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Legacy effects of *Acacia* species on amphibian pond communities

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RESUMO ALARGADO

As invasões biológicas constituem uma das principais ameaças à biodiversidade a nível global, provocando impactes significativos a nível ecológico, económico e social. Os impactes de várias espécies de árvores invasoras, por exemplo *Acacia* spp., podem persistir no ecossistema mesmo após a sua remoção, através de mecanismos que possibilitam que estes legados permaneçam no solo. Podemos encontrar alguns destes mecanismos nas acácias, pertencentes à família das leguminosas, que têm a capacidade de fixar azoto no solo, o que lhes confere uma vantagem na colonização de solos pobres, e que produzem grandes quantidades de sementes que se acumulam no solo e mantêm viáveis durante vários anos; isto permite que os legados das suas invasões persistam a longo prazo. Têm ocorrido em todo o mundo ações de controlo e erradicação de espécies invasoras, sendo que também estas ações podem causar impactes. Neste sentido, após o corte, a lixiviação do azoto fixado em torno das raízes das acácias pode impactar negativamente as águas superficiais e as suas comunidades. Por outro lado, a abertura de clareiras resultante da remoção das acácias permite que uma maior quantidade de luz atinja o solo e os corpos de água, aumentando, de forma generalizada, a produtividade dos mesmos. Com o avançar da sucessão ecológica, o aumento da cobertura arbórea levará ao ensombramento dos corpos de água e, consequentemente, a uma diminuição dessa produtividade. No entanto, a alteração das espécies arbóreas existentes provocará também uma alteração da qualidade da manta morta, o que poderá resultar em comunidades diferentes no pré e no pós-intervenção.

O presente estudo tem como objetivo avaliar o impacte das ações de gestão florestal na comunidade de anfíbios da Serra de Sintra, nomeadamente em propriedades geridas pela Parques de Sintra – Monte da Lua, S. A.. Para tal, foi selecionado um conjunto de corpos de água distribuídos por locais com as seguintes características: áreas ainda invadidas com acácias, áreas com diferentes idades após remoção de acácias e áreas já com flora nativa mais desenvolvida. Nestes locais investigou-se se ocorreram alterações nas concentrações de compostos azotados nos corpos de água; se as ações de remoção de acácias incrementaram a produtividade do corpo de água; se a condição corporal das larvas de anfíbios melhorou em áreas abertas comparativamente a áreas florestadas, identificando também os fatores que mais influenciam a condição corporal.

Os resultados mostraram que após a ação de remoção de acácias houve um aumento acentuado na concentração de compostos azotados nos corpos de água, com tendência a diminuir ao longo do tempo. A produtividade dos corpos de água, em geral, aumentou após as intervenções; um aumento comprovado pela maior abundância de zooplâncton e de pequenos macroinvertebrados. No entanto, com o desenvolvimento da flora arbórea nativa, esta produtividade tende a diminuir. A comunidade de anfíbios apresentou maior riqueza específica após a remoção de *Acacia* spp., com uma ligeira diminuição à medida que a floresta nativa se desenvolve. Focando especificamente na espécie mais comum, a salamandra-de-pintas-amarelas (*Salamandra salamandra*), foi possível verificar alterações na condição corporal da suas formas larvares após as intervenções, sendo que 1) o tipo de corpo de água e a sua largura e 2) a profundidade máxima e cobertura herbácea em redor do corpo de água são as variáveis que melhor explicam essa variação relativamente à robustez da larva e proporção da musculatura da cauda, respetivamente.

No geral, com a exceção de um charco, não foram atingidas concentrações de compostos azotados potencialmente perigosas para a comunidade de anfíbios após a remoção das árvores invasoras. O aumento da produtividade dos corpos de água após as intervenções pode ser consequência do aumento de luz solar que chega ao solo e à água, ou, por outro lado, ser consequência indireta do aumento de compostos azotados, ou até de uma interação entre estes. O aumento da robustez das larvas de salamandra em corpos de água artificiais quando comparado com corpos de água naturais e com o aumento da largura do corpo de água poderá estar relacionado com o hidroperíodo do mesmo, uma vez

que em charcos temporários será mais importante a aceleração da metamorfose. A diminuição da proporção da musculatura da cauda em charcos mais profundos poderá ser uma estratégia de sobrevivência à predação, pois membranas maiores aumentam as probabilidades de sobrevivência a um ataque. Já a relação entre a cobertura herbácea e a proporção da musculatura da cauda não é linear, diminuindo até aos 40% de cobertura, o que pode ser resultado de condições ambientais favoráveis até para larvas menos musculadas, ou ser uma resposta à predação. A partir dos 40% de cobertura herbácea, e até aos 80%, essa proporção tende a aumentar, o que pode ser resultado de uma diminuição na produtividade do corpo de água como consequência do aumento da sombra, favorecendo larvas mais musculadas, com maior velocidade natatória, capazes de evitar o canibalismo, caçar mais presas ou até de canibalizar larvas mais pequenas.

A remoção de *Acacia* spp. e o restauro ecológico dos locais previamente invadidos são importantes para a conservação das comunidades de anfíbios (como os mais raros rã-de-focinho-pontiagudo (*Discoglossus galganoi*) ou salamandra-de-costelas-salientes (*Pleurodeles waltl*)). De futuro, a manutenção de clareiras em redor de alguns destes corpos de água contribuirá para uma maior diversidade das comunidades dos charcos e para o aumento das populações de espécies de anfíbios ameaçadas nas propriedades da PSML e na serra de Sintra. Estas intervenções poderão mesmo beneficiar espécies de anuros como a rela-mediterrânica (*Hyla meridionalis*) e o raro, e talvez localmente extinto, sapo-parteiro-comum (*Alytes obstetricans*). Um projeto de monitorização, no seguimento do que já vem a ser feito pela PSML com o lagarto-de-água (*Lacerta schreiberi*), será importante para avaliar a expansão destas duas espécies nas zonas sem acácia. Para além da comunidade de anfíbios, também a comunidade de répteis, incluindo *L. schreiberi*, poderá beneficiar da manutenção de algumas destas clareiras.

Palavras-chave: Gestão florestal, invasões biológicas, comunidade de anfíbios, compostos azotados, condição corporal larvar

ABSTRACT

Biological invasions are amongst the main threats to biodiversity. Alien invasive tree species, such as *Acacia* spp. often cause impacts on the invaded ecosystem that persist after their removal. *Acacia* spp. are able to fix nitrogen and are prolific producers of seeds that remain viable in the soil for several years; these mechanisms guarantee the persistence of their legacies in the long term. The removal of *Acacia* spp. may lead to a release of nitrogenous compounds, present in their roots, to ground and surface waters, affecting pond and stream communities. On the other hand, the removal of trees results in clearings, which allows more light to reach the forest ground and water bodies, increasing their productivity. These conditions will change over time as succession takes place and, eventually, the shade over the water bodies will again decrease their productivity. However, if the eradication is successful the leaf litter and root systems of the new trees will be different, and therefore the soil and pond communities before and after the interventions may differ.

The main goal of this study is to assess the influence of forest management actions (removal of *Acacia* spp.) on the amphibian community of the Serra de Sintra areas managed by Parques de Sintra – Monte da Lua, S. A.. We selected ponds located in plots still occupied by invasive *Acacia* spp., in plots of different ages after *Acacia* spp. removal, and in plots with native flora, and investigated: if there are differences in the nitrogenous compounds concentrations among ponds; if plot clearing promotes an increase in pond productivity; if amphibian larvae improve their body condition in cleared areas when compared with the forested plots.

The results showed that after the removal of *Acacia* spp. there is a notorious increase in the nitrogenous compounds concentration, and then it tends to decrease over time. The increase in zooplankton and small macroinvertebrates abundance indicated that the water bodies productivity increased after the interventions. However, with the development of native trees, this productivity tended to decrease. We found that the amphibian community presented a higher species richness after the removal of the invasive trees, decreasing afterwards as the native forest develops. The body conditions of the larvae of the most common amphibian, *Salamandra salamandra*, change after the interventions. The type of water body and its width were the factors that better explained the variation of the ratio between maximum abdominal width and snout-vent length (A/SVL) and the maximum pond depth and bank cover by short vegetation were the factors that better explained the variation of the ratio between middle tail muscle depth and middle tail fin depth (M/F).

In general, the nitrogenous compounds concentration did not rise to potentially harmful levels for the amphibian community, except for one of the sampled ponds. The increase in water bodies productivity after the interventions may be a result of the increase of sunlight reaching the water bodies, an indirect result of the increase of nitrogenous compounds concentration, or it may result from the interaction between both factors. The increase of larval salamanders A/SVL ratio in artificial pools when compared to natural ponds and with the increase of the water body's width may be related to hydroperiod, since in natural ponds with shorter hydroperiods larvae need to invest earlier in metamorphosis. The decrease of M/F ratio in deeper ponds may be a strategy to predation. This ratio decreases in response to a low bank cover by short vegetation, which may be due to favourable environmental conditions to less muscled larvae or as a response to predation. As vegetation cover increases, this ratio also increases, which may be a result of the decrease in productivity by increased shading; in this less productive environment, muscled larvae, which can swim faster, better avoid cannibalism, catch more preys or cannibalise their conspecifics, are more likely to survive.

The removal of alien invasive plants and restoration of the invaded sites are extremely important to preserve native amphibians. Maintaining forest clearings around at least some of the ponds will

contribute to a higher diversity of pond communities and to increase the populations of threatened amphibians.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

FIGURES

TABLES

LIST OF ABBREVIATIONS

A/SVL – Maximum abdominal width / Snout-vent length

ANOVA – One-way analysis of variance

GAMM – Generalised additive mixed effects models

M/F – Middle tail muscle depth / Middle tail fin depth

N – Nitrogen

NO_x^- – Nitrogenous anions (in this case, referring to nitrates and nitrites)

Nx – Nitrogenous compounds

PSML – Parques de Sintra – Monte da Lua, S. A.

SVL – Snout-vent length

INTRODUCTION

It is frequently assumed that the ecological impacts of invasive species are strongly reduced or are eliminated immediately after their control or removal (Wittenberg and Cock 2005). That is not always the case and some invasive species can alter the environment in such a way that their effects remain for long periods of time; those are called "the legacy effects" (Ehrenfeld and Scott 2001; D'Antonio and Meyerson 2002). Those effects are also identified as an important factor for the success of invasions (Davis and Pelsor 2001; Blumenthal 2005), and can even prevent the recolonisation by native species after the invader's removal (Vitousek et al. 1987). There are several mechanisms that can lead to the persistence of these legacies in the soil. These can result from changes in the seed banks (D'Antonio and Meyerson 2002), in soil biota (Eppinga et al. 2006; Pringle et al. 2009) or in nutrient availability, with the invasive species consuming the available resources or, on the other hand, increasing nutrient availability through nitrogen fixation or increased decomposition rates (Allison and Vitousek 2004; Rothstein et al. 2004).

Invasive alien plants can alter ecosystem dynamics, posing a threat to the local biodiversity (Richardson et al. 1995). These species may change geomorphological processes (Vitousek et al. 1987), fire regime, both in intensity and frequency (D'Antonio 2000), or even water availability (Le Maitre et al. 2002). Often the diverse original communities are replaced by monospecific communities of the invasive species. A typical example is that of *Acacia* species that have dispersed over large areas of native fynbos in South Africa, resulting in stands with a small number of species and leading to a decline in biodiversity (Richardson et al. 1995). This type of substitutions can lead to the extinction of plant species and can also have impacts on native fauna (Cronk and Fuller 2001). There are also plants that alter the chemical properties of the soil; this is the case of nitrogen-fixing plants, which correspond to a large proportion of invasive alien plants worldwide (Cronk and Fuller 2001). A higher availability of nitrogen in the soil increases plant production in several ecosystems (Vitousek and Howarth 1991) and can even promote a change in their plant species composition (Bobbink, 1991; Wedin and Tilman, 1996), including the spread of other invasive species (e.g. Huenneke et al. 1990; Vinton and Burke 1995; Maron and Connors 1996). Moreover, community composition is known to be sensitive to relatively small changes in nitrogen circulation rates (e.g. Aerts and Berendse 1988; McLendon and Redente 1991; Pascke et al. 2000). For instances, *Morella faya* (Aiton) Wilbur is a nitrogen-fixing species, invasive in nutrient-poor volcanic soils in Hawaii. This invasive plant completely changed nitrogen availability in invaded areas, where the nitrogen concentration quadrupled and appears to have favoured the spread of other exotic species (Vitousek et al. 1987). In another example, *Mesembryanthemum crystallinum* L., an annual species, alters the soil properties in a different way, since it releases large quantities of salt after its death. This leads to the salinisation of the invaded areas, limiting the colonisation by native species, even after its removal (Vivrette and Muller 1977).

The genus *Acacia* belongs to the *Leguminosae* family, one of the families with a higher number of invasive species (Cronk and Fuller 2001), having the ability to fix nitrogen, an advantage when invading nutrient-poor environments (Vitousek and White 1981). The traits of most *Acacia* species, such as their rapid growth, the capacity of growth by vegetative reproduction, prolific seed production, which remain viable for several years, and efficient dispersal strategies, give them a competitive advantage over other species. In many cases they are stimulated by fire, either by resprouting and/or by seed germination. A well-studied example of the impact of acacias occurs on fynbos, a Mediterranean-type ecosystem in South Africa, where they became dominant in the invaded area (Stock et al. 1995). In this ecosystem the nitrogen levels in soil and groundwater are higher in the invaded areas than in areas with native vegetation (Jovanovic et al. 2009). In Portugal, research on the invasion of *Acacia longifolia* (Andrews) Willd in coastal dune ecosystems evidenced a decrease in plant diversity (Marchante et al. 2003) and

changes in available nitrogen and in soil enzymatic activity (Marchante 2001; Marchante et al. 2004). Besides fixing nitrogen, *Acacia* species also depict a high concentration of phenolic compounds (Tindale and Roux 1974), among which tannins, reactive phenols that tend to accumulate in the leaves (Rier et al. 2005). Considering that *Acacia* species have high litterfall production, rich in nitrogen, leaf decomposition will contribute to soil enrichment in nitrogen and other nutrients (Marchante 2001). Moreover, nitrogen entering the soil can be leached and contaminate groundwaters (Jovanovic et al. 2009).

Several campaigns for the eradication of invasive alien species have been taking place worldwide; however, the legacies of those invasions may persist for many years. Jovanovic et al. (2009) concluded that the removal of *Acacia* spp. may lead to a fast release of nitrates to groundwater, due to the high content of nitrogenous compounds present in their roots, also leading to a decrease in evapotranspiration. Nevertheless, the long-term effect of the removal of these invaders is a decrease in the concentration of nitrogenous compounds in groundwater (Jovanovic et al. 2009). Marchante et al. (2009) showed that, after the removal of *Acacia longifolia*, the nitrogen concentration remained high for at least four and a half years, persisting in the soil as a long-term legacy.

Nitrogen impacts on amphibians

Nitrogen can enter water bodies by fixation of atmospheric nitrogen, surface leaching, rainfall or through groundwater. A large amount of the nitrogen existing in natural ponds is organic; however, it also occurs in its inorganic forms, as molecular N_2, nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+). The processes of transforming ammonium into nitrate (nitrification) and nitrate into molecular nitrogen (denitrification) are regulated mostly by bacteria and fungi. Nitrite is present in freshwaters in small concentrations (Eddy and Williams 1987) and can be a product of both nitrification, which may then oxidise into nitrate, and denitrification, which may then reduce to molecular nitrogen (Hutchinson 1957).

Most ponds are so shallow that there is little variation in dissolved oxygen and nutrients along the water column. These systems may be oligotrophic, presenting a small concentration of ammonium that varies little with depth, since there is little variation on oxygen (Brönmark and Hansson 2005). An increase in the concentration of nitrogenous compounds will affect pond communities (Temmink et al. 1989; Rouse et al. 1999). For instance, most amphibians depend on aquatic habitats during the early stages of their lives and they are therefore vulnerable to toxic compounds and materials in water (Cooke 1981; Baker and Waigths 1993). Nitrogen and nitrogenous compounds negatively affect not only adults but also amphibian larvae (e.g. Baker and Waights 1993; Baker and Waights 1994; Egea-Serrano et al. 2012). Marco and Blaustein (1999) concluded that tadpoles exposed to low nitrite concentrations metamorphosed slower than control tadpoles, and Egea-Serrano et al. (2012), using meta-analyses, demonstrated that nitrogenous compounds significantly decreased amphibian body mass and survival rate, both of embryos and larvae, and also caused an increase in malformation frequency. The interaction between nitrogenous compounds and other environmental factors can have more severe impacts on embryos and larvae (Hatch and Blaustein 2000; Boone et al. 2005; Macías et al. 2007), but the effects will vary according to the species (Marco et al. 1999).

On the other hand, de Wijer et al. (2003) found no detrimental effects of nitrates on tadpoles; in fact, they found an increase in mass at metamorphosis and survival, and a decrease in time to metamorphosis, which they explained to be indirect results of increases of periphyton and phytoplankton.

Other effects of tree removal

Removing trees, regardless of the species, has other profound effects on the dynamics of forest ponds and on pond fauna, such as amphibians. The removal of trees results in clearings, which may have similar conditions to gaps resulting from canopy trees' collapse. When a gap is created environmental conditions change within it: higher light intensity and duration, higher temperatures and lower amount of leaf litter, among others.

These conditions will change over time as succession takes place. Firstly, pioneer species develop to create a dense herbaceous stratum with a small number of woody species, followed by the development of a well differentiated second stratum, with a larger number of shrubs. These two stages of the succession allow light to reach water bodies. In shallow ponds, an increase in light availability facilitates photosynthesis, allowing the development of periphyton and algae, followed by phytoplankton and aquatic plants (Brönmark and Hansson 2005). Winder et al. (2012) found that an increase in light supply led to an increase in phytoplankton and densities of primary consumers such as *Daphnia*, and concluded that plankton succession in lentic systems was greatly affected by light limitation. Hocking and Semlitsch (2008) found a strong positive relationship between the amount of periphyton and anuran tadpoles' performance.

As succession continues, a new stratum of trees develops, increasing shade, decreasing temperature and increasing leaf litter input. The increase of canopy cover reportedly has a negative impact on pond fauna, as it decreases amphibian abundance, species richness, survival, growth and development (Werner and Glennemeier 1999; Skelly et al. 2002; Halverson et al. 2003; Skelly et al. 2005; Schiesari 2006; Binckley and Resetarits 2007; Thurgate and Pechmann 2007; Werner et al. 2007; Williams et al. 2008). This trend is also verified on zooplankton and macroinvertebrates, with a decrease in colonisation probability, richness, abundance, biomass and density (Nilsson and Svensson 1995; Palik et al. 2001; Batzer et al. 2004; Binckley and Resetarits 2007). However, anurans and caudates are not affected in the same way since they have different traits. In fact, since the main prey of caudates appear not to be negatively affected by a denser canopy cover (McWilliams and Bachmann 1989; Regester et al. 2008) and may benefit from an increase of input of leaf litter (Batzer and Palik 2007), caudates may not be affected by canopy cover or even thrive in closed canopy areas.

Serra de Sintra is the main forested area of the Lisbon region, however most of the current tree cover is composed of exotic species. Vegetation consists mainly of evergreen forests dominated by invasive *Acacia dealbata* Link, *Acacia melanoxylon* R. Br., *Acacia longifolia* and *Pittosporum undulatum* Vent. According to Marchante (2001), the first specimens of *A. melanoxylon* were planted in Serra de Sintra in 1880, and these were responsible for the first invasion outbreaks. A cyclone in 1941 opened several clearings, which were quickly occupied by *A. melanoxylon*, and a wildfire in 1966 further increased its spread (Ferreira and Reis 1999). It is also reported that the same wildfire promoted the invasion by *A. dealbata* (Costa 1981). Currently, the two species, along with *A. longifolia*, are widely spread across Serra de Sintra. In order to revert this situation, Parques de Sintra – Monte da Lua, S. A., has been promoting forest management actions that include the removal of these invasive trees in its properties.

Objectives

The main goal of this study is to assess the influence of forest management actions on the amphibian community of the Serra de Sintra areas managed by Parques de Sintra – Monte da Lua, S. A..

We selected ponds located in plots still occupied by invasive *Acacia* spp., in plots of different ages after clearing of *Acacia* spp. trees, and in plots with native flora, and investigated if: 1) there are differences in the nitrogenous compounds concentrations among ponds; 2) plot clearing promotes an increase in

pond productivity; 3) amphibian larvae improve their body condition in cleared areas when compared with forested plots, regardless of the tree species.

We expect that ponds in *Acacia*-invaded plots will present a higher water concentration of nitrogen and nitrogenous compounds than ponds in managed plots or with native forest; we also expect that, after clearing, the concentrations of these compounds will increase due to an increase in the decomposition rate of the remaining leaf litter. Given the increase of light intensity after the plot clearing, general pond productivity shall increase until native forest develops. Finally, an improvement in body conditions of the larvae is also expected due to the increase in food resources, but only several years after *Acacia* spp. removal, due to their potential legacy effects.

METHODS

Study area

This study was conducted in several properties of Parques de Sintra – Monte da Lua, S. A. (PSML), covering around 911,4 ha (Fig. 2.1). Located in Serra de Sintra, this area is integrated into Sintra–Cascais Natural Park and the Natura 2000 Network (PTCON0008). Altitude ranges from 120 to 528 m a.s.l.. The climate is mesomediterranean (Alcoforado et al. 2009), with an average annual temperature of 15.3ºC and with an accumulated annual precipitation of ~726 mm (data from Colares meteorological station; SNIRH 2019). Vegetation is dominated by the abovementioned invasive exotic and there are also small patches of mixed forests of native trees and conifers (mainly *Pinus pinaster*).
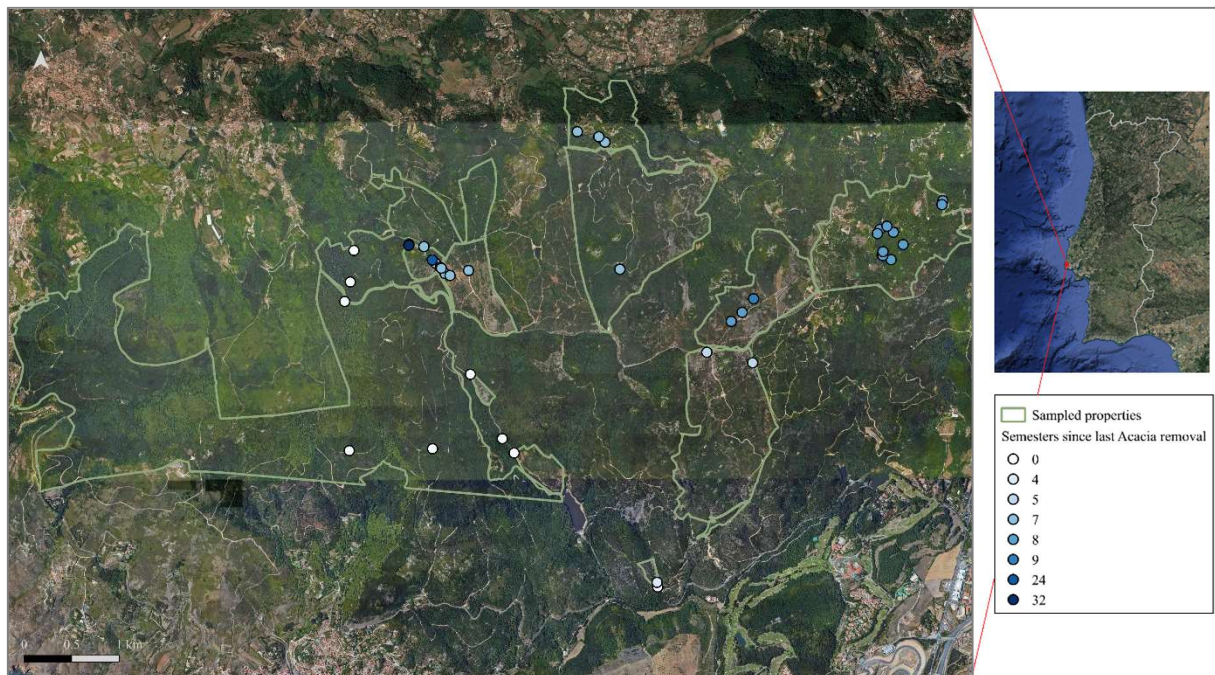
Figure 2.1 – Study area. Green lines represent the sampled properties. Circles represent sampling sites and their different colours represent the number of semesters since last *Acacia* spp. removal.

Since 2001, PSML has been promoting forest management actions that include the removal of all invasive trees on its properties, through a combination of mechanical and chemical control (PMSL 2019). The removal process starts with the smaller trees being sprayed with herbicide, after which the tallest trees (the ones that contribute the most with leaf litter) are the last ones to be removed. For this last step the cut stump method is used: the plants are cut down and the stump is immediately painted

4

with herbicide. When all the plants are removed, it is necessary to control the germination of the seeds that remain in the soil bank, by hand pulling or, if at a later stage, the spraying method.

Experimental design

PSML has conducted asynchronous forest management actions in different properties and in different plots inside a property. The records of these interventions were organised by plot. To attribute an age after *Acacia* spp. removal, we considered the number of semesters since the last *Acacia* spp. removal because that was the moment when the tallest trees were removed. By considering semesters (and not months or years), we tried to balance sample size and number of age categories.

The sampling sites (ponds, stream pools and artificial pools) were chosen based on a previous study conducted in the same area (Aguilar and Rebelo, 2017). In that study, Aguilar and Rebelo (2017) mapped thoroughly the existing water bodies in all properties, providing a precise location of the potential sampling sites. Ponds and stream pools were chosen according to the number of semesters since the last *Acacia* spp. removal in the drainage basin of each water body, and all existing ages were considered. The distance between sampling sites was also considered, to avoid spatial autocorrelation. Only ponds/stream pools with no fishes and where the bottom could be reached with a dipnet were sampled.

The sampling period was divided into two rounds: the first during the winter, from late-February to mid-March 2019 (1st round) and the second during spring, May 2019 (2nd round).

Environmental characterisation

The 32 variables summarised in Table 2.1 were registered to characterise all ponds, stream pools and their surroundings. The variables were divided into three categories: i) pond characteristics, which include abiotic variables, leaf litter and aquatic vegetation; ii) characteristics of the vegetation of the surrounding area within 5m; and iii) semesters since the last intervention. Some variables were chosen based on previous studies (Egan and Paton 2004; Couto et al. 2017) and others considering their potential impact on the amphibian community of the area.

Table 2.1 – List of variables used for the environmental characterisation.

Variables	Description	Range (unit)
Pond characteristics		
Type		1: Natural 2: Artificial
Length	Maximum length, measured with a 20-meter measuring tape	0.5-10 (m)
Width	Maximum width, measured with a 20-meter measuring tape	0.5-7 (m)
Maximum depth	Maximum depth, measured with wooden stick and a ruler	0.02-1.1 (m)
Turbidity	Ratio of the Secchi depth and maximum pond depth	0.3-1
Flow		0: Absent 1: Present
Hydroperiod		1: Permanent 2: Temporary
Edge slope	Mode of the slope of 4 margins	1: <20 (º) 2: 21º-60 (º) 3: >61 (º)
Leaf litter	Average of four 50 cm transects, perpendicular to the margins of the pond/pool, assessing the presence/absence of leaf litter every 10 cm	0-100 (%)
Shadow	Photo taken from the center of the pond/pool towards the canopy. The percentage of area occupied by the canopy was calculated using *ImageJ*	0-100 (%)
<u>Bottom substratum</u>		
Rock		0-100 (%)
Boulder		0 (%)
Cobble	Average of four 50 cm transects, perpendicular to the margins of the pond/pool, assessing the grain size every 10 cm	0-5 (%)
Gravel		0-100 (%)
Sand		0-100 (%)
Silt and Clay		0-100 (%)

(cont.)

Variables	Description	Range (unit)
Pond characteristics (*cont.*)		
<u>Aquatic vegetation</u>		
Floating	Average of four 50 cm transects, perpendicular to the margins of the pond/pool, assessing the vegetation type every 10 cm	0-100 (%)
Submerged		0-100 (%)
Emergent		0-50 (%)
Surrounding environment (within 5m)		
<u>Vegetation type</u>		
Arboreal	Average of four 5 m transect, perpendicular to the margins of the pond/pool, assessing the vegetation type, excluding *Acacia* spp., every 1 m	0-60 (%)
Shrubs		0-30 (%)
Herbaceous		0-100 (%)
Bare ground		0-90 (%)
<u>*Acacia*</u>		
Acacia < 10cm	Average of four 5 m transects, perpendicular to the margins of the pond/pool, assessing *Acacia* spp. height every 1 m	0-15 (%)
10 < *Acacia* < 50cm		0-40 (%)
50 < *Acacia* < 200cm		0-35 (%)
Acacia > 200cm		0-15 (%)
Arboreal + *Acacia* > 200cm	Average of four 5 m transects, perpendicular to the margins of the pond/pool, assessing the type of vegetation, including *Acacia* spp., every 1 m	0-60 (%)
Shrubs + 10 < *Acacia* < 200cm		0-80 (%)
Herbaceous + *Acacia* < 10		0-100 (%)
Acacia	Average of 4 transects with 5 m length, perpendicular to the margins of the pond/pool, assessing the presence of *Acacia* spp. every 1 m	0-70 (%)
Age since *Acacia* removal	Calculated in semesters until February 2019	0-32 (semesters)

 We opted to measure length and width instead of the surface area of the pond because the different shapes of the ponds did not allow a straightforward estimation of their areas. Moreover, most of the ponds are not visible from satellite images, making it impossible to estimate the surface area using those images.

Water temperature also plays an important role on amphibian reproduction and development (Álvarez and Nicieza 2002), however the measurement of this variable during the sampling period of each pond/pool would only reflect that specific moment and would not be representative of the fluctuations to which the larvae are exposed. Due to a lack of data loggers, this was not attempted.

Water and soil

Water samples were collected from 11 ponds using a sterile tube sampler (500 mL), which was submerged until full, minimising the input of organic matter. Water samples were analysed for Kjeldahl N (by oxidative pyrolysis), NO_3^- (by ionic chromatography), NO_2^- (by molecular absorption spectrometry - NED), NH_4^+ (by molecular absorption spectrometry - indophenol) and total inorganic nitrogen; the analyses were performed at LAIST - Analysis Laboratory at Instituto Superior Técnico.

Soil samples were collected from the same 11 ponds using a core (*ca.* 250 mL) and sieved through 4 and 2 mm sieves to remove large debris. Then, the content retained on a 63 μm sieve was collected to a tube until it reached *ca.* 20 g of soil. Soils were analysed for Kjeldahl N (by a combination of digestion, distillation and oxidative pyrolysis), also at LAIST.

Zooplankton and small macroinvertebrates

The zooplankton and small macroinvertebrates present in the water column of 10 ponds were sampled by collecting water from the ponds, at middle depth, using a 400 mL tube sampler. To sample these communities in the leaf litter a core of the same volume was used to collect the substrate of each pond.

The water and soil samples were filtered over 4 and 2 mm sieves to remove large debris and then through a 63 μm sieve. All samples (N=20) were transported in plastic tubes and preserved in alcohol until identification in the laboratory.

For each sample, either of water or soil, 5 subsamples of 1 mL were taken using a 1mL pipette. A 1x1cm grid was drawn in Petri dishes and 9 regularly spaced grid cells were chosen for counting. All individuals present in the grid cells were counted and identified using a stereo microscope. The organisms were identified to the class or order, when possible, using Tachet et al. (2006).

Amphibians

For this group, a total of 40 water bodies was sampled between February and May 2019, 19 of which were sampled twice. The surveys were conducted during daytime, by active search using dipnets. All observations of amphibians (adults and larvae) were recorded, however, for this study we considered only the species whose larvae were present at the sampling sites (see Appendix E).

The larvae were identified and photographed in top and side views, against a millimetric grid background. After that, all individuals were released at the capture site.

The identification of the developmental stage followed Gosner (1960) for anurans, and Miaud (1990) for caudates.

Metrics such as larval growth and development rates can be used to evaluate the condition of amphibian populations. Since the former can vary with resource availability (Travis 1984) and the latter can be adjusted as a response to environmental conditions (Wilbur and Collins 1973), these allow inferring the conditions under which larvae are living (Wilbur and Collins 1973). For these reasons, six morphological traits of fire salamander (*Salamandra salamandra* L.) larvae (total length, snout-vent length (SVL), tail length, maximum abdominal width, middle tail muscle depth and middle tail fin depth) (Fig. 2.2) were measured to the least 0.001 mm, using *ImageJ* software (v. 1.52k). Additionally, larval salamanders are prone to cannibalism (Warburg et al. 1979) and their ability to predate conspecific is related to gape size (Warburg 1992) therefore we also measured the maximum head width of these larvae. The ratios maximum abdominal width/SVL (A/SVL) and middle tail muscle depth/middle tail fin depth (M/F) were used as indicators of body condition. A high M/F ratio may also be an indication that the larvae are reaching the time for metamorphosis. Nevertheless, while sampling and analysing the photos, only a small number of individuals showed gill reduction, an external sign of metamorphosis.
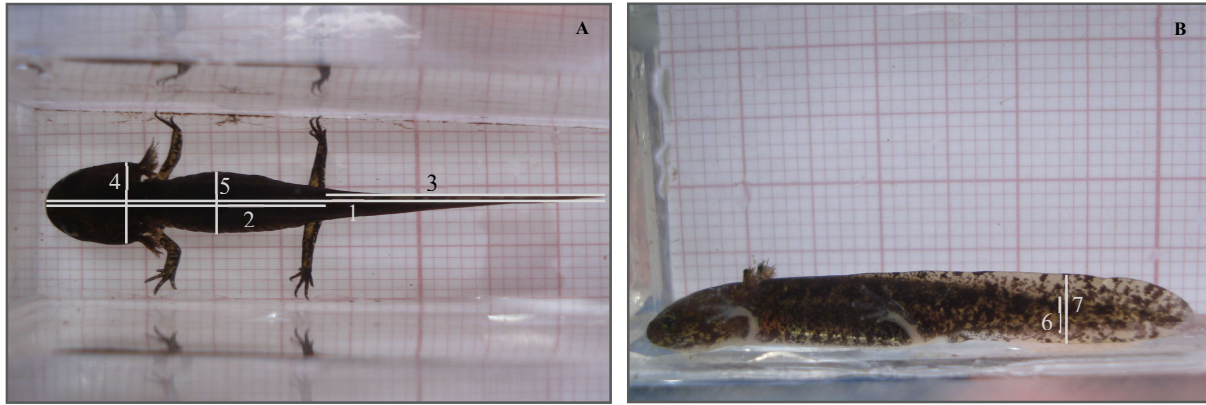
Figure 2.2 – Morphological traits measured on *S. salamandra* larvae. A) 1 – Total length; 2 – Snout-vent length (SVL); 3 – Tail length; 4 – Maximum head width; 5 – Maximum abdominal width; B) 6 – Middle tail muscle depth; 7 – Middle tail fin depth.

The kurtoses of the distributions of these ratios within each pond were calculated in order to assess the general condition of the pond's population. Populations with high kurtosis values indicate that most individuals are similar, which leads to a low trait diversity (Enquist et al. 2015). Populations with low kurtosis values indicates that there is an even distribution of trait values (Le Bagousse-Pinguet et al. 2017), which means there is a higher trait diversity (Enquist et al. 2015).

These analyses were only conducted for the most common species and not for other species due to the small sample size.

Data analysis

Before running any other analyses, we verified if there was any natural grouping of ponds/pools by performing a PCA with all variables. For this we used *ggbiplot* R package (Vu 2011), which standardises the values of the different variables.

To assess the changes through the semesters since the last *Acacia* spp. removal, several graphical analyses were conducted on the distribution of *Acacia* size classes, on the results of the chemical analyses, and on the zooplankton and macroinvertebrates abundance estimates.

Due to the small sample size of all species but fire salamander (Appendix B), a simple graphic depiction of the presence and absence of the species along the gradient of ages was built. To build this graph we complemented our data with data from Aguilar and Rebelo (2017).

To compare *S. salamandra* SVL and body ratios among plots, one-way ANOVAs followed by post-hoc Tukey tests were performed.

 We used Pearson correlation tests to verify if the maximum abdominal width and the middle tail muscle depth were correlated with the maximum head width.

For each round we tested spatial autocorrelation using each pond average values of the *S. salamandra* body ratios, the kurtosis of those ratios and SVL as dependent variables. We considered the Euclidian distance between each pair of ponds/stream pools. Spatial autocorrelation was tested with the Moran's I statistic. In this test, the null hypothesis is that the tested points are randomly distributed (Moran, 1948). For this analysis we used ESRI ArcGIS® software (v. 10.4.1) (ERSI 2016) spatial autocorrelation tool.

After building histograms of the salamanders' body conditions *per* sampling point for each round and for the combination of the two rounds, the kurtosis of each curve was calculated using *moments* R package (Komsta and Novomestky 2015). A Pearson correlation was used to verify if the kurtoses were correlated with the abundance of zooplankton and small macroinvertebrates. For this we used kurtosis

values of the 2nd round and three estimates of zooplankton and small macroinvertebrate abundance: total zooplankton and small macroinvertebrates abundance (water column, leaf litter and the sum of both), Ostracoda and Cladocera — primary consumers — abundance (water column, leaf litter and the sum of both) and Copepoda — secondary consumers — abundance (water column, leaf litter and the sum of both). These analyses only considered data from the 2nd round because the zooplankton and small macroinvertebrates community was only sampled during this round.

To test the effects of environmental variables (Table 2.1) on fire salamander larvae body ratios we first tested all the correlations among them using Pearson's correlation; for highly correlated pairs (coefficient >70%) we retained the variable with more biological and ecological importance. Results of both rounds were used to calculate body ratios; we did not test the effect of the abundance of zooplankton and small macroinvertebrates on these ratios because these were sampled during the 2nd round.

After visualising the plots of the relation between the response variables (ratios as indicators of body conditions, considering both rounds) and the independent variables (environmental variables), we verified that not all the relations were linear. We therefore tested for the combined effects of the independent variable with a generalized additive mixed model (GAMM), being the sampling site the random-effect variable, because the larvae present at each point are not independent from each other (several will presumably be siblings). Using *gamm4* R package (Wood and Scheipl 2017), we ran univariate GAMMs with a Gaussian error distribution and identity link function to determine which variables were relevant to each response variable. Following that, we built models and, given that GAMMs outputs cannot be compared with Akaike Information Criterion, we selected the best models based on the significance of each variable, refining them with a stepwise approach, i.e., removing non-significant variables one by one, always retesting the new sets of variables. Concurvity is essentially the non-linear form of collinearity and was measured in the potentially final models using the *mgcv* R package (Wood 2004), verifying if any smooth term in the models could be approximated by any other simpler smooth terms. With the same package we verified the fitting of the models and if their predictions were in the expected range.

Statistical significance was set as *p*<0.05. All graphs were made using the *ggplot2* R package (Wickham 2016). All the analyses were made using R statistical software (v. 3.5.1) (R Core Team 2018). The maps were made using QGIS software (v. 2.14.3) (QGIS Development Team 2016).

RESULTS

Overall, the efficacy of the *Acacia* removal operations is confirmed by the reduction of all size-classes along the removal procedures. However, and despite the removal of all the trees, there are still a few plants of 10 cm or more, even after 24 semesters since the last *Acacia* spp. removal (Fig. 3.1). When considering the environmental variables, the PCA showed that ponds were not sorted into groups (Appendix F).
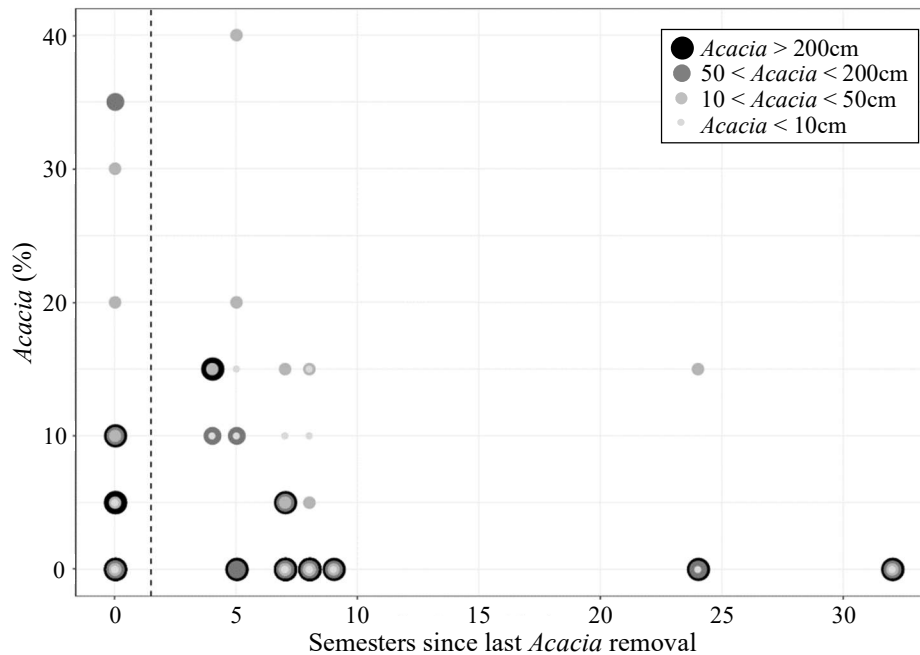
Figure 3.1 – Percentage of cover by different size classes of *Acacia* spp. per semester since the final intervention. The vertical dashed line is used to separate the sampling plots that have not yet been cleared (to the left of the dashed line) from the sampling plots that have been cleared (to the right of the dashed line). The values of the uncleared areas are used as starting point with which the other values will be compared to. In several sampling points there is as overlap of the cover of *Acacia* spp. with different ranges of heights.

Chemical analyses

There is some variation in the concentration of nitrogenous compounds in the first years after *Acacia* spp. removal (Fig. 3.2A), but in general the values are similar to those of ponds in uncleared areas, with the exception of the nitrate concentration on a single pond, where the intervention took place 7 semesters ago. The overall trend of nitrogenous compounds' concentration is to diminish over time. Following the same pattern, there are fluctuations of inorganic nitrogen in the water (Fig. 3.2B), with the pond with peak values coinciding in both plots. All but one sampling site had NH_4^+ concentrations under the detectability threshold, which is why this nitrogenous compound is not represented in the graphic.

The soil total Kjeldahl nitrogen shows more variability (Fig. 3.3); however, in the first years after the removal actions, the nitrogen concentration is overall higher than in places where the trees have not yet been cut. The trend is to diminish over time.
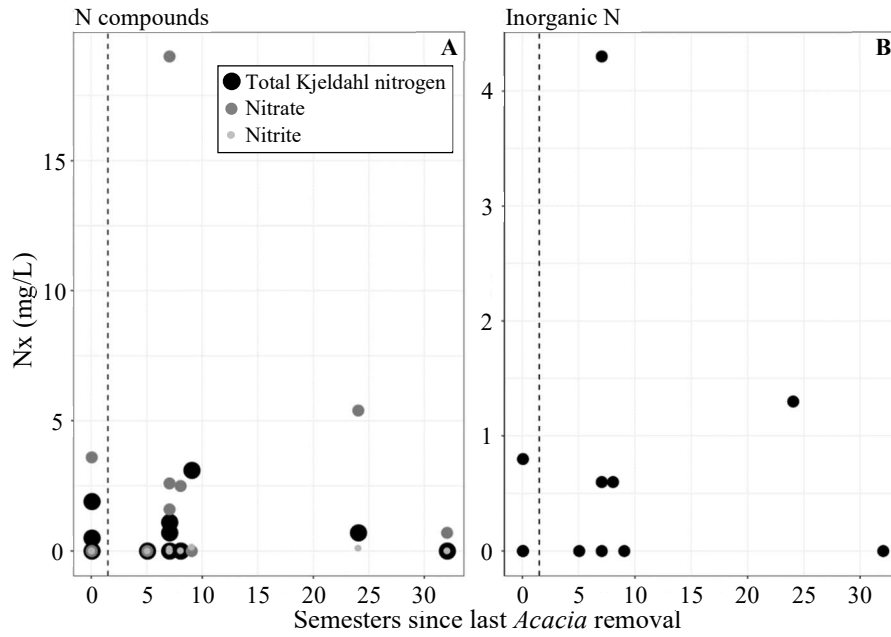
Figure 3.2 – Variation in nitrogenous compounds (A) and inorganic nitrogen (B) concentration (mg/L of water) across time. Note that the scales are different. The vertical dashed lines separate uncleared (left) plots from cleared plots (right).
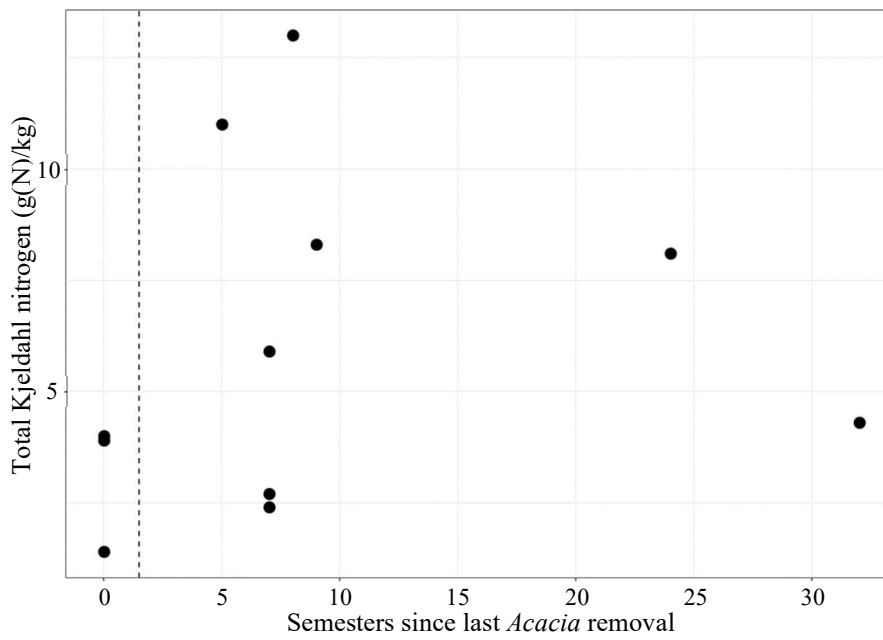
Figure 3.3 – Variations in the concentration of Total Kjeldahl nitrogen (g(N)/kg of soil) across time. The vertical dashed line separates uncleared (left) plots from cleared plots (right).

Zooplankton and small macroinvertebrates

There is an increase in both leaf litter and water column zooplankton and small macroinvertebrates abundance after the *Acacia* spp. removal (Fig. 3.4). This effect decreases over time, with values being close to those of uncleared areas 32 semesters after removal.

The overall trend is similar for different taxa, with a decrease over time (Fig 3.5 A and B). Among the leaf litter samples, the more abundant are the primary consumers Ostracoda and Cladocera (Fig. 3.5 B).
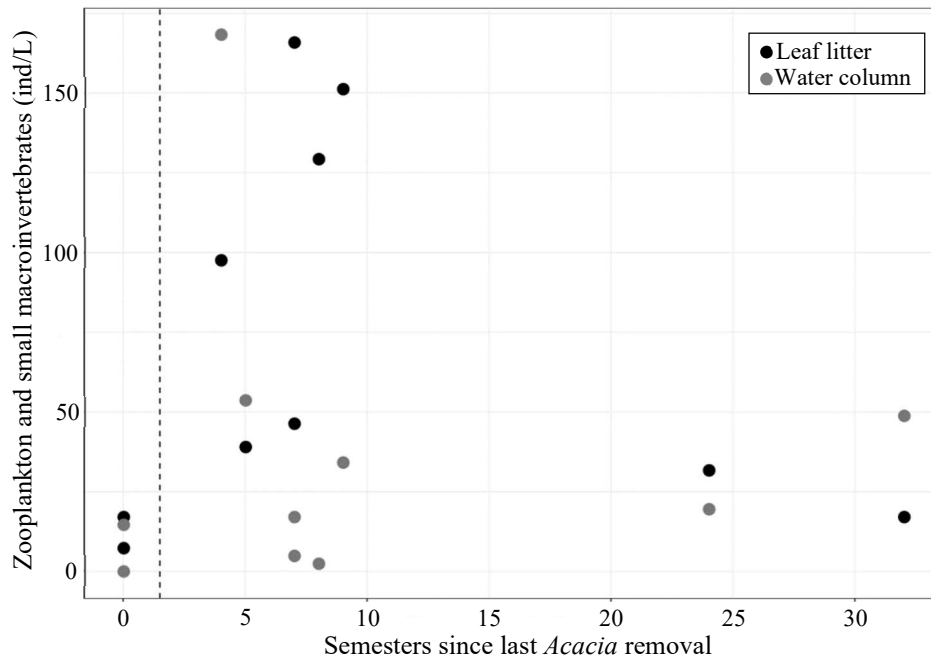
Figure 3.4 – Variation in abundance of zooplankton and small macroinvertebrates in the leaf litter and in the water column across time. The vertical dashed line separates uncleared (left) plots from cleared plots (right).
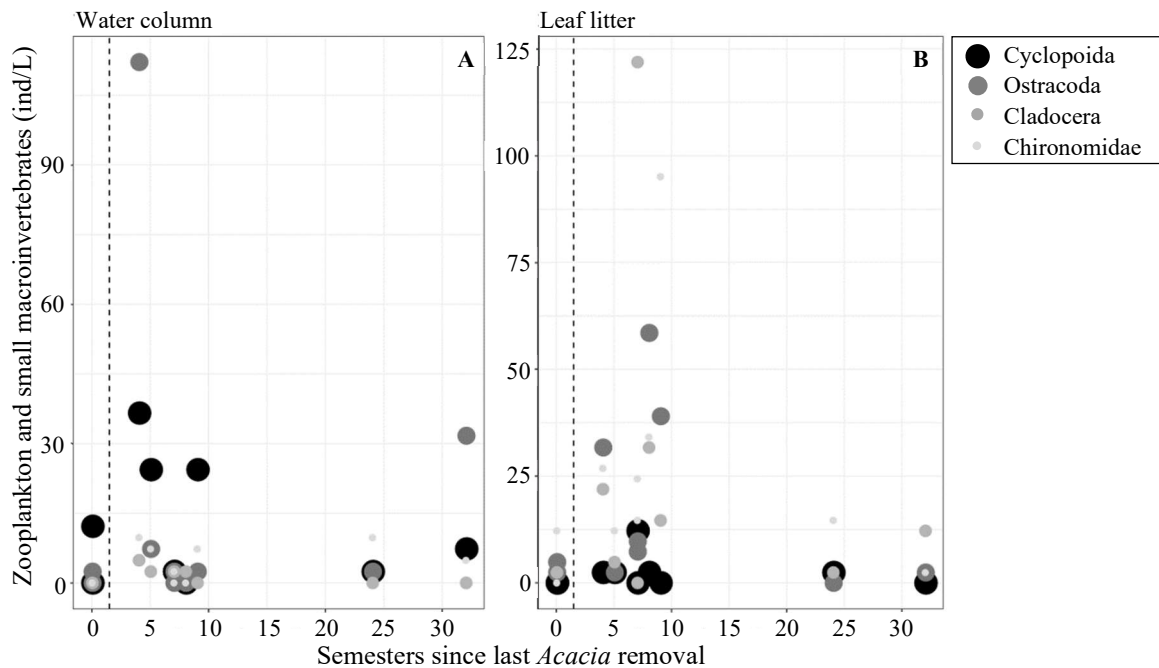
Figure 3.5 – Variation in different zooplankton and small macroinvertebrates taxa over time. A) Zooplankton and small macroinvertebrates in the water column and B) Zooplankton and small macroinvertebrates in the leaf litter. Note the difference in the scales. The vertical dashed lines separate uncleared (left) plots from cleared plots (right). Different sized circles do not reflect differences in abundance of each taxa; they were adopted to allow the visualization of overlapping results.

Amphibians

Figure 3.6 shows the presence of different amphibian species arranged according to semesters since last the *Acacia* spp. removal (N=1221). *Pleurodeles waltl* is a rare species. Other caudata larvae (*S. salamandra*, *Lissotriton boscai* and *Triturus marmoratus*) occur in every age. *Bufo spinosus* was the only anuran found to be reproducing in uncleared areas. *Discoglossus galganoi* reproduces in plots that have been cleared and not in uncleared *Acacia* stands or where the forest is more developed.
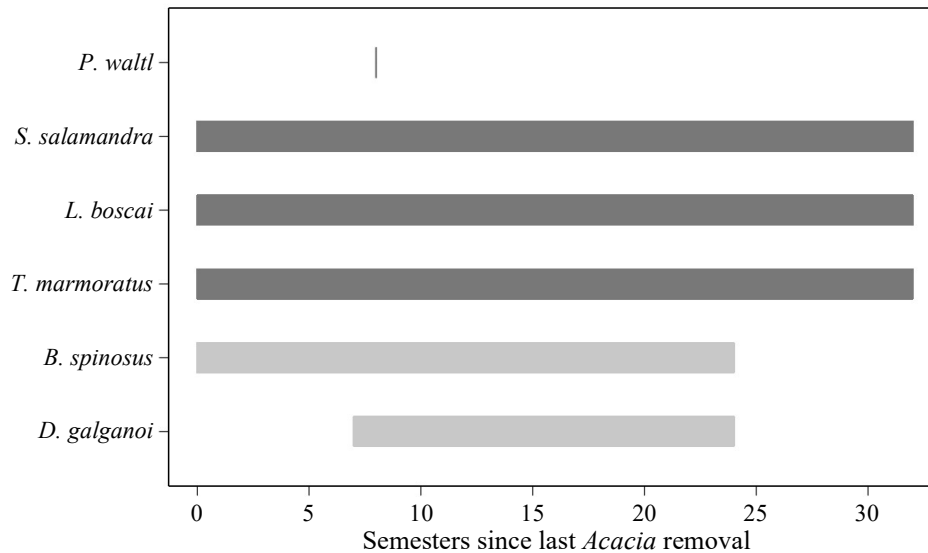
Figure 3.6 – Presence of amphibian species over time. The different colours refer to the order: caudates (dark grey) and anurans (light grey).

Salamandra salamandra

A total of 910 *S. salamandra* larvae were photographed (N$_{\text{1st round}}$=646; N$_{\text{2nd round}}$=264).

We tested for spatial autocorrelation for A/SVL ratio, M/F ratio, kurtosis of A/SVL ratio, kurtosis of M/F and SVL, for each round and found it to be non-existent for any ratio or measurement (Appendix G).

The average SVL was statistically different among semesters (ANOVA: $F_{7,902} = 6.678$, $p = <0.001$) (Fig. 3.7). We found that larvae developing in areas cleared for 5 semesters are significantly smaller than those devoloping in areas cleared for 4 and 9 semesters. The average A/SVL ratio was not statistically different among semesters (ANOVA: $F_{7,902} = 1.301$, $p = 0.247$) (Fig. 3.8). However, the average M/F ratio of was statistically different among semesters (ANOVA: $F_{7,902} = 8.256$, $p = <0.001$) (Fig. 3.9). We found that larvae developing in uncleared plots and plots cleared for 32 semesters are statistically different, having the latter a thicker tail muscle. We also found that larvae developing in plots cleared for 8 semesters have a significantly thinner muscle when compared to those developing in uncleared plots, plots cleared for 7, 9 and 32 semesters.
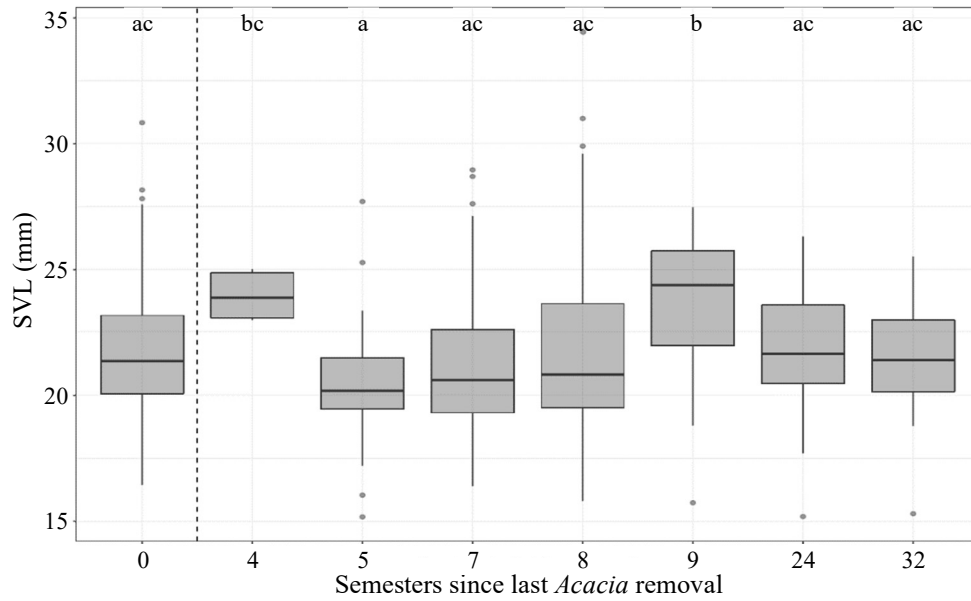
Figure 3.7 – SVL (mm) of *S. salamandra* larvae in different semesters since the last *Acacia* spp. removal. The thick horizontal lines represent the median; the lower and upper boundaries of the box represent the 25th and 75th percentiles, respectively; whiskers represent the range of observations within 1.5 times the interquartile range from the edge of the box; and outliers (grey circles with transparency) represent observations farther than 1.5 times the interquartile range. Boxes with the same letter are not statistically different (Tukey test, p > 0.05). The vertical dashed line separates uncleared (left) plots from cleared plots (right).
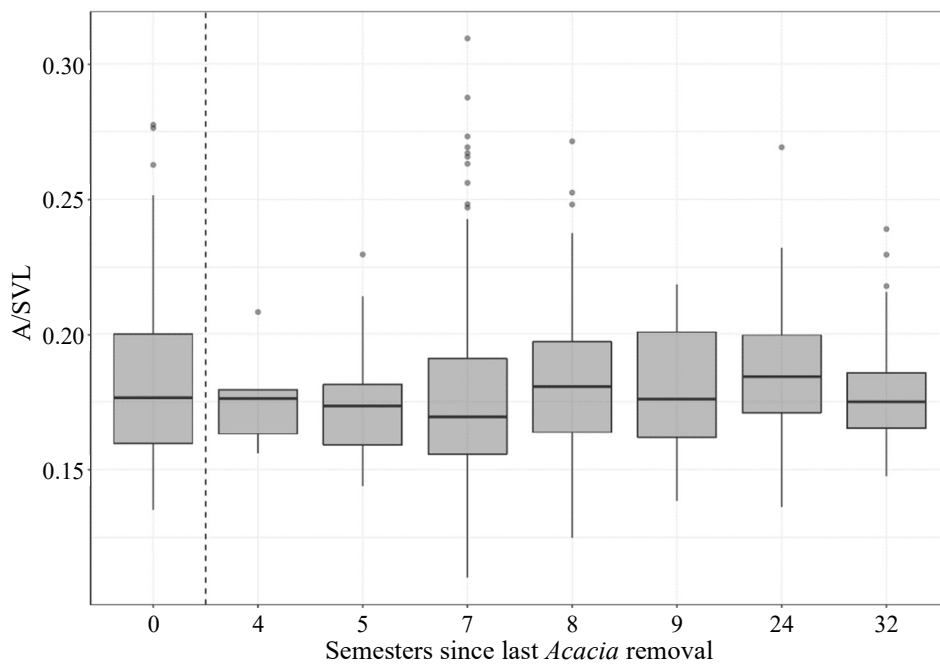
Figure 3.8 – A/SVL ratios of S. *salamandra* larvae in different semesters since the last *Acacia* spp. removal. The vertical dashed line separates uncleared (left) plots from cleared plots (right).
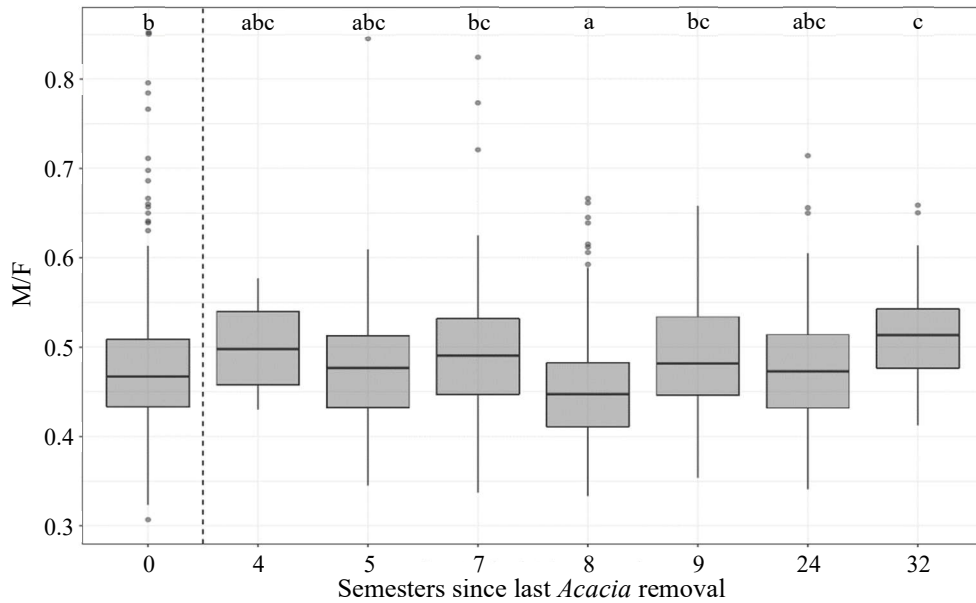
Figure 3.9 – M/F ratios of *S. salamandra* larvae in different semesters since the last *Acacia* spp. removal. The vertical dashed line separates uncleared (left) plots from cleared plots (right).

We found a positive and statistically significant relation between maximum head width and maximum abdominal width ($r = 0.830$, $p < 0.001$). We also found a positive and statistically significant relation between maximum head width and middle tail muscle depth ($r = 0.667$, $p < 0.001$).

The relationships between the abundance of the different types of zooplankton and small macroinvertebrates and the kurtoses of A/SVL and M/F ratios were tested. We found a positive and statistically significant relation between the abundance of Copepoda in the leaf litter and the kurtosis of A/SVL ratio. The second strongest relation found is negative, but not significant, and concerns the abundance of zooplankton and small macroinvertebrates in the water column and the kurtosis of M/F ratio (Table 3.1).

Table 3.1 – Pearson's correlations output between the kurtoses of the ratios and the abundance of the different types of zooplankton and small macroinvertebrates.

	Kurtosis A/SVL ratio			Kurtosis M/F ratio		
	r	n	*p*	*r*	n	*p*
Zooplankton and small macroinvertebrates in the leaf litter	-0.522	5	0.367	-0.493	5	0.398
Copepoda in the leaf litter	**0.889**	5	**0.044***	0.670	5	0.216
Ostracoda and Cladocera in the leaf litter	-0.587	5	0.298	-0.489	5	0.404
Zooplankton and small macroinvertebrates in the water column	-0.168	5	0.787	**-0.739**	5	0.154
Copepoda in the water column	-0.473	5	0.421	-0.700	5	0.188
Ostracoda and Cladocera in the water column	0.444	5	0.454	-0.214	5	0.730
Total zooplankton and small macroinvertebrates	-0.510	5	0.380	-0.639	5	0.246
Total Copepoda	-0.078	5	0.901	-0.477	5	0.417
Total Ostracoda and Cladocera	-0.538	5	0.349	-0.536	5	0.352

16

Regarding the identification of the variables that explain the variation of the A/SVL ratio, the main results of the GAMM are presented on Table 3.2.

The smooth terms of the responses of A/SVL ratio to the selected predictors are shown in Figure 3.10. According to this model, larvae developing in artificial ponds would have higher values of the ratio (Table 3.2), i.e., would be larger than those developing in natural ponds. The ratio also has a positive linear relationship with the width of the pond, whether they are natural or artificial. With the increase of the width, the ratio appears to increase at a higher rate in natural ponds when compared to artificial ponds.

Table 3.2 – Predictors selected by GAMM for A /SVL of fire salamanders. Parametric coefficients depict values of estimate, standard error, t-values and p-values. Smooth terms depict the estimate degrees of freedom, F-values and p-values Each final model is associated with R-square and number of samples used to build the model.

Response variable	Predictors	(-)lmer.REML	R-sq. (adj)	n
A/SVL	Type Width	-4018.300	0.049	910
A. Parametric coefficients	**Estimate**	**Std. error**	**t-value**	**p-value**
(Intercept)	0.173	0.002	70.435	<0.001
Type2	0.012	0.003	3.726	<0.001
B. Smooth terms	**edf**	**F-value**	**p-value**	
s(Width):Type1	1.000	4.000	0.046	
s(Width):Type2	1.000	1.884	0.170	
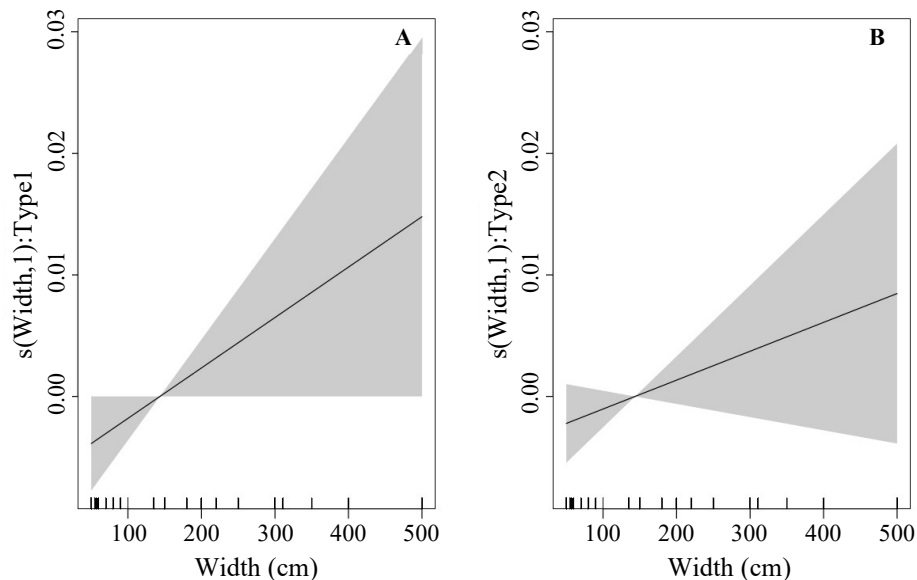
Figure 3.10 – GAMM results with A/SVL as response variable. The relations represented are: A) A/SVL and width (measured in cm) of the pond (type 1 = natural pond). B) A/SVL and width of the pool (type 2 = artificial pool). Fitted smooth terms indicated as s(name of the predictor, degrees of freedom) for the A/SVL ratio in solid lines. Shading correspond to 95% confidence intervals. The marks in the x-axis represent the location of the observations along the predictors. This plot ("partial dependence plot") shows the residuals regression of A/SVL ratio on width, therefore it shows how the response variable is expected to vary with the predictor, in this case according to pond type.

Regarding the identification of the variables that explain the M/F ratio, the main results of the GAMM are presented on Table 3.3.

Figure 3.11 shows the smooth terms of the responses of M/F ratio to the selected predictors. The M/F ratio declined linearly when the maximum depth increases. This ratio declined in areas with herbaceous plants and *Acacia* spp. under 10 cm up to 40% cover, then there is a turning point and the ratio increases up to approximately 80% cover.

Table 3.3 – Most relevant predictors selected after using GAMM for modelling M/F of fire salamanders. Parametric coefficients are associated with values of estimate, standard error, t-values and p-values. Smooth terms are associated with values of estimate degrees of freedom, F-values and p-values Each final model is associated with R-square and number of samples used to build the model.

Response variable	Predictors	(-)lmer.REML	R-sq. (adj)	n
M/F	Max_Depth Herb_Ac_10	2242.600	0.088	910
A. Parametric coefficients	**Estimate**	**Std. error**	**t-value**	**p-value**
(Intercept)	0.479	0.003	138.300	<0.001
B. Smooth terms	**edf**	**F-value**	**p-value**	
s(Max_Depth)	1.000	12.358	<0.001	
s(Herb_Ac_10)	3.430	4.462	<0.01	
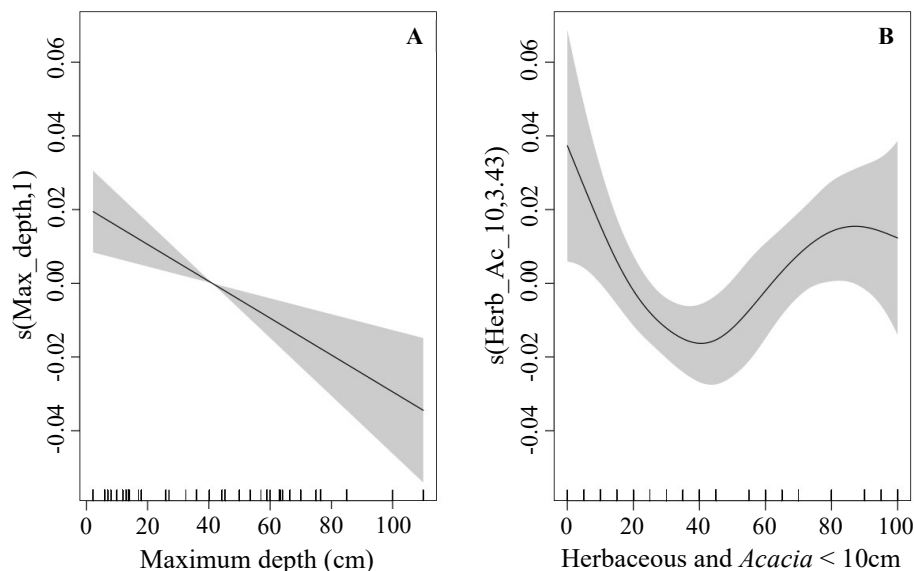
Figure 3.11 – Result of the GAMM with M/F as response variable. A) relation between the response variable and maximum depth. B) relation between the response variable and small plants (Herbaceous plants and *Acacia* spp. under 10cm). These plots show the residuals regression of M/F ratio on maximum depth (A) and on herbaceous plants and *Acacia* spp. under 10 cm cover (B), therefore they show how the response variable is expected to vary with the predictors.

DISCUSSION

With this study we verified that tree removal can acutely affect freshwater ecosystems, changing the dynamics of pond habitats. Not only have we found that nitrogenous compounds' concentration varies rapidly after *Acacia* spp. removal, but also that after this major event zooplankton and small macroinvertebrates communities and salamanders' body conditions change markedly. However, disentangling the effects of the increase in nitrogenous compounds and of an increase in sunlight availability was not possible, and overall our results lead us to suggest that the strong NO_x^- influx that happened after *Acacia* spp. removal may affect pond communities indirectly, by improving pond productivity, with little toxic effects.

Indeed, there was a higher concentration of zooplankton and small macroinvertebrates in most ponds in the first 9 semesters after *Acacia* spp. removal. The clearings created by the tree removal allowed more light to reach the ground and pond water, and therefore, an increase in primary production was expected (Brönmark and Hansson 2005). The high concentrations of zooplankton in these intermediate semesters, specifically of primary consumers, is a strong suggestion of that effect.

NO_x^- toxicity in amphibians

The increase in the concentration of nitrogenous compounds recorded after *Acacia* spp. removal was expected, as this was already documented in soils (Marchante et al. 2009) and groundwater (Jovanovic et al. 2009). This study shows that even 24 semesters after the felling of the tallest trees, the concentration of nitrogenous compounds can be higher in cleared plots than in invaded areas, indicating that the N pools can remain high for several years.

In the studied plots the NO_x^- concentrations varied from <0.01 to 0.3 mg/L for nitrite and from <0.3 to 19 mg/L for nitrate. As for other animal species (Jensen 1995), nitrites are more toxic to amphibians than nitrates, which are, nevertheless, slightly toxic (Odum and Zippel 2008). Marco et al. (1999) found that nitrite concentration of 1.0 mg/L can be moderately to highly toxic to some amphibian species. Marco and Blaustein (1999) also found that tadpoles exposed to concentrations of nitrite of 3.5 mg/L developed and metamorphosed slower than control tadpoles. Since the concentrations measured in this study were never higher than 0.3 mg/L, we can conclude that the *Acacia* spp. invasion in Sintra did not raise the values of nitrite to harmful levels.

Baker and Waights (1993, 1994) found that concentrations of nitrate between 9 and 22.6 mg/L can lead to reduced growth, behavioural changes and increased mortality of *Bufo bufo* tadpoles. Marco et al. (1999) found that a concentration of 10 mg/L can be moderately toxic to some amphibian species. Nonetheless, de Wijer et al. (2003) found no detrimental effects of nitrates on tadpoles; in fact, they found an increase in mass at metamorphosis and survival, and a decrease in time to metamorphosis, which they explained as indirect results of the increase of periphyton and phytoplankton. In our study, the indirect effect on plants was not measured but seems to exist, since an increase in primary consumer abundance was detected. However, it is not possible to distinguish if it is a result of the increase in nitrate concentration or the increase of light reaching the ground.

Only in one pool did the nitrate values reach values higher than the minimum concentrations referred to in these previous studies, which means that larvae developing in that pool could have been affected to some extent. We speculate that this area had previously been heavily invaded and that clearing of *Acacia* spp. led to a leaching of high amounts of nitrogenous compounds. The morphological traits of the salamander larvae found at this site were not different from all the others (Santos I, data not shown), therefore we cannot conclude if hazardous levels were reached. All other sampling sites did not reach potentially harmful nitrate levels.

Amphibians

With this study we showed that *D. galganoi* is a species that prefers areas with low tree cover. This is probably because a less dense canopy cover allows more light to reach the ground, inducing periphyton growth. Since *D. galganoi* presents a shorter larval development period than other anuran species (García-París et al. 2004), it is important that this food resource is readily available. The capacity of *B. spinosus* to breed in invaded areas demonstrates that it may not be very selective; according to Lizana (2002) this species only requires stagnant or slow flow water, preferably permanent and vegetated. Furthermore, their larval development is much slower, which means that these tadpoles can live in low productivity habitats like shaded ponds.

As for caudates, and except *P. waltl*, a rare species in the properties of PSML, we found that all other species are present in all stand ages, which corroborates their preference for forest habitats, i.e., they need forested areas in the surroundings of their breeding ponds (Schabetsberger et al. 2004; Marty et al. 2005; Denöel and Lehmann 2006), where they spend the post-breeding phase (Marty et al. 2005). Moreover, the existence of carnivorous caudata larvae in all stand ages may indicate that the zooplankton communities are abundant enough to maintain larval amphibian communities. Besides their forest habits, *S. salamandra gallaica* — the subspecies occurring in Sintra — is ovoviviparous, giving birth to very developed larvae, nearing metamorphosis (Rebelo and Leclair 2003). This allows the larvae to include larger invertebrates in their diet early on in their free larval development and to thrive under different conditions, even those that are not optimal.

Recently hatched salamander larvae feed on zooplankton, especially on small crustaceans such as water fleas (Cladocera) and copepods (Copepoda). After a while they start feeding on larger invertebrates, which includes midges (Chironomidae) and snails (Gastropoda) (Richter and Azous 2000). Larger larvae can even feed on other amphibian larvae (Richter and Azous 2000). The positive correlation between maximum head width and maximum abdominal width indicates that larger larvae are capable of eating larger preys or even cannibalise their conspecifics. In fact, the absence of any relation between zooplankton and small macroinvertebrate abundances and *S. salamandra* larvae body size or body ratios (appendices I and J) may be an indicator that, at the time of sampling, the larvae were already feeding on larger prey. However, the generally low values of kurtoses of the biometric distributions of *S. salamandra* larvae indicate that there is a high trait diversity (Enquist et al. 2015), which means that Sintra ponds environments may be suitable for all types of body shapes, either larger or thinner individuals and for either more or less muscled individuals.

There was a significant decrease in larval SVL shortly after tree removal. The larvae developing in those intermediate age plots have more food resources (Fig. 3.4 and 3.5) and therefore larvae of all sizes may fulfil their energy needs without incurring the risk of cannibalism. The increase in SVL that occurs in older plots (Fig. 3.7) may reflect either extremely advantageous environmental conditions for growth or, conversely, that intraspecific competition reached a level where bigger larvae over compete smaller ones. In old plots, 32 semesters after *Acacia* spp. removal, larval sizes are similar to those of larvae developing in uncleared areas, which may be a result of the increase of canopy cover.

Regarding body ratios, the A/SVL ratio trend showed no statistically significant differences among plots, with similar values among different ages. As for the changes in the M/F ratio, we found that the extreme ages were different and that larvae developing in areas cleared for 32 semesters presented a higher proportion of tail muscle. The main difference in these areas concerns the type of vegetation, i.e., native instead of alien invasive, and so the difference may be due to leaf litter quality. There is also a significant difference between areas cleared for 8 semesters and uncleared areas on one side, and areas cleared for 7, 9 and 32 semesters on the other. This difference may be due to ecological succession in

pond vegetation. A more vegetated pond may present more refuges to larvae (Egan and Paton 2004), where they can hide better from predators or sit-and-wait for prey.

We found a positive relation between the abundance of Copepoda in the leaf litter and the kurtosis of the A/SVL ratio, which means that as the abundance of Copepoda increases, larvae tend to have similar values of A/SVL ratio. However, this does not give us a hint of the range of values benefiting from that higher Copepoda abundance. Blaustein et al. (1996) found that ponds with salamanders had their copepods population significantly reduced when compared with ponds without salamanders, and that copepod abundance recovered after salamander metamorphosis, when the individuals left the ponds. Copepods play an important role in the larvae's diet, perhaps contributing to a general higher A/SVL ratio when more abundant.

There was a negative relation between the abundance of zooplankton and small macroinvertebrates in the water column and the kurtosis of the M/F ratio. This means that when the abundance of zooplankton and small macroinvertebrates in the water column increases, the larvae tend to have a larger diversity of M/F ratios, meaning that both more muscled larvae and less muscled larvae have the same chances of survival, which may be due to the higher availability of different types of food resources.

To explain the variation in A/SVL ratios, the most important variables were waterbody type and width. Larvae from man-made, artificial water bodies are larger than those growing in natural ponds. This type of sampling point was strongly correlated with hydroperiod — in general, artificial pools are permanent. When larvae develop in temporary ponds they tend to metamorphose earlier than those developing in permanent ones, possibly as a response to cues of pond drying (Semlitsch et al. 1988), investing in traits needed to survive outside water, such as the development of lungs, instead of investing in body mass. In Sintra, the natural ponds resulting from surface run-off are relatively narrow and the wider ponds are typically artificial, i.e., in general, a wider pond is also an artificial one. Therefore, the fact that larvae are larger in wider ponds, is in accordance with the previously discussed result.

Maximum depth and herbaceous stratum were the most important variables to explain the M/F ratio. This ratio declined linearly when the maximum depth increased. Pond depth can be seen as a proxy to pond permanence, i.e., the deeper the pond, the more likely it is for it to be permanent. A decrease in this ratio may mean that the tail fin is deeper, and this can be seen as a defence against predators, since it lowers the probability of a lethal attack (Yurewicz 2004) from predators that can exist in higher densities in the bottom of deeper ponds. On the other hand, a study with newts found that in permanent ponds newts tend to not only to grow deeper tail fins, but also deeper tail muscles (Van Buskirk 2009), which would contribute to higher ratio values than those which develop only deeper tail fins. That could also be advantageous against predators, since the muscle would allow a stronger escape response (Van Buskirk et al. 1997).

The M/F ratio declined in areas with herbaceous plants and *Acacia* spp. under 10 cm up to 40% cover, and then there is a turning point, and the ratio increases in areas with up to approximately 80% of herbaceous cover (Fig. 3.11). In this figure, plots with *Acacia* spp. and native forest are represented by the same values, that is, to the left of the figure, since where trees are taller, there is a lower herbaceous cover. In these closed forests, regardless of the tree species, the abundance of zooplankton and small macroinvertebrates is lower when compared to cleared areas, where the sunlight can reach the ponds. From 0% to 40% of herbaceous cover, the M/F ratio tends to decrease, that is, the larvae are less muscled, or they develop a deeper fin. If the first case is true, then the environmental conditions may be favourable even to larvae that are less muscled. If the latter case is true, then they may have developed a strategy to overcome cannibalism, since a deeper fin is usually linked to sites with a high number of predators (Storfer and White 2004). On sites with 40% to 80% of herbaceous cover the M/F ratio tends to increase, i.e., the larvae tend to have a more developed tail muscle. The herbaceous cover may be already high

enough to overshadow the ponds, decreasing their primary productivity and, consequently, the number of fire salamander prey. If this is the case, developing a stronger, wider muscle may reveal advantageous to survive, probably because these larvae can swim faster, better avoid cannibalism, catch more preys or even cannibalise their conspecific, which would allow them to gain access to a high-energy prey, and allocate resources to the production of more muscle.

Final considerations

In conclusion, this study reveals that *Acacia* spp. removal impacts freshwater habitats, whether by changing nitrogenous compounds' concentration — which remains as a legacy effect of the invasion for several years —, or light availability at the soil level. In the ponds, we found changes in the zooplankton communities, on amphibian species richness and on larval fire salamanders' body condition. However, we could not discriminate the main driver of pond community changes, either water chemistry, increase of sunlight reaching the ponds or their interaction. In fact, as most laboratory studies cited above do not consider the interactions between several factors, the effect of Nx pollutants on amphibians in natural conditions are not yet clear and deserve further studies (Boone and Bridges 2003; Gomez-Mestre and Tejedo 2003).

In general, the legacy effect of nitrogenous compounds surface leaching did not reach dangerous levels at the sampling sites, with a single exception of a plot where *Acacia* spp. removal took place 3.5 years ago. The lack of information concerning the nitrogenous compounds' concentrations in more recently cleared plots, or repeatedly in the same pond along the succession trajectory, does not allow a full understanding of the toxicity levels potentially reached and their toxic effects for the pond community. Therefore, it would be interesting to understand under which conditions the toxicity thresholds are exceeded (e.g., depending on density of *Acacia* spp. before the intervention), and to follow the concentrations of the nitrogenous compounds during the first semesters after *Acacia* spp. removal, at least for 8 semesters.

We found *Acacia* spp. sprouts even 12 years after the interventions, which indicates germination from the soil-stored seed bank. Seeds from this genus have a high longevity and remain viable in the soil for long periods of time (Farrell and Ashton 1978; Cavanagh 1980; Leino and Edqvist 2010). This is one of the consequences of removing trees, since seed germination can then be stimulated by the sunlight (Marchante et al. 2008). This result corroborates the importance of continued management efforts so that these interventions can be effective in the long term.

The removal of alien invasive plants and restoration of the invaded sites are extremely important to preserve native amphibians in Sintra (e.g., *D. galganoi* or the rare *P. waltl*). Nevertheless, these interventions change significantly macro and micro habitats, therefore those taking place near ponds should be followed not only by reforestation, but also by creation of shelters (e.g., fallen logs) that protect adult amphibians from desiccation and predation during their migration.

The maintenance of forest clearings around at least some of the ponds would certainly contribute to a higher diversity of pond communities and to increase the populations of threatened amphibians at the PSML properties. In fact, these interventions might benefit anuran species such as the Mediterranean tree frog (*Hyla meridionalis*) and the rare, or even locally extinct, common midwife toad (*Alytes obstetricans*). A monitoring program, similar to that conducted for the Iberian emerald lizard (*Lacerta schreiberi*), would be important to track the expansion of these two species into the cleared areas. Additionally, the reptile community, including *L. schreiberi*, would also benefit from the maintenance of some forest clearings.

REFERENCES (according to Biological Invasions)

Aerts R, Berendse F (1988) The effect of increased nutrient availability on vegetation dynamics in wet heathlands. Vegetatio 76:63-69. https://doi.org/10.1007/BF00047389

Aguilar F, Rebelo R (2017) Inventariação de anfíbios nas áreas sob gestão da PSML - Relatório final.

Alcoforado MJ, Alegria MF, Ramos-Pereira A, Sirgado C (2009). Domínios bioclimáticos em Portugal definidos por comparação dos índices de Gaussen e de Emberger. Centro de Estudos Geográficos, Universidade de Lisboa.

Allison SD, Vitousek PM (2004) Rapid nutrient cycling in leaf litter from invasive plants in Hawai'i. Oecologia 141:612–619. https://doi.org/10.1007/s00442-004-1679-z

Álvarez D, Nicieza AG (2002) Effects of temperature and food quality on anuran larval growth and metamorphosis. Funct Ecol 16:640-648. doi:10.1046/j.1365-2435.2002.00658.x

Baker J, Waights V (1993) The effect of sodium nitrate on the growth and survival of toad tadpoles (*Bufo bufo*) in the laboratory. Herpetol J 3:147-148.

Baker J, Waights V (1994) The effects of nitrate on tadpoles of the tree frog (*Litoria caerulea*). Herpetol J 4:106-108.

Batzer DP, Palik BJ (2007) Variable response by aquatic invertebrates to experimental manipulations of leaf litter input into seasonal woodland ponds. Fundam Appl Limnol 168:155-162. https://doi.org/10.1127/1863-9135/2007/0168-0155

Batzer DP, Palik BJ, Buech R (2004) Relationships between environmental characteristics and macroinvertebrate communities in seasonal woodland ponds of Minnesota. J North Am Benthol Soc 23:50-68. https://doi.org/10.1899/0887-3593(2004)023<0050:RBECAM>2.0.CO;2

Binckley CA, Resetarits WJ (2007) Effects of forest canopy on habitat selection in treefrogs and aquatic insects: implications for communities and metacommunities. Oecologia 153:951–958. https://doi.org/10.1007/s00442-007-0780-5

Blaustein L, Friedman J, Fahima T (1996) Larval salamandra drive temporary pool community dynamics: evidence from an artificial pool experiment. Oikos 76:392-402. https://doi.org/10.2307/3546211

Blumenthal D (2005) Interrelated causes of plant invasion. Science 310:243–244. https://doi.org/10.1126/science.1114851

Bobbink R (1991) Effects of nutrient enrichment in Dutch chalk grassland. J Appl Ecol 28:28-41. https://doi.org/10.2307/2404111

Boone MD, Bridges CM (2003) Effects of pesticides on amphibian populations. In: Semlitsch RD (ed) Amphibian conservation. Smithsonian Books, Washington, DC, pp 152–167.

Boone MD, Bridges CM, Fairchild JF, Little EE (2005) Multiple sublethal chemicals negatively affect tadpoles of the green frog, *Rana clamitans*. Environ Toxicol Chem 24:1267–1272. https://doi.org/10.1897/04-319R.1

Brönmark C, Hansson LA (2005) The biology of lakes and ponds. Oxford University Press Inc., New York.

Cavanagh AK (1980) A review of some aspects of the germination of acacias. Proc Roy Soc Vic 91:161-180. In: Holmes PM (1989) Decay rates for buried alien *Acacia* seed populations of different density. S Afr J Bot 55:299–303. https://doi.org/10.1016/S0254-6299(16)31179-6

Cooke AS (1981) Tadpoles as indicators of harmful levels of pollution in the field. Environ Pollut A Ecol Biol 25:123–133. https://doi.org/10.1016/0143-1471(81)90012-X

Costa AML (1981) A acção de produtos fitocidas no combate do acacial infestante. II Simpósio Nacional de Herbologia. In: Marchante H (2001) Invasão dos ecossistemas dunares portugueses por *Acacia*: uma ameaça para a biodiversidade nativa. Dissertation, University of Coimbra.

Couto AP, Ferreira E, Torres RT, Fonseca C (2017) Local and landscape drivers of pond-breeding amphibian diversity at the Northern edge of the Mediterranean. Herpetologica 73:10-17. https://doi.org/10.1655/HERPETOLOGICA-D-16-00020.1

Cronk QC, Fuller JL (2001) Plant invaders: the threat to natural ecosystems. Routledge, London.

D'Antonio C (2000) Fire, Plant invasion and global changes. In: Mooney HA, Hobbs RJ (ed) Invasive species in a changing world. Island Press, Washington DC, pp 65-93.

D'Antonio C, Meyerson LA (2002) Exotic plant species as problems and solutions in ecological restoration: a synthesis. Restor Ecol 10:703–713. https://doi.org/10.1046/j.1526-100X.2002.01051.x

Davis MA, Pelsor M (2001) Experimental support for a resource-based mechanistic model of invasibility. Ecol Lett 4:421–428. https://doi.org/10.1046/j.1461-0248.2001.00246.x

de Wijer P, Watt PJ, Oldham RS (2003) Amphibian decline and aquatic pollution: effects of nitrogenous fertiliser on survival and development of larvae of the frog *Rana temporaria*. Appl Herpetol 1:3-12. https://doi.org/10.1163/157075403766451180

Denoël M, Lehmann A (2006) Multi-scale effect of landscape processes and habitat quality on newt abundance: implications for conservation. Biol Conserv 130:495-504. https://doi.org/10.1016/j.biocon.2006.01.009

Eddy FB, Williams EM (1987) Nitrite and freshwater fish. Chem Ecol 3:1-38. https://doi.org/10.1080/02757548708070832

Egan RS, Paton PWC (2004) Within-pond parameters affecting oviposition by wood frogs and spotted salamanders. Wetlands 24:1–13. https://doi.org/10.1672/0277-5212(2004)024[0001:WPAOBW]2.0.CO;2

Egea-Serrano A, Relyea RA, Tejedo M, Torralva M (2012) Understanding of the impact of chemicals on amphibians: a meta-analytic review. Ecol Evol 2:1382-1397. https://doi.org/10.1002/ece3.249

Ehrenfeld JG, Scott N (2001) Invasive species and the soil: effects on organisms and ecosystem processes. Ecol Appl 11:1259–1260. https://doi.org/10.1890/1051-0761(2001)011[1259:ISATSE]2.0.CO;2

Enquist BJ, Norberg J, Bonser et al. (2015) Scaling from traits to ecosystems: developing a general trait driver theory via integrating trait-based and metabolic scaling theories. Adv Ecol Res 52: 249-318. https://doi.org/10.1016/bs.aecr.2015.02.001

Eppinga MB, Rietkerk M, Dekker SC, De Ruiter PC, Van der Putten WH (2006) Accumulation of local pathogens: a new hypothesis to explain exotic plant invasions. Oikos 114:168–176. https://doi.org/10.1111/j.2006.0030-1299.14625.x

ESRI (2016) ArcGIS Desktop: Release 10.4.1. Redlands, CA: Environmental Systems Research Institute.

Farrell TP, Ashton DH (1978) Population studies on *Acacia melanoxylon* R. Br. I. variation in seed and vegetative characteristics. Aust J Bot 26:365-379. https://doi.org/10.1071/BT9780365

Ferreira J, Reis L (1999) Cálculo do volume total de *Acacia melanoxylon* R. Br. na Tapada do Mouco (Serra de Sintra). Ed SPCF/ADERE. 1º Encontro Sobre Invasoras Lenhosas. Gerês. In: Marchante H (2001) Invasão dos ecossistemas dunares portugueses por *Acacia*: uma ameaça para a biodiversidade nativa. Dissertation, University of Coimbra.

García-París M, Montori A, Herrero P (2004) Amphibia, vol 24. CSIC, Madrid. In: Escoriza D, Boix D (2014) Reproductive habitat selection in alien and native populations of the genus Discoglossus. Acta Oecologica 59:97-103. https://doi.org/10.1016/j.actao.2014.06.005

Gomez-Mestre I, Tejedo M (2003) Local adaptation of an anuran amphibian to osmotically stressful environments. Evol 57:1889-1899. https://doi:10.1111/j.0014-3820.2003.tb00596.x

Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183-190.

Halverson MA, Skelly DK, Kiesecker JM, Freidenburg LK (2003) Forest mediated light regime linked to amphibian distribution and performance. Oecologia 134:360–364. https://doi.org/10.1007/s00442-002-1136-9

Hatch AC, Blaustein AR (2000) Combined effects of UV-B, nitrate, and low pH reduce the survival and activity level of larval cascades frogs (*Rana cascadae*). Arch Environ Contam Toxicol 39:494–499. https://doi.org/10.1007/s002440010132

Hocking DJ, Semlitsch RD (2008) Effects of experimental clearcut logging on gray treefrog (*Hyla versicolor*) tadpole performance. J Herpetol 42:689–698. https://doi.org/10.1670/07-294R1.1

Huenneke LF, Hamburg SP, Koide R, Mooney HA, Vitousek PM (1990) Effects of soil resources on plant invasion and community structure in Californian serpentine grassland. Ecology 71:478-491. https://doi.org/10.2307/1940302

Hutchinson GE (1957) A Treatise on limnology. Vol 1: Georgraphy, Physics and Chemistry. Wiley, New York. In: Eddy FB, Williams EM (1987) Nitrite and freshwater fish. Chem Ecol 3:1-38. https://doi.org/10.1080/02757548708070832

Jensen FB (1995) Uptake and effects of nitrite and nitrate in animals. In: Walsh PJ, Wright PA (ed) Nitrogen metabolism and excretion. CRC Press LLC, Florida (pp 289-303).

Jovanovic NZ, Israel S, Tredoux G, Soltau L, Le Maitre D, Rusinga F, Rozanov A, Van der Merwe N (2009) Nitrogen dynamics in land cleared of alien vegetation (*Acacia saligna*) and impacts on groundwater at Riverlands Nature Reserve (Western Cape, South Africa). Water SA 35:37-44. https://doi.org/10.4314/wsa.v35i1.76653

Komsta L, and Novomestky F (2015) moments: Moments, cumulants, skewness, kurtosis and related tests. R package (Version 0.14). https://CRAN.R-project.org/package=moments

Le Bagousse-Pinguet Y, Gross N, Maestre FT et al. (2017) Testing the environmental filtering concept in global drylands. J Ecol 105:1058-1069. https://doi.org/10.1111/1365-2745.12735

Le Maitre DC, van Wilgen BW, Gelderblom CM, Bailey C, Chapman RA, Nel JA (2002) Invasive alien trees and water resources in South Africa: case studies of the costs and benefits of management. For Ecol Manag 160:143-159. https://doi.org/10.1016/S0378-1127(01)00474-1

Leino M, Edqvist J (2010) Germination of 151-year old *Acacia* spp. seeds. Genet Resour Crop Evol 57:741-746. https://doi.org/10.1007/s10722-009-9512-5

Lizana M (2002) *Bufo bufo*. In Atlas y Libro Rojo de los Anfibios y Reptiles de España. Dirección General de Conservación de la Naturaleza, Madrid, pp 103-106.

Macías G, Marco A, Blaustein AR (2007) Combined exposure to ambient UVB radiation and nitrite negatively affects survival of amphibian early life stages. Sci Total Environ 385:55–65. https://doi.org/10.1016/j.scitotenv.2007.06.016

Marchante E, Freitas H, Marchante H (2008) Guia prático para a identificação de plantas invasoras de Portugal Continental. Imprensa da Universidade de Coimbra, Coimbra.

Marchante E, Kjøller A, Struwe S, Freitas H (2009) Soil recovery after removal of the N2-fixing invasive *Acacia longifolia*: consequences for ecosystem restoration. Biol Invasions 11:813-823. https://doi.org/10.1007/s10530-008-9295-1

Marchante H (2001) Invasão dos ecossistemas dunares portugueses por *Acacia*: uma ameaça para a biodiversidade nativa. Dissertation, University of Coimbra.

Marchante H, Marchante E, Buscardo E, Maia J, Freitas H (2004) Recovery potential of dune ecosystems invaded by an exotic *Acacia* species (*Acacia longifolia*). Weed Technol 18:1427-1433. https://doi.org/10.1614/0890-037X(2004)018[1427:RPODEI]2.0.CO;2

Marchante H, Marchante E, Freitas H (2003) Invasion of the Portuguese dune ecosystems by the exotic species *Acacia longifolia* (Andrews) Willd.: effects at the community level. In: Child LE, Brock JH, Brundu G, Prach K, Pyšek P, Wade PM, & Williamson M (ed) Plant invasion: ecological threats and management solutions. Backhuys Publishers, Leiden (pp 75-85).

Marco A, Blaustein AR (1999) The effects of nitrite on behavior and metamorphosis in Cascades frogs (*Rana cascadae*). Environ Toxicol Chem 18:946-949. https://doi.org/10.1002/etc.5620180519

Marco A, Quilchano C, Blaustein AR (1999) Sensitivity to nitrate and nitrite in pond-breeding amphibians from the Pacific Northwest, USA. Environ Toxicol Chem 18:2836-2839. https://doi.org/10.1002/etc.5620181225

Maron JL, Connors PG (1996) A native nitrogen-fixing shrub facilitates weed invasion. Oecologia 105:302-312. https://doi.org/10.1007/BF00328732

Marty P, AngÉlibert S, Giani N, Joly P (2005) Directionality of pre- and post-breeding migrations of a marbled newt population (*Triturus marmoratus*): implications for buffer zone management. Aquatic Conserv Mar Freshw Ecosyst 15:215-225. https://doi.org/10.1002/aqc.672

McLendon T, Redente EF (1991) Nitrogen and phosphorus effects on secondary succession dynamics on a semi-arid sagebrush site. Ecology 72:2016-2024. https://doi.org/10.2307/1941556

McWilliams S, Bachmann M (1989). Foraging ecology and prey preference of pond-form larval small-mouthed salamanders, *Ambystoma texanum*. Copeia 1989:948-961. https://doi.org/10.2307/1445981

Miaud C (1990) La dynamique des populations subdivisées: étude comparative chez trois amphibiens urodèles (*Triturus alpestris*, *T. helveticus* et *T. cristatus*). Dissertation, University of Lyon.

Moran PAP (1948) The interpretation of statistical maps. J R Stat Soc Ser B Methodol 10:243-251. https://doi.org/10.1111/j.2517-6161.1948.tb00012.x

Nilsson AN, Svensson BW (1995) Assemblages of dytiscid predators and culicid prey in relation to environmental factors in natural and clear-cut boreal swamp forest pools. Hydrobiologia 308:183–196 (1995). https://doi.org/10.1007/BF00006870

Odum RA, Zippel KC (2008), Amphibian water quality: approaches to an essential environmental parameter. International Zoo Yearbook 42:40-52. https://doi.org/10.1111/j.1748-1090.2008.00053.x

Palik BJ, Batzer DP, Buech R et al. (2001) Seasonal pond characteristics across a chronosequence of adjacent forest ages in northern Minnesota, USA. Wetlands 21:532–542. https://doi.org/10.1672/0277-5212(2001)021[0532:SPCAAC]2.0.CO;2

Paschke MW, McLendon T, Redente EF (2000) Nitrogen availability and old-field succession in a shortgrass steppe. Ecosystems 3:144-158. https://doi.org/10.1007/s100210000016

PMSL (2019) Resumo Combate Invasoras.

Pringle A, Bever JD, Gardes M, Parrent JL, Rillig MC, Klironomos JN (2009) Mycorrhizal symbioses and plant invasions. Annu Rev Ecol Evol Syst 40:699–715. https://doi.org/10.1146/annurev.ecolsys.39.110707.173454

QGIS Development Team (2016) QGIS Geographic Information System. Open Source Geospatial Foundation Project.

R Core Team (2018) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rebelo R, Leclair MH (2003) Differences in size at birth and brood size among Portuguese populations of the fire salamander, Salamandra salamandra. Herpetol J 13:179-188.

Regester KJ, Whiles MR, Lips KR (2008) Variation in the trophic basis of production and energy flow associated with emergence of larval salamander assemblages from forest ponds. Freshw Biol 53:1754-1767. https://doi.org/10.1111/j.1365-2427.2008.02073.x

Richardson DM, Cowling RM, Bond WJ, Stock WD, Davis GW (1995) Links between biodiversity and ecosystem function in the Cape Floristic Region. In: Davis GW, Richardson DM (ed) Mediterranean-Type Ecosystems. Springer-Verlag, Berlin (pp 285–333).

Richter KO, Azous AL (2000) Amphibian distribution, abundance, and habitat use. In: Azous AL, Horner RR (ed) Wetlands and urbanization: implications for the future. CRC Press LLC, Florida (pp 143-165).

Rier ST, Tuchman NC, Wetzel RG (2005) Chemical changes to leaf litter grown under elevated CO_2 and the implications for microbial utilization in a stream ecosystem. Can J Fish Aquat Sci 62:185–194. https://doi.org/10.1139/f04-148

Rothstein DE, Vitousek PM, Simmons BL (2004) An exotic tree alters decomposition and nutrient cycling in a Hawaiian montane forest. Ecosystems 7:805–814. https://doi.org/10.1007/s10021-004-0009-y

Rouse JD, Bishop CA, Struger J (1999) Nitrogen pollution: an assessment of its threat to amphibian survival. Environ Health Perspect 107:799-803. https://doi.org/10.1289/ehp.99107799

Schabetsberger R, Jehle R, Maletzky A, Pesta J, Sztatecsny M (2004) Delineation of terrestrial reserves for amphibians: post-breeding migrations of Italian crested newts (*Triturus c. carnifex*) at high altitude. Biol Conserv 117:95-104. https://doi.org/10.1016/S0006-3207(03)00268-4

Schiesari L (2006) Pond canopy cover: a resource gradient for anuran larvae. Freshw Biol 51:412-423. https://doi.org/10.1111/j.1365-2427.2005.01497.x

Semlitsch RD, Scott DE, Pechmann JHK (1988) Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. Ecology 69:184-192. https://doi.org/10.2307/1943173

Skelly DK, Freidenburg LK, Kiesecker JM (2002) Forest canopy and the performance of larval amphibians. Ecology 83: 983-992. https://doi.org/10.1890/0012-9658(2002)083[0983:FCATPO]2.0.CO;2

Skelly DK, Halverson MA, Freidenburg LK, Urban MC (2005) Canopy closure and amphibian diversity in forested wetlands. Wetl Ecol Manag 13:261–268. https://doi.org/10.1007/s11273-004-7520-y

SNIRH (2019) Sistema Nacional de Informação de Recursos Hídricos. Available at: https://snirh.apambiente.pt/snirh/_dadosbase/site/simplex.php?OBJINFO=DADOS&FILTRA_BACIA=138&FILTRA_COVER=920123704&FILTRA_SITE=920685260

Stock WD, Wienand KT, Baker AC (1995) Impacts of invading N_2-fixing *Acacia* species on patterns of nutrient cycling in two Cape ecosystems: evidence from soil incubation studies and 15N natural abundance values. Oecologia 101:375-382. https://doi.org/10.1007/BF00328825

Storfer A, White C (2004) Phenotypically plastic responses of larval tiger salamanders, *Ambystoma tigrinum*, to Different Predators. J Herpetol 38:612-615. https://doi.org/10.1670/24-04N

Tachet H, Richoux P, Bournaud M, Usseglio-Polatera P (2006) Invertébrés d'eau douce: systématique, biologie, écologie (Vol. 15).CNRS éditions, Paris.

Temmink JHM, Field JA, Van Haastrecht JC, Merkelbach RCM (1989) Acute and sub-acute toxicity of bark tannins in carp (*Cyprinus carpio* L.). Water Res 23:341-344. https://doi.org/10.1016/0043-1354(89)90100-0

Thurgate NY, Pechmann JHK (2007) Canopy closure, competition, and the endangered dusky gopher frog. J Wildl Manag 71:1845-1852. https://doi.org/10.2193/2005-586

Tindale MD, Roux DG (1974) An extended phytochemical survey of Australian species of *Acacia*: chemotaxonomic and phylogenetic aspects. Phytochemistry 13:829-839. https://doi.org/10.1016/S0031-9422(00)91147-6

Travis J (1984) Anuran size at metamorphosis: experimental test of a model based on intraspecific competition. Ecology 65:1155–1160. https://doi.org/10.2307/1938323

Van Buskirk J (2009) Natural variation in morphology of larval amphibians: Phenotypic plasticity in nature?. Ecol Monogr 79:681-705. https://doi.org/10.1890/08-1692.1

Van Buskirk J, McCollum SA, Werner EE (1997) Natural selection for environmentally induced phenotypes in tadpoles. Evolution 51:983-1992. https://doi.org/10.1111/j.1558-5646.1997.tb05119.x

Vinton MA, Burke IC (1995) Interactions between individual plant species and soil nutrient status in shortgrass steppe. Ecology 76:1116-1133. https://doi.org/10.2307/1940920

Vitousek PM, Howarth RW (1991) Nitrogen limitation on land and in the sea: how can it occur?. Biogeochemistry 13:87-115. https://doi.org/10.1007/BF00002772

Vitousek PM, Walker LR, Whiteaker LD, Muellerdombois D, Matson PA (1987) Biological invasion by *Myrica faya* alters ecosystem development in Hawaii. Science 238:802–804. https://doi.org/10.1126/science.238.4828.802

Vitousek PM, White PS (1981) Process studies in succession. In: West DC, Shugart HH, Botkin DB (ed) Forest succession. Springer, New York (pp 267-276).

Vivrette NJ, Muller CH (1977) Mechanism of invasion and dominance of coastal grassland by *Mesembryanthemum crystallinum*. Ecol Monogr 47:301-318. https://doi.org/10.2307/1942519

Vu VQ (2011) ggbiplot: A ggplot2 based biplot. R package (Version 0.55). http://github.com/vqv/ggbiplot

Warburg MR (1992) Breeding patterns in a fringe population of fire salamanders, *Salamandra salamandra*. Herpetol J 2:54-58.

Warburg MR, Degani G, Warburg I (1979) Growth and population structure of *Salamandra salamandra* (L.) larvae in different limnological conditions. Hydrobiologia 64:147–155. https://doi.org/10.1007/BF00023190

Wedin DA, Tilman D (1996) Influence of nitrogen loading and species composition on the carbon balance of grasslands. Science 274:1720-1723. https://doi.org/10.1126/science.274.5293.1720

Werner E, Glennemeier K (1999) Influence of forest canopy cover on the breeding pond distributions of several amphibian species. Copeia 1999:1-12. https://doi.org/10.2307/1447379

Werner EE, Skelly DK, Relyea RA and Yurewicz KL (2007) Amphibian species richness across environmental gradients. Oikos 116:1697-1712. https://doi.org/10.1111/j.0030-1299.2007.15935.x

Wickham H (2016) ggplot2: Elegant graphics for data analysis. Springer-Verlag, New York.

Wilbur HM, Collins JP (1973) Ecological aspects of amphibian metamorphosis: nonnormal distributions of competitive ability reflect selection for facultative metamorphosis. Science 182:1305-1314. https://doi.org/10.1126/science.182.4119.1305

Williams BK, Rittenhouse TAG, Semlitsch RD (2008) Leaf litter input mediates tadpole performance across forest canopy treatments. Oecologia 155:377–384. https://doi.org/10.1007/s00442-007-0920-y

Winder M, Berger SA, Lewandowska A et al. (2012) Spring phenological responses of marine and freshwater plankton to changing temperature and light conditions. Mar Biol 159:2491–2501. https://doi.org/10.1007/s00227-012-1964-z

Wittenberg R, Cock MJW (2005) Best practices for the prevention and management of invasive alien species. In: Mooney HA, Mack RN, McNeely JA, Neville LE, Schei PJ & Waage JK (ed) Invasive alien species: a new synthesis. Island Press, Washington DC (pp 209-232).

Wood SN (2004) Stable and efficient multiple smoothing parameter estimation for generalized additive models. J Am Stat Assoc 99:673-686. https://doi.org/10.1198/016214504000000980

Wood SN, Scheipl F (2017) gamm4: Generalized Additive Mixed Models using 'mgcv' and 'lme4'. R package (Version 0.2-5). https://CRAN.R-project.org/package=gamm4

Yurewicz KL (2004) A growth/mortality trade-off in larval salamanders and the coexistence of intraguild predators and prey. Oecologia 138:102–111. https://doi.org/10.1007/s00442-003-1395-0

APPENDICES

Appendix A – Sampling sites coordinates, property and samples conducted on each site. Reference system: WGS84 / Pseudo Mercator - EPSG: 3857.

X-coordinate	Y-coordinate	Property	Chemical analyses	Zooplankton and small macroinvertebrates	Amphibians	
-9.43554	38.78323	Convento dos Capuchos			X	CC1L
-9.43600	38.78354				X	CC2C
-9.43631	38.78378		X		X	CC3L
-9.43851	38.78491		X	X	X	CC4C
-9.43853	38.78485				X	CC5C
-9.39335	38.78626	Parque da Pena			X	PP1C
-9.39401	38.78603				X	PP2C
-9.39425	38.78572				X	PP3C
-9.39372	38.78439				X	PP4C
-9.39374	38.78410				X	PP5C
-9.39381	38.78419				X	PP6C
-9.39296	38.7838		X	X	X	PP7C
-9.39182	38.78491				X	PP8C
-9.39274	38.78582				X	PP9C
-9.38815	38.78785				X	PP10C
-9.38807	38.78814				X	PP11C
-9.41999	38.79245	Parque de Monserrate			X	PM1C
-9.4226	38.79322		X	X	X	PM2C
-9.42057	38.79285				X	PM3C
-9.42857	38.76957	Perímetro Florestal	X	X	X	AC1C
-9.42969	38.77062				X	AC2C
-9.43270	38.77536				X	AC3C
-9.44458	38.78072				X	PF1C
-9.44405	38.78216		X	X	X	PF2L
-9.44371	38.78448				X	PF3L
-9.4371	38.78476				X	PF4L
-9.43628	38.76988		X		X	PF5C
-9.44415	38.76974				X	PF6C
-9.43509	38.78278	Tapada de D. Fernando			X	TF1L
-9.43463	38.78263				X	TF2C
-9.43288	38.78300		X	X	X	TF3C
-9.43545	38.78317				X	TF4C
-9.41858	38.78309	Tapada de Monserrate	X	X	X	TM1L
-9.40806	38.77926	Tapada do Mouco			X	TO1C
-9.40703	38.77992				X	TO2C
-9.40595	38.78092		X	X	X	TO3C
-9.41037	38.77698	Tapada do Saldanha	X	X	X	TS1C
-9.40604	38.77621				X	TS2L
-9.41507	38.76004			X	X	TS3C
-9.41501	38.7597				X	AC4L

Appendix B – Total number of amphibian larvae captured and photographed at each site. Note: sampling involved active search, trying to capture as many larval amphibians as possible, and therefore these values do not reflect amphibian abundance at each site. To build this matrix, data from Aguilar and Rebelo (2017) was used and is depicted in **bold**.
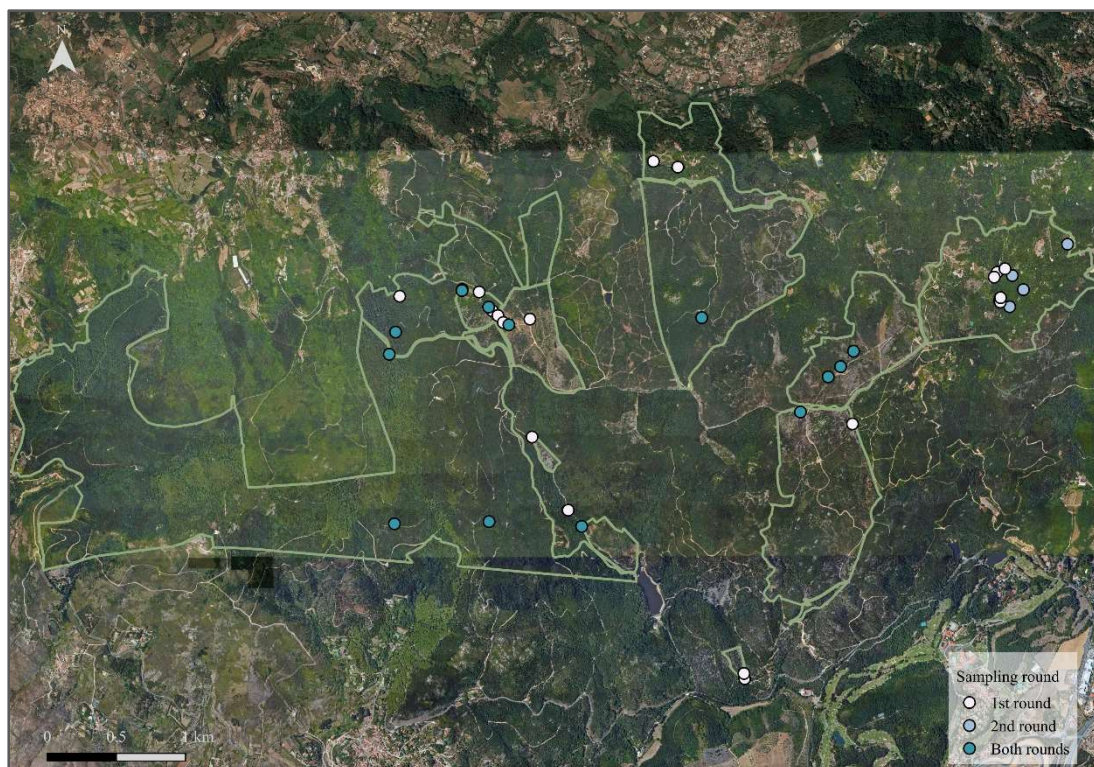
Sampling site	Sample size					
	Pleurodeles waltl	*Salamandra salamandra*	*Lissotriton boscai*	*Triturus marmoratus*	*Bufo spinosus*	*Discoglossus galganoi*
CC1L	0/**0**	28/**1**	0/**0**	0/**0**	0/**6**	0/**0**
CC2C	0/**0**	38/**8**	0/**1**	0/**0**	0/**0**	0/**0**
CC3L	0/**0**	42/**0**	1/**3**	2/**0**	0/**92**	0/**26**
CC4C	0/**0**	19/**12**	1/**0**	0/**0**	0/**0**	0/**0**
CC5C	0/**0**	25/**0**	1/**4**	0/**11**	0/**0**	0/**0**
PP1C	0	3	0	0	0	0
PP2C	0	21	0	0	0	0
PP3C	0/**0**	12/**4**	0/**0**	0/**0**	0/**0**	0/**0**
PP4C	0	60	0	0	0	0
PP5C	0	12	0	0	0	0
PP6C	2	4	0	0	0	0
PP7C	0/**0**	7/**4**	0/**2**	0/**0**	0/**0**	0/**0**
PP8C	0/**0**	20/**12**	3/**2**	0/**1**	0/**0**	0/**0**
PP9C	0/**0**	6/**7**	0/**3**	0/**0**	0/**0**	0/**0**
PP10C	0	4	0	6	0	0
PP11C	0	0	0	19	0	0
PM1C	0/**0**	0/**0**	0/**0**	3/**0**	64/**42**	0/**0**
PM2C	0	1	2	0	0	0
PM3C	0	15	0	0	0	0
AC1C	0	45	32	6	0	0
AC2C	0	32	0	0	0	0
AC3C	0	22	0	0	0	0
PF1C	0	33	0	0	0	0
PF2L	0/**0**	9/**0**	0/**0**	0/**0**	41/**0**	0/**0**
PF3L	0/**0**	12/**5**	0/**0**	0/**0**	0/**0**	0/**0**
PF4L	0/**0**	6/**0**	0/**3**	0/**0**	0/**0**	0/**0**
PF5C	0	82	0	5	0	0
PF6C	0	42	0	0	0	0
TF1L	0/**0**	31/**4**	0/**0**	0/**0**	0/**21**	0/**0**
TF2C	0	51	0	0	0	0
TF3C	0/**0**	8/**0**	30/**2**	0/**3**	0/**0**	0/**0**
TF4C	0/**0**	0/**3**	0/**0**	0/**0**	0/**12**	25/**0**
TM1L	0/**0**	38/**13**	0/**0**	0/**0**	33/**16**	0/**0**
TO1C	0	52	0	0	0	0
TO2C	0	34	0	0	0	0
TO3C	0	41	0	0	0	0
TS1C	0/**0**	24/**1**	25/**13**	0/**0**	0/**0**	0/**0**
TS2L	0/**0**	24/**12**	0/**0**	0/**0**	0/**0**	0/**0**
TS3C	0/**0**	6/**0**	10/**15**	3/**3**	0/**1**	0/**0**
AC4L	0	1	6	1	0	0

Appendix C – Study area. Green lines represent the sampled properties. Circles of different sizes represent fire salamander, zooplankton and small macroinvertebrates and chemical analyses sampling sites.
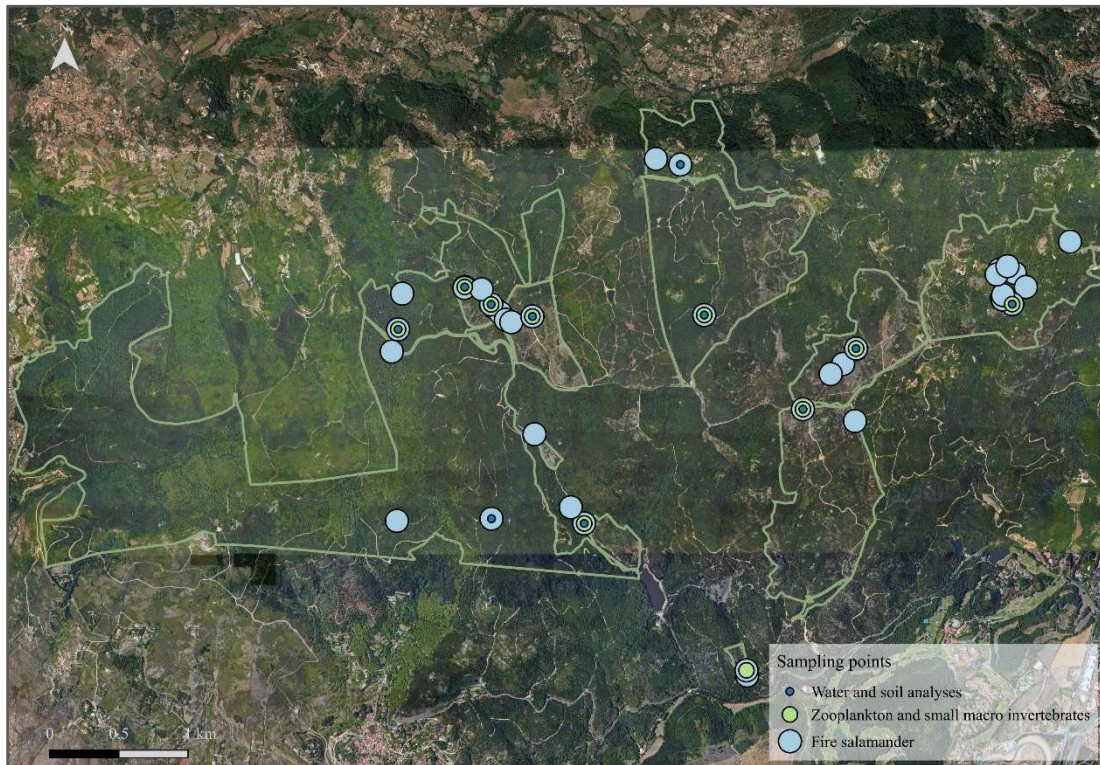
Appendix D – Study area. Green lines represent the sampled properties. Circles represent fire salamander sampling sites sampled on each round.

Appendix E – Larvae of the different amphibian species sampled during this study.
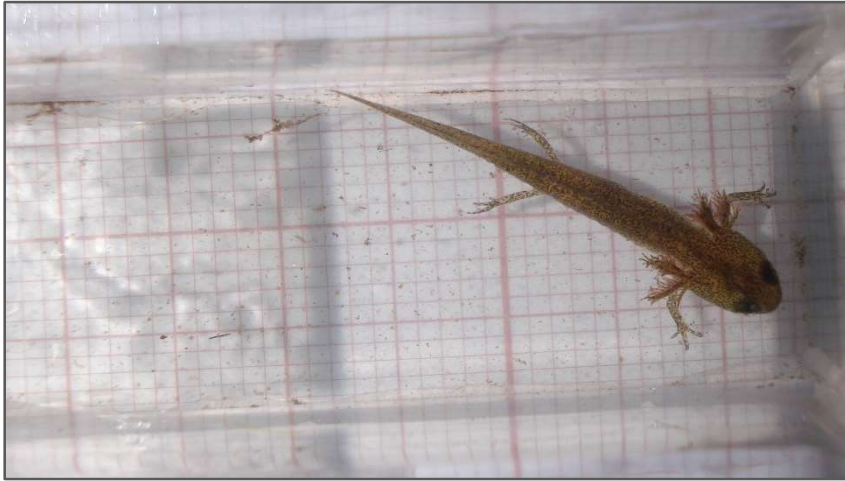

Discoglossus galganoi Capula, Nascetti, Lanza, Bullini and Crespo
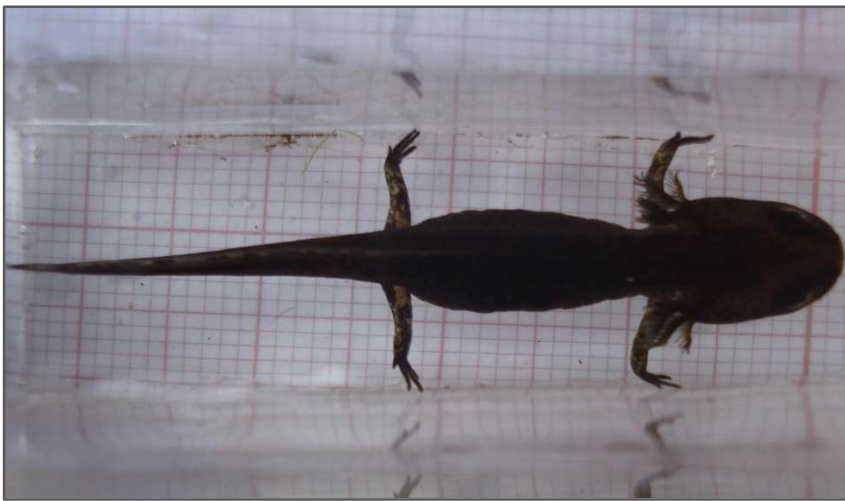

Bufo spinosus Daudin

Triturus marmoratus Latreille
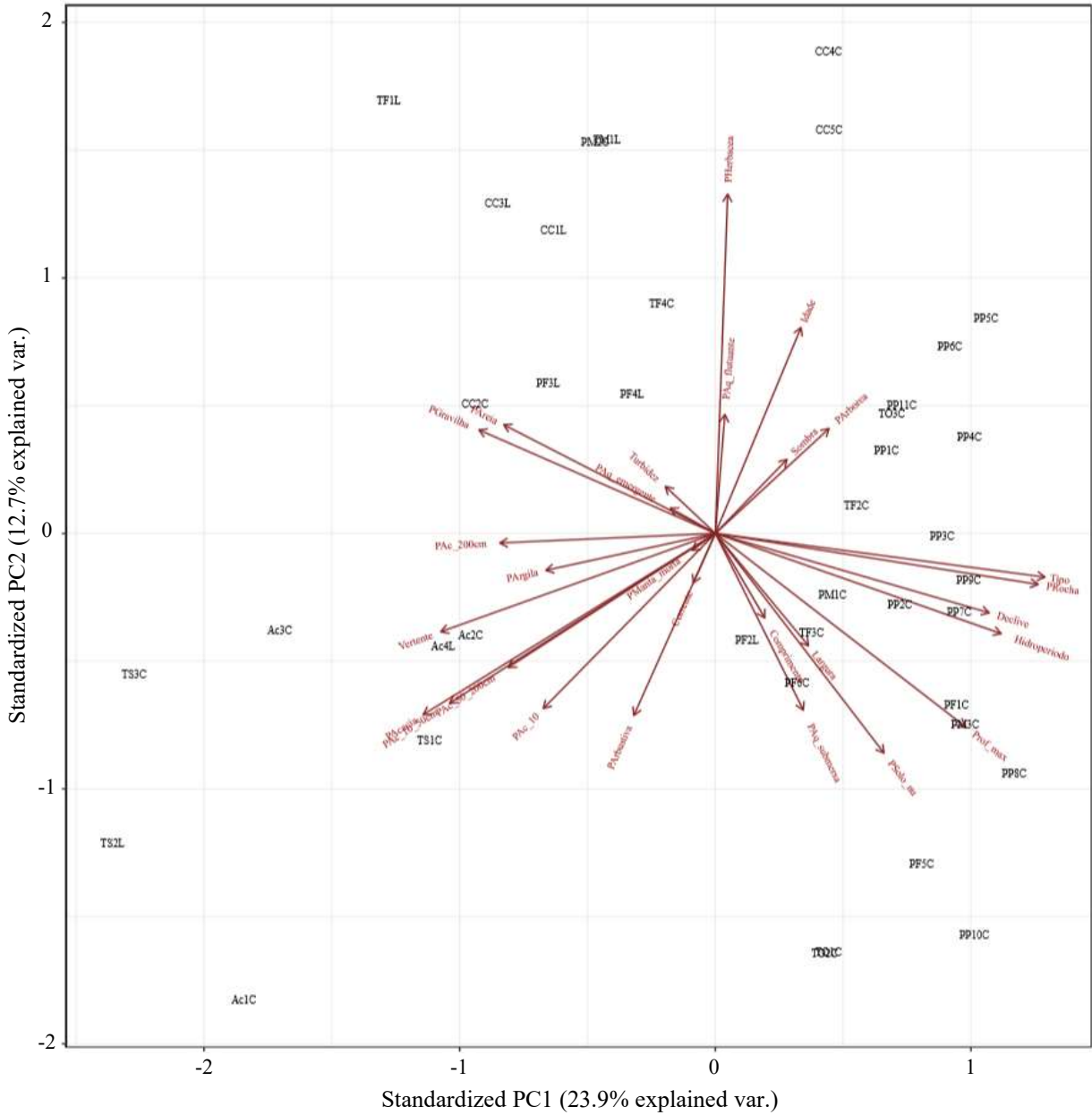
Lissotriton boscai Lataste

Salamandra salamandra L.

Pleurodeles waltl Michahelles

Appendix F – Graphic depiction of the PCA result. PC1 explains 23.9% and PC2 explains 12.7% of the variability. The code of all sampling sites is depicted in black. Ponds are not sorted into groups.

Appendix G – Spatial autocorrelation tests' results. No spatial autocorrelation was found.

	SVL	Maximum abdominal width/Snout-vent length	Middle tail muscle depth/Middle tail fin depth	Kurtosis Maximum abdominal width/Snout-vent length	Kurtosis Middle tail muscle depth/Middle tail fin depth
			1st round		
Moran's Index	0.207392	0.126439	0.297126	0.053011	0.048214
z-score	1.274004	0.833262	1.751883	0.420650	0.413180
p-value	0.202662	0.404697	0.079794	0.674010	0.679475

	SVL	Maximum abdominal width/Snout-vent length	Middle tail muscle depth/Middle tail fin depth	Kurtosis Maximum abdominal width/Snout-vent length	Kurtosis Middle tail muscle depth/Middle tail fin depth
			2nd round		
Moran's Index	0.270474	0.341663	0.362064	-0.020435	0.022141
z-score	1.479223	1.832529	1.936596	0.047589	0.273068
p-value	0.139081	0.066873	0.052795	0.962044	0.784801

Appendix H – Kurtoses values of the A/SVL and M/F ratios. "–": number of salamander larvae sampled not high enough to calculate the kurtosis; Blank cells: no salamander larvae were found in that round. In that case, we considered the single kurtosis value available.

Sampling site	A/SVL ratio Kurtosis values			M/F ratio Kurtosis values		
	1st round	2nd round	Both rounds	1st round	2nd round	Both rounds
AC1C	0.885	-0.106	0.412	-0.051	2.292	4.488
AC2C	0.602			0.246		
AC3C	1.047			7.295		
AC4L	–			–		
CC1L	-0.195			-0.851		
CC2C	6.472			5.844		
CC3L	4.347	-0.498	2.085	-0.346	0.134	0.663
CC4C	-0.546	–	-0.823	-0.532	–	-0.783
CC5C	1.356	–	0.607	0.317	–	1.183
PF1C	2.703	–	0.433	1.556	–	1.236
PF2L	–	–	–	–	–	–
PF3L	-0.129			-0.904		
PF4L	–			–		
PF5C	4.427	0.061	-0.633	8.424	2.442	3.760
PF6C	-1.012	-1.126	-1.178	2.033	-1.172	1.392
PM2C	–			–		
PM3C	0.243			0.151		
PP1C	–			–		
PP2C	2.032			-1.043		
PP3C	-1.056			-0.504		
PP4C	3.120			-0.189		
PP5C	-0.314			-0.835		
PP6C	–			–		
PP7C		–			–	
PP8C		-0.297			-0.923	
PP9C		–			–	
PP10C		–			–	
TF1L	3.808			-0.118		
TF2C	0.561	3.304	-0.109	-0.651	0.595	2.891
TF3C	–			–		
TM1L	1.177	1.592	2.958	-0.092	3.473	3.879
TO1C	0.032	-0.548	-0.381	-0.067	0.434	1.199
TO2C	3.664	-0.141	1.035	0.005	1.704	3.400
TO3C	-0.349	-1.168	-1.174	0.800	-0.947	-0.021
TS1C	-1.504	0.296	1.797	-0.459	-0.770	-0.201
TS2L	-0.827			6.137		
TS3C	–			–		

Appendix I – Variation of the 2nd round A/SVL ratio as a response to the abundance of zooplankton and small macroinvertebrates.
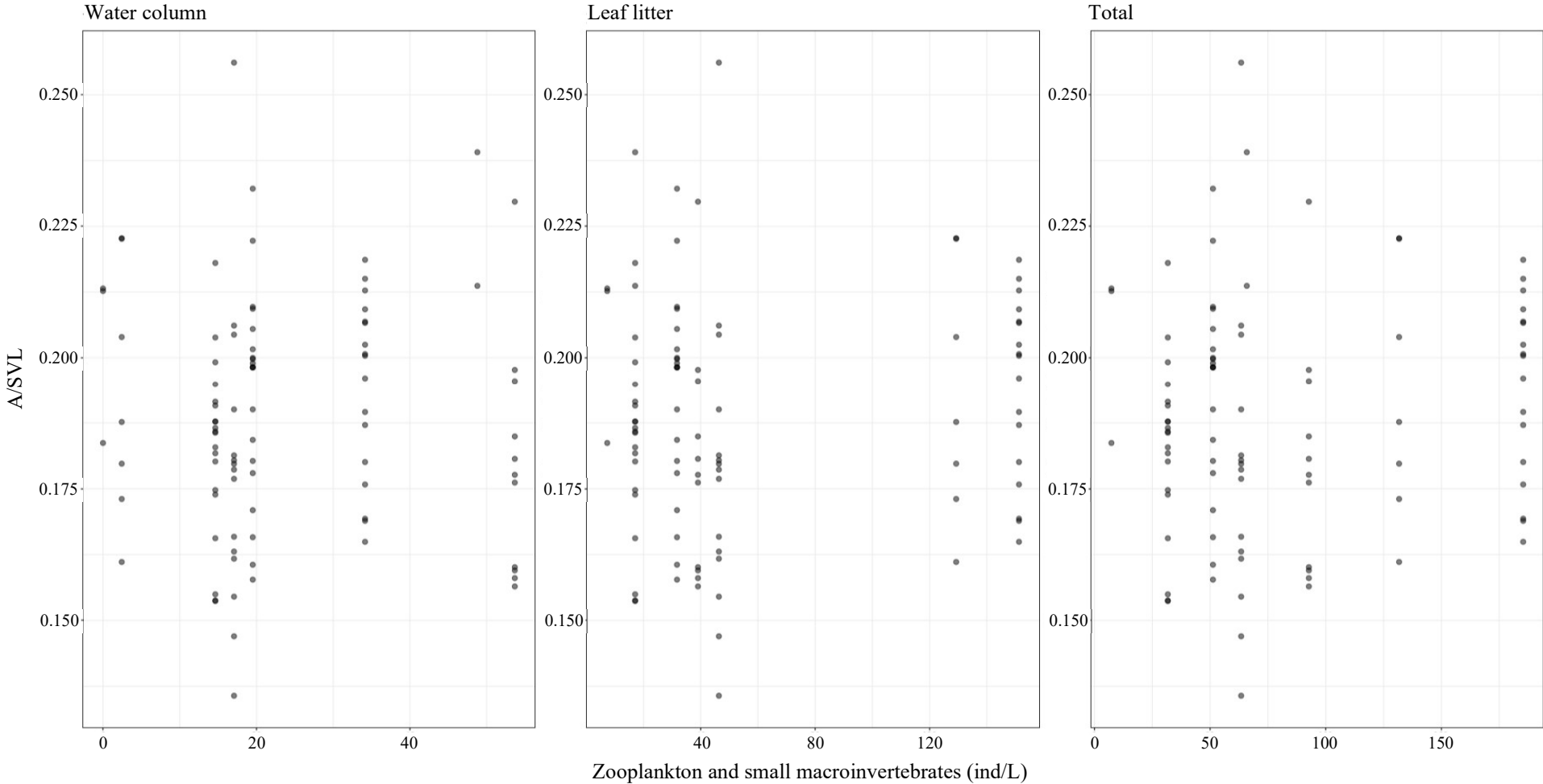
Appendix J - Variation of the 2nd round M/F ratio as a response to the abundance of zooplankton and small macroinvertebrates.