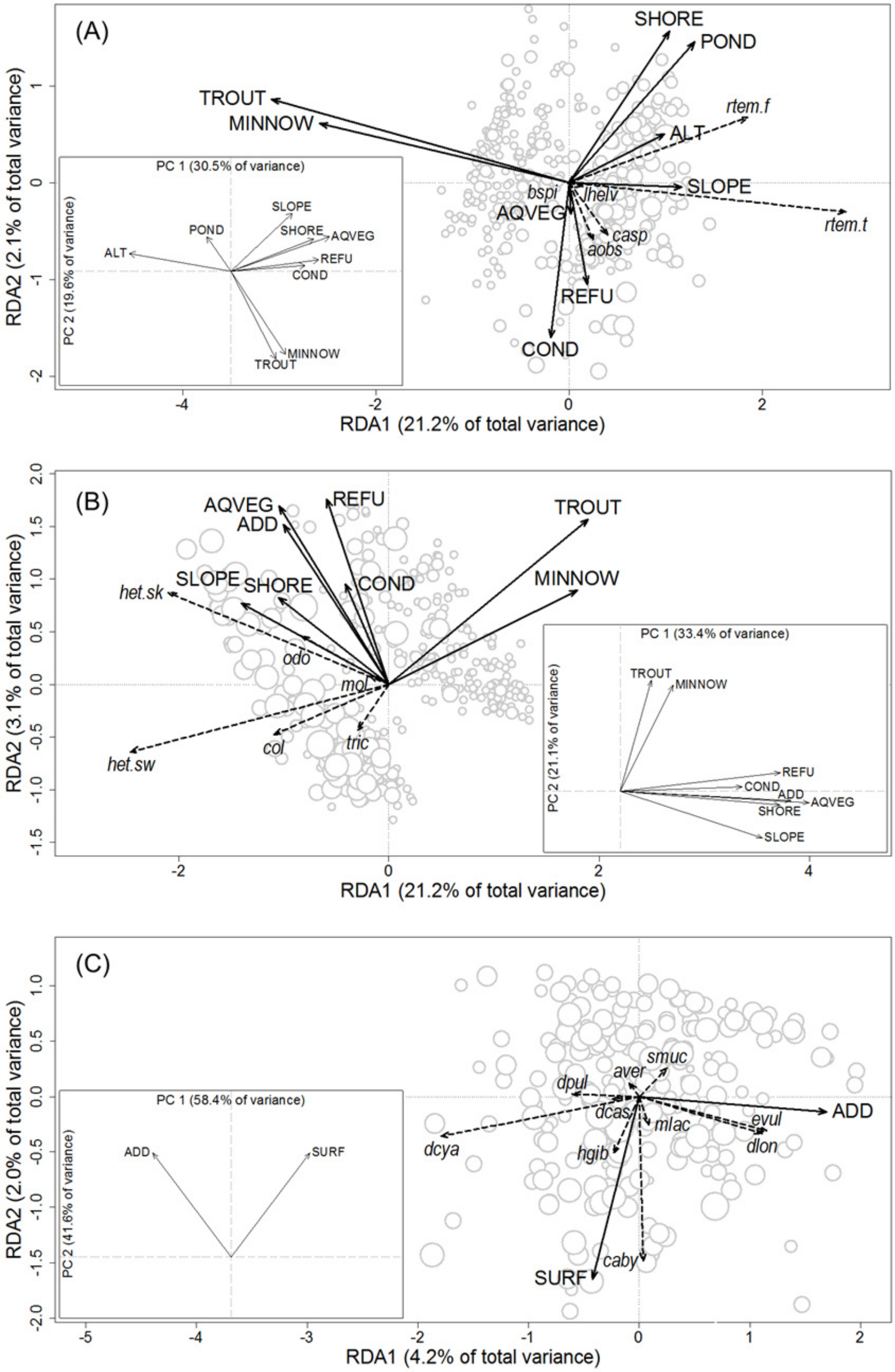

(A)
RDA2 (2.1% of total variance)
RDA1 (21.2% of total variance)
1
0
-1
-2
-4
-2
0
2
SHORE
POND
TROUT
MINNOW
rtem.f
ALT
SLOPE
rtem.t
bspi
lhelv
AQVEG
casp
aobs
REFU
COND
PC 1 (30.5% of variance)
PC 2 (19.6% of variance)
SLOPE
POND
SHORE
AQVEG
ALT
REFU
COND
MINNOW
TROUT
(B)
RDA2 (3.1% of total variance)
RDA1 (21.2% of total variance)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2
0
2
4
AQVEG
ADD
REFU
TROUT
SLOPE
SHORE
COND
MINNOW
het.sk
odo
mol
col
tric
het.sw
PC 1 (33.4% of variance)
PC 2 (21.1% of variance)
TROUT
MINNOW
REFU
COND
ADD
AQVEG
SHORE
SLOPE
(C)
RDA2 (2.0% of total variance)
RDA1 (4.2% of total variance)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-5
-4
-3
-2
-1
0
1
2
smuc
aver
dpul
dcas
mlac
evul
ADD
dlon
dcya
hgib
caby
SURF
PC 1 (58.4% of variance)
PC 2 (41.6% of variance)
ADD
SURF

Table 4. Percentage of variance explained by each component in the variation partitioning analyses for the three assemblages. Variation partitioning analyses were made with partial RDAs on each of the sub-sets of variables representing the components listed in Table 2: Environment, stressor and spatial broad-scale. No variable was identified from the spatial fine-scale component in the forward selection procedures for all three assemblages (see results section for more details). The *P* values from the ANOVA tests of the partial RDAs are written between brackets. The % of explained variance was computed on the Adjusted R^2 values in all cases.

Component	Amphibians	Conspicuous macroin-vertebrates	Planktonic crustaceans
Environment (pure)	2.8 (0.001)	5.4 (0.001)	2.9 (0.001)
Stressor (pure)	5.4 (0.001)	5.6 (0.001)	0 (0.616)
Broad-scale (pure)	11.6 (0.001)	7.6 (0.001)	20.9 (0.001)
Environment & Stressor	2.4	2.6	2
Stressor & Broad-scale	3.5	2.7	2
Environment & Broad-scale	2.7	4.3	1.6
Environment & Stressor & Broad-scale	5.1	3.2	0.7
Residuals (not explained)	66.5	68.6	73.5

In the forward selections made *a priori* for variation partitioning, 7 variables in the environmental fraction were selected: surface (9.4%), aquatic vegetation (12.2%), accumulate temperature (13.1%), shore substrate (13.7%), altitude (14.5%), littoral slope (15.1%) and conductivity (15.6%); 2 variables in the stressor fraction: trout (12.2%) and minnows (14.1%); and 28 MEMs in the spatial broad-scale fraction. Level fluctuation wasn't retained in the stressor fraction, and no MEMs were selected in the spatial fine-scale fraction. Variation partitioning (Table 4 and Fig. A4B) identified a significant pure broad-scale spatial fraction (adjusted R^2 = 0.076, P = 0.001): a significant pure stressor fraction (adjusted R^2 = 0.056, P = 0.001) that was of similar magnitude to the significant pure environmental fraction (adjusted R^2 = 0.054, P = 0.001). Trout and minnows together as a stressor fraction, explained approximately 30% of the exclusive explained variance for the conspicuous macroinvertebrate assemblage.

Between 70% and 88% of the conspicuous macroinvertebrate occurrences were placed in the contingency where there were no stressors present, except Mollusca which also occurred if there was presence of trout or minnows (Table A3; Fig. A5). In the Log-linear models (Table 5), the factor 'trout' showed significant negative effect on the occurrence of Notonectidae, Odonata, Coleoptera and Trichoptera (Table 5; Fig. 3G1, 3H1, 3I1 and 3J1) but not on Gerridae and Mollusca (Table 5). The factor 'minnows' had significant negative effect on the occurrence of Gerridae, Notonectidae, Odonata and Coleoptera (Table 5; Fig. 3F1, 3G2, 3H2 and 3I2), but not on Trichoptera and Mollusca (Table 5). The factor 'level fluctuation' had significant negative effect only on Gerridae (Table 5; Fig. 3F2). We also found a three-way significant interaction among Gerridae, trout and minnows (Table 5; Fig. 3F3), where trout showed a notable negative effect on the taxon occurrence, irrespective of whether there was presence or absence of minnows. However, when trout were absent, the presence of minnows caused a larger negative effect on Gerridae occurrence.

3.3. Planktonic crustaceans

We obtained a significant RDA (F = 8.04, P = 0.001) which explained 6.2% of the variance of the planktonic crustacean assemblage (adjusted R^2 = 0.05) with 2 variables selected by the forward selection process: accumulated temperature (3.8) and surface (5.4). We found two significant canonical axes RDA1 and RDA2 (F = 10.96, P = 0.001 and F = 5.11, P = 0.002 respectively) which explained 4.2% and 2.0% of the total variance respectively. Trout, minnows and level fluctuation were all three excluded in the forward selection process and were not related to the planktonic crustacean assemblage. The two selected variables exhibited equidistance from the two principal unconstrained axes (PCA), while in the constrained configuration (RDA), accumulated temperature was situated near RDA1 and surface was quite strongly linked with RDA2 (Fig. 2C; Table A1). In the RDA diagram, *D. longispina* and *E. vulgaris* showed quite good correlation with high values of accumulated temperature, while *D. cyaneus* and *D. pulicaria* were more associated with cold sites. *H. gibberum* and to a degree *C. abyssorum* were more correlated with high values of surface area, while *S. mucronata* was found to be better connected with small sites. *D. castaneti*, *A. vernalis* and *M. laciniatus* appeared in the central undefined part of the chart. Planktonic crustacean richness did not display any clear pattern (Fig. 2C). The RDA analysis including significant MEMs to account for space showed roughly the same pattern and importance of the selected environmental variables, but it explained a substantially higher amount of variance (33%; adjusted R^2 = 0.25; Fig. A3).

Table 5. Significance of the chi-squared test (*P*-value) for the interest interaction terms in the log-linear model for each taxon included in the assemblages. The interest interaction terms are shown between brackets in the head of the table; *tx* means the taxa in each line; T the factor trout presence/absence; M the factor minnow presence/absence; L the factor artificial water-level fluctuation presence/absence. The significant terms (*P*< 0.05) are written in bold type. NA indicates that it was not possible to compute the p-value of the chi-squared test due the existence of cells with zero cases.

	Interest interaction terms						
	Two-way			Three-way			Four-way
Taxon	[T*tx*]	[M*tx*]	[L*tx*]	[TM*tx*]	[TL*tx*]	[ML*tx*]	[TML*tx*]
Rana temporaria tadpole	**<0.001**	**<0.001**	0.569	0.143	**0.046**	**0.014**	1
Rana temporaria frog	**<0.001**	0.143	**0.028**	0.053	0.188	0.523	1
Alytes obstetricans	**0.032**	0.382	0.348	0.349	NA	1	1
Bufo spinosus	0.975	0.569	0.433	**0.025**	0.551	0.556	NA
Calotriton asper	**0.005**	0.283	0.335	0.137	1	1	1
Lissotriton helveticus	0.317	0.125	0.659	1	1	NA	1
Gerridae	0.198	**<0.001**	**0.015**	**0.049**	0.380	0.068	1
Notonectidae	**0.001**	**<0.001**	0.356	0.088	0.183	0.158	1
Odonata	**0.016**	**0.005**	0.176	NA	0.189	1	1
Coleoptera	**<0.001**	**0.010**	0.614	0.136	0.079	0.078	NA
Trichoptera	**0.023**	0.137	0.297	0.401	NA	1	1
Mollusca	0.399	0.085	0.151	0.613	NA	1	1
Daphnia longispina	0.792	0.465	0.077	0.840	**0.033**	0.903	1
Daphnia pulicaria	0.363	**0.017**	0.110	0.448	1	NA	1
Holopedium gibberum	0.755	0.478	0.136	0.132	0.819	0.192	NA
Scapholeberis mucronata	0.360	0.103	0.689	0.710	NA	0.547	1
Acanthocyclops vernalis	0.306	0.277	0.233	0.398	0.337	0.960	1
Cyclops abyssorum	0.727	0.069	0.238	**0.009**	0.128	0.749	1
Diaptomus cyaneus	0.225	0.106	**0.012**	**0.025**	0.761	0.695	NA
Diaptomus castaneti	0.984	**0.049**	0.452	0.589	0.176	0.274	1
Eudiaptomus vulgaris	0.675	**0.009**	0.888	0.836	1	0.291	1
Mixodiaptomus laciniatus	0.546	0.945	0.232	0.052	0.540	0.105	NA

In the forward selections made before variation partitioning, 2 environmental variables were selected: accumulated temperature (3.8%) and surface (5.4%). In the case of the stressor component, minnow was selected, but the later partial RDA was not significant. Trout and level fluctuation were not selected. In the spatial broad-scale fraction 31 MEMs were retained, but no MEM was identified in the spatial fine-scale fraction. Variation partitioning (Table 4; Fig. A4C) identified a large significant pure broad-scale spatial fraction (adjusted R^2 = 0.209, P = 0.001) and a small significant pure environmental component (adjusted R^2 = 0.029, P = 0.001). The analysis did not distinguish a significant stressor portion (Table 4).

The occurrences of all planktonic crustacean taxa were evenly distributed in the contingency table where there were no stressors present and the contingencies with presence of trout, minnows and level fluctuation (Table A3; Fig. A5). In the Log-linear models (Table 5), the factor 'trout' did not show any significant effect on any planktonic crustacean species. In contrast, the factor 'minnows' had significant negative effects on the occurrence of *D. pulicaria* and *D. castaneti* (*Table 5*; Fig. 3L, 3O) and a significant positive effect on *E. vulgaris* (Table 5; Fig. 3P). The factor 'level fluctuation' had a significant negative effect on the occurrence of *D, cyaneus* (Table 5; Fig. 3N1). We also found 3 three-way significant interactions involving *D. longispina*, *C. abyssorum* and *D. cyaneus* (table 5). In the interaction between *D. longispina*, trout and level fluctuation, artificial water-level fluctuation showed a negative effect on the species occurrence, irrespectively of the presence or absence of trout (Fig. 3K). In the interaction between *C. abyssorum,* trout and minnows, we found maximum occurrence when there were both trout and minnows present. In contrast, minnows had a strong negative effect on *C. abyssorum* occurrence in the absence of trout but not when trout were present (Fig. 3M). When minnows were absent there was a moderate occurrence of *C. abyssorum* irrespective of presence or absence of trout. We found a similar pattern in the significant interaction between *D. cyaneus*, trout and minnows, but in this case the highest occurrence of the crustacean was found when both trout and minnows were absent (Fig. 3N2)**.**

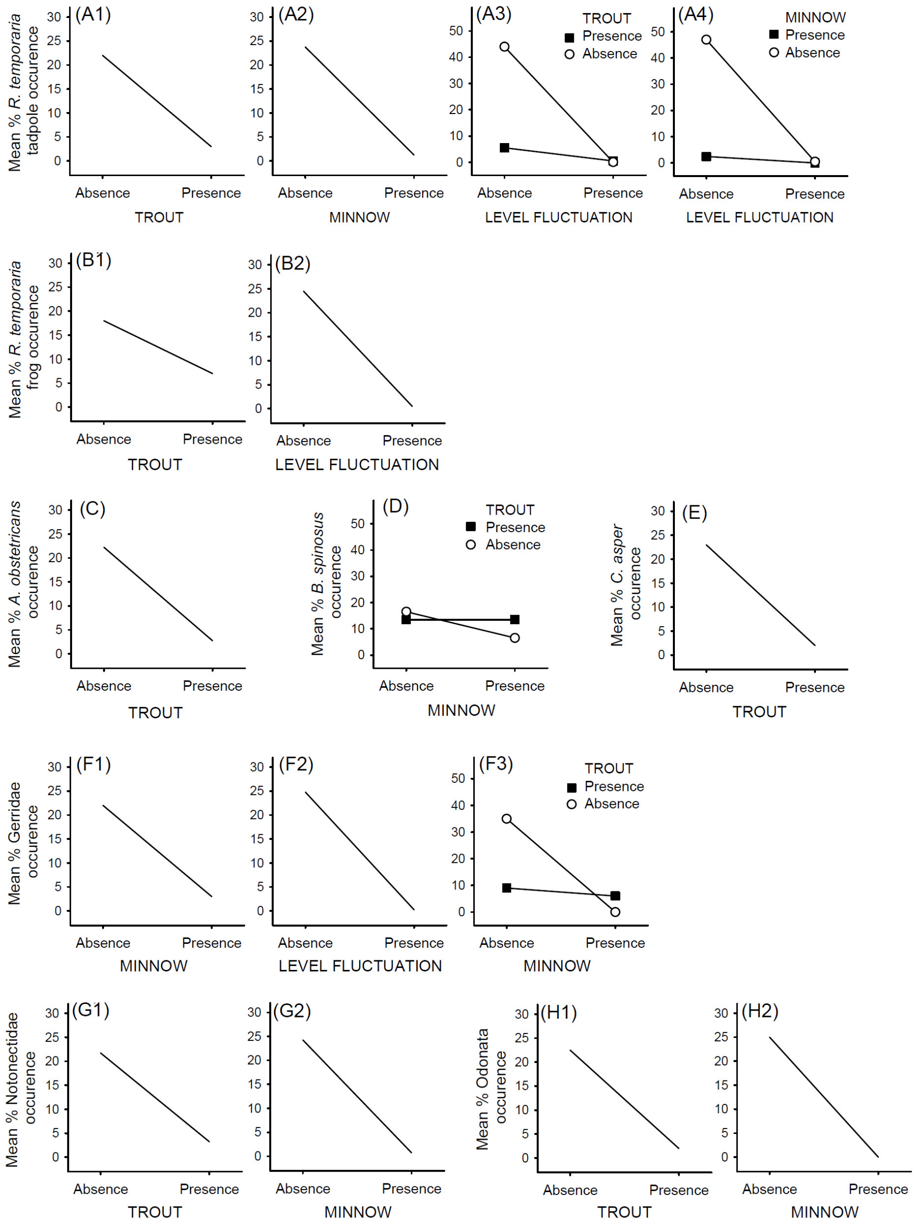
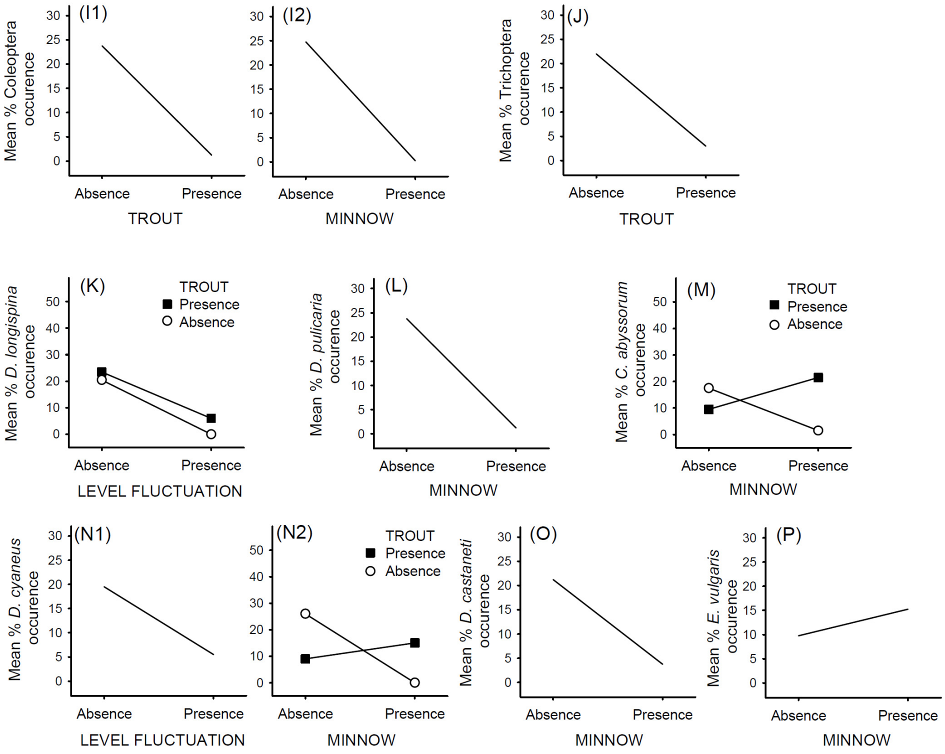


Figure 3 (two pages). Interaction plots of all significant interaction terms found in the log-linear models. The Y axis shows the % of the taxon occurrence for (A) *R. temporaria* tadpole, (B) *R. temporaria* frog, (C) *A. obstetricans*, (D) *B. spinosus*, (E) *C. asper*, (F) Gerridae, (G) Notonectidae, (H) Odonata, (I) Coleoptera, (J) Trichoptera, (K) *D. longispina*, (L) *D. pulicaria*, (M) *C. abyssorum*, (N) *D. cyaneus*, (O) *D. castaneti* and (P) *E. vulgaris*.

Figure 3. (continued).



4. Discussion

In this study we described the effects of three stressors on high mountain lake fauna: the presence of introduced trout and minnows and habitat alteration by artificial water-level fluctuation associated with human exploitation. Our results clearly indicate that, although the presence of exotic trout has a substantial negative impact, both exotic minnows and artificial water-level fluctuation are also two clear local stressors of these ecosystems. RDA and Variation Partitioning analysis showed that at assemblage level, minnows together with trout have a strong negative impact on amphibian and macroinvertebrate occurrence. At taxon level, log-linear models showed that trout was the most impactful factor on taxa occurrence with 14 negative interactions (8 two-way and 6 three-way), but minnow was also involved in 12 negative interactions (7 two-way and 5 three-way), and level fluctuation in 6 negative interactions (3 two-way and 3 three-way). Several studies carried out in high mountain lakes during recent decades have described exotic trout as a strong local stressor due its impact on different components of the ecosystem (e.g. Knapp et al. 2001b, Orizaola and Braña 2006, Pilliod et al. 2010, Tiberti et al. 2014b). Apart from introduced trout, the exposure to pesticides has also been tested as a stressor on high mountain watersheds, showing stronger impact than trout on the distribution of the Sierra Nevada, USA anuran *Lithobates muscosa* (Davidson and Knapp 2007).

4.1. Stressors interaction

The type of interaction among the three stressors of this study, either additive, synergistic or antagonistic (e.g. Darling et al. 2010), can be described as being antagonistic. The impact of trout, minnows and water-level fluctuation is clearly cumulative, because in some cases they affect the different species of different assemblages. We found that, while trout impacts on amphibian occurrence but not on zooplankton, minnows impact on some zooplankton species but much less on amphibians. Level fluctuation also impacted on the occurrence of a few of zooplankton species that appear not to be affected by trout and minnows. Nevertheless, the high degree of field overlap and correlation among the three stressors suggests that the total impact may not be equivalent to the sum of the separate impacts (additive effect) nor higher (synergistic interaction), rather indicating an antagonistic response that leads to an impact greater than the effect of each stressor separately, but being less than the sum of them (Didham et al. 2007).

4.2. Trout

The impact caused by trout on Pyrenean high mountain lake fauna is clear and similar to that found in the lakes of other mountain ranges. At assemblage level, trout is the factor that most determines the presence of amphibians and conspicuous macroinvertebrates, as well as the taxonomic richness of both assemblages. The RDA of amphibians showed a direct impact on *R. temporaria* either as tadpoles or frogs, but also indirectly on *A. obstetricans* and *C. asper* by linking the existence of predation refuge to them. Moreover, trout and minnows together as a stressor fraction explained approximately a third of the exclusive explained variance for amphibian and conspicuous macroinvertebrate assemblages. At taxon level, the presence of trout impacts most on amphibian and conspicuous macroinvertebrate taxa occurrence, but not on planktonic crustaceans. Our findings are in general consistent with the results of previous studies on salmonid impact on high mountain lakes. Strong effects of trout on some amphibians and reptiles have been also found in several mountain ranges of the US and Europe (Knapp 2005, Orizaola and Braña 2006, Pope et al. 2008, Pilliod et al. 2010, Tiberti and von Hardenberg 2012). The lack of impact that we found on *B. spinosus* can be explained by the unpalatability and toxicity of this species' tadpole (Kruse and Stone 1984, Benard and Fordyce 2003). In fact, those species of the genus *Bufo* studied so far in high mountain lakes seem not to be adversely impacted by trout, but rather favored (Knapp 2005, Orizaola and Braña 2006, Welsh et al. 2006). Habitat structure was also previously found as important to give shelter to some species such as *A. obstetricans* in mountain lakes of the north of the Iberian peninsula (Orizaola and Braña 2006). The lack of impact on *L. helveticus* is likely related to its low occurrence in our data set: *L. helveticus* was found in 8 of the 388 sampled lakes, and only in one of them in sympatry with trout.

Previous studies also showed a clear effect of trout on the macroinvertebrate community, mainly affecting the largest taxa, as was found in the Sierra Nevada, California (Knapp et al. 2001b, Parker et al. 2010), in the Pyrenees (de Mendoza et al. 2012) and in the western Italian Alps (Tiberti et al. 2014b). The absence of impact on Gerridae is likely to be related to their living mainly in the shore fringe, where they can easily find shelter from trout predation. The lack of detection of trout effect on Mollusca may be associated with the shortcomings of the visual method, but it is consistent with previous studies where burrowing taxa, such as the genus *Pisidium,* showed the same distribution in the presence or absence of trout (Knapp et al. 2001b). In the case of planktonic crustaceans, although we did not find significant effects of trout on their occurrence, other studies in other high mountain lakes have found an effect on abundance (Knapp et al. 2001b, Parker et al. 2010, Tiberti et al. 2014a).

4.3. Minnows

Minnows had similar impacts to trout on the littoral fauna, but greater impact on the planktonic crustaceans. Our results showed a strong impact on amphibian and conspicuous macroinvertebrate richness. Minnows were also the second most influent variable (after trout) for both assemblages in

Pyrenean lakes. At taxon level, minnows had a negative effect on the occurrence of tadpoles of *R. temporaria* and *B. spinosus* and on all conspicuous macroinvertebrates except Trichoptera and Mollusca. Although it is hard to find information about the impact of minnows on the amphibians and macroinvertebrates of high mountain lakes, in lowland and boreal lakes minnow species were shown to negatively affect these organisms (e.g. Werner et al. 2007, Naestad and Brittain 2010). Minnows have been reported as a cause of the decline of frogs, paedomorphic newts and the species richness of amphibian tadpoles (Denoel et al. 2005, Eaton et al. 2005, Werner et al. 2007). Opposite to the case of trout, the faint negative effect of minnows on the occurrence of *B. spinosus* that we found in this study, seems especially remarkable, since the genus *Bufo* is well known to be unpalatable for fish (e.g. Welsh et al. 2006). Minnows have also been described as drivers of change in the composition of the littoral invertebrate community, causing the decrease of Gammaridae, Ephemeroptera, Plecoptera and Trichoptera and favoring the increase of Chironomidae and Oligochaeta (Naestad and Brittain 2010).

Our findings indicate that in contrast with trout, minnows also impact on the occurrence of some planktonic crustaceans, mainly the largest species. This has been described before in two lakes of the Northern Calcareous Alps of Austria, where it was suggested that European minnow caused the disappearance of the larger crustaceans, leading to the predominance of smaller zooplankton (Schabetsberger et al. 2006). In contrast, the same authors found that the autochthonous newt *Ichthyosaura alpestris* had no effect on zooplankton despite being a top predator. From the contingency table analyses we found an interesting interaction between minnows and trout as stressors. While trout did not show any individual negative impact on planktonic crustacean occurrence, minnow only showed a negative effect in absence of trout. Actually, in this interaction, the most severe situation in terms of *C. abyssorum* and *D. cyaneus* occurrence was in the absence of trout, and when minnows were the only fish in the lakes. This finding is consistent with previous studies from Scandinavian boreal lakes where the authors had found that minnows had no impact on zooplankton when trout was present in the lake (e.g. Larsson et al. 2010). Minnows live in the littoral fringe of the lake when trout is also present (Museth et al. 2007, Borgstrom et al. 2010), where they are unable to feed on planktonic crustaceans. Moreover, experimental and field studies have found that the size selective predation of minnows on zooplankton leads to a trophic cascade to the phytoplanktonic community, increasing its biomass and the proportion of small cells or nanoplankton (Proulx et al. 1996, Labaj et al. 2013). Minnows experimented a worrisome widespread during the late 1800s and throughout the twentieth century in the mountain and high mountain lakes of several areas, for instance Scotland, Scandinavia, and the Pyrenees where they have been introduced to the 27-46% of the lakes, related to live-bait trout angling (Maitland and Campbell 1992, Museth et al. 2007, Miró and Ventura 2015). In some Pyrenean lakes, trout have been reported to disappear some decades after the arrival of minnows (Miró and Ventura 2015). Since minnows are becoming a fast-spreading invasive species in high mountain lakes, their control will be a great challenge for the future.

4.4. Artificial water-level fluctuation

Water-level fluctuation has a certain impact on high mountain lake fauna, usually masked by introduced fish. We have not detected any impact of water-level fluctuation at assemblage level. At taxon level, we found a negative effect on the occurrence of two taxa linked with the littoral habitat, *R. temporaria* frogs and Gerridae, and also on the planktonic crustaceans *D. longispina* and *D. cyaneus*. Previous studies have shown that artificial water-level fluctuation greatly damages the littoral community of macrophytes (e.g. Gacia and Ballesteros 1998, Krolova et al. 2013). Furthermore, on regulated boreal lakes, a decrease larger than 3.46 m during winter prevented lakes from being assessed as of good ecological status based on macrophytes, macroinvertebrate, and fish assemblages sampled in littoral zone (Sutela et al. 2013). Dammed lakes of the Pyrenees can experience a summer drawdown ranging from 1 to 89 m depending on the lake, with an overall mean of 18.5 m (data from MOPU 1988, Catalan et al. 1997). Hence, this suggests that the impact on amphibian and conspicuous macroinvertebrate assemblages in the littoral zone of some Pyrenean lakes most likely exists, but is masked by the co-occurrence of trout and minnows in the same lakes. It is highly probable that there are negative impacts on the benthos of high-mountain lakes caused by water-level fluctuation, as was described during the filling of Lake Baciver in the Pyrenees in 1991-1992, following damming in 1990; the strong attenuation of light, and the winter oxygen depletion, appeared to be the factors responsible for the complete disappearance of the benthic macrophyte community (Gacia and Ballesteros 1996, 1998).

Taxon-level analyses in relation to habitat alteration due to artificial water-level fluctuation showed a negative effect on the occurrence of the planktonic crustaceans *D. longispina* and *D. cyaneus*. Specifically in the case of *D. longispina* (the second most frequently encountered crustacean in the Pyrenees), the significant interaction with trout and level fluctuation showed that water-level fluctuation had a negative effect on the species occurrence, irrespective of whether trout were present or not. Since the surface of the lake was one of the two environmental variables that we found to be important for planktonic crustaceans, the impact could result through the drastic reduction of the size and water volume during the lower level period. For some months during the summer or autumn, impounded high mountain lakes can become small lakes, or even ponds. Although it is hard to see from our dataset, due to the high coincidence of stressors and the lack of sites with level fluctuation and without fish, the reduction of depth may also prevent the vertical migration of planktonic crustaceans. This in turn probably prevents predator avoidance (Loose and Dawidowicz 1994) and allows introduced fish to catch them easily (Gonzalez and Tessier 1997). From this point of view, artificial water-level fluctuation through anthropogenic habitat modification, can enhance the impact of invasive trout and minnows on native fauna, as has been previously described (Didham et al. 2007). The damming of Pyrenean lakes began at the end of the nineteenth century in order to supply hydroelectric power to the nearby industrialized cities. Nowadays *ca.* 15% of Pyrenean high mountain lakes are impounded, mostly the largest and the deepest. Their management in combination with nature conservation is becoming a great challenge for the future too, particularly in some of the highly protected areas such as the Aigüestortes i Estany de Sant Maurici National Park, where 29% of the lakes >0.5ha are impounded or directly impacted by a dam.

4.5. Restoration

Restoration of high mountain lake fauna has been shown to be rapid when trout are removed (e.g. Knapp et al. 2007). It is well-known that high mountain lake fauna have low resistance, but high resilience, to fish introductions (Knapp et al. 2001b). Actually, several studies have demonstrated that removal of introduced fish allows a fast recovery of declining, and even lost, amphibians and macroinvertebrates (Vredenburg 2004, Knapp et al. 2007, Pope 2008, Pope et al. 2009). In the case of planktonic crustaceans, the species with parthenogenetic reproductive mode and greater longevity of diapausing eggs can easily recover after fish eradication even after long periods of fish presence but not obligatory sexual species and with lower longevity of diapausing eggs (Knapp and Sarnelle 2008).

On the other hand, an on-going initiative of lake restoration through minnow removal in the Pyrenees (within the framework of the LIFE+ LimnoPirineus project) suggests that high mountain lakes recover some sensitive fauna even before complete minnow eradication (M. Ventura, personal observation).

Eventually, we have realized that it is hard to find recorded experience or information on the possibilities of restoration of more natural regimes and limnological characteristics on artificial water-level fluctuation in high mountain lakes. This would therefore be an interesting field to explore in the future.

Acknowledgements

The authors would like to acknowledge all people who helped us during the field work. Also, we want to sincerely thanks the valuable assistance and facilities given in the field work by management teams, employees and nature wardens of the Catalan, Aragonese and French environmental agencies and from the Pyrenean protected areas of Aigüestortes i Estany de Sant Maurici National Park, Pyrénées National Park, Alt Pirineu Natural Park, Posets-Maladeta Natural Park and Valles Occidentales Natural Park. David O'Brien kindly assisted in editing the manuscript and made useful suggestions to improve it. Economic support was provided by the Spanish Government projects Fundalzoo (CGL2010-14841) and Invasivefish (427/2011) and by the European Commission LIFE+ project LimnoPirineus (LIFE13 NAT/ES/001210). We also benefited from the Biodiversity Conservation Plan from ENDESA S. A. with the project number 6900014499.

Appendix Chapter 3

Supplementary tables and figures

1. Materials and Methods

1.1. Supplementary tables

Table A1. Spearman correlations between each variable included in the RDA analyses and the two most explanatory canonical axes obtained. For each assemblage we performed one RDA with all available environment and stressor variables together. The symbol − means that the variable was included in the analysis but was not selected by the forward selection process. X marks the variables related to the littoral zone, not included in the planktonic crustaceans' analyses. High values of principal component SLOPE are related to low littoral slope and vice versa. Also, high values of principal component SHORE are related to vegetated shore and low values are related to bare rocky shore (Table 3). * denote correlations significantly different from zero (P <0.05). The total variation explained by each axis is written between brackets below the axis name.

Variable name	Amphibians		Conspicuous MInvertebrates		Planktonic crustaceans	
	RDA1 (21.2%)	RDA2 (2.1%)	RDA1 (21.2%)	RDA2 (3.1%)	RDA1 (4.2%)	RDA2 (2.0%)
Altitude (ALT)	0.28*	0.06	−	−	−	−
Surface area (SURF)	−	−	−	−	-0.26*	-0.96*
Conductivity (COND)	0.09	-0.35*	-0.06	0.36*	−	−
Accumulated temperature (ADD)	−	−	-0.32*	0.57*	0.99*	-0.06
Littoral slope (SLOPE)	0.30*	-0.01	-0.46*	0.22*	X	X
Littoral substrate (LITTO)	−	−	−	−	X	X
Shore substrate (SHORE)	0.32*	0.42*	-0.41*	0.30*	X	X
Aquatic vegetation (AQVEG)	-0.01	-0.07	-0.32*	0.57*	X	X
Predation refuge (REFU)	0.05	-0.29*	-0.17*	0.64*	X	X
Ponds around (POND)	0.38*	0.39*	−	−	X	X
Trout (TROUT)	-0.84*	0.23*	0.75*	0.64*	−	−
Minnows (MINNOW)	-0.71*	0.16*	0.69*	0.37*	−	−
Level variation (LEVEL)	−	−	−	−	−	−

able A2. Spearman correlations between each variable included in the variation partitioning analyses and the two most explanatory canonical axes obtained in each partial RDA. For each assemblage we performed four partial RDAs, one for each component with the available variables. The symbol − means that the variable was included in the analysis but was not selected by the forward selection process. X marks the variables related to the littoral zone, not included in the planktonic crustaceans' analyses. High values of principal component SLOPE are related to low littoral slope and vice versa. Also, high values of principal component SHORE are related to vegetated shore and low values are related to bare rocky shore (Table 3). * denotes correlations significantly different from zero (P <0.05). The total variation explained by each axis is written between brackets below the axis name.

Component Environment Variable name	Amphibians		Conspicuous MInvertebrates		Planktonic crustaceans	
	RDA1 (3.9%)	RDA2 (0.1%)	RDA1 (7.1%)	RDA2 (0.1%)	RDA1 (2.7%)	RDA2 (2.0%)
Altitude (ALT)	0.11*	-0.29*	0.41*	0.22*	−	−
Surface area (SURF)	0.21*	-0.38*	0.47*	-0.28*	-0.65*	-0.58*
Conductivity (COND)	0.28*	-0.02	-0.28*	-0.27*	−	−
Accumulate temperature (ADD)	−	−	-0.61*	0.01	0.69*	-0.30*
Littoral slope (SLOPE)	-0.32*	0.69	-0.51*	0.17*	X	X
Littoral substrate (LITTO)	−	−	−	−	X	X
Shore substrate (SHORE)	-0.60*	-0.23*	-0.45*	0.47*	X	X
Aquatic vegetation (AQVEG)	−	−	-0.61*	-0.10*	X	X
Predation refuge (REFU)	−	−	−	−	X	X
Ponds around (POND)	-0.34*	0.06	−	−	X	X

Component Stressors Variable name	Amphibians		Conspicuous MInvertebrates		Planktonic crustaceans	
	RDA1 (8.0%)	RDA2 (0.01%)	RDA1 (6.9%)	RDA2 (0.1%)	RDA1	RDA2
Trout (TROUT)	-0.74*	-0.08	0.68*	-0.25*		
Minnows (MINNOW)	-0.56*	0.55*	0.65*	0.37*		
Level variation (LEVEL)	−	−	−	−		

Table A3. Taxon distribution for each combination of absence (A) and presence (P) of the three anthropogenic stressors considered (trout, minnows and artificial water-level fluctuation). Total occurrences of each taxon were written as absolute values and as percentage between brackets.

		% Occurrence for each contingency							
	TROUT:	A				P			
	MINNOW:	A		P		A		P	
	LEVEL FLUCTUATION:	A	P	A	P	A	P	A	P
Taxa	Total occurrence (%)								
Rana temporaria tadpole	136 (35.1)	87	0	1	0	7	1	4	0
Rana temporaria frog	132 (34)	67	0	5	0	15	1	11	1
Alytes obstetricans	18 (4.6)	89	0	0	0	6	0	5	0
Bufo spinosus	15 (3.9)	33	0	13	0	27	0	20	7
Calotriton asper	24 (6.2)	88	0	4	0	8	0	0	0
Lissotriton helveticus	8 (2.1)	88	0	0	0	13	0	0	0
Gerridae	103 (26.5)	70	0	0	0	17	1	12	0
Notonectidae	119 (30.7)	87	0	0	0	9	1	3	0
Odonata	21 (5.4)	90	0	0	0	5	5	0	0
Coleoptera	56 (14.4)	95	0	0	0	2	1	2	0
Trichoptera	25 (6.4)	88	0	0	0	8	0	4	0
Mollusca	16 (4.1)	56	0	0	0	31	0	13	0
Daphnia longispina	168 (68.6)	36	0	5	0	20	2	27	10
Daphnia pulicaria	17 (6.9)	71	0	0	0	24	0	5	0
Holopedium gibberum	15 (6.1)	20	7	0	0	7	7	39	20
Scapholeberis mucronata	16 (6.5)	31	0	13	0	13	0	31	13
Acanthocyclops vernalis	16 (6.5)	31	0	0	0	25	6	19	19
Cyclops abyssorum	180 (73.5)	33	2	3	0	17	2	30	13
Diaptomus cyaneus	50 (20.4)	48	4	0	0	14	4	16	14
Diaptomus castaneti	13 (5.3)	46	8	0	0	31	0	8	7
Eudiaptomus vulgaris	41 (16.7)	27	0	7	0	12	0	37	17
Mixodiaptomus laciniatus	30 (12.2)	30	4	0	0	13	7	33	13

1.2. Supplementary Figures

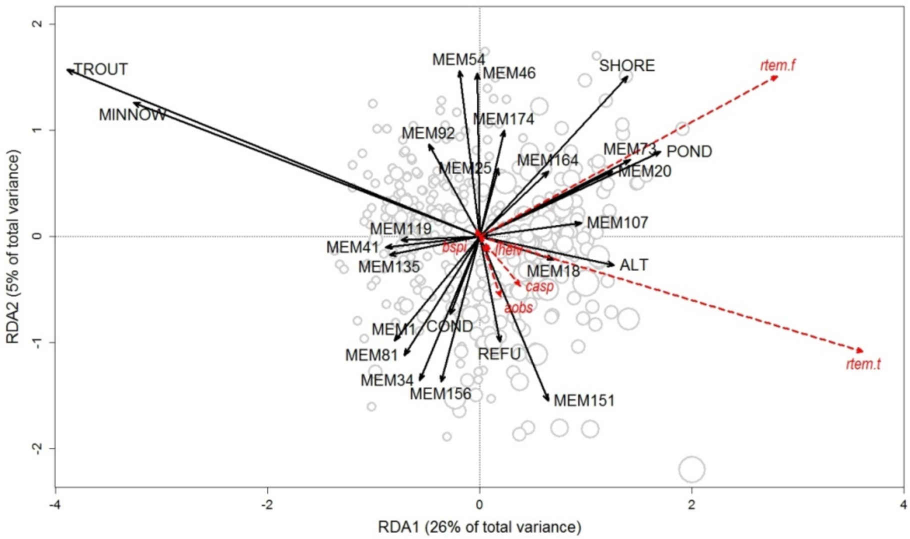


Figure A1. RDA correlation triplot of amphibian assemblage using MEMs to account for space. Variables are written with black capital letters. Taxa are written with red low case Italic font. Lakes are drawn with circles as site scores (linear constraints scores). Circle size indicates the richness, between 0 and 5 taxa (smaller and larger circles respectively). Frogs of *R. temporaria* were not considered as a taxon to compute amphibian richness. Variable name abbreviations are given in Table 2. Taxon name abbreviations are: *R. temporaria* tadpole (*rtem.t*), *R. temporaria* frog (*rtem.f*), *A. obstetricans* (*aobs*), *B. spinosus* (*bspi*), *L. helveticus* (*lhelv*) and *Calotriton asper* (*casp*). Note that high values of principal component SHORE are related to vegetated shore and low values are related to bare rocky shore (Table 3). RDA analysis was significant (F = 7.95, P = 0.001), and explained 35% of the variance (adjusted R^2 = 0.31). Canonical axes RDA1 and RDA2 were significant (F = 153.4, P = 0.001 and F = 29.27, P = 0.001 respectively). 25 variables were identified by the forward selection process (in the selection order and followed by the cumulative percentage of fit based on the adjusted R^2 value): TROUT (15.4%), SHORE (17.7%), ALT (19.1%), POND (20%), REFU (20.9%), MINNOW (21.8%), MEM73 (22.5%), COND (23.2%), MEM20 (23.9%), MEM151 (24.6%), MEM34 (25.2%), MEM25 (25.6%), MEM41 (26.2%), MEM174 (26.7%), MEM156 (27.2%), MEM107 (27.7%), MEM54 (28.1%), MEM1 (28.5%), MEM46 (28.9%), MEM81 (29.3%), MEM164 (29.7%), MEM18 (30.1%), MEM92 (30.4%), MEM119 (30.7%), and MEM135 (30.9%).

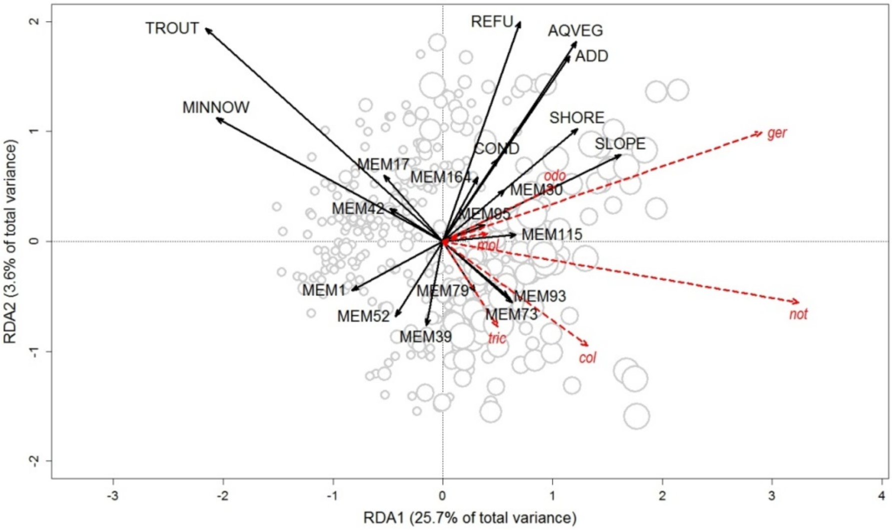


Figure A2. RDA correlation triplot of the conspicuous macroinvertebrate assemblage using MEMs to account for space. Variables are written with black capital letters. Taxa are written with red low case Italic font. Lakes are drawn with circles as site scores (linear constraints scores). Circle size indicates the richness, between 0 and 5 taxa (smaller and larger circles respectively). Variable name abbreviations are given in Table 2. Taxon name abbreviations are: Gerridae (*ger*), Notonectidae (*not*), Odonata (*odo*), Coleoptera (*col*), Trichoptera (*tri*) and Mollusca (*mol*). Note that high values of principal component SLOPE are related to low littoral slope and vice versa. Also, high values of principal component SHORE are related to vegetated shore and low values are related to bare rocky shore (Table 3). RDA analysis was significant (F = 9.59, P = 0.001), and explained 34% of the variance (adjusted R^2 = 0.31). Canonical axes RDA1 and RDA2 were significant (F = 149.2, P = 0.001 and F = 20.59, P = 0.001 respectively). 20 variables were identified by the forward selection process (in the selection order and followed by the cumulative percentage of fit based on the adjusted R^2 value): TROUT (12.2%), AQVEG (17%), MINNOW (19.4%), ADD (21.6%), MEM42 (22.7%), MEM1 (23.8%), MEM73 (24.8%), MEM95 (25.5%), MEM164 (26.2%), REFU (26.8%), MEM93 (27.3%), MEM17 (27.9%), SHORE (28.4%), MEM39 (28.9%), SLOPE (29.3%), MEM115 (29.6%), COND (29.9%) MEM79 (30.2%), MEM52 (30.5%), and MEM30 (30.7%)

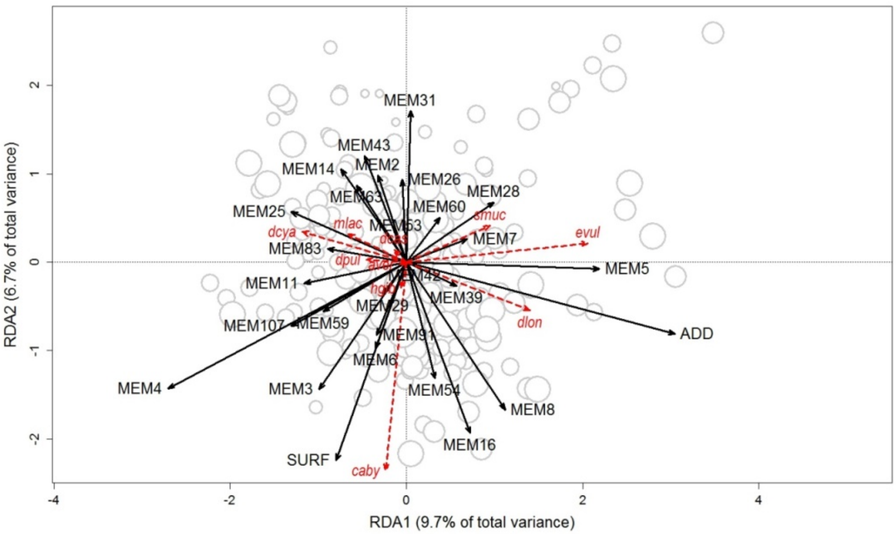


Figure A3. RDA correlation triplot of the planktonic crustacean assemblage using MEMs to account for space. Variables are written with black capital letter. Species are written with red low case Italic font. Lakes are drawn with circles as site scores (linear constraints scores). Circle size indicates the richness, between 0 and 5 species (smaller and larger circles respectively). Variable name abbreviations are given in Table 2. Taxon name abbreviations are: *Daphnia longispina* (*dlon*), *Daphnia pulicaria* (*dpul*), *Holopedium gibberum* (*hgib*), *Scapholeberis mucronata* (*smuc*), *Acanthocyclops vernalis* (*aver*), *Cyclops abyssorum* (*caby*), *Diaptomus cyaneus* (*dcya*), *Diaptomus castaneti* (*dcas*), *Eudiaptomus vulgaris* (*evul*) and *Mixodiaptomus laciniatus* (*mlac*). RDA analysis was significant (F = 3.89, P = 0.001), and explained 33% of the variance (adjusted R^2 = 0.25). Canonical axes RDA1 and RDA2 were significant (F = 24.19, P = 0.001 and F = 23.71, P = 0.001 respectively). 28 variables were identified by the forward selection process (in the selection order and followed by the cumulative percentage of fit based on the adjusted R^2 value): ADD (3.8%), MEM4 (6.9%), MEM8 (8.5%), SURF (10%), MEM5 (11.1%), MEM14 (12.1%), MEM39 (12.9%), MEM3 (13.8%), MEM26 (14.6%), MEM6 (15.3%), MEM16 (16.1%), MEM83 (16.8%), MEM107 (17.5%), MEM7 (18.1%), MEM11 (18.8%), MEM2 (19.4%), MEM63 (20.1%), MEM31 (20.6%), MEM91 (21.1%), MEM25 (21.6%), MEM43 (22.1%), MEM42 (22.6%), MEM60 (23.1%), MEM59 (23.5%), MEM28 (23.9%), MEM54 (24.2%), MEM53 (24.6%),and MEM29 (25%).

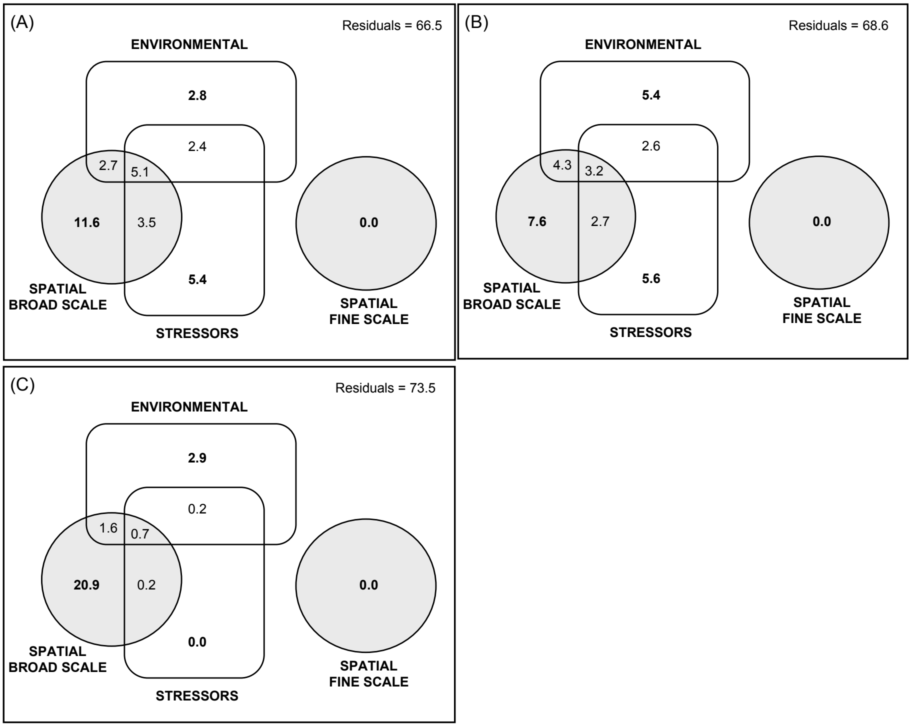
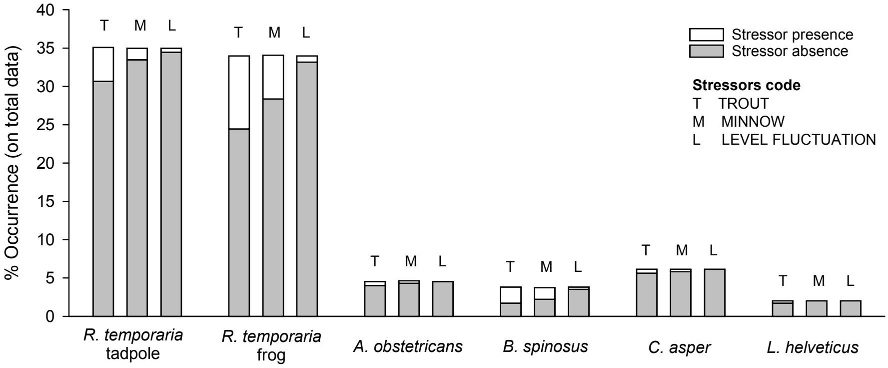


Figure A4. Venn diagrams showing the percentages of the variation partitioning of the three assemblages: (A) amphibians, (B) conspicuous macroinvertebrates and (C) planktonic crustaceans. Diagrams show the variation partitioning schemes with 4 data matrices, 2 of them containing random explanatory variables (environmental and stressors) and the other 2 containing eigenfunction sub-models (MEMs of broad- and fine-scales). Structure of the diagrams based on Legendre et al. (2012).

(A)



(B)

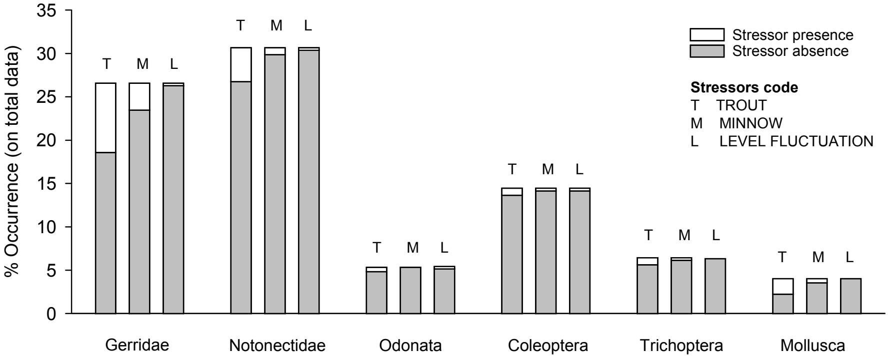
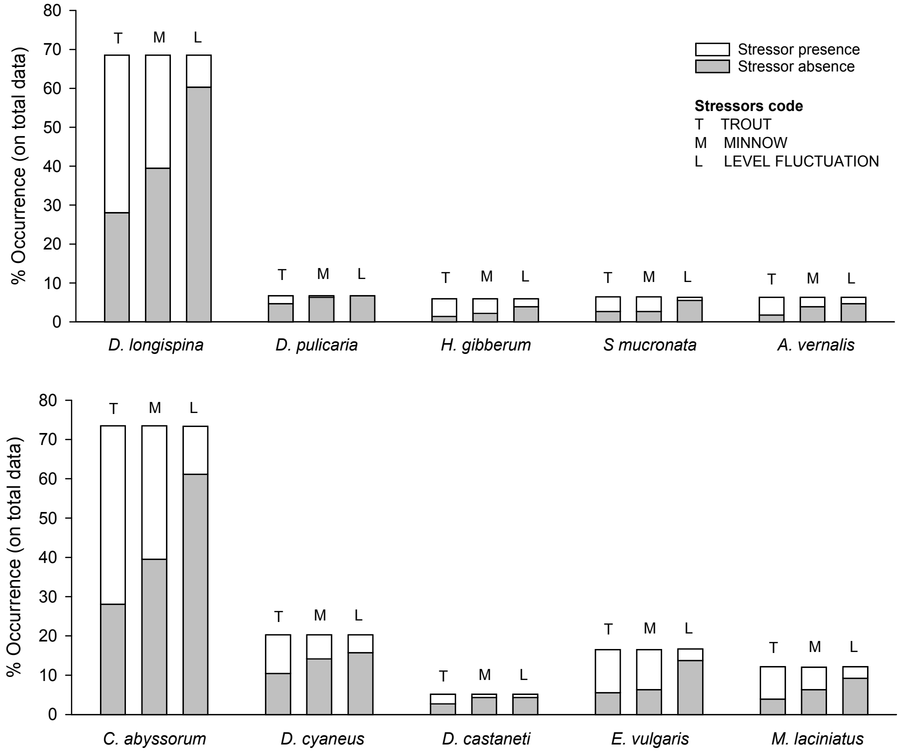


Figure A5 (two pages). Taxon distribution in presence or absence of each stressor for our three assemblages: (A) Amphibians, (B) conspicuous macroinvertebrates and (C) planktonic crustaceans. All data were used to draw each bar.

Figure A5 (continued).

(C)



Chapter 4

Amphibian distribution in Pyrenean high mountain lakes and ponds is best explained by spatial patterns and fish introductions[5]

Abstract

Introduced fish have been reported as a major worldwide threat for amphibian communities in high mountain lakes and ponds. However, the interaction of introduced fish with spatial environmental patterns at the scale of the mountain range has not previously been studied. This paper presents an investigation of the impact of these factors on amphibian distribution. We surveyed 1736 Pyrenean lakes and ponds between 2008 and 2014, for amphibian occurrence, fish presence, and environmental variables. We regressed the data from each lake and pond with the occurrence of the five amphibian species which were sufficiently frequent, using generalized additive models (GAM). We then investigated the existence of spatial patterns comparing the western and eastern regions of the mountain range for all six of the amphibian species found as well as the environmental variables. Fish presence was significant for four of the five amphibian species modelled and the most explanative factor for one of them. Geographical location was significant for three species, but the most explanative for two of them. Five of the six amphibian species studied were more frequent in the western than in the eastern region. Moreover, the western region had fewer sites with introduced fish, a higher proportion of sites with predation refuges, a higher conductivity, lower duration of ice-

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[5] Miró, A. & Ventura, M. 2015. Amphibian distribution in Pyrenean high mountain lakes and ponds is best explained by spatial patterns and fish introductions.

cover, steeper littoral slopes, more vegetated shores and higher macroinvertebrate richness. Although introduced fish had a high impact locally, western-eastern patterns of some environmental variables were the main drivers of amphibian communities at range scale. Therefore, the most successful amphibian conservation policy should be designed at range scale, ignoring political boundaries, and including spatial environmental patterns and management of fish stocks.

Keywords

Amphibian biogeography, amphibian decline, generalized additive models, habitat features, high altitude lakes and ponds, minnow, range scale, trout

1. **Introduction**

Amphibians have strongly decreased in recent decades, and are one of the most threatened animal groups worldwide (Beebee and Griffiths 2005). Since 1900, the proportion of critically endangered amphibian species has reached almost 10%, twice the proportion of critically endangered birds or mammals (Pimm et al. 2014). Factors found to be related to amphibian declines locally and globally include: habitat loss or fragmentation (Becker et al. 2007, Todd et al. 2009), land use change (Loman and Lardner 2009, Curado et al. 2011), disease (Walker et al. 2010, Price et al. 2014), invasive species (Simon and Townsend 2003, Adams et al. 2011), climate change (Alford et al. 2007, Shoo et al. 2011, McCaffery et al. 2012), pesticides (Bishop et al. 2010), increase in UV radiation (Adams et al. 2005) and road mortality (Beebee 2013). Since these threats are extremely difficult to reduce, a reversal of amphibian declines is unlikely in the short or medium term (Beebee and Griffiths 2005).

Within mountain regions, amphibians are amongst the most conspicuous native animals of the high altitude watersheds, where they are threatened by introduced trout, but are also influenced by environmental variables such as altitude, maximum depth, littoral and shore substrate or irradiation (e.g. Knapp 2005, Pilliod et al. 2010). Other factors with negative impacts on amphibians in high mountains are chytridiomycosis and pesticides in the Sierra Nevada, California (Davidson and Knapp 2007, Vredenburg et al. 2010), as well as introduced minnows and artificial water-level fluctuation in the Pyrenees (Miró et al. 2015).

High mountain lakes are naturally fishless ecosystems due to natural barriers that prevent the colonization of fish species from lower streams or dispersal between interconnected lakes (Knapp et al. 2001a, Pister 2001). However, there have been numerous trout introductions to such ecosystems around the world since the 19th century, mainly for angling (Cambray 2003). Since the 1980s, several studies have reported strong negative effects of introduced trout on autochthonous amphibians (among other impacts) in mountain ranges of North America (e.g. Pilliod and Peterson 2001, Knapp 2005) and Europe (e.g. Orizaola and Braña 2006, Tiberti and von Hardenberg 2012). Most studies showed that direct trout predation on larvae can lead to the elimination of some native amphibians (e.g. Knapp 2005, Tiberti and von Hardenberg 2012); other works highlighted competition between introduced trout and adult amphibians for invertebrate prey (Finlay and Vredenburg 2007) or increases in shared predators leading to increased predation pressure on amphibians (Pope et al. 2008). The impacts of introduced trout can extend beyond the boundaries of individual water bodies and extinguish some amphibians from whole catchments if there is not enough suitable habitat without fish (Pilliod et al. 2010). However, the role of spatial environmental patterns and their interaction with introduced fish at mountain range scale have not been considered yet.

The Pyrenees are a continuous montane biogeographic area in southwest Europe, forming the northern boundary of the Iberian Peninsula, running from the Atlantic ocean to the Mediterranean sea (2°05’W–3°15’E, 43°18’–42°16’N). The range is roughly oriented W-NW to E-SE occupying c. 20000 km^2, with a width of c. 450 km and a maximum breadth of c. 150 km. Maximum elevation is 3404 m (a.s.l.) and some glaciers are present. The eastern part of the Pyrenees consists mainly of granite and gneissose materials, but in the western part the granite rocks are mixed with limestone. Air temperature and precipitation follow a marked altitudinal gradient, with lower temperatures and higher precipitation at higher altitudes. Rainfall (from 600-2700 mm/year), also declines from the north-west, (under continental and Atlantic influence) to the south-east (under Mediterranean influence). The tree line ranges from 2000 to 2500 m altitude, being higher in the central and most continental part of the range and lower in the east and west.

There are approximately 4500 high mountain lakes and ponds in the Pyrenees, either of glacial origin or modified by the activity of quaternary glaciations, and most are potentially suitable habitat for amphibians. Although amphibian distribution is generally well known in the region (e.g. Pleguezuelos 2002, Pottier 2008), their biogeography and ecology has been poorly studied at a Pyrenean scale. The influence of geological and climatic gradients on the vegetation is clearly apparent, and corresponding differences in amphibian distribution can also be expected. Moreover, although high mountain areas are naturally fishless, approximately 25% of Pyrenean lakes >0.5 ha were stocked in historical periods, from some centuries ago to 1950. Since then a further wave of introductions has taken place with minnows, as well as exotic trout, affecting between 35% and 85% of the lakes >0.5 ha depending on the valley, although the south western region of Aragon has been less affected (Miró and Ventura 2013).

For this study, we used an extensive dataset of 1736 Pyrenean high mountain lakes and ponds to investigate if environmental spatial patterns at mountain range scale influence amphibian distribution, in addition to the possible local effect of introduced fish. First, we applied multiple generalised additive models (GAM) to investigate the relative importance of environmental variables, introduced fish and space (geographical location), on the distribution of each amphibian species of Pyrenean lakes and ponds. Second, we took advantage of the environmental differences between the western and eastern parts of the Pyrenees to investigate the existence of latitudinal gradients (west-east), both for amphibian species occurrence and for environmental variables.

2. Methods

2.1. Amphibian and environment surveys

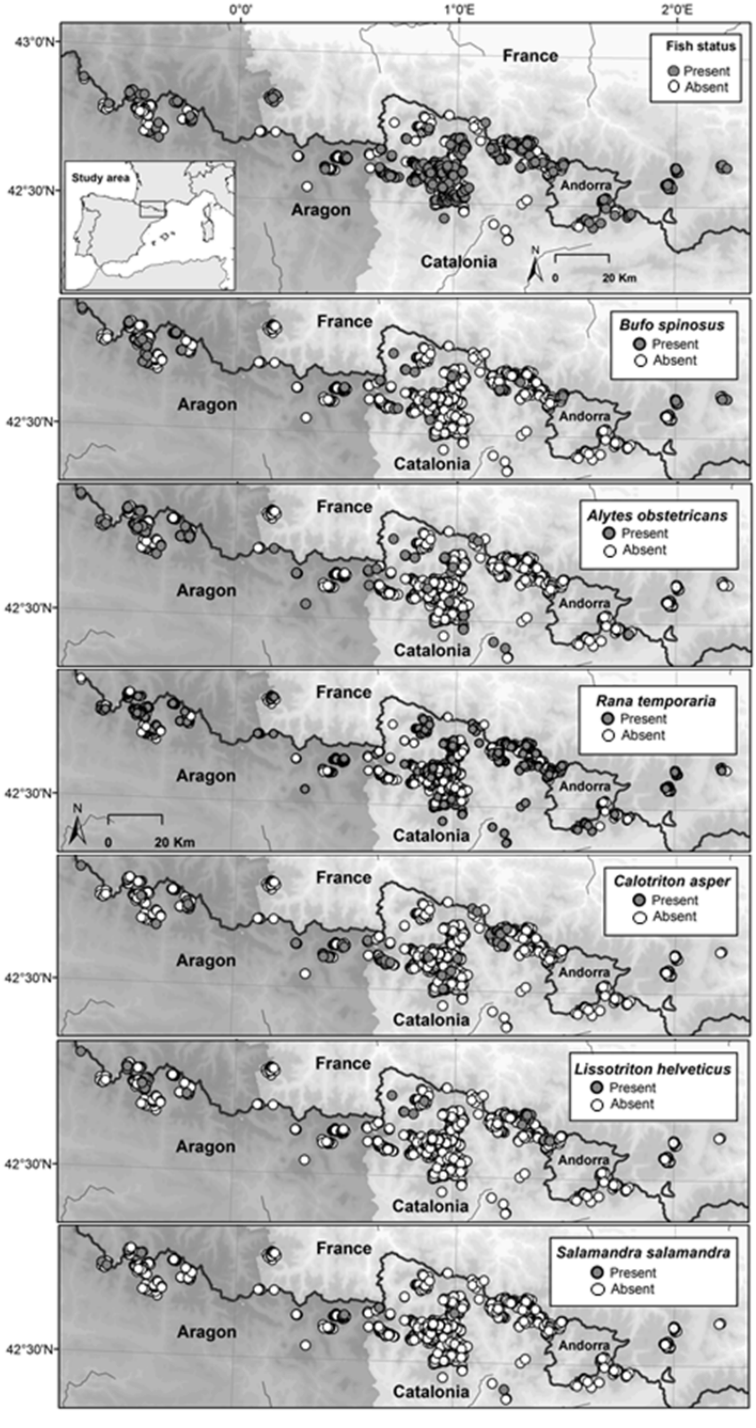
We surveyed 1736 lakes and ponds along the Pyrenean mountain range (but mainly on the southern slope), representing approximately 30% of the total number in the range (Fig. 1) (A detailed description of lake characteristics is provided in the Appendix). The field surveys were conducted during the lakes' ice-free periods from 2008 to 2014. To avoid seasonal variations in species occurrence, as found in some studies of high mountain and boreal lakes (e.g. Laugen et al. 2003, Knapp 2005), we carried out surveys following altitudinal and insolation gradients, from warmer to colder sites. Moreover, when there were doubts about validity of data, we repeated the survey in the following years. In total we conducted 2384 amphibian surveys in the 1736 lakes and ponds studied.

The studied amphibian species comprised three anurans and three caudatans: common frog *Rana temporaria* Linnaeus, 1758; midwife toad *Alytes obstetricans* (Laurenti, 1768); common toad *Bufo spinosus* Daudin, 1803; palmate newt *Lissotriton helveticus* (Razoumovsky, 1789); fire salamander *Salamandra salamandra* (Linnaeus, 1758); and Pyrenean newt *Calotriton asper* (Dugès, 1852), the last of which is endemic to the region. The occurrence of the species *A. obstetricans* was collected and analyzed as a single taxon, although two subspecies have been found (*A. o. obstetricans* more in the western part and *A. o. almogavarii* in the eastern part) with a poorly described distribution in the Pyrenees (Gonçalves et al. 2015). We assessed the occurrence of *R. temporaria*, *A. obstetricans* and *B. spinosus* based on the presence/absence of egg masses or larvae and the occurrence of *C. asper*, *L. helveticus* and *S. salamandra* on the presence/absence of at least one of the different life stages (eggs, larvae, sub-adults or adults).

The occurrence of amphibians was evaluated by visual encounter surveys (Crump and Scott Jr. 1994) of sufficient shore segments to cover all the habitat variability along the perimeter of each site (between 10 and 30 depending on the water body perimeter). Shore segments were 10 m long, parallel to the shoreline (2 m into the water and 2 m into the next terrestrial strip) and were proportionally distributed for the different substrates found in the water-bodies and along their edges. When no amphibians were detected in the segments, the entire shoreline of the lake was resurveyed. Because high mountain lakes are extremely clear, large areas could be surveyed from the shore, and macroscopic animals could be easily detected, as has been shown by previous studies (e.g. Knapp 2005, Pilliod et al. 2010).

To quantify which factors best explain amphibian distribution, we generated a set of 10 variables summarizing the environmental characteristics of the sampled lakes and ponds.

Figure 1 (next page) Distribution of studied lakes along the Pyrenean range with the presence or absence of fish, or the different amphibian species found in the Pyrenees.


0°0'
1°0'E
2°0'E
43°0'N
42°30'N
France
Aragon
Catalonia
Andorra
Study area
Fish status
Present
Absent
N
0
20 Km
Bufo spinosus
Present
Absent
Alytes obstetricans
Present
Absent
Rana temporaria
Present
Absent
Calotriton asper
Present
Absent
Lissotriton helveticus
Present
Absent
Salamandra salamandra
Present
Absent

Apart from the presence of introduced fish and geographical location, the dataset included eight environmental variables: water conductivity, estimated mean of the difference between daily maximum and minimum temperatures of the water body (diurnal water temperature variation), ice-cover duration, sites with cold water inflow during the summer (e.g. from cold springs or melt-water), littoral slope, shore substrate type, existence of refuge areas from fish predation, and the richness of the more conspicuous macroinvertebrate taxa or general groups (Table 1).

We chose descriptive habitat variables, trying to include characteristics which are biogeographically and ecologically meaningful for amphibians. For instance, we used water conductivity and temperature-related features such as the ice-cover period and the diurnal water temperature variation instead of associated variables such as elevation, surface, maximum depth or shore perimeter. A detailed description of how these variables were obtained is provided in Appendix 1.

Table 1 Description of environmental variables used in the analyses. Variable name abbreviations are given in parentheses.

Variable type	Variable name	Description
Habitat	Conductivity (COND)	Water conductivity (µS/cm)
	Diurnal water temperature variation (VARI)	Estimated mean of the difference between daily maximum and minimum temperatures of the water body (ºC)
	Ice-cover duration (ICECO)	Estimated duration of the annual ice-cover of the lake (Days)
	Cold sites (COLD)	Binary factor determined by the presence of cold springs or late ice-snow in the water body
	Littoral slope (SLOPEPC1)	First principal component for littoral zone slope. High values are associated with low littoral slope and vice versa
	Shore substrate (SHOREPC1)	First principal component for shore zone substrate types. High values are associated with bare rocky shores, while low values are associated with vegetated shores
	Predation refuge (REFU)	Binary factor determined by the presence of predation refuge areas from fish in the water body
	Macroinvertebrate richness (MIRICH)	Number of taxa of more conspicuous macroinvertebrates (Family, Order or Phylum level) detected in the water body
Fish	Fish presence (FISH)	Binary factor determined by the presence of introduced fish in the water body
Spatial	Geographical location (LOCATION)	Smooth function of UTM easting and northing of the water body

2.2. Statistical analysis

The relative importance of introduced fish, environmental variables and geographic location on amphibian occurrence was investigated through semiparametric generalized additive models (GAM; Hastie and Tibshirani 1990, Wood 2006). For each amphibian species (except *S. salamandra* as there were too few samples), one GAM of binomial distribution was made. Smooth penalized thin plate regression spline class was used for continuous variables, as they tend to give the best mean square error performance (Wood 2003). The optimal amount of smoothing was determined by general cross validation error a method that allowed us to recognize either linear or non-linear relationships with the response variable (Wood 2008). To account for spatial autocorrelation, but also to establish the relative importance of the geographical location, we included the smooth function of UTM easting and northing of the water body as a covariate (e.g. Knapp, 2005; Pilliod et al., 2010). We determined the relative weight of each variable by dropping it from the model whilst maintaining all other variables and evaluating the resultant change in deviance. The relationship between the significant predictor variables ($P < 0.05$) and the probability of the species' occurrence is shown graphically in separate plots. The response curves in each plot show the relative influence of the predictor variable on the probability of the species' occurrence. This response curve is based on partial residuals, is plotted on a log-scale and is standardized to have a mean value of 0 (Knapp 2005). Before performing the regressions, we examined the dataset for linear dependencies, computing pairwise Pearson's correlations (r) among the 6 continuous variables and Variance Inflation Factor (VIF) for the whole dataset. All r and VIF values obtained were below $|0.80|$ and 5, the respective thresholds indicative of worrisome collinearity (Zuur et al. 2009). UTM easting and northing were centred around 0 to keep the intercept of the models in low values (Zuur et al. 2009).

Spatial patterns at Pyrenean scale were investigated by comparing the data available in our dataset between the western and eastern regions of the Pyrenees. The western region was determined by the hydrological borders of the catchments of the rivers: Adour in the northern side, and Aragón, Gállego, Cinca and Ésera in the southern side. These five catchments encompass approximately 30% of the Pyrenees' area. The other catchments of the range were assigned to the eastern region (shaded area and non-shaded area in Fig. 1). Individual analyses comparing each amphibian species and environmental variable among regions were made applying Chi-square (χ^2) and U Mann-Whitney tests for categorical and continuous variables respectively.

All analyses were performed with R statistical software (R Development Core Team 2014) using the basic functions and the package *mgcv* to perform and plot GAMs (Wood 2011).

3. Results

3.1. Amphibian occurrence

Among the 6 amphibian species present in Pyrenean lakes and ponds, *R. temporaria* was the most widely distributed, being found in 1025 of the 1736 lakes and ponds (59%). *A. obstetricans* was found in 68 sites (3.92%), *B. spinosus* in 44 (2.53%), *C. asper* in 42 (2.42%), *L. helveticus* in 61 (3.51%) and *S. salamandra* in 9 (0.52%).

3.2. Fish effect

Overall amphibian occurrence in fishless sites was 75.6% (n=1041), but only 20.3% (n=73) in lakes and ponds with fish ($\chi2$ = 378.2, $P < 0.001$). In 63% of the sites (n=46) where amphibians and fish were present in the same lake, amphibians were only found in littoral predation refuges inaccessible to fish (e.g. shallow areas with macrophytes or rocks), and not in the main body of the lake ($\chi2$ = 39.8, $P < 0.001$). Moreover, almost half of the lakes and ponds where amphibians and fish shared the main body of the lake, had only tadpoles of *B. spinosus*, and no other amphibians (n=19).

3.3. Environmental variables

GAMs showed geographical location to be a significant predictor variable for three of the five species, while the presence of introduced fish was significant for four species. However, geographical location was the most explanative variable in two species and the presence of fish in one.

The GAM developed for the occurrence of *R. temporaria* had eight significant variables and highlighted the negative effect of introduced fish, which had more than twice the explained deviance (16.1%) of any other variable (Fig. 2; Table S4 in the Appendix). Geographical location explained 7.9% of deviance and showed a slightly higher probability of occurrence in the south east of the mountain range (Fig. 2; Fig. S2 (a) in the Appendix). Other significant predictors for *R. temporaria* presence were: macroinvertebrate richness (positively associated), summer cold inflow site (negatively associated), ice-cover duration and mean diurnal water temperature variation (both associated with the centres of unimodal curves), littoral slope (positively associated with low slope angle) and presence of predation refuge (positively associated) (Fig. 2; Table S4).

The GAM performed for *A. obstetricans* occurrence had five significant predictor variables. Geographical location had the greatest explained variance (39%), showing a higher probability of finding the species in the western Pyrenees (Fig. 3(a); Fig. S2 (b)). Introduced fish had a significant negative effect, but explained just 0.7% of deviance. Other significant predictors for *A. obstetricans* presence were: water conductivity (positive), littoral slope (positively associated with high slope angle) and macroinvertebrate richness (positive) (Fig. 3(a); Table S4 in the Appendix).

The GAM computed for *B. spinosus* occurrence had two significant variables, both temperature-related: ice-cover duration (negative) and mean of diurnal water temperature variation (positive) (Fig. 3(b); Table S4 in the Appendix).

The GAM performed for *C. asper* occurrence had seven significant predictor variables. Geographical location had approximately three times the explained deviance (30.1%) of any other variable, showing higher probability of finding the species in the western Pyrenees (Fig. 4(a); Fig. S2 (c) in the Appendix). Introduced fish had a significant negative effect, explaining 10.5% of deviance. Other significant predictors for *C. asper* presence were: water conductivity (associated with the centre of a unimodal curve), littoral slope (positively associated with high slope angle), macroinvertebrate richness (positive), diurnal water temperature variation (negative) and presence of predation refuge (positive) (Fig. 4(a); Table S4 in the Appendix).

The GAM developed for *L. helveticus* occurrence had three significant variables: two environment-related variables and the presence of introduced fish. The environment-related variables were shore substrate (associated with the centre of a unimodal curve) and littoral slope (positively associated with high slope angle) which had most of the explained deviance (7.1% and 1.2%). The presence of introduced fish had a significant negative effect, but only 0.4% of explained deviance (Fig. 4(b); Table S4 in the Appendix).

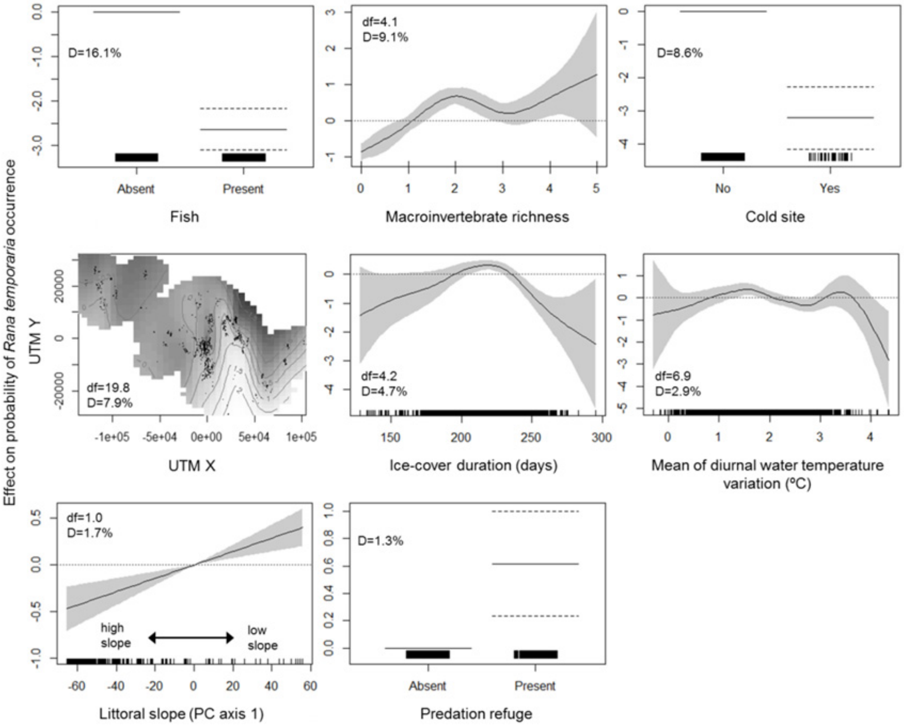


Figure 2 Estimated effect of each of the significant variables ($p < 0.05$) on the probability of occurrence by *Rana temporaria*, determined from the generalized additive model (GAM). Response curves are based on partial residuals and are standardized to have a mean probability of zero. The contour of the shaded areas and the error dashed lines are approximately +/−2 SE (95% CI) relative to the main estimate, and hatch marks at the bottom are a descriptor of the frequency of data points along the gradient in continuous variables, or within each category for categorical variables. The effect of UTM easting and northing is shown as an isoline map, where light gray indicates positive effect and dark gray indicates negative effect. Note that high values of SLOPEPC1 are associated with low littoral slope and vice versa. “df” indicates the estimated degrees of freedom of the smooth curve: df=1 is equivalent to a linear relationship. Smoothing parameters were estimated by general cross validation error (Wood, 2004). “D” indicates the percentage of explained deviance. Dotted gray lines in smooth terms indicates the Y value = 0, allowing one to see the regions where effect on accurrence is positive or negative. Variables are ordered from highest to lowest explained deviance.

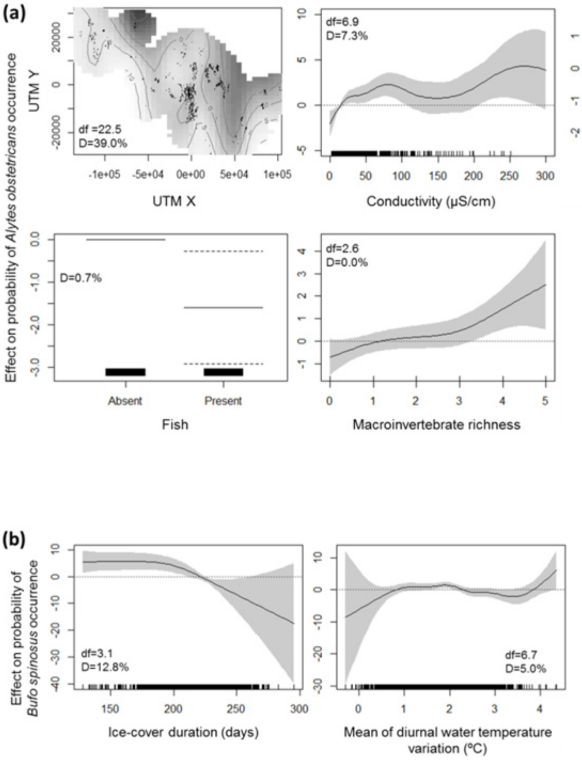


Figure 3 Estimated effect of each of the significant variables ($p < 0.05$) on the probability of occurrence for (a) *Alytes obstetricans* and (b) *Bufo spinosus,* determined from the generalized additive model (GAM). Response curves are based on partial residuals and are standardized to have a mean probability of zero. The contour of the shaded areas and the error dashed lines are approximately +/–2 SE (95% CI) relative to the main estimate, and hatch marks at the bottom are a descriptor of the frequency of data points along the gradient in continuous variables, or within each category for categorical variables. The effect of UTM easting and northing is shown as an isoline map, where light gray indicates positive effect and dark gray indicates negative effect. Note that high values of SLOPEPC1 are associated with low littoral slope and vice versa. “df” indicates the estimated degrees of freedom of the smooth curve: df=1 is equivalent to a linear relationship. Smoothing parameters were estimated by general cross validation error (Wood, 2004). “D” indicates the percentage of explained deviance. Dotted gray lines in smooth terms indicates the Y value = 0, allowing to see the regions where effect on accurrence is positive or negative. Conductivity chart for *Alytes obstetricans* is drawn up to 300 µS/cm for clarity; the default graphic can be seen in the Appendix 1. Variables are ordered from highest to lowest explained deviance.

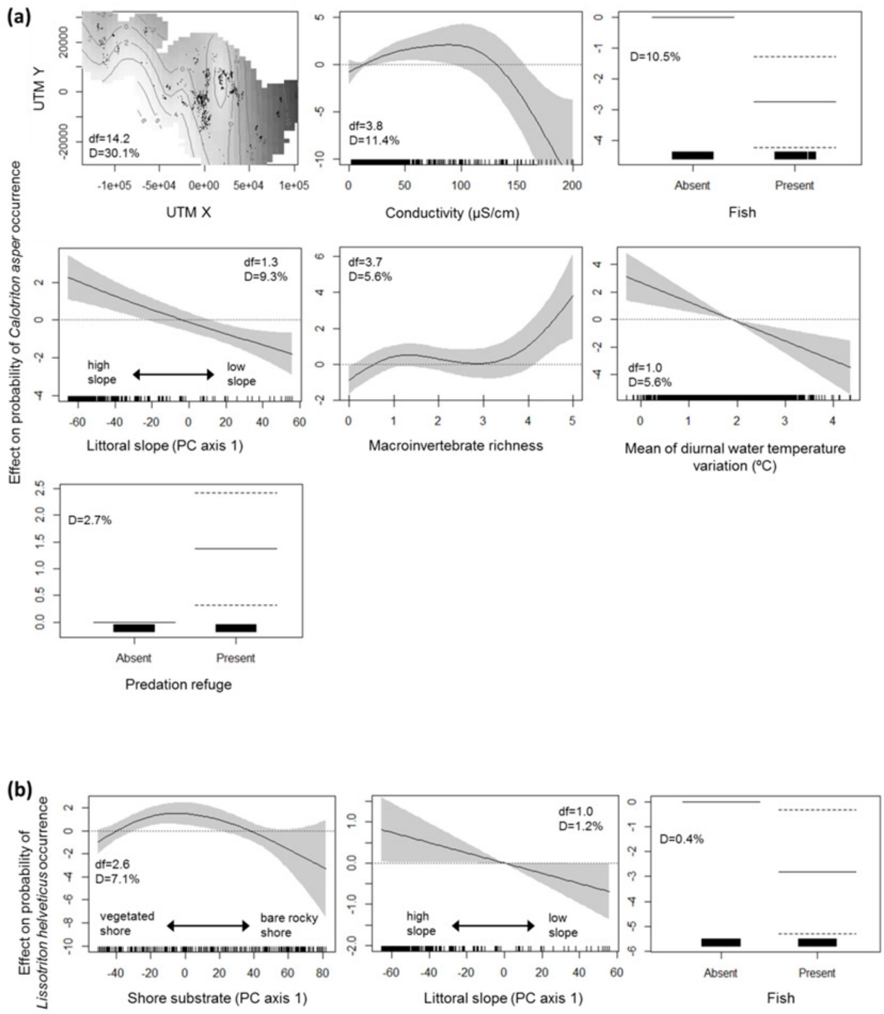

(a)
Effect on probability of *Calotriton asper* occurrence
UTM Y
UTM X
df=14.2
D=30.1%
-1e+05 -5e+04 0e+00 5e+04 1e+05
-20000 0 20000
df=3.8
D=11.4%
Conductivity (µS/cm)
0 50 100 150 200
D=10.5%
Absent
Present
Fish
df=1.3
D=9.3%
high slope
low slope
Littoral slope (PC axis 1)
-60 -40 -20 0 20 40 60
df=3.7
D=5.6%
Macroinvertebrate richness
0 1 2 3 4 5
df=1.0
D=5.6%
Mean of diurnal water temperature variation (ºC)
0 1 2 3 4
D=2.7%
Absent
Present
Predation refuge
(b)
Effect on probability of *Lissotriton helveticus* occurrence
df=2.6
D=7.1%
vegetated shore
bare rocky shore
Shore substrate (PC axis 1)
-40 -20 0 20 40 60 80
df=1.0
D=1.2%
high slope
low slope
Littoral slope (PC axis 1)
-60 -40 -20 0 20 40 60
D=0.4%
Absent
Present
Fish

Figure 4 (last page) Estimated effect of each of the significant variables ($p < 0.05$) on the probability of occurrence by (a) *Calotriton asper* and (b) *Lissotriton helveticus,* determined from the current generalized additive model (GAM). Response curves are based on partial residuals and are standardized to have a mean probability of zero. The contour of the shaded areas and the error dashed lines are approximately +/−2 SE (95% CI) relative to the main estimate, and hatch marks at the bottom are a descriptor of the frequency of data points along the gradient in continuous variables, or within each category for categorical variables. The effect of UTM easting and northing is shown as an isoline map, where light gray indicates positive effect and dark gray indicates negative effect. Note that high values of SLOPEPC1 are associated with low littoral slope and vice versa; and high values of SHOREPC1 are associated with bare rocky shores, while low values are associated with vegetated shores. “df” indicates the estimated degrees of freedom of the smooth curve: df=1 is equivalent to a linear relationship. Smoothing parameters were estimated by general cross validation error (Wood, 2004). “D” indicates the percentage of explained deviance. Dotted gray lines in smooth terms indicates the Y value = 0, allowing to see the regions where effect on accurrence is positive or negative. Conductivity chart for *Calotriton asper* is drawn up to 250 µS/cm for clarity; the default graphic can be seen in the Appendix. Variables are ordered from highest to lowest explained deviance.

3.4. Comparison of western and eastern Pyrenean regions

The western and the eastern regions of the Pyrenees showed clear differences between their amphibian populations, most species being more frequent in the western region. Only *R. temporaria* was equally frequent ($\chi 2 = 0.098$, $P = 0.768$) in both Pyrenean regions. In contrast, *A. obstetricans*, *B. spinosus*, *C. asper*, *L. helveticus* and *S. salamandra* were more frequently present in the western region ($\chi 2 = 207.4$, $P < 0.001$; $\chi 2 = 44.8$, $P < 0.001$; $\chi 2 = 42.2$, $P < 0.001$; $\chi 2 = 5.97$, $P = 0.029$; $\chi 2 = 35.2$, $P < 0.001$ respectively) (Table 2).

Most of the predictor variables in our dataset showed differences between the Pyrenean regions. The western region showed a lower proportion of sites with introduced fish ($\chi 2 = 5.41$, $P < 0.020$) and a higher proportion of lakes or ponds with predation refuges ($\chi 2 = 13.8$, $P < 0.0001$). The western region also showed higher conductivity ($W = 235805$, $P < 0.0001$), lower ice-cover duration ($W = 98362$, $P < 0.0001$), steeper littoral slope ($W = 134228$, $P < 0.0001$), more vegetated shores ($W = 119153$, $P < 0.0001$) and higher macroinvertebrate richness ($W = 185264$, $P = 0.002$). However, the diurnal water temperature variation ($W = 166683$, $P = 0.786$) and summer cold inflow sites ($\chi 2 = 0.336$, $P = 0.528$) showed no statistical differences (Fig. 5; Table S5 in the Appendix).

Table 2 Occurrence of amphibian species in the two Pyrenean areas studied: western and eastern. We show the percentage presence within the geographical area and the number of sites in parentheses. Asterisks after species name indicate the results of Chi-square tests between both geographical areas: NS, not significant (P ≥0.05); *P <0.05; **P <0.01; ***P <0.001.

Species occurrence	Western Pyrenees (n= 217)	Eastern Pyrenees (n= 1519)
Rana temporaria^{NS}	58.1% (126)	59.2% (899)
*Alytes obstetricans****	21.7% (47)	1.4% (21)
*Bufo spinosus****	9.2% (20)	1.6% (24)
*Calotriton asper****	8.8% (19)	1.5% (23)
*Lissotriton helveticus**	6.5% (14)	3.2% (48)
*Salamandra salamandra****	3.2% (7)	0.1% (2)

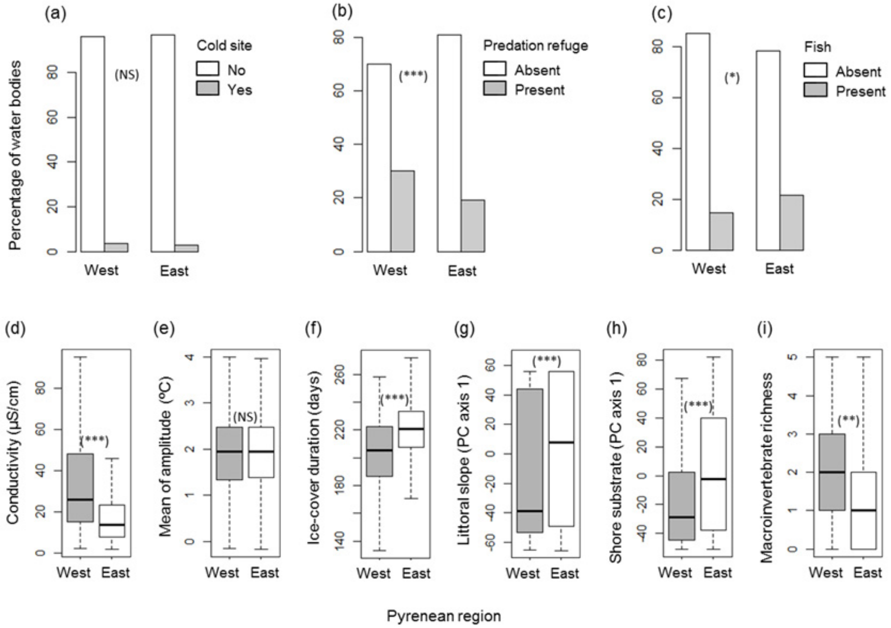


Figure 5 Differences between the two Pyrenean areas studied for categorical (a-c) and continuous (d-i) environmental variables used in the analyses. The line within each box marks the median; the bottom and top of each box indicate the 25th and 75th percentiles and the whiskers below and above each box indicate the 10th and 90th percentiles. Asterisks provide the results of Chi-square and U Mann-Whitney tests respectively, between both geographical areas: NS, not significant (P ≥0.05); *P <0.05; **P <0.01; ***P <0.001.

4. Discussion

4.1. Spatial patterns

Despite the high presence of introduced fish in Pyrenean high mountain lakes and ponds, spatial patterns of the environment explained more deviance than fish on amphibian distribution at range scale. The data from the western Pyrenees identify some natural environmental characteristics in this area that are clearly associated with the higher frequency of amphibians. We identified water conductivity, water temperature-related variables (duration of the ice-cover, the diurnal water temperature variation and sites with summer cold inflows) and environmental variables associated with the quality of the habitat (littoral and shore conditions, macroinvertebrate richness and presence of predation refuge) as important for some of the five amphibian species modelled. Spatial environmental patterns have also been recognized by previous studies as the main driver of amphibian presence in several habitats, such as tropical forests (Keller et al. 2009) or grassland (Searcy et al. 2013), as well as at regional and global scale (e.g. Qian 2010).

4.2. Fish effect

With the exception of *B. spinosus,* amphibians are almost absent from sites with introduced fish. As well as the spatial patterns described above, the western Pyrenees shows a lower occurrence of introduced fish, an important variable for four of the five species for which GAMs were produced. This was the most explanative variable for *R. temporaria*, the most frequent Pyrenean amphibian and the only one equally distributed over the range. In total, of 360 sites with fish, only 46 had amphibians in the main water body, excluding amphibians present only in littoral predation refuge areas. Furthermore, 19 sites of these 46 had only tadpoles of *B. spinosus*. These results concerning the impact of introduced fish on the amphibian assemblage of lakes and ponds are consistent with those obtained by previous studies. Fish have been reported as one of the most important factors for amphibian communities in many studies carried out in lowland lakes and ponds (Indermaur et al. 2010, Cosentino et al. 2011, Jeliazkov et al. 2014); as well as in high altitude lakes and ponds, which are well known for their poorly structured habitat and high water transparency (Knapp 2005, Pilliod et al. 2010, Tiberti and von Hardenberg 2012). The lack of impact that we found on *B. spinosus* can be explained by the unpalatability and toxicity of this species’ tadpoles (Kruse and Stone 1984, Benard and Fordyce 2003). In fact, all species of the genus *Bufo* studied so far seem to benefit from the presence of fish (Orizaola and Braña 2006, Welsh et al. 2006, Indermaur et al. 2010).

4.3. Environmental variables

Together with introduced fish and geographical location of the water body, the spatial heterogeneity of some environmental variables play a strong role in the distribution of Pyrenean amphibians at mountain range scale. The most interesting to discuss are: water conductivity, temperature-related features and littoral slope, all of them poorly used so far to describe the habitat of high mountain lakes and ponds. Water conductivity, which can be used as a surrogate for concentration of solutes, appeared to be important for *C. asper* and especially for *A. obstetricans*. The minimum conductivity for ponds and lakes with *A. obstetricans* was 10.4 µS/cm (95% of the species’occurrences; Fig. S3 and S4 in the Appendix). This species was mostly present in the western Pyrenees, where conductivity was in general higher (Fig. S5 in the Appendix). These results contrast with the much lower conductivity tolerated by *R. temporaria* or *L. helveticus*, which are found at conductivities of 3.9 and 3.2 µS/cm respectively (95% of the species’ occurrence; Fig. S3 in the Appendix). Several mechanisms to keep internal osmolarity under control in changing environments are known in amphibians (Suzuki et al. 2007, Gomes et al. 2009). Aquaporins and other channels allow hydromineral transport through the epithelium and other membranes, and some organs, such as the urinary bladder and kidneys, are involved in amphibian homeostasis (Gomes et al. 2009, Suzuki and Tanaka 2009). However, our results show that amphibian species have different tolerances to water conductivity in the field, with *A. obstetricans* the least tolerant to low values. This results in substantial limitations of suitable habitat for *A. obstetricans*, a species which is also highly threatened by chytridiomycosis in the region (Walker et al. 2010).

As amphibians are ectothermic we expected to find the water temperature-related variables were particularly important. The species most affected by these variables appears to be *B. spinosus*, which seems to benefit from short ice-cover durations. Since the duration of ice-cover is highly correlated with the altitude of the site (Table S2 in the Appendix), this may explain why we only found *B. spinosus* at relatively low altitudes, almost always below 2200 m. This finding is consistent with those obtained by previous studies from the central part of the Pyrenees, which only found *B. spinosus* below 2160 m (Vences et al. 2003). Nevertheless, the altitudinal limit of this species may also be influenced by the high sensitivity of its tadpoles to UV-B radiation (Häkkinen et al. 2001).

The model of *R. temporaria* also selected ice-cover duration, but in this case, the highest probability of finding the species was at intermediate values. The study of Vences et al. (2003) in the central Pyrenees, also found a polynomic relationship between altitude and the log(probability of *R. temporaria* occurrence). The altitudinal gradient of the ice-cover duration did not show any effect on the caudatans *C. asper* and *L. helveticus,* both of which have overwintering larvae, or on *A. obstetricans*, which is the only anuran found in Pyrenees lakes and ponds that has overwintering tadpoles.

The diurnal water temperature variation was selected by the *R. temporaria* and *B. spinosus* models, but its effect on *C. asper* was even clearer. Experimental work from the second half of the twentieth century showed that high variations in the diurnal water temperature increased the rate of growth of *R. temporaria* (Balcells 1975). Our results show that the diurnal water temperature variation has a complex relationship with this species, with highest probability of finding *R. temporaria* at intermediate values. However, diurnal water temperature variation had a slightly positive effect on the occurrence of *B. spinosus*. In contrast, the variable showed a marked negative effect on *C. asper*. Since diurnal water temperature variation is strongly correlated with the surface area of the water body (and therefore with the maximum depth, perimeter and water volume), this feature may be associated with the high occurrence of *C. asper* in lakes, in comparison with its low occurrence in ponds. Finally, the presence of cold water inflow during the summer (from cold springs or late melt-water), was the third most strongly weighted variable in the *R. temporaria* model. This species is known to select microhabitats with favourable temperatures (Kohler et al. 2011), so its discrimination against excessively cold sites is to be expected.

The PC1 of the littoral slope was selected by four models, but showed different sign. While *R. temporaria* was related to shallow littoral, *A. obstetricans*, *C. asper* and *L. helveticus* were associated with sloping littoral. The littoral slope is a robust proxy of other environmental variables. For instance, shallower littoral in Pyrenean lakes and ponds are quite well associated with fine-grain substrate or high macrophytes coverage. Previous studies made in high mountain lakes had shown that, after accounting for fish presence, some caudatans such as *Taricha torosa sierrae* or *Ambystoma macrodactylum* are more frequent on coarse-grain littoral substrates (Knapp 2005, Welsh et al. 2006).

4.4. Biogeographic distribution limitation

The fact that all six amphibians are present throughout the length of the Pyrenees, suggests that there might be low dispersal limitations for most of these species over the range, and the current distributions are mainly associated with spatial gradients of some environmental variables.

The only exception being *A. obstetricans*, for which it has been suggested that the current distribution in the Pyrenees and northern areas is the result of several independent colonisations from Iberian Pleistocene glacial refuges (Martinez-Solano et al. 2004). Recent genetic studies have reported the existence of three different linages in the Pyrenees: in the western tip there is *A. o. obstetricans*; in the central Pyrenees there is a haplogroup not associated with the subspecies described to date; and in the eastern Pyrenean region there is *A. o. almogavarii*, which appears to be highly differentiated and probably immersed in an incipient speciation process (Gonçalves et al. 2015).

A different physiological tolerance of these subspecies or haplogroups might be an important factor explaining the contrasting difference in the occurrence between the western and eastern regions of the Pyrenees. The minimum conductivity threshold that we found for *A. obstetricans* in the western region was 8.1 µS/cm (95% of the species' occurrence), while in the eastern region, concordant with the distribution area of *A. o. almogavarii*, the value was 18.1µS/cm. As a consequence, considering this ecological factor alone, while the suitable habitat for *A. obstetricans* in the western region encompass approximately 88% of the lakes and ponds (>8.1 µS/cm), the suitable habitat for *A. o. almogavarii* in the eastern region represents approximately only 35% (>18.1 µS/cm; Fig. S5). Therefore it seems that the strong geographical gradient depicted by the factor geographical location could be explained by a different tolerance to conductivity by the different subspecies, and therefore the variance explained by geographical location for this species is likely related with environmental variability.

The endemic newt *C. asper* was the other species with a strong gradient in its distribution with geographical location as the most explanative variable like *A. obstetricans.* Contrasting with the latter present genetic studies have not found any significant genetic differentiation along the Pyrenees that might be associated with a different colonisation history (Valbuena-Urena et al. 2013). However there seem to be different morphological characteristics between stream and lake populations, at least in the western Pyrenees (Oromi et al. 2014). Therefore it is unlikely that the contrasting difference between the eastern and western areas that we found would be a result of a biogeographic distribution limitation. Rather it is possible that an environmental factor or particular feature of the species not accounted by in this study lies behind the geographical location variance accounted by this variable. For example, inability of the species to survive in ponds might have had a stronger consequence of fish introductions in the eastern part, where in many valleys fish introductions have reached nearly all the lakes. Other studies have shown that the number of lakes in a given catchment can be an important factor for the survival of amphibian species (Pilliod et al. 2010).

4.5. Conservation implications

We believe that the most successful way to avoid adverse impact on amphibians in Pyrenean high mountain lakes should be a conservation policy at a Pyrenean scale, transcending political boundaries and incorporating spatial environmental patterns and fish stocking management. Our results show that suitable amphibian habitat in Pyrenean high mountain lakes is naturally highly fragmented for *A. obstetricans*, *B. spinosus*, *C. asper*, *L. helveticus* and *S. salamandra*, particularly in the eastern part due to the spatial environmental patterns. In addition to the potential impact of emerging diseases on some species such as *A. obstetricans* and *S. salamandra* (Walker et al. 2010, Martel et al. 2013), the high presence of introduced fish (i.e. 20.1% in our dataset) is locally making many lakes and ponds unsuitable and thus, markedly increasing the degree of isolation of breeding sites. In general, it has been reported that species may be negatively affected by habitat fragmentation, when suitable habitat have become too isolated; either by natural or anthropogenic causes (Fahrig 2003, Fischer and Lindenmayer 2007). This fragmentation may lead to loss of fitness through inbreeding or local extinction through stochastic effects (Griffiths and Williams 2000). This negative effect may be still stronger on species with a small geographic range of distribution, which have to be urgently targeted for conservation

(Sodhi et al. 2008), such as *C. asper* or the different subspecies of *A. obstetricans*.

There are six different countries or regions that have environmental responsibilities in the Pyrenees range: with few exceptions, the northern side is part of France and the southern is part of the Spanish regions of Basque Country, Navarre, Aragon and Catalonia. In the southern and eastern part there is also Andorra. Therefore, there is a strong heterogeneity of protection agencies and environmental management policies across the range. Moreover, fish stocking actions are mostly regulated by different agencies from those responsible for conservation policies. Taking a regional overview of Pyrenean amphibian ecology and biogeography can be a sound base to inform conservation policies and actions at mountain range scale, removing artificial borders and involving all countries and administrations present. Nevertheless, it will be necessary to include spatial patterns criteria and stocked-angling management within a regional conservation policy for amphibians.

Acknowledgements

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Appendix Chapter 4

Supplementary methods, tables and figures

1. Supplementary methods

1.1. Additional description of the study area

The studied lakes and ponds are spread along the Pyrenean mountain range (0°42’W - 2°09’E, 42°52’ - 42°23’N; Fig. 1). There are 1080 lakes >0.5 ha and approximately 4500 small lakes and ponds either of glacial origin or modified by the activity of quaternary glaciations. They range in altitude between 1500 and 2960 m with the highest frequency found at *ca.* 2300 m. The unmodified lakes are relatively small and deep, with average surface area of 4.5 ha and average maximum depth of 15 m, with the largest being 44 ha and the deepest 105 m. Approximately 15% of the total lakes are impounded, mostly the biggest. Among these semi-natural dammed lakes, the largest has an area of 160 ha and the deepest a maximum depth of 123. The ponds can range in surface area from between a few square meters and 0.5 ha, and in maximum depth from between a few centimetres and 1-2 m. Some of them are temporary and may dry up in the peak of the ice-free season.

Since one of the aims of the work was to compare the western and the eastern regions of the Pyrenees range, to enhance the differences between both areas, we spent slightly more sampling effort on the distal parts of the mountain range, and less on the central part. As a result of that, the lakes and ponds sampled in the western region had lower altitudes, but quite similar surface areas than those sampled in the eastern region (Fig. S6 and S7).

Due to their common glacial origin, there is a close positive relationship between surface area and maximum depth (Catalan et al. 2009b). Most of the lakes are above the tree line with catchments partially covered by meadows, although some of them are on or below the tree line. Approximately half of the lakes have catchments on granodiorite bedrock, the remaining being located in catchments with metamorphic (25%), detrital (15%) or carbonate (10%) bedrock, with a minority on Silurian slate

(Casals-Carrasco et al. 2009). The latter bedrocks, due to their high sulphate content, give natural acidity to the waters (pH < 5.5). The ionic content is generally low, being the chemical factor most strongly related to bedrock composition (Catalan et al. 1993). The lakes have low phosphorous and chlorophyll content, being of oligotrophic nature (Buchaca and Catalan 2007). A general description of the climate, physic-chemical and biological characteristics of Pyrenean lakes and a comparison with other European high mountain lakes can be found elsewhere (Camarero et al. 2009, Catalan et al. 2009a, Kernan et al. 2009a, Thompson et al. 2009).

1.2. Detailed description of the predictor variables

To quantify the biogeographical specificities and assess which factors best explained amphibian distribution, we generated a set of environmental variables summarizing the environmental variables of the sampled lakes and ponds (Table 1).

Water conductivity (COND), referred to 20ºC, was gathered during field surveying with handheld meter WTW Cond 340i.

To describe the thermal characteristics of the lakes and ponds studied, we used the mean of the diurnal water temperature variation (VARI), the ice-cover duration (ICECO) and the categorization of the sites with cold water during the summer (COLD). Diurnal water temperature (VARI) and ice-cover duration (ICECO) were modelled using field data of the water temperatures obtained from the years 2010 to 2014 by 48 automatic thermometers (Vemco Minilog-T) situated at a depth of *ca.* 1m in 58 Pyrenean lakes and ponds, covering a wide range of altitudes, surface areas and catchment sizes. Eventually we had 192 complete annual datasets: 44 from 2010, 40 from 2011, 36 from 2012, 40 from 2013 and 32 from 2014. This temperature dataset was assumed to be representative of the epilimnion, following previous limnological knowledge of these lakes and calibration measurements performed seasonally at different depths. Both variables for all studied lakes were interpolated using mixed regression models on various morphometric and thermal parameters (Sabas et al. 2016). This was possible since the temperature variability of lakes in alpine areas follows a close relationship with lake altitude, but also with lake size among other morphometric variables (Thompson et al. 2009). We also made a factor to highlight the sites with cold water inflow throughout the summer (COLD). This binary categorical variable was determined by the presence of cold springs or late ice/snow in the water body during the Summer.

Littoral slope and shore habitats were characterized by visually estimating substrate features and composition of the same shore segments where we had looked for amphibians (see Amphibian and habitat surveys section, in the main body of the paper). Data were expressed as % coverage for each category of littoral slope and shore substrate composition. Littoral slope was categorized as shallow (<10 cm deep, <15º slope), slight slope (15º–30º slope), sloping (30º–70º slope) or sub vertical (70º–90º slope). Shore substrate composition was categorized as cobble-boulder (2.5 cm–1 m), big boulder-bedrock (>1 m) or terrestrial vegetation. We adapted these classifications from previous studies of high mountain and boreal lake fauna (e.g. Knapp et al. 2001b, Johnson et al. 2004). To reduce the dimensionality of the substrate data, we subjected littoral slope and shore substrate data to separate principal component analyses (Knapp 2005). In all three analyses, axis 1 explained a substantial amount of the total variation in substrate characteristics and composition (littoral slope: 77.6%; shore substrate: 79.4%), so the scores were used as the independent variable representing littoral zone slope (SLOPEPC1) and shore substrate composition (SHOREPC1). High values of SLOPE were associated with low littoral slope and vice versa; low values of SHORE were associated with vegetated shores, while high values of SHORE were associated with bare rocky shores (Table S1).

The binary variable predation refuge (REFU) was created to reflect the presence in the lake of littoral areas where fish were assumed to be unable to access: shallow areas either with or without macrophytes and stones.

A simple characterization of the macroinvertebrate community was done confirming the presence/absence of the more conspicuous macroinvertebrate taxa or general groups that can be found in Pyrenean high mountain lakes. The gathered groups were: adult pond skaters (Family Gerridae), adult back-swimmers (Family Notonectidae), adults and larvae of beetles (Order Coleoptera), larvae of dragonflies (Order Odonata), caddisflies (Order Trichoptera) and molluscs (Phylum Mollusca). Their occurrence was assessed by visual encounter surveys (Crump and Scott Jr. 1994) of the same shore segments where we looked for amphibians (see Amphibian and habitat surveys section, in the main body of the paper). As all macroinvertebrate taxa were found to be positively correlated with most of the amphibians studied and to summarize the structure of the macroinvertebrate community, the macroinvertebrate richness (MIRICH) based on these groups was subsequently calculated for each water body.

The occurrence data for fish (FISH) were obtained from historical documents, from interviews with local elderly fishermen or nature reserve wardens, from local reports of fishing or walking societies and from our own visual encounter and field surveys (Miró and Ventura 2013, 2015). The different trout species found in Pyrenean lakes are *Salmo trutta, Oncorhynchus mykiss*, *Salvelinus fontinalis*, *Salvelinus alpinus* and *Salvelinus namaycush* (Miró and Ventura 2013). Currently the only minnow species found in Pyrenean lakes is *Phoxinus phoxinus* (Miró and Ventura 2015).

Geographical coordinates easting (UTM X) and northing (UTM Y) of the lakes were obtained from a GIS generated from 1:25000 maps from various governmental agencies (Casals-Carrasco et al. 2009). To account for spatial autocorrelation in the GAM models, the smooth function of UTM easting and northing of the water body (LOCATION) was also included as predictor variable (e.g. Knapp 2005, Pilliod et al. 2010).

2. Supplementary tables

Table S1 Pearson's correlations among the variables littoral slope, littoral substrate and shoreline substrate categories with the principal component axis 1. The variance contained in the first axis is given in parentheses and above the correlations (% explained variance).

Habitat characteristics	Axis 1	Sig.
Littoral slope (SLOPE)	*(77.6% explained variance)*	
Shallow (<10 cm deep, <15º slope)	0.99	<0.001
Slight slope (15º–30º slope)	-0.67	<0.001
Sloping (30º–70º slope)	-0.79	<0.001
Vertical (70º–90º slope)	-0.49	<0.001
Shoreline substrate (SHORE)	*(79.4% explained variance)*	
Terrestrial vegetation	-0.99	<0.001
Cobble-boulder (2.5 cm–1 m)	0.86	<0.001
Big boulder-bedrock (>1 m)	0.59	<0.001

Table S2 Pearson correlation matrix of the 6 continuous variables used in the analyses. Traditional variables altitude (ALT) and surface area (SURF) were also added. Variable name abbreviations are given in the section “Detailed description of the predictor variables” in this Appendix. Note that high values of SLOPEPC1 are associated with low littoral slope and vice versa; and high values of SHOREPC1 are associated with bare rocky shores, while low values are associated with vegetated shores. See table S3 for abbreviations of environmental variables. *denotes correlation significantly different from zero ($p < 0.05$). The degrees of freedom for all pairwise correlation tests were 1734.

	VARI	ICECO	SLOPEPC1	SHOREPC1	MIRICH	ALT	SURF
COND	0.04	-0.41*	0.05*	-0.25*	0.09*	-0.41*	0.03
VARI		-0.20*	0.57*	-0.27*	0.37*	-0.27*	-0.48*
ICECO			-0.30*	0.44*	-0.12*	0.84*	-0.16*
SLOPEPC1				-0.28*	0.33*	-0.16*	-0.28*
SHOREPC1					-0.29*	0.47*	0.09*
MIRICH						-0.18*	-0.24*
ALT							-0.10*

Table S3 Variance Inflation Factors (VIF) of the dataset used in the analyses.

Variable name	VIF value
Conductivity (COND)	1.25
Diurnal water temperature variation (VARI)	1.86
Ice-cover duration (ICECO)	1.79
Cold sites (COLD)	1.02
Littoral slope (SLOPEPC1)	1.64
Shore substrate (SHOREPC1)	1.43
Predation refuge (REFU)	1.14
Macroinvertebrate richness (MIRICH)	1.43
Fish presence (FISH)	1.73

Table S4 Results of current generalized additive models (GAM) performed for the occurrence of each amphibian species. The increase in deviance resulting from dropping the selected variable from the model is shown. The percentage increases are given in parentheses, and were calculated as (deviance increase/(null deviance-model deviance[a]))x100 (Knapp 2005). [a]Sometimes referred to as ''residual'' deviance. Asterisks indicate the level of statistical significance associated with each variable: NS, not significant ($p \geq 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The model for *Salamandra salamandra* was not build due the low number of localities where present, 9 on 1736 lakes and ponds.

Parameter	*Rana temporaria*	*Alytes obstetricans*	*Bufo spinosus*	*Calotriton asper*	*Lissotriton helveticus*
Null deviance	2349.5	573.9	410.3	395.6	528.3
Degrees of freedom (null model)	1735	1735	1735	1735	1735
Model deviance[a]	1512.1	233.7	151.9	183.4	156.2
Degrees of freedom (full model)	1691.3	1691.2	1689.5	1702.9	1688.8
Explained deviance (% of total)	35.6%	59.3%	62.9%	53.6%	70.4%
Deviance increase					
COND	1.1 (0.1) NS	24.8 (7.3)*	0.4 (0.2) NS	24.2 (11.4)**	25.6 (6.9) NS
VARI	24.5 (2.9)*	14.5 (4.3) NS	12.9 (5.0)*	11.8 (5.6)***	8.2 (2.2) NS
ICECO	39.0 (4.7)***	0.1 (0.0) NS	33.2 (12.8)**	18.2 (8.6) NS	14.6 (3.9) NS
COLD	71.7 (8.6)***	0.1 (0.0) NS	7.3 (2.8) NS	0.0 (0.0) NS	0.0 (0.0) NS
SLOPEPC1	14.6 (1.7)**	12.8 (4.3)***	8.6 (3.3) NS	19.7 (9.3)***	4.6 (1.2)*
SHOREPC1	7.2 (0.9) NS	0.4 (0.1) NS	4.2 (1.6) NS	5.7 (2.7) NS	26.6 (7.1)**
REFU	10.6 (1.3)**	2.4 (1.2) NS	7.0 (2.7) NS	5.8 (2.7)**	1.9 (0.5) NS
MIRICH	76.2 (9.1)***	0.0 (0.0)*	0.0 (0.0) NS	11.9 (5.6)**	13.3 (3.6) NS
FISH	134.6 (16.1)***	2.3 (0.7)*	4.3 (1.7) NS	22.2 (10.5)***	1.6 (0.4)*
LOCATION	65.8 (7.9)**	132.8 (39.0)***	79.4 (30.7) NS	63.9 (30.1)**	159.8 (42.9) NS

Table S5 Differences between the two Pyrenean areas studied for the variables used in the analyses. In the continuous variables, the mean and the range values are shown. In the presence/absence variables, we show the number of sites with presence and the % of the sites of the geographic area in parentheses. Note that high values of SLOPEPC1 are associated with low littoral slope and vice versa; and high values of SHOREPC1 are associated with bare rocky shores, while low values are associated with vegetated shores. Geographical UTM coordinates were previously centred to 0. Variable name abbreviations and units are given in parentheses. Asterisks after variables' names indicate the results of U Mann-Whitney and Chi-square tests for continuous and categorical variables respectively, between both geographical areas: ***P <0.001; **P <0.01; *P <0.05; NS (not significant, P ≥0.05).

Variable	Western Pyrenees (n= 217)	Eastern Pyrenees (n= 1519)
Conductivity (COND) (µS/cm)***	44.15 (2.3-429.0)	20.50 (1.9-333.3)
Mean of the diurnal water temperature variation (VARI) (ºC)^{NS}	1.93 (-1.47-4.36)	1.89 (-0.29-4.12)
Ice-cover duration (ICECO) (Days)***	202.1 (127.7-258.1)	220.1 (134.4-295.6)
Cold sites (COLD)^{NS}	8 (3.7%)	45 (3.0%)
Littoral slope (SLOPEPC1)***	-13.52 (-65.18-55.86)	1.89 (-65.44-55.86)
Shore substrate (SHOREPC1)***	-16.62 (-50.77-80.81)	2.41 (-50.77-82.02)
Predation refuge (REFU)***	65 (30%)	290 (19.1%)
Macroinvertebrate richness (MIRICH)**	1.76 (0-5)	1.49 (0-5)
Fish presence (FISH)*	32 (14.7%)	328 (21.6%)

3. Supplementary figures

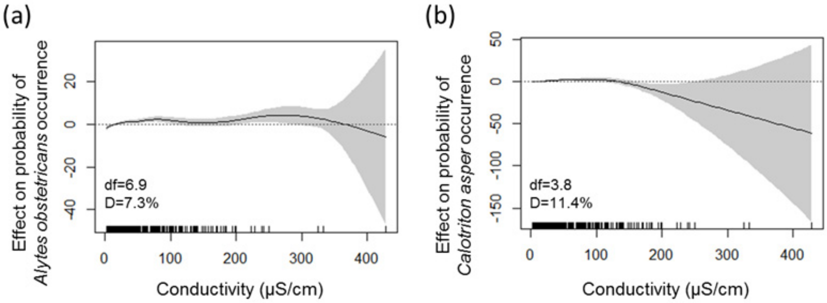

Figure S1 Default charts of conductivity terms for (a) *Alytes obstetricans* and (b) *Calotriton asper*.

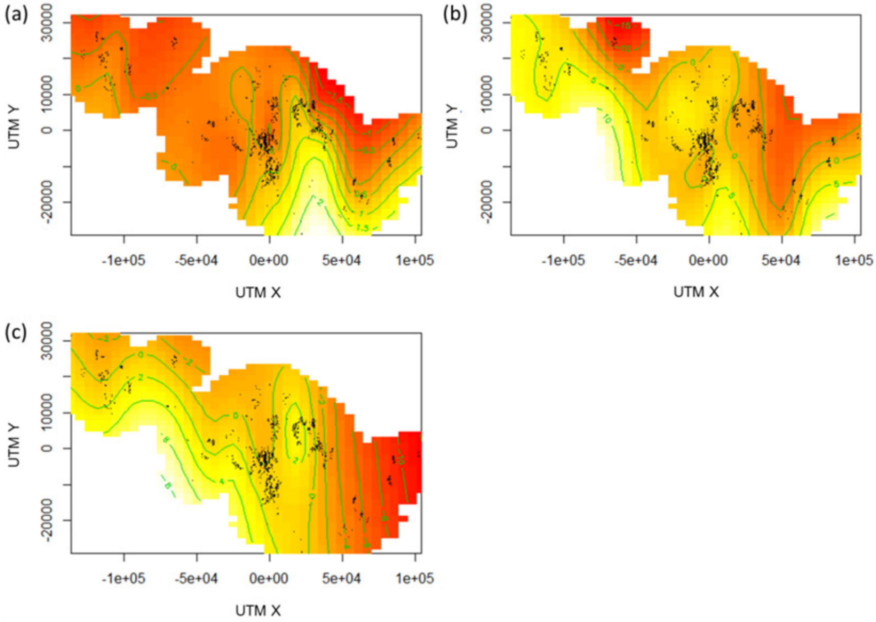

Figure S2 Colour version of the GAM isoline maps for the estimated effect of the significant (*P* <0.05) smoothing term UTM easting and northing on the occurrence of: (a) *Rana temporaria*, (b) *Alytes obstetricans*, and (c) *Calotriton asper*. Yelow colour indicates positive effect and red colour indicates negative effect.

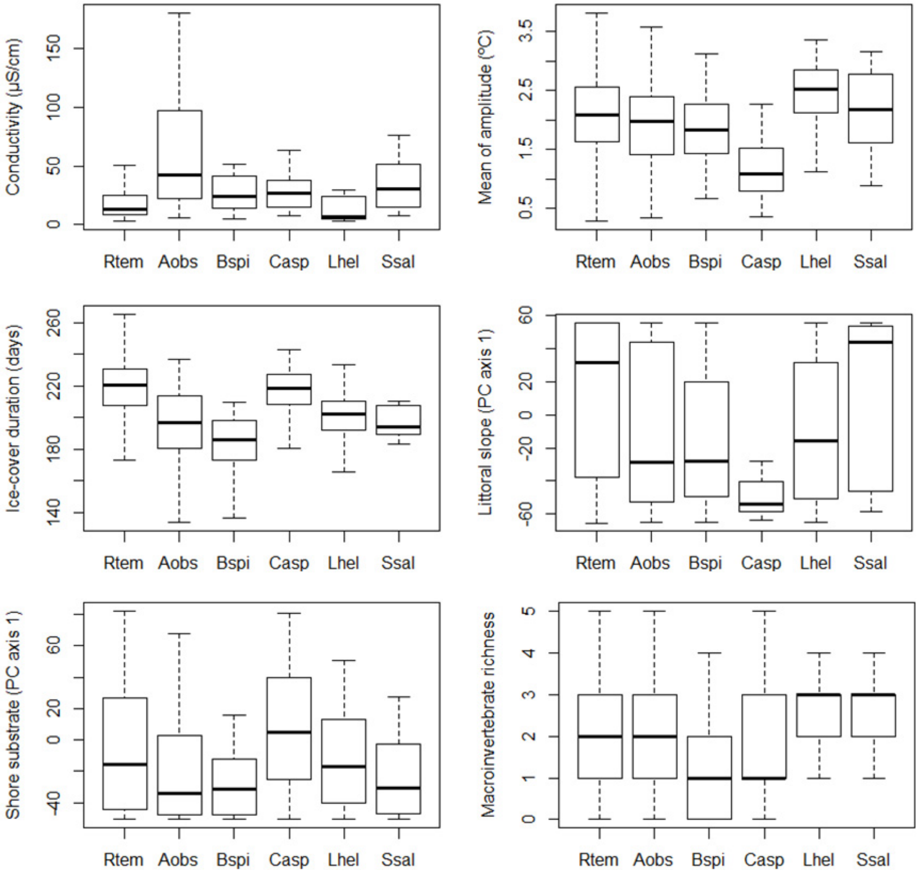

Figure S3 Range of presence of the 6 amphibian species found in the Pyrenean high mountain lakes for the continuous variables used in the analyses. Species abreviations are: (Rtem) *Rana temporaria*, (Aobs) *Alytes obstetricans*, (Bspi) *Bufo spinosus*, (Casp) *Calotriton asper*, (Lhel) *Lissotriton helveticus* and (Ssal) Sala*mandra salamandra*. The boxplots are made using only the presence of each species. The line within each box marks the median; the bottom and top of each box indicate the 25th and 75th percentiles and the whiskers below and above each box indicate the 10th and 90th percentiles. Note that high values of SLOPEPC1 are associated with low littoral slope and vice versa; and high values of SHOREPC1 are associated with bare rocky shores, while low values are associated with vegetated shores.

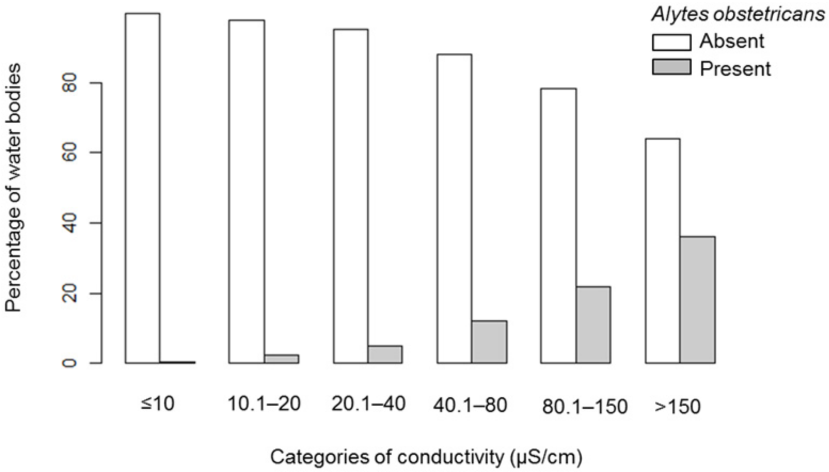

Figure S4 Percentage of presence and absence of *Alytes obstetricans* within different categories of water conductivity.

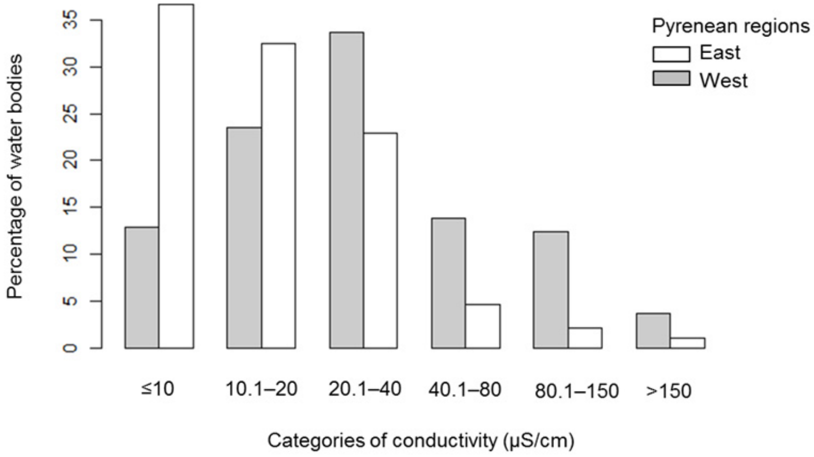

Figure S5 Percentage of sampled lakes and ponds by different categories of water conductivity, within the western and the eastern Pyrenean regions.

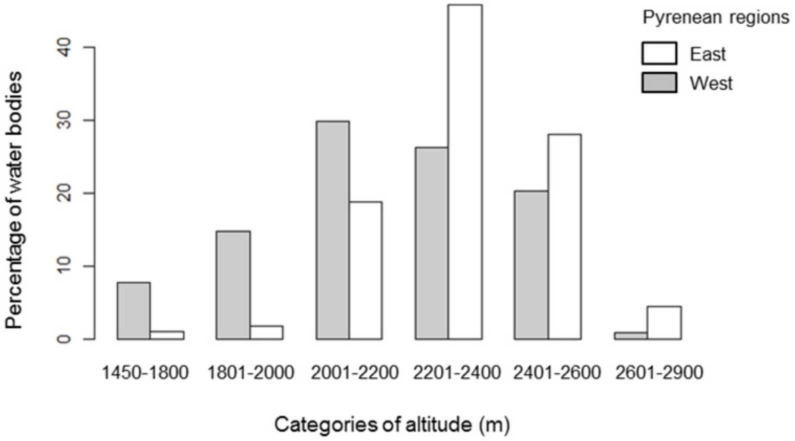

Figure S6 Percentage of sampled lakes and ponds by different categories of altitude, within the western and the eastern Pyrenean regions.

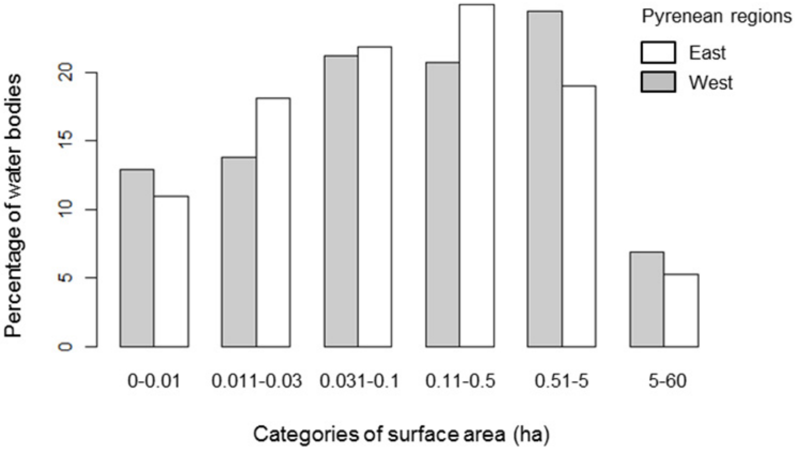

Figure S7 Percentage of sampled lakes and ponds by different categories of surface area, within the western and the eastern Pyrenean regions.

Chapter 5

Introduced fish mediate trophic cascade on the epilithon of high mountain lakes and ponds through tadpole eradication[6]

Abstract

Indirect effects of top predators on primary producers by the elimination of herbivorous consumers (trophic cascade) have been reported in many terrestrial and aquatic ecosystems during last decades. Food web alterations have been described, in lakes and ponds either in pelagic or in benthic habitats. In this study, we have combined field data from 75 Pyrenean lakes and ponds and experimental data from two field experiments to investigate the indirect effect of introduced fish on the epilithon community of the littoral of high mountain lakes and ponds, through the predation on amphibian tadpoles. Specifically, we applied ANOVAs, redundancy analyses and principal component analyses to disentangle the indirect effect of introduced fish on the biomass, the relative taxonomic abundance, the diversity patterns and the degree of activity and senescence of the littoral epilithon. The response variables used were the Chl-a concentration, the assemblages of algal and cyanobacterial groups, diatom species, and bacteria and archaea OTUs, as well as the stoichiometric composition of the whole littoral epilithic community. Both field and experimental data showed a large indirect effect of introduced fish on the littoral epilithon community of high

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mountain lakes in the Pyrenees. The epilithon biomass was higher in the presence of fish (6.6-fold higher biomass in terms of Chl-a), than in sites without fish. Fish was the main variable defining the biomass of the different algal and cyanobacterial groups. Chemical variables and nutrient availability were the most important characteristics in defining the composition of diatoms and bacteria, but tadpole abundance was also selected. The diversity patterns for epilithic diatom and bacteria assemblages showed higher α and γ diversities when fish were present and tadpole absent, but no difference in or lower β diversity, which suggested the existence of a process of biota homogenization in sites with fish. Finally, the epilithon community was less senescent and more active in presence of tadpoles. Therefore, there exists a strong trophic cascade caused by the indirect effect of introduced fish that is globally structuring the littoral epilithic community of high mountain lakes and ponds.

Keywords: ecosystem shift; littoral; biofilm; tadpole grazing; pigments; Chlorophyll-a; algal groups; diatoms; bacteria; archaea; stoichiometry; Pyrenees.

1. Introduction

Gradual environmental changes often lead to smooth shifts in ecosystems up to a threshold after which there is a drastic change to a markedly different state (Scheffer and Carpenter 2003). Lower ability to recover from perturbations (loss of resilience) has been reported as a conspicuous symptom of the upcoming severe change (Scheffer et al. 2009). An increasing number of studies are showing that introduced organisms can transform community composition and ecosystem properties through trophic cascades (Simberloff et al. 2013). Indeed, clear indirect effects of predators on primary producers, mediated by herbivores (trophic cascade), have been described in marine, terrestrial and riparian ecosystems during recent decades (e.g. Schmitz et al. 2006, Myers et al. 2007, Beschta and Ripple 2009, 2015).Trophic cascades impacting benthic communities have also been assessed in fluvial habitats (e.g. McIntosh and Townsend 1996, Usio 2000). Therefore, trophic cascades can cause large changes in ecosystems including impacts on ecosystem function and biodiversity, as well as living resources and ecosystem services with implications for human well-being. Understanding the conditions that facilitate trophic cascades is an issue of global interest (Carpenter et al. 2010).

In lentic habitats, several studies have shown that large fluctuations in top predator biomass have caused considerable changes in prey assemblages, primary producers and ecosystem processes (Carpenter et al. 2010). In addition to the influence of abiotic factors such as light or nutrients on the pelagic habitat, an increase in zooplanktivorous fish leads to a reduction in large-bodied herbivorous zooplankton, which in turn causes an increase in planktonic primary production, as well as phytoplankton richness, biomass and biovolume (Carpenter et al. 2001, Mette et al. 2011). The benthic habitat is often affected by trophic cascades, as it is permanently coupled with the rest of the ecosystem (Genkai-Kato et al. 2011). For example, the loss of submerged plants in shallow lakes is a result of the increase in phytoplankton biomass after cultural eutrophication (Jeppesen et al. 1998) or in some cases because of the increase in epiphyte biomass mediated by fish predation on grazing invertebrates (Martin et al. 1992, Jones and Sayer 2003). Furthermore, the increase in benthic algal biomass in lakes and ponds has been linked to the activity of fish or crayfish predators on herbivorous grazers (Bernot and Turner 2001, Nyström et al. 2001). The most widely studied intermediate grazer taxa have been freshwater snails and macroinvertebrates, although there have been some studies of anuran tadpoles (Schindler et al. 1990, Nystrom et al. 1999, Bernot and Turner 2001, Nyström et al. 2001).

High mountain lakes are remote freshwater ecosystems where most studies of trophic cascades have focused on the pelagic habitat in relation to the indirect effects of introduced fish. Although these lakes are naturally fishless, there have been numerous trout introductions to such ecosystems around the world since the 19th century, mainly for angling (Pister 2001, Miró and Ventura 2013). In recent decades, minnows have also been introduced as live-bait for recreational fishing to some mountain areas, including the Pyrenees (Miró and Ventura 2015). Previous studies from small alpine lakes have found an increase of herbivorous zooplankton biomass after removal of introduced trout, followed by a reduction of phytoplankton biomass, mainly due to colonisation by the cladoceran herbivore *Daphnia* (Sarnelle and Knapp 2005, Parker and Schindler 2006). The response of the phytoplankton biomass can be relatively fast or there may be a lag of several years (Parker and Schindler 2006). Comparatively little is known about the indirect effect of introduced fish on the benthic community of high mountain lakes, especially that resulting from the reduction or disappearance of tadpole grazers. High mountain lakes have high water transparency and often little or no coverage of submerged macrophytes, facilitating predation by introduced fish on local fauna such as anuran tadpoles. In fact, several studies have reported strong negative impacts of introduced trout on tadpoles of high mountain lakes (Knapp and Matthews 2000, Knapp 2005).

The drastic reduction in tadpole grazers has been shown to be the major factor leading to the restructuring of epilithic communities of streams (Whiles et al. 2013, Connelly et al. 2014). Similarly, in high mountain lakes a strong indirect effect of introduced fish on epilithic community might be expected after reduction in numbers or extinction of tadpoles. Our overall aim was to investigate whether introducing fish to high mountain lakes causes a trophic cascade affecting the littoral epilithic communities through eradication of tadpole grazers. In particular we characterized changes in the eplithic community through evaluating its development, changes in the species composition of different algal, bacterial and archaeal assemblages, patterns of diversity and homogenization of communities, and the degree of activity and senescence. We hypothesized that introduced fish were responsible for a trophic cascade affecting the epilithon of high mountain lakes and ponds through removing tadpole grazers. Specifically we hypothesized that with as a result of the presence of fish and absence of tadpole grazers, (*i*) the epilithic community biomass would increase, (*ii*) introduced fish would be one of the main factors affecting taxonomic composition of epilithic community, (*iii*) diversity patterns would change towards a more homogeneous community, and (*iv*) the epilithon community would become less active and more senescent.

2. Methods

2.1. Description of the study Area

We sampled lakes and ponds spread along the Pyrenean mountain range (0°42’W - 2°09’E, 42°52’ - 42°23’N; Fig. 1). There are 1 080 lakes > 0.5 ha and approximately 4500 small lakes and ponds either of glacial origin or modified by the activity of quaternary glaciations. They range in altitude between 1 500 and 2 960 m with the highest frequency found at *ca.* 2 300 m. The natural lakes are relatively small and deep, with mean surface area of 4.5 ha and mean maximum depth of 15 m, with the largest being 44 ha and the deepest 105 m. Approximately 15% of the total lakes are impounded, mostly the biggest. Among these semi-natural dammed lakes, the largest has an area of 160 ha and the deepest a maximum depth of 123 m. The ponds can range in surface area from a few square meters to 0.5 ha, and in maximum depth from a few centimeters to 1-2 m. Some of them are temporary and may dry up in the peak of the ice-free season.

Due to their common glacial origin, there is a close positive relationship between surface area and maximum depth (Catalan et al. 2009b). Most of the lakes are above the tree line with catchments partially covered by meadows, although some of them are on or below the tree line. Approximately half of the lakes have catchments on granodiorite bedrock, the remaining being located in catchments with metamorphic (25%), detrital (15%) or carbonate (10%) bedrock with a minority on Silurian slate (Casals-Carrasco et al. 2009). The latter bedrocks, due to their high sulfate content, give natural acidity to the waters (pH < 5.5). The ionic content is generally low, being the chemical factor most strongly related to bedrock composition (Catalan et al. 1993). The lakes have low phosphorous and chlorophyll content, being of oligotrophic nature (Buchaca and Catalan 2007). A general description of the climate, physical-chemical and biological characteristics of Pyrenean lakes and a comparison with other European high mountain lakes can be found elsewhere (Camarero et al. 2009, Catalan et al. 2009a, Kernan et al. 2009a, Thompson et al. 2009).

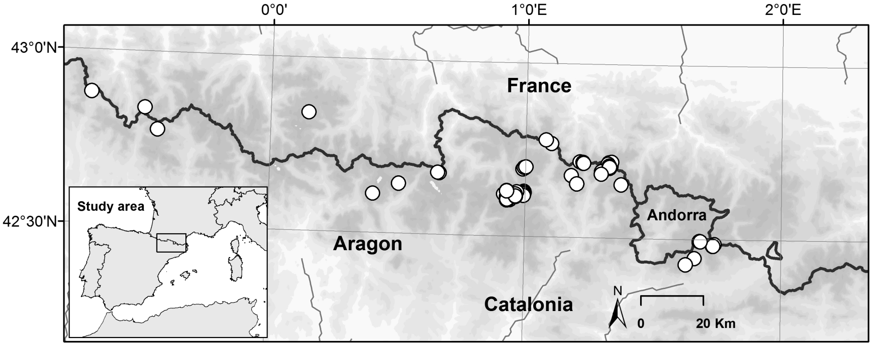


Fig. 1. Distribution of studied lakes and ponds along the Pyrenean range

2.2. Study design

To disentangle whether introduced fish cause a trophic cascade on the littoral of high mountain lakes and ponds through eradication of tadpole grazers, we chose as response variables four assemblages of the littoral epilithon community: algal and cyanobacterial groups (chlorophytes, diatoms, rhodophytes, N2-fixing cyanobacteria and other cyanobacteria), diatoms (at species level), bacteria and archaea; in addition to the total concentration of Chl-a, the Chl-a concentration from epilithic diatoms and the stoichiometric composition of the whole littoral epilithic community.

We worked on a main dataset of samples from each of 75 lakes and permanent ponds. The main features of the sampled lakes and ponds are summarized in Table 1. The sites were chosen according to four situations: presence of fish and absence of tadpoles, absence of fish and presence of tadpoles, presence of both fish and tadpoles and absence of both fish and tadpoles. The number of sites in each category was roughly proportionate to the real number of lakes and ponds that exist in the Pyrenean range, being 26, 33, 5 and 11 respectively. The sites with fish had brown trout (*Salmo trutta*), or European minnow (*Phoxinus* sp.), or both species. The sites with tadpoles had at least one of the three anuran species that live in Pyrenean high mountain lakes, mainly common frog (*Rana temporaria*), but also midwife toad (*Alytes obstetricans)* and common toad (*Bufo spinosus*) in some cases.

For the analyses of algal and cyanobacterial groups and diatoms the data were gathered from the complete dataset of 75 sites. For bacteria and archaea analyses the data were gathered from a subset of 26 sites (12 with fish only and 14 with only tadpoles). For stoichiometric analyses the data were gathered from a subset of 31 sites (15 with fish only and 16 with only tadpoles) (Fig. 2A).

2.3. Field experiments

Additionally to the field sampling, two exclusion experiments of fish and tadpoles were carried out in two different lakes: Lake Clotada, fishless and with tadpoles of common frog and Lake Naorte, with introduced European minnow and without tadpoles. The overall aim of exclusion experiments was to demonstrate the link between introduced fish presence and degree of epilithon grazing activity and also the existence of a direct grazing effect of tadpoles on the epilithon. The mesocosms used were semitransparent plastic boxes 52x42x35 cm. We placed a net of 1 mm mesh size in a hole of 22x26 cm in the two longer sidewalls, which allowed water exchange between the lake and the mesocosms. The open tops of the boxes were covered by a lid made with 1 cm diameter mesh, mainly to prevent predator access and fish escape. The bottoms of the boxes were covered by cobbles collected from the lakes, handled carefully to not remove the epilithon. Field experiments were installed from 6th to 27th August 2013 in Lake Clotada and from 4th to 26th August 2013 in Lake Naorte, remaining in situ for 22 and 23 days respectively.

The Lake Clotada experiment aimed specifically to verify if tadpoles prevented the epilithon growth and comprised three replicates with 6 tadpoles of common frog from the lake in each and three replicates without tadpoles. No fish were used in the Lake Clotada experiment. Control samples were gathered directly from the lake, which had tadpoles of common frog but not fish (Fig. 2B).

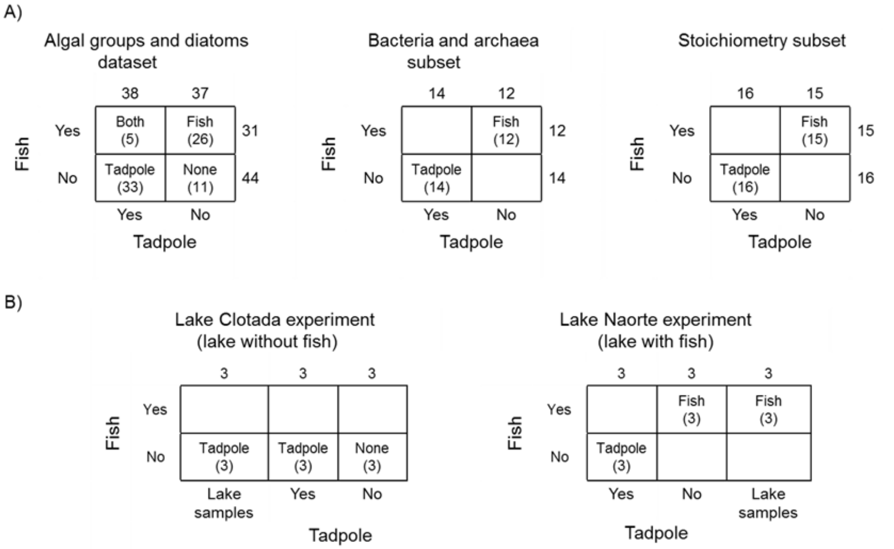


Fig. 2. Plan of the study design, showing the distribution of the sampled sites on the different datasets, subsets and categories used.

Table 1. Main characteristics (median and range values) of the complete dataset of 75 lakes and ponds studied, with and without fish.

	Lakes and ponds	
Variable	With fish (n=31)	Without fish (n=44)
Tadpole abundance (ind/m shoreline)	0 (0-102.5)	3.6 (0-113.5)
Altitude (m)	2158 (1952-2441)	2282 (1875-2551)
Surface area (ha)	0.31 (0.01-14.44)	0.1 (0.001-17.95)
Total catchment (ha)	71.01 (0.15-457.9)	7.85 (0.1-156.9)
Maximum depth (m)	2.1 (0.25-63)	0.7 (0.15-46)
Conductivity (µS/cm)	16.1 (3.3-48.1)	10.9 (3.2-108)
pH	6.93 (5.67-8.21)	6.2 (5.4-8.02)
Alkalinity (µeq/L)	108 (14-341)	76 (1-817)
Nitrate (µg N/L)	22.8 (1.6-233.4)	17.3 (0-354.2)
Phosphate (µPg/L)	1.5 (0-24)	1 (0-27.5)

The Lake Naorte experiment aimed specifically to verify if fish prevented grazing activity and if tadpoles significantly reduced the biomass of epilithon and changed the structure of the epilithic community. The experiment comprised three replicates with two European minnows from the lake in each and three replicates with six tadpoles of common frog from Lake Clotada in each. Control samples were gathered directly from the lake, which had introduced minnows but not tadpoles (Fig. 2B).

During the installation periods, the field experiments were visited weekly to confirm the stability of the water level and the well-being of fish and tadpoles in the mesocosms. Since the water level of Lake Clotada naturally decreased during the experiment period, the placement of the mesocosms was moved slightly towards the center of the lake. The water level of Lake Naorte remained unchanged. No problems were detected in the presence or condition of the fish and tadpoles in either experiment. When the experiments were dismantled, all fish and tadpoles were found to be in good condition, except one tadpole in one Lake Clotada mesocosm, which was not found. No other incidents happened during the field experiments.

On the day that experiments ended, we collected samples for Chl-a, algal and cyanobacterial groups and diatoms from the cobbles that covered the bottoms of the mesocosms. The samples were gathered following the methods described in the following paragraphs.

2.4. Collection of epilithic samples

We collected the samples of the epilithon community during the ice-free seasons from 2010 to 2013. Samples were gathered from the upper side of 4-6 cobbles per site. Cobbles measured 5-10 cm and were collected from different places within the permanently submerged zone of littoral perimeter at 1 to 0.5 m depth. The epilithon was detached by scraping with a toothbrush and washed into polyethylene containers. The cobble surface brushed (from 300–1000 cm^2 per site) was delimited using aluminum foil that was dried and weighted in the lab and converted to surface by a linear regression function between aluminum foil surface and weight. Samples to identify diatoms were preserved in Lugol iodine (4%) prior to counting. Samples to analyze Chl-a, algal and cyanobacterial groups, bacteria, archaea and stoichiometry were kept cold at 4ºC during the field day and frozen the same evening for preservation.

2.5. Epilithic pigment analysis

Pigments were extracted from a known weight of liofilized epilithon with a probe sonicator (50 W, 2 min) using 90% acetone. The extract was filtered through Whatman Anodisc filters (0.1 µm) and analysed using a Waters AQUITY UPLC system (Waters Corporation, USA). Samples (ca. 4.5 µL) were resolved with 100 % solvent B (0.3 M ammonium acetate in methanol:acetonitrile:MilliQ water, 51:36:13 (v/v/v)) to 75 % B and 25 % A (ethyl acetate:acetonitrile, 70:30, (v/v)) for 3 min, 0.45 min of isocratic hold at 75% B and 2 min linear gradient to100% solvent A and a Waters ACQUITY C18 SB 1.8µm HSS particle, 2.1 mm x 100 mm column. The flow rate was 0.7 mL min^{-1}. The solvent composition was returned to initial conditions over a 0.55 min gradient, followed by 1 min of system equilibration before injection of the next sample. The detector was set at 440 and 660 nm for carotenoid and phorbin peak integration, respectively. Pigments were identified by comparison with a library of pigment spectra obtained from extracts of pure algae cultures from the Culture Collection of Algae and Protozoa (CCAP, Oban, Scotland, UK). The extinction coefficients used for calculations were obtained from the literature (Davies 1976, Rowan 1989, Jeffrey et al. 1997). Pigment molecular weight was obtained from the literature (Proteau et al. 1993, Jeffrey et al. 1997, Borrego et al. 1999). The pigment concentration in the epilithon samples was calculated as nmols cm^{-2}.

2.6. Algal and cyanobacterial group composition from marker pigments

To estimate the contribution of each algal and cyanobacterial group most pigment-based methods use ratios between a given marker pigment and Chl-a. One of those methods is a computer program for estimating class abundance from chemical markers using factor analysis (CHEMTAX) (Mackey et al. 1996). The method works by algorithm iteration and requires an initial estimate of the marker pigment to Chl-a ratios (input ratio matrix) appropriate for the algal and cyanobacterial classes that may happen to be in the sample. An initial value for the abundance of algal and cyanobacterial class is then derived. After that, the pigment ratio matrix is varied by a predetermined amount and the class abundance recalculated. A steep descent algorithm is used to obtain a best estimate of class abundance and pigment ratios. The process is repeated until the least squares fit satisfies predetermined limits, CHEMTAX then gives the best fit of contributions of the predefined taxa to total Chl-a. The advantage of this method is that it distinguishes between algal and cyanobacterial groups with qualitatively identical pigment compositions by differences in pigment ratios and gives the best fit of contributions of the predefined taxa to total Chl-a. We used between 1 and 4 marker pigments per algal and cyanobacterial group including biomarkers of chlorophytes, diatoms, cyanobacteria and rhodophytes. The initial estimation of pigment ratios (initial ratio matrix; H0) was obtained from the output matrix of previous studies in alpine lakes (Buchaca 2009) and references of river biofilms (Caramujo et al. 2008, Majdi et al. 2011) and cultures (Schagerl and Donabaum 2003, Schluter et al. 2006).

2.7. Diatom processing and analysis

Samples for diatom analysis were processed for the oxidation of organic matter (33 % Hydrogen Peroxide), and carbonates were removed by adding 2 ml HCL 1M (ca. 10%) using standard techniques following Battarbee et al. (2001). Diluted slurries were permanently mounted for diatom enumeration using Naphrax®. In order to homogenize valve counting effort, exactly five hundred diatom valves were identified at 1,000x magnification under oil immersion using a Zeiss® Axio Imager A1 microscope under interferencial contrast. Diatom identifications were based on (Lange-Bertalot and Metzeltin 1996, Krammer 2000, Lange-Bertalot 2001, Krammer 2002, Krammer 2003, Hofmann et al. 2011, Lange-Bertalot et al. 2011, Levkov 2013)

2.8. Bacteria and archaea analysis

The structure of bacteria and archaea assemblages were analyzed through ARISA method (Automated Ribosomal Intergenic Spacer Analysis, Brown et al. 2005). Intergenic Transcribed Spacers (ITS) from DNA samples were amplified using PCR with primers TSF/ITSReub (5'-GTCGTAACAAGGTAGCCGTA-3' / 5'-GCCAAGGCATCCACC-3') for Bacteria and 1389F/71R (5’-ACGGGCGGTGTGTGCAAG-3’ / 5`-TCGGYGCCGAGCCGAGCCATCC-3’) for Archaea. The forward primer was fluorescently labeled (5-FAM) in both cases. The PCR mixture (40 µl) contained a final concentration of 0.25 ng µl-1 of DNA template, 250 nM of each primer, 250 µM of each dNTP, 2.5 mM MgCl2, 3 units of a Taq DNA polymerase (Invitrogen-Life Technologies), 40 ng µl-1 of BSA and the enzyme buffer. PCR cycling, carried out in an automated thermocycler (BioRad), was: initial denaturation at 94ºC for 2 min; 32 cycles with denaturation at 94ºC for 15 sec, annealing at 55ºC for 30 sec and extension at 72ºC for 3 min; and a final extension at 72ºC for 9 min. PCR products, stored at 4ªC, were purified with the QIAquick PCR Purification Kit (Qiagen) and quantified with NanoDrop 1000 (Thermo Fisher Scientific Inc., Wilmington, DE). Each purified PCR product was added to a mix composed of 10 µl of Hi-Di formamide, 0.3 µl of the internal size standard X-Rhodamine MapMarker 1000 (ROX) (BioVentures). PCR product final concentration was 1 ng µl-1. Samples were run using a genetic analyzer with 36 cm Capillary Array and 3130 POP-7 Polymer (Applied Biosystems). The electropherograms were then analyzed using the GeneMarker analysis software (Softgenetics) for size calibration. Binning of the peaks into Operative Taxonomic Units (OTUs) was done using R scripts as in Ramette (2009) and available at http://www.mpi-bremen.de/en/Software_2.html. A minimum RFI cutoff value of 0.001% and a window size of 2 bp was used.

2.9. Stoichiometry processing and analysis

Epilithic samples were dried to constant mass for subsequent analysis of elemental C, N and P. Elemental C and N were analyzed at the Stable Isotope Facility, University of Davis, California, U.S.A., using a Europa Hydra 20 ⁄20 continuous flow isotope ratio mass spectrometer (CFIRMS) (PDZ Europa, Cheshire, U.K.) coupled with an elemental analyzer. Elemental P was analysed after persulfate digestion (Grasshoff, 1983) and phosphorus determined by the ascorbic acid-molybdenum blue method according to Murphy & Riley (1962).

2.10. Habitat and water chemistry descriptors

In addition to epilithon samples, we gathered several descriptor variables of the study design, habitat descriptors and water chemistry from each site, to be used in the different analyses carried out (Table A1).

The occurrence data for fish (FISH) were obtained from historical documents, from interviews with local elderly fishermen or nature reserve wardens, from local reports of fishing or walking societies and from our own visual encounter and field surveys (Miró and Ventura 2013, 2015). The occurrence and abundance of tadpoles (TADPOLE) were obtained by visual encounter surveys of the entire shoreline (Crump and Scott Jr. 1994). As high mountain lakes are extremely clear, large areas could be surveyed from the shore and tadpoles could be easily detected, as had been shown by previous studies (e.g. Knapp 2005, Pilliod et al. 2010). To avoid the temporal differences in amphibian occurrences during the lake ice-free period as found in some studies in high mountain lakes (e.g. Knapp 2005), we performed the surveys during the season by following the altitudinal and insolation gradients, from warmer to colder sites. Moreover, when there were doubts about the representativeness of the date for tadpoles, we repeated the survey in the following years and took the greatest abundance found.

Altitude (ALT), surface area (SURF), shore perimeter (PERIM), total catchment (TCAT), direct catchment or proportion of catchment not shared with other lakes that are upstream of the lake (DCAT), and geographical coordinates UTM x (utmx) and UTM y (utmy) of the lakes were obtained from a GIS generated from 1:25000 maps from the various national agencies (Casals-Carrasco et al. 2009). Maximum depth (DEPTH) was measured during the field surveying by echosounding.

We used the accumulated degree days (ADD) during the ice-free period calculated from daily max-min temperature data using the sine-wave method, to describe the thermal characteristics of the lakes, assuming the trigonometric sine curve as an approximation of the diurnal temperature curve and adding the area under the curve and above the lower threshold for each day (Baskerville and Emin 1969). As the lower threshold, we used the minimum developmental temperature of *R. temporaria,* 7.64°C (Balcells 1975) below which growth is not feasible, and computed the estimated ADD for each site following the model developed by Miró et al. (2015). Four variables related to solar radiation were also obtained from

GIS: total catchment direct radiation duration (CATCH-RAD) which is the annual mean duration of direct solar radiation income in the total catchment of the lake; total catchment solar global radiation (CATCH-SOL) which is the annual mean solar radiation, direct and diffuse, income in the total catchment of the lake; lake direct radiation duration (LAKE-RAD) which is the annual mean duration of direct solar radiation income in the lake or pond surface; and lake solar global radiation (LAKE-SOL) which is the annual mean solar radiation, direct and diffuse, income in the lake surface.

Littoral and shore habitats were characterized by visually estimating substrate features and composition of the entire shoreline. Data were expressed as % coverage for each category of 3 characteristics: littoral slope, littoral substrate composition and shore substrate composition. Littoral slope was categorized as shallow (<10 cm deep, <15º slope), slight slope (15º–30º slope), sloping (30º–70º slope) or sub vertical (70º–90º slope). Littoral substrate composition was categorized as organic material, silt-sand (<0.5 cm), gravel (>0.5–2.5 cm), cobble (>2.5–25 cm), boulder (>25 cm–1 m), big boulder (>1 m) or bedrock. Shore substrate composition was categorized as cobble-boulder (2.5 cm–1 m), big boulder-bedrock (>1 m) or terrestrial vegetation. We adapted these classifications from previous studies about high mountain and boreal lake fauna (e.g. Knapp et al. 2001b, Johnson et al. 2004). To reduce the dimensionality of the substrate data, we subjected the littoral slope, littoral substrate and shore substrate data to separate principal components analyses (Knapp 2005). In all three analyses, axis 1 explained a substantial amount of the total variation in substrate characteristics and composition (littoral slope: 75.4%; littoral substrate: 68.6%; shore substrate: 78.3%), so the scores were used as the independent variable representing littoral zone slope (SLOPE), littoral zone substrate composition (LITTO) and shore substrate composition (SHORE). High values of SLOPE were associated with low littoral slope and vice versa; high values of LITTO were associated with fine grain substrate, while low values of LITTO were associated with coarse grain substrate; high values of SHORE were associated with vegetated shores, while low values of SHORE were associated with bare rocky shores (Table A2). Aquatic vegetation (AQVEG) is the % of macrophyte coverage in the littoral zone.

A sample for Chl-a (CHLA.SES) was taken from the water column of deepest point of the lake at a depth of 1.5-times the Secchi depth, using a polyethylene tube connected to a flask and a manual vacuum pump. In lakes where the Secchi depth reached the sediment surface, or in ponds, the sample was taken between 1 and 2 m above sediment surface. From this sample, between 1.5 and 2 L were filtered using a manual vacuum pump and GF/F filters. The filters were kept cold at 4ºC during the field day and frozen the same evening for preservation. Pigments were extracted from frozen filters with a probe sonicator (50 W, 2 min) using 90% acetone. The extract was filtered through Whatman Anodisc filters (0.1 µm) and analyzed using a Waters AQUITY UPLC system as described before in the "Epilithic pigment analysis" subsection. Total epilithic concentration of Chl-a (CHLA.EPI) and Chl-a concentration of epilithic bacillariophyta (BACILL) were also used in some analyses.

Water samples for chemical analyses were collected from the lake outlet and analyzed using the methods described in Ventura et al. (2000). The chemical features obtained for each site were the pH (pHlab), and concentrations of ammonium (NH4), calcium (ca), magnesium (Mg), sodium (Na), potassium (K), alkalinity (Alk), sulphate (SO4), nitrate (NO3), chloride (Cl), phosphate (PO4), total nitrogen (TN), total phosphorus (TP), silica (Si), total organic nitrogen (TON), total organic phosphorus (TOP) and dissolved inorganic nitrogen (DIN).

2.11. Statistical analyses

We analyzed the epilithon response variables in four ways, matching the four aims and hypotheses of the study: (1) impact of introduced fish on of epilithon development, (2) relative importance of introduced fish on taxonomic composition, as well as (3) impact on the diversity patterns and community homogenization, and (4) impact on the degree of activity and senescence of the epilithic community. The data from the exclusion experiments were analyzed to confirm the field results of the first and second hypotheses.

The impact of introduced fish on the biomass of epilithon was tested by two univariate two-way ANOVA of total concentration of Chl-a and concentration of Chl-a from diatoms. Factors comprised Fish (Yes and No, N = 2) and Tadpole (Yes and No, N = 2). Main and interaction effects between both factors Fish and Tadpole were tested. One univariate one-way ANOVA was also made on the abundance of bacteria cells of the epilithon using the factor Fish/Tadpole (presence of fish plus absence of tadpole and vice versa, *N* = 2). To avoid heterogeneity in the variance, total

Chl-a concentration and bacteria cell abundance were log(100x+1) transformed and Chl-a concentration from diatoms was fourth root transformed. The relative importance of fish on the biomass of epilithon was assessed by a redundancy analysis (RDA; Wollenberg 1977) using Chl-a concentration of algal and cyanobacterial groups as response variables, and fish, tadpoles, habitat and chemistry features as explanative variables (Table A1). To focus on the quantity of epilithon, we used the concentration values of Chl-a, which were fourth root transformed to be brought closer to the normal distribution. To search for parsimony and prevent the problem of inflation of the overall type I error, before the RDA analysis we performed an appropriate variable forward selection process using a double stopping criterion for each candidate variable: (a) traditional significance level α= 0.05 and (b) global adjusted R^2 threshold of the RDA made with all potential explanatory variables, only if that test was significant (Blanchet et al. 2008). In addition, we examined for linear dependencies among the explanatory variables in the RDA which could render the regression coefficients of the explanatory variables unstable (Legendre and Legendre 1998), using Variance Inflation Factor (VIF, Borcard et al. 2011). After the forward selection processes, all VIF values obtained were below 10, the VIF threshold value indicative of worrisome collinearity (Table A3). We tested the significance of all RDAs and their axes through permutation tests with 999 permutations. Before being introduced in the RDA, all variables were standardized to zero mean and unit variance to correct their heterogeneous dimensions (Borcard et al. 2011). Binary variables of presence/absence were used directly as dummy (1/0) variables (Legendre and Legendre 1998). To visualize RDA results, we drew a correlation triplot diagram (scaling 2), which allows one to focus on the variables' ordination (Legendre and Legendre 1998). For each RDA, we plotted the two most explanatory canonical axes found in the RDA analyses, RDA1 and RDA2. The site scores were drawn as linear combinations of explanatory variables (lc scores). The relative importance of the selected variables was estimated by computing the explained variance of them through successive partial RDAs using each evaluated variable as covariable each time. Individual explained variance represents the percentage of the total variance not explained by the RDA when the examined variable is held constant (Borcard et al. 2011).

The relative importance of introduced fish on the taxonomic structure of the epilithic community was assessed by distance-based RDA (dbRDA) performed for the three assemblages: diatoms, bacteria and archaea. The dbRDA were made by variable forward selection process following the RDA method detailed in the previous paragraph, but using as response variables the principal coordinates obtained from the euclidean distances between sites based on the taxonomic matrices normalized through Hellinger transformations (Legendre and Gallagher 2001). The VIF values for the sets of explanatory variables introduced in the final analyses are shown in Appendix A (Tables from A4-A6). The relative importance of the variables was estimated also by individual partial RDA as we explained above.

The impact of introduced fish on the diversity patterns and species homogenization of the epilithon was investigated by comparing α, β and γ diversity patterns in presence/absence of fish or tadpoles for diatom, archaea and bacteria assemblages. Taxonomic richness was used as α diversity measure and was tested by ANOVA as described above. Interaction effect between fish and tadpoles was also tested for on diatom assemblage. β diversity was assessed by computing the distances of the group members to the group centroid (spatial median) in a principal coordinate space (Anderson et al. 2006). The effects of the factors were explored through analyses of the multivariate dispersion (variance), testing by ANOVA the differences between-categories for the distances of the category members to the category centroid. The groups used were the categories of the same factors detailed above for α diversity analyses. The similarities among sites used were the euclidean distances among sites based on the taxonomic matrices normalized through Hellinger transformations (Legendre and Gallagher 2001). As the distances to the centroid were computed separately for each category, interaction effect between fish and tadpole factors could not be computed for diatom assemblage. γ diversity was evaluated by building species accumulation curves (SAC) for diatom, bacteria and archaea assemblages, made by randomly addition of sites through 9999 permutations (Gotelli and Colwell 2001). For the diatom assemblage, we included only the sites that had exclusively fish or tadpoles and excluded the sites that had both or none of them.

Impact of introduced fish on the degree of activity and senescence of the epilithic community was investigated through several analyses. Firstly we analyzed the stoichiometric composition of the epilithon by computing separately three one-way ANOVA on the proportions of total epilithic C, N and P. The factor used was Fish/Tadpole (presence of fish and absence of tadpole and vice versa, $N = 2$). To assess the relative importance of fish on the stoichiometric composition of the epilithon, we performed one RDA using the proportions of total epilithic C, N and P as response variables and fish presence, tadpole abundance and habitat and chemistry variables that did not contain C, N or P as explanatory variables. The RDA was made by a variable forward selection process following the RDA method detailed in the previous paragraphs. To achieve normality and avoid heterogeneity in the variance, the ANOVA and the RDA were computed on the percentages of C, N and P normalized by the transformation arcsine(square root(x/100)). The VIF values for the set of explanatory variables introduced in the final RDA are shown in Appendix A (Table A7). The relative importance of the variables was estimated also by individual partial RDA as we explained above. Secondly, the effect of introduced fish on the degree of senescence of epilithic Chl-a was tested by one univariate two-way ANOVA on the Chl-a preservation index (Chl-a expressed as a percentage of a-phorbins). Factors comprised Fish (Yes and No, N = 2) and Tadpole (Yes and No, N = 2). The main and interaction effects between both factors Fish and Tadpole were tested. Again, to avoid heterogeneity in the variance, Chl-a preservation index was arcsine(square root(x/100)) transformed. Eventually, we assessed the degree of activity of the epilithon versus the degree of grazing by linearly regressing total epilithic Chl-a concentration and tadpole abundance for the 33 sites that have tadpoles and do not have fish. Both variables were log(100x+1) transformed to bring them closer to the normal distribution.

The data of the exclusion experiments were used to confirm the field results of the impact of introduced fish on the biomass of the epilithic community and its taxonomic structure. The impact on the biomass of epilithon was tested by univariate one-way ANOVA on the concentration of total Chl-a and the concentration of Chl-a from diatoms. Factors comprised Tadpole exclusion in Lake Clotada (Tadpole from the lake and Tadpole/No tadpole from mesocosms, N = 3) and Fish exclusion in Lake Naorte (Tadpole/Fish from mesocosms and Fish from the lake, N = 3). Tukey post hoc tests were applied to see differences among categories when ANOVA was significant. To avoid heterogeneity in the variance, total Chl-a concentration was log(100x+1) transformed and Chl-a concentration from diatoms was fourth root transformed. The impact of fish on the taxonomic structure of the epilithon of the exclusion experiments was investigated by PCA representations of the concentration of Chl-a from algal and cyanobacterial groups and for the taxonomic composition of the diatoms assemblage. Data were previously normalized by fourth root transformation for algal and cyanobacterial groups and Hellinger transformation for diatom assemblage.

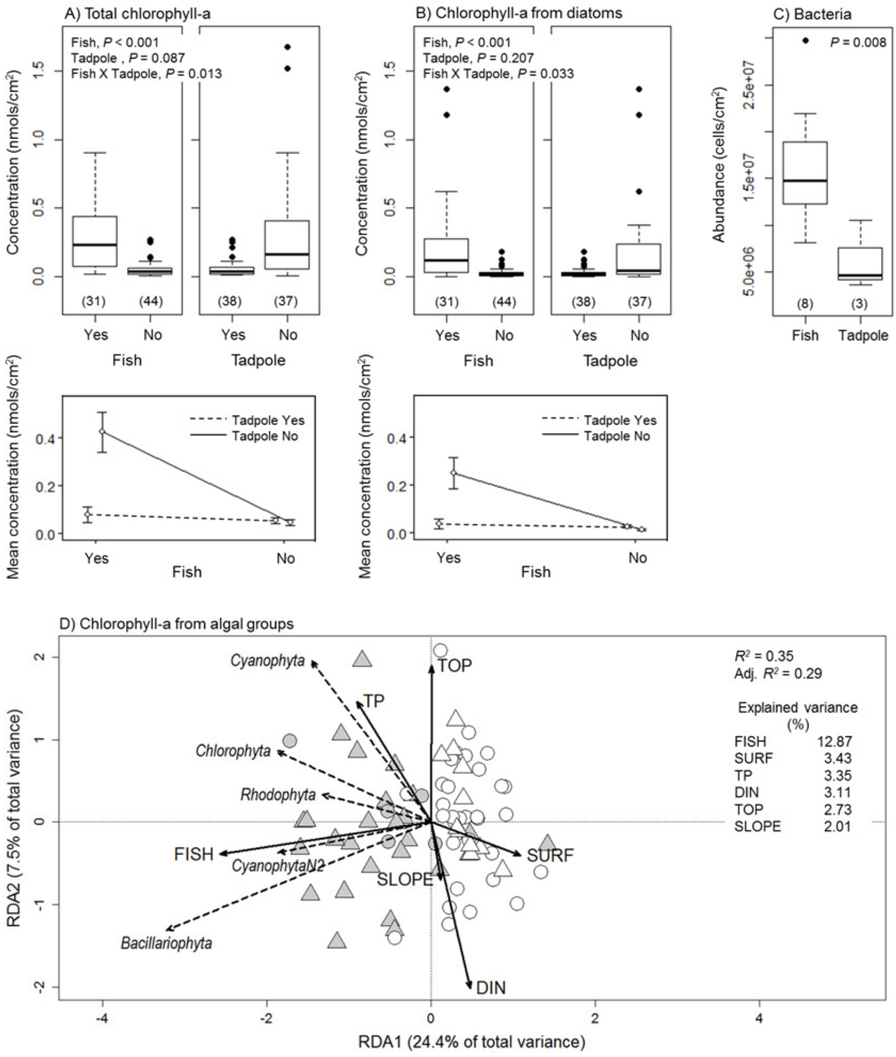
All analyses were performed with R statistical software (R Development Core Team 2014) using the basic functions and the packages *car* to compute Levene's tests of homogeneity of variance (Fox and Weisberg 2011), *ggplot2* to draw interaction plots of ANOVA analyses (Wickham 2009), *packfor* to perform multivariate forward selection procedures (Dray et al. 2013) and *vegan* to compute RDA and diversity analyses (Oksanen et al. 2013a).

3. Results

3.1. Biomass of epilithon

The overall median for total epilithic Chl-a in our dataset was 0.052 nmols/cm^2. Two-way ANOVA revealed that sites with fish had higher concentration of total Chl-a (median of 0.231 nmols/cm^2) than sites without fish (median of 0.035 nmols/cm^2); tadpole factor did not show significant main effects for concentration of total epilithic Chl-a (Fig. 3A; Table A8). Fish and Tadpole had a significant interaction effect, in that the concentration of total epilithic Chl-a was higher when fish were present and tadpoles were absent (Fig. 3A; Table A8 in the Appendix).

Fig. 3. (next page) Effect of tadpoles and introduced fish on the biomass of epilithon. The upper panel shows boxplots and interaction plots of ANOVA results for (A) total concentration of Chl-a, (B) Chl-a concentration from diatoms, and (C) abundance of bacteria. The p-values of the two-way (A and B) and one-way (C) ANOVAs are shown on the top of the graphics. The number of sites for each category are written between brackets and below the boxes. The vertical bars of the interaction plots represent the standard error of the mean. The lower chart (D) is the RDA performed on the quantity of Chl-a from different algal and cyanobacterial groups of the epilithon. To focus on the quantity of each algal and cyanobacterial group, the RDA was computed directly on Chl-a concentrations normalized by forth root transformations. Symbols of gray color indicate fish presence; white color, fish absence; circles, tadpole presence; triangles, tadpole absence. The explained variance of the variables was estimated by successive partial RDAs using each selected variable as a covariable each time, and represents the percentage of the total variance not explained by the RDA when the examined variable is held constant. Note that High values of SLOPE are associated with low littoral slope and vice versa. A complete ANOVA table can be seen in the Appendix A (Table A8).



The overall median for Chl-a from diatoms was 0.024 nmols/cm^2. As with total Chl-a, two-way ANOVA revealed that sites with fish had higher concentration of Chl-a from diatoms (median of 0.115 nmols/cm^2) than sites without fish (median of 0.015 nmols/cm^2); tadpole presence/absence did not show significant main effects on Chl-a concentration from diatoms, but fish and tadpole had a significant interaction effect: the concentration of Chl-a from diatoms being higher when fish were present and tadpoles were absent (Fig. 3B; Table A8). Two-way ANOVA also revealed fish main effects on Chl-a concentration from chlorophyta, rhodophyta, N_2-fixing cyanobacteria and other cyanobacteria, with the biomass of all groups being higher in the presence of fish than in the absence. Nevertheless, the concentrations of Chl-a from these algal and cyanobacterial groups were not statistically different for tadpole presence/absence or for the interaction between both factors fish and tadpole (Table A9 and Fig. A3 in the Appendix A).

The overall median for bacteria cell abundance obtained was 12.4 x 10^6 cells/cm^2. Bacteria cell abundance was higher when fish was present (median of 14.8 x 10^6 cells/cm^2) than when tadpoles were present (median of 4.7 x 10^6 cells/cm^2) (Fig. 3C; Table A8).

Analyzing the quantity of Chl-a from all five algal and cyanobacterial groups, we obtained a significant RDA (F = 6.023, P = 0.001) that explained 34.7% of the variance (Fig. 3D). We found two significant canonical axes RDA1 and RDA2 (F = 25.82, P = 0.001 and F = 7.97, P = 0.001 respectively) which explained 24.4% and 7.5% of the total variance respectively. Six variables were identified by the forward selection process, with the presence/absence of fish being the most explanative of them (approximately 4 times more than any other) and the most associated with RDA1. High concentrations of Chl-a from all five algal and cyanobacterial groups were situated closely to RDA1 and clearly associated with fish presence (Fig. 3D).

Thus, fish presence/absence was the factor that mainly determined the biomass of epilithon in the littoral of Pyrenean high mountain lakes.

3.2. Relative importance of introduced fish

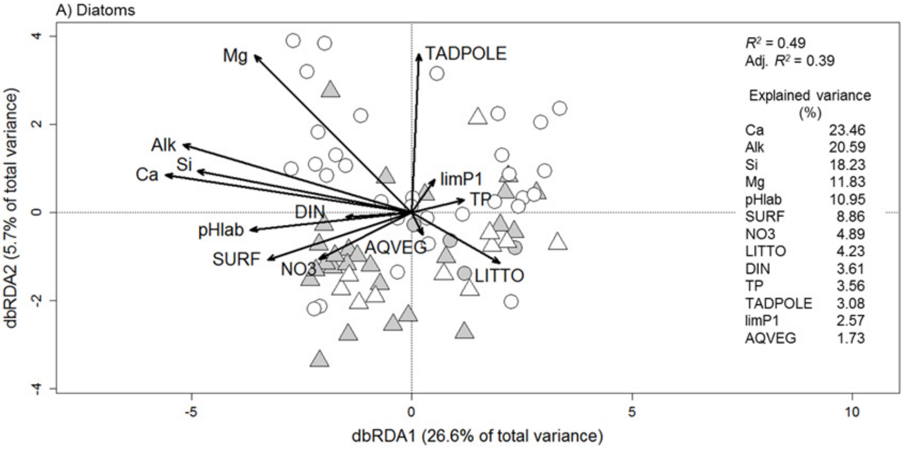
Analyzing the taxonomic structure of the diatom assemblage, for % of species abundance within each site, we obtained a significant dbRDA (F = 4.589, P = 0.001) which explained 49% of the variance (Fig. 4A). We found two significant canonical axes dbRDA1 (F = 32.06, P = 0.001) and dbRDA2 (F = 6.87, P = 0.001) which explained 26.6% and 5.7% of the total variance respectively. Thirteen variables were identified by the forward selection process, with some chemistry features being the more explanative and the most associated with RDA1: calcium, alkalinity, silica, magnesium and pH. Tadpole abundance was the variable most associated with RDA2, which also discriminates between the sites with and without fish quite well (Fig. 4A).

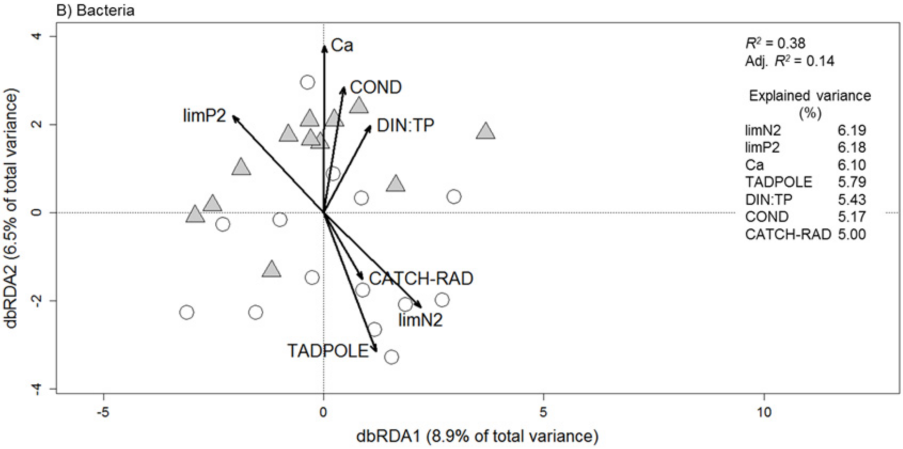
Analyzing the taxonomic structure of bacteria assemblage, for % of OTUs abundances within each site, we obtained a significant dbRDA (F = 1.566, P = 0.001) which explained 37.8% of the variance (Fig. 4B). We found two significant canonical axes dbRDA1 (F = 2.57, P = 0.001) and dbRDA2 (F = 1.88, P = 0.001) which explained 8.9% and 6.5% of the total variance respectively. Seven variables were identified by the forward selection process, with the following nutrient and chemistry features being the more explanative: limitation by N (DIN:TP ≤ 3.4), limitation by P (DIN:TP ≥ 1.5), and calcium. Tadpole abundance was highly associated with RDA2 which also discriminates between the sites with and without fish quite well (Fig. 4B).

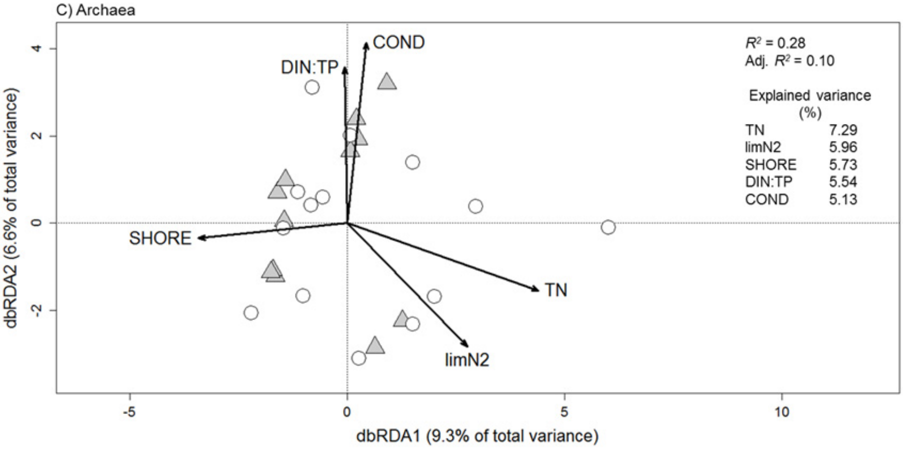
Analyzing the taxonomic structure of archaea assemblage, for % of OTUs abundances within each site, we obtained a significant dbRDA (F = 1.566, P = 0.001) which explained 38% of the variance (Fig. 4C). We found two significant canonical axes dbRDA1 (F = 2.58, P = 0.001) and dbDA2 (F = 1.84, P = 0.001) which explained 9.3% and 6.6% of the total variance respectively. Five variables were identified by the forward selection process, with the following nutrient and habitat features being the more explanative: Total N, limitation by N (DIN:TP ≤ 3.4), shore habitat, dissolved inorganic nitrogen/total phosphorous and conductivity. Neither tadpole abundance, nor fish presence/absence, were selected by the forward selection process (Fig. 4C).

Thus, tadpole abundance was one of the variables that determined the taxonomic structure of the diatoms and bacteria assemblages, but not archaea. Fish presence/absence was not selected by the forward selection process for any assemblage.

Fig. 4. (next page) Relative importance of tadpoles and introduced fish on the assemblage taxonomic structure of epilithic (A) diatoms, (B) bacteria and (C) archaea. To focus on the community structure, the dbRDA were computed on the euclidean distance of the Hellinger transformation for each assemblage matrix. Symbols of gray color indicate fish presence; white color, fish absence; circles, tadpole presence; triangles, tadpole absence. The explained variance of the variables was estimated by successive partial dbRDAs using each selected variable as a covariable each time, and represents the percentage of the total variance not explained by the dbRDA when the analyzed variable is held constant. For other graph components and abbreviations, see Fig. 3. Note that high values of LITTO are associated with fine grain substrate, while low values of LITTO are associated with coarse grain substrate and that high values of SHORE are associated with vegetated shores, while low values of SHORE are associated with bare rocky shores.


A) Diatoms
Mg
TADPOLE
Alk
Si
Ca
limP1
TP
DIN
pHlab
AQVEG
SURF
NO3
LITTO
R^2 = 0.49
Adj. R^2 = 0.39
Explained variance (%)
Ca 23.46
Alk 20.59
Si 18.23
Mg 11.83
pHlab 10.95
SURF 8.86
NO3 4.89
LITTO 4.23
DIN 3.61
TP 3.56
TADPOLE 3.08
limP1 2.57
AQVEG 1.73
dbRDA2 (5.7% of total variance)
dbRDA1 (26.6% of total variance)
-4
-2
0
2
4
-5
0
5
10


B) Bacteria
Ca
COND
limP2
DIN:TP
CATCH-RAD
limN2
TADPOLE
R^2 = 0.38
Adj. R^2 = 0.14
Explained variance (%)
limN2 6.19
limP2 6.18
Ca 6.10
TADPOLE 5.79
DIN:TP 5.43
COND 5.17
CATCH-RAD 5.00
dbRDA2 (6.5% of total variance)
dbRDA1 (8.9% of total variance)
-4
-2
0
2
4
-5
0
5
10


C) Archaea
COND
DIN:TP
SHORE
TN
limN2
R^2 = 0.28
Adj. R^2 = 0.10
Explained variance (%)
TN 7.29
limN2 5.96
SHORE 5.73
DIN:TP 5.54
COND 5.13
dbRDA2 (6.6% of total variance)
dbRDA1 (9.3% of total variance)
-2
0
2
4
-5
0
5
10

3.3. Diversity patterns

The measures used to explore the diversity patterns were: (α) richness, (β) distance to the group centroid, and (γ) species accumulation curve (SAC).

The overall median for diatom richness was 49 species. Two-way ANOVA revealed that sites with fish had higher diatom richness (median of 54 species) than sites without fish (median of 45 species); the Tadpole factor did not show statistical main effects for diatom richness, nor did the interaction fish x tadpoles (Fig. 5A1; Table A10). The overall median for bacteria richness was 104 OTUs. Bacteria richness was higher when fish was present (median of 114 OTUs) than when tadpoles were present (median of 96 OTUs) (Fig. 5B1; Table A10). The overall median for archaea richness was 60 OTUs. There was no statistical difference for archaea richness between sites where fish were present and sites where tadpoles were present (Fig. 5C1; Table A10).

One-way ANOVA revealed that the β diversity, in terms of distances to the group centroid, was statistically higher for diatoms when tadpoles was present than when tadpoles was absent (Table 2; Table A11). The analyses of factor Fish for diatoms β diversity and factor Fish/Tadpole for bacteria and archaea β diversity did not show significant effects (Table 2; Table A11).

The overall numbers of recorded taxa in our samples were 448 diatom species, 464 bacteria OTUs and 410 archaea OTUs. The sites with fish had higher overall number of recorded diatom species and bacteria OTUs than the sites with tadpoles, with their SACs being more vertical in presence of fish (Fig. 5A2; Fig, 5B2). For archaea, however, there was no difference between the SAC of the sites with fish and the sites with tadpoles (Fig. 5C2).

Thus, although sites with fish had higher richness and accumulated number of taxa for diatoms and bacteria, the diatom assemblage was more homogenized among sites when tadpoles were absent.

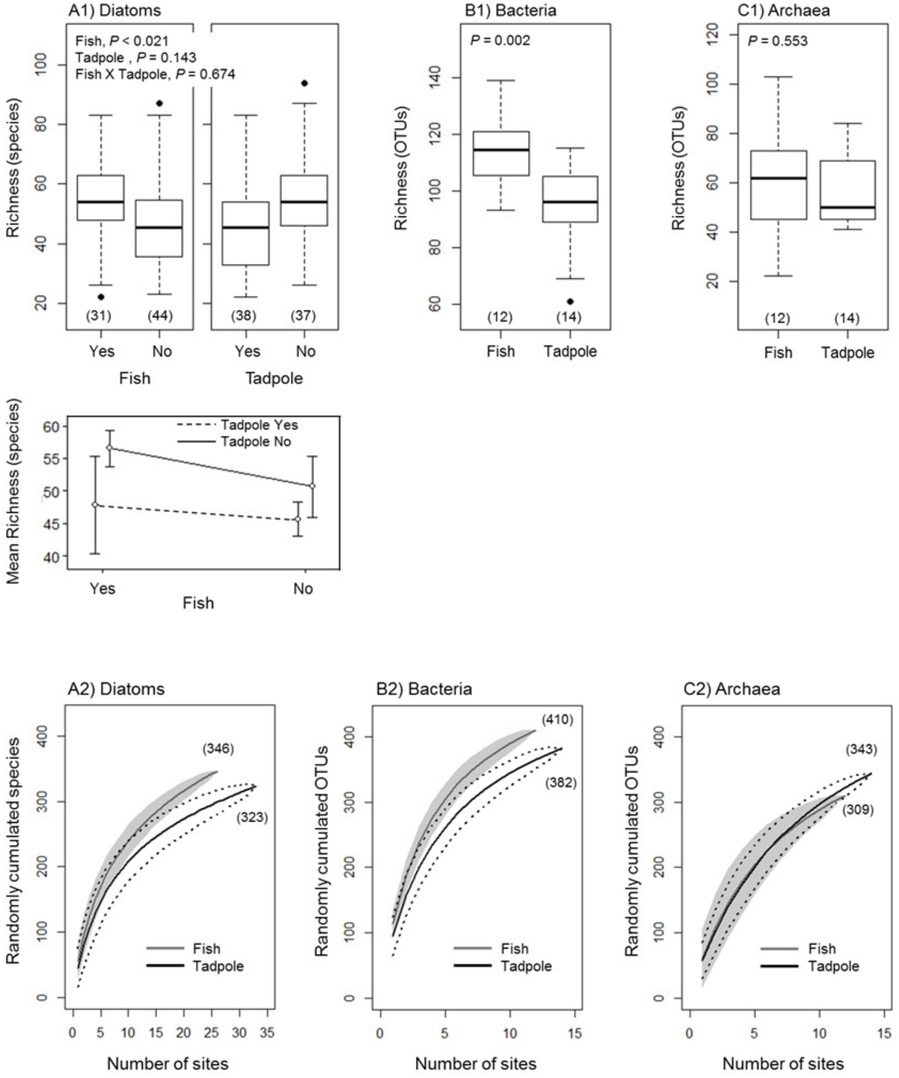

A1) Diatoms
Fish, P < 0.021
Tadpole , P = 0.143
Fish X Tadpole, P = 0.674
Richness (species)
20
40
60
80
100
(31)
(44)
(38)
(37)
Yes
No
Yes
No
Fish
Tadpole
B1) Bacteria
P = 0.002
Richness (OTUs)
60
80
100
120
140
(12)
(14)
Fish
Tadpole
C1) Archaea
P = 0.553
Richness (OTUs)
20
40
60
80
100
120
(12)
(14)
Fish
Tadpole
Mean Richness (species)
40
45
50
55
60
Tadpole Yes
Tadpole No
Yes
No
Fish
A2) Diatoms
Randomly cumulated species
0
100
200
300
400
(346)
(323)
Fish
Tadpole
0
5
10
15
20
25
30
35
Number of sites
B2) Bacteria
Randomly cumulated OTUs
0
100
200
300
400
(410)
(382)
Fish
Tadpole
0
5
10
15
Number of sites
C2) Archaea
Randomly cumulated OTUs
0
100
200
300
400
(343)
(309)
Fish
Tadpole
0
5
10
15
Number of sites

Fig. 5. (last page) α and γ diversity of (A) diatoms, (B) bacteria and (C) Archaea of the epilithon in presence/absence of fish or tadpoles. The upper panel (1) shows the ANOVA results using boxplots and interaction plot for taxonomic richness. The *P*-values of the two-way (A1) and one-way (B1 and C1) ANOVA are shown on the top of the graphic. The number of sites for each category is written between brackets and below the boxes. The vertical bars of the interaction plot represent the standard error of the mean. The lower panel (2) shows the species accumulation curves (SAC), made by randomly addition of sites through 9999 permutations. In the case of diatoms, only sites that had exclusively fish or tadpoles were used: sites that had both or none of them were excluded. The CI 95% is drawn as gray areas for fish categories and as dashed lines for tadpole categories. The total number of taxa of each category is written between brackets in the end of the lines. For other graph components and abbreviations, see Fig. 3.

Table 2. β diversity as average distance to the group centroid of (A) diatoms, (B) bacteria and (C) archaea of the epilithon in presence/absence of introduced fish or tadpoles. The distances of the group members to the group centroid (spatial median) in a principal coordinate space were used as β diversity measure. The factors' effects were explored through analyses of dispersion (variance), and testing the differences between-categories for the distances of the category members to the category centroid (spatial median) by ANOVAs. In the case of diatoms, the distances to centroid were obtained separately for both factors, Fish and Tadpole. Then, two one-way ANOVAs were performed separately, so the interaction term between both factors could not be computed. Bold type indicates significant differences in the ANOVA tests at α = 0.05. The number of sites in each category is written between brackets. A complete ANOVA table can be found in Appendix A (Table A11).

Factor	Categories	A) Diatoms	Factor	B) Bacteria	C) Archaea
Fish	Yes (31)	0.803	Fish (12)	0.761	0.762
	No (44)	0.832	Tadpole (14)	0.768	0.814
	p value	*0.194*	*p* value	*0.525*	*0.112*
Tadpole	Yes (38)	0.843			
	No (37)	0.798			
	p value	***0.044***			

3.4. Degree of activity and senescence

The overall median for the percentage of total C in our dataset was 22.2%. One-way ANOVA revealed that percentage of total C was lower when fish was present (median of 20.3%) than when tadpoles were present (median of 24.7%) (Fig. 6A; Table A12). The overall median for the percentage of total N obtained was 2.2%. One-way ANOVA revealed that percentage of total N was lower when fish was present (median of 2.1%) than when tadpoles were present (median of 2.4%) (Fig. 6B; Table A12). For percentage of total P, however, there was no difference between the sites with fish and the sites with tadpoles (Fig. 6C; Table 12).

Analyzing the stoichiometric data, we obtained a significant RDA ($F = 7.084$, $P = 0.002$) which explained 33.6% of the variance (Fig. 6E). We found a significant canonical axis RDA1 (F = 14.15, P = 0.001) which explained 33.6% of the total variance and a non-significant canonical axis RDA2 ($F = 0.014$, $P = 0.986$). Two variables were identified by the forward selection process, with the presence/absence of fish being the more explanative of them (approximately 6 times more than the other) and the most associated with RDA1. High proportions of C, N and P were closely related to RDA1 and clearly associated with fish absence (Fig. 6E).

The overall median for Chl-a preservation index (Chl-a expressed as a percentage of a-phorbins) was 73.2%. Two-way ANOVA revealed that sites with fish had lower Chl-a preservation index (median of 71.2%) than sites without fish (median of 74.1%); Tadpole factor did not show statistical main effects for Chl-a preservation index, nor did the interaction fish x tadpoles (Fig. 7A; Table A12).

In the linear regression between total concentration of epilithic Chl-a and tadpole abundance, we found a significant positive relationship between both variables; although the data range for total Chl-a concentration had been significant lower in the presence of tadpoles than in absence, when they were present the values of Chl-a were higher when the tadpole abundance increased (Fig. 7B).

Thus, fish presence/absence was the factor that mainly determined the percentage of total C and total N in the epilithon. Sites without fish showed less degradation products, suggesting that the eplithon is less senescent in terms of Chl-a expressed as a percentage of a-phorbins; and sites with higher abundance of tadpoles had more active epilithon in terms of total concentration of Chl-a.

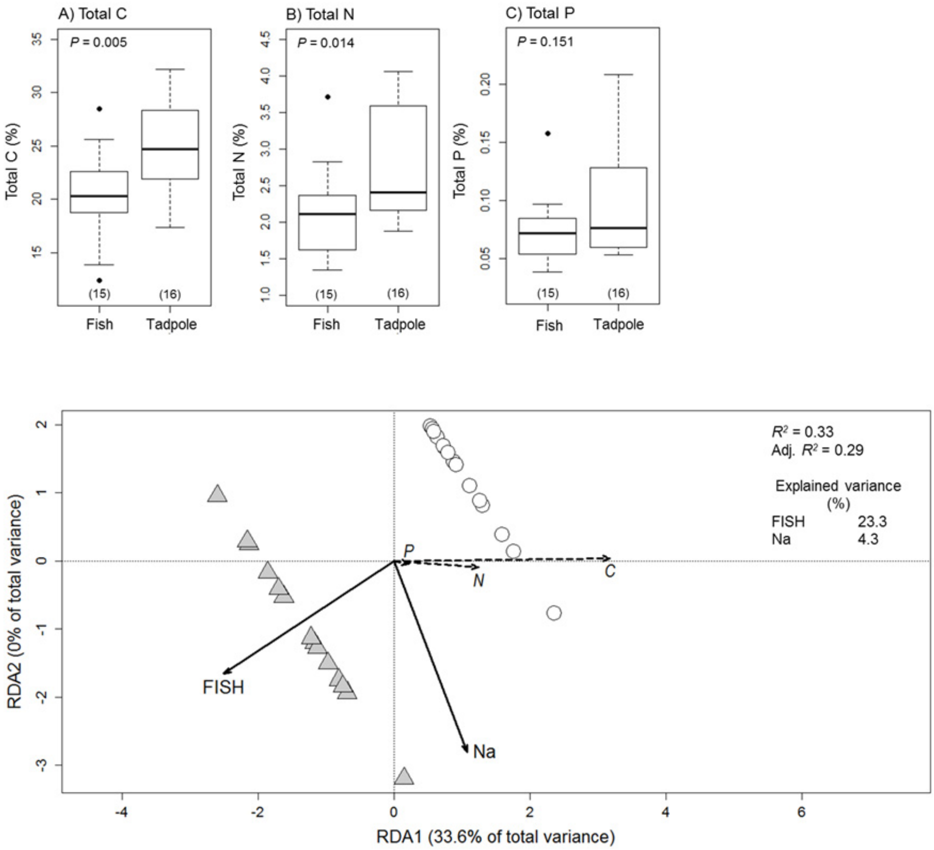


Fig. 6. Stoichiometric composition of the epilithon in presence/absence of introduced fish or tadpoles. The boxplots in the upper panel show the ANOVA made on the percentage of (A) total carbon, (B) total nitrogen, (C) total phosphorous, for sites that had only fish or only tadpoles. The lower chart shows the RDA performed also on the percentages of total C, N and P of the epilithon. To achieve normality and avoid heterogeneity in the variance, the ANOVA and the RDA were computed on the percentages of C, N and P normalized by the transformation arcsine(square root(x/100)). As explanatory variables we used fish presence, tadpole abundance and habitat and chemistry variables excluding those containing C, N or P. Symbols of gray color indicate fish presence; white color, fish absence; circles, tadpole presence; triangles, tadpole absence. The explained variance of the variables was estimated by successive partial RDAs using each selected variable as a covariable each time, and represents the percentage of the total variance not explained by the RDA when the examined variable is held constant. For other graph components and abbreviations, see Fig. 3.

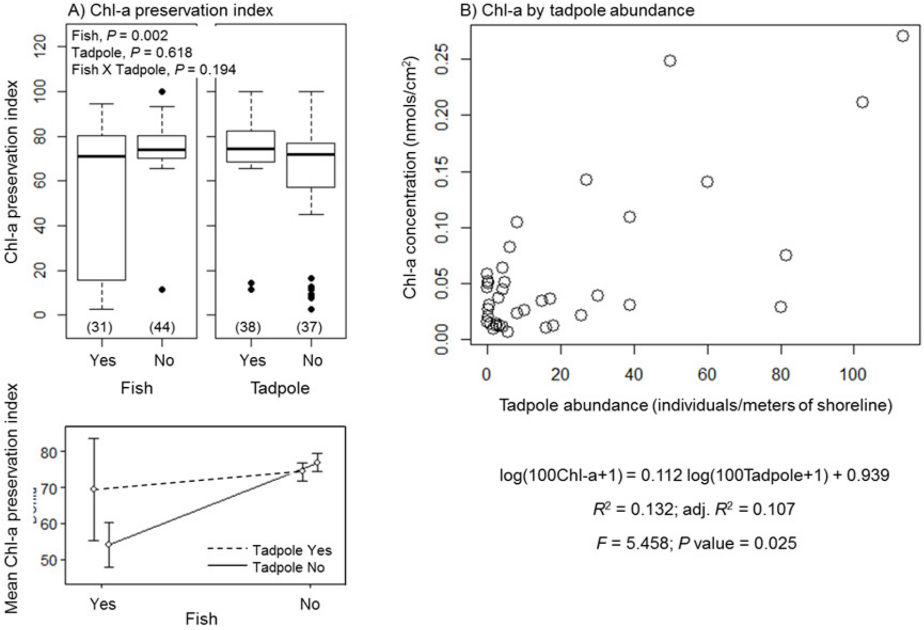


Fig. 7. Degree of activity and senescence of the epilithon in the presence/absence of introduced fish or tadpoles. The left panel (A) shows the boxplots and the interaction plot of the ANOVA results for Chl-a preservation index (Chl-a expressed as a percentage of a-phorbins). The right chart (B) shows the lineal model of total concentration of Chl-a of the epilithon and tadpole abundance, computed only using the sites where there were tadpoles. Epilithic Chl-a concentration and tadpole abundance data are shown in their original units, although they were normalized by log(100x+1) transformation to make the model. For other graph components and abbreviations, see Fig. 3.

3.5. Exclusion experiments

In the tadpole exclusion experiment (Lake Clotada) we did not find any significant differences between the treatment (no tadpole) and control (tadpole) or lake sample for Chl-a ($F_{2,6} = 3.16$, $P = 0.115$) (Fig. 8A1) or of Chl-a from diatoms ($F_{2,6} = 0.53$, $P = 0.611$) (Fig. 8B1).

There were, however, significant differences in the fish exclusion/tadpole grazing experiment (Lake Naorte) between the lake sample, the control and treatment for total Chl-a and Chl-a from diatoms concentrations ($F_{2,6} = 39.13$, $P < 0.001$ for total Chl-a and $F_{2,6} = 55.78$, $P < 0.001$for Chl-a from diatoms); Tukey's post hoc tests revealed that natural and experiment control (fish present) had higher concentrations for both total Chl-a and Chl-a from diatoms than the treatment (tadpole present) (Fig. 8A2 and 8B2).

PCAs performed on the algal and cyanobacterial groups abundance and taxonomic composition of diatoms, highlighted that fish presence/absence was the strongest variable that determined the group or taxonomic composition of these assemblages. PC1, which gave most of the explained variance for both assemblages, clearly discriminated between the samples where fish was present or absent in the exclusion experiment in Lake Naorte (Fig. 8C and 8D).

Thus, field exclusion experiments confirmed the strong role of fish on the biomass of epilithon that exist in the littoral of high mountain lakes, as well as on structure of the epilithic communities.

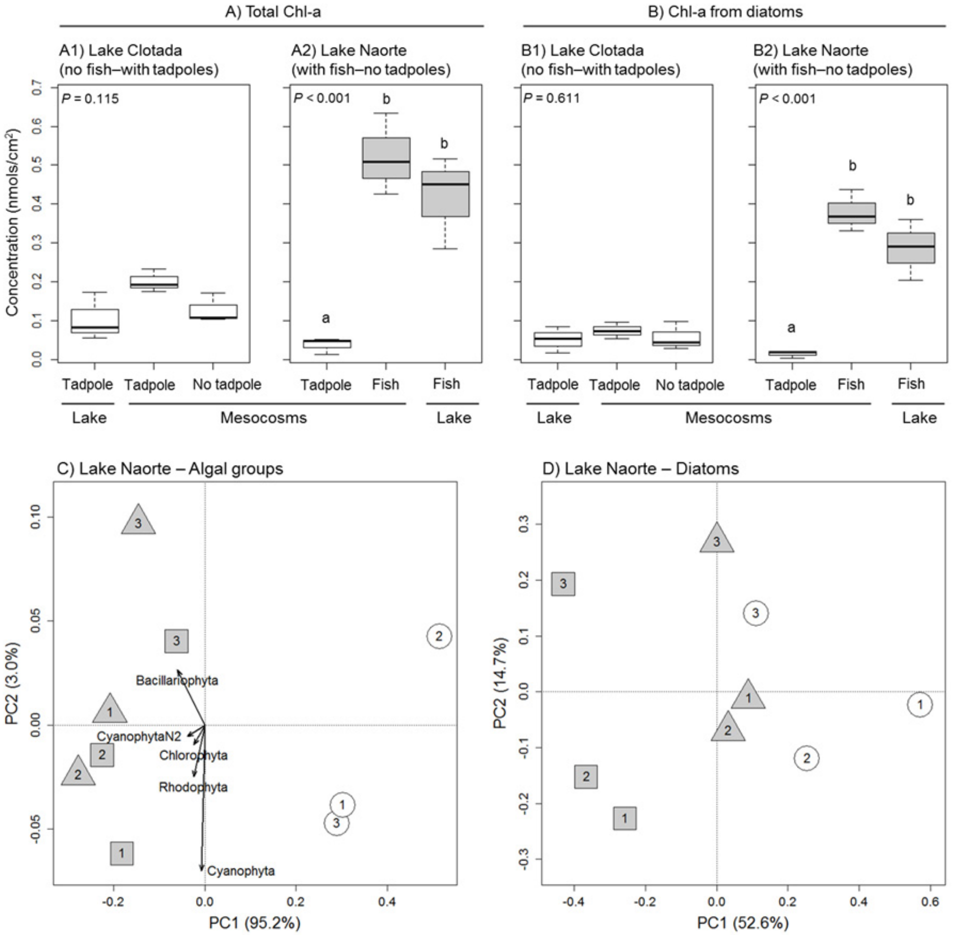


Fig. 8. Results of the exclusion experiments. The upper panel contains the boxplots of the one-way ANOVA results of the epilithon for (A) the total concentration of Chl-a and (B) the concentration of Chl-a from diatoms obtained in the experiments in (1) Lake Clotada and (2) Lake Naorte. The p-values of the ANOVAs are written on the top of the graphic. Categories with different lowercase letters above the boxes indicate significant differences at the $p < 0.05$ level in Tukey post hoc tests. White boxes denote absence of fish and presence of tadpoles, while grey boxes denote presence of fish and absence of tadpoles. The lower panel shows the representations by PCAs of the epilithic experimental data obtained from Lake Naorte for (C) total concentration of Chl-a from algal and cyanobacterial groups and for (D) the taxonomic composition of the diatoms assemblage. Data were previously normalized by fourth root transformation (algal and cyanobacterial groups) and Hellinger transformation (Diatom assemblage). Symbols of gray color indicate fish presence; white color, fish absence; circles, tadpole presence; triangles, tadpole absence. For other graph components and abbreviations, see Fig. 3.

4. Discussion

4.1. Trophic cascade

We found a trophic cascade mediated by introduced fish in the littoral of high mountain lakes, which altered the epilithon community at several levels, including the biomass, diversity and the degree of activity or senescence of the epilithon community. The absolute abundances of cyanobacterial and algal groups were also almost entirely explained by the presence of introduced fish in the lake or pond. In contrast, the relative taxonomic abundances of cyanobacteria and algal groups, diatom species, and bacteria and archaea OTUs, were mostly explained by chemical and environmental variables, highlighting the existence also of some bottom-up control caused by resource availability. The coexistence of both top-down and bottom-up forces have been described before in the food webs of lakes and ponds (Fitter and Hillebrand 2009, Mette et al. 2011). Nevertheless, top-down control has been found more important in clear lakes and in shallow lakes with low coverage of submerged macrophytes (Jeppesen et al. 1997, Liboriussen et al. 2005), characteristics also often observed in high mountain lakes and ponds.

4.2. Biomass of epilithon

Tropic cascade mediated by fish predation on grazing tadpoles had a large impact on the biomass of epilithon community in high mountain lakes and ponds. Our results showed that lakes and ponds with introduced fish had a median biomass of total Chl-a of 6.6:1 relative to the sites without fish. The same ratio for Chl-a from diatoms was even higher at 7.6:1. All algal and cyanobacterial groups had higher biomass in presence of fish than in absence. These values are similar to those obtained in other ecosystems, as for example in tropical upland streams, where algal biomass increased between 6.3-fold and 2.2-fold in pools and riffles, after a drastic disease-driven amphibian decline (Connelly et al. 2014). Our results also agree with those found in a meta-review of 865 studies across all aquatic ecosystems, where grazers were found to remove, on average, 59% of the periphyton biomass, without significant differences between lotic, lentic, and coastal habitats, but with significant differences between laboratory (65%) and field (56%) experiments (Hillebrand 2009).

In contrast to the significant results of the fish factor in our ANOVAs, the tadpole factor was not significant. This is probably mainly related to the high coincidence of fish presence and tadpole absence in our dataset and hence, to the amount of variance shared by both factors. Although the presence/absence of tadpoles was not significant for Chl-a biomass in our ANOVAs, the interaction between fish and tadpole factors was significant. This means that the presence of tadpoles always caused a reduction of Chl-a either with or without introduced fish. Nevertheless, in the absence of tadpoles, the Chl-a biomass was also lower if introduced fish were absent. Tadpole grazing in the presence of fish was mostly by *B. spinosus* tadpoles, which are not predated by fish due the unpalatability and toxicity of this species' tadpoles (Kruse and Stone 1984, Benard and Fordyce 2003). The low biomass of periphyton found in absence of both fish and tadpoles can be explained by the grazing activity of benthic herbivorous invertebrates, as had been found, for example, in artificial channels fed by a boreal forest stream (Bechara et al. 2007), or in experimental enclosures in shallow lakes (Liboriussen et al. 2005).

4.3. Differential impact on the assemblages

The littoral trophic cascade mediated by introduced fish in high mountain lakes has less impact on the relative taxonomic composition (percentage of taxonomic abundance) of the epilithon community than on its biomass. Availability of nutrients and chemistry appear to be more important than fish presence or tadpole abundance on this aspect. Furthermore, the intensity of the direct grazing activity seems more important than fish presence in defining the relative taxonomic abundance of the epilithon. In fact, while tadpole abundance was significant for diatoms and bacteria assemblages, fish presence was not selected for any assemblage. Previous research suggested that grazing activity had a specific effect on different algal or cyanobacterial groups, causing the predominance of prostrate diatoms and the reduction of chlorophytes and cyanobacteria (McCollum et al. 1998, Bechara et al. 2007). High levels of grazing activity kept the epilithic community in younger stages even in the presence of toxic cyanobacteria (Bechara et al. 2007, Zhang et al. 2012).

Archaea assemblage responded less to the effect of the littoral trophic cascade mediated by introduced fish. In contrast to the effect found on diatom and bacteria assemblages, the relative taxonomic structure of archaea assemblage seems to be absolutely independent of direct grazing, since neither tadpole abundance nor fish presence were significant. This pattern could also be seen in the results of the diversity analyses. While the trophic cascade, and hence the grazing intensity were associated with changes in the diversity values of diatoms and bacteria assemblages, diversity values of archaea were not.

4.4. Homogenization of the biota

The littoral trophic cascade mediated by introduced fish in high mountain lakes is homogenizing the epilithon community. One of the more widely reported impacts of invasive species is the homogenization of the biota (Clavero and García-Berthou 2006, Simberloff et al. 2013). Our data show that introduced fish is causing an indirect homogenization process on the epilithic community by the large-scale reduction of grazing activity. Sites with introduced fish and without tadpoles have higher α diversity (taxonomic richness) and γ diversity (species accumulation curves), but no difference in or lower β diversity (average distance to the group centroid), for the diatom and bacteria assemblages. When introduced fish are absent and tadpoles present, epilithic communities seem to be more sensitive to the different natural chemistical conditions and to the different levels of resource availability. In contrast, when fish are present and tadpole absent, epilithon community is strongly conditioned by the absence of grazing and the sites mostly lost their specificities. Previous research showed that high mountain lake epilithon had a high bacterial genetic diversity (richness) in comparison with high mountain lake plankton or soils, and that the community composition changed along the altitudinal gradient, particularly related to the relative location regarding to the treeline (Bartrons et al. 2012a). Experimental studies had also reported that grazing activity and low nutrient levels reduce the algal diversity of the periphyton (Simpson diversity index), though different levels of light do not (Liess and Kahlert 2007). However, to our knowledge, the community homogenization process in the epilithon of high mountain lakes indirectly caused by introduced fish, have been not reported so far.

4.5. Elemental content and senescence

The littoral trophic cascade mediated by introduced fish in high mountain lakes is causing high levels of senescence and inactivity of the epilithon community. Our results show that the percentage content of elemental C, N and P is higher when fish are absent and when tadpoles are present, although the difference is not significant for P. This likely indicates that there is a higher proportion of organic matter when there is tadpole grazing. Previous studies have described that tadpoles, in addition to reducing significantly the standing stock of periphyton, also effectively remove settled particulate matter from benthic surfaces, becoming real ecosystem engineers (Wood and Richardson 2010). The fact that in Pyrenean lakes and ponds the content of all three elements C, N and P varied in the same direction, meant that the C:P and N:P ratios had no significant differences in presence or absence of introduced fish. This result differs from experimental findings, where grazing activity reduced C:P and N:P ratios (Hillebrand and Kahlert 2001, Knoll et al. 2009). This suggests that in field samples of epilithon from high mountain lakes and ponds, it is more difficult to see the P enrichment of the algal fraction of the epilithon attributable to excretion by grazers, as has been reported in experimental conditions. However, we found that within the lower range of Chl-a concentration at sites with tadpoles and without fish, the abundance of tadpoles was slightly correlated with Chl-a concentration. This result suggests that, although the epilithon biomass is lower in the absence of tadpoles, their grazing activity might result in an increase of productivity and growth rate, as has been reported by previous studies (Kupferberg 1997).

Acknowledgements

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Appendix Chapter 5

Supplementary tables and figures

1. Supplementary tables

Table A1. Description of predictor variables used in the analyses. Variable name abbreviations are given in parentheses. The transformation made to normalize the variable, when necessary, is also given.

Variable type	Variable name	Description
Study design	Fish presence (FISH)	Binary factor determined by fish presence in the lake
	Tadpole presence (TADPOLE)	Binary factor determined by tadpole presence in the lake. Used for ANOVA analyses
	Tadpole abundance (TADPOLE)	Abundance of tadpoles in the lake (ind/m shoreline), log100x+1 transformed. Used for RDA analyses
Habitat descriptors	Altitude (ALT)	Elevation of the site a.s.l. (m)
	Surface area (SURF)	Area of the lake (ha), log100x+1 transformed
	Shore perimeter (PERIM)	Perimeter of the shoreline of the lake, log100x+1 transformed
	Total catchment (TCAT)	Area of the total catchment of the lake (ha), log100x+1 transformed
	Direct catchment (DCAT)	Area of the proportion of catchment not shared with other lakes that are upstream of the lake (ha), log100x+1 transformed
	Maximum depth (DEPTH)	Maximum depth of the water body (m), log100x+1 transformed
	Coordinate UTM x (utmx)	Longitude coordinate of Universal Transverse Mercator coordinate system (m), fourth root transformed
	Coordinate UTM y (utmy)	Latitude coordinate of Universal Transverse Mercator coordinate system (m), fourth root transformed

Variable type	Variable name	Description
	Accumulated temperature (ADD)	Estimated Accumulated Degree Days of the site above 7.64ºC
	Total catchment direct radiation duration (CATCH-RAD)	Annual mean duration of direct solar radiation income in the total catchment of the lake (hours/day)
	Total catchment solar global radiation (CATCH-SOL)	Annual mean solar radiation, direct and diffuse, income in the total catchment of the lake (Wh/m^2day), log100x+1 transformed
	Total lake direct radiation duration (LAKE-RAD)	Annual mean duration of direct solar radiation income in the lake or pond surface (hours/day)
	Total lake solar global radiation (LAKE-SOL)	Annual mean solar radiation, direct and diffuse, income in the lake or pond surface (Wh/m^2day), log100x+1 transformed
	Littoral slope (SLOPE)	First principal component for littoral zone slope. High values of SLOPE are associated with low littoral slope and vice versa.
	Littoral substrate (LITTO)	First principal component for littoral zone substrate types. High values of LITTO are associated with fine grain substrate, while low values of LITTO are associated with coarse grain substrate.
	Shore substrate (SHORE)	First principal component for shore zone substrate types. High values of SHORE are associated with vegetated shores, while low values of SHORE are associated with bare rocky shores.
	Aquatic vegetation (AQVEG)	% of site surface area with submerged or emergent aquatic vegetation, fourth root transformed
	Chl-a of seston (CHLA.SES)	Total Chl-a concentration of the water column (µg/L), log100x+1 transformed
	Chl-a of epilithon (CHLA.EPI)	Total Chl-a concentration of the epilithon ($nmol/cm^2$), log100x+1 transformed
	Chl-a of bacillariophyta (BACILL)	Chl-a concentration of epilithic bacillariophyta ($nmol/cm^2$), fourth root transformed
Water chemistry	Conductivity (COND)	Lake water conductivity (µS/cm), log100x+1 transformed
	pH (pHlab)	pH of the water
	Ammonium (NH4)	Ammonium concentration of the water (µgN/L), logx+1 transformed
	Calcium (ca)	Calcium concentration of the water (mg/L), log100x+1 transformed
	Magnesium (Mg)	Magnesium concentration of the water (mg/L), log100x+1 transformed
	Sodium (Na)	Sodium concentration of the water (mg/L), logx+1 transformed

Variable type	Variable name	Description
	Potassium (K)	Potassium concentration of the water (mg/L), logx+1 transformed
	Alkalinity (Alk)	Alkalinity of the water (μeq/L), fourth root transformed
	Sulfate (SO4)	Sulfate concentration of the water (mg/L), log100x+1 transformed
	Nitrate (NO3)	Nitrate concentration of the water (μgN/L), logx+1 transformed
	Chloride (Cl)	Chloride concentration of the water (mg/L), square root transformed
	Phosphate (PO4)	Phosphate concentration of the water (μg/L), third root transformed
	Total nitrogen (TN)	Total nitrogen concentration of the water (μgN/L), third root transformed
	Total phosphorus (TP)	Total phosphorus concentration of the water (μgP/L), third root transformed
	Silica (Si)	Silica concentration of the water (mgSi/L), third root transformed
	Total organic nitrogen (TON)	Total organic nitrogen concentration of the water (μgN/L), third root transformed
	Total organic phosphorus (TOP)	Total organic phosphorus concentration of the water (μgP/L), square root transformed
	Dissolved inorganic nitrogen (DIN)	Dissolved inorganic nitrogen concentration of the water (μgN/L), log100x+1 transformed
	Total nitrogen/total phosphorus (TN:TP)	Ratio between total nitrogen and total phosphorus concentrations of the water
	Dissolved inorganic nitrogen/total phosphorus (DIN:TP)	Ratio between dissolved inorganic nitrogen and total phosphorus concentrations of the water
	Threshold TN:TP ≥ 19 (limP1)	Binary factor determined by the existence of P limitation (Bergström 2010)
	Threshold TN:TP ≤ 41 (limN1)	Binary factor determined by the existence of N limitation (Bergström 2010)
	Threshold DIN:TP ≥ 1.5 (limP2)	Binary factor determined by the existence of P limitation (Bergström 2010)
	Threshold DIN:TP ≤ 3.4 (limN2)	Binary factor determined by the existence of N limitation (Bergström 2010)

Table A2. Pearson's correlations among the variables: littoral slope, littoral substrate and shoreline substrate categories with the principal component axis 1. The variance contained in the first axis is given in parentheses and above the correlations (% explained variance).

Habitat characteristics	Axis 1	Sig.
Littoral slope (SLOPE)	*(75.4% explained variance)*	
Shallow (<10 cm deep, <15º slope)	0.99	<0.001
Slight slope (15º–30º slope)	-0.63	<0.001
Sloping (30º–70º slope)	-0.8	<0.001
Vertical (70º–90º slope)	-0.53	<0.001
Littoral substrate (LITTO)	*(68.6% explained variance)*	
Organic material	0.17	0.141
Silt-sand (<0.5 cm)	0.99	<0.001
Gravel (>0.5–2.5 cm)	-0.58	<0.001
Cobble (>2.5–25 cm)	-0.65	<0.001
Boulder (>25 cm)	-0.67	<0.001
Bedrock	-0.42	<0.001
Shoreline substrate (SHORE)	*(78.3% explained variance)*	
Terrestrial vegetation	0.99	<0.001
Cobble-boulder (2.5 cm–1 m)	-0.79	<0.001
Big boulder-bedrock (>1 m)	-0.68	<0.001

Table A3. Variance Inflation Factors (VIF) of the 6 selected variables to be included in the RDA for algal groups (Fig. 3D). Numerical variables were previously normalized by the transformations detailed in table A1. Variable abbreviations used in the figures are given in brackets.

Variable type	Variable name	VIF value
Study design	Fish presence (FISH)	1.305
Habitat descriptors	Surface area (SURF)	1.641
	Littoral slope (SLOPE)	1.721
Water chemistry	Total phosphorus (TP)	8.270
	Total organic phosphorus (TOP)	8.472
	Dissolved inorganic nitrogen (DIN)	1.047

Table A4. Variance Inflation Factors (VIF) of the 13 selected variables to be included in the dbRDA for diatom assemblage (Fig. 4A). Numerical variables were previously normalized by the transformations detailed in the table A1. Variable abbreviations used in the figures are given in brackets.

Variable type	Variable name	VIF value
Study design	Tadpole abundance (TADPOLE)	1.522
Habitat descriptors	Surface area (SURF)	1.816
	Littoral substrate (LITTO)	1.823
	Aquatic vegetation (AQVEG)	1.311
Water chemistry	pH (pHlab)	2.149
	Calcium (Ca)	8.057
	Magnesium (Mg)	2.794
	Alkalinity (Alk)	8.533
	Nitrate (NO3)	3.693
	Total phosphorus (TP)	2.166
	Silica (Si)	3.802
	Dissolved inorganic nitrogen (DIN)	3.827
	Threshold TN:TP ≥ 19 (limP1)	1.305

Table A5. Variance Inflation Factors (VIF) of the 7 selected variables to be included in the dbRDA for bacterial assemblage (Fig. 4B). Numerical variables were previously normalized by the transformations detailed in table A1. Variable abbreviations used in the figures are given in brackets.

Variable type	Variable name	VIF value
Study design	Tadpole abundance (TADPOLE)	1.578
Habitat descriptors	Total catchment direct radiation duration (CATCH-RAD)	1.874
	Conductivity (COND)	3.021
Water chemistry	Calcium (Ca)	3.318
	Dissolved inorganic nitrogen/total phosphorus (DIN:TP)	1.608
	Threshold DIN:TP ≥ 1.5 (limP2)	3.346
	Threshold DIN:TP ≤ 3.4 (limN2)	4.416

Table A6. Variance Inflation Factors (VIF) of the 5 selected variables to be included in the dbRDA for archaea assemblage (Fig. 4C). Numerical variables were previously normalized by the transformations detailed in table A1. Variable abbreviations used in the figures are given in brackets.

Variable type	Variable name	VIF value
Habitat descriptors	Shore substrate (SHORE)	1.392
	Conductivity (COND)	1.762
Water chemistry	Total nitrogen (TN)	1.399
	Dissolved inorganic nitrogen/total phosphorus (DIN:TP)	1.427
	Threshold DIN:TP ≤ 3.4 (limN2)	1.688

Table A7. Variance Inflation Factors (VIF) of the 2 selected variables to be included in the RDA for stoichiometry data (Fig. 6E). Numerical variables were previously normalized by the transformations detailed in table A1. Variable abbreviations used in the figures are given in brackets.

Variable type	Variable name	VIF value
Study design	Fish presence (FISH)	1.050
Water chemistry	Sodium (Na)	1.050

Table A8. ANOVA results for the effect of fish and tadpoles on the epilithon biomass, for (A) total Chl-a concentration, (B) Chl-a concentration from diatoms and (C) abundance of bacteria cells (Fig. 3).

Factor	df independent term	*F* ratio	*P* value	df error term
A) Total chlorophyll-a				
FISH	1	58.007	<0.001	71
TADPOLE	1	3.001	0.087	71
FISH x TADPOLE	1	6.548	0.013	71
B) Chlorophyll-a from diatoms				
FISH	1	25.752	<0.001	71
TADPOLE	1	1.621	0.207	71
FISH x TADPOLE	1	4.720	0.033	71
C) Bacteria abundance				
FISH/TADPOLE	1	11.38	0.008	9

Table A9. ANOVA results for the effect of fish and tadpoles on the epilithon biomass for Chl-a concentration from (A) chlorophyta, (B) rhodophyta, (C) N_2-fixing cyanobacteria, and (D) other cyanobacteria (Fig. A3).

Factor	df independent term	*F* ratio	*P* value	df error term
A) Chlorophyll-a from chlorophyta				
FISH	1	4.484	0.038	71
TADPOLE	1	0.193	0.661	71
FISH x TADPOLE	1	0.669	0.416	71
B) Chlorophyll-a from rhodophyta				
FISH	1	4.349	0.041	71
TADPOLE	1	2.059	0.156	71
FISH x TADPOLE	1	3.076	0.084	71
C) Chlorophyll-a from N_2 – fixing cyanobacteria				
FISH	1	8.708	0.004	71
TADPOLE	1	0.085	0.771	71
FISH x TADPOLE	1	0.715	0.401	71
D) Chlorophyll-a from other cyanobacteria				
FISH	1	4.926	0.029	71
TADPOLE	1	0.024	0.876	71
FISH x TADPOLE	1	0.947	0.334	71

Table A10. ANOVA results for the effect of fish and tadpoles on the taxonomic richness of the assemblages of (A) diatoms, (B) bacteria, (C) archaea (Fig. 5).

Factor	df independent term	*F* ratio	*P* value	df error term
A) Diatoms				
FISH	1	5.567	0.021	71
TADPOLE	1	2.195	0.143	71
FISH x TADPOLE	1	0.179	0.675	71
B) Bacteria				
FISH/TADPOLE	1	11.91	0.002	24
D) Archaea				
FISH/TADPOLE	1	0.362	0.553	24

Table A11. ANOVA results of the effect of fish and tadpoles on β diversity, in terms of distances to the group centroid, for the assemblages of (A) diatoms, (B) bacteria, (C) archaea (Table 2).

Factor	df independent term	*F* ratio	*P* value	df error term
A) Diatoms				
FISH	1	1.721	0.194	73
TADPOLE	1	4.187	0.044	73
B) Bacteria				
FISH/TADPOLE	1	0.416	0.525	24
D) Archaea				
FISH/TADPOLE	1	2.722	0.112	24

Table A12. ANOVA results for the effect of fish and tadpoles on the degree of activity and senescence. (A) total C, (B) total N, (C) Total N, (D) C from bacteria and (E) Chl-a preservation index (Chl-a expressed as a percentage of a-phorbins) (Fig. 6 and 7).

Factor	df independent term	*F* ratio	*P* value	df error term
A) Total C				
FISH/TADPOLE	1	9.1	0.005	29
B) Total N				
FISH/TADPOLE	1	6.775	0.014	29
C) Total P				
FISH/TADPOLE	1	2.172	0.151	29
D) C from bacteria				
FISH/TADPOLE	1	14.69	0.004	9
E) Chl-a preservation index (Chl-a expressed as a percentage of a-phorbins)				
FISH	1	10.052	0.002	71
TADPOLE	1	0.251	0.618	71
FISH x TADPOLE	1	1.722	0.194	71

2. Supplementary figures

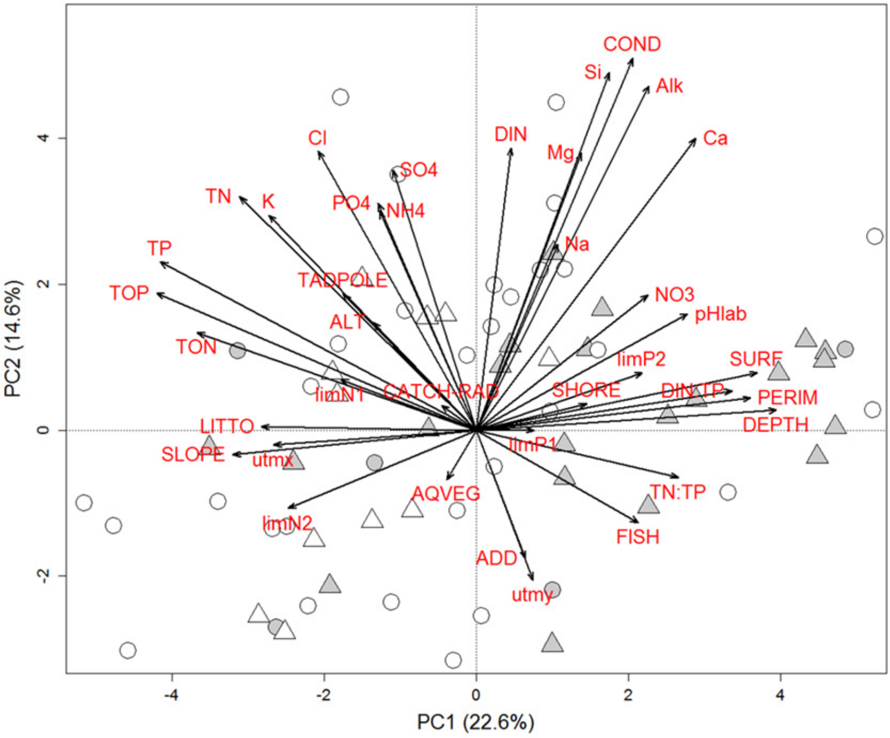


Fig. A1. Representation of the variables for algal groups and diatoms dataset by a PCA. Symbols of gray color indicate fish presence; white color, fish absence; circles, tadpole presence; triangles, tadpole absence.

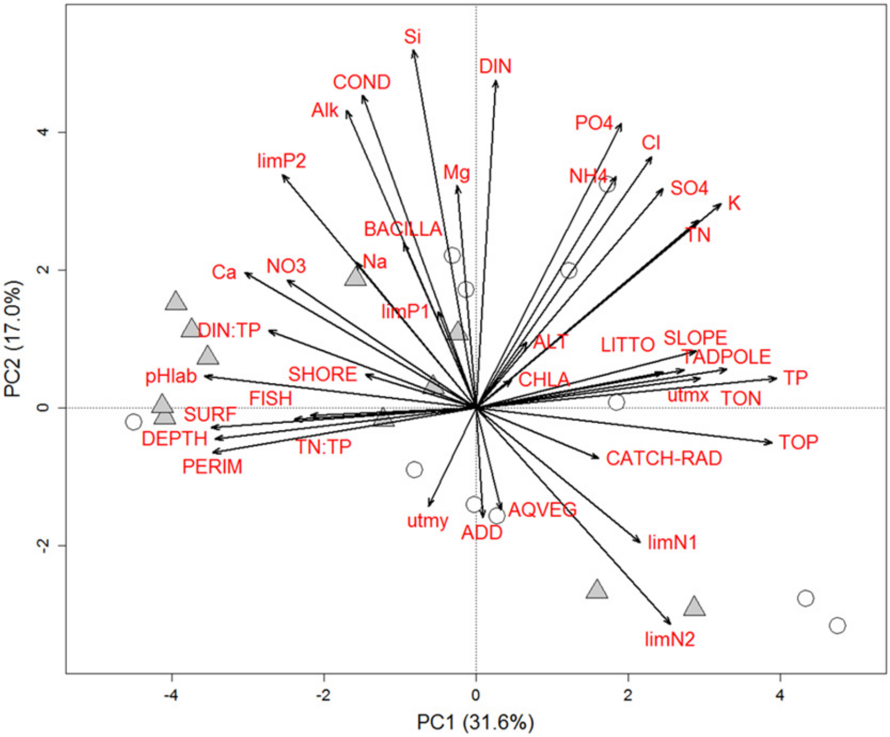


Fig. A2. Representation of the variables for bacteria and archaea dataset by a PCA. Symbols of gray color indicate fish presence; white color, fish absence; circles, tadpole presence; triangles, tadpole absence.

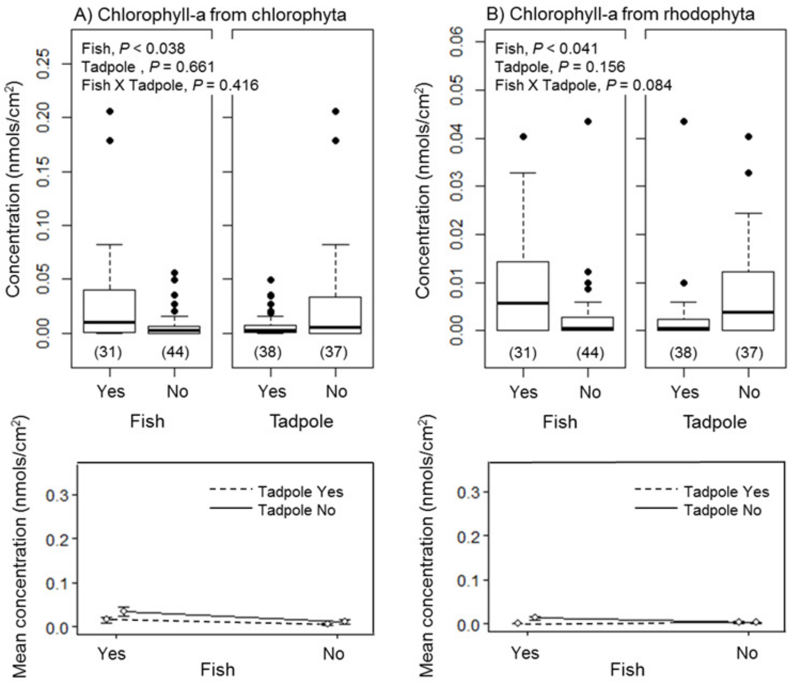
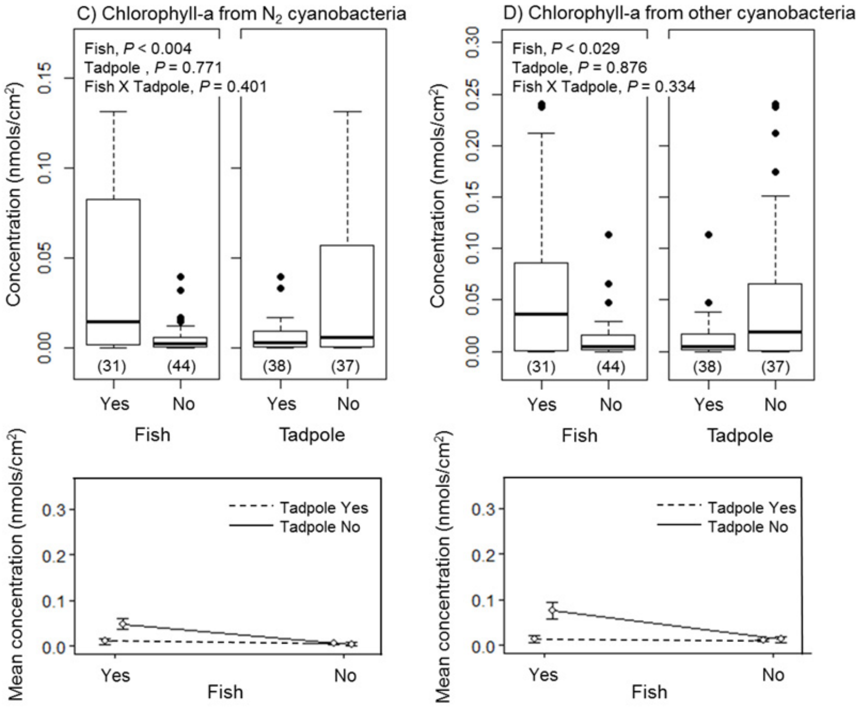


Fig. A3. (two pages) Effect of introduced fish and tadpoles on the epilithon biomass. Boxplots and interaction plots of ANOVA results for the Chlorophyll-a concentration from (A) chlorophyta, (B) rhodophyta, (C) N_2-fixing cyanobacteria, and (D) other cyanobacteria of the epilithon. The p-values of the two-way ANOVAs are shown on the top of the graphics. The number of sites for each category are written between brackets and below the boxes. The vertical bars of the interaction plots represent the standard error of the mean.


C) Chlorophyll-a from N_2 cyanobacteria
Fish, $P < 0.004$
Tadpole , $P = 0.771$
Fish X Tadpole, $P = 0.401$
Concentration (nmols/cm^2)
0.00
0.05
0.10
0.15
(31)
(44)
(38)
(37)
Yes
No
Yes
No
Fish
Tadpole
D) Chlorophyll-a from other cyanobacteria
Fish, $P < 0.029$
Tadpole, $P = 0.876$
Fish X Tadpole, $P = 0.334$
Concentration (nmols/cm^2)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
(31)
(44)
(38)
(37)
Yes
No
Yes
No
Fish
Tadpole
Mean concentration (nmols/cm^2)
0.0
0.1
0.2
0.3
Tadpole Yes
Tadpole No
Yes
No
Fish
Mean concentration (nmols/cm^2)
0.0
0.1
0.2
0.3
Tadpole Yes
Tadpole No
Yes
No
Fish

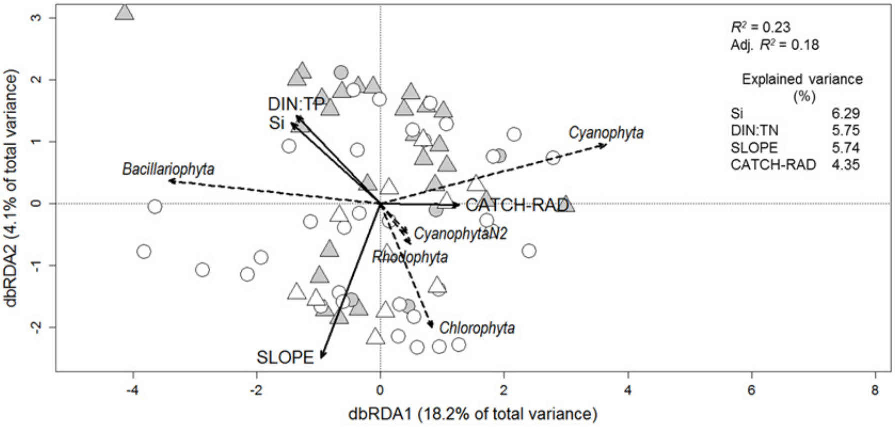


Fig. A4. Relative weight of introduced fish and tadpoles on algal group abundance. To focus on the community structure, the dbRDA was computed on the Hellinger distance of algal group abundance matrix. Symbols of gray color mean fish presence; white color, fish absence; circles, tadpole presence; triangles, tadpole absence. The explained variance of the variables was estimated by successive partial dbRDAs using each selected variable as covariable each time, and represents the percentage of the total variance not explained by the dbRDA when the analyzed variable is held constant.

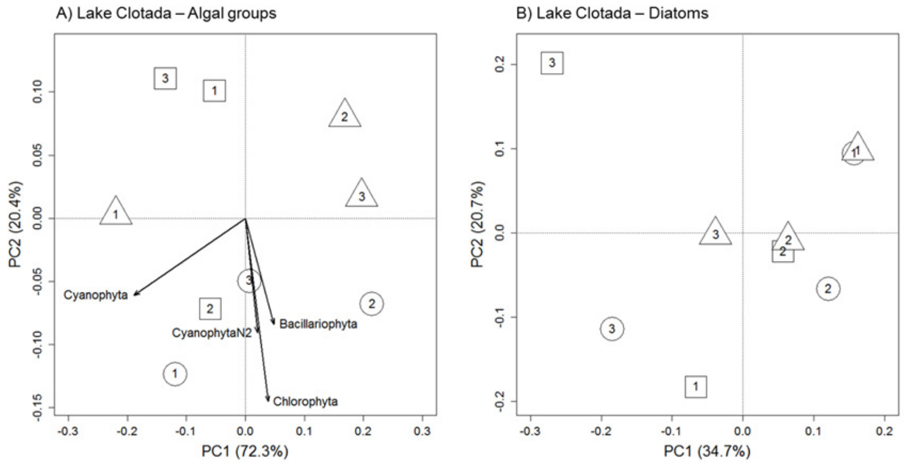


Fig. A5. PCA Representations of the epilithic experimental data obtained from Lake Clotada for (A) quantity of chlorophyll-a from five algal groups and for (B) the diatom assemblage structure. Data were previously normalized by fourth root transformation (algal groups) and Hellinger transformation (Diatom assemblage). Symbols of gray color mean fish presence; white color, fish absence; circles, tadpole presence; triangles, tadpole absence. Note the different scale in the PC2 axes.

General discussion

Traditional and modern stocking of Pyrenean lakes

Fish stocking in Pyrenean high mountain lakes is an old practice that has increased during the last half of the 20th century. The results of Chapter 1 of the thesis show that traditional exploitation dated back at least to the 14th and 15th centuries. These initial introductions affected 26.5% of the lakes, which already had introduced trout by 1900. However, since 1950 a wave of introductions with exotic species has affected 35% to 85% of the Pyrenean lakes, depending on the valley, similar to many other worldwide mountain ranges. Our findings suggest that human exploitation of some of these lakes might have originated further back in time, possibly to the Neolithic, when primitive residents already used the high altitude pastures. However, the written evidence suggests that the first major historical introductions occurred within the medieval warm period (1000–1300 AD) when the human population in the Pyrenees was at its highest.

This result contrasts with most of the available literature for other mountain ranges across the world, where trout introductions are relatively recent. In North America, introductions started in the 19th century with the arrival of European colonists (Schindler 2000, Wiley 2003) and in most of Europe it was not until the end of the 19th and 20th centuries that most alpine areas were populated with trout (Gliwicz and Rowan 1984, Sostoa and Lobón-Cerviá 1989, Martinez-Solano et al. 2003, Toro et al. 2006).

The existence of traditional fishing rights for local citizens in the Pyrenees, as described in the historical written documents (Miró 2011), probably contributed strongly to lake stocking in historical periods. In addition, lakes traditionally used for fishing were typically closer to towns and hence easier to exploit, were at lower altitude, and had higher surface areas.

Our results clearly support this hypothesis. The data from 1900 to 1950 showed that walking distance from nearby urban centres was the dominant factor explaining 29-60% of the variation in trout distribution. In contrast, for the period 1960–2000, the most significant factors were management practices associated with modern recreational fishing, and lake characteristics likely to be related to the probability of survival of the fish populations. These results are similar to those found in other areas of the world, including mountain areas of the western USA, where trout species are present in ca. 60% of lakes (Bahls 1992, Knapp 1996a, Wiley 2003).

In recent decades, minnows have also been introduced to some mountain areas, including the Pyrenees, a process associated with their use as live-bait for recreational fishing. The results of Chapter 2 strongly suggest that the introduction of minnows to the high mountain lakes of the Pyrenees is mediated by a preceding invasive species and facilitated by human activity. We found that the introduction of minnow is a more recent and more rapid process than that of salmonids. Since 1970s, when the first introduction took place, it has now spread to 27% of the lakes of the southern Pyrenees with an introduction rate of 4.7 lakes *p.a.*, compared to 2.2 lakes *p.a.* for trout over the period between 1940 and 2000.

The results of Chapter 2 also indicate that the presence of trout before minnow introduction is by far the most important variable explaining minnow distribution. In fact, we have not found any case of minnow introduction in naturally fishless lakes, strongly supporting the hypothesis that recreational fishing with live-bait is responsible for these introductions.

Unlike trout, minnow introductions are in general not authorised by governmental authorities and their introduction is often an illegal angling practice. In many cases fish unused as live-bait are released at the end of the fishing expedition (Maitland and Campbell 1992, Kerr et al. 2005, Winfield et al. 2011, Kilian et al. 2012), which makes their spread more difficult to control.

Another concerning aspect is that the minnow, like other small widely-distributed freshwater fish, displays a remarkable variability in its life history depending on the site temperature. For example, minnows have a maximum life-span of between 3 and 13 years, and the age of maturity varies from 1–2 to 5–7 years in hot and cold places respectively, while showing significantly lower growth increments in cold summers (Mills 1988).

This plasticity in their life history is an important factor in the species' ability to easily adapt from living in streams, its natural habitat, to high mountain lakes, where it shows higher resistance to harsh conditions than trout. In fact, in the Pyrenees we have found that in eight lakes (and 12 ponds) pre-existing trout populations have disappeared since minnow introductions. In some of these lakes brown trout were introduced centuries ago, while others were stocked more recently.

Introduced fish invasiveness in Pyrenean lakes

Fish introduced in Pyrenean high mountain lakes, both trout and minnows, are invasive species. The results obtained in Chapters 3, 4 and 5 clearly show a large impact on the Pyrenean lake biota. In Chapter 3 we demonstrated that the impact of trout on Pyrenean high mountain lake fauna is clear and similar to that found in the lakes of other mountain ranges. Trout was the most important factor determining the presence of amphibians and conspicuous macroinvertebrates in the lakes, as well as the taxonomic richness of both assemblages.

At taxon level, the presence of trout impacts most on amphibian and conspicuous macroinvertebrate taxa occurrence, but not on planktonic crustaceans. Our findings are, in general, consistent with the results of previous studies on salmonid impact on high mountain lakes. Strong effects of trout on some amphibians and macroinvertebrates have also been found in several mountain ranges of the US and Europe (Knapp et al. 2001b, Knapp 2005, Orizaola and Braña 2006, Pope et al. 2008, Parker et al. 2010, Pilliod et al. 2010, de Mendoza et al. 2012, Tiberti and von Hardenberg 2012, Tiberti et al. 2014b).

Minnows had similar impacts to trout on the littoral fauna, but greater impacts on the planktonic crustaceans. Our results showed a strong impact on amphibian and conspicuous macroinvertebrate richness. Although it is hard to find information about the impact of minnows on the amphibians and macroinvertebrates of high mountain lakes minnow species have been shown to negatively affect these organisms in lowland and boreal lakes (e.g. Werner et al. 2007, Naestad and Brittain 2010). Minnows have been reported as a cause of declines of frogs, paedomorphic newts and the species richness of amphibian tadpoles (Denoel et al. 2005, Eaton et al. 2005, Werner et al. 2007).

In contrast with trout, minnows also affect the occurrence of some planktonic crustaceans, mainly the largest species. We found an interesting interaction between minnows and trout as stressors. While trout did not show any individual negative impact on planktonic crustacean occurrence, minnow only showed a negative effect in absence of trout. Actually, in this interaction, the most severe situation in terms of *Cyclops abyssorum* and *Diaptomus cyaneus* occurrence was in the absence of trout, and when minnows were the only fish in the lakes.

This finding is consistent with previous studies from Scandinavian boreal lakes where minnows had no impact on zooplankton when trout was present in the lake (e.g. Larsson et al. 2010). In fact, when trout is also present, minnows live in the littoral fringe of the lake (Museth et al. 2007, Borgstrom et al. 2010), where they are unable to feed on planktonic crustaceans.

In Chapter 3 we also showed that water-level fluctuation associated with hydraulic exploitation, which affects 15% of Pyrenean lakes, has a marked impact on high mountain lake fauna, and is becoming an important third stressor of Pyrenean high mountain lakes locally together with trout and minnow. At taxon level, we found a negative effect on the occurrence of two taxa linked with the littoral habitat, *Rana temporaria* frogs and Gerridae, and also on the planktonic crustaceans *Daphnia longispina* and *D. cyaneus*. Previous studies have shown that artificial water-level fluctuation also greatly damages the littoral community of macrophytes (e.g. Gacia and Ballesteros 1998, Krolova et al. 2013).

Nevertheless, the findings of Chapter 4 highlighted that environmental spatial patterns explained more amphibian distribution at range scale, despite a large local effect of introduced fish in Pyrenean high mountain lakes and ponds,. The data from the western Pyrenees identified some natural environmental characteristics in this area that were clearly associated with the higher frequency of amphibians. Notable environmental factors included water conductivity for *Alytes obstetricans*, water conductivity together with diurnal water temperature variation for *Calotriton asper* and ice-cover duration (likely a proxy for water temperature) for *Bufo spinosus*.

Finally, in Chapter 5 we reported the existence of a trophic cascade mediated by introduced fish in the littoral of high mountain lakes, which altered the epilithon community at several levels, including biomass, diversity and the degree of activity or senescence. Despite the large top-down influence of introduced fish on the littoral food web of Pyrenean high mountain lakes, the analyses of the relative taxonomic abundances of diatoms and bacteria, showed some bottom-up control through availability of resources. The coexistence of both top-down and bottom-up forces have been described before in the food webs of lakes and ponds (Fitter and Hillebrand 2009, Mette et al. 2011). However, top-down control has been found to be more important in clear lakes, and in shallow lakes with low coverage of submerged macrophytes (Jeppesen et al. 1997, Liboriussen et al. 2005), characteristics often observed in high mountain lakes and ponds.

Conservation implications at Pyrenean range

There is a clear need for improving the conservation status of Pyrenean high mountain lakes and ponds; 35% to 85% of the lakes >0.5ha, depending on the valley, are impacted by invasive fish, and 15% on average are affected by artificial water-level fluctuation.

Our results show that suitable habitats for freshwater organisms in Pyrenean high mountain lakes are highly fragmented, by both natural and anthropogenic factors. In addition to natural spatial environmental patterns, the degree of isolation of breeding sites is markedly increased locally by presence of introduced fish, artificial water-level fluctuations, and potentially by the impact of emerging diseases on some species such as *A. obstetricans* and *Salamandra salamandra* (Walker et al. 2010, Martel et al. 2013). In general, species may be negatively affected by habitat fragmentation, either by natural or anthropogenic causes (Fahrig 2003, Fischer and Lindenmayer 2007). This fragmentation may lead to loss of fitness through inbreeding or local extinction through stochastic effects (Griffiths and Williams 2000). This negative effect may be still stronger on species with a small geographic distribution range, which should be urgently targeted for conservation (Sodhi et al. 2008), such as *C. asper* or the different subspecies of *A. obstetricans*.

Since the Pyrenees are shared by several countries and regions, there is a strong heterogeneity of protection agencies and environmental management policies across the range. Moreover, fish stocking actions are mostly regulated by different agencies from those responsible for conservation policies, and hydro-electrical power plants are linked to many of the largest Pyrenean high mountain lakes even within highly protected areas. Taking a regional overview of Pyrenean lake and pond ecology can provide a sound basis to inform conservation policies and actions at mountain range scale, overcoming artificial borders and involving all countries and administrations present.

On a positive note, restoration of high mountain lake fauna has been shown to be rapid when trout are removed (e.g. Knapp et al. 2007), since high mountain lake fauna have low resistance, but high resilience, to fish introductions (Knapp et al. 2001b). Several studies have demonstrated that removal of introduced fish allows rapid recovery of declining, and recolonization of lost, amphibians and macroinvertebrates, and also the progressive recovery of the pelagic habitat (Parker et al. 1996, Parker et al. 2001, Sarnelle and Knapp 2004, Vredenburg 2004, Sarnelle and Knapp 2005, Parker and Schindler 2006, Knapp et al. 2007, Pope 2008, Pope et al. 2009). In addition, an on-going initiative of lake restoration through minnow removal in the Pyrenees (within the framework of the LIFE+ LimnoPirineus project; www.lifelimnopirineus.eu) suggests that high mountain lakes recover some sensitive fauna even before complete minnow eradication. In contrast, it is hard to find data on restoration of more natural regimes and limnological characteristics in high mountain lakes subject to artificial water-level fluctuation. This would therefore be an interesting field to explore in the future.

In conclusion, a successful conservation policy for Pyrenean high mountain lakes and ponds, should be designed at a range scale, transcending political boundaries and incorporating spatial environmental patterns, fish stocking and water-level fluctuation management, as well as adequate restoration actions.

General conclusions

Arrival, distribution and ecology of introduced fish

1 The presence of trout in 52% of high mountain lakes of the southern slope of the Pyrenees is entirely associated with human stocking actions, either from historical or recent periods.

2. The distribution of *Salmo trutta* is best explained by both anthropogenic factors and lake characteristics, while only anthropogenic factors are associated with the distribution of *Salvelinus fontinalis* and *Oncorhynchus mykiss*.

3. Since the 1970s minnows have been introduced to 27% of the high mountain lakes of the southern slope of the Pyrenees, which represents the fastest spread of all introduced fish in these ecosystems.

4. The previous presence of trout in the lake is the most explanative variable for minnow occurrence, confirming its association with recreational fishing using minnows as live-bait.

5. A fishing ban in protected areas has resulted in a stabilization of the number of lakes with trout or minnows, suggesting that this is a reliable management policy for avoiding new introductions and preserving the conservation status of high mountain lakes.

Impacts on amphibians and other organisms

6. Trout, minnows and water-level fluctuation have a cumulative and antagonistic impact on Pyrenean high mountain lake fauna.

7. At assemblage level, trout and minnows are the factors that most strongly determine the presence/absence of amphibians and conspicuous macroinvertebrates, as well as their taxonomic richness, but do not determine that of the planktonic crustacean assemblage.

8. At taxon level, presence of trout and minnows is associated with a lower occurrence of most amphibian species and conspicuous macroinvertebrate taxa, while minnows alone reduced the occurrence of some planktonic crustaceans. The largest impact of minnows on these crustaceans was seen when minnows were the only fish in the lake.

9. Artificial water-level fluctuation also reduced the occurrence of some planktonic crustaceans that appeared unaffected by trout and minnows.

10. Presence of introduced fish is an important variable explaining the presence/absence of four of the five amphibian species found in the Pyrenean high mountain lakes and ponds, and the most important for *Rana temporaria*.

11. Five of the six amphibian species studied are more frequent in the western than in the eastern region of the Pyrenees. This appears to be related to spatial patterns of environmental variables.

12. Although introduced fish have a high local impact, western-eastern patterns of some environmental variables are the main drivers of amphibian communities at Pyrenean range scale.

13. To be successful, an amphibian conservation policy should be designed at range scale, ignoring political boundaries, and including spatial environmental patterns and management of fish stocks.

14. There is a strong trophic cascade caused by the indirect effect of introduced fish that is globally structuring the littoral epilithic community of high mountain lakes and ponds through predation on grazing tadpoles.

15. The epilithon biomass is higher where fish are present. Introduced fish is the main variable explaining the biomass of the different algal and cyanobacterial groups of epilithon in Pyrenean high mountain lakes and ponds.

16. Chemical variables and nutrient availability are more important than tadpole abundance or presence of fish in defining the composition of epilithic diatoms and bacteria assemblages. This highlights that, although there is strong top-down control of the littoral food web in the Pyrenean high mountain lakes, there is also some bottom-up influence coexisting.

17. Patterns of diversity in epilithic communities suggest a process of biota homogenization in lakes and ponds with fish and without tadpoles.

18. The littoral epilithon communities of Pyrenean high mountain lakes are less senescent and more active in the absence of fish and the presence of grazing tadpoles.

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Publications

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Historical use, fishing management and lake characteristics explain the presence of non-native trout in Pyrenean lakes: Implications for conservation

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ABSTRACT

High mountain lakes are naturally fishless, although many have had introductions of non-native fish species, predominantly trout. Predation on native fauna by introduced trout involves profound ecological changes. The objective of this study was to reconstruct the historical process of trout introduction in 520 high mountain lakes >0.5 ha of the southern Pyrenees and quantify which particular factors either environmental or anthropogenic, best explained their present distribution and lake conservation status. The first written evidence of trout introductions dated back to 1371 AD. By 1900, trout had been introduced to 26.5% of lakes during preceding centuries. A subsequent wave of introductions began in 1960 when stocking led to trout becoming established in 52.5% of lakes. From 1900 to 1950, walking distance from nearby urban centres was the dominant factor explaining 29–60% of the variation in trout distribution, indicating that trout introductions were in lakes closer to human settlements. In contrast, with the onset of modern fish management that took place during the period 1960–2000, the most significant factors were both the management practices and lake characteristics which are likely related with the probability of survival of the fish populations. After 2000, the remaining fishless lakes were those of highest altitudes, and the shallowest and those with the lowest surface area. The recent fishing ban in protected areas of National Parks has resulted in a stabilization of the number of lakes with fish, suggesting that this is the only reliable management policy to avoid new introductions and preserve the conservation status of high mountain lakes.

1. Introduction

Previous studies of the effects of invasive species in freshwater ecosystems have shown negative ecological consequences (Vitule et al., 2009). Freshwater fishes are one of the animal groups with higher number of invasive species, as has been shown in Europe (Hulme et al., 2009). At present, the introduction of freshwater fishes is closely related to human activities (Marchetti et al., 2004), and particularly with angling in the case of salmonid fishes (Cambray, 2003). Within high mountain lakes, the introduction of non-native fish species (mainly salmonids), is a global threat with common origins, mainly related with recreational angling and often promoted by different relevant governmental agencies (Pister, 2001; Schindler and Parker, 2002). These ecosystems are originally fishless due to natural barriers that have prevented the natural colonisation of fish species from lower streams (Knapp et al., 2001a; Pechlaner, 1984).

Introduced trout become the top predators of high mountain lake ecosystems leading to profound ecological changes. Predation on native fauna can lead to the elimination of amphibian and reptile populations (Knapp, 2005; Knapp et al., 2001b; Martinez-Solano et al., 2003; Orizaola and Brana, 2006; Pope et al., 2008), changes in zooplankton and benthic macro-invertebrate species composition and size structure (Brancelj, 2000; de Mendoza et al., 2012; Knapp et al., 2001b; Toro et al., 2006), alteration of ecosystem process such as nutrient cycling (Schindler et al., 2001) and indirect effects through resource depletion (Epanchin et al., 2010). Trout introductions are therefore a threat for the conservation of high mountain lake biodiversity in general and in particular for the most conspicuous animal groups.

In order to implement effective conservation measures, it is necessary to have a precise knowledge of trout distribution and the factors that determine their presence. Detailed information on the causes of the spread of salmonid fishes exist for some high mountain areas such as western North America (Bahls, 1992; Schindler,

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2000; Wiley, 2003), where introductions took place between the end of the nineteenth and beginning of the 20th century. Trout introductions were undertaken initially by individual fishermen and, a few decades later, by governmental agencies responsible for fisheries management (Schindler, 2000). In contrast to the American continent, in European high mountain lakes the colonisation process has not been studied in detail (Gliwicz and Rowan, 1984; Pechlaner, 1984; Sostoa and Lobón-Cerviá, 1989). The first introductions in the Alps were carried out at the end of the 16th century (Pechlaner, 1984) and in the Tatra mountains at the end of the 19th century (Brancelj, 2000; Gliwicz and Rowan, 1984). In the Cantabric mountains (Iberian Peninsula) introductions also occurred at the end of the 19th century (Terrero, 1951) and even more recently in the Sistema Central and Sistema Iberico, also in the Iberian Peninsula (Martinez-Solano et al., 2003; Toro et al., 2006).

In the Pyrenees it has been suggested that most fish introduction events are relatively recent (Sostoa and Lobón-Cerviá, 1989).The different trout species introduced in the Pyrenees include *Salmo trutta*, *Oncorhynchus mykiss* and *Salvelinus fontinalis* in the southern (Spanish) Pyrenees (Sostoa and Lobón-Cerviá, 1989) and these species together with *Salvelinus alpinus* and *Salvelinus namaycush* in the northern (French) Pyrenees (Delacoste et al., 1997). At a global scale, *S. trutta* and *O. mykiss* are considered among the 100 most invasive alien species of the world (Lowe et al., 2000). Within Europe, *O. mykiss*, *S. fontinalis* and *S. namaycush* have been introduced from North America, while *S. trutta* and *S. alpinus* which are native European species, have been widely introduced beyond their native range, mostly in high mountain areas. Therefore they are all classified as European alien species (Hulme et al., 2009).

Among the different approaches used to reconstruct the fish introduction processes in lakes, the review of historical information has been very useful (Le Cren et al., 1972; Pechlaner, 1984). Studies with a historical perspective are necessary to understand the impacts of introduced species (Kulhanek et al., 2011), which in turn are the basis for establishing more appropriate management policies. For example, the historical perspective has been used successfully in the North American Great Lakes, where it has had a significant potential for establishing restoration goals (Steedman et al., 1996). The combined analysis of historical information and the factors that might explain the distribution of high mountain lake fishes has not yet been conducted.

The objective of this study was to reconstruct the historical process of trout introduction events in high mountain lakes of the southern slope of the Pyrenees, to quantify which particular factors, either environmental or anthropogenic, best explain their present distribution and to evaluate which conservation measures have been most effective. In particular, we had the following specific hypotheses: (i) we expected that lakes with historical presence of trout populations would be those closer to towns and only hold the autochthonous species *S. trutta*; (ii) that modern introductions would be more likely in areas where active fish management has taken place and would involve the occurrence of other species than *S. trutta*; (iii) the absence of trout would be more likely in lakes with acid pH, higher altitudes and lower lake sizes; and finally (iv) prohibitions on fishing in National Parks would result in stopping fish introductions.

2. Materials and methods

2.1. Study area

The studied lakes are spread along the Pyrenean mountain range (0°42′W–2°09′E, 42°52′–42°23′N; Fig. 1). From among 1062 lakes >0.5 ha we surveyed 520 water bodies which comprise all those lying within the Catalan-Aragoneese (Spanish or southern) Pyrenees. Pyrenean lakes originated from glacial processes or were modified by the activity of quaternary glaciations. A more detailed description of the lake characteristics is provided in Appendix 1.

2.2. Data collection and environmental variables

The occurrence data for the different fish species were obtained from various sources depending on the period. Historical information of fish introductions (before 1900) was obtained from historical documents from local, regional and national archives. For introductions between 1900 and 2000 we collected ca. 6000 citations of species presence from interviews with local elderly fishermen or nature reserve guards, from local reports of fishing or walking societies and from our own visual encounter surveys. In a few cases we used gill nets to validate the information from the interviews. On average, we collected 11.6 citations per lake. We chose this method instead of only using our own surveys, since our aim was to provide a historical perspective of fish introductions and to be able to provide an objective account of the main factors responsible for these introductions.

To quantify which factors were responsible for fish introductions we generated a set of environmental variables summarizing both physical lake characteristics that might have affected the distribution of fish species and anthropogenic factors. Physical attributes from each water body included altitude, surface area, temperature and pH. Anthropogenic factors were divided into two groups, those describing or indicators of the mode of fish introductions (walking distance from the nearest town, the size of the population of the nearest town and their number of hotel beds, records of helicopter stocking and the presence of forestry roads), and those describing the management practice where the lake is situated (Table 1). A detailed description of how the variables were obtained is described in Appendix 1.

2.3. Statistical analysis

We compared the data on the presence/absence of the different fish species in the Pyrenean lakes with the different environmental variables including lake characteristics, site location variables, mode of introduction variables and fish management practice descriptors. We used generalized additive models for the analyses because they are similar to generalized linear models, but relax the assumption that the relationships between the dependent variable (when transformed to a logit scale) and predictor variables are linear by estimating a nonparametric loess smooth function for each continuous predictor variables (Hastie and Tibshirani, 1991; Knapp, 2005). See Appendix 1 for details on the regression procedure. The analysis was run first to obtain a historical perspective every decade from 1900 until present (year 2000) with all the taxa together and secondly with the present day data for each species separately.

Finally, we compared the presence/absence of the different fish species with the categorical predictor variables by 2 × 2 contingency tables. With the different continuous predictor variables, we tested if there were significant differences between the lakes that are fishless, those stocked before the year 1900 and those after 1900 with one-way ANOVA and a Tukey post hoc test. Data had previously been normalized.

3. Results

3.1. Historical process

More than 98% of the 520 studied lakes had actually natural barriers left after the glaciers retreated that had prevented the natural

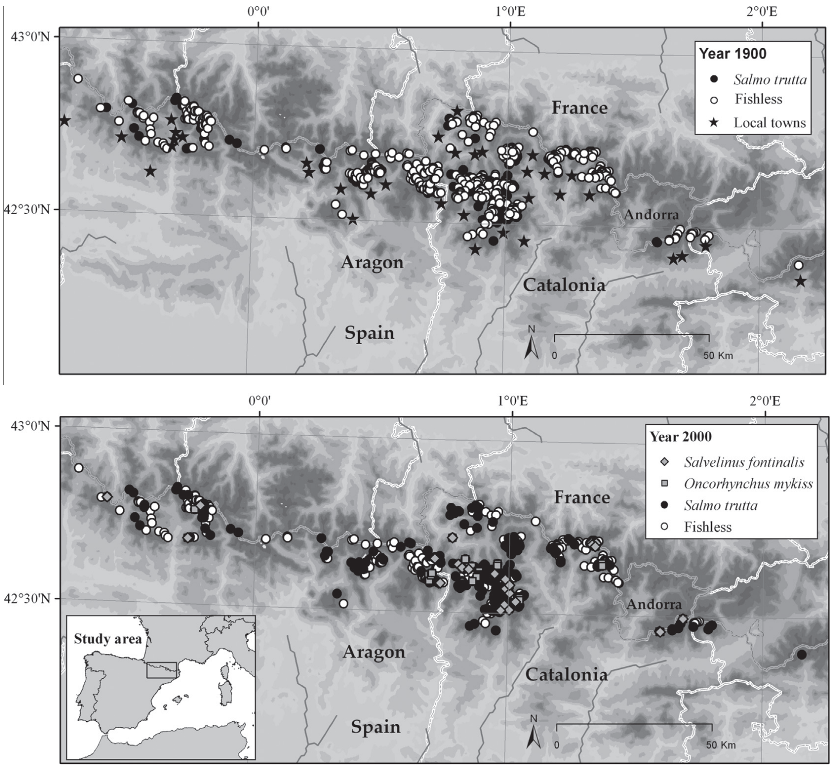

Fig. 1. Fish species occurrence and distribution of studied lakes along the Pyrenean range at the year 1900 (upper panel) and at the year 2000 (lower panel). Note that in 43 lakes there are more than one trout species per lake that are not visible in the map. In particular we have found that 32 lakes have two trout species, mainly *S. trutta* with *S. fontinalis* or *O. mykiss*. In addition, 11 lakes have the three trout species. Asterisks in the upper panel are the local towns.

colonisation of fishes. Therefore, most fish populations found in them at present are presumed to have come from non-natural sources. Research in historical archives confirmed that exploitation of lakes by local citizens was a common traditional activity in the Pyrenees. In this case, lakes were stocked with brown trout from nearby streams where it is native. The first explicit citations dated back to the 14th and 15th centuries in Lake Evol (1371 AD and 1423 AD), in Lakes Naut and Major de Saboredo (1581 AD), in Piedrafita Lake (1624 AD) and Escrita and Peguera catchments (1674 AD) (Miró, 2011). These old documents describe both the presence of trout in some lakes, and fishing for commercial exploitation of trout. After these initial historical documents, we found an increase in the number of citations, probably due to the increased amount of preserved written information. Our findings suggest that human exploitation of some of these lakes might have originated further back in time, possibly with the human colonisation of the Pyrenees.

We found sufficiently detailed information to be able to perform a detailed historical reconstruction of introductions in the 20th century. As of 1900, there were 138 lakes (26.5% of the total) that had been stocked with trout during preceding centuries for traditional exploitation (Fig. 1). The first modern introductions using large stocks of juvenile trout grown in local fisheries occurred occasionally at the beginning of the 20th century, but it was not until 1960 that most fish stockings were recorded. During this period the number of lakes with introduced trout increased to 272 (52.5% of the sample). Prior to 1900, the trout species introduced was exclusively brown trout, which was also the species with the highest increase during the last decades of the 20th century, increasing from 153 lakes in 1950 (29.4%) to 262 (50.4%) in 2000 (Fig. 2). The other species found, *O. mykiss* and *S. fontinalis*, originally from North America, were first recorded in 1950 being present in 32 (6.2%) and 33 (6.3%) lakes respectively (Fig. 2). These latter introductions were carried out mostly by governmental agencies but also by hydroelectric exploitation companies and local fishermen's societies.

Those lakes that had been stocked before 1900 had significantly higher surface area, shorter walking distance from the nearest town and higher catchment area compared to the lakes stocked after 1900. In contrast, fishless lakes had the highest altitudes, greatest walking distances and lowest temperatures (Fig. A1). Lakes stocked after 1900 had intermediate values for these variables.

Among the different factors explaining trout introductions during the 20th century, there was a clear difference between the first and second half of the 20th century. From 1900 to 1950, walking distance from the nearest settlement was clearly the dominant factor explaining from 29% to 60% of deviance: the lakes where fish were introduced were those closer to human settlements. This factor was followed in significance by lake surface area (13–30% of deviance increase) and lake location and altitude (Fig. A2). In contrast, with the onset of modern fish management, walking distance

Table 1
Description of predictor variables used in the generalized additive models.

Variable type	Variable name	Description
Physical and chemical	Altitude (ALT)	Elevation of the lake (m)
	Surface area (SURF)	Surface of the lake (ha)
	Water body location (LOCATION)	Latitude and longitude of the lake, UTM reference system
	Accumulated degree days (ADD)	Thermal accumulation in degree days >7.8 °C (°CDay)
	pH < 5.5 (pH)	Lakes with pH < 5.5
Mode of introduction	Walking distance (WALK)	Walking distance from the nearest town (min)
	Population (POP)	Inhabitants of the nearest town where the lake is situated
	Hotel beds (HOT)	Number of hotel beds in the nearest town where the lake is situated
	Helicopter stocking (HEL)	Helicopter stocking in the lake
	Forestry road (FROAD)	Lakes with forestry road access
Management practice	Fishing zone (FIZ)	Active fish management, at present or in the past
	National Park (NATP)	Part of the National Park where no fishing is allowed at present
	Hydroelectrical power (HEP)	Lakes with water level regulation
Biological parameters	*Salmo trutta* presence (STRU)	Presence of *S. trutta*. Only for *S. fontinalis* and *O. mykiss* models
	Salvelinus fontinalis presence (SFON)	Presence of *S. fontinalis*. Only for *S. trutta* and*O. mykiss* models
	Oncorhynchus mykiss presence (OMYK)	Presence of *O. mykiss*. Only for *S. fontinalis* and *S. trutta* models

ceased to be the most explicative factor during the period 1960–2000. Management factors, such as fishing zone or helicopter stocking area (only during the last decade, 1990–2000), and lake characteristics such as lake surface area, altitude and temperature were the most relevant factors. These results indicated that both the management practices and lake characteristics were the most relevant factors for explaining the presence of the fish population in modern fish introductions.

3.2. Factors explaining the present distribution of the three introduced salmonids

Among the three trout species found in south Pyrenean lakes, *S. trutta* is clearly the most widely distributed. It was found in 258 (49.6%) lakes and had a significantly higher chance of being found in the lakes within active fish management areas ($\chi^2 = 138.7$, $p < 0.0001$), in lakes with HEP ($\chi^2 = 44.7$, $p < 0.0001$), with nearby forestry roads ($\chi^2 = 44.7$, $p < 0.0001$) or in helicopter stocking areas ($\chi^2 = 77.65$, $p < 0.0001$). In contrast, the probability of occurrence in lakes within National Parks was not statistically different than outside them ($\chi^2 = 0.55$, $p = 0.457$).

The generalized additive model selected nine of the thirteen predictor variables (helicopter stocking, active fish management, surface area, altitude, location, pH < 5.5, walking distance from nearest settlement, forestry road and HEP) which were significantly associated with the probability of *S. trutta* occurrence (Table A1). The relationship between the probability of *S. trout* occurrence (on a logit scale) and the important continuously distributed predictor variables were all significantly nonlinear (Fig. 3). The response curve describing the estimated effect of lake surface area on the probability of *S. trutta* occurrence (p_i) indicated that p_i was low at the smallest lakes, but increased steadily until ca. 3 ha when it was constant. *S. trutta* had an increasing function of altitude until 2300 m, when it decreased progressively, and it had a constant, decreasing function with walking distance from the nearest town. Categorical variables representing different management practices contributed to approximately half of the deviance explained by the model (28.1%), with morphometric variables representing lake characteristics the other half (23.9%). The response surface for water body location is not provided in this or subsequent figures as it was often complex and offered no additional insights into the effects of the different predictor variables on species occurrence.*O. mykiss* was found in 32 (6.2%) of the lakes and had a significantly higher chance of being found in lakes within active fish management areas ($\chi^2 = 196$, $p < 0.0001$), in lakes with HEP ($\chi^2 = 5.2$, $p = 0.023$), with nearby forestry roads ($\chi^2 = 43.2$, $p < 0.0001$), and within National Parks ($\chi^2 = 17.9$, $p < 0.0001$), but its occurrence was independent of the lake being within helicopter stocking areas ($\chi^2 = 0.05$, $p = 0.817$). The generalized additive model selected two of the thirteen predictor variables (active fish management area and forestry roads) which were significantly correlated with the probability of *O. mykiss* occurrence (Fig. A3a). Both variables were equally important within the model.*S. fontinalis* was found in 33 (6.3%) of the lakes and had a significantly higher chance to be found in lakes within active fish management areas ($\chi^2 = 9.62$, $p = 0.002$), in lakes with HEP ($\chi^2 = 22.4$, $p < 0.0001$), with nearby forestry roads ($\chi^2 = 51.3$, $p < 0.0001$), and within National Parks ($\chi^2 = 4.66$, $p = 0.031$), but its occurrence was independent of the lake being within helicopter stocking areas ($\chi^2 = 0.43$, $p = 0.518$). The generalized additive model selected two of the thirteen predictor variables (to have a forestry road access and to be within an active fish management area) which were significantly correlated with the probability of *S. fontinalis* occurrence (Fig. A3b). Access to a forestry road had a stronger weight in the model explaining 71.3% of deviance compared to the other significant variable (20.1%).

Finally, for the three trout species, none of the biological variables (e.g., the occurrence of other trout species) were significant, suggesting that the probability of finding one particular trout species is not directly linked with the previous presence of another species.

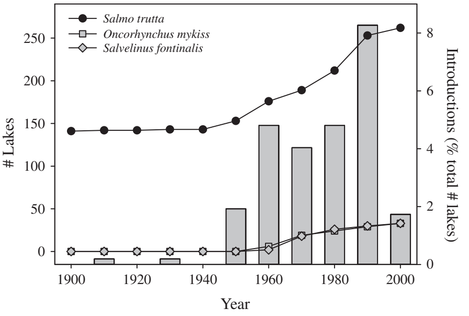

Fig. 2. Introduction process of non-native trout species in the southern Pyrenees during the 20th century. Vertical gray bars are the decadal total number of lakes that have suffered fish introductions in percentage of the total number of lakes >0.5 ha of the study area ($n = 520$).

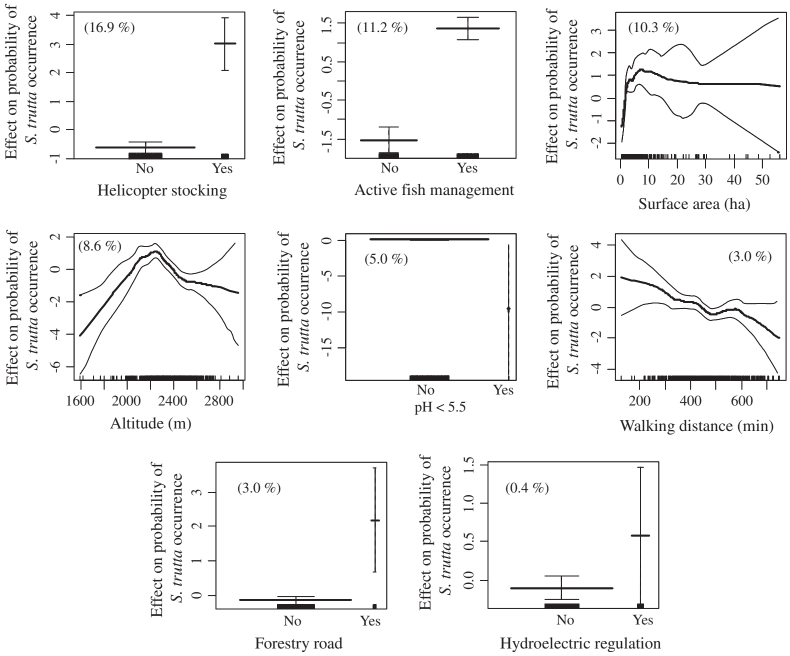

Fig. 3. Estimated effect of each of the highly significant ($p \leqslant 0.01$) predictor variables on the probability of occurrence by *Salmo trutta*, as determined from the generalized additive model (span = 0.5). Response curves are based on partial residuals and are standardized to have an average probability of zero. Thin lines are approximate 95% confidence intervals and hatch marks at the bottom are a descriptor of the frequency of data points along the gradient in continuous variables or within each category for categorical variables. The width of horizontal lines in categorical variables is proportional to the frequency of the data within each category. Numbers in parenthesis are the percentage of explained deviance of each variable. See Table A1 for model details.

3.3. The role of National Parks in the conservation of lakes

In the previous statistical models, National Parks did not appear as a significant variable in explaining the historical changes or the present trout distribution. Therefore, in order to evaluate the particular role of National Parks in lake conservation, we compared the changes in the number of lakes with fish during the 20th century in Aigüestortes i Estany de Sant Maurici National Park with the other areas, distinguishing the two management areas of the National Park (Fig. 4a). We found that among the three areas, the number of lakes with trout only stopped increasing at the time of the ban (1988) in the area where fishing was prohibited, while at the other areas it continued to increase.

The increase in the number introductions could partially be masked if, at the same time, there were some lakes in which trout populations had gone extinct. Therefore we counted the number of lakes that had been stocked during the 20th century and the number that at present are fishless in the three areas. The percentage of lakes that lost salmonid populations was lowest in the fishing area of the National Park, while it was higher in the area of the National Park where fishing is not allowed (Fig. 4b), although, these differences were not significant in the chi square tests ($p > 0.1$ in all cases). In contrast, the lakes where fish disappeared had a significantly higher altitude and only half of the surface area (Fig. 4c and d respectively), suggesting that the disappearances basically occurred in lakes where populations cannot persist and that had not been re-introduced, independently of the type of protection area. Within the National Park, the slightly higher rate of population disappearances in the fishing-prohibited area was likely due to re-stocking in the fishing-allowed area.

4. Discussion

4.1. First introductions: traditional exploitation of lakes

The first written documents describing the existence of fishing rights in high mountain lakes of the Pyrenees dated back to the 14th and 15th centuries. These initial introductions for traditional exploitation resulted in 26.5% of the lakes having introduced trout by 1900. Similarly, in the northern side of the Pyrenees, it has been described that ca. 25% of the lakes had fish prior to the onset of widespread introductions after 1936 (Delacoste et al., 1997), which might also be attributed to traditional fishing activities by local fishermen. Our findings suggest that human exploitation of some of these lakes might have originated further back in time, possibly back to Neolithic times, when primitive residents already used the high altitude pastures (Miró, 2011). However, written evidence found suggest that the first major historical introductions occurred within the medieval warm period (1000–1300 AD) when the human population in the Pyrenees was highest (Miró, 2011). Our results contrast with what has been described from other alpine areas of the world where trout introductions are a relatively recent process. In North America, introductions started in the 19th century with the arrival of European colonists (Schindler, 2000; Wiley, 2003) similar to most areas of Europe where it was not until the end of the 19th and 20th centuries that most alpine areas were

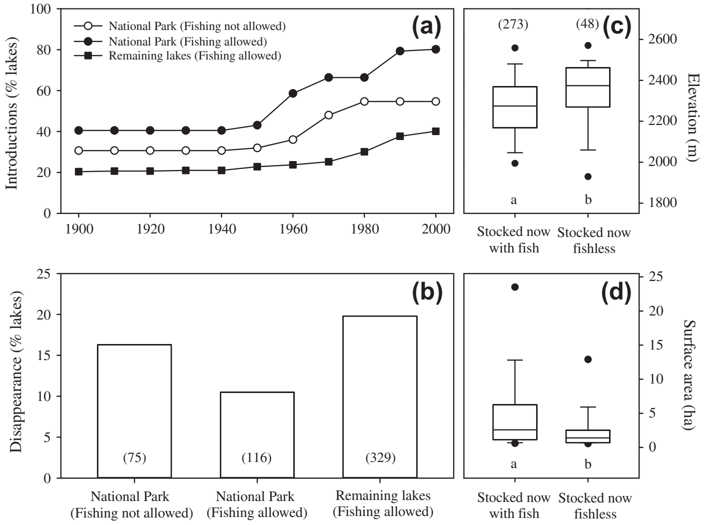

Fig. 4. (a) Effect of Aigüestortes i estany de Sant Maurici National Park on the introduction of non-native trout species in high mountain lakes of the Pyrenees. Circles are the lakes within the National Park, and squares are lakes outside the National Park. White circles are the area of the National Park where fishing is not allowed and black circles are the lakes in fishing allowed areas. (b) Percentage of lakes (respect to the total number of each area, in brackets) in the two management areas of the National Park compared to the rest of the study area. (c and d) Box plots of the altitude and surface area of the lakes that have been stoked with trout after 1900 distinguishing those that are still with fish from those that are now fishless. The lines within each box follow Fig. 3. Sample sizes are given in parentheses in (c). Categories with different letters are significantly different at the $p = 0.05$ level (Mann Whitney U test).

populated with trout (Gliwicz and Rowan, 1984; Martinez-Solano et al., 2003; Sostoa and Lobón-Cerviá, 1989; Toro et al., 2006). Similarly, in northern European regions with many alpine lakes, such as Scotland or Scandinavia, trout introductions have occurred since the Middle Ages but were mainly at low altitudes, and it was not until the late 19th century that introductions to remote high mountain lakes began, mainly related with angling activities (Maitland and Campbell, 1992; Nilsson, 1972). The only exception is in Tyrol, in the Alps, where there are records of introduction of Arctic char (*S. alpinus*) at the end of the 15th and beginning of the 16th centuries (Pechlaner, 1984). The main difference between the introductions in the Alps and the Pyrenees was that in the Alps the introductions and fishing were performed by the nobility (e.g., King Maximillian I; Pechlaner, 1984), while in the Pyrenees the rights of exploiting the lakes were given to local towns in order to keep the population in the area. As a result of this historical process those lakes that had been traditionally exploited for fish were typically those closer to towns, and were also those of higher surface area, lower altitude and bigger catchment size (Figs. 1 and A2).

4.2. Modern introductions and the role of management practices

With the onset of modern fish management that took place during the period 1960–2000, the most significant factors explaining trout distribution were related with management practices. However, different factors were important for the different species. For *S. trutta*, the probability of occurrence was highest in helicopter stocking areas, and in active fish management areas. The repeated use of helicopter stocking in recent decades has resulted in extensive occurrence of trout in these areas and, as a result, in a high percentage of lakes with fish (52.5%). In these areas the inverse relationship between trout presence and altitude is lost. This result is similar to that found in other areas of the world where helicopters and aeroplanes have been used for stocking trout, such as several mountain areas of West USA, where trout is present in ca. 60% of lakes (Bahls, 1992).

In contrast to *S. trutta*, *S. fontinalis* was preferentially found in lakes with forestry road access, while for *O. mykiss* it was preferentially found in lakes that had a forestry road access but in areas that active fish management has been carried out. These two last species are present in a much lower number of lakes, and also were introduced mainly between 1960 and 1980 (Fig. 2). Their presence is closely linked with the majority of construction of hydroelectric schemes at high altitudes in the Pyrenees (Catalan et al., 1997). Hydroelectric companies compensated local citizens by developing local fisheries that were used to introduce these two species to the lakes around the area where hydroelectric schemes were developed. The same hydroelectric development was responsible for the construction of forestry roads that were in turn, the main routes serving for introducing these two species. Amongst the species, *S. fontinalis* was mainly introduced in lakes with vehicle access. In the 1980s administrative changes shifted stocking responsibilities to local fishermen's societies together with governmental agencies. This favoured the closure of *S. fontinalis* and *O. mykiss* hatcheries and the consolidation of North and Central European strains of *S. trutta* in official fish hatcheries. As a result, there has been a shift from using local stocks to using races from different parts of central and northern Europe (Araguas et al., 2009).

Modern management practices have therefore resulted in high fish introductions during the last few decades with a maximum during 1990s (Fig. 2). As a result, trout have been introduced in most lakes with higher fishing interest (e.g., lower altitude or bigger surface area). This also explains that fish introductions dropped considerably during 2000.

4.3. Limits to trout survival: the role of lake characteristics

Lake characteristics were the second most important group of variables (altogether explaining 23.9% of deviance; Table A1) after those related with management. Amongst them lake surface area was the most important variable. Similarly, in the Sierra Nevada (USA) Knapp (1996) also found that those lakes with fish were significantly bigger than those without. The possible role of lake surface area on trout occurrence is likely related with the probability of population persistence. We found that those lakes that lost their fish populations had significantly smaller surface area and higher altitude. Lake surface area and lake depth are highly correlated in alpine lakes due to their common glacial origin (Catalan et al., 2009). Smaller, and therefore shallower, lakes are those where ice-cover thickness (e.g., 2–4 m, typical of these lakes; Ventura et al., 2000) can reach lake bottom or close to it, and therefore kill all fish either directly due to disappearance of water or indirectly through disappearance of oxygenated layers below the ice-cover. The second most important factor is lake altitude, strongly correlated with lake temperature in alpine areas (Thompson et al., 2009). However, temperature itself does not seem to be the main factor under altitude, since we explicitly incorporated the accumulated summer temperature (Table 1) in the model and this variable was not selected in the analysis. It is possible that the fraction of deviance explained by altitude is more related with lake characteristics, such as benthic substrate composition, or the probability of finding inlet streams, both needed for trout reproduction and therefore population persistence (Wiley, 2003).

The presence of lakes with pH <5.5 also explained a significant proportion of deviance of *S. trutta*, being the probability of occurrence positively associated with pH. This species cannot survive below this pH (Rosseland et al., 1999). The Pyrenees has some lakes with such a naturally low pH occurring due to substrate geology (Casals-Carrasco et al., 2009), where although they have been stocked in the past, we do not find the presence of trout in any of them. Other trout species such as *S. fontinalis* potentially would be able to survive in some of these lower pH lakes (Korsu et al., 2007; Westley and Fleming, 2011). However, the history of *S. fontinalis* introduction was closely linked with hydroelectric exploitations (see Section 4.2), and therefore the species has never been introduced in these lakes.

4.4. The role of National Parks

In this study we have found no significant effect of National Parks in the occurrence of *S. trutta*, and surprisingly the probability of finding *O. mykiss* and *S. fontinalis* was higher inside than outside National Parks. This finding contrasts with previous studies where the probability of finding trout is lower in National Parks where fishing is forbidden (Knapp, 1996; Wiley, 2003). For the two species with higher occurrences inside the National Park, this apparent contradiction is likely due to the higher abundance of hydroelectric exploitation inside the Park. For these two species the presence of forestry roads (build almost exclusively during lake impoundments) is one of the two most important factors explaining their distribution. In addition, before prohibition of fishing in the National Park, these areas were included within normal fish management areas, and therefore National Parks before the 1980s, had the coincidence of the two most relevant variables for explaining the occurrence of these two species.

A closer examination of the number of lakes with fish in Aigüesteortes i Estany de Sant Maurici National Park compared to the rest of the area (Fig. 4a) showed that in the area of the National Park where fishing is forbidden, the number of lakes with trout stopped increasing just at the time of the prohibition, while at the other areas, it continued to increase. This finding illustrates that the only management practice that had an effect in the protection of lakes is the prohibition of fishing. Although the most obvious route to avoid introductions would be for governmental agencies to stop stocking trout, this is complicated by the fact that through the history of trout introductions in the Pyrenees there have been several other agents involved in these introductions in addition to governmental agencies (e.g., local citizens in the past or local fishermen's societies in the last decades). As a result we have found some lakes that have been stocked recently without the collaboration of the administration.

The social implications of prohibiting fishing would not be relevant if the prohibition is implemented in those lakes that are at present fishless (at the moment there is the paradox that some lakes have fishing rights while never being stocked with fish). It could also be implemented in some particular lakes or groups of lakes that are especially important for the conservation of some animal groups (e.g., Pyrenean newt *Calotriton asper*). Finally, prohibition on fishing has already been performed in the Aigüestortes i estany de Sant Maurici National Park, with no documented adverse social impact: basically local citizens realized that the social benefits of nature preservation (through higher tourism) have been greater than those that were obtained via fishing.

Although the purpose of this paper was not to explicitly quantify the relative proportion of established (self-sustainable) populations, our results suggest that there is a high proportion of the populations that are established. This is obvious with the historical introductions prior to 1900 (the lakes still have trout at present) but less clear with the introductions that occurred along the 20th century. By comparing the data of the recorded introductions with the persistence of populations at the year 2000, we were able to quantify the number of lakes where trout populations have not been able to survive. We found a small percentage of stocked lakes where trout disappeared (Fig. 4b). As we discussed in Section 4.3. the disappearance was not related with any of the protection figures, but with physical lake characteristics, such as higher altitudes and smaller surface areas (Fig. 4c and d). Moreover, previous studies have suggested that fish naturalisation is relatively infrequent, due to the requirement of stream inlets connected with the lakes (Lek et al., 1996). It is also possible that we did not find a higher reduction of trout disappearances in the non-fishing area of the National Park due to the relatively recent date of fishing prohibition. Previous studies have described that it takes several decades (between 20 and 30 years, but in some circumstances even 40) before populations disappear from the lakes where they have been introduced and that have unsuitable characteristics for trout populations to persist (Knapp et al., 2005). We would expect, therefore, that in the next few decades there will be a higher increase of fish disappearances in the protected area of the National Park.

5. Conclusion

The results found in this study, together with those of other areas (Knapp, 1996; Wiley, 2003), show that when fishing or fish stocking is forbidden, the number of lakes with trout stop increasing and tend to decrease. In contrast, in National Parks where fishing and stocking are allowed, the number of lakes with trout increase until nearly all have fish (fishing allowed area of Aiguestortes i estany de Sant Maurici National Park, Fig. 4; and National Park of Pyrénées; Delacoste et al., 1997). In this latter case the conservation status of those animal groups affected by fish introductions will be affected.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2013.07.016.

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ORIGINAL PAPER

Evidence of exotic trout mediated minnow invasion in Pyrenean high mountain lakes

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Abstract Although high mountain lakes are naturally fishless, there have been numerous trout introductions to such ecosystems in many areas of the world with negative ecological consequences. In recent decades other fishes, such as minnows, have been introduced to some mountain areas, including the Pyrenees. These introductions may cause further ecological problems, since minnows also occupy the top of the food chain, and are difficult to manage since such introductions occur without permission from the authorities. In this study we have analyzed the process of minnow introductions in all high mountain lakes of the southern slope of the Pyrenees to find out which particular factors best explained their present distribution and to evaluate which management measures have been most effective for stopping introductions. We found 27 % of the lakes had minnows (*Phoxinus* sp.) present, 52 % had trout and 47 % were fishless. Trout presence was the most significant variable explaining 27 % of deviance of minnow presence data in a generalized additive model. Recreational fishing using minnows as live bait is likely responsible for these introductions. Minnow introductions are therefore mediated by a preceding invasive species and facilitated by human activity. We also compared the number of minnow introductions in non-fishing areas of National Parks with other areas where managed fishing takes place. We found that the number of lakes with minnow introductions was increasing in all areas except those where fishing was prohibited, indicating that prohibiting fishing is an effective management practice for stopping minnow introductions.

Keywords *Phoxinus phoxinus* · Minnow · Invasive species · National Park · Lake conservation · High mountain lakes

Introduction

Invasions by human-introduced non-indigenous species are one of the main threats to biodiversity. They have been identified as the main cause of extinction of wildlife in many occasions, but also as a potential phenomenon generating evolutionary stress and moreover causing biotic community homogenization (Clavero and García-Berthou 2005; Lockwood et al. 2013; Marr et al. 2013; Simberloff et al. 2013). Freshwater fishes are one of the animal groups with a high number of invasive species (Hulme et al. 2009). Ecological impact of invasive species in aquatic environments has been described as severe when they become top predators (Vitule et al. 2009), since in addition to decreasing the relative abundances or completely

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eradicating their prey, they also alter the trophic relationships inducing trophic cascades to the base of the food web (e.g. Byström et al. 2007; Wahl et al. 2011). Furthermore, the introduction of multiple predator species can have cumulative effects on the receiving ecosystems (Nyström et al. 2001).

Angling practices with live bait represents a worrying pathway for alien species introductions (Kerr et al. 2005; Webb 2007; DiStefano et al. 2009; Ward et al. 2012). The largest organisms used as live bait are several species of small fishes, but other animals such as amphibians, earthworms, crayfishes, grubs and insects are also used (Lindgren 2006; Keller and Lodge 2007). Improper disposal of live bait has been attributed as the source of introduction of at least 14 species of fishes in Ontario (Kerr et al. 2005). In the English Lake District, individuals of at least 12 native and non-native fish species have been brought to Windermere for the purpose of live-baiting (Winfield et al. 2011). Live-bait use was also responsible for the introduction of 47 known freshwater species in United States Mid-Atlantic slope drainage systems, among which are at least 5 non-native fishes, 4 non-native crayfishes and 9 non-native earthworm species (Kilian et al. 2012). Live baits may arrive in the environment by accidental escape or more often, by being released into the water at the end of the fishing trip (Winfield et al. 2011; Kilian et al. 2012). In some cases, it has been shown that bait-related introductions have resulted in established populations of invasive species (e.g. Callaham et al. 2006; Migge-Kleian et al. 2006).

The use of small fishes as live bait, mainly used to catch salmonids, has previously been reported in boreal and mountain lakes. For example, during the second half of the twentieth century, Scottish lochs have received, at least 7 non-native species used as live-bait, minnows among them (Maitland and Campbell 1992). Also, minnows have expanded outside their native distribution in Scandinavia since the beginning of the twentieth century, mainly in mountain areas and in association with angling practices (Museth et al. 2007). However, invasions linked with released live-bait have not been very well described in high mountain watersheds so far.

High mountain lakes are originally fishless ecosystems due to natural barriers that have prevented the natural colonization of fish species from lower streams or dispersal among interconnected lakes (Knapp et al. 2001a; Pister 2001). However, the Pyrenees like many other high mountain areas of the world nowadays have introduced trout in more than half of the lakes (Miró and Ventura 2013). These introductions first took place in lower altitude lakes (ca. 25 % of the lakes) historically associated with traditional fish exploitation dating back at least to the fourteenth century. More recently, during the second half of the twentieth century, introductions have taken place in another 25 % of the lakes, mainly for recreational fishing purposes and promoted by local fishermen associations, environmental administrative offices and hydroelectrical power companies (as compensatory actions) (Miró and Ventura 2013).

Complementary to the spread of trout, minnows have also been detected in many Pyrenean lakes during the last few decades (Miró 2011). Although minnows apparently are used as live-bait for trout angling, this type of angling is forbidden in the southern part of the Pyrenees since 1966. The illegal introduction of minnows, therefore, occurs without the control of public authorities and generates a challenge for resource managers tasked with the conservation of high mountain lake ecosystems. Within the Pyrenees, there are areas with different degrees of protection (e.g. National Parks where fishing is prohibited and other areas where fish management takes place) that offer an interesting opportunity to explore how different fish management practices (authorized or prohibited trout angling) have affected the spread of minnows.

The objective of this study was to understand the distribution and spread of minnows in high mountain lakes of the southern slope of the Pyrenees, to find out which particular factors, either environmental or anthropogenic, best explained their present distribution and to evaluate which conservation measures have been most effective. We expected to confirm that successful minnow introductions were closely related with their use as live-bait for trout fishing. In particular, we had the following specific hypotheses: (1) we would only find minnows in lakes where trout had been previously introduced; (2) minnow introductions would be more likely in areas where active fish management has taken place; (3) the probability of finding minnows would be higher in lower altitude lakes and in those with larger sizes; and finally (4) fishing prohibition (for trout) in National Parks should result in stopping minnow introductions.

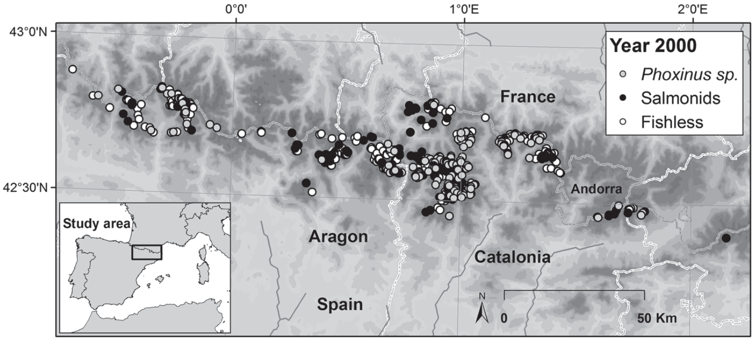


Fig. 1 Distribution of the 520 studied lakes along the southern Pyrenean range and fish presence at year 2000. Minnows (*Phoxinus* sp.) usually share the lake with trout or are alone in some cases; see "Results" section to more details

Materials and methods

Description of the study area and species

The studied lakes are spread along the Pyrenean mountain range (0°42′W–2°09′E, 42°52′–42°23′N; Fig. 1). From among 1,062 lakes >0.5 ha, we surveyed 520 water bodies which comprise all those lying within the Catalan-Aragoneese (Spanish or southern) Pyrenees. Pyrenean lakes originated from glacial processes or were modified by the activity of quaternary glaciations. The surveyed lakes range in altitude between 1,600 and 2,960 m with the highest frequency found at ca. 2,400 m and are relatively small and deep (average surface area of 2 ha and average maximum depth of 17 m, with the largest of 54 ha and deepest 105 m). Due to their common origin, there is a close positive relationship between surface area and maximum depth (Catalan et al. 2009). Most of the lakes are above the tree line with catchments partially covered by meadows, although some of them are within or below the tree line. Approximately half of the lakes have catchments on granodiorite bedrock, the remaining being located in catchments with metamorphic (25 %), detrital (15 %) or carbonate (10 %) bedrock with a minority with Silurian slate (Casals-Carrasco et al. 2009). The latter bedrocks, due to their high sulphate content, give natural acidity to the waters (pH <5.5).

Within the study area there is a National Park, Aigüestortes i Estany de Sant Maurici National Park which has 37 % of the total studied lakes. These lakes are under two different management regimes: a core area with strict regulations where fishing has been prohibited since 1988 (with 75 lakes) and a peripheral area where fishing is allowed (with 116 lakes).

The study species is the European minnow (*Phoxinus phoxinus* (L. 1758)), that was considered a single species until recently, and therefore most citations were referred as *P. phoxinus*. A recent taxonomical revision has distinguished various species: *P. phoxinus* sensu stricto in the northern (French) Pyrenean slope, *Phoxinus bigerri* Kottelat, 2007 native in the north and south-western Pyrenean streams, and *Phoxinus septimaniae* Kottelat, 2007 native in the north-eastern Pyrenean streams (Kottelat 2007). During summer of 2013 we were able to examine the species present in fifteen lakes of both north and south slopes of Pyrenees, and only found *P. phoxinus* sensu stricto. To avoid taxonomical confusion, in this paper we use the term minnow to refer to *Phoxinus* sp. found in the Pyrenean lakes.

Data collection and environmental variables

We collected 435 citations of minnow presence and 379 of minnow absence in the study area from interviews with local elderly fishermen or nature reserve wardens, from local reports of fishing or walking societies and from our own littoral's visual encounter surveys. In fifteen lakes, we also used minnow traps to validate the information from the interviews, confirming all cases. Historical information of minnow absence was also obtained from historical documents from local, regional and national archives. The collected data from the different complementary sources covered the whole twentieth

Table 1 Description of predictor variables used in the generalized additive models

Variable type	Variable name	Description
Physical and chemical	Altitude (ALT)	Elevation of the lake (m)
	Surface area (SURF)	Surface of the lake (ha)
	Water body location (LOCATION)	Latitude and longitude of the lake, UTM reference system
	Connectivity (CONN)	Binary factor that has a value of 1 in each lake that has a direct stream connection without any natural barrier with another lake with an established minnow population
	Accumulated degree days (ADD)	Thermal accumulation in degree days >7.8 °C (°C day)
	pH < 5.5 (pH)	Binary factor indicating lakes with pH < 5.5
Mode of introduction	Walking Effort (WALK)	Walking effort from the nearest town (minutes)
	Population (POP)	Inhabitants of the nearest town to the lake
	Hotel beds (HOT)	Number of hotel beds in the nearest town to the lake
	Helicopter stocking (HEL)	Binary factor determined by the existence of helicopter stocking in the lake
	Forestry road (FROAD)	Binary factor marking the lakes with forestry road access
Management practice	Fishing zone (FIZ)	Binary factor determined by the existence of active fish management, at present or in the past
	National Park (NATP)	Binary factor indicating the lakes which belonging to the part of the National Park where fishing is prohibited at present
	Hydroelectrical power (HEP)	Binary factor marking the lakes with water level regulation
Trout facilitation	Trout presence (TROUT)	Binary factor determined by trout presence in the lake

century and allowed us to reconstruct the decade when minnows arrived to each lake. We chose this method instead of only using our own surveys, since our aim was to provide a historical perspective of minnow introductions and to be able to provide an objective account of the main factors responsible for these introductions.

To detect which factors were associated for minnow introductions, we generated a set of environmental variables summarizing both physical lake characteristics and anthropogenic factors that might have affected the distribution of minnows (Table 1). Altitude and surface area were obtained from a GIS generated from 1:25,000 maps from the Spanish, Aragonese an Catalan geographical agencies (Casals-Carrasco et al. 2009). A binary factor of connectivity (CONN) was included to assess the possible natural dispersion of minnows through streams or their secondary spread after introduction in one lake, coding the presence/absence for a given lake of a stream connection with another lake with an established minnow population. Water temperature was the accumulated degree days (ADD) during the ice-free period calculated from daily max–min temperature data using the sine-wave method, assuming the trigonometric sine curve as an approximation of the diurnal temperature curve and adding the area under the curve and above the lower threshold for each day (Baskerville and Emin 1969). As a lower threshold we used 7.8 °C, the lower developmental temperature below which *Salmo trutta* reproduction is not feasible (Elliott et al. 1995). Daily water temperatures were obtained from 27 automatic thermometers (Vemco Minilog-T) deployed at a depth of ca. 1 m in lakes covering a wide range of altitudes, surface areas >0.5 ha and catchment sizes from 2008 to 2009. The calculated ADDs from those lakes with automatic thermometers were extrapolated to all other lakes using a multiple linear regression model between ADD and various morphometric parameters and choosing the minimum combination of morphometric variables that described most of the variance with stepwise forward selection procedure. The following model obtained was:

$$\begin{aligned}&\text{Degree days}(>7.8\,^{\circ}\text{C}) = 4410.4874 - 1.39\ \text{ALT}\\&- 21.73\ \text{SURF} - 117.72\ln(\text{CS}/\text{SURF})\\&- 0.08\ \text{CS} - 53.71\ln(\text{DC}/\text{CS})\\&R^2 = 0.833;\ F_{5,20} = 19.9, P < 0.0001\end{aligned} \tag{1}$$

where ALT and SURF are lake altitude and surface area (Table 1), CS is the lake total catchment size and DC is the lake direct catchment size (i.e. the proportion of catchment not shared with other lakes that are upstream of the lake). This latter parameter equals CS when there are no lakes upstream and the ratio DC:CS is relevant since the presence of other lakes in the catchment changes the temperature of the streams flowing out of them. The ratio CS:SURF is indicative of the rate of water renewal.

When using pH as an environmental variable, we used a binary variable which separated out lakes with pH <5.5, as below this pH fish are known to be unable to survive (Rosseland et al. 2000).

Among the factors indicating the mode of fish introductions, we estimated the walking effort from the nearest town (WALK; in minutes) which could be a good predictor of the likelihood of a lake having fish introduced. To estimate it, we chose a representative subset of 72 lakes from different areas, for which we quantified the walking time from the nearest town by ourselves climbing to each of these lakes. Then we regressed the walking time with two predictor variables: the altitudinal difference between the lake and the town (ALTDIFF; in meters) and the linear distance between them (DIST; in meters), both obtained from a GIS and 1:25,000 cartographic maps. Since climbing uphill takes more time than downhill, we used two regressions, one for uphill (UPWALK) and the other for downhill (DOWNWALK):

$$\text{UPWALK} = -4.279 + 0.157\ \text{ALTDIFF} + 0.008\ \text{DIST} \quad (2)$$

$$R^2 = 0.942;\ F_{2,72} = 563.6;\ p < 0.001$$

$$\text{DOWNWALK} = -5.732 + 0.078\ \text{ALTDIFF} + 0.013\ \text{DIST} \quad (3)$$

$$R^2 = 0.885;\ F_{2,69} = 258.9;\ p < 0.001$$

The total walking effort was then obtained by adding UPWALK with DOWNWALK. We also used the size of the nearest town (POP) and the number of hotel beds (HOT) at the time of the minnow introduction, both obtained from national twentieth century historical inventory surveys. We also recoded if there was helicopter stocking of trout (HEL) in the area nearby to the lake during a certain period (1970s–2000s) or if the lake had a forestry road giving car access to the lake (FROAD). The management practice variables considered were whether the location was within an area with active fish management (FIZ), whether lakes belonged to fishing prohibited areas of National Park of Aigüestortes i Estany de Sant Maurici (NATP) or if the lake had water fluctuations for hydroelectric production (HEP). Hydroelectric production (HEP) is not only relevant for the potential direct effect of changing water levels on minnow breeding, but also because HEP companies performed trout introductions in order to compensate local towns from the potential negative effects of the impounding. Finally we also used the presence of trout species (TROUT) as a categorical variable to explore potential interferences and relationships between minnow and trout species.

Statistical analyses

First of all we calculated the introduction rate of minnow and trout for the twentieth century by dividing the number of lakes where they have been introduced by the period in years that the introductions lasted. In the case of trout we only included the modern trout introductions that took place during the second half of the twentieth century to compare the rate with those of minnows. Then, we used generalized additive models (GAMs) as statistical analyses to compare the data on the presence/absence of minnows in the Pyrenean lakes with the different environmental variables described above. We used GAMs for the analyses because they are similar to generalized linear models, but distend the assumption that the relationships between the dependent variable (when transformed to a logit scale) and predictor variables are linear by estimating a nonparametric loess smooth function for each continuous predictor variable (Hastie and Tibshirani 1990; Knapp 2005). Prior to analyses with the GAMs, we tested for collinearity among the predictor variables by Pearson correlation coefficients (r) for all pair-wise combinations of continuous predictor variables. The strongest correlation coefficient was $|0.72|$, below the suggested cut-off of $|r| \geq 0.85$ that would indicate collinearity for the sample size used in these analyses (Berry and Felman 1985). Therefore, all predictor variables were included initially in the regression models. In the regression models, p_i is the probability of finding the species at location i, and is defined as:

$$p_i = \frac{e^{\theta_i}}{1 + e^{\theta_i}},$$

where the linear predictor (i.e., logit line) θi is a function of the independent variables. For minnow, the specific relationship we used for θ was the following function of covariates:

$$\begin{aligned}\theta = {} & \mathrm{lo(ALT)} + \mathrm{lo(SURF)} + \mathrm{lo(LOCATION)} \\ & + \mathrm{CONN} + \mathrm{lo(ADD)} + \mathrm{pH} + \mathrm{lo(WALK)} \\ & + \mathrm{lo(POP)} + \mathrm{lo(HOT)} + \mathrm{HEL} + \mathrm{FROAD} + \mathrm{FIZ} \\ & + \mathrm{NATP} + \mathrm{HEP} + \mathrm{SALM}\end{aligned} \tag{4}$$

where $\mathrm{lo}(\cdot)$ represents a nonparametric loess smoothing function that characterizes the effect of each continuous independent variable on p_{i}. The location covariate lo(LOCATION) was a smooth surface of UTM easting and northing (see Table 1 for variable abbreviations).

From Eq. (4) we selected a subset of significant variables explaining the greatest proportion of deviance. The best combination of independent variables was selected by stepwise forward selection using AIC criteria. The use of this procedure ensured that at each selection step only those variables explaining a significant proportion of previously non-explained variance would be selected. In other words, while altitude, temperature (ADD) and surface area share a significant amount of variance, their inclusion in the model would only take place if each variable would explain a fraction of variance not explained by the others. The proportion of variance explained by each variable was determined by evaluating the change in deviance resulting from dropping each variable from the model in the presence of all other variables. Analysis of deviance and likelihood ratio tests (based on the binomial distribution) were used to test the significance of the effect of each predictor variable on the probability of occurrence by minnow. Because the large sample sizes used in the regression models could result in predictor variables being statistically significant despite very weak associations with species presence/absence, predictor variables were considered to have significant effects only when $p \leq 0.01$.

The relationship between the significant predictor variables and the probability of minnow occurrence is shown graphically in separate plots separating the unshared fraction of variance that each factor explains (Fig. 3). Each plot depicts a response curve that describes the contribution of the predictor variable to the logit line. More generally, the response curve shows the relative influence of the predictor variable on the probability of minnow occurrence. This response curve is based on partial residuals, is plotted on a log-scale, and is standardized to have an average value of 0. For example, a hump-shaped response curve for the predictor variable "elevation" indicates that minnow was, in a relative sense, most likely to be detected at sites at low elevations and less likely to be detected at sites at high elevations (Knapp et al. 2003).

We used the estimated effect of previous trout presence in the binomial equation to approximate the change in the likelihood (i.e. odds ratio) of finding minnow in the presence versus absence of trout after having controlled for the effects of habitat and spatial variables (Hastie and Tibshirani 1990; Welsh et al. 2006).

All regression-related calculations were conducted using R statistical software (R Development Core Team 2013) with the function library *gam* (Hastie 2013). The analyses were run with the data up to the year 2000.

To analyse in more detail the factor variables, we compared the presence/absence of minnows with the categorical predictor variables by 2 × 2 contingency tables. To test if the morphometric characteristics of the lakes where minnows were introduced were different from the other lakes, we compared the values of the continuous predictor variables with one-way ANOVA and a Tukey post hoc test, for distinguishing among three groups: fishless lakes, lakes stocked with trout only and lakes stocked with trout and minnows. Data had previously been normalised.

Finally, in order to assess the effectiveness of banning fishing to prevent new introductions of minnows, we compared the changes in the number of lakes with minnows in Aigüestortes i Estany de Sant Maurici National Park during the twentieth century. Drawing line charts, we compared the number of lakes with new minnow introductions since the fishing ban (1988) in the 75 lakes where fishing was banned and the 116 lakes where fishing continued. We grouped the rest of the lakes, outside of the National Park, in a third group (with 329 lakes) and plotted the changes in each category during the twentieth century.

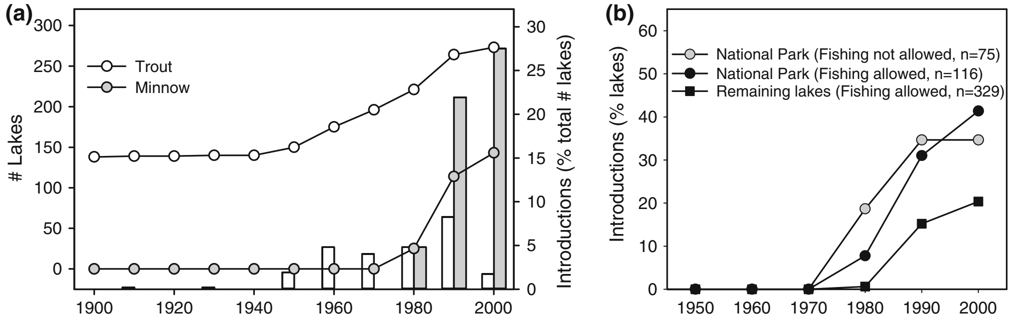


Fig. 2 **a** Introduction process of the minnow compared to trout in the southern Pyrenees during the twentieth century. *Vertical gray bars* are the decadal total number of lakes that were stocked with trout in percentage of the total number of lakes >0.5 ha of the study area (n = 520). **b** Effect of Aigüestortes i Estany de Sant Maurici National Park on the introduction of minnows in high mountain lakes of the Pyrenees. *Circles* are the lakes within the National Park, and *squares* are lakes outside the National Park. *Grey circles* are the area of the National Park where fishing is not allowed and *black circles* and *squares* are the lakes in fishing allowed areas

Results

We found minnows in 141 of 520 (27 %) of the surveyed high mountain lakes (Fig. 1). In 133 of these lakes there were also at least one trout species. The remaining 8 lakes had trout in the past which had gone extinct but still had minnows present. In addition, we found 132 lakes (25.4 %) with only trout (mainly *S. trutta*, but also in some lakes *Salvelinus fontinalis* and *Onchorynchus mykiss*) and 247 lakes (47.5 %) that were fishless. All the minnow introductions registered up to the year 2000 took place during the last three decades of the twentieth century (Fig. 2a). Before 1970 there was no Pyrenean high mountain lake with minnow. Since then, the number of minnow introductions has been much higher than those of trout. The introduction rate for minnow was 4.7 pa (141 lakes introduced between 1970 and 2000) and 2.2 pa for trout (133 lakes introduced between 1940 and 2000). The documented causes of introduction of minnows were transport to the lake in water containers by fishermen to be used as live bait. According to our interviews, minnows were then introduced to the lakes by throwing the remaining individuals into the lake in the belief that this species would be food for trout.

The forward selection procedure for the generalized additive model selected six of the fourteen predictor variables by order of importance which were significantly correlated with the probability of minnow occurrence: trout presence, surface area, location, altitude, helicopter stocking and forestry road (Table 2). The relationship between the probability of minnow occurrence (on a logit scale) and the important

Table 2 Results of generalized additive models developed for minnows (*Phoxinus* sp.) introduced in the Pyrenean lakes

Parameter	*Phoxinus* sp.	
Null deviance	608	
Degrees of freedom (null model)	519	
Model deviance[a]	288	
Degrees of freedom (full model)	502	
Explained deviance (% of total)	53	
Deviance increase[b]		
Trout presence	86.8	(27.2)***
Surface area	38.8	(12.1)***
Location	37.1	(11.6)***
Altitude	34.3	(10.8)***
Helicopter stocking	15.5	(4.8)***
Forestry road	2.9	(0.9)***

Only variables that were significant at the stepwise procedure are included in the table

[a] Sometimes referred to as "residual" deviance

[b] Deviance increase means the increase in deviance resulting from dropping the selected variable from the model. The percentage increase is given in parentheses, and was calculated as [deviance increase/(null deviance-model deviance)] × 100 (Knapp 2005)

Asterisks indicate the level of statistical significance associated with each variable: * $p \leq 0.01$ and $p > 0.001$; ** $p \leq 0.001$; $p > 0.0001$; *** $p \leq 0.0001$ and *NS* not significant ($p > 0.01$)

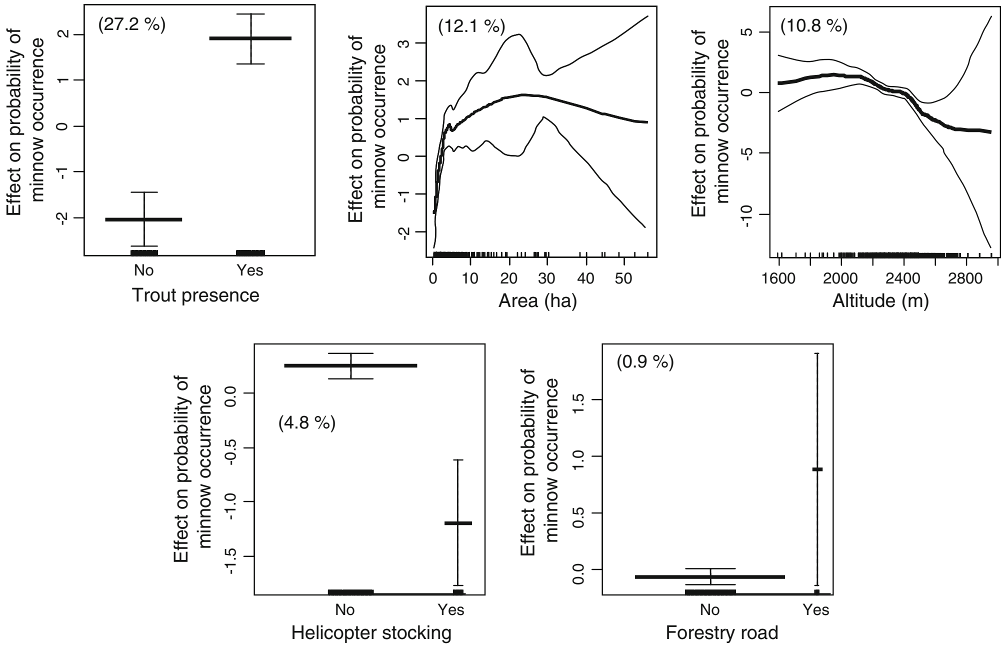


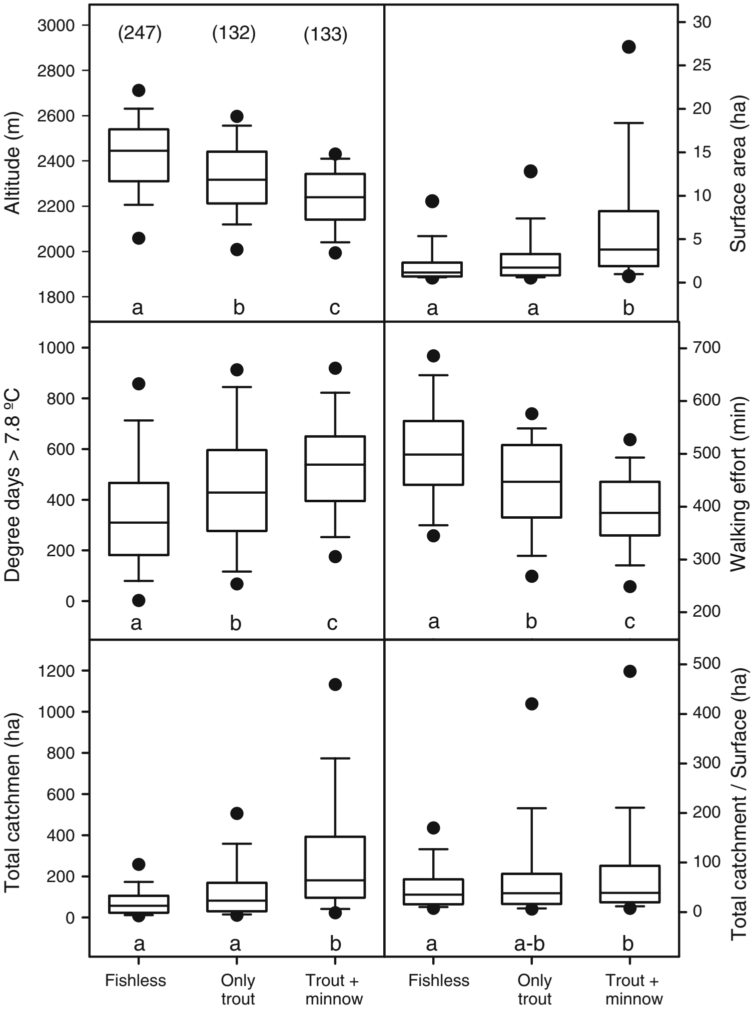
Fig. 3 Estimated effect of each of the highly significant ($p \leq 0.01$) predictor variables on the probability of occurrence by minnow, as determined from the generalized additive model (span = 0.5). Response curves are based on partial residuals and are standardized to have an average probability of zero. Thin *lines* are approximate 95 % confidence intervals and hatch marks at the bottom are a descriptor of the frequency of data points along the gradient in continuous variables or within each category for categorical variables. The width of *horizontal lines* in categorical variables is proportional to the frequency of the data within each category. *Numbers* in *parenthesis* are the percentage of explained deviance of each variable. See Table 2 for model details

continuously distributed predictor variables were all significantly nonlinear (Fig. 3). The presence of trout explained 27.2 % of deviance of minnow occurrence (higher probability when trout were present either now or previously in the lake), more than twice than the second most important variable. The response curve describing the estimated effect of lake surface area on the probability of minnow occurrence (p_i) indicated that p_i was low at the smallest lakes, but increased steadily until ca. 3 ha when it increased slowly until ca. 25 ha and then decreased gradually. Minnow presence was a constant function of altitude until 2,300 m, when it decreased progressively. Categorical variables representing different management practices (helicopter stocking and forestry road) contributed only a little part on the deviance explained by the model. The response surface for water body location is not provided in this figure as it was complex and offered no additional insights into the effects of the different predictor variables on species occurrence.

Minnow appears to be particularly linked to the previous trout presence in the lake. After controlling for the effects of habitat and spatial variables, minnows were 54 times more likely to be found in trout-containing water bodies than lakes without trout (odds ratio, with approximate 95 % confidence limits 17–169).

The lakes with introduced minnows had significantly lower altitude, greater surface area, higher accumulated temperature, shorter walking effort from the nearest town and greater catchment area compared to the lakes stocked only with trout. In contrast, fishless lakes had the highest altitudes and walking effort and lowest temperatures and catchment areas (Fig. 4). Lakes stocked only with trout had intermediate values for these variables.

Fig. 4 Box plots showing elevation, surface area, accumulated degree days above 7.8 °C, total catchment, the ratio of total catchment to lake surface area, and the walking effort from the nearest town to each lake, of fishless lakes and stocked lakes with trout and with trout + minnow. The *line* within each box marks the median, the bottom and *top* of each box indicate the 25th and 75th percentiles, the *whiskers* below and above each box indicate the 10th and 90th percentiles, and the points above and below the *whiskers* indicate the 5th and 95th percentiles. Sample sizes for each category are given between brackets in the first panel. Categories with *different letters* are significantly different at the $p < 0.05$ level (one way ANOVA, Tukey post hoc)



Minnows were significantly more likely to be found in the lakes with prior trout presence ($\chi^2 = 159.9$, $p < 0.0001$), within active fish management areas ($\chi^2 = 49.8, p < 0.0001$), in lakes with hydroelectrical power exploitation ($\chi^2 = 44.1$, $p < 0.0001$), or with nearby forestry roads ($\chi^2 = 44.8$, $p < 0.0001$). In contrast, the probability of occurrence in lakes within National Parks was not statistically different from the lakes outside them ($\chi^2 = 1.36, p = 0.244$), as well as in lakes with helicopter-based trout introductions compared to lakes where trout were introduced with other methods ($\chi^2 = 2.36, p = 0.124$).

In the previous statistical models, National Park does not appear as a significant variable in explaining minnow distribution. However, if we examine the accumulated percentage of lakes with minnow at the final part of the twentieth century (Fig. 2b), we can see that only in the non-fishing area, the number of lakes with minnows stopped increasing from the time of the prohibition, while in the other areas it continued to increase.

Discussion

Introduction mediated by invasive trout

The results of this study strongly suggest that the invasion of minnows in the high mountain lakes of the Pyrenees is mediated by a preceding invasive species and facilitated by human activity. We found that the introduction of minnow is a more recent and faster process than those of salmonids (Fig. 2a). Since 1970, when the first introduction took place, it has now spread to 27 % of the lakes of the southern Pyrenees with an introduction rate of 4.7 pa, compared to those of trout, at 2.2 pa for the period between 1940 and 2000. The results of GAM indicate that the presence of trout before minnow introduction is by far the most important variable explaining minnow's distribution. In fact, we have not found any case of minnow introduction in naturally fishless lakes, indicating that recreational fishing with live-bait is likely to be responsible for these introductions (ca. half of the lakes with salmonids; Miró and Ventura 2013). The higher probability of finding minnows at lower altitude lakes and closer to forestry roads also support this idea, since both are elements facilitating the access of fishermen to lakes.

In high mountain lakes, trout are the only species group authorised for introduction by governmental agencies worldwide (e.g. Sostoa and Lobón-Cerviá 1989; Wiley 2003), mainly associated with recreational fishing (Cambray 2003). Unlike trout, minnow introductions are in general not authorised by governmental authorities and their introduction is often an illegal angling practice. The end result in many cases is that fish unused as live-bait are released at the end of the fishing expedition (Maitland and Campbell 1992; Kerr et al. 2005; Winfield et al. 2011; Kilian et al. 2012). This has been quantified to be done by 36 % of the fishermen in Michigan and Wisconsin, 41 % in Ontario and 65 % in Maryland (Litvak and Mandrak 1993; Kerr et al. 2005; Kilian et al. 2012). To prevent the widespread release of non-native species used as live-bait, many US states and Canadian territories have restricted the use, sale, or transport of bait (Kerr et al. 2005; Peters and Lodge 2009). A similar situation exists on the southern slope of Pyrenees, where the release of any organism to the environment without government authorization is also strictly prohibited (Miró 2011). Nevertheless, similar to our findings from the southern valleys of the Pyrenees, in some regions of North America a large proportion of anglers appear to be unaware of, or choose to ignore, the current regulation prohibiting the release of live organisms because they believe their actions are compassionate and that the released unused bait is suitable food for angling fishes (Kerr et al. 2005; Kilian et al. 2012). Our results strongly suggest the need to intensify preventive actions by giving accurate information of the potential negative effects for the local environment of the release of non-native organisms to fisheries boards and local communities. These actions are one of the best guiding principles to prevent the spread of invasive species together with regulation and legislation (Simberloff et al. 2013).

Live-bait related introductions of minnows have occurred in lower, boreal and arctic lakes where trout are present, but they have not been previously described in high mountain lakes. The distribution of minnow expanded considerably throughout the 1900s in the north European lakes of Scotland and Norway, especially in mountain areas, due mainly to the use of minnows as live bait for angling (Maitland and Campbell 1992; Museth et al. 2007). When minnow is introduced in lakes with autochthonous trout, it reduces recruitment and annual growth rates of trout, causing a decrease of the trout abundance by 35 % on average; however, the effect on other native fauna takes place primarily in the shallow littoral areas (Museth et al. 2007).

In the particular case of extreme habitats such as high mountain lakes that are naturally fishless, such as those of the Pyrenees, the introduction of trout results in a substantial impact for native fauna, especially for the more conspicuous organisms such as amphibians and macroinvertebrates which may be extirpated (e.g. Knapp et al. 2001b). However, minnows, like other small cyprinids, have an omnivorous and opportunistic diet similar to trout (Oscoz et al. 2008; Museth et al. 2010). They predate on zooplankton, benthic macroinvertebrates (Vinebrooke et al. 2001; Naestad and Brittain 2010) and also feed on fish eggs and hatchlings (Kottelat 2007). Their small size allows them to access shallow areas which trout cannot reach, thus eliminating the possibility that other taxa such as amphibians can shelter in littoral areas of the lake. Consequently, the introduction of minnows as a second top predator, will likely result in a stronger negative effect on the native fauna as has been found in other aquatic ecosystems (Nyström et al. 2001). In fact, preliminary results obtained in Pyrenean lakes seem to confirm the negative effects of this small fish (A. Miró, Pers. Comm.).

Minnow invasive potential

Differences in minnow life history characteristics compared to those of trout give them a higher acclimation success and therefore an extraordinary invasive potential. The minnow, like other small widely-distributed freshwater fish, displays a remarkable variability in its life history depending on the site temperature. For example, minnows have maximum age ranges between 3 and 13 years reaching maturity between 1–2 and 5–7 years in hot and cold places respectively, while show significantly lower growth increments in cold summers (Mills 1988). This plasticity in their life history is what has allowed the species to easily adapt to high mountain lakes, showing higher resistance to harsh conditions than trout. In our dataset, we have not found any lake where minnows have disappeared once established. In contrast, in lakes with trout, between 10 and 44 % of the populations go extinct after 20–30 years due to a lack of favourable conditions (Knapp et al. 2005; Miró and Ventura 2013). Moreover, in Pyrenees, we have found that in eight lakes, preexisting trout populations have disappeared after minnow introductions. Some of these lakes had brown trout introduced centuries ago while others were stocked recently.

Also, minnow presence was more likely at lakes with lower elevations, greater surface areas and higher temperatures. This could be a result of the pattern of introductions (e.g. fishermen using live bait do not fish so often at the upper altitude lakes) or to acceptable habitat conditions for released minnows to become established. Taking into account the high adaptability of minnows, it seems more likely that our finding is a result of the pattern of introduction.

As well as minnows, other species might be used as live bait, increasing the number of invasive species to lakes. This is the case for gudgeon (*Gobio* sp.), which is now found in some lakes of the northern slope of the Pyrenees (Miró 2011). This fish has similar size and flexible life-history features to those of minnows (Tang et al. 2011). Thus the ecological effects of fish introductions in high mountain lakes can result in stronger unpredicted consequences.

Management practices

In this study we have found that the only area where minnow introductions have stopped increasing is at that area of the Aigüestortes i estany de Sant Maurici National Park where fishing has been prohibited since 1988 (Fig. 2b). Similar results have also been found for trout (Miró and Ventura 2013) and this indicates that to date the only management practice that is effective in stopping minnow introductions in high mountain lakes is to ban fishing. This is especially the case for the southern Pyrenean lakes, where the use of live-bait is prohibited, and therefore minnow introductions occur out of the control of governmental agencies. It is necessary to improve the regulation of the activity, and in the cases where maximum protection are required, authorities might even consider the prohibition of fishing.

Conclusions

Minnows have been introduced in 27 % of high mountain lakes of the Southern slope of the Pyrenees as a result of releases by fishermen using it as live-bait. This invasion took place in only the last three decades of the twentieth century. Although this fishing technique is forbidden in the Southern slope of the Pyrenees since 1966, this has not prevented their introduction in high mountain lakes and the spread of the species. In contrast, trout fishing prohibition since 1988 in the core area of Aigüestortes i Estany de Sant Maurici National Park has been an effective measure to stop minnow introductions. Our results show that there is almost no natural minnow spread beyond the lake of introduction. Therefore, if new introductions are prevented, spread may be contained to only those lakes where introductions already occurred. In order to prevent further minnow introductions we suggest, apart from to studying fishing prohibition in high protected areas, conducting intensive information campaigns to fishermen, local communities and administrative offices in the areas where trout fishing is allowed.

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