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Anthropic habitats as novel environments: consequences for evolution and conservation

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A mis padres y abuela

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Summary

This Thesis explores how the rapid transformation of the natural habitat to anthropic environments alters the behavioural responses and life-history traits of amphibians and reptiles. This project focuses on two of the most widely extended anthropic habitats of the world: urban habitats and exotic tree plantations. Anthropic environments are actual new habitats, with selective pressures different from natural ones. The populations inhabiting these disturbed habitats need to quickly evolve or to show phenotypic plasticity to adapt and survive in the new environmental conditions. In that sense, anthropic habitats provide a unique opportunity to study the adaptation process (or the lack of it) and the ability of the species to plastically respond to the new environmental pressures, so in the last years the interest of the scientist on this research area has increased drastically.

This Thesis delves into the effect of the presence of these two novel environments on the functional ecology of individuals, using amphibians and reptiles as model study species. The Thesis includes both field work and laboratory experiments, sometimes combining both techniques in a single chapter to refine the results.

Exotic plantations

First, field work showed that the richness and abundance of amphibians was lower in pine and eucalypt plantations compared to the natural oak forest. We combined this data with an experimental study in mesocosms were we found that the presence of leachates released by the exotic trees may explain, at least in part, the results found in the field (Chapter 1).

The next three chapters (Chapters 2-4) are based on the palmate newt, the only species that appeared frequently in both exotic plantations and oak forests. First, we tested the condition-dependent trade-offs between sexual traits, body condition and immunity in male newts (Chapter 2). Although we did not find changes in the reliability of sexual signals among habitats, males inhabiting exotic plantations showed a reduced ability to develop secondary sexual traits and had lower immune responses. To test if these results were driven by the presence of the toxic leachates in the water we performed a mesocosms experiment. We found that a short exposure to these chemical

compounds reduced the immune response, but not the body condition and sexual traits of males, suggesting that other characteristics of the habitat, or the ecology of the species, may also explain part of the variance found in the field.

In the next experiment (Chapter 3), a two choice experiment in the lab, we found that the exposure to eucalypt leachates reduces the ability of females to choose the best males. However, female preferences were affected after a long exposure of 21 days, but not after an immediate exposure to the water containing eucalypt leachates. These results suggest that the effect is driven by long-term changes in female physiology rather than by the immediate inhibition of transmission or reception of pheromonal signals. This contrasts with the next study (Chapter 4), where we found that immediate exposure to eucalypt leachates affected the ability of male newts to detect ponds with female odour and conspecific alarm cues, but did not affect the ability of newts to find food items. These contrasting results are a reminder that it is often difficult to predict the exact effects of novel evolutionary pressures on species.

Urbanization

The last two chapters of this Thesis explore the effect on some life-history traits of amphibians and the behaviour of a lizard species inhabiting urban areas.

In the case of the amphibians (Chapter 5), we found that some traits, such as body size were larger in urban environments (in 3 of the 4 studied species), while differences in body condition depended on the studied species (it was better in the urban area in the case of the aquatic newts). However we found no effect of urbanization on the immune response of individuals. These results suggest that different species of amphibians may be affected differently by anthropogenic habitat alteration depending on their specific ecology.

Finally, we explored the potential habitat dependent trade-off between lowering predation risk and elevating body temperature to fight infection in the Catalonian wall lizard (Chapter 6). We found that while such trade-off exists in the case of the natural population, the differences in the relative risk of predation versus that of acquiring or countering an infection in the urban population eliminates the existence of this trade-off.

Introduction

1. Anthropic environments as new habitats

In the last century, the natural environment has been transformed by human activities on an unprecedented scale. Rapid and profound anthropogenic alterations, such as habitat destruction, climate change and the worldwide introduction of invasive species, are forcing wildlife to confront new environmental conditions within a very short time-frame. Anthropic habitats often impose a variety of novel conditions ranging from reduced predation risk (Gering and Blair 1999), to unusual food sources (Ditchkoff et al. 2006), or drastic alterations of the chemosphere through eutrophication or spillage of pollutants like herbicides or pesticides (Candolin et al. 2008). In response to such perturbations, some species produce phenotypic changes that are seemingly well adapted to these new habitats (e.g. Slabbekoorn and Peet 2003), while others do not (e.g. Lengagne 2008) or might even respond in seemingly maladaptive ways (Battin 2004).

Natural populations are, at least, partially adapted to their environment. However, the intensity and direction of the selection fluctuates locally over time, so the adaptation process is a permanent necessity (Carroll et al. 2007). Anthropic environments are actual new habitats, with selective pressures different from natural ones, and that are exploited by certain species which are simultaneously found in natural habitats. Some examples of the challenges wildlife have to face in our changing world include urbanization, the increase in area covered by agrosystems (e.g. crop fields and exotic tree plantations), climate change, the introduction of exotic species, the eutrophication and the spillage of chemical compounds. The comparison of animal populations found in both their natural habitat and in anthropic environments allows us to examine how the constant dynamic adaptation to the environment occurs and whether it can keep pace with the dramatic appearance of anthropized habitats. Numerous studies document rapid phenotypic changes as a consequence of adaptation to new environments (Räsänen et al. 2003). Anthropic habitats provide a unique opportunity to study the adaptation of the species that occupy them, and in the last years the interest of the scientist in this area has increased drastically (see reviews Brockerhoff et al. 2008; Candolin and Heuschele 2008; Lowry et al. 2013).

This Thesis focuses on two of the most drastic transformations of the habitat that are reducing diversity and driving wildlife populations to extinction all over the word: the growth of urban areas and the massive replacement of natural forests by exotic tree plantations.

1.1 Urbanization

The most drastic and widespread of the habitat alterations nowadays is urbanization, and it is one of the leading causes of species extinction (Czech et al. 2000). Urbanization is the conversion of natural habitats into areas partly covered by buildings, heavily fragmented, and with a high level of edge effects. Cities are expanding worldwide and predictions suggest that approximately 60% of the world's human population will be living in urbanized landscapes in 2030. The effects of urbanization are longer, lasting and more extreme (often land alteration intensifies with time so that there is no opportunity for successional recovery) than those accompanying other anthropogenic land uses (Marzluff and Ewing 2001). In cities, animal and plant diversity is usually lower (*see section 2.1*), because urbanization i) reduces and fragments the area available for organisms (e.g. over 80% of most downtown urban areas are covered by pavement and buildings; Blair and Launer, 1997), ii) simplifies vegetation structure (due to removal of shrubs and dead wood and an increase in grasses and herbs; Marzluff and Ewing, 2001), iii) faces wildlife with novel challenges such as changes in natural resources (e.g. habitat and food) and elevated anthropogenic disturbance levels (e.g. louder noise) (Fernandez-Juricic and Telleria 2000). However, some aspects of urbanization, such as a high spatial habitat heterogeneity (Savard et al. 2000) or the high rate of dispersal of non-native species related to human activities (Mack and Lonsdale 2001) can promote increasing levels of biodiversity, usually by the addition of non-native species that replace native species faster than they are lost (*see section 2.1*) (McKinney 2002).

In the last decade, a growing amount of studies have focused not only on the loss/gain of diversity in urban areas, but on the ecological and evolutionary mechanisms that drive the differences among species to cope with urban alteration. The two chapters of the present Thesis about urbanization are also set in this research area and focus on how urbanization can affect the immune response and life-history traits (Chapter 5) and induce behavioural changes (Chapter 6). We selected these traits because they are likely to be affected by the new environmental conditions and may reflect the trade-offs faced by individuals in these novel habitats. For example, changes in the allocation of resources due to differences in the environmental pressures may affect both the immune response and other life-history traits. The reduced number of species in anthropic habitats may alter the interspecific relationships, which may affect the intensity of parasites; it could be lower if the number of intermediate hosts is fewer; or higher if the transmission increases due to higher animal densities (derived from the absence of competitors or predators). These higher parasite rates, or a lower predatory pressure, may, at the same time, alter the resources allocate to each trait. Likewise, other factors, as the pollution, may negatively affect the immune response. Finally, behaviour is the most plastic characteristic of individuals, and may change rapidly to compensate the effect of some environmental alterations. Behaviour may help soften some trade-offs and facilitate the mentioned allocation of resources among different traits related to survival (*see section 2.5*). We now have evidence about the importance of phenotypic plastic behaviours of the species (*see section 2.5*), and animal personalities to respond to urban conditions and inhabit novel environments. However, although behavioural plasticity helps some species, the persistence of these species in human-induced novel environments is being threatened by increasingly common maladaptive behaviours called ‘evolutionary traps’ (*see section 2.2*).

1.2 Exotic tree plantations

Other habitat alterations, such as the massive replacement of the natural forests by exotic tree plantations, have apparently attracted less interest among scientists. However, these habitats occupy a total of 7% of the global forested area is occupied by plantations (MacDicken 2015), and are mainly used to produce paper, timber or charcoal. The replacement of the natural forest can cause important changes in diversity and community composition at local and regional scales (Brockerhoff et al. 2001). However, there is a strong controversy about the damage or benefits for the diversity and species conservation in these habitats (*see below, section 2.1*).

Strikingly, studies about tree plantations have focused mainly on patterns of abundance and diversity of different taxa (Carrascal and Tellería 1990; Sax 2002; Zurita et al. 2006), but very few researches have progressed into mechanistic ecology, such as the behavioural ecology of species interactions and evolution. One of the aims of the present Thesis is to address this gap in knowledge by exploring the effects of exotic plantations not only on the diversity (see Chapter 1), but also on some ecological and behavioural aspects of amphibians (see Chapters 1, 2, 3 and 4). In these plantations, wildlife have to deal with changes in the habitat structure, such as a higher density, but lower diversity, of trees than in natural forests (Lindenmayer and Hobbs 2004), and often with the appearance of recently introduced chemicals released by plants (Iglesias-Carrasco et al. 2016b) (Chapter 2).

Eucalypt (*Eucalyptus* sp.) and pine plantations (*Pinus* sp.) are the two most common types of exotic plantations worldwide. As with many non-native plants, eucalypt and pine plantations release toxic substances into the soil (Souto et al. 1994) and waterways (Bärlocher and Oertli 1978; Pozo et al. 1998), reduce water yields, and change physical characteristics of soil (Florence 1986; Farley et al. 2004). The combined effect of the structure change and the exposure to novel chemical compounds (Maerz et al. 2005) produced by exotic plants may represent cryptic threats to native fauna inhabiting plantations. For example, allelophatic compounds from exotic plants are known to inhibit the growth of other plants (Ahmed et al. 2008) and affect native vegetation community diversity and composition (Tererai et al. 2013). Invertebrate species composition of stream and riverine communities also changes under eucalyptus plantations, due to the combined toxicity and low quality of leaf litter (Larrañaga et al. 2009). Four of the chapters of the present Thesis focus on stationary (lentic) water systems under pine and eucalypt plantations. It can be expected that the effects of the leachates (secondary compounds, such as tannins, released by leaves) will be exacerbated due to the steady accumulation of leaf litter and the lack of water renewal (Canhoto and Laranjeira 2007). Likewise, this kind of ponds may be used by both amphibians with fast and slow larval development times. However, due to their accelerate metabolism we may expect the effects of leachates to be stronger in the species with fast development. In that sense, species with different life histories may show different vulnerability in the face of environmental alteration.

2. Adaptive and maladaptive responses of animals to anthropic environments

2.1 Effects on animal diversity

Critical transformation of the habitat (i.e. destruction of the habitat of native species) alters the composition of wildlife communities. Habitat alteration usually leads to an overall loss of native biodiversity. However, when the alteration causes increased resource availability (Marzluff 2001), simplified habitat structures, and changes in the trophic interactions (Faeth et al. 2005), it may promote the establishment and increased abundances of a small number of species that do well in these environments, frequently non-native species. The replacement of wide ranges of native species by a few species well-adapted to human-dominated landscapes is repeated in many regions all over the world, and has been termed "biotic homogenization" (Blair 2001).

Urban areas as the most homogenizing habitats

Urbanization has been suggested as the most homogenizing of human activities (McKinney 2006). Cities are uniform habitats comprising roads, skyscrapers and residential housing in the suburbs, which makes them physically very similar throughout the world. Effect of urbanization on native fauna is known to be more critical in the urban core, and researchers usually analyse urban impacts on natural systems by using the urban–rural gradient (Figure 1). As we approach to the urban core, physical changes such as human population density, road density, fragmentation of natural vegetation, air and soil pollution and average ambient temperature exponentially increase (McKinney 2002).Consequently, most of the species are excluded from the city centre, while others can take advantage, attaining large population sizes and often reaching higher densities than their natural counterparts (Buijs and Van Wijnen 2001; Donnley and Marzluff 2004).

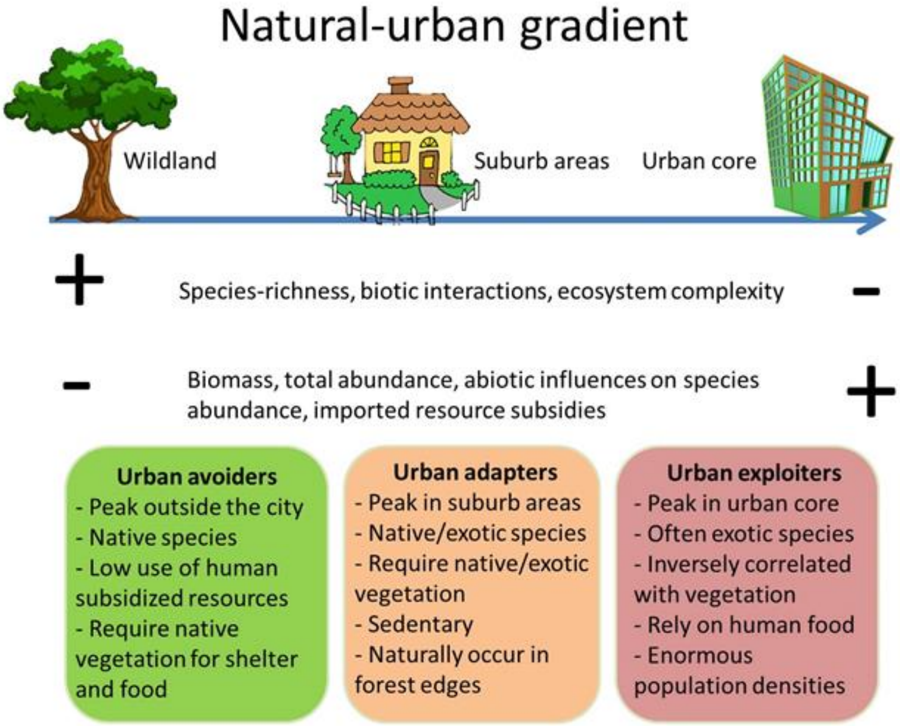

Figure 1. In base of their response human alteration, species have been classified into three distinct categories (Blair 2001): avoiders, the species that are extremely sensitive and disappear quickly when urbanization occurs; adapters, species that can adapt to urban habitats but also use natural resources; exploiters, the species that become dependent on urban resources.

Successful colonisation and invasion of urban areas has been related to high dispersal abilities because, to establish permanent populations, individuals need to reach novel environments at a sufficient rate (Clobert et al. 2001). Likewise, high feeding innovation rates to exploit available resources (ways of foraging that deviate from those commonly recognised for a species) may allow successful exploitation of novel environments (Møller 2009). These two characteristics of urban colonisers and the environmental disturbance in urban areas promote the establishment of more non-native than native species (D’Antonio and Meyerson 2002). Introduced species often have strong competitive abilities, so in human settlements native species are at competitive disadvantage since disturbance alters the natural selection regimes (Byers 2002). In urban areas, exotic species attain population densities far above those found under natural conditions (Buijs and Van Wijnen 2001) because they exploit human resources, have reduced threat of natural enemies, and find an adequate physical environment (e.g. temperature). The same non-native species tend to establish in cities all over the world since they are imported by humans for several reasons (Mack and Lonsdale 2001). Accordingly, highly urbanized habitats should be very similar across the planet, increasing the biotic homogenization.

The controversy about exotic plantations

Tree plantations have been suggested to be favourable habitats for the native wildlife compared to other economically productive land uses, such as agricultural land (Brockerhoff et al. 2008). More than a half of the world animal species live in forests, so the loss of biodiversity due to deforestation is an important conservation concern (Laurance 2007). Natural forests’ area decreases annually, mostly by the conversion to agriculture (FAO 2015). Although plantations of forests only occupy around the 3.5 % of the forested area, the area of tree plantations is increasing annually (FAO 2015). The main aim of tree plantations is the production of timber and wood, so most of the plantations are often monospecific. Moreover, most of the seeds or small trees are planted at the same time and age, so they grow creating a very homogenous habitat with less microhabitat diversity and complexity than natural forests, reducing the quality of plantations as suitable habitats for many species. Moreover, tree plantations are usually associated with intensively managing, such as thinning and clear-cut harvesting and often have short rotations. All these characteristics of the newly created environment make plantation forestry often not compatible with biodiversity conservation goals. For example, some studies have shown that plantations reduce the number of species of important animal groups, such as arthropods and birds (Zurita et al. 2006; Fork et al. 2015). In plantations, forest specialist richness is often lower than in natural forests, but the differences for generalist species is often lower (Magura et al. 2000; Raman 2006). This sometimes leads to similar diversity, but to a loss of important specialist species that changes species composition (Sax 2002).

Some researchers, however, have suggested that tree plantations should be explore in a wider context, and take into account numerous factors that determine the likely effects on biodiversity (Brockerhoff et al. 2008). Exotic plantations may work as corridors in fragmented environments. Forest fragmentation may contribute to species extinction by reducing natural forest patches area, increasing the isolation among patches and increasing the edge effect (Yates et al. 2004). However, the presence of exotic plantations in this fragmented landscape has been proposed to enhance native diversity by improving the connectivity between isolated natural patches (Norton 1998) and by buffering the edge effect of small natural forest remnants (Fischer and Lindenmayer 2006).

Several studies have shown that, if managed appropriately and in extreme cases where natural forests become scarce, plantations can paradoxically contribute to the conservation of diversity harbouring some forests species (Brockerhoff et al. 2008; Mortelliti and Lindenmayer 2015). Even uncommon and threatened species may take advantage of the presence of exotic plantations in some cases.

However, the mere presence of a species may not be a good indicator of the suitability of the habitat. For example, palmate newts inhabit both natural oak forests and exotic plantations, but those in plantations have a poorer immune response and the reduced sexual characters (Iglesias-Carrasco et al. 2016b). Therefore, research may focus not only on the presence and abundance of species in a specific habitat, but on the effects at individual level. Exploring the ecological aspects is critical to fully understand whether and how tree plantations may drive populations to extinction by, for example, the creation of ecological traps (*see section 2.2*).

2.2 Anthropic habitats as ecological and evolutionary traps

Evolutionary traps are low-quality resources that are preferred by animals over better quality resources (Robertson et al. 2013). Natural selection tends to favour the individuals that are capable of using environmental cues to reliably asses resource quality to increase their fitness, so the existence of these traps is surprising. However, human activities alter the environmental conditions at higher rates than animals' evolutionary capacity to respond to change. Behavioural plasticity has buffered, in part, some species against these rapid changes, but maladaptive behavioural responses are increasing.

Animals rely on environmental cues to make decisions about the state of the environments. Anthropic-related environment disturbance can change the relationship between the environmental cue and the ecological conditions that have evolutionarily been associated with. In these cases, the accuracy with which animals assess the fitness value of habitats, mates, or resources will decrease, affecting their fitness. When this happens and organisms prefer to exploit lower quality resources reducing the rewards on fitness, animals are trapped. An evolutionary trap is a form of maladaptation, where any resource (e.g., mate, food, or habitat) is found by organisms equally or more attractive than other available resources despite a reduced fitness value (Robertson et al. 2013).

An ecological trap is an evolutionary trap in which the low quality resource preferred by individuals is the habitat (Donovan and Thompson 2001) (see figure 2). One example of an ecological trap may be found in harvested forests mimicking the appearance, but not the quality of natural habitats. For instance, the olive-sided fly-catcher (*Contopus cooperi*) densities and nestling rates are greater in a managed forest than in naturally burned habitat. However, this human-managed habitat is an ecological trap for the species, since the nest success was roughly half that found in the natural habitat, probably due to a relatively high abundance of nest predators found in the artificially disturbed forest (Robertson and Hutto 2007). Another clear example is when animals draw into the cities to nest, because of plentiful of food and that have fitness consequences. This seems to be the case of Cooper's Hawks (*Accipiter cooperii*) nesting in Tucson, Arizona (Boal and Mannan 1999). The densities of this species are higher in the city, individuals begin nestling earlier and clutches are larger than in natural areas. However, after hatching, nestling mortality is much higher in the city (>50%) than in exurban areas (<5%), probably due to a disease carried by their main preys, pigeons and doves. Urban population should be experiencing significant declines (Boal 1997), but it appears to be stable or increasing, probably due to the immigration of individuals from outside the cities (an attractive sink).

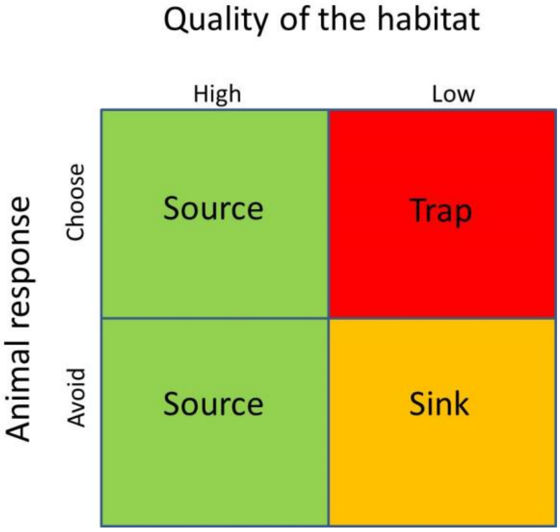

Figure 2. A ecological trap is a sink habitat that is preferred instead of being avoided by individuals (also called attractive sink, Delibes et al. 2001) (Figure modified from Battin 2004).

In the absence of rapid counter-adaptation, evolutionary traps may lead populations to extinction, because animals usually abandon superior resources to exploit and settle in poorer ones (Battin 2004). Human activities can increase the mismatch between the environmental cue and the ecological conditions by, for example, altering the cues in which animals rely on. In that sense, some human created surfaces can appear attractive, while reduce the fitness and increased mortality of individuals. For example, wayward orientation of sea turtle hatchlings in the presence of artificial lights (Witherington 1997) or oviposition of aquatic invertebrates in polarized-reflecting glass buildings that resembles aquatic ponds (Kriska et al. 2008). In other cases, the environmental cue does not change, but the fitness benefit associated with the preferred resource is reduced. Examples of that phenomenon can be found in managed forests, where some saproxilic species use the forest fuel pills to lay eggs that die when the wood is collected (Hedin et al. 2008).

Strikingly, not all the species are prone to being trapped (reviewed in Robertson et al. 2013). It has been suggested that the susceptibility to getting trapped will increase if the cues for novel poor options closely resemble what had indicated good options in the past; and if in the past the costs of rejecting good options was high, but the cost of accepting a poor option was low. However, the susceptibility to getting trap may also depend on the age, sex, condition and personality (*see section 2.5*). For example, bold individuals may be more likely to use novel poor resources and require stronger cues to avoid danger. Intraspecific relationships may also promote individuals to be trapped by coping others that are already trapped (López-Sepulcre and Kokko 2012); or if high-quality and dominant individuals get trapped into low-quality resources, they can exclude lower-quality subordinates and push them into better habitats (Hollander et al. 2011). Finally, the susceptibility to being trapped will depend also of cue reliability, which depends on the transmission of the cue in the environment and the perception abilities of animals (McNamara et al. 2011). So many human stressors that block cue transmission or alters cue reception (chemical pollution of the water, for example. see Chapter 4, Fisher et al. 2006) may, therefore, increase the susceptibility of individuals to be trapped.

It is not always easy to identify an evolutionary trap and lots of studies report findings that are consistent with the presence of a trap, but ignore the concept (Battin

2004). However, identifying such traps is critical for the conservation of species. Different studies have suggested measures to eliminate the impact of evolutionary traps (Gilroy and Sutherland 2007; Robertson et al. 2013). The main three ideas are: i) reduction of the attractiveness and misleading cues of the bad-quality resources; ii) increase habitat quality (e.g. control of predators) to increase fitness values of falsely attractive resources; and iii) limit access to traps. For example, to reduce misleading cues that trigger sea turtles navigation up the beach it has been suggested reducing the number and intensity of light and applying red lighting filters, and physically block artificial light sources (Tuxbury and Salmon 2005). Increasing the habitat connectivity between attractive patches or reducing the connectivity between high-quality resources and low-quality resources may also help to mitigate the effect of traps. However, although evolutionary traps are seen as a potential conservation concern, removal of the traps has been suggested to be impossible in most cases (Battin 2004).

2.3 Sensory disturbance

In most aquatic and terrestrial species, communication and detection based on chemical, visual and auditory signals are important for social interactions among conspecifics and interspecific relationships. This sensory exchange can affect many key life-history traits including those involved in predator avoidance, prey detection, mate searching, mate choice and social grouping (Todd et al. 1967; Johansson and Jones 2007; Ferrari et al. 2010). Anthropic environments produce a number of sensory disturbances, such as chemical pollution in exotic plantations, or light pollution and constant background noise in urban areas.

Research in urban environments has mostly focused on bird vocalization changes due to urban noise. We now know that animals can improve signal transmission in noisy habitats by altering or adjusting their acoustic communication patterns (Catchpole and Slater 2008). However, modification of behaviour in response to noise may involve costs. For example, it has been proposed that short-term adjustments can be energetically costly, so some species are likely to lack the physiological capacity to modify their behaviour (Oberweger and Goller 2001). In addition, noise can negatively affect reproduction and population growth by negatively impacting on sexual signals (Laiolo 2010), or by changing females preferences (Slabbekoorn and Ripmeester 2008).

Similarly, alteration of chemical detection can have drastic consequences for predator detection, foraging success, the ability to locate and discriminate between potential mates or, in extreme cases, to recognize conspecifics (Wolf and Moore 2002,Gill and Raine 2014, Polo-Cavia et al. 2016). Both in urban environments and in exotic plantations, contaminants released by human activities are an increasingly important source of environmental modification. These contaminants (e.g. herbicides) usually disrupt chemical communication by binding to pheromones and/or block chemoreceptors (Mesquita et al. 2003), potentially having a direct effect on the ability of organisms to detect important chemical signals or cues. For example, recognition and rapid reaction to cues related to predation are key to survival (Lima and Dill 1990). However, some studies have shown a reduced ability to detect predators in the presence of artificial chemical compounds in larval amphibians (Polo-Cavia et al. 2016) and after the exposure to leachates released by exotic tree plantations in adult newts (Chapter 4), which may increase the mortality of individuals and reduce the populations inhabiting ponds with these chemical compounds.

There could also be indirect effects if these contaminants increase stress levels and thereby affect the physiology of individuals or lower body condition, as many key behaviours are condition-dependent (e.g. mate searching, courtship signalling: Candolin 1999; Hunt et al. 2005). For instance, altered breathing physiology changes the behaviour of growing amphibian tadpoles and make them more prone to predation risk (Hickman and Watling 2014). Human induced disruption of chemical communication can have drastic evolutionary effects for species that discriminate among potential mates using chemical cues. For example, changes in the physiology due to the exposure to exotic eucalypt leachates alter female newts mating preferences, so that they reduce their interest for the higher quality males (Chapter 3). In extreme cases, it can even lead to hybridization. Female poeciliid fish exposed to humic acid do not appear to exhibit mating preferences for conspecific males, and are more likely to hybridize with a related species (Fisher et al. 2006).

2.4 Disease and pathogens

Anthropic environments have been related to a loss of diversity, but also to densities of some species that exceed those found in more natural areas (*see section 2.1*). As the main hosts disappear, most of the parasites will also be missing in anthropic habitats. However, the infection dynamics of the remaining parasites can change across a gradient of habitats, leading to, in some cases, higher prevalence of diseases in human-modified habitats than what is found in natural environments (Ditchkoff et al. 2006).

The presence of abundant resources (both accidental and intentional) makes big densities of animals to feed in specific places. These densities may elevate contact rates of individuals of one or various species, favouring the transmission of pathogens by direct contact or oral-faecal routes (Bradley and Altizer 2007). For example, in urban populations of racoons (*Procyon lotor*), disease-related deaths were higher than in natural populations, due to the aggregated distribution of animals that increases the contact between individuals, and hence the transmission of pathogens (Prange et al. 2003). Other characteristics, such as poor physical condition or immune defences, may also increase the susceptibility of animals to pathogens. For example, the use of human resources may lead to malnourishing, reducing the immune response and increasing mortality rates after infection (Ezenwa 2004). Susceptibility to pathogens may also increase in stress situations (due to high intraspecific competition for example), or after the exposure to toxic substances (e.g. pesticides). Both chronic stress (Padgett and Glaser 2003) and the presence of chemical substances (Iglesias-Carrasco et al. 2016b) are known to be detrimental for animals' immune responses, which can lower the resistance to infection and intensify the harmful effects of pathogens (Bradley and Altizer 2007). Finally, climate changes related to urban areas, such as temperature increases or lack of seasonality may also increase diseases transmission by the alteration of pathogen activity periods.

2.5 Behavioural responses to changing conditions

In general anthropic habitats are characterized by having a reduced animal diversity but both “adapters” and “exploiters” (see figure 1) are able to prosper and occur in high numbers in these environments (McKinney 2006). But why some species are successful in these newly created environments while others are excluded?

A growing amount of urban ecology studies have shown behavioural modifications of the wildlife inhabiting cities and suburban areas (e.g. Bókony et al. 2010; Iglesias-Carrasco et al. 2016a; Lapiedra et al. 2016). However, research focused in the transformation of the habitat by exotic plantations has been limited to study diversity, abundance and community of different taxa. Behaviour is one of the most plastic characteristics of animals, and it has been suggested that the ability to adjust the behaviour to new conditions is crucial to be successful in human created habitats. Phenotypic plasticity allows an organism to adjust its morphology and physiology to suit the conditions of its immediate environment and thus potentially increase its fitness (Thibert-Plante and Hendry 2011; Van Buskirk 2012). Accordingly, lack of phenotypic plasticity has been pointed out as cause of exclusion of species from novel or deeply altered environments (Badyaev 2009).

Human modified environments have distinct biotic and abiotic characteristics, which can present different challenges for animals that are found in native habitats. For example, urban populations experience a variety of new conditions such as lower or higher predation risk (Gering and Blair, 1999; e.g. pest species Sorace 2002), and unusual food sources (Ditchkoff et al. 2006) and breeding sites (Møller 2010). Likewise, wildlife inhabiting exotic plantations has to deal with plant leachates only experienced since the establishment of the new habitat. Thus, the individuals that can respond to these selective pressures should be more successful establishing in the new environment.

Alteration of foraging and reproduction

Changes in foraging patterns, the duration of breeding seasons and human structure exploitation have been documented in anthropic habitats, mostly in urban areas (reviewed in Lowry et al. 2013). For example, human disturbance (e.g. vehicular traffic) is known to negatively affect the feeding efficiency of urban animals. Feeding related activities are risky for some animal groups, since the probability of collision with cars is high. Consequently, some species alter their foraging patters to avoid the periods of higher human activity (Tigas et al. 2002; Dowding et al. 2010).

The exploitation of human food resources have led to a reduced seasonality in terms of food availability. These stable food supplies usually drives animals to reduce their range sizes in urban areas due to localized and greater food resources (Herr et al. 2010). Foraging stability in urban environments has also been suggested to alter the onset and duration of breeding. Several studies have shown that urban individuals start breeding earlier (Beck and Heinsohn 2006) and breed for longer periods (Smith and Carlile 1992). Changes in the sexual behaviour are critical, since reproductive success is directly related to fitness and population continuity. However, alteration of breeding timing and extension in urban habitats do not lead to greater productivity. Different studies have demonstrated that birds in cities have poorer fledging success and slower growth rates than their natural counterparts (Richner 1989). The reduced nesting success has been attributed to increase predation events (Beck and Heinsohn 2006). If this is the case, human trophic subsidies that simultaneously attract focal species and their predators may cause evolutionary traps (*see section 2.2*) that may lead populations to extinction.

The importance of animal personalities

A growing amount of studies are demonstrating the existence of consistent individual behavioural differences, or animal personalities. Animal personality has been defined as individual differences in their behaviour that are consistent in the time and among situations (Réale et al. 2007). An animal's personality is known to affect it's fitness, since the personality affects the way the individual cope with new challenges (Réale et al. 2007). The presence of animal personalities suggests that animals sometimes show limited flexibility in behaviour (Dingemanse and Réale 2005). However, some kind of personalities may be favoured by certain circumstances. For instance, bold individuals are those that thrive on risk and novelty (Sloan Wilson et al. 1994). This behaviour seems to be crucial to be successful in human-induced environmental changes. For example, in urban environments being bolder and more tolerant of humans may be beneficial (Sol et al. 2013), because they save energy and increase feeding and mating time when humans do not represent a significant threat. A recent study has shown that *Anolis sagrei* lizard vary consistently in their behaviour across different times and contexts and that the individuals from urban areas differ from nearby forest individuals in several behavioural traits related to boldness: urban lizards were more tolerant of humans, less aggressive, bolder after a simulated predator attack, and they spent more time exploring new environments (Lapiedra et al. 2016). Bolder behaviour has been related not only to risk-taking and exploration, but also with the ability to disperse. Birds with bold personality disperse further than their shier counterparts (Dingemanse et al. 2003); and invader fish individuals are bolder and exhibit greater dispersal tendencies than the non-invader individuals (Rehage and Sih 2004). Both innovation rates and the capacity to disperse are key traits of the invaders of human-modified environments (*see above section 2.2*), so boldness seems critical to successfully invade anthropic environments.

3. Amphibians and reptiles as study species

Amphibians and reptiles are the main groups studied in this Thesis. Due to their high sensitivity to the physical environment, the ecology of both groups is likely to be affected by any change in the natural habitat, such as those experimented by anthropic habitats. For example, the highly permeable skin of amphibians makes them sensitive to toxic substances (Rowe et al. 2003) that are present both in urban areas (Allinson et al. 2015) and exotic plantations (Maerz et al. 2005). Consequently, small changes in the characteristics of their terrestrial or aquatic habitats (depending on the life history phase) can have major effects on amphibian survival, life-history traits and breeding success. Alteration of the water chemistry due to the presence of secondary compounds released by exotic plants is known to affect amphibian larval traits, such as performance, survival, growth and behaviour (Watling et al. 2011a; Watling et al. 2011b; Hickman and Watling 2014). For instance, changes in the aquatic habitat structure can influence duration of the larval development and survival rate for some larval anuran species (Purrenhage and Boone 2009); while small fluctuations in their preferred terrestrial microhabitats alters amphibian communities (Watling et al. 2011c). Likewise, lizards depend on basking and the environment temperature for adequate metabolism, so any microclimatic change related to, for example, the life in the city, could affect the allocation of time and resources to different activities. On the other hand, both amphibians and reptiles have low mobility and reduced dispersion ability compared to other groups present in the cities, such as birds. For example, amphibian and reptile communities in urban landscapes are often impacted by roads, houses and buildings. These structures fragment the remnant habitat and present barriers to dispersal, impairing ecological connectivity among ponds and suitable habitats (Vos and Chardon 1998), so populations inhabiting cities are likely to only interact with urban environments. Finally, amphibians and reptiles have the highest global extinction rate among vertebrates (Stuart et al. 2004). Nowadays 32.5 % of the 1856 amphibian species and 20 % out of

10,000 reptiles are globally threatened, according to the categories of vulnerable, endangered, or critically endangered of the IUCN Red List. This is largely due to habitat loss (Houlahan et al. 2000), so understanding how the transformation of the habitat by urban areas and exotic plantations affects these groups would be critical to successfully conserve and manage endangered populations.

4. Conservation interest of anthropic habitat

The growth of anthropic areas and the biotic homogenization associated with these habitats are a huge challenge to conservation. The fragmentation of large habitats into smaller and more isolated areas (the isolation and edge increase, while habitat complexity decreases) results in a loss of species. Interestingly, in our constantly changing world, urban green areas, such as parks and gardens, and exotic tree plantations have been suggested to serve as refuge for native wildlife (Goddard et al. 2010).

4.1 Urban areas

Several studies have proposed multiple management measures to develop in urban areas to increase the quality of cities as biodiversity refuges. For example, size of green patches are known to influence beetle species distribution (Watts and Lariviere 2004), so city development should consider what is the optimal patch sizes for parks. Another important characteristic of urban green areas is the connectivity with big natural forest patches. For instance, in less isolated patches, insect assemblage composition are known to be similar to that found in continuous forests (Burke and Turin 1998). For some animals, vegetation structure is critical for their survival, so providing optimal vegetation patches along the city would increase the connectivity between natural assemblages and urban wildlife. However, it is known that “not all green is as good” (Chong et al. 2014), so the management of green areas needs to coordinate with the surrounding landscape (e.g. by planting native vegetation) to enhance native diversity. On the other hand, maintenance of suitable habitat features that ensure reproduction and food availability seems to be important for high mobile animals (e.g birds), while less mobile species would take advantage of a correct configuration and composition of landscape (Croci et al. 2008).

However, not only the hostile nature of cities, but the human perception of nature has to be considered to increase the value of cities as wildlife reservoirs (McKinney 2006). Most people live in cities, where an important part of the fauna is exotic. As consequence, people are becoming disconnected from the native wildlife. This lack of emotional connection to indigenous species makes difficult to educate and persuade public opinion of the importance of conservation of native species (Miller 2005).

4.2 Exotic plantations

Management measures related to the structure and composition may enhance the biodiversity present in exotic plantations. For example, one of the main problems for the native fauna is the continuous disturbing human activities, such as land preparation and harvest. Some forest species take advantage from the herbaceous vegetation that is destroyed when intensive site preparation starts. Likewise, due to short rotation lengths animals that are old forest specialists may not be able to colonise or reproduce in plantations. Thus, avoiding site preparation and increasing the rotation lengths have been suggested as important management measures to enhance the presence of native wildlife (e.g. Humphrey 2005).

Finally, the age at which trees are harvested and the tree species selected are also often seen as key issues for native biodiversity (Lindenmayer and Hobbs 2004). Native biodiversity increases in older stands, so older harvested trees may help on the rapid establishment of native old forest specialist. Finally, greater diversity of trees have been shown to increase the range of habitat types available (Hartley 2002), increase habitat diversity and dependent species (Spellerberg et al. 1996), and provide a more stable and resilient environment (Jactel and Brockerhoff 2007).

5. References

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Objectives

The main objective of this thesis is to deepen our knowledge about the changes in animal behaviour driven by the transformation of the habitat to anthropic areas. Importantly, the objective of the present thesis is to progress into organisms' mechanistic ecology, such as the behavioural ecology of species interactions and evolution, and not just focus on patterns of species abundance and diversity on of different taxa. To accomplish this, this thesis is organized in six chapters. The first four explore the effect of exotic plantations (pine and eucalypt) and the leachates released by these trees in different aspects of amphibian ecology. The last two chapters, however, test the effect of the urbanization on such ecological traits of amphibians and reptiles.

Chapter 1. What effect do tree plantations have on species richness and the ecology of native organisms? The objectives of this chapter are to explore 1) whether the transformation of the natural oak forest to pine and eucalypt plantations reduces the richness and abundance of native amphibian species, 2) which are the physical characteristics of the ponds that drive this reduction, and 3) to what extent the leachates released by eucalypt leaves are behind the differences found in richness and abundance.

Chapter 2. What effect do tree plantations have on the condition-dependent sexual traits and immune response of the palmate newt? In this chapter we aim to explore 1) whether male palmate newts inhabiting pine and eucalypt plantations have a reduced expression of secondary sexual traits and a lower immune function, 2) if the reliability of these sexual traits differed among habitats, and 3) to what extent the differences found in the field are driven by the presence in the water of leave leachates.

Chapter 3. What effect do tree plantations have on female mate choice of the palmate newt? We aim to know 1) whether the presence of eucalypt leachates in the water alters the choosiness and preferences of female palmate newts, and 2) differentiate whether this potential alteration results from indirect effects of environmental changes on physiology (e.g. body condition or neurological pathways), or from an immediate, direct effect on the ability of females to discriminate between potential mates.

Chapter 4. What effect do tree plantations have on the chemosensory ability of male palmate newts? In this chapter our aim is to explore whether eucalypt leachates entering waterways affect three key life-history traits in male palmate newts by adversely affecting their chemosensory ability to: 1) locate mates, 2) detect conspecific alarm cues indicative of the presence of predators, and 3) find food. We also want to explore if this effect varies depending on whether the individuals tested had previous exposure to these chemicals in their habitat of origin.

Chapter 5. Does urbanization affect key life-history traits in native organisms? We aim to test if the different evolutionary pressures encountered in the city affect three important life-history traits related to fitness: body size, body condition and immune response of three anuran and an urodele species.

Chapter 6. Does urbanization affect antipredatory responses of lizards after an immune challenge? Our purpose is to explore 1) whether morphological traits of the Catalonian wall lizard differed between natural and urban populations, 2) whether these morphological traits are related to the antipredatory behaviour of lizards, and 3) whether the city environment alters the trade-off between hiding in a safe refuge and combat an immune challenge.

Chapter 1

Exotic plantations lead to lower species richness, density and condition of amphibians: linking field observations and physiological responses in mesocosms

Maider Iglesias-Carrasco, Aitor Larrañaga, Iraide Artetxe, Ion Garin-Barrio, Carlos Cabido

ABSTRACT

What effect do tree plantations have on species richness and the ecology of native organisms? Some studies show that plantations reduce the diversity and abundance of forest biota, while others suggest that they preserve biodiversity. Pine and eucalypt plantations are among the most widespread exotic plantations, and they have negative effects on many taxa. But how these exotic plantations affect species richness, density, and traits related to survival of amphibians have been poorly explored. Here, we first sampled amphibians in ponds under patches of native oak forests, and pine and eucalypt plantations. We found significantly fewer amphibian species and lower densities in plantations (mainly under eucalypts) than in native forests. Land use explained most of the variance in both variables (57 and 36%, respectively), suggesting that factors regulated by habitat type, such as the accumulation of leachates in the water, may be responsible for the observed differences. Second, we chose three anuran species with markedly different responses to the presence of plantations and reared them in mesocosms to test how exposure to eucalypt leachates affects key survival traits: growth and immune response of tadpoles, and the jumping performance and size of froglets. The three species showed a reduced immune response after being exposed to eucalypt leachates, but the effect on growth, size and jumping ability varied across species. We conclude that leachates appear to be an important characteristic affecting amphibians under plantations, but effects of other variables that are also dependent on the habitat type could not be ruled out.

Keywords: amphibian conservation, anthropic environment, eucalypt plantations, immune response, performance, pine plantations.

INTRODUCTION

Vast areas of the world's native forests have been converted to exotic tree plantations (MacDicken 2015). Plantations have a higher density, but lower diversity, of trees than natural forests (Lindenmayer and Hobbs 2004). Many studies have shown that plantations reduce the number of species of important animal groups, such as arthropods and birds (Zurita et al. 2006; Fork et al. 2015). However, in extreme cases where natural forests become scarce, plantations can paradoxically contribute to the conservation of diversity (Brockerhoff et al., 2008). To date, studies about the mass replacement of natural forest with exotic tree plantations have focused mainly on patterns of species abundance and diversity on different taxa (Carrascal and Tellería 1990; Sax 2002; Zurita et al. 2006), but very few studies have investigated the functional ecology of individuals (for an exception see Iglesias-Carrasco et al., 2016).

Eucalypt (*Eucalyptus* sp.) and pine plantations (*Pinus* sp.) are the two most common types of exotic plantations worldwide. They negatively affect the diversity of many taxa, such as mites (Rieff et al. 2016), fish (Oliveira et al. 2016) and amphibians (Cruz et al. 2015). The quality of detritus in plantations is usually lower than in native deciduous forests (Martínez et al. 2016) and the presence of toxic compounds in that detritus is higher (Canhoto and Laranjeira 2007). When released from the detritus, toxic substances enter the soil (Souto et al. 1994) and waterways (Bärlocher and Oertli 1978; Pozo et al. 1998), changing their physical characteristics (Florence 1986; Farley et al. 2004). The exposure to these novel chemical compounds produced by exotic plants may represent cryptic threats to native fauna. For example, it is already documented that the presence of these toxins, and the low quality of the detritus affects the composition of animal communities in freshwater systems within plantations (Ferreira et al. 2006; Martínez et al. 2016). Even species that naturally occur in eucalypt forests are affected detrimentally by the toxic leachates found in these habitats (Morrongiello et al. 2013). Strikingly, few experimental studies have targeted the effects on traits that are directly related to the survival (e.g growth or immune response) of species that have only recently come into contact with the chemicals released by exotic eucalypts and pines.

One of the animal groups that has been most affected by loss of habitat globally are amphibians. Habitat loss is particularly devastating as amphibians often have low mobility and high philopatry (Cushman 2006). Small, ephemeral ponds are common in forests and usually support a high diversity and abundance of amphibians. Ephemeral ponds are essential for the breeding of many amphibians, which stay there until they complete larval development (Semlitsch and Bodie 2003). Given the importance of ponds for the life-cycle of amphibians, any changes to their physical or structural characteristics (e.g. shorter hydroperiods) may have important consequences for larval survival and development (Brodman et al. 2003; Orizaola and Braña 2003).

Amphibians are also sensitive to toxic substances, due to their highly permeable skin (Rowe et al. 2003). Consequently, small changes in the chemical characteristics of their terrestrial or aquatic habitats (depending on the life history phase) can have major effects on their survival and breeding success. Exposure to leachates released by exotic plants can affect the survival, growth and performance of amphibian larvae (Watling et al. 2011a; Watling et al. 2011b). Likewise, adult newts inhabiting pine and eucalypt forests have a reduced immune response and expression of secondary sexual characters (Iglesias-Carrasco et al. 2016). The expression of several costly morphological traits is widely understood to depend on the condition of individuals (*sensu* Rowe and Houle, 1996; e.g. Tomkins et al., 2004). This condition, hence the optimal expression of most life-history traits, usually depends on the quality of the environment (e.g. Greenfield and Rodriguez, 2004). Exotic plantations can be considered low quality environments with limited resources (e.g. due to the lower water quality and toxic detritus). If there are fewer resources for important life-history traits, such as tadpole growth, the survival of larvae and fitness of adults might be compromised in exotic plantation habitats.

Here we designed a field and a mesocosm experiment with two purposes: (i) to investigate species richness and density of amphibians across natural and exotic plantation habitats; (ii) to test whether the toxicity of detritus leachates drives the differences in amphibian richness in the field. We sampled amphibians and measured physico-chemical characteristics from ponds in three habitats: pine plantations, eucalypt plantations, and native oak forest. We then used a mesocosm experiment which controlled food supply and varied the type of leachate of leaf litter (either oak or eucalypt). We reared three anuran species with different ecological requirements and that showed different responses to the presence of plantations in an earlier observational study: the Iberian green frog (*Pelophylax perezi*), the European common frog (*Rana temporaria*) and the common midwife toad (*Alytes obstetricans*). We hypothesized that: 1) plantations are associated with a lower species richness and abundance of amphibians 2) eucalypt leachates reduce growth rates, immune response, jumping performance and body size, and 3) the magnitude of the negative effect on those traits in specific taxa parallels their abundances in the field under different types of vegetation.

MATERIAL AND METHODS

Field data: amphibian species richness and density in exotic plantations

Study site and amphibian sampling

We sampled amphibians in ponds under patches of native oak forest (*Quercus robur*), eucalypt plantations *(Eucalyptus globulus)* and pine plantations (*Pinus radiata)* in Basque Country (North Spain).The ponds were totally surrounded by at least 400 meters of the corresponding habitat type. We sampled each pond twice, in mid-March and in late May 2015, to increase the likelihood of finding both winter and spring/summer breeding amphibians. Each sampling campaign was less than a week long to reduce confounding effects of weather or seasonality. All ponds were dipnetted by MIC for invertebrates and amphibians (larvae and adults) in a standardized way (effort: 1 minute m^{-2}; net size: 1 mm mesh, 26 x 21 cm frame). We identified all amphibian species *in situ*, recorded the number of individuals and determined their sex and life cycle stages (larvae, metamorphic, juvenile or adult). We then released them back into the pond. We stored collected macroinvertebrates in 70% ethanol and transported them to the laboratory for identification to the family level following Tachet et al., 2010. We calculated two biological indices designed to assess water quality that are based on the sensitivity of invertebrate families to water pollution: IBMWP (Iberian Biomonitoring Working Party) and IASPT (Iberian Average Score Per Taxon Alba-Tercedor et al., 2002). Each pond was then characterized with a set of variables that are important in determining the presence and absence of amphibians and macroinvertebrates (Brodman et al. 2003; Orizaola and Braña 2003). We measured the pH and conductivity with field WTW multi-parametric sensors. We measured the longest axis of the pond (a) and the length of a defined number of equidistant and perpendicular transects (b_1 to b_n) (total transects depended on the irregularity of the pond: range 2-8). The area of the pond was estimated as a*average (b_1 to b_n). Finally, we estimated the percentage of the pond covered by terrestrial, submerged and emergent aquatic vegetation.

Data analysis

We used linear models to test how the measured physical pond characteristics varied among habitats. We also ran linear models to test for differences among the three habitat types in total amphibian richness (number of species in each habitat), total density (sum of all amphibians / m^2 of pond) and each species density (individuals of each species/ m^2 of pond). We checked the residuals of all models to ensure that they met the assumption of normality, linearity and homoscedasticity, and, when necessary, data were log-transformed to meet model requirements. Pairwise comparisons were planned and made using Tukey's honestly significant difference tests. As data were not normal, we used Kruskal-Wallis tests, and Dunn tests for pairwise comparisons to explore between-habitat differences in species'density.

Finally, we performed multiple stepwise linear regressions with a forward selection procedure (a reduction in AIC-Akaike's Information Criteria > 2 to enter, Legendre and Legendre, 1998) to look for the combination of physio-chemical variables of ponds, including habitat type, that best explained amphibian species richness and total density. We first standardized each variable to a mean of zero and a standard deviation of one to correct for collinearity between the habitat and each variable. We then checked for collinearity and discarded for our models the only variable (pond size) which showed high correlation parameters with the others (based on Pearson's correlation parameters).

Mesocosm experiment. Effect of eucalypt leaves on the life-history traits and immune response of three amphibian species

In the mesocosm experiment we only tested eucalypt leachates because the effects of Eucalypt plantations on amphibian density are stronger than pine plantations (*see online Appendix* Fig A.1 and Iglesias-Carrasco et al. 2016). We chose three amphibian species that exhibited different responses to eucalypt plantations in our field observations (*online Appendix* Table A1): *A. obstetricans* did not appear in Eucalypt plantations; *R. temporaria* appeared in both natural forests and Eucalypt plantations, but with a reduced density of tadpoles in eucalypt plantations; *P. perezi* appeared both in oak and eucalypt plantations without a detectable change in density.

During the breeding season of each species (January for *R. temporaria*, May for *P. perezi* and *A. obstetricans*) we collected three clutches from a pond in a native deciduous forest. We placed each clutch into 4 L plastic tanks (one clutch/tank) filled with mineral water (ca. 12:12 light-dark cycle and 12 º C of temperature). We moved hatchlings in groups of 20 individuals to new tanks filled with 4 L of clean mineral water until they reached the free feeding stage (Gosner stage 25; Gosner 1960). Males of *A. obstetricans* carry the clutch with them until the eggs are close to hatching, so we collected recently hatched tadpoles from a natural pond and maintained them in groups of 20 individuals with 4 L of mineral water until they reached Gosner stage 25.

To simulate natural pond conditions we used 470 L mesocosms containing 200 L of mineral water. A hundred grams of dried leaves of either oak (*Q. robur*) or eucalypt (*E. globulus*) were placed in each of the 6 outdoor mesocosms (3 per leaf species) (see Maerz et al., 2005b for doses). We also added 5 L of natural pond water containing zooplankton and algae at the beginning of the experiment. The mesocosms were covered with mesh to prevent the entry of predators. After 48 h we introduced 80 tadpoles to each mesocosm. Tadpoles were fed rabbit chow daily (5 g day^{-1}). We reared the tadpoles in the mesocosms until metamorphosis. The initial 100 g leaves remained in the mesocosms until the end of the experiment.

On day 21 we randomly selected 20 tadpoles from each mesocosm and they were laterally photographed and weighed. We later measured larval body and tail fin length, tail crest depth and tail muscle depth from photographs with ImageJ 1.47v. We calculated body condition as the residuals from the regression of log transformed body mass (g) on log transformed total length (mm). This estimate for body condition has been widely for amphibians (Denöel et al. 2002; Janin et al. 2011).

When tadpoles reached Gosner stage 43 (Gosner 1960), we randomly chose another 15 tadpoles per mesocosm and individually placed them in opaque aquaria with 1 L of mineral water. To estimate the physiological condition of the tadpoles we used phytohaemagglutinin injection assay (PHA test), which measures T-cell dependent immunocompetence in vivo (Lochmiller et al. 1993). This assay has been used in many vertebrate species, including amphibians (e.g. Brown et al., 2011). We first measured the thickness of the base of the tail 5 mm from the vent with a pressure-sensitive spessimeter (accuracy: 0.01 mm; we use the average of 5 measurements made to each tadpole). All individuals were anaesthetized by immersion in Tricaine methane sulphonate (0.15 g MS-222/1 L dechlorinated water) for 5-10 minutes. Immediately after, we injected 0.01 mg of PHA dissolved in 0.01 ml of phosphate-buffered saline (PBS). Tadpoles were then placed in their corresponding plastic containers. The effect of the PHA injection is a slight swelling of the skin, caused by the immune response, which disappears after 48 h. After 24 h, we again measured the thickness of the tail base at the same point to calculate the difference between pre- and post-injection measures (inflammation). None of the tadpoles showed any sign of stress or pain during these tests, and all started swimming and feeding after recovering from anesthesia. We collected data blind to the water treatment of tadpoles.

Thereafter, mesocosms were checked daily for the presence of metamorphics. Froglets with a tail shorter than their body were placed individually in small boxes with mineral water. Upon completed tail resorption we measured the jumping performance of each metamorphic. Environmental temperature can cause differences in jumping performance (Van Buskirk and Saxer 2001), so all trials were conducted in standardize conditions at 17ºC for *R. temporaria* and 24 ºC for *P. perezi*, temperatures commonly experienced in the field. Metamorphosis results of *A. obstetricans* are not included because they overwinter in the water, extending the exposure to the leachates for more than a year, and reducing the comparability with the other two species. The jumping trials were recorded using a camera located directly above the trial bench. We stimulated the froglet gently with the blunt end of a pen to make it jump (5 times). We measured the longest horizontal jump distance using the software Image J by measuring the linear distance between the positions of the frog's vent in each frame (Van Buskirk and Saxer 2001). We repeated the jumping assay in two consecutive days and used the average value for the analyses. After the second trial we weighed and photographed the froglets face up with the leg completely extended. We later measured the snout vent-length (SVL) and the length of the femur with Image J. Again, we calculated the body condition of individuals as the residuals from the regression of log transformed body mass (g) on log transformed SVL (mm). At the end of the experiment we euthanized all individuals by immersion in lethal concentration of MS-222.

All capture and handling of amphibians complied with the existing laws regulating the treatment of animals in Spain. The study was performed under a capture and handling license from Alava, Bizkaia and Gipuzkoa Administrations (numbers 1695 and 2222). M. I-C and C.C conducted this work with Spanish accreditation to conduct experiments with animals R.D 53/2013 (ref number 10/096442.9/13).

Data analysis

Maximum tail depth, tail muscle depth and femur length were size corrected as the residuals of the regression of the log transformed variable against log transformed total length or SVL of froglets (all *p*-values of the regressions < 0.001). The PHA immune response was calculated as the residuals of the regression of the inflammation against snout-vent length (both variables log-transformed). Maximum jump length is correlated with the leg size (Ficetola and De Bernardi 2006), so we calculated the jumping performance as the residuals of the regression of log jumping distance against log femur length. We used linear mixed models (LMM) to test for differences in the measured variables between the two mesocosm treatments. Models were run in R 3.2.2. Mesocosm identity was included as a random factor, and the leaf treatment as a fixed effect. As the size, body condition, and jumping performance may be related to the time needed to reach metamorphosis (residuals of the regression p < 0.001), we included the number of days until this stage as covariate in the models (leaf litter type did not affect development time in either *P. perezi*, $X^2 = 0.08$, $p = 0.77$, or *R. temporaria* $X^2 = 0.00$, $p = 0.97$). To meet model assumption of normality, linearity and homoscedasticity data were log-transformed when necessary.

RESULTS

Field experiment

We found that pond surface area ($F_{2,5.62} = 7.78$, $p = 0.004$) and depth ($F_{2,3.6} = 6.45$, $p = 0.009$) were higher in native forests than in pine and eucalypt plantations (Table 1). We also found a marginal significant effect of land use on terrestrial vegetation coverage ($F_{2,8108} = 3.94$, $p = 0.041$). The Tukey's analysis suggests that this is because pine plantations differs from eucalypt plantations ($p = 0.062$) and native oak forest ($p = 0.073$), but that eucalypt plantations and native are similar ($p = 0.995$). Finally, we found that the index based on the sensitivity of invertebrates to water contamination, the IBMWP ($F_{2,3600} = 4.94$, $p = 0.022$), was significantly higher in native forests than in eucalypt plantations (Tukey HSD $p = 0.021$), but there were no differences between pine plantations and native forests (Tukey HSD $p = 0.094$) or eucalypt plantations (Tukey HSD $p = 0.752$) (Table 1).

During our sampling, we detected all seven species expected in the kind of ponds we studied. We found 5494 individuals (larvae and adults) from these seven species in ponds under native forests, 885 individuals of two species under pine plantations and 168 individuals of four species under eucalypt plantations (Fig 1a). Species richness was significantly higher in ponds in native forests than in either pine and eucalypt plantations ($F_{2,15} = 9.95$, $p = 0.002$, Fig. 1a), but there was no difference between the two plantation types (Tukey HSD test: $p = 0.701$). *A. obstetricans*, *Rana dalmatina* and *Triturus marmoratus* were absent in exotic plantations (Table 2), while *Lissotriton helveticus*, *R. temporaria*, *P. perezi*, and *Salamandra salamandra* occurred in both native forests and eucalypt plantations (Table 1). Among the physical characteristics measured, the model included habitat type (57.1% of the variance explained; $p < 0.001$), and conductivity (26.5%; $p < 0.001$) as important drivers of amphibian richness (Table 3).

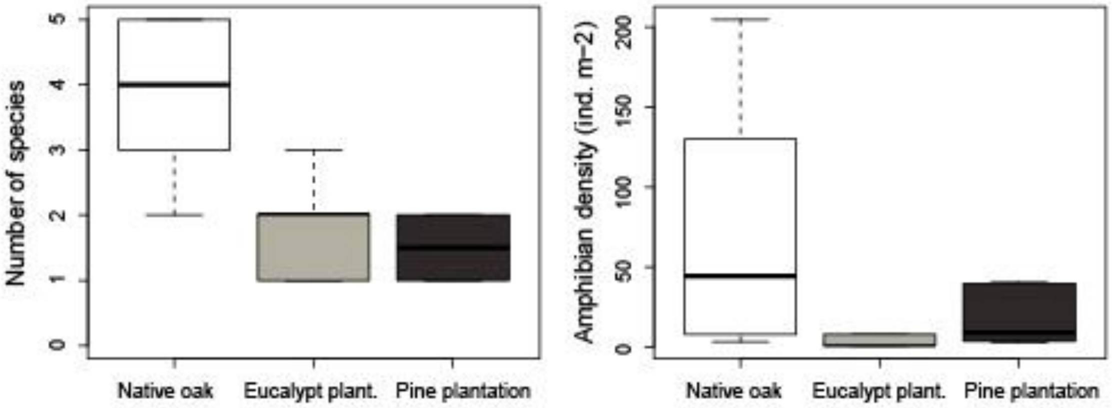

Figure 1. (a) Number of species and (b) total density of individuals per pond. ANOVA results for Richness: $F_{2,15} = 9.95$, $p = 0.002$; Tukey HSD comparison: Native > Eucalypt = Pine, and for Total density: $F_{2,15} = 4.29$, $p = 0.034$; Tukey HSD: Native > Eucalypt (the other pairwise tests are non-significant). Note the logarithmic y-axis for total density.

There were also differences in total density between native forests and plantations ($F_{2,15} = 4.29$, $p = 0.034$, Fig 1b), but the only statistically significant pairwise difference was between native forests and eucalypt plantations (Tukey HSD: $p = 0.026$). Looking at each species separately, only densities of *A. obstetricans* ($X^2 = 9.52$, $p = 0.008$) and *R. temporaria* ($X^2 = 7.10$, $p = 0.028$) varied significantly with habitat type. *A. obstetricans* only inhabited native forests, but the only significant density difference was between the natural forest and eucalypt plantation (both Dunn tests $p < 0.02$, Table

2). *R. temporaria* occurred in all three habitats but its density was higher under oak forest, intermediate under pine plantations and lowest under eucalypt plantations (significant pairwise difference between oak forests and eucalypt plantations, Dunn test: p = 0.016).. The variables that best predicted total amphibian density were habitat type (36.4% of the variance explained; p = 0.008), pond depth (14.2%, p = 0.033), submerged vegetation cover (11.6%, p = 0.051) and emerged vegetation cover (8.11%; p = 0.094; this variable was retained by the AIC criterion, despite not being significant in the ANOVA) (Table 3).

Mesocosms experiment

Effect of eucalypt leachates on A.obstetricans

A. obstetricans (not present in exotic plantations) was strongly affected by exposure to leachates of eucalypt leaves for 21 days (Fig 2A). Tadpoles in eucalypt water had shorter bodies ($F_{1,118}$ = 114.66, p < 0.001), tail fins ($F_{1,116}$ = 134.51, p < 0.001), worse body condition ($F_{1,118}$ = 9.78, p = 0.002), and shallower tail muscles ($F_{1,118}$ = 5.61, p = 0.019) than those in oak-like water. No differences in the maximum tail depth were found ($F_{1,116}$ = 0.65, p = 0.420). The immune response was also 26.4 % lower in eucalypt-exposed than oak-exposed tadpoles ($F_{1,85}$ = 21.85, p < 0.001, Figure 2A).

Effect of eucalypt leachates on R.temporaria

Despite the lower density of *R. temporaria* tadpoles in eucalypt plantations, this species was less affected by exposure to eucalypt leachates than the other two species (Figure 2A). Tadpoles from mesocosms containing oak or eucalypt leaf litter had similar body lengths ($F_{1,118}$ = 0.22, p = 0.638), tail fin lengths ($F_{1,116}$ = 0.11, p = 0.737), body condition ($F_{1,116}$ = 0.001, p = 0.974), maximum tail depth ($F_{1,116}$ = 1.76, p = 0.187) and muscle depth ($F_{1,116}$ = 1.11, p = 0.294). However, the immune response of tadpoles exposed to eucalypt water was 28.3 % poorer than in oak water ($F_{1,83}$ = 32.86, p < 0.001, Fig. 2A). In addition, froglets exposed to eucalypt leachates had a worse jumping performance ($F_{1,109}$ = 14.97, p < 0.001) than those reared in oak leachates (Figure 2B). We did not find an effect of the treatment on froglets' body condition ($F_{1,107}$ = 2.07, p = 0.153), SVL ($F_{1,107}$ = 0.37, p = 0.543) or femur length ($F_{1,108}$ = 3.68, p = 0.057).

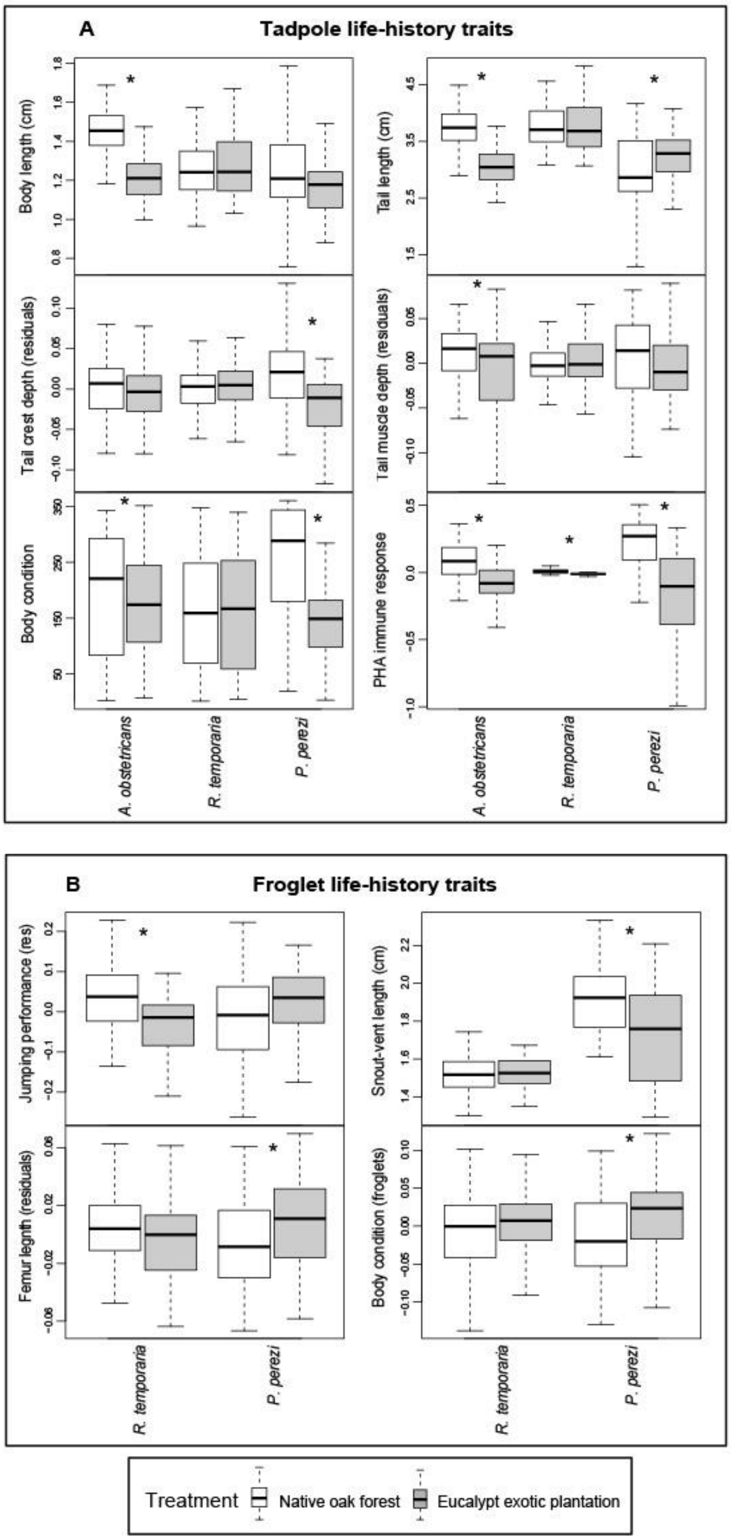

Figure 2. Morphological and physiological traits measured (A) to tadpoles of *A. obstetricans* (not present in plantations), *R. temporaria* (reduced diversity in plantations) and *P. perezi* (same presence in natural forest and plantations), after 21 days (or Gosner stage 43 for the PHA immune response) of exposure to oak native leachates (white boxes) or exotic eucalypt leachates (grey boxes); and (B) to *R. temporaria* and *P. perezi* metamorphics after finishing their larval period in native oak (white boxes) or exotic eucalypt (grey boxes) treated water.

Effect of eucalypt leachates on P. perezi

P. perezi (similar presence and density in both plantations and natural forest) showed an intermediate negative effect when tadpoles were exposed to eucalypt water (Figure 2A). These tadpoles also had worse body condition ($F_{1,116}$ = 41.74, $p < 0.001$), and shallower tail crests ($F_{1,114}$ = 23.74, $p < 0.001$). *P. perezi* tadpoles reared in eucalypt water had longer tail fins ($F_{1,114}$ = 16.13, $p < 0.001$), but similar body length ($F_{1,114}$ = 2.94, $p = 0.089$), compared to those grown in oak. We did not find any effect of the treatment on the depth of the tail muscle ($F_{1,114}$ = 2.77, $p = 0.099$). The immune response of *P. perezi* was more strongly affected by the exposure to eucalypt leachates than the other two amphibian species, with a 51.1 % reduction in immune response compared to oak exposed individuals ($F_{1,83}$ = 38.08, $p < 0.001$). Surprisingly, when they reached the metamorphosis, tadpoles of *P. perezi* reared in eucalypt leachates showed a higher body condition ($F_{1,122}$ = 4.88, $p = 0.029$) and had longer femur lengths ($F_{1,121}$ = 6.07, $p = 0.015$) than those reared in oak leachates (Figure 2B). However, the snout-vent length (SVL) was longer in froglets reared in oak leachates than those reared in eucalypt leachates ($F_{1,120}$ = 59.57, $p < 0.001$). There were no significant differences in jumping performance between the two treatments ($F_{1,120}$ = 1.75, $p = 0.188$).

DISCUSSION

Field data

Species richness and density of amphibians was lower in ponds in eucalypt plantations than in natural oak forests. The species richness in pine plantations was also lower than that in natural oak forest, but the density of amphibians was similar. We found that the surrounding land use was the property of ponds that explained most of the variance in species richness and density (57 and 36%, respectively). This suggests that some factors that we did not measure in the field which are regulated by habitat type (e.g. water toxicity), are partly responsible for the observed differences in amphibian species richness.

In our field data amphibian species richness was negatively related to water conductivity (see Hamer and Parris, 2011). Water conductivity, an indicative of the accumulation of chemicals in ponds, is known to increase when eucalypt leaves are present (Canhoto and Laranjeira 2007). So the accumulation of leachate chemicals may play a role in amphibian diversity. Further, the IBMWP (Iberian Biomonitoring Working Party) was significantly higher in ponds in native forests than in eucalypt plantations. IBMWP was designed to detect water pollution (Jáimez-Cuéllar et al. 2002), and could thus be related to the organic enrichment created by the leachates. In contrast, species richness was not dependent on physical vegetation characteristics of the ponds. These findings suggest that water chemistry might be the driving factor that determines differences in species richness between habitat types (see also our discussion of the mesocosm experiment below).

In contrast to our results for species richness, amphibian density did not depend on water chemistry, but rather on physical characteristics of the ponds, such as depth and pond vegetation. Pond depth may be particularly important in determining amphibian density in species that spend an extended amount of time as tadpoles, due to the potential for ponds to dry out prior to metamorphosis. Several of the species found in natural oak forests, but not in the exotic plantations, such as *R. dalmatina* and *T. marmoratus* have a long larval period of about three months that extends until summer (García-París et al. 2004); and for *A. obstreticans* the tadpoles overwinter in the water, extending the larval cycle to a year (García-París et al. 2004). Crucially, we found that ponds in oak natural forests were significantly deeper than those in plantations. In addition to pond depth both submerged and emergent vegetation were important in determining amphibian density. This result is in line with previous studies which have found that amphibian density increases with vegetation-related structural complexity of the habitat (Vallan 2002), and that the submerged vegetation provides refuge, food, protection against UV-B radiation and support for oviposition in newts (Waringer-Löschenkohl 1988; Alarcos et al. 2003).

Mesocosms experiment

We predicted that the extent to which the presence of toxic leaf leachates affects amphibians would reflect the findings from presence/abundance field data. We found partial support for this prediction. Of the three species we tested, *A. obstetricans*, which was not found in ponds within eucalypt plantations, was the most negatively affected by exposure to leachates. *A. obstetricans* tadpoles exposed to eucalypt leachates were smaller, had worse body condition and had smaller tail muscles than those grown in oak water. This sensitivity to eucalypt leachates, and more generally their water chemistry, could explain the absence of *A. obstetricans* from the two exotic plantations.

Our findings are similar to other studies which show that leachates from invasive plants negatively alter behaviour and performance (Watling et al. 2011a; Watling et al. 2011b), and increase mortality (Watling et al. 2011a) in some larval amphibians. Morrongiello et al., (2011) suggested that fish females exposed to eucalypt leachates might reduce the resources available to allocate in reproduction, since fish have to expend energy to resist the effects of leachates. Tadpoles inhabiting exotic plantations might also need to invest more resources into coping with toxins, reducing the allocation of resources to costly morphological structures (e.g. deep crests) or growth. Smaller morphological structures and reduced body sizes may make tadpoles inhabiting plantations more vulnerable to predation (Van Buskirk and Mccollum 2000), increase mortality rates and thereby reduce the number of tadpoles reaching metamorphosis.

In *R. temporaria,* which was found in both natural forests and eucalypt plantations, albeit with a lower density of tadpoles in eucalypt plantations, we found that froglet traits, but not tadpole traits, were harmed by exposure to eucalypt leachates. Previous studies have shown that the conditions encountered by amphibians during aquatic development can influence not only larval growth, but also froglets' survival, morphology, and jumping performance (Álvarez and Nicieza 2002; Relyea and Hoverman 2003; Vonesh 2005). Poor performance of froglets has been suggested to negatively impact the persistence of amphibian populations (Vonesh and De la Cruz 2002), increase mortality in the terrestrial environment (Morey and Reznick 2001) and decrease locomotor performance (Ficetola and De Bernardi 2006) and might therefore explain the decreased density of *R. temporaria* in eucalypt plantations.

In contrast to our predictions, *P. perezi,* which appeared at similar densities in eucalypt plantations and natural forests, showed negative effects of eucalypt leachates on many of the tadpole traits we measured, including body condition, tail crests and immune response. Surprisingly, however, tadpoles reared in water with eucalypt leaves had higher condition and larger femur size as froglets. This result could indicate that *P. perezi* froglets can somehow compensate for a poor quality larval environment, which could potentially explain its similar density in eucalypt plantations and natural forests despite the negative effects of eucalypt leachates on tadpoles. An alternative explanation may lie in the ecology of *P. perezi*. It is mainly an aquatic species and the froglets travel long distances to colonize new ponds (García-París et al. 2004). As the individuals detected in field samples were juvenile froglets, we might have recorded individuals that had emigrated from ponds surrounded by native vegetation. If this is the case then field observations may underestimate the effect of plantations on *P. perezi*. Genetic data should reveal whether *P. perezi* populations in eucalypt plantations constitute source or sink populations.

Finally, changes in the terrestrial environment should be considered to fully understand how habitat transformation affects amphibian richness and density. Our main aim was to study the extent to which amphibian richness was affected by alteration of the aquatic habitat and the presence of toxic leaf leachates, so we did not explore how changes in the terrestrial environment might drive our results. The introduction of exotic plants can alter amphibian communities due to changes in the temperature of the terrestrial microhabitat (Watling et al. 2011c), reduce foraging success of adult frogs (Maerz et al. 2005a), and the introduction of exotic fauna (Maerz et al. 2009). Likewise, in plantations, the places available for terrestrial refuges may be fewer, and feeding opportunities scarcer due to a reduced prey availability (Zahn *et al.*, 2010). If so, the number of metamorphics and adult frogs and newts that survive and reproduce in ponds would be lower, explaining the reduced richness and density of some species in pine and eucalypt plantations.

Conclusions

Our results are consistent with previous studies showing that the replacement of natural forests by plantations is harmful for amphibian diversity (e.g. Gallmetzer and Schulze, 2015). The fact that the loss of diversity we observed was stronger for amphibians in eucalypt plantations corresponds with previous studies that suggest that eucalypt plantations are more harmful to newts than pine plantations (Iglesias-Carrasco et al. 2016). The negative effects on the growth of tadpoles partly support our field data and our predictions that the presence of toxic leachates is a driver of the presence and density of amphibian species and body condition of individuals. Our results highlight that different species can be influenced by habitat changes in different ways and that the negative effects of habitat variables on individuals need not always be apparent at the population or community level. Such variability should be taken into account when designing conservation management plans.

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Table 1: Mean and SE of descriptors of the ponds sampled. Comparisons after Tukey HSD tests are shown for each variable. Statistically significant differences among habitat types highlighted in bold.

Descriptor	Native forest		Pine plantations		Eucalypt plantations		Tukey HSD
	Mean	SE	Mean	SE	Mean	SE	
Mean water depth (cm)	**23.55**	**6.85**	**7.79**	**0.83**	**8.44**	**2.22**	**Nat > Pin = Euc**
Area (m^2)	**22.69**	**5.90**	**7.16**	**0.20**	**5.95**	**1.59**	**Nat > Pin = Euc**
Submerged vegetation (%)	23.33	11.38	0.83	0.83	20.00	10.88	Nat = Pin = Euc
Emerged vegetation (%)	1.67	1.05	1.67	1.05	10.00	4.28	Nat = Pin = Euc
Terrestrial vegetation (%)	**73.33**	**14.70**	**29.17**	**12.81**	**75.00**	**11.55**	**Nat = Euc > Pin**
pH	7.34	0.15	6.74	0.45	7.35	0.26	Nat = Pin = Euc
Conductivity (µS cm^{-1})	136.52	41.15	91.53	21.25	147.17	42.43	Nat = Pin = Euc
IBMWP*	**56.67**	**9.85**	**31.83**	**6.26**	**23.33**	**6.77**	**Nat = Pin >Euc**
IASPT*	4.51	0.36	4.18	0.29	4.56	0.59	Nat = Pin = Euc

*IBMWP, Iberian Biomonitoring Working Party and Iberian Average Score Per Taxon

Table 2: Density (mean ± SE, ind. m^{-2}) and frequency (out of 6 ponds) for each amphibian species separated in adults and larvae. Comparisons among the three habitat types after Tukey HSD tests are shown. In bold are species that differ in density among habitats. (N = 6 ponds per habitat type).

Species	Stage	Native forest			Pine plantations			Eucalypt plantations			Tukey HSD
		Density		Frequency	Density		Frequency	Density		Frequency	
		Mean	SE		Mean	SE		Mean	SE		
L. helveticus	Adults	2.16	0.45	6	3.95	0.76	6	4.66	2.52	6	Pin = Euc = Nat
	Larvae	4.96	2.57	4	3.27	2.02	3	2.69	2.69	1	Nat = Pin = Euc
T. marmoratus	Adults	0.04	0.04	1							Nat = Pin = Euc
S. salamandra	Larvae	0.14	0.12	3				0.44	0.43	2	Euc = Nat = Pin
A. obstetricans	**Larvae**	**1.77**	**0.91**	**4**							**Nat > Pin = Euc**
R. temporaria	**Larvae**	**62.99**	**33.2**	**5**	**10.37**	**6.34**	**3**	**0.11**	**0.11**	**1**	**Nat = Pin > Euc**
P. perezi	Juvenile	0.44	0.24	3				0.24	0.16	2	Nat = Euc = Pin
R. dalmatina	Larvae	0.02	0.02	1							Nat = Euc = Pin

Table 3: Model supported by AIC for mean density and total richness of amphibians in the ponds.

Response variable	Variable	DF	SS	MS	F	P	Variance %	AIC	Comparison/Slope
Total amphibian richness	Habitat type	2	3.05	1.52	24.34	<0.001	57.11	22.05	Nat > Euc = Pin
	Conductivity (µS cm^{-1})	1	1.42	1.42	22.70	<0.001	26.59	6.970	-
	Residuals	14	0.87	0.06			16.29		
Mean amphibian density	Habitat type	2	16.28	8.14	7.36	0.008	36.4	67.32	Nat = Pin > Euc
	Pond depth (cm)	1	6.37	6.37	5.77	0.033	14.24	64.76	+
	Sumerged vegetation (%)	1	5.19	5.19	4.69	0.051	11.6	61.93	+
	Emerged vegetation (%)*	1	3.63	3.63	3.29	0.094	8.11	59.57	+
	Residuals	12	13.25	1.10			29.63		

*Although non-significant in the ANOVA, AIC supported the inclusion of this variable in the model.

Chapter 2

Condition-dependent trade-offs between sexual traits, body condition and immunity: the effect of novel habitats

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ABSTRACT

The optimal allocation of resources to sexual signals and other life history traits is usually dependent on an individual's condition, while variation in the expression of sexual traits across environments depends on the combined effects of local adaptation, mean condition, and phenotypic responses to environment-specific cues that affect resource allocation. A clear contrast can often be drawn between natural habitats and novel habitats, such as forest plantations and urban areas. In some species, males seem to change their sexual signals in these novel environments, but why this occurs and how it affects signal reliability is still poorly understood. The relative size of sexual traits and level of immune responses were significantly lower for male palmate newts *Lissotriton helveticus* caught in pine and eucalyptus plantations compared to those caught in native forests, but there was no habitat-dependent difference in body condition (n= 18 sites, 382 males). The reliability with which sexual traits signalled body condition and immune responses was the same in all three habitats. Finally, we conducted a mesocosm experiment in which males were maintained in pine, eucalypt or oak infused water for 21 days. Males in plantation-like water (pine or eucalypt) showed significantly lower immune responses but no change in body condition. This matches the pattern seen for field-caught males. Unlike field-caught males, however, there was no relationship between water type and relative sexual trait size. Pine and eucalyptus plantations are likely to be detrimental to male palmate newt because they are associated with reduced immune function and smaller sexual traits. This could be because ecological aspects of these novel habitats, such as high water turbidity or changes in male-male competition, drive selection for reduced investment into sexual traits. However, it is more probable that there are differences in the ease of acquisition, hence optimal allocation, of resources among habitats. Our mesocosm experiment also provides some evidence that water toxicity is a causal factor. Our findings offer insights into how plantations affect amphibian life histories, and how novel habitats might generate long-term selection for new resource allocation strategies in native species.

Keywords: novel habitats, *Lissotriton helveticus*, sexual selection, PHA, resource allocation.

INTRODUCTION

Sexual selection drives the evolution of elaborate male traits that increase mating and/or fertilization success (Kokko et al. 2006). However, these sexually selected traits can also be costly, and reduce other fitness components (Kotiaho 2001). For example, sexual traits can decrease the ability to avoid predators, increase energy expenditure, and lower immune responses, which ultimately reduces survivorship (Liker and Székely 2005; Bonduriansky et al. 2008). Adaptive responses to these trade-offs can occur over evolutionary time (i.e. selection for genotypes), and/or be expressed in the short-term (i.e. phenotypic plasticity).

At the individual level there is abundant evidence that condition-dependence (*sensu* Rowe and Houle, 1996) drives phenotypic plasticity in the expression of sexual traits (reviews: Hill, 2011; Tomkins et al., 2004). This is assumed to be adaptive because males in better condition can afford to invest more into sexual traits because they pay lower marginal costs (Grafen, 1990 but see Getty, 2006). Of course, the optimal expression of sexual signals also depends on the environment (Greenfield and Rodriguez 2004; Hunt and Hosken 2014). The most intensively studied source of environment–dependent selection on sexual traits is associated with their efficacy as signals (i.e. signal-to-noise ratio for communication; e.g. Moiron et al., 2015). However, the optimal expression of most life history traits also depends on ecological aspects of the environment. For example, the risk of mortality is partly determined by factors that affect predation risk (Cooper and Blumstein 2015), resource availability (Davison et al. 2014) and parasite loads (Studer et al. 2013). Consequently, the survival costs of a given level of sexual trait expression, as with most life history traits, will vary across habitats (Roff 2002).

Species appear to vary in whether and how they alter their sexual signals in response to variation in environmental conditions, particularly that associated with novel habitats (Wong and Candolin 2015). Some species produce signals that are seemingly well adapted to new habitats (Slabbekoorn and Peet 2003), others do not (Lengagne 2008), and some even respond in apparently maladaptive ways, resulting in so-called "evolutionary traps" (Battin 2004; Rodewald et al. 2011). Environmental factors determine the net benefit of sexually selected traits because they alter the relative returns from investing into different fitness components. The extent of variation in sexual traits across environments will depend on the combined effects of local adaptation (i.e. genetic evolution of new allocation decisions) due to differences in mean condition (i.e. via condition-dependence) and/or changes in trade-off relationships (i.e. new cost/benefit ratios); and through adaptive phenotypic responses based on environment-specific cues that affect optimal resource allocation decisions (e.g. Winandy and Denoël, 2015).

Male sexual traits are often positively correlated with greater immune responses or lower parasite loads (Møller et al., 1999; Schmid-Hempel, 2003, but see e.g. Faivre et al., 2003) and females tend to prefer males with lower parasite loads and/or greater immune function (Milinski and Bakker 1990). Experimental studies suggest that the immune system competes with sexually selected ornaments for resources (Folstad et al. 1992; Alonso-Alvarez et al. 2004; Boonekamp et al. 2008). This leads to a trade-off between sexual trait expression and immune function. It is well known that the observed phenotypic correlation between traits that trade-off can be positive or negative (see Jennions et al., 2001; Slatyer et al., 2012) for meta-analyses of the outcome of trade-offs involving sexual traits). The sign of the relationship is usually attributed to whether there is greater variation in resource acquisition or allocation (van Noordwijk and de Jong 1986). Equally importantly, however, is how the costs and benefits of sexual and other fitness-enhancing traits differ among environments. For example, immune function is less important when parasites are rare (Vergara et al. 2012b); and sexual traits are less important when competition for mates is low (Martínez-Padilla et al. 2014), or when they are more costly to produce because males have fewer resources (i.e. poorer condition), or even when the benefit of investing in signals is reduced because the environment makes it harder to discriminate between high and low quality males (Candolin et al. 2007). The environment should therefore affect optimal investment into traits and the phenotypic relationships between these traits.

To date, few field studies have explicitly reported on the extent to which the phenotypic relationship between sexual trait expression, body condition and immune response varies among habitats (P. Vergara et al., 2012, but see Hunt and Hosken, 2014 for laboratory studies). To explore this question in a focused manner it is necessary to study contrasting environments. Anthropogenically created novel habitats, such as forest plantations, provide an ideal contrast with native habitats. These are newly created, seemingly lower quality, environments that often affect individuals' body condition and can alter the strength of sexual selection (e.g. Candolin et al., 2007; Järvenpää and Lindström, 2004; Tuomainen and Candolin, 2011). Species appear to vary in whether their sexual signals change in response to novel environmental conditions (Wong and Candolin 2015). To date, the effect of novel habitats on trade-offs between sexual traits and other fitness-enhancing traits, such as immune function, has been poorly explored. This is important because some studies report seemingly adaptive changes in sexual trait expression (Moiron et al. 2015), which could actually be maladaptive if they shift resources away from other fitness-enhancing traits that have a greater effect on fitness in the new environment. Here we address this shortfall.

We studied the palmate newt *Lissotriton helveticus* (Razoumowsky, 1789), a common urodele in Western Europe. During the mating season males develop distinct visual secondary sexual traits, such as hind-feet webs, a caudal crest and a caudal filament (e.g. Cornuau et al., 2012; Haerty et al., 2007). These newts breed in a wide range of waterbodies (from ponds to lakes) and occur in a range of habitats, from intensively managed agricultural lands to natural forests. Among forested areas, they are most common in native forests, but are also found in exotic eucalypt and pine plantations. These plantations have been established in the study area in the last 50 years, so the evolutionary pressures faced by species inhabiting them are new. Both eucalypt and pine plantations are consistently associated with reduced species richness (Carrascal and Tellería 1990; Zahn et al. 2010; Martínez et al. 2016) and altered community structure (Watling et al. 2011). In addition, eucalypt plantations are characterized by the release of toxic substances into the substrate (Souto et al. 1994) and waterways (Pozo et al. 1998). There is, however, little data about the effect that these habitats have on the ecology of individuals (for an exception see Morrongiello et al., 2013).

We sampled males from 18 populations in pine or eucalyptus plantations, or in native oak forest. Different types of habitat patches were usually close to each other, so that gene flow due to the movement of individuals between habitats is likely. Both the mobility of newts and the short period of time that has elapsed since the establishment of plantations suggest that any differences in newt phenotypes among habitats are primarily due to plastic changes related to the characteristics of the habitat, rather than to local adaptation. If pine/eucalyptus plantations negatively affect male newts, we predict:

(a) a lower expression of sexual characters, a lower immune response and poorer body condition in pine/eucalyptus plantations due to habitat-related characteristics, such as fewer food resources and/or greater toxicity; (b) a difference in the relationship between condition and immune response and/or in the extent of sexual trait development among habitats (arising from different allocation strategies). Next, we created experimental mesocosms, where we controlled food supply and manipulated the type of leaf litter (eucalypt, pine or oak leaf). We tested the extent to which observed habitat differences in male traits might be driven by changes in water toxicity that affect males during their aquatic breeding phase. If negative effects of pine/eucalyptus plantations are primarily due to leaf toxicity, we predict that (c) differences among mesocosms would mirror those seen in field-caught males.

MATERIAL AND METHODS

Field study

From April 3-10, 2013 we captured 18-23 adult male newts from each of 18 ponds: six in native deciduous forest patches (*Quercus robur* L.), six in eucalypt plantations (*Eucalyptus globulus* Labill.) and six in pine plantations (*Pinus radiata* D.Don) in Basque Country. The vegetation in the study area is highly fragmented. The natural oak and beech forests are reduced to small patches surrounded by pine and eucalypt plantations. The ponds sampled were in habitat patches ranging in size from 0.6 km^2 to 1.2 km^2. Forests of different types were chosen to be as close to each other as possible given the available distribution of habitats to minimize differences between populations due to microclimate characteristics of the area (i.e. precipitation, substrate, temperature; Fig. 1). The distance between the closest ponds in different habitats was between 400 and 1000m. After an aquatic larval period of about 90 days (Montori and Herrero 2004), juvenile newts metamorphose and enter a long terrestrial phase (2 years), which they spend living in the forest. After this terrestrial phase the newts return to ponds as adults to breed. Once adulthood is reached, individuals usually remain within a few meters of the ponds (Graham 1977), but they can also disperse several kilometres (Montori and Herrero 2004), a sufficient distance to readily move between sampled ponds and to colonize new habitats. All ponds were small (ranging from 2.4 to 10.7 m^2), temporary and situated in the middle of the woodland.

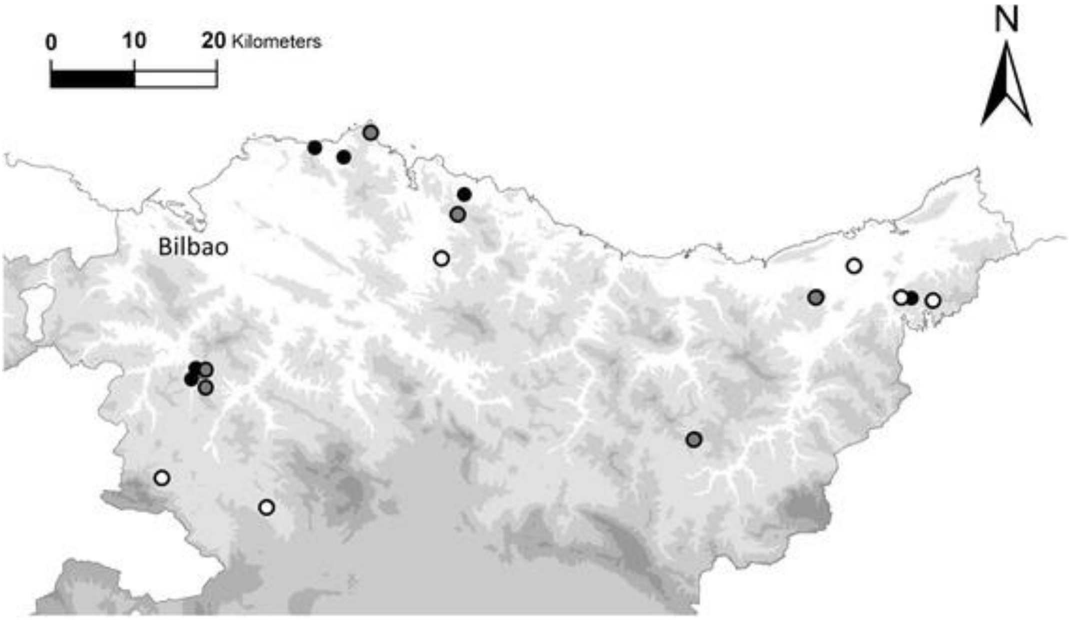

Figure 1. Map showing the 18 capture sites. White: natural oak forest; grey: pine plantations; black: eucalyptus plantations

We measured each male's body condition, immune function, four sexually dimorphic traits and total body length (n = 382). We calculated body condition as the residuals from the regression of body mass (g) on total length (mm), both variables were first Box-Cox transformed. When the relationship between these variables is linear (as it was here: field data $p < 0.001$, $r^2 = 0.48$; mesocosms data $p < 0.001$, $r^2 = 0.51$) this measure is often used as an index of the relative amount of fat stored, and, hence, of nutritional status (reviewed in Green, 2001). It is possible that the body mass of newts is related to the amount of food they have recently eaten and hence, in part reflects gut load. To counter this concern we reduced the influence of recent feeding on body mass measures by waiting 16 h after capture before weighing animals. The regression of body mass on body length is commonly used in newts as a non-invasive method to measure condition, and has successfully been used to investigate differences in food availability and habitat quality (MacCracken and Stebbings 2012; Sztatecsny et al. 2013).

To measure immune function we used a phytohaemagglutinin injection assay (PHA test), which is a delayed-type hypersensitivity test. This test is a reliable measure of T-cell dependent immunocompetence *in vivo* (Lochmiller et al. 1993), and has been used and validated in many studies including those on amphibians (Brown et al. 2011;

Clulow et al. 2015) (Note, however, that it does not capture all aspects of immune function: see (Adamo 2004)). We measured the thickness of the base of the tail 5mm from the vent with a pressure-sensitive spessimeter (+0.01 mm). The spessimeter closes to the point at which it touches the skin of the animal. It is important not to press the skin, because the inflammation caused by the PHA can disappear with the pressure of the spessimeter and lead to an incorrect post-injection measure. Our measurements were highly repeatable based on being taken five times. We used the average measurement for our statistical analyses. Immediately after the initial measurements, we then injected males with 0.01 mg of PHA dissolved in 0.01 ml of phosphate-buffered saline (PBS). All individuals were first anaesthetized by immersion in Tricaine methane sulphonate (0,15 g MS-222/1 L dechlorinated water) for 5-10 minutes (Cakir and Strauch 2005). Once recovered post-injection, newts were placed into plastic containers with 1 L of dechlorinated water. After 24 h, we measured the thickness of the tail base at the same point to calculate the difference between pre- and post-injection measures (inflammation). The cellular immune response index (hereafter 'PHA immune response') was calculated as the residuals of the regression of the inflammation against snout-vent length (all variables Box-cox transformed; Lochmiller et al., 1993; Smits et al., 1999). The only appreciable effect of the PHA injection was a slight swelling of the skin, caused by the immune response, which disappeared after 48 h. None of the newts showed any sign of stress or pain during these tests, and all looked healthy after the trials. All newts were fed after the second measure and were returned to their capture sites 48 h after being captured.

Male *Lissotriton helveticus* have several secondary sexual morphological characters that are involved in female mate choice: hind feet webs, caudal crests and caudal filaments (e.g. Cornuau et al., 2012; Haerty et al., 2007). Body dimensions were taken from photos of anaesthetized newts placed on a glass board with a measurement scale. We measured four secondary sex characters (caudal crest area, caudal crest depth, caudal filament length and hind feet webbing) and total body length. After the second measurement for the PHA test, animals were maintained in water to allow them to freely open their hind feet webs and another photo was taken from above to measure hind feet web area. All measurements were made using Image J (Abràmoff et al. 2004).

Mesocosms study

We had three experimental treatments (oak, pine or eucalyptus leaves) with three replicates of each. To simulate natural pond conditions we used 470 L mesocosms containing 200 L of tap water. Extracts were prepared by placing 100 g of dried leaves in each mesocosm. We also added 5 L of natural pond water from natural oak forest containing zooplankton and algae. The mesocosms were covered with a mesh to prevent the entry of predators. Then, after 48 h, we captured 180 adult male palmate newts from three nearby natural ponds in a mixed beech-oak natural forest. We randomly assigned 20 males to each of the 9 mesocosms, along with five females per mesocosm so that males stayed in the mesocosm, and maintained their secondary sexual characters (i.e. did not enter the terrestrial phase because of a lack of mating possibilities). Every day 5 g of blood worms were added to each mesocosm. We kept the animals in the mesocosms for 21 days to look for short-term responses to our treatments. Based on previous observations in the lab, the sexual traits of males become significantly reduced after a few days in captivity, presumably due to the initial stress (ICM personal observation). Moreover, 21 days is similar to the period of exposure used in other toxicological assays of amphibians (Sparling et al. 2010). Thereafter all animals were individually placed in aquaria with 1 L of clean water to take the same morphological and immune measurements described for the field study. The individuals used for the mesocosms study were captured in three adjacent ponds, between which individuals were likely to have free movement. Afterwards, animals were returned to the capture sites.

Statistical analyses

The measured traits (PHA immune response, body condition; and four sexual traits: tail crest depth, caudal filament, tail crest area and hind-feet web) were Box-Cox transformed to better meet assumptions of normality. We then calculated the residuals for each sexual trait from its regression on snout vent length. To reduce the number of independent variables for sexual traits (and decrease potential problems associated with multiple testing), we ran a Principal Component Analysis on these residuals. The residuals of all four sexual traits loaded strongly and positively on PC1, which explained 71% of the variance for the field data and 59 % of the variance for the mesocosms. PC1 was used as our measure of the relative expression of sexual traits (hereafter 'relative sexual trait size'). It is, however, possible that the different sexual signals measured are differentially influenced by habitat type and/or condition. To explore this possibility we repeated all the models for each trait separately (*post hoc* test results are presented as supplementary material). The statistical analyses and results presented in the main text are based on PC1 of all four sexual traits.

We used Linear Mixed Models (LMM) to test for the effect of habitat type on our male measurements. Models were run in R 3.2.2, or with Statistica 13.0. We treated replicate (i.e. forest) as a random factor, and habitat as a fixed effect. We conducted post-hoc pairwise comparison using Tukey tests. We checked the residuals of all models using q-q plots to ensure that they met the assumption of normality, linearity and homoscedasticity.

To test whether the relationship between relative sexual trait size and PHA or body condition respectively differed among the three forest types, we ran separate LMMs with PHA response or body condition as the dependent variables, and relative sexual trait size, and forest type as fixed effects. We first standardized relative sexual trait size to a mean of zero and a standard deviation of one to assist in interpretation of the results (Schielzeth 2010).

We repeated the same statistical analyses for the effect of experimental treatment in the mesocosms study.

RESULTS

Field experiment

Summary statistics for each forest type are presented in Table 1. Relative sexual trait size differed significantly among the three forest types ($F_{2,15.06} = 23.25$, $P < 0.001$, Table1a). Sexual trait expression was highest in newts from oak forest, intermediate in pine plantations and much lower in those from eucalyptus plantations (all pairwise tests, $p < 0.001$). We also found significant habitat differences in male immune response to PHA ($F_{2,14.78} = 25.57$, $P < 0.001$, Table 1a). The PHA immune response of males from oak forest was much higher than that of males inhabiting pine or eucalyptus plantations (both pairwise tests, $p < 0.001$). PHA immune response was also higher in pine than eucalyptus plantations (pairwise test p < 0.001). Surprisingly, however, male body condition did not differ among the three habitats ($F_{2,15.19} = 1.90$, $P = 0.183$, Table 1a).

Table 1. Differences in the relative sexual traits, PHA response and body condition among habitats in a) field data and b) mesocosms. Significant values are in bold. Sample sizes in text.

	Mean ± SD				
Trait	Oak	Pine	Eucalyptus	F	P - value
A) Field					
Relative sexual traits	0.740 ± 0.76	-0.245 ± 0.82	-0.742 ± 0.75	23.25	**< 0.001**
PHA response	0.034 ± 0.03	0.002 ± 0.04	-0.050 ± 0.03	25.57	**< 0.001**
Body condition	0.002 ± 0.02	0.007 ± 0.02	-0.011 ± 0.02	1.9	0.18
Total length (cm)	6.804 ± 0.45	7.036 ± 0.43	7.013 + 0.38	1.92	0.18
Weight (g)	1.468 ± 0.24	1.587 ± 0.24	1.468 ± 0.24	1.25	0.31
B) Mesocosms					
Relative sexual traits	0.303 ± 0.97	-0.187 ± 0.80	-0.117 ± 1.14	2.52	0.16
PHA response	0.242 ± 0.21	0.018 ± 0.22	-0.265 ± 0.22	29.65	**< 0.001**
Body condition	-0.006 ± 0.11	-0.004 ± 0.14	0.010 ± 0.13	1.29	0.275

Males with larger sexual ornaments for their body size had stronger PHA immune responses in all three habitats (PC1: estimate ± SE: 0.012 ± 0.004; $t_{369.8} = 2.945$, $P = 0.003$, Fig 2a), and the strength of the relationship did not differ among the three forest types (interaction: estimate ± SE: 0.005 ± 2.1e $^{-5}$; $t_{373.8} = 0.985$, $P = 0.25$; Fig 2a).

Similarly, males with larger sexual ornaments for their body size were in better body condition (estimate ± SE: 0.009 ± 0.003, t_{368} = 3.156, P = 0.001, Fig. 2b), but, again, there was no difference in the strength of the relationship across the three forest types (estimate ± SE: 0.003 ± 1.6 e $^{-5}$, t_{371} = 0.973, P = 0.56, Fig. 2b).

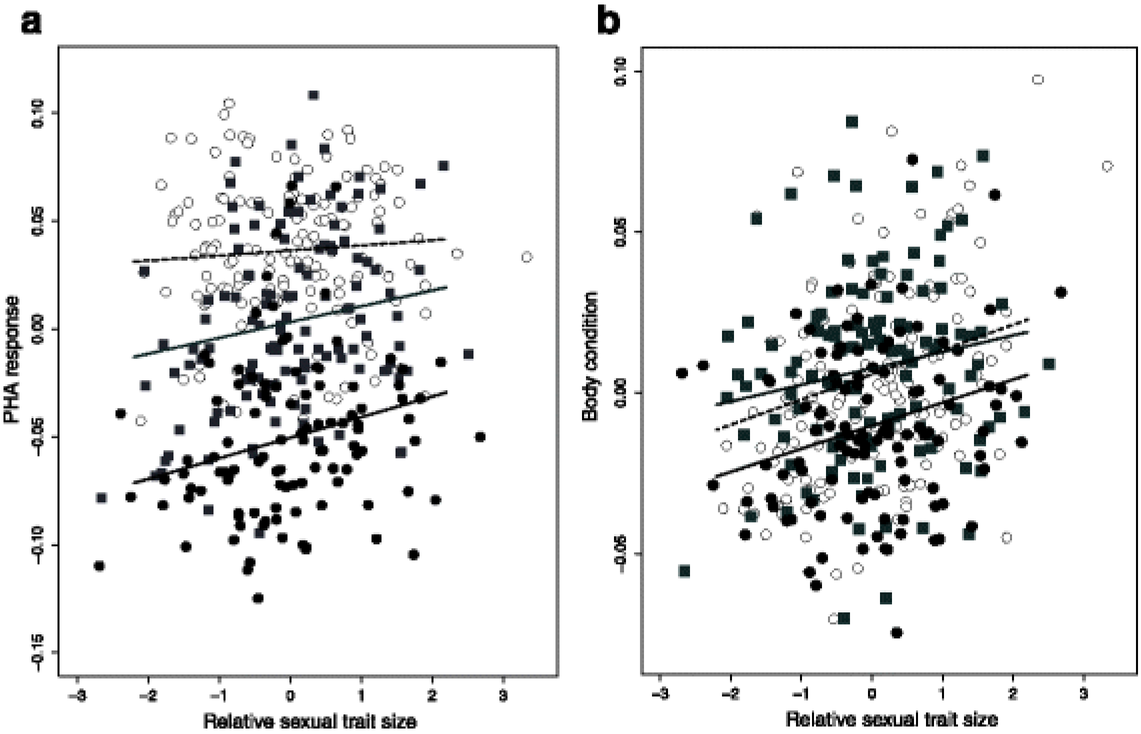

Figure 2. The relationship between relative sexual trait expression and: (**a**) PHA immune response was positive in all three habitats, model R^2: 0.62; (**b**) body condition was positive in all three habitats, model R^2: 0.42. The strength of the relationship did not differ among forests for either PHA immune response or body condition (see main text). Oak forest: white circles, dashed line; pine plantations: grey squares, grey solid line; eucalyptus plantations: black circles, black solid line

Mesocosms experiment

There was no effect of treatment on either relative sexual trait size or body condition when males were maintained for 21 days in mesocosms (sexual traits: $F_{2,5.97}$ = 2.52, P = 0.160; body condition: $F_{2,179}$ = 1.29, P = 0.275; Table 1b). However, this short period of time was sufficient to affect male PHA immune response ($F_{2,6.12}$ = 29.65, P < 0.001; Table 1b). As with field-caught males the PHA immune response was highest in the oak treatment males, intermediate in pine treatment males and lowest in males maintained in eucalyptus leaf litter (all pairwise tests < 0.01). Summary statistics are presented in Table 1b.

Unlike the case for field caught males, there was no relationship between sexual trait expression and PHA immune response (estimate ± SE: 0.024 ± 0.024, $t_{177.1}$ = 0.978, P = 0.328; Fig. 3a). There was, however, still a positive relationship between sexual trait expression and body condition (estimate ± SE: 0.042 ± 0.013, t_{179} = 3.120, P = 0.002; Fig 3b), and, as in the field collected males, the strength of the relationship did not differ among treatments (estimate ± SE: -0.013 ± 0.002, t_{179} = -0.553, P = 0.374; Fig 3b).

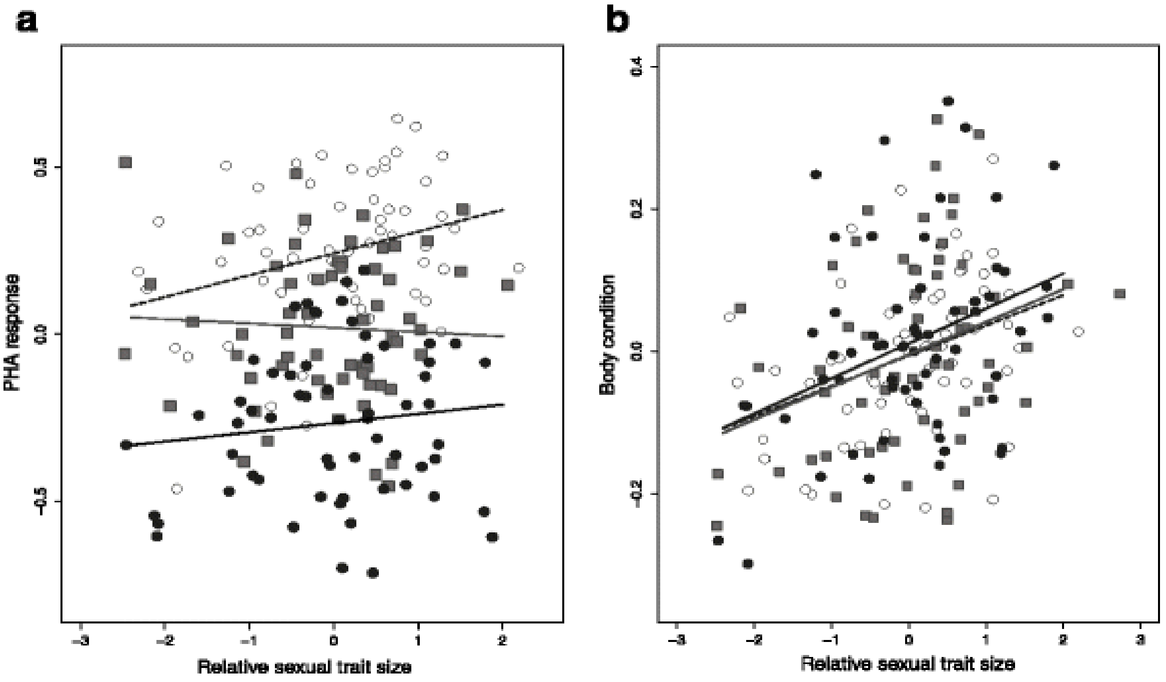

Fig. 3
In males in mesocosms there was (**a**) no relationship between sexual trait expression and PHA immune response in any of the three treatments, model R^2: 0.51; (**b**) a significant positive relationship with body condition that did not differ in strength among the three treatments (see text), model R^2: 0.12. Oak forest: white circles, dashed line; pine plantations: grey squares, grey solid line; eucalyptus plantations: black circles, black solid line

In general, we observed the same general pattern seen for PC1 (i.e. relative sexual trait size) when we ran the models separately for the relative size of each sexual trait (Supplementary Material). However, we found slight differences among traits when looking at the relationship between each trait and body condition or PHA response (Supplementary Tables 2 and 3). These findings suggest that some traits might be less costly to produce and, hence, less reliable indicators of male quality. These results are a reminder of the importance of studying several traits when assessing the effect of habitat alteration on male sexual signalling.

DISCUSSION

We predicted that inhabiting novel forest plantations would change the resource acquisition of male palmate newts (*Lissotriton helveticus*) due to specific characteristics of these habitats (e.g. reduction of food both in the aquatic and terrestrial phases (Zahn et al. 2010; Martínez et al. 2016), or confronting toxic substances (Escartin and Mariani 2014) and that this would reduce the amount of resources that males can invest in traits that are sexually selected and traits that enhance viability. In support, we found a significant reduction in relative sexual trait size and immune response in wild caught males from Eucalyptus or pine plantations compared to those from natural forests. There was, however, no effect of forest type on male body condition. We did not find differences among habitats in the relationship between sexual traits and either body condition or immune response. These findings suggest that the reliability of male sexual ornaments as signals does not change and that they are good predictors of immune response and body condition in both native and novel habitats. Finally, the similarities between the field and mesocosms data show that water toxicity might play an important role in the observed habitat differences. Although palmate newts can inhabit forest plantations these habitats seem to have a harmful effect (inferred from smaller sexual traits and weaker immune responses), which could affect the evolution of reproductive strategies (e.g resource allocation, mate choice) in the long-term.

Mean trait values

The lack of a detectable difference in body condition between novel and native forests seemingly contradicts our assumption that plantations are a low quality habitat. It is, however, important to distinguish between the theoretical concept of condition (i.e. total resources available *prior* to allocation to life history traits; see (Rowe and Houle 1996) and measured body condition (i.e. some measure of the ratio of fat or body mass to body length) (reviews: Hill, 2011; Tomkins et al., 2004). Measured body condition is itself a life-history trait, and variation in body condition could adaptively vary (or remain constant) among environments depending on the relative fitness returns from investing in it as opposed to other traits (e.g. sexual traits or immune function).

Plantations could have negative effects on trait expression not only due to lower resource availability, but also due to changes in other environmental factors that select for different optima. These factors include the greater prevalence of diseases, or higher parasite loads (e.g. Carey et al., 2003). If so, it might be adaptive for males to invest less into sexual signals and more into immune response. Compared to males from native habitat, however, the immune response to PHA was lower for field-caught males from plantations. The same was true for males kept in mesocosms mimicking the water conditions in the respective habitats. Previous laboratory experiments show that immune function can change rapidly when exposed to toxins as a stress-induced response (Burraco et al. 2013). The allelopathic substances in eucalyptus and pine leaves might therefore directly reduce immune function. However, the observed immune response to PHA is not necessarily reflective of total investment into immune function. It has to be discounted by the extent to which resources have already been used to counter earlier infections and/or dealing with toxins (for a thorough review see Adamo, 2004).

In our field data, males from pine plantations reduced their investment in both sexual traits and immune response compared to those from natural oak forests, but less so than males from eucalyptus plantations. We observed the same pattern for immune responses (PHA), but not for sexual traits, in the mesocosms where the amount of food provided was kept constant across treatments. The toxicity of eucalyptus leaves is known to have a strong effect on some animal communities (Ferreira et al. 2015). If eucalypt leaves are more toxic than pine leaves we expected newts from eucalyptus plantations to invest more of their resources into trying to expel toxins from their body. If males invest resources into expelling these toxins, the resources that are then available to invest into other traits, such as sexual characters and immune response, should be reduced. The toxicity of the water, especially in the case of eucalyptus, might therefore partly explain the differences in immune response to PHA found between oak forests, pine and eucalypt plantations in both our field and mesocosms experiments. On the other hand, sexual trait expression might only change plastically over a longer time scale than the 21 days of our study, or in response to environmental characteristics not manipulated in our mesocosms study. For example, investment in sexual traits might depend on the conditions encountered on land and the resources acquired during the terrestrial phase. Males develop sexual traits only during the breeding aquatic phase, while during the terrestrial stage they have no secondary traits. In plantations, the availability of terrestrial prey seems to be lower (Zahn et al. 2010) and the environmental conditions harsher (i.e. less refuge availability or presence of toxic substances), affecting the availability of resources for sexual characters upon entering the water phase. Once the investment in sexual traits is completed, even if the water conditions worsen (as in our mesocosms experiment), animals might strategically maintain their investment in sexual characters to avoid losing mating opportunities, but in so doing they might then compromise other fitness-related traits.

Differences in sexual trait expression between habitat types could also be explained by other ecological variables, such as water turbidity or male competition. For example, water turbidity is known to have a negative effect on the expression of sexual traits in *L. helveticus* (Secondi et al. 2007). In turbid environments, visual signals are less effective, so males might show an adaptive phenotypically plastic response, and reduce investment into costly visual sexual signals and reallocate resources elsewhere (Candolin et al. 2007). Social communication in newts depends on both visual and chemical signals (Halliday 1975; Houck 2009). Water turbidity could reduce the advantage of developing visual sexual signals traits, so that males will benefit from greater investment into pheromones. However, the leachates released by tree plantations, can also disrupt mate choice based on chemical cues (e.g. disruption of mate choice in newts by endosulfan (Park et al. 2001) or realistic nitrate concentrations (Secondi et al. 2009)), which could have a strong effect on sexual selection in these novel habitats. Moreover, when male-male competition for females is higher we generally expect stronger investment in sexually selected traits. In another study, however, we found that neither water turbidity nor the population density differs among study ponds in different habitat types (Iglesias-Carrasco et al, unpublished).

Adaptive responses to a trade-off between elevating reproductive success and decreasing survival can occur over evolutionary time by adaptation and in the short-term by phenotypic plasticity. However, the pine and eucalyptus plantations in our study were established less than 50 years ago. Gene flow between populations in different habitats is highly likely because the populations are not isolated (there are patches of mixed plantations with natural forests throughout the region). The observed changes in trait expression in plantations are therefore most likely to be due to phenotypic plasticity and differential acquisition allocation of resources and not to genetic adaptation to the new environment.

Relationships between traits

Environmental differences can create variation in the level of condition-dependence of sexual traits (Cothran and Jeyasingh 2010). This variation arises when the optimal resolution of trade-offs, such as investing into self-maintenance or the production of costly ornaments, differs between contrasting environments (Robinson et al. 2008). This could result in the long-term evolution of population differences in allocation decisions (i.e. the relationship between condition and a given trait), as predicted by life history theory (Roff 2002). It is, however, necessary to be careful when talking about adaptive phenotypic plasticity and optimal resource allocation. Some populations and species exhibit pre-adaptations to novel environments, especially when the conditions in the original habitat are similar to those in the novel habitat (Blackburn et al. 2009). Life-history strategies seem to play an important role in the capacity to invade new habitats (Sol et al. 2012). In general, however, organisms will only show an adaptive phenotypic response in, say, sexual trait expression or immune function if the optimal allocation *and* the proximate cues for how to allocate resources are similar in both the novel and historically encountered environments (i.e. those that have shaped phenotypic plasticity to make it adaptive). It is also plausible that being in an novel habitat amplifies or reduces the relative difference in condition between low and high quality individuals (e.g. a stressful environments can similarly affect the magnitude of inbreeding depression (Armbruster and Reed 2005; Fox and Reed 2011). This could alter the strength, and possibly even the direction, of relationships between sexual traits and other fitness components (Fargallo et al. 2007; Cothran and Jeyasingh 2010; Vergara et al. 2012a).

In general we found that field-caught males with relatively larger sexual traits were in better body condition and had a stronger immune response to PHA. Assuming that plantations are a lower quality habitat, and given the observed lack of differences in mean body condition between plantations and native forests, we expected the phenotypic relationship between body condition, sexual trait size and immune response to differ among habitats (i.e. resources being diverted away from the latter two traits to maintain body condition in plantations). There was, however, no difference among habitats in the mean strength of the relationship for either trait (Fig. 2a, b). One explanation for this finding is that we only measured a few life history traits. The relative allocation to other unmeasured traits might change across habitats based on cues other than condition, such as perceived predation risk or food availability, so that the relative investment into sexual traits and body condition or immunity remains constant.

Stronger sexual selection for condition-dependent male sexual traits has been suggested to occur in both favorable (Cockburn et al. 2008) and stressful environments (Fisher and Rosenthal 2006). In contrast to other empirical studies (Vergara et al. 2012a), however, our findings suggest that male sexual ornaments are equally reliable predictors of immune response and body condition in both low and high quality habitats. Reliable signaling is a key concept to understand how male ornamentation has co-evolved with female preferences (Greenfield and Rodriguez 2004). However, due to environmental heterogeneity (Greenfield and Rodriguez 2004; Higginson and Reader 2009), a mismatch between a male's sexual trait expression and his underlying fitness can compromise the benefits of female mating preferences (Holman and Kokko 2013). Although the reliability of male signals of specific life history traits did not change (i.e. body condition and immune response to PHA), it is unknown whether these traits are themselves equally valuable in all habitats, hence whether males are always reliably signalling their *net* fitness. In addition, we need to consider the effect of the habitat on female mate choice, which could possibly be mediated by changes in condition that affect her own mate choice decisions (e.g. Gillespie et al., 2014; Hunt et al., 2005; Judge et al., 2014).

Conclusions

Male *L. helveticus* sexual signals differ in mean expression between a native and two novel habitats. Despite this, the reliability of these sexual traits as signals of body condition and immune responses was the same across all three habitats. The extent to which this is maladaptive and generates selection for the evolution of new male allocation strategies will ultimately depend on the net fitness of males who continue to use allocation strategies that evolved in very different environments to these novel habitats.

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Chapter 3

Secondary compounds from exotic tree plantations change female mating preferences in the palmate newt (*Lissotriton helveticus*)

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ABSTRACT

Selection can favor phenotypic plasticity in mate choice in response to environmental factors that alter the costs and benefits of being choosy, or of choosing specific mates. Human-induced environmental change could alter sexual selection by affecting the costs of mate choice, or by impairing the ability of individuals to identify preferred mates. For example, variation in mate choice could be driven by environmentally induced differences in body condition (e.g. health) that change the cost of choosiness, or by environmental effects on the ability to detect or discriminate sexual signals. We teased apart these possibilities experimentally, by comparing female mate choice in the palmate newt *Lissotriton helveticus* between environments that mimic water from either native oak forests or exotic eucalypt plantations. In laboratory two-choice mate trials in clean water, females with long exposure (21 days) to waterborne chemicals leached from eucalypt leaves did not preferentially associate with the male with a stronger immune response, but females exposed to water with chemicals from oak leaves did. In contrast, female choice was unaffected by the immediate presence or absence of eucalypt leachates during mate choice (using only females previously held in oak-treated water). The habitat-related change in female choice we observed is likely to be driven by effects of eucalypt leachates on female physiology, rather than immediate inhibition of pheromone transmission or blocking of pheromone reception.

Key words: anthropogenic disturbance, female choice, *Lissotriton helveticus*, sexual selection, Urodeles.

INTRODUCTION

If mating non-randomly confers benefits, then mate choice can lead to sexual selection for costly but attractive traits. Mate choice is, however, itself costly due to the additional time, energy and predation risks that arise when assessing and then rejecting potential mates (Kokko et al. 2015). In some species, an increase in the costs of mate choice is associated with a reduction in choosiness by both females (Hunt et al. 2005; Head et al. 2010; Tomkins et al. 2016) and males (Wong and Jennions 2003). Ultimately, mate choice is only maintained by selection if the benefits of expressing mating preferences exceed the costs of choosiness. As with most life history traits (review: DeWitt et al. 1998), there are therefore circumstances where selection will favor phenotypic plasticity in mating preferences in response to environmental factors that alter the costs and benefits of mate choice.

Increasingly, species are being confronted with novel environments due to human habitat disturbance. These new environments could change the intensity and direction of sexual selection (Candolin et al. 2007). One factor that could drive these changes is the costs of mate choice, such that previously adaptive preferences or choosiness (for the distinction see Jennions and Petrie 1997) become maladaptive (i.e. greater costs favor reduced choosiness; Head et al. 2010). This could have long-term consequences because sexual selection can affect the viability and rate of adaptation of populations to new conditions (review: Candolin and Heuschele 2008; Power and Holman 2015).

Many studies report differences among populations in female mating preferences (e.g. Boughman 2001; Kwiatkowski and Sullivan 2002), but relatively few studies relate these differences to habitat-dependent effects (Maan et al. 2006; Rafferty and Boughman 2006; Heuschele et al. 2009) Even fewer studies take the final step of conducting experiments to directly test which aspects of the habitat drive differences in mate choice. There are therefore few studies that document habitat variation in female mate choice, and then provide a functional account for its occurrence. Here we pursue this goal by comparing female mate choice in European palmate newts (*Lissotriton helveticus* Razoumowsky, 1789), a common urodele in Western Europe, in two well-defined environments: native oak forest and a recently established exotic eucalypt plantation used by our study species. Eucalypt plantations are anthropogenic habitats, created in the 1950s in the study area. Eucalypt plantations are characterized by the release of secondary compounds into waterways, such as polyphenols and tannins. These compounds can be toxic to fish (Steinberg et al. 2006), aquatic invertebrates (Larrañaga et al. 2009b) and amphibians (Iglesias-Carrasco et al. 2016) and can affect growth (Canhoto and Laranjeira 2007), development (Martin and Blossey 2013), reproduction (Morrongiello et al. 2011) and immune response (Iglesias-Carrasco et al. 2016) of aquatic taxa. Experiments further show that these chemicals also affect the behaviour of Australian species that have a long history of co-existence with Eucalyptus (McMaster and Bond 2008). However, how plantations affect the sexual behaviour of species, such as many in Europe, that have only recently come into contact with the chemicals released by eucalypts has not been explored.

Variation in female choice could result from physiological differences that arise due to environmental effects (e.g. high parasite loads reducing body condition); or it could arise because environmental factors have an immediate, direct effect on the ability of females to discriminate between potential mates. To tease apart these two hypotheses, we performed two experiments using two-choice mate trials in which each female was allowed to choose between two males that differed in their immune response. We based our experiments on this male physiological trait because it is condition-dependent and positively correlated with the expression of a number of other sexually selected characters (Iglesias-Carrasco et al., 2016). In the first experiment we compared female mate choice in clean water after females had long (21 day) exposure to waterborne chemicals from either native oak or exotic eucalypt leaves. Female newts exposed to chemicals released by eucalypt plantations might allocate stored resources to combat toxicity, so that other life-history traits are adversely affected. We therefore predicted that females exposed to eucalypt chemicals would be less discriminating, as is generally the case for females under stress (Hunt et al. 2005). Second, we took females that had only been exposed to waterborne chemicals from native oak (21 day) and compared their mate choice in water that contained waterborne chemicals from either oak or eucalypt leaves. Female newts use both visual signals and pheromones to choose mates (Houck 2009), so a decline in water quality due to toxic substances released by eucalypt leaves might impair chemical communication between the sexes (Park et al. 2001). We therefore predicted that females tested in water containing eucalypt chemicals, when deprived of visual cues, would be less capable of discriminating between males. This could occur because waterborne eucalypt chemicals reduce the efficiency with which pheromones are transmitted, or because they lower the ability of females to detect pheromones (i.e. alter their sensory capabilities). From these experiments we use trials where female mate choice occurred under natural conditions (i.e. native oak water) to establish a baseline of female choice for male ability to respond to an immune challenge. Under natural conditions females are expected to choose males with a stronger immune response.

MATERIAL AND METHODS

We conducted two experiments. First, we exposed females to eucalypt-infused water for a period of 21 days in mesocosms (hereafter called "long-term exposure" to differentiate from the "immediate" or "short-term exposure" in experiment 2) (*see experiment 1*) and then tested their mate choice. Second, we tested whether mate choice differed when females maintained in native oak-infused water were tested in water from their natural habitat or in water that was eucalypt-infused (*see experiment 2*). We conducted dichotomous choice tests where females were able to choose between two males. We measured male immune responses (following a phytohaemagglutinin (PHA) injection assay, *see below*) as an index of mate health. We ensured that male pairs covered a range of differences in PHA immune responses (from 0.60 to 0.02), so females had to choose between pairs of males that varied in how much they differed in immune response (mean difference and SE for each treatment: Experiment 1: eucalypt 0.215 ± 0.149; oak 0.203 ± 0.139. Experiment 2: eucalypt = 0.148 ± 0.07; oak = 0.164 ± 0.09). We expected that the more the males differed, the stronger the choice would be for the male with a greater immune response. All the males were adults and there was no difference in body size between treatments (mean size (mm) and SE: Experiment 1 eucalypt = 35.78 ± 2.01; oak = 35.65 ± 1.78. Experiment 2 eucalypt = 35.44 ± 1.73; oak = 35.62 ± 1.61).

Female's maintenance. Palmate newts in Spain breed in spring and summer (February to June) in a range of waterbodies, from ponds to lakes, and occur in a variety of habitats, from intensively managed agricultural lands to natural forests. In forested areas, newts are far more common in native forests than eucalypt plantations, but they are found in both habitats. On March 7th and 8th 2014, in the middle of the mating season, we captured by dipnetting 182 adult female newts (March 7th experiment 1: n = 120; March 8th experiment 2: n = 62) from ponds in native deciduous forests (mainly oak *Quercus robur*). We sampled three ponds within a habitat patch of 1.2 km^2. Given that newts move between ponds (up to several km apart, Montori & Herrero, 2004) the collected individuals can be considered to come from a single population. For experiment 2, it would also have been interesting to test if there is adaptation or acclimation to eucalypt leachates by testing females from eucalypt plantations. However, females were rare in ponds under eucalypt, and the sample size we obtained was insufficient (n = 18) to conduct the experiment. After collection female newts were brought back to the laboratory at Aranzadi Science Society and housed in mesocosms. To simulate natural pond conditions we used 470L outdoor mesocosms containing 200L of mineral water and 5L of natural pond water containing zooplankton and algae. We set up three mesocosms per habitat type (3 oak replicates and 3 eucalypt replicates for experiment 1; 3 oak replicates for experiment 2). In each mesocosm we added 100g of dried leaves (see Maerz *et al.*, 2005 for dose rates), either eucalypt (treatment) or oak (control). To prevent the entry of predators, we covered each mesocosm with mesh. After 48h we placed 20 females into each mesocosm. We added 5g of blood worms daily. Females were kept in the mesocosms for 21 days, which is a standard period for behavioural ecotoxicology studies (Sparling et al. 2010). This 21 day period has been shown to be ecologically meaningful for amphibians, since the exposure to plant leachates for three weeks increases mortality and reduces growth of amphibian larvae (Maerz et al. 2005; Watling et al. 2011), and it reduces the immune response of adult newts (Iglesias-Carrasco et al. 2016).

Male immune response. We used male immune function as a likely predictor of female mate choice for two reasons. First, it is better than measuring any given sexually selected trait because these traits often show phenotypic trade-offs. In contrast, immune function gives an overall estimate of health that is correlated with key traits assessed by females when choosing mates in both habitats (for field data see Iglesias-Carrasco *et al.*, 2016). Second, measuring immune responses on the large number of males we tested is quicker than measuring numerous sexually selected traits. This allowed us to use males shortly after capture from the wild, rather than having to keep them in captivity for a long period prior to testing.

We captured 240 adult male newts in native oak forest patches three days prior to behavioural trials. We measured their immune function as inflammation following a phytohaemagglutinin injection assay (“PHA immune response”). PHA immune response is a reliable measure of T-cell dependent immunocompetence in vivo (Lochmiller et al. 1993), that has been used and validated in several studies, including ones on amphibians (Clulow et al. 2015; Murillo-Rincón et al. 2016).We measured the thickness at the base of the tail 5mm from the vent with a pressure-sensitive spessimeter (+0.01 mm). Immediately afterwards, we injected 0.01 mg of PHA dissolved in 0.01 ml of phosphate-buffered saline (PBS). After 24 hours, we again measured tail thickness and calculated the difference between the pre- and post-injection measurements (i.e. inflammation). Tail inflammation is related to body size ($R^2 = 16.67$, $P < 0.001$) so we calculated relative immune response as the residuals of the regression of log transformed inflammation on log transformed snout-vent length. By doing this we controlled for the relationship between PHA response and body size in our analysis. In addition, the paired males were similar in size in both treatments and in both experiments (mean difference in size (mm) ± SE between the male with the stronger and the weaker immune response: Experiment 1 eucalypt: -0.15 ± 2.67; oak: 0.16 ± 2.35. Experiment 2 eucalypt: 1.41 ± 3.01; oak: 0.13 ± 2.66).

Experiment 1: Long-term exposure to eucalypt leaf litter and female mate choice

Behavioural trials. We removed females from mesocosms with an aquarium net 21 days after first housing them in the mesocosms. We placed them individually in 36 x 42 cm aquaria with 10L of mineral water, with a natural photoperiod and constant temperature of 12 ºC. Females were allowed 1 h to acclimate to these conditions, before two males were added. To ensure female choice depended on chemical cues, males were placed in an opaque 1L cup (7 x 7 x 20 cm) pierced with numerous small holes to prevent tactile and visual stimuli, but allow water exchange. A cup was placed at each end of the aquarium and females could freely move between them. We allowed 20 min for the newts to acclimate before observations began. Then, every 10 minutes for 150 min, we noted if the female was <5cm from a cup. Previous studies have shown that the relative time spent with males is a good predictor of female mate choice (Amundsen and Forsgren 2001; Milinski et al. 2005; Heuschele et al. 2009). Moreover, using a subset of females, we confirmed that females mate with the male with whom they preferentially associated ($n = 18/20$; Binomial test, $P < 0.001$). Placement of aquaria was randomized to avoid potential environmental gradients within the laboratory confounding our treatments. All the trials were conducted on the same day and began at 9pm (i.e. after dark) to ensure that females were active and searching for mates (based on activity patterns in a similar species Griffiths, 1985 and confirmed in our study species by MIC). The behavioural trials were made in darkness at a constant temperature of 12 ºC, mirroring the air temperature in the field on the night of the experiment. MIC collected all data blind to both the females' treatment and the males' PHA immune response. All animals remained healthy during the trials and were eventually re-released at the capture sites.

Experiment 2: The immediate effect of eucalypt leaf litter on female mate choice

To test the immediate effects on female mate choice of the presence of secondary compounds from eucalypt leaf litter we conducted a second experiment where *all* females (n = 62) were housed in mesocosms containing oak-treated water for 21 days. We then quantified female choice for males that differed in their immune response as in experiment 1 (see above), except that the test aquarium now contained either 10L of oak-infused water (control) or eucalypt-infused water (n = 31, 31). We opted for the preparation of new extracts, and did not directly use the water from the mesocosms from experiment 1 to eliminate other factors (e.g. female and food odour) that might affect the behaviour of our study animals. Infusions were prepared 48 h prior to the trials by adding 5 g of dried leaves to the water (dose rates Maerz *et al.*, 2005). Previous studies looking at leachate composition of water show that 48 h is enough time for plant secondary compounds to be leached into the water (Maerz *et al.*, 2005; Watling *et al.*, 2011). Leaves were strained with cheesecloth from the leachate solution to remove solid material immediately before the choice trial. After 1h of acclimation a pair of males was added to the aquarium and female choice was recorded.

Statistical analyses

In our dataset tail inflammation was correlated with body size (R^2 = 0.16, P < 0.001). However, given that the paired males were similar in size in both treatments and in both experiments (mean difference in size (mm) ± SE between the male with the stronger and the weaker immune response: Experiment 1 eucalypt: -0.15 ± 2.67; oak: 0.16 ± 2.35. Experiment 2 eucalypt: 1.41 ± 3.01; oak: 0.13 ± 2.66) and that preliminary analyses showed no effect of male size difference on female preferences (P-values of the model for both experiments > 0.27), we chose not to control for body size in our analyses (Freckleton, 2001). Note however, that controlling for body size did not alter our results.

First, we tested with Chi-square tests whether there was a difference in female interest in males between the two habitats. We compared the proportion of females from each treatment that showed no interest in males (i.e. hung at the surface and did not explore the aquaria) (experiment 1: *n* =16/120; experiment 2: *n* = 5/62).

For the rest of the analyses we discarded trials where the females showed no interest in males. The final sample sizes were 53 eucalypt- and 51 oak-treatment females in experiment 1, and 28 eucalypt- and 29 oak-treatment females in experiment 2. We initially analyzed our data with a generalized linear mixed model (GLMM) with a binomial error distribution, but the data were highly overdispersed (the scaling parameter was 4.26 in exp.1 and 1.57 in exp. 2 for models that included the interaction term). A quasibinomial error distribution cannot be used when models include random effects, so we opted to analyze the proportion of observations a female spent with the higher quality male (i.e. male with the stronger immune response) using linear mixed models (LMM) for both experiments. We checked and confirmed that the residuals of all models met the assumptions of normality, linearity and homoscedasticity. First, to test if the time females spent associating with males differed among treatments, we ran a model for each experiment that included the treatment as a fixed term and the mesocosms replicate as a random factor. Then, for each experiment we ran separate models that included the water treatment, the difference in PHA immune response between the pair of males and the interaction between these variables as fixed terms. We included mesocosm replicate identity as a random factor. All the analyses were run in R 3.2.2. The two key statistical tests ask: (i) Is there a positive effect of the magnitude of the difference in male PHA immune response on the proportion of time females spent with the male with the stronger immune response? If so, this indicates that females spent relatively more time with the male with a greater immune response when the two males are more readily distinguished; and (ii) Is there an interaction between water treatment and the difference in PHA immune response that affects the proportion of time spent with the male with the stronger immune response? If so, this indicates that ability of females to discriminate between males that differ in their PHA immune response depends on the water treatment.

RESULTS

Experiment 1: Long-term exposure to eucalypt leaf litter and female mate choice

There was no effect of the water treatment on the level of female interest in males (X^2 = 0.062, P = 0.802). Neither was there an effect of the treatment on the average time that females spent associating with males ($F_{1,101}$ = 1.20, P = 0.275).

However, the water treatment did affect how females responded to the difference in males' PHA immune response (interaction estimate ± SE = 96.10 ± 36.55, t = 2.62, P = 0.009). To look at female preference for male PHA immune response within each treatment we ran separate analyses for each water treatment. Females from oak-treated mesocosms spent significantly more time near the male with the stronger PHA immune response as the difference between the two males increased (estimate ± SE = 65.82 ± 26.85, t = 2.45, P = 0.016). In contrast, eucalypt-treated females showed a non-significant preference in the opposite direction (estimate ± SE = -30.18 ± 24.80, t = -1.22, P = 0.225) (Fig. 1).

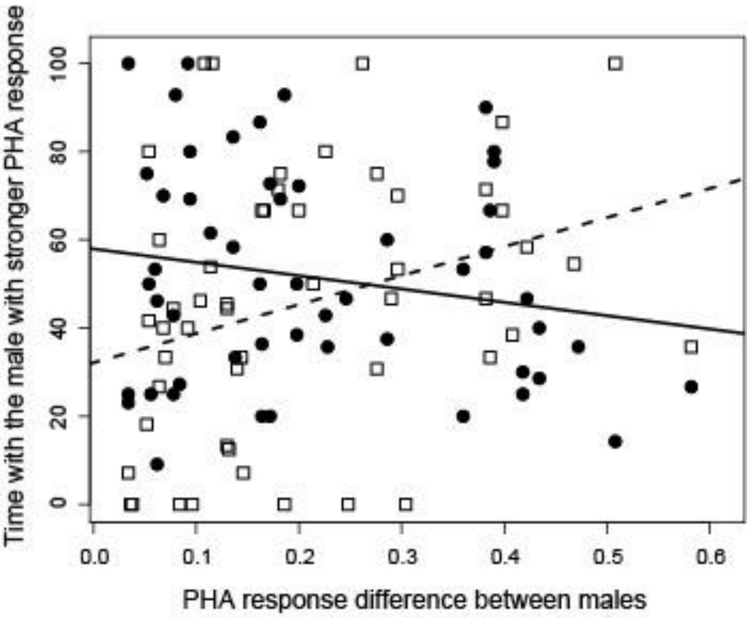

Figure 1. The relative time spent near the male with the stronger PHA immune response (% of point samples) by females housed in either eucalypt-treated mesocosms (solid line, black circles) or oak-treated mesocosms (dashed line, white squares). Graph shows the raw data.

Experiment 2: The immediate effect of eucalypt leaf litter on female mate choice.

Again, we did not find any effect of the water treatment on the level of interest females had in males ($X^2 = 0.065$, $P = 0.797$), or in the time females spent associating with males ($F_{1,48.79} = 0.25$, $P = 0.617$)

Contrary to expectation, females tested in oak-infused and eucalypt-infused water behaved similarly when choosing males (estimate ± SE = -37.30 ± 61.09, $t = -0.20$, $P = 0.841$). Females spent significantly more time near the male with the stronger PHA immune response as the difference between the two males increased, regardless of the water type they were tested in (oak: estimate ± SE = 111.00 ± 34.55, $t = 3.21$, $P = 0.002$; eucalypt: estimate ± SE = 148.30 ± 50.12, $t = 2.95$, $P = 0.004$) (Fig. 2).

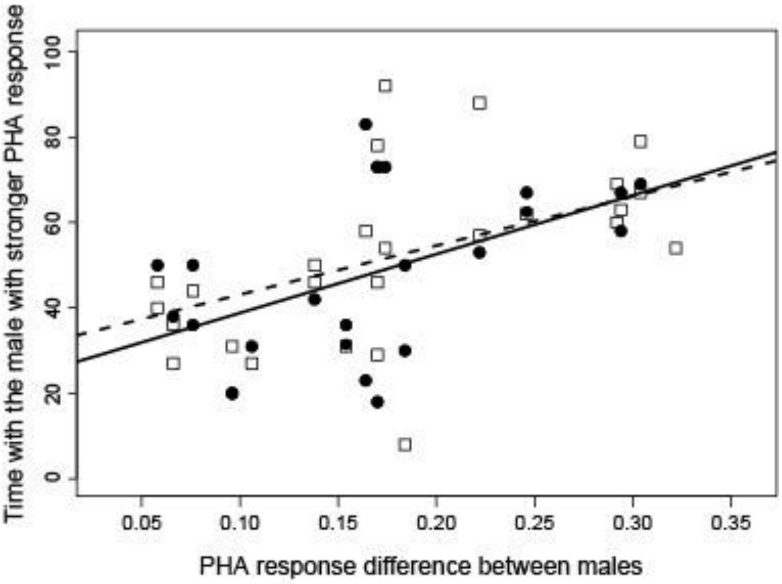

Figure 2. The relative time spent near the male with the stronger immune response (% of point samples) by females housed in oak treated water, but tested in either eucalypt-treated water (solid line, black circles) or oak-treated water (dashed line, white squares). Graph shows the raw data.

DISCUSSION

Both natural and sexual selection can differ between native and human-modified environments (Candolin and Wong 2012). In two-choice laboratory mating trials we found that female palmate newts kept and tested in water that mimics their natural habitat prefer males with a stronger immune response to a phytohaemagglutinin injection assay. Females exposed to eucalypt-infused water for a long period (21 days) showed reduced levels of mate choice for these males compared to females exposed for the same period to water mimicking their natural habitat. In contrast, when females were initially kept in water mimicking their natural habitat, the immediate presence of eucalypt-infused water during mate choice did not reduce their choice for males with a stronger immune response. We also found that the interest females had in males and the amount of time spent with males by females was similar for the treatments in both experiments. Our results suggest that: females generally prefer males with a stronger immune response; female olfactory capacity and the diffusion of pheromones is unaffected by the immediate presence of eucalypt leachates; but that long term exposure to eucalypt leachates may reduce a female's ability to, or interest in, mating with males with a stronger immune response. If these results apply in the wild, the strength of sexual selection on male pheromones will differ between native forests and exotic eucalypt plantations.

Effect of long-term exposure to eucalypt water on female mate choice.

Females showed weaker mate choice for males with a strong immune response after long-term exposure to eucalypt-infused water compared to water that mimicked natural oak forests. Eucalypt trees leach chemical compounds into the water (e.g. polyphenols and tannins) which can adversely affect the diversity of aquatic animals and their investment in life history traits (Maerz et al. 2005; Larrañaga et al. 2009a; Morrongiello et al. 2013). Here, we show that long-term exposure to these compounds can also alter behaviours that directly affect sexual selection. Across both experiments, in three of the four treatment groups, females significantly preferred to associate with (and would mate with, see *Methods*) males that had a higher PHA immune response: the relative amount of time spent with the male that had a greater immune response increased when the difference in immune response between the two males was greater. In contrast, female newts that were exposed to eucalypt leachates for 21 days showed no tendency to associate with males with a stronger immune response (Fig 1). Males with a higher PHA immune response are generally considered to be in better condition, and tend to have larger secondary sexual traits (e.g. Møller et al., 1999; Navarro et al., 2003; Schmid-Hempel, 2003), so this change in mate choice by females that have had long-term exposure to leachates could be considered a reduction in preference for high quality males. If this is the case the lack of mate choice after long-term exposure to eucalypt leachates might have consequences for long-term population viability of palmate newts inhabiting eucalypt plantations.

It has been suggested that exposure to plant leachates can cause physiological stress. For example, carp extracts can damage fish gills, and possibly interfere with respiration (Temmink et al. 1989). Likewise, exposure to eucalypt leachates is known to affect survival and reproduction of some Australian fish species. For instance, chronic exposure to eucalypt leachates negatively affected growth and survival in relation to body size and source population of juvenile pygmy perch (*Nannoperca australis*) (Morrongiello et al. 2013); while leachate-exposed females of the same species were less likely to reach maturity and reproduce (Morrongiello et al. 2011). Amphibians are also highly susceptible to habitat alteration and the presence of toxic substances in the water (e.g. Watling *et al.*, 2011). For example, the body condition and immune response of both adult and larval amphibians may be reduced by the presence of crops and exotic eucalypt plantations (Brodeur *et al.*, 2011; Iglesias-Carrasco *et al.*, 2016; Iglesias-Carrasco *in prep*.). We hypothesize that the absence of female mate choice for higher 'quality' males after long-term exposure to eucalypt leachates may be due to changes in female physiology. Previous studies have suggested that female fish exposed to eucalypt leachates have fewer resources available to allocate to reproduction since they expend energy to resist the effects of leachates (Morrongiello et al. 2011). For instance, combating toxins derived from eucalypt leachates might make females less choosy because they have fewer resources to allocate to mate sampling (Hunt et al. 2005). However, the fact that female association time with males did not differ between the water treatments suggests that female responsiveness or choosiness (when defined as the time invested by a female in making a choice, see Brooks & Endler, 2001) was unaffected. Alternatively, long-term exposure to toxins might reduce a female's investment into traits that are required to assess mates (e.g. neurological pathways), or it might simply damage a female's sensory apparatus, and thereby lower her ability to discern differences among males. Teasing apart these competing proximate explanations is an interesting avenue for future research.

In a previous study we showed that male palmate newts in eucalypt plantations have a reduced immune response and smaller sexual characters than those in native oak forests (Iglesias-Carrasco et al. 2016). We suggested that this was due to lower male body condition given the stress of living in eucalypt plantations (Iglesias-Carrasco et al. 2016). However, our current results suggest that reduced expression of male sexual characters could partly reflect weaker sexual selection due to a lack of mate choice (Candolin et al. 2007): females exposed to eucalypt infused water no longer discriminate between males. An alternative is that the difference in mate choice by females housed in the two water types might represent adaptive plasticity in mating preferences if the traits that signal male fitness differ between habitats. It is generally assumed that mating preferences are adaptive and, in the absence of direct benefits, signal male genetic quality (i.e. net offspring fitness; Kokko *et al.*, 2006). However, genetic benefits are context-dependent if the performance of different genotypes varies across environments (Roff 1997). A genotype that is advantageous in one environment may be disadvantageous in another (e.g. Welch, 2003; Hunt & Hosken, 2014). If there are fewer immune threats in eucalypt plantations than native forests then natural selection might favour males that invest less in costly immune defenses. This could favour females that show no preference for males with a strong immune response. We suggest this argument is biologically implausible, however, as male immune response and sexual trait expression are positively correlated in both habitats (Iglesias-Carrasco et al. 2016) and the relatively recent establishment of eucalypt plantations in the area (<70yrs) seems unlikely to have allowed sufficient time for such a fine-scale adaptive response. Likewise, the distances between natural forests patches and exotic plantations are short enough to be crossed by newts. Even so, it is likely that plantations are acting as sinks for individuals from natural forests, so that migration to natural forests of females previously adapted to eucalypts seems implausible.

The immediate effect of eucalypt leaf litter on female mate choice

Several studies have shown that toxic substances can impair female mate choice, especially in aquatic vertebrates (e.g. Park and Propper 2002; Tomkins et al. 2016). The transmission and reception of sexual signals partly depends on the channel of transmission (Endler 1992), and male-female communication via pheromones is known to be hindered by the presence of some contaminants. For example, naturally occurring chemicals can bind pheromones together (Mesquita et al. 2003) and block chemoreceptors (Hubbard et al. 2002), thereby affecting the ability to select preferred mates. We showed, however, that mating preferences and the resultant mate choice did *not* change when female palmate newts were tested in eucalypt-infused water. Even when eucalypt leachates were present, females presented with a pair of males still chose the one with the stronger PHA immune response. This result suggests that neither immediate transmission nor reception of palmate newt pheromone signals are inhibited by the presence of eucalypt leachates.

Conclusions

Sexual selection is affected by environmental factors that influence the condition of choosy individuals. It has been argued that this is especially important in human-modified environments because they often differ dramatically from native habitats in food availability, parasite loads and pollution levels (review: Ditchkoff et al. 2006). We found that leachates reduce the ability of female palmate newts to identify mates with a stronger immune response. Importantly, however, this effect was only detected after females had spent a 21 day period in eucalypt-infused water. This suggests that the effect is driven by changes in female physiology, rather than by the immediate inhibition of transmission or reception of pheromonal signals. Future studies are needed to tease apart the exact effects of eucalypt leachates on female newt physiology if we are to better understand the proximate causes. More generally, human-modification of habitat through the planting of eucalypts provides a large-scale ‘natural experiment’ to test how mating preferences evolve when the costs and benefits of mate choice change; and to test the broader effects of long-term consequences of changes in sexual selection at the population level (Power and Holman 2015).

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Chapter 4

Leaf extracts from an exotic tree affect responses to chemical cues in the palmate newt (*Lissotriton helveticus)*.

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ABSTRACT

Chemical communication in aquatic species can affect many key life-history traits, such as prey and predator detection and mate searching. However, changes in the environment can disrupt the effectiveness of signals and the ability of individuals to detect these signals. Many studies have examined the effect of secondary compounds from exotic plants on the ecology and physiology of a range of taxa. But whether the replacement of natural forests with exotic trees influences the behavioural responses of animals by disrupting chemical communication is rarely investigated. We experimentally tested how eucalypt tree chemicals influenced three key aspects of chemical communication in adult male palmate newts (*Lissotriton helveticus*). We tested both for effects of exposure to eucalypt water (i.e. extracts obtained by soaking leaves in mineral water) and of the origin of newts (eucalypt plantations and natural oak forests). We examined whether exposure to eucalypt water altered the chemosensory ability of males to: detect pools containing females, detect pools containing conspecific alarm cues, find food. We found that eucalypt leachates had different effects on each behavioural trait. Fewer males detected female chemicals when exposed to the eucalypt water treatment compared to the oak water treatment, independent of the males' habitat of origin. Newts from oak forest were less able to detect conspecific alarm cues signaling predatory events when exposed to eucalypt water than when exposed to oak water, or compared to newts from eucalypt plantations for either water treatment. The ability of males to find food using chemical cues was similar in oak and eucalypt treatments. Our results suggest that chemical compounds not previously encountered during the evolutionary history of the species can influence the ability to respond to predators and locate mates. Future studies should explore the fitness costs associated with a reduced ability to respond to predator or detect mates.

Keywords: alarm cues, *Eucalyptus* plantations, mate searching, novel habitats, palmate newt, predator detection, prey detection.

INTRODUCTION

In many aquatic species, chemical cues are important to maintain social interactions among conspecifics and interspecific relationships. In both vertebrate and invertebrate animals, communication based on chemical cues can affect many key life-history traits, including those involved in predator avoidance, prey detection, mate searching, mate choice, and social grouping (e.g. Todd et al. 1967, Johansson and Jones 2007, Ferrari et al. 2010). For example, many aquatic predators have evolved sophisticated chemical receptors to detect prey. In turn, prey species have become more efficient at reducing their vulnerability to predation by detecting and avoiding places that contain substances emitted by predators (Turner, Bernot, & Boes, 2000), or by recognizing 'alarm cues' from conspecifics that have been attacked by a predator (Gonzalo, López, & Martín, 2007; Schoeppner & Relyea, 2005). Further, in many aquatic vertebrates, females release waterborne pheromones that males use to locate potential mates. The importance of the use of chemical cues is especially relevant in aquatic species where vision is limited due to turbidity or low light levels (e.g. Ferrari et al., 2010).

Specific environmental conditions (e.g. water pH) have shaped the evolution of waterborne chemical signals (Ferrari et al., 2010). Similarly, receivers are under selection to detect signals or relevant cues under the prevailing conditions. For example, fringe-lipped bats, *Trachops cirrhosus*, are able to detect very low-frequency calls and use túngara frog mating calls to detect their prey, but their ability to locate frogs decreases with increased complexity of the environment and greater background noise (Page & Ryan, 2008). The recognition of predator chemical cues in aquatic systems is critical for survival. Some species learn about new predators based on, for example, conspecific alarm cues (Ferrari et al., 2010), changes in the environment, such as water acidification, may reduce the potential for this kind of learning (Ferrari et al., 2012). Chemical cues released by conspecifics are usually mixed with other 'background' chemical substances in the environment, that could either diminish or exacerbate the perception and consequent response of the receptor (Hale, Swearer, & Downes, 2009). Consequently, rapid changes in the environment (e.g. the introduction of new substances) can negatively alter ('disrupt') the effectiveness of signals and the ability of receivers to detect signals or, more generally, perceive informative cues (Wolf and Moore 2002, Fabian et al. 2007).

Disruption of chemical communication can have drastic consequences for predator detection, foraging success, the ability to locate and discriminate between potential mates or, in extreme cases, to recognize conspecifics (Wolf and Moore 2002,Gill and Raine 2014, Polo-Cavia et al. 2016). Contaminants released by human activities are an increasingly important source of environmental modification that disrupts chemical communication. These contaminants can bind to pheromones and/or block chemoreceptors (Mesquita, Canário, & Melo, 2003), potentially having a direct effect on the ability of organisms to detect important chemical signals or cues. There could also be indirect effects if these contaminants increase stress levels and thereby lower body condition, as many key behaviours are condition-dependent (e.g. mate searching, courtship signaling: Candolin 1999; Hunt et al. 2005). Human induced disruption of chemical communication can have drastic evolutionary and conservation effects for species that discriminate among potential mates using chemical cues. In extreme cases, it can even lead to hybridization. For example, female poeciliid fish exposed to humic acid do not appear to exhibit mating preferences for conspecific males, and are more likely to hybridize with a related species (Fisher, Wong, & Rosenthal, 2006).

Similar to the adverse effects arising from the presence of contaminants in the water, the chemical changes caused by exotic plants and their leaf litter can negatively affect several key life history traits of aquatic organisms. For instance, the introduction of exotic plants in native areas has been shown to alter amphibian communities (Watling, Hickman, & Orrock, 2011a). The presence of secondary compounds can reduce growth and survival in amphibians, as well as change food resources and thereby alter foraging behaviour (Brown, Blossey, Maerz, & Joule, 2006; Cohen, Maerz, & Blossey, 2012; Maerz, Brown, Chapin, & Blossey, 2005; Watling, Hickman, Lee, Wang, & Orrock, 2011). However, the extent to which the replacement of natural forests by exotic plantations has affected the chemical communication of native fauna remains underexplored.

Among artificial forests, eucalypt tree plantations stand out due to their worldwide occurrence. Eucalypt plantations are characterized by the large amounts of secondary compounds (e.g. polyphenols and tannins) that they release into the substrate (Souto, Gonzales, & Reigosa, 1994) and nearby aquatic ecosystems (Pozo et al., 1998). It is now clear that these substances can be toxic and adversely affect several ecological and physiological aspects of fish (Steinberg et al., 2006), aquatic invertebrates (Larrañaga, Basaguren, Elosegi, & Pozo, 2009) and amphibians (Iglesias-Carrasco, Head, Jennions, & Cabido, 2016). Even species that naturally occur in waterways in eucalypt forests, such as some fish, have reduced growth and survival when they are exposed to the toxic leachates found in these habitats (Morrongiello, Bond, Crook, & Wong, 2013). The high levels of tannins and essential oils in eucalypt leaves may also change the local biochemistry (e.g. decreasing water oxygen level and pH, Canhoto & Laranjeira, 2007). These changes in water chemistry may reduce the ability of individuals to detect and respond to chemical cues (Leduc, Munday, Brown, & Ferrari, 2013). To date, however, the effects of chemicals from eucalypt trees leaching into waterways and disrupting the use of chemical communication for key behaviours, such as mate searching or feeding activity, has not been investigated experimentally. To fully understand how exotic plantations affect the conservation status of native fauna, it is critical to delve not only into the direct effects on species diversity, but also into the indirect effects on the ecology of individuals. One of these indirect effects on ecology is the impairment of olfactory sensory modality by the presence of new chemical substances in the water. Olfactory disruption can have important consequences for the conservation of populations if it affects important life-history traits and the fitness of individuals.

In many amphibians, detecting chemical cues is crucial during their juvenile and adult aquatic phases. For example, the survival of tadpoles often depends on their ability to detect predators and produce an effective behavioural (e.g. reducing their activity) or morphological (e.g. decreasing the relative length of the body) response (Van Buskirk & Arioli, 2002). In newts, chemical communication through pheromones plays a key role in locating and choosing a suitable mate (Houck, 2009). While in other species, such as some urodeles, individuals that forage in the water use chemical cues to locate their prey (e.g. tiger salamander, Lindquist & Bachmann, 1982). The presence of secondary compounds from exotic plants in the water might induce changes in the behaviour of individuals that are exposed to these compounds. For example, the exposure of larval amphibians to leachates from an exotic shrub can induce a risk-prone response by increasing surfacing, even in the presence of potential predators (Hickman & Watling, 2014). In the same way, chemicals found in eucalypt plantations might negatively affect predator detection, mate recognition and foraging ability. Here we address this gap in knowledge by combining, in a single study, the effects of habitat alteration on three key behaviours that are usually closely related to evolutionary fitness.

We designed three experiments to investigate whether chemicals entering waterways from eucalypt tree plantations affect three key life-history traits in an amphibian by adversely affecting the ability to: 1) locate mates; 2) detect conspecific alarm cues indicative of the presence of predators; and 3) find food. In addition, we tested whether these negative effects vary depending on whether the individuals tested had previous exposure to eucalypt leachates in their habitat of origin. We studied males of the palmate newt (*Lissotriton helveticus*), a common urodele in Western Europe that inhabits both eucalypt plantations and native oak forests. These newts breed in a wide range of waterbodies, from ponds to lakes. They are most common in native oak forest, but are now also found in eucalypt plantations created in the 1950s. We captured newts from both eucalypt plantations and oak natural forests and then tested their ability to use chemical cues to locate females, detect conspecific alarm cues that signal the presence of predators, and find food when they were in water that contained leaf chemicals from either eucalypt plantations or oak forests. We generally predicted that chemicals released by eucalypt should impair the ability to: 1) detect females, 2) detect predators and 3) find food. It is more difficult to make predictions about the effect of prior exposure to eucalypt chemicals on individuals. Making predictions in this system is difficult because newts from eucalypt plantations might perform better when exposed to eucalypt chemicals than those from oak forests due to habituation, developmental phenotypic plasticity, or local adaptation. Alternatively, newts might perform worse if their past exposure to eucalypt leachates has lowered their body condition or directly damaged their olfactory abilities.

MATERIAL AND METHODS

The palmate newt has an aquatic reproductive period lasting from January to June. In February 2014, we captured, by dipnetting, 135 adult males from native Oak forests (*Quercus robur* L.) and 135 males from eucalypt plantations (*Eucalyptus globulus* Labill.) over four consecutive days (one day per experiment 1 and 2; two days for experiment 3) and brought them to the nearby Aranzadi Society of Sciences laboratory. Although it would be interesting to look at sex specific effects of water quality, we opted to only test males because there is no evidence that females move between ponds during the mating season. The vegetation in the study area in Basque Country (North Spain) is highly fragmented. The ponds sampled were located in forest patches ranging in size from 0.6 km^2 to 1.2 km^2. Given the available distribution of habitats we chose forests as close to each other as possible (maximum distance of 400 m) to minimize differences between population characteristics. During the reproductive period, newts feed in the water and copulate frequently. Despite their reduced mobility, males still move between nearby ponds during this reproductive period (Montori & Herrero, 2004). Consequently, the ability to detect ponds containing females should be advantageous for males due to increased mating opportunities (Aragón, López, & Martín, 2000). Similarly, using chemical cues to detect and then avoid ponds with predators would be advantageous due to increased survival (Mathis & Vincent, 2000; Secondi, Haerty, & Lodé, 2005). For experiment 1 and 2 we captured the males in the morning and we maintained them in laboratory conditions in groups of ten individuals / 10 litres of original pond water during the afternoon until the experiment started. All experimental trials were conducted after dark at 9:00 pm to ensure that males were active (Montori & Herrero, 2004). The three experiments were conducted in darkness at a constant temperature of 12 ºC, mirroring the night air temperature in the field on the days of the experiment. Males used in experiment 3 (foraging test) were maintained individually in 1 litre of pond water without feeding for 48 hours before starting the trial to ensure that individuals were hungry after a short, controlled starvation period.

To simulate the water chemistry of ponds located inside oak forests and eucalypt plantations, we collected senescing oak or eucalypt leaves that had recently fallen to the ground (in small 20 m^2 areas inside each forest), which were transferred to, and dried immediately in, a heated room (at 20 ºC) for 48 h. Dried leaves are widely used in studies that test the effects of exotic plants on amphibians (e.g. Hickman & Watling, 2014) as a standardized method to control leaf biomass. Some of these studies have also analyzed the composition of leachates from dried leaves (Maerz et al., 2005), so it is known that the secondary compounds are not removed by drying. Leachates were created by soaking 0.5 g dried leaves/1 litre of mineral water (concentration doses based on Maerz et al. 2005) for 48 h prior to our experimental trials. Leaves were then strained with cheesecloth from the leachate solution to remove solid material 1h before the behavioural trials. We opted for the preparation of the extracts in the laboratory, and did not directly collect water from natural ponds to eliminate other factors (e.g. predator kairomones) that might affect the behaviour of our study animals. The same methodology we used has also been used for studies on invasive plants(e.g. Maerz et al. 2005; Hickman and Watling 2014). Our goal was to test for disruption of chemical communication at the beginning of the mating period, because this is the most active period for males in searching for females and for new ponds. We collected males in February and conducted our experiments over a four day period to eliminate any effects of seasonal variation in male newt behaviour.

Experiment 1. Does water chemistry affect a male's ability to locate ponds with females?

We conducted a 2x2 factorial experiment that varied both the habitat males were collected from (oak forest or eucalypt plantation) and water chemistry type (oak or eucalypt extract) (*N* = 27 males/combination, Total *N* = 108). Males were randomly allocated to each treatment and did not show any morphological differences, such as body size, that could drive behavioural differences. We conducted the trials in 108 plastic aquaria, 50 x 42 x 36 cm, with two 1 litre plastic pools located at opposite ends at a 15 cm distance between them. One pool had 0.5 litres of mineral water and 0.5 litres of eucalypt or oak extract. The other pool had 0.5 litres of mineral water with female odour, obtained by placing 10 females in 10 litres of mineral water for 72 h, and 0.5 litres of eucalypt or oak extract. We removed the females from the stimulus water, with an aquarium net, 15 min before starting the trial. All females were removed in less than 10 sec to avoid the release of stress substances to the water. We mixed the stimuli into the water 10 min before starting the behavioural trial. The inside of the pools were covered with steps (1 mm high, 5mm wide) to allow the newt to leave the pool easily. Pools were filled to the top with a water depth of 14 cm. A polystyrene sheet was used to raise the floor and to ensure a ground level entrance to the pools. From prior observation we determined that newts exhibit a full range of natural behaviours (including mating and reproduction) in 1 litre pools (MIC personal observation). We changed the water, cleaned the pools with water and dried them between trials so that each individual was tested in newly prepared water. We used a total of 120 females and 120 litres of mineral water. These females were captured in the same oak forests as males.

To start a trial, a male was placed in a bowl of 5 cm diameter and 5 cm depth, with 150 ml of mineral water. We used this small bowl to ensure the male was motivated to seek out a larger pond. The entrance to the bowl was also at ground level. The bowl was shallow (5 cm depth) to allow a quick and easy exit. It was located 10 cm from the nearest entrance to each pool (i.e. equidistant to both pools). We noted the identity of the first pool that the male entered ('initial choice'). The following morning during daylight (12h later), we recorded which pool the male was using ('final choice'). We believe measuring "final choice" the following morning is biologically meaningful. Newts are crepuscular and nocturnal animals that mate and feed mostly at night (Griffiths, 1985). Personal observations in the field have shown that movement between ponds in *L. helveticus* occurs during the night, when the air humidity is high. The same observer (M.I.C.) collected data blind to the habitat of collection of males and the water treatment. For statistical analyses, we discarded trials when the male did not leave the small bowl (N = 0/54 oak origin males; N = 13/54 eucalypt origin males, $X^2_2 = 0.24$, P = 0.62*)*.

Experiment 2. Does water chemistry affect a male's ability to detect ponds with predators?

To simulate a recent predator attack, we used conspecific chemical alarm cues rather than predator kairomones because the former have a stronger effect on behaviour in related newt species (Gonzalo, Cabido, López, & Martín, 2012). By using alarm cues we also avoided the possibility that differences in newt behaviour were influenced by perceived vulnerability (Mathis, Murray, & Hickman, 2003) or previous predation experience (Murray, Roth, & Wirsing, 2004). For instance, in eucalypt plantations the presence and diversity of predators (such as dragonfly larvae) might be lower than in oak forests (e.g. eucalypt plantations change macroinvertebrate communities, Ferreira et al., 2015), which could influence subsequent male behaviour due to their previous interactions (Fraker, 2009). In newts, the recognition of alarm cues is believed to be innate (Ferrari et al., 2010), so we expected this stimulus to be perceived similarly by males independent of their habitat of origin. Alarm cues were prepared using six adult newts (following Gonzalo et al. 2012). They were cold anesthetized at 4°C for 25 min, and then euthanized with a quick blow to the head to avoid suffering (ASIH, 2004). The extract was then prepared by mixing 0.8 g of skin tissue with 100 ml of aged tap water, filtered through absorbent paper to remove solid particles.

We again conducted a 2x2 factorial experiment in which we varied the habitat of collection of males (oak or eucalypt) and water chemistry type (oak or eucalypt) (N = 20 males/combination, Total N = 80; these males were different from those used in experiment 1 and were captured the morning of the experiment). For the behavioural trials we used the same tank set up as in Experiment 1. One of the pools contained 0.5 litres of mineral water and 0.5 litres of oak or eucalypt water. The other pool was filled with 0.485 litres of mineral water, 15 ml of alarm cue extract, and 0.5 litres of oak or eucalypt extract. We noted males' initial and final pool choice as in experiment 1. M.I.C. collected data blind to a male's habitat of collection and water treatment. For statistical analyses, we discarded trials if the male did not leave the small bowl (N = 6/40 newts of oak origin; N = 10/40 newts of eucalypt origin; $X^2{}_2 = 0.02$, $P = 0.87$).

Experiment 3. Does water chemistry affect a male's ability to find food?

Newts often live in turbid habitats with abundant vegetation and locate prey through olfaction (Lindquist & Bachmann, 1982). We again conducted a 2x2 factorial experiment with 80 new males, and we varied the habitat of collection of males (oak or eucalypt) and water chemistry type (oak or eucalypt) (N = 20 males/combination of treatments, Total N = 80). Males were not fed for 48 h prior to experimental trials to ensure that they were hungry and looking for food. For a trial each male was individually placed in a 40 x 32 x 22 cm aquarium, filled with 2 litres of mineral water mixed with 2 litres of either oak or eucalypt extract. We used blood worms as prey because they a common prey item (Fontanet 1992), and are readily consumed by newts in captivity (MIC personal observation). We placed a thawed blood worm 20 cm from the male and noted how long it took him to locate the worm (i.e. to start eating). We used dead prey to avoid the possibility that prey detection was due to prey movement. In this experiment, behavioural trials were video recorded from above. We started recording just before placing the worm in the aquarium. The "capture" moment of the prey is very conspicuous since newts perform a sudden, distinctive lunge forward with their whole body. We stopped the recording after 30 min. The only identification of individual newts was a random number in the aquarium, so data collection was blind to a male's habitat of collection and water treatment. We tested each male twice on consecutive days. As the time taken to start eating the prey was statistically repeatable (r = 0.63, $P < 0.01$) (i.e. consistent across days) we used the mean time in our analyses. For statistical analyses, we excluded males that did not start to eat within 30 min (N = 7/40 of oak origin; N = 5/40 of eucalypt origin, $X^2{}_2 = 0.008$, $P = 0.92$).

Ethical note

All capture and handling of newts complied with the existing laws regulating the treatment of animals in Spain and the internal Aranzadi institutional ethical guidelines (number 2014/007). The study was performed under a capture and handing license from, Bizkaia and Gipuzkoa Administrations (numbers 1695 and 2222). M. I-C and C.C conducted this work with Spanish accreditation to conduct experiments with animals R.D 53/2013 (ref number 10/096442.9/13). None of the newts showed any sign of stress or pain during these tests, and all looked healthy after the trials. Every group of newts was released at their capture site within 24 or 72h (for experiment 3) of being captured.

Statistical analyses

For experiments 1 and 2 we used the initial and final pool choice as binary response variables in separate generalized linear models (GLM) with binomial error distribution. We included the habitat of collection of newts, water extract type and their interaction as fixed factors. We further conducted simple binomial tests to test whether males detected the pond with the predator cues or entered the pond with cues of female presence more often than expected by chance. For experiment 3, we ran a generalized linear model with a quasi-poisson distribution of errors, with time until the male started to eat as the response variable, while habitat of collection, water extract type and their interaction were included as fixed factors. Pairwise comparisons were planned using Tukey's honestly significant difference tests. Analyses were conducted in *R 3.2.2* with $\alpha = 0.05$. All tests were two-tailed.

RESULTS

Experiment 1. Does water chemistry affect a male newt's ability to locate ponds with females?

When males were tested in water with oak extract they initially chose the pool that contained female odour significantly more often than when they were tested in water with eucalypt extract (Table 1, Fig. 1a, Tukey's test: *P* = 0.011). This effect did not depend on the male's collection habitat (Tukey's test: *P* = 0.681), nor was there an interaction between the habitat of collection and the water extract type (Table 1, Fig. 1a). However, when each extract treatment was analyzed separately, and male collection site was ignored, there was no preference for ponds with female odour in either water type in the initial choice (Binomial tests, both *P* > 0.20). Thus, although males more often chose the pond with the female when exposed to oak extract versus eucalypt extract, the likelihood of choosing the pond with the female within each extract type did not differ from 50:50. For their final choice, males tested in oak and eucalypt water made similar choices in terms of which pool contained female odour (Tukey's pairwise comparison: *P* = 0.914). There was again no effect of the habitat of collection (Tukey's test: *P* = 0.157), nor was there an interaction between the male's habitat of collection and the water extract type (Table 1, Fig. 1b). Given the lack of effects of either habitat of collection or water extract type, we pooled the data to compare the total number of final choices for the pool with the female odour. There was no tendency towards choosing pools with the female odour in either the oak-extract or eucalypt-extract water (Binomial tests, both *P* > 0.50).

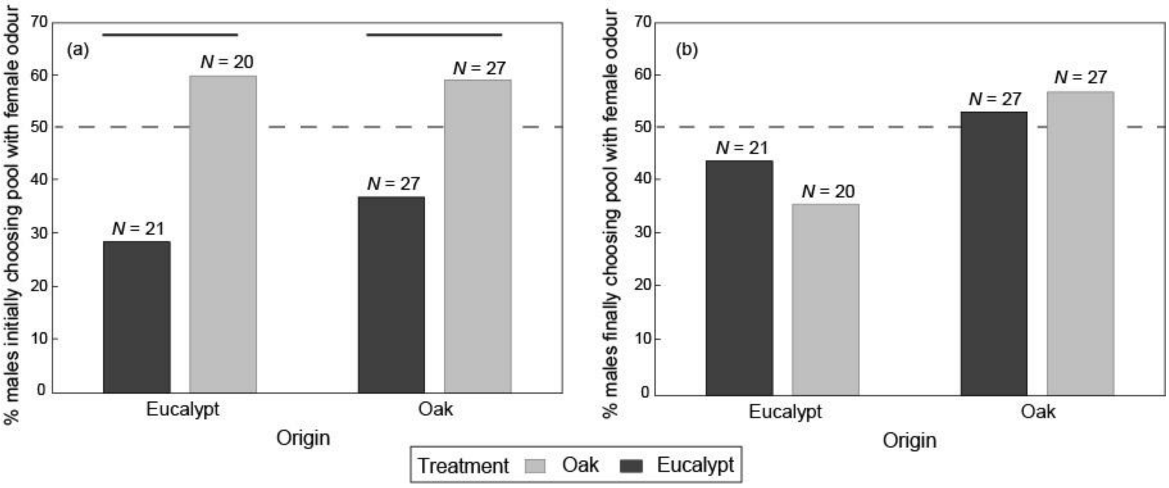

Figure 1. Percentage of newts from different collection habitats and water treatments (dark grey: eucalypt; light grey: oak) that chose the pool with female odour as their a) initial choice and b) final choice. Dashed line indicates no choice (i.e. 50:50). Horizontal bars show statistically significant differences.

Experiment 2. Does water chemistry affect a male's ability to detect ponds with predators?

The initial pool choice of males did not depend on the male's habitat of collection (Tukey's test: *P* = 0.813), the water extract type (pairwise comparison: *P* = 0.842) or their interaction (Table 2, Fig. 2a). When combining all treatment groups, males did not show a statistically significant ability to detect ponds containing alarm cues during their initial choice (Binomial test, *P* = 0.08). In contrast, the males' final choice depended on a significant interaction between habitat of collection and water extract type (Table 2, Fig. 2b). Males from the oak forests showed a strong ability to detect the pool with alarm chemicals when tested in water with oak extract (Binomial test, *P* < 0.001), but not when tested in water with eucalypt extract (*P* > 0.90). In contrast, males from the eucalypt plantations showed the same non-significant tendency to detect pools with alarm chemicals in water with either oak (Binomial test, *P* = 0.13) or eucalypt extract (Binomial test, *P* = 0.50). Pooling across extract types, males from eucalypt plantations did not detect pools with alarm cues based on their final choice more often than expected by chance (Binomial test, *P* = 0.10).

Table 1. Effect of collection habitat, water treatment and their interaction on the initial and final choice of the pool with cues of female presence (Experiment 1). The intercept represents the estimate for males from the Eucalypt collection site tested in the eucalypt water.

		Cues of female presence			
Trait	Predictors	Estimate	SE	z-value	*P*-value
Initial choice	Intercept	-0.91	0.48	-1.87	0.057
	Collection Oak	0.38	0.62	0.61	0.538
	Treatment Oak	1.32	0.66	1.98	**0.046**
	Collection Oak x treat Oak	-0.41		-0.48	0.631
Final choice	Intercept	-0.28	0.44	-0.65	0.514
	Collection Oak	0.36	0.58	0.61	0.537
	Treatment Oak	-0.33	0.64	-0.51	0.607
	Collection Oak x treat Oak	0.48	0.84	0.56	0.569

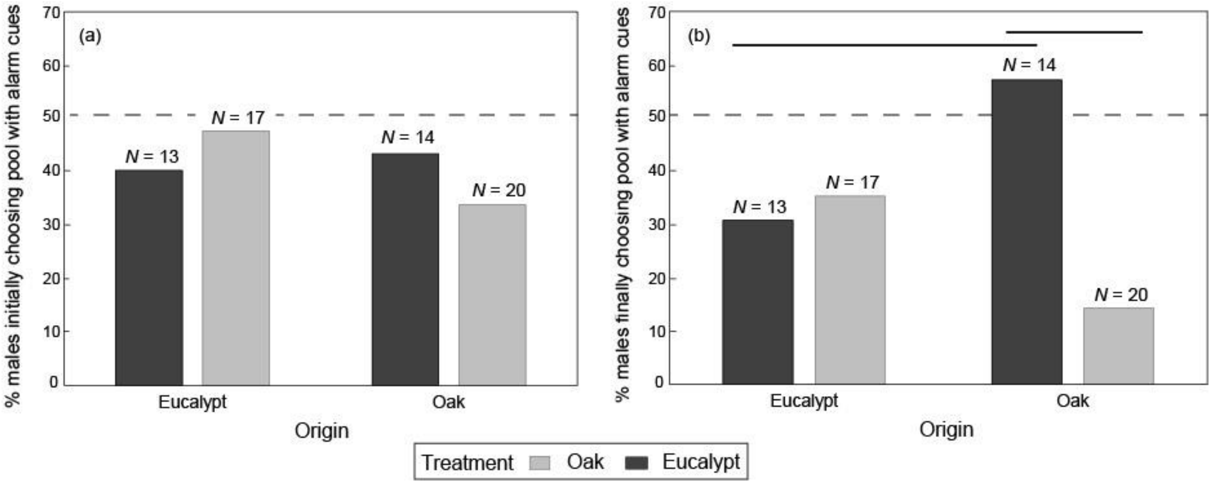

Figure 2. Percentage of newts from different habitat of collection and treatment (dark grey: eucalypt; light grey: oak) that chose the pool with conspecific alarm cues a) initially b) finally. Dashed line indicates no choice (i.e. 50:50). Horizontal bars show the significant differences.

Table 2. Effect of the habitat of collection, treatment and their interaction on the initial and final choice of the pool with cues of predator presence (Experiment 2). The intercept represents the estimate for males from the Eucalypt collection site tested in the eucalypt water.

		Cues of predator presence			
Trait	Predictors	Estimate	SE	z-value	*P*-value
Initial choice	Intercept	-0.81	0.60	-1.34	0.177
	Collection Oak	0.52	0.80	0.64	0.517
	Treatment Oak	0.69	0.77	0.89	0.37
	Collection Oak x treat Oak	-1.09	1.05	-1.04	0.296
Final choice	Intercept	-0.81	0.60	-1.34	0.177
	Collection Oak	1.09	0.80	1.36	0.173
	Treatment Oak	0.20	0.78	0.26	0.794
	Collection Oak x treat Oak	-2.28	1.13	-2.00	**0.045**

Experiment 3. Does water chemistry affect a male's ability to find food?

The time that newts took to locate a food item was not statistically affected by the male's habitat of collection, the water extract type or their interaction (Table 3, Fig. 3, all pairwise tests: $P > 0.873$).

Table 3. Effect of the habitat of collection, treatment and their interaction on the time newts need to find and eat the prey (Experiment 3).

Feeding activity of newts

Trait	Predictors	Estimate	SE	t-value	*P*-value
Time to find the prey	Intercept	6.67	0.09	68.87	< **0.001**
	Collection Oak	-0.11	0.14	-0.75	0.451
	Treatment Oak	-0.01	0.14	-0.11	0.905
	Collection Oak x treat Oak	0.10	0.20	0.48	0.628

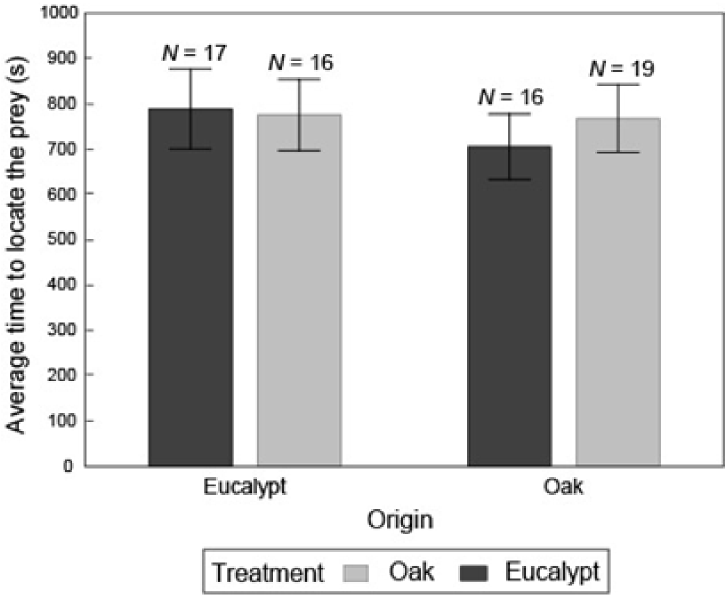

Figure 3. Average time (mean + SE) that newts from different collection habitat and treatment (dark grey: eucalypt; light grey: oak) took to find the prey. Horizontal bars show the significant differences.

DISCUSSION

In our experiments, leachates released by eucalypt leaves interfered with both mate finding and predator detection of male palmate newts, but not with the ability to locate a food item. It appears that eucalypt leachates have different effects on each of the three behaviours. Male palmate newts had greater difficulty identifying pools with female chemical cues when tested in water containing eucalypt extracts, regardless of the male's habitat of collection. In contrast, males from eucalypt plantations (69.3 %) avoided the pools with predator chemical cues more often than males from oak forests (43.9 %) when the test water contains eucalypt extracts. One explanation for the increase in performance of males from eucalypt forests when tested in eucalypt treated water is that they become habituated to the presence of leachates. This claim is further bolstered by the fact that males from oak forests have a greater tendency than males from eucalypt plantations to choose pools without predator cues when the test water contains oak extracts. Males from eucalypt forests are likely to have an altered ability to detect chemical cues of predation risk. Finally, the time taken to locate prey is unaffected by eucalypt leachates, regardless of the male's habitat of collection, so male foraging ability does not appear to be lower in eucalypt plantations.

Cues of female presence

Independent of their habitat of collection, males tested in eucalypt water identified pools which contained chemical cues of females less often than males tested in oak extracts. However, we did not find a significant effect of the water type on the males' final choice. Cue detectability and the consequent response of animals depends on the chemical background where these cues are released (Hale et al., 2009). A possible explanation for the observed difference between the initial and final choice of males is that eucalypt chemicals change the natural background interfering with, and thus diminishing, the detectability of pheromones from outside the water. During the 12 hours of the experiment males changed pools several times (M.I.C personal observation), so they might be able to detect cues when in the water that indicate which pool is more likely to contain a female. Why then did all males not finally reside in the pool with female cues? One possibility is that even if males can detect the odour of females (irrespective of water extract type), the absence of an actual female meant that males eventually 'decided' that the current female chemical cues were uninformative of the likely future presence of a female in a pool. Another possibility is that the female cues diminish over time and can no longer be detected after 12 hours, even under natural (i.e. oak extract) conditions.

Focusing on the initial choice of males, it seems plausible that leachates from eucalypt trees might disrupt sexual communication by binding to pheromones released by females. The binding of pheromones with leachates would be consistent with evidence that water contamination with humic acid adversely affects the tendency of pheromones to dissolve in organic matter (Mesquita et al., 2003). If males cannot identify pools with females before entering the water, eucalypt leachates might lead to lost mating opportunities and increase the time that males spend searching for females. If the effectiveness of pheromone signaling is disrupted, we might also expect changes in female mate choice (e.g. hybridization increases in the presence of humic acid; Fisher et al., 2006). Indeed, we have recently shown experimentally that exposure to eucalypt leachates alters female choice in palmate newts (Iglesias-Carrasco et al. in prep.).

Cues of predator presence

In contrast to our finding on the ability to locate females based on male's initial and final choices, males detected pools with alarm cues based on their final choice, but not on their initial choice. In addition, there was an effect of the collection habitat. For their final choice, males collected in oak forests failed to identify the pools with alarm cues when tested in water with eucalypt extracts, but did so successfully when tested in water with oak extracts. In contrast, males collected in eucalypt plantations did not respond differently depending on whether the water contained oak or eucalypt extract, and in both cases they failed to detect the pool with predatory cues more often than expected by chance.

The use of alarm cues has been shown in many taxa (Chivers & Smith, 1998), including newts, that are similar to our study species (Gonzalo et al., 2012). Aquatic prey receive and respond to these chemical cues to reduce their predation risk (Ferrari et al., 2010). Recognition and rapid reaction to cues related to predation are key to survival (Lima & Dill, 1990). Being able to detect pools with active predators should be beneficial both for adult male survival and that of any offspring they sires. Our results are consistent with previous studies suggesting that the presence of pesticides or changes in the water chemistry affect amphibian predator-prey interactions (e.g. Relyea, 2005). Individuals of several taxa exposed to chemically impaired environments fail to respond to predation cues (Dixson, Munday, & Jones, 2010), have increased predation rates (Ortiz-Santaliestra, Fernández-Benéitez, Marco, & Lizana, 2010), and have impaired predator detection (Lürling & Scheffer, 2007). Oak males in our study might fail to identify pools with alarm cues because leachates released by eucalypt leaves disrupt the dispersion and/or detection of relevant predator cues. Notably, the only trials in which males showed a statistically significant ability to detect the pool with alarm cues were those with males from oak forests tested in water with oak extracts (i.e. the natural setting). The effect of eucalypt leachates on the ability of newts to respond to predators could affect their mortality rates and population sizes in these plantations.

Males collected in eucalypt plantations did not detect the pools with alarm cues when tested in water with oak extracts. Receiving and responding to alarm cues depends not only on the environment, but also on intrinsic factors of individuals (Ferrari et al., 2010). The inability to detect the pool with alarm cues by males from eucalypt plantations might be due to a reduction in their body condition, or to changes in their physiology after exposure to toxic substances (e.g. male newts inhabiting eucalypt plantations show poorer immune responses, Iglesias-Carrasco et al., 2016). Poor male condition in eucalypt plantations could lead to different trade-offs in the allocation of resources to different behaviours (e.g. Hunt et al., 2005; Iglesias-Carrasco, Head, & Cabido, 2016). For example, physiological costs related to exposure to secondary compounds of invasive plants provoke changes in the behaviour of amphibian tadpoles, elevating their exposure to predators (Hickman & Watling, 2014). Independent of their collection habitat, male newts did not detect alarm cues when tested in water with eucalypt extract. Again, leachates may bind to these cues, potentially impeding the recognition of a predatory event (Turner & Chislock, 2010). Another possible explanation is that substances released by eucalypt overwhelm the newts' senses, so that the odour of the alarm cues is camouflaged.

Effect of eucalypt extracts on feeding ability

Contrary to our predictions, males tested in water with eucalypt extract did not take longer than males tested in oak water to locate prey. We also found no effect of the collection habitat of males on their prey detection ability. Our results contrast with studies in other aquatic taxa that show disruption of prey localization ability due to the alteration of the chemical environment (e.g. Sherba et al. 2000). However, we cannot be sure that the exposure to eucalypt leachates does not affect the ability of newts to locate prey as newts rely on both chemical and visual signals to detect prey (Sullivan, Frese, & Mathis, 2000). One caveat of our study is that we assume that male searching rates did not differ across collection habitats. Eucalypt plantations alter invertebrate communities in streams, by increasing or decreasing the densities of groups with different ecological requirements (Larrañaga, Basaguren, Elosegi, & Pozo, 2009). A lower density of invertebrates might reduce foraging opportunities for newts in ponds in plantations. Fewer foraging opportunities are often associated with greater activity levels (Anholt, Werner, & Skelly, 2010), so newts collected from eucalypt plantations might have been more active during the trials. If so, they might have encountered the worm sooner by chance alone, countering any negative long-term effect of eucalypt chemicals on the ability to chemo-locate prey.

If we extrapolate from our laboratory findings, it appears that newts in eucalypt plantations do not have lower foraging abilities than those in native oak forests, at least with respect to the detection of prey chemical cues. Further work directly measuring newt activity and foraging levels, and using live prey is needed to confirm this statement. Moreover, it is important to note that our study examined prey detection ability only in adult newts. We do not know whether, or how, the exposure to eucalypt leachates might influence larval amphibians. An examination of the effects of eucalypt secondary compounds on the aquatic early stages of the life cycle is crucial to understand the full effects on interactions between predator and prey.

Conclusions

Replacing natural oak forest by eucalypt plantations may have both ecological and conservation implications for amphibians. Some studies have shown that secondary compounds from exotic plants have a negative effect on tadpole performance, survival and behaviour (Watling et al. 2011a, Watling et al. 2011b). To our knowledge, however, ours is the first study to explore how alteration of the chemical environment due to plant secondary compounds affects three key behaviours related to fitness in adult amphibians. Our results lead to the testable prediction that the effects of chemical compounds not previously encountered during the evolutionary history of the species potentially lower the fitness of individuals by altering their anti-predator and mating behaviour. The difference in habitat of collection mediated effects on these two behaviours, in combination with no detectable negative effect on foraging behaviour, is a reminder that it is often difficult to predict the exact effects of novel evolutionary pressures on species. Our study also highlights the importance of integrating behavioural studies into conservation programs to fully understand how the transformation of habitats affects endangered species. We show that large-scale habitat transformation can affect amphibian behaviours which are important for survival and reproduction. Such individual level effects may have consequences for the persistence of populations. For instance, exotic plantations could lead to a reduction in population size due to the inability of individuals to detect potential mates and predators. Using behavioural approaches to understand these indirect and cryptic effects (such as the alteration of the sensory environment) may be important for explaining and preventing loss of species in exotic plantations (e.g. Carrascal & Tellería, 1990; Sax, 2002; Zurita, Rey, Varela, & Villagra, 2006).

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Chapter 5

Urban habitats can affect body size and body condition but not immune response in amphibians.

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ABSTRACT

Does urbanization affect key life-history traits in native organisms? Some studies show that urban areas reduce diversity in certain taxa, but there is little insight into how these environments affect physiological and ecological traits. Urban areas have distinct physical structure and ecological processes compared to original habitats. The environmental changes associated with urban areas can influence the costs and benefits of different traits and behaviours of local organisms. Some of these effects have been explored in groups such as birds, but we might expect stronger effects in animals with reduced mobility, such as amphibians. Importantly, the effects of urban habitats on amphibians have not been explored, in spite that these are the most threatened vertebrate group in the world. Here, we compared three main traits related to the fitness of amphibians in urban and natural habitats: body size, body condition and immune response. To test the generality of our results, we assessed adult males of four amphibian species. We found that the body size was larger in urban environment populations in three of four studied species, while the body condition was better in the urban populations of two aquatic newt species. Finally, we found no effect of urbanization on the immune response of individuals of any species. In conclusion, we show that different species of amphibians may be affected differently by anthropogenic habitat alteration depending on their specific ecology.

Keywords: amphibian conservation, *Lissotriton helveticus, Pelophylax perezi,* PHA, *Salamandra salamandra, Triturus marmoratus.*

INTRODUCTION

Urbanization of natural landscapes is occurring on an unprecedented scale worldwide. An important consequence of urbanization is the simplification of the natural landscape and the emergence of artificial structures, such as buildings and roads (Marzluff 2001). Urban environments are usually characterized by a reduced diversity of species (Shochat 2004) and altered communities, for example due to the introduction of exotic species (Marzluff 2001). Species respond differently to human-related habitat alteration, so that some species take advantage of the new characteristics while other species experience population declines. Animal populations inhabiting urban areas experience a variety of new conditions, such as different predatory pressure (lower predation risk, Gering and Blair 1999 or higher risk, Murphy et al. 2016b), increased temperatures, and unusual food sources (Ditchkoff et al. 2006). These environmental changes can influence the costs and benefits of different traits and behaviors, which in turn can alter trade-offs between traits (e.g. McGlothlin et al. 2007; Iglesias-Carrasco et al. 2016a). The investment in different traits across environments will depend on local adaptation and adaptive phenotypic responses, driven by environment-specific cues.

The effect of urbanization has largely been studied in highly mobile animals such as birds (Møller 2009; Bókony et al. 2010; LaZerte et al. 2016), but little attention has been given to animals with reduced mobility. Critically, we might expect the effects of urbanization to be stronger on animals that have limited abilities to avoid these habitats, such as amphibians. Amphibians are one of the most threatened groups of animals in the world, affected by the loss and alteration of their habitat (Houlahan et al. 2000), pollution (Egea-Serrano et al. 2012), invasive predators (Kats and Ferrer 2003) and emerging diseases (Daszak et al. 2003). In general, alteration of the landscape with urban areas promotes reduced amphibian diversity compared to the original habitat (Rubbo and Kiesecker 2005). Amphibians are prone to the effects of urbanization for several reasons. For example, 1) they have highly permeable skin that renders them sensitive to toxic substances, both in the terrestrial and aquatic stages; 2) their ability to disperse may be hindered in urban landscapes; 3) the scarce suitable breeding habitats might constrain breeding opportunities, leading to usage of habitats that are poor or have low-suitability; 4) different stressors, such as noise, light pollution or increased temperatures in urban areas may have behavioral and ecological consequences by the disruption of acoustic signaling or the reduction of the immune response. Most studies have focused on the presence/absence of species, species richness and abundance of populations in urban habitats (Hamer and McDonnell 2008). However, the effect of urban-induced environmental changes on fitness enhancing life-history traits in amphibians remains unexplored.

We examined here whether the urban environment affects body size, body condition and immune response in four amphibian species. Body size of individuals from urban areas often differs from the surrounding natural areas, with both increases and decreases in the body size of organisms in urban habitats being reported in the literature (Ruiz et al. 2002; Murphy et al. 2016a). Differences in temperature, resource availability, or the chemistry of the water have been proposed as explanations for differences in body size across taxa (Kozłowski et al. 2004; Walters and Hassall 2006; Barrett et al. 2010). On the other hand, high food predictability and low mortality in urban habitats can elevate reproductive rates and population densities in some species (Møller 2009). Changes in population density can in turn affect the body condition of individuals, which is a crucial trait that determines fitness and is indicative of environmental stress (Peig and Green 2010). Increases in population densities are predicted to lead to poorer body condition of individuals, due to over exploitation of food that may reduce foraging success (Liker et al. 2008). However, a negative effect of urbanization on body condition is likely not to be universal (e.g. house sparrows Bókony et al. 2012), probably due to stable and abundant food supplies, such as human resources, that are exploited by many generalist species in urban areas (e.g. Herr et al. 2010). Finally, the immune response may also differ between urban and natural habitats for many reasons. For example, the poor quality (e.g. low protein intake) or reduced quantity of food available for many species in urban areas could constrain the amount of resources allocated by individuals to each physiological trait, including the immune response. Similarly, chronic stress (e.g. chronic noise or high intraspecific competence) (Padgett and Glaser 2003) and the presence of toxic chemical substances (Iglesias-Carrasco et al. 2016b) are detrimental for individual immune responses. The immune system is expensive to maintain and use, so energy deficits generated by the need to face toxins may alter the resource allocation decisions, resulting in urban populations being in worse health (i.e. lower immune responses) than natural populations (e.g. lizards Cabido et al. 2008). Contrarily, some studies have found enhanced immune responses in urban populations compared to natural ones (Audet et al. 2016) as a response to the exposure to novel pathogens.

We studied three urodele and one anuran species that are frequently found in cities. Different amphibian species may exhibit markedly different responses to urban habitats. Habitat-generalist amphibians or those with relatively low dispersal requirements appear to do better in urban areas (Hamer and McDonnell 2008). Two of our study species, the palmate newt (*Lissotriton helveticus*) and the strictly aquatic common green frog (*Pelophylax perezi*) are generalist amphibian species that breed in a wide range of aquatic environments, ranging from big lakes, to streams and flooded ditches on the roads (Montori and Herrero 2004). Both species, but mostly the common green frog, have high resistance to water pollution and show few requirements in relation to water quality (Montori and Herrero 2004). The other two of our study species, the marbled newt (*Triturus marmoratus*) and the fire salamander (*Salamandra salamandra*), are more restricted in their ecological requirements (Montori and Herrero 2004). During the terrestrial stage the marbled newt uses rocks and mammal refuges to hide, usually sited in forest or shrub areas. For reproduction, it uses several still water aquatic habitats, but usually these are ponds with high volume of water and abundant aquatic vegetation (Montori and Herrero 2004). The fire salamander is a terrestrial species that requires high humid and gloomy environments. It can be found in any type of plant community, but populations are more abundant in deciduous forests (Montori and Herrero 2004). Among urodeles, we used two species in aquatic phase (the palmate newt and the marbled newt) and a mostly terrestrial salamander (fire salamander) to test if the urban environment has different effects depending on the species ecology. We compared natural and urban populations of these four species and predicted that inhabiting the city can result in changes in 1) body size and 2) body condition, and 3) reduction of the immune response of individuals. Due to the contrasting results found in studies based on other animal groups (e.g. Liker et al. 2008; Bókony et al. 2012) and the lack of this kind of studies in amphibians, it is difficult to make predictions about the direction of the effect of urban areas on the body size and body condition on the species studied.

MATERIAL AND METHODS

Study area and species

In April 2013, we captured 18-24 adult males of each of the four amphibian species (specific sample sizes shown in Figure 1) in an urban parkland in the city of Donostia/San Sebastián in Northern Spain (43º 18’ 18.06’’ N, 1º 59’ 29.97’’ W), and in a nearby natural oak forest (1.7 km in a straight line to the city, 43º 18’ 8.91’’ N, 2º 02’ 37.19’’ W). The urban area is isolated, surrounded by the city buildings and attracts large crowds of tourists and citizens. The distance to the nearest natural habitat is approximately 1.9 km. This distance combined with several obstacles, such as roads, poses a substantial degree of isolation for the amphibian urban populations from the natural populations. Amphibians in the city occupy human constructions. In our urban population the palmate newt, the marbled newt and the common green frog breed in concrete artificial ponds, surrounded by exotic garden trees. The urban population of fire salamander studied here is viviparous and, hence, independent of the water for reproduction. This characteristic allows the survival of salamanders in isolated and somewhat extreme environments such as urban areas, where they use the walls of historic buildings as refuge. In the natural populations, individuals of the four studied species occupy naturally created ponds inside native oak forests and find shelter under rocks and logs.

It would have been interesting to look for the effect of urbanization in more than one population. However, urban populations of some of the species are very limited in numbers and restricted to the few available suitable breeding habitats that remain inside the city, so the sample size we obtained in other urban ponds was insufficient to conduct the experiment.

All the animals were captured in April and during the night, coinciding with the breeding season of the four species. To avoid potential confounding effects of weather or seasonality all the animals were captured within a single week. The aquatic newts were captured by dip netting, while the terrestrial salamander and the frog were collected by hand. Animals were individually housed in 4 L terraria with a 0.75 L water pool and moss as refuge (for terrestrial salamanders and common frogs) or in 2 L aquaria (for the aquatic newts) during the 48 h of the trials. We maintained the animals in a constant temperature of 15 ºC and natural photoperiod in a laboratory. They were fed twice a day with three mealworms (for the terrestrial species) or three bloodworms (for the aquatic species) during the two days of the experiment.

Measure of morphological traits and immune response.

We measured three traits that are likely to be altered by urban habitats: body size, body condition and immune response. Body size was measured from photos of individuals placed on a glass board with a reference measurement scale. We used the snout-vent length (SVL: measured in mm from the tip of the snout to the ventricular opening) as a measure of the size for the anuran species, while for the urodeles we used total length (i.e. TL: measured in mm from the tip of the snout to the tip of the tail). All measures were made using Image J software (Abràmoff et al. 2004).

We calculated body condition as the residuals from the regression of log body mass (g) on log TL or log SVL. When the relationship between these variables is linear, this measure is often used as an index of the relative amount of fat stored, and hence of nutritional status (reviewed in Green 2001). This index has successfully been used to investigate the effects of differences in food availability and habitat quality in amphibians (MacCracken and Stebbings 2012; Sztatecsny et al. 2013).

Finally, to estimate the immune response we used the delayed-type hypersensitivity phytohaemagglutinin injection assay (PHA test). This assay was described as a reliable measure of T-cell dependent immunocompetence *in vivo* (Lochmiller et al. 1993). However, recent studies have shown that the PHA-induced swelling might instead be considered as a multifaceted index of the cutaneous immune activity (Salaberria et al. 2013). We used this test as a standard index of the immunocompetence in order to avoid differences related to the type of the immune cells involved (Kopena et al. 2014). This test has been used and validated in many studies including those on amphibians (Brown et al. 2011; Clulow et al. 2015).

All individuals were anaesthetized by immersion in Tricaine methane sulphonate (0.15 g MS-222/1 L dechlorinated water) for 5-10 min (Cakir and Strauch 2005) immediately before the measurements. Then for newts we measured the thickness of the base of the tail 5 mm from the vent (based on Iglesias-Carrasco et al. 2016) with a pressure-sensitive spessimeter (±0.01 mm). We let the spessimeter to gently touch the skin of the animal without pressing it to avoid the post-injection inflammation caused by the PHA to disappear with the pressure. We made our measurements five times and used the average measurement for statistical analyses. Immediately after, we injected 0.01 mg of PHA dissolved in 0.01 ml of phosphate-buffered saline (PBS). Once recovered from the anaesthesia, newts were placed into plastic containers with 2 L of dechlorinated water. After 24 h, we measured the thickness of the tail base at the same point (this time without anaesthesia) to calculate the difference between pre- and post-injection measures (inflammation). We used a similar procedure with the common green frog and the fire salamander. In this case, the measure of the thickness and the injection was made in the left hind footpad (commonly used in lizards, see e.g. Husak et al. 2016). Animals were individually maintained in a 4 L aquarium. The immune response index (hereafter 'PHA immune response') was calculated as the residuals of the regression of the inflammation against SVL or LT. The only appreciable effect of the PHA injection was a slight swelling of the skin, caused by the immune response, which disappeared after 48 h. None of the animals showed any sign of stress or pain during these tests, and all looked healthy after the trials. All individuals were returned to their capture sites 48 h after being captured.

Statistical analyses

To test whether males from the different habitat types differed in morphology or PHA immune response, we used one-way ANOVAs with habitat (natural vs urban) as a fixed factor. We ran a separate model per each species and trait, such that there were 12 models tested. We checked the residuals of all our models to ensure that they met the assumption of normality and homoscedasticity. To test whether variances in body size were statistically different between populations, we ran an F-test for each species. All statistical tests were conducted using R 3.2.2.

RESULTS

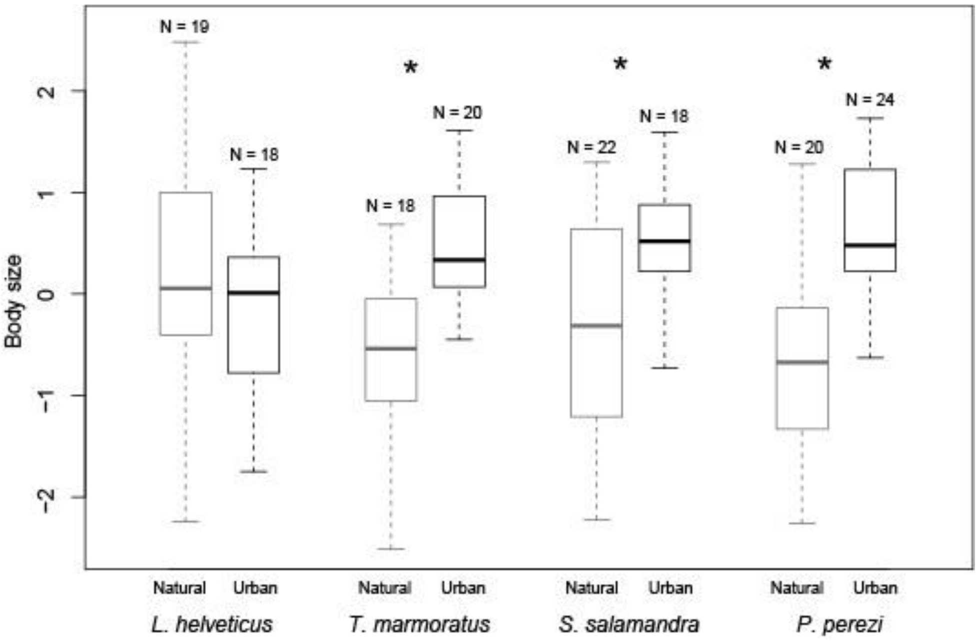
We found significant differences between populations in body size in *T. marmoratus* ($F_{(1,36)} = 15.51$, $p < 0.001$), *S. salamandra* ($F_{(1,38)} = 5.91$, $p = 0.001$) and *P. perezi* ($F_{(1,42)} = 35.16$, $p < 0.001$), with individuals from the urban area being 6.29, 8.47 and 17.73 % respectively larger in body size than the ones from the natural population (Table 1, Fig. 1a). However, we did not find any significant effect of urbanization on the body size of *L. helveticus* ($F_{(1,35)} = 1.05$, $p = 0.310$). We also found a significant difference between populations in the variances of body size of *T. marmoratus* (F test, $F = 3.508$, $p = 0.009$), so that individuals from the natural population showed a significant higher size variability than urban individuals. However, we did not find a significant effect of the population on body size variability in any of the other three species (F tests: *S.salamandra*, $F = 2.282$, $p = 0.088$; *L. helveticus*, $F = 1.877$, $p = 0.200$; *P. perezi*, $F = 2.048$, $p = 0.102$).

Table 1. Differences in body size, body condition and immune response between urban and natural habitats. Results (F, P) of ANOVA tests are shown. Significant differences are marked in bold.

Trait	**Species**	**mean ± SE**		**F**	**P**
		Urban	**Natural**		
Body size (cm)	*L. helveticus*	7.23 ± 0.28	7.35 ± 0.40	1.05	0.310
	T.marmoratus	15.23 ± 0.49	14.30 ± 0.92	15.51	< **0.001**
	S. salamandra	15.10 ± 1.20	13.87 ± 1.87	5.91	**0.001**
	P. perezi	7.98 ± 0.58	6.68 ± 0.83	35.16	< **0.001**
Body condition	*L. helveticus*	0.05 ± 0.03	-0.04 ± 0.04	60.88	< **0.001**
	T.marmoratus	0.05± 0.03	-0.04 ± 0.04	50.24	< **0.001**
	S. salamandra	-0.009 ± 0.04	0.007 ± 0.05	1.18	0.283
	P. perezi	0.01 ± 0.02	-0.01 ± 0.06	2.14	0.150
Immune response (mm)	*L. helveticus*	0.03 ± 0.11	-0.03 ± 0.11	3.29	0.078
	T.marmoratus	-0.02 ± 0.25	0.02 ± 0.21	0.46	0.500
	S. salamandra	0.01 + 0.15	-0.01± 0.13	0.47	0.497
	P. perezi	0.02 ± 0.17	-0.01± 0.18	0.63	0.434

Body condition of the two aquatic newts were significantly greater in the urban populations (*T. marmoratus*: $F_{(1,36)} = 50.24$, $p < 0.001$; *L. helveticus*: $F_{(1,35)} = 60.88$, $p < 0.001$) (Table 1, Fig. 1b). However, there were not significant differences in body condition between habitats in the terrestrial salamander ($F_{(1,38)} = 1.18$, $p = 0.283$) and the frog ($F_{(1,42)} = 2.14$, $p = 0.15$) (Table1, Fig. 1b).

Contrary to expectations, there were no significant differences between habitats in the PHA immune response in any of the species (*L. helveticus*: $F_{(1,35)} = 3.29$, $p = 0.078$; *T. marmoratus*: $F_{(1,36)} = 0.46$, $p = 0.500$; *S. salamandra*: $F_{(1,38)} = 0.47$, $p = 0.497$; *P. perezi*: $F_{(1,43)} = 0.63$, $p = 0.434$) (Table 1, Fig. 1c). Nevertheless, in *L. helveticus* the data suggest a trend for a stronger PHA immune response in the individuals from the natural population which might have reached statistical significance with a bigger sample size.


Body size
2
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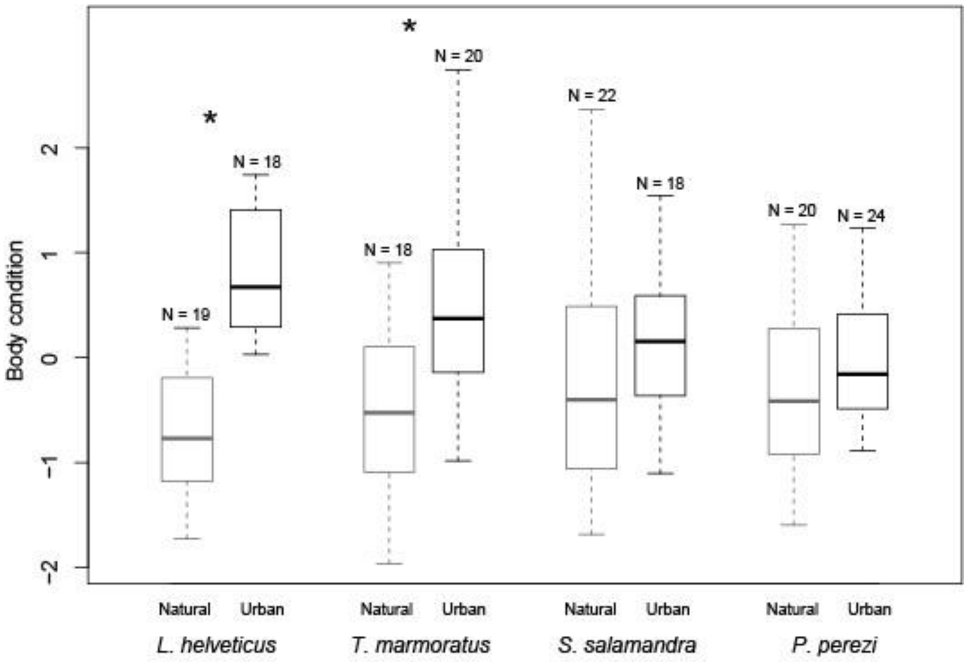
L. helveticus
T. marmoratus
S. salamandra
P. perezi


Body condition
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T. marmoratus
S. salamandra
P. perezi

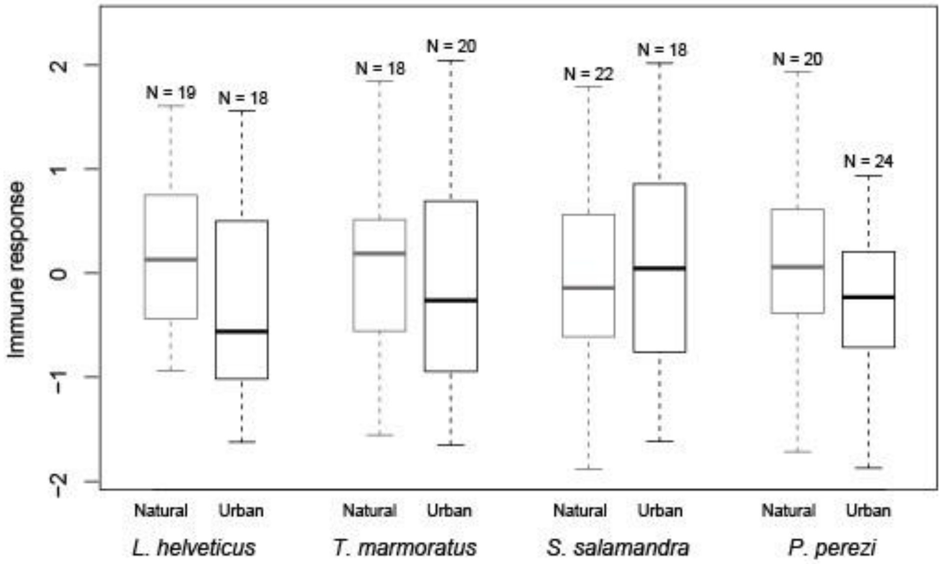

Figure 1. Habitat differences in a) the body size, b) the body condition and c) the immune response of the four studied amphibian species. All measures were scaled to 0 within species for illustration. Significant comparisons are marked with an asterisk.

DISCUSSION

Urbanization can affect several life-history traits of animals by imposing new evolutionary and environmental conditions. Our results suggest that some traits are more prone to be affected by urban conditions than others. Likewise, the different responses on body condition and body size among species suggest that the effect of urbanization depends on the species in question. Specific ecological requirements of each species may influence the direction of the effect, making it difficult to predict species responses under urban environmental conditions. However, it is important to take into account the limitation of our results, since we only explored one population per each species and habitat. Future studies in other populations are needed to test if our findings are general trends.

Effect of urbanization on body size

We found that in three of the four studied species, males inhabiting the urban environment had larger body sizes than those inhabiting the natural habitat. Changes in foraging behaviour, reproductive decisions, survival, and increased temperatures in urban habitats have been suggested as explanations of the body size differences between urban and natural habitats in some animal groups, such as birds or amphibians (Shochat 2004; Murphy et al. 2016a). As such, one possible explanation for our findings is that individuals inhabiting the city were older than those in the natural habitat, and thus they had had more time to grow. In the parks where we carried out the study, there are no invasive fish species that prey on amphibians, and avian and mammal predators are almost absent. The relatively small number of predators could lead to increased survival and longer lifespan, and hence larger body size. Finally, habitat-specific costs and benefits of a specific body size may drive the adaptation of populations in different habitats. For example, strong selection due to predation may favour small adult body size (Gosler et al. 1995), so that the release from predation in the urban population leads to the evolution of larger body sizes. Interestingly, in our data the variation in body size of *T. marmoratus* individuals from the natural population was higher than in the urban population. This could be simply because in the natural habitat the population size of individuals is higher, so, despite that the captured animals were all adults, the variability in body size may be higher. However, we cannot exclude that some unknown specific ecological pressure of the urban habitat is selecting for a limited body size range that might make more effective the life in this new environment.

Prey availability has also been hypothesized as an explanatory variable of larger body size in urban areas (Shochat 2004). This could also explain the differences found in our study, as exaggerated densities of some exotic invertebrate species have been observed in the studied urban habitats (MIC and CC, personal observation). On the other hand, lower abundances of conspecifics in urban streams could lead to reduced competition and increased growth rates (Petranka and Sih 1986). However, reduced competition seems unlikely in our urban population; although we did not measure the population density of adults in urban parkland, it did not seem smaller than in the natural population (MIC personal observation). Finally, Murphy et al. (2016) showed that higher temperatures experienced in urban environments can increase metabolic rates, leading to larger amphibian larval sizes. It would be interesting to compare physiology of amphibians from the different habitats to determine whether changes in metabolic rates could explain the differences found in our study.

Effect of urbanization on body condition

We found that the body condition of the two aquatic newts was better in the urban population, but no differences were found in the anuran species or the terrestrial salamander. These findings seemingly contradict the assumption that urban areas are a low quality habitat, at least for the aquatic species. The two newt species spend a long breeding period of time in the water, during which they feed on aquatic invertebrates and other amphibian eggs and larvae (Montori and Herrero 2004). Then these newts spend several months in terrestrial phase during which they change their diet completely. These changes in the ecology of the species might explain the differences found between the terrestrial and aquatic amphibians. Some invertebrate groups are known to have lower diversity and population densities in urban environments (Weller and Ganzhorn 2004), but others can increase densities, in particular exotic ones (see e.g. ants Guénard et al. 2015). Having an amphibious diet can be beneficial when it comes to adaptation to new environments because these species can more easily take advantage of recently created exotic communities of invertebrates. However, it seems implausible that resources provided by humans drive increased body condition in amphibians. This is because, as opposed to some birds and mammals (e.g. Ross 2004; Bateman and Fleming 2012), the amphibians' diet composition (i.e. live insects, crustaceans and worms) could not be based on human resources and waste.

One alternative explanation for a better body condition in urban areas is a reduced population density or diversity of predators. Escaping from predators usually trades-off with other fitness enhancing activities, such as foraging or mating (e.g. Turney and Godin 2014). If aquatic predators of adults are scarcer in urban areas, newts inhabiting the urban pools could spend more time feeding than their natural counterparts and improve their body condition.

Effect of urbanization on the PHA immune response

Both increases and decreases of the immune function associated with urbanization have been reported in the literature in several vertebrate taxa (Bradley and Altizer 2007; French et al. 2008), but to our knowledge this has not been explored in amphibians. Novel arrays of pathogens encountered in urban habitats have been shown to enhance immunocompetence in some bird species (Audet et al. 2016), while the exposure to toxic compounds that are present in urban areas reduce the immune response of others (Lewis et al. 2013). Contrary to expectations, we did not find any effect of living in the city on the immune response of any of our four amphibian species. Amphibian populations inhabiting anthropic habitats are expected to face chemical compounds not previously encountered during the evolutionary history of the species (Croteau et al. 2008; Iglesias-Carrasco et al. 2017). Previous laboratory and field experiments show that the immune function in tadpole and adult amphibians can change rapidly when exposed to toxins as a stress-induced response (Burraco et al. 2013; Iglesias-Carrasco et al. 2016b). Other stressors derived from anthropic activities (e.g. acoustic pollution; Barber et al. 2010) may also affect the immune response of urban populations. For example, increased average temperatures in cities, the chronic light pollution or the increase in the background noise can increase physiological stress (Tennessen et al. 2014), increase the mating call effort (Sun and Narins 2005) and alter feeding behaviours of amphibians (Perry et al. 2008), which may, in turn, constrain the immunity (e.g. Raffel et al. 2006). Air pollutants are also suggested to reduce the immune function of urban populations of lizards (Cabido et al. 2008). However, the urban habitat we sampled here had low levels of pollution, potentially explaining the lack of a significant effect of habitat on immune response.

Conclusions

Unique environmental pressures in urban areas seem to promote changes in individual investment toward different traits. Intriguingly, the direction of the changes is hard to predict and appears to depend on each species' ecology, such as feeding behavior related to aquatic or terrestrial habits, or predation pressure. As eggs are more limiting than sperm, females are often the limiting factor for population growth and persistence. Comparing the studied traits in females would be also interesting and will provide information on the total effect on the specific species. Further studies comparing additional urban and natural populations are needed to determine whether the results presented here are caused primarily by urbanization and if they apply generally to amphibians.

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Chapter 6

Habitat dependent effects of experimental immune challenge on lizard anti-predator responses.

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ABSTRACT

Lizards often respond to predators by hiding in sunless refuges, but this eliminates opportunities for thermoregulatory basking. Hiding can therefore lower body condition. Furthermore, in ectotherms basking is important to induce fever and activate an immune response. A potential trade-off therefore exists between lowering predation risk and elevating body temperature to fight infection. Such a trade-off could be habitat dependent if habitats differ in the relative risk of predation versus that of acquiring or countering an infection. Here we take an experimental approach to test whether lizard basking behaviour is affected by a trade-off between predator avoidance and fighting an infection. We quantified the antipredator behaviour of male lizards (*Podarcis liolepis)* both before and after they were immune challenged (injected with LPS) or not (injected with PBS control). To test the generality of any trade-off we tested lizards from both an urban and a natural habitat. We found that males spent less time hiding following a simulated predator attack after they had been immune challenged than before, but this decline was only significant for males from the natural habitat. We also tested whether morphological traits, body condition and immune response level explained variation in male hiding time. In the natural habitat, but not in the urban habitat, males with relatively small heads hid for significantly longer. In conclusion, we show that lizard anti-predator behaviour is affected by an immune-challenge. Habitat differences in the factors that predict hiding time offers potential insights into why this might be the case.

Key words: immunity, life history, *Podarcis liolepis,* predation costs, trade-off, urban habitat.

INTRODUCTION

How animals allocate resources and time to different activities (e.g. predator avoidance, foraging, reproduction, immune defense) is determined by trade-offs between those activities. The exact trade-off varies when the costs and benefits of each activity differ for specific individuals (e.g. McGlothlin et al. 2007). Often these costs and benefits vary depending on both an individual's phenotype (e.g. French et al. 2007a), and on the local environment. For example, larger bodied individuals who are less vulnerable to predators spend more time foraging. Similarly, many studies have shown that individuals spend more time foraging when predation risk is low. Quantifying variation in trade-offs between behaviours that have important fitness consequences is key to understanding how these behaviours evolve. Human-modified environments, such as urban areas, differ from natural environments in many aspects, such as predatory pressure or resource availability (Gering and Blair 1999; Ditchkoff et al. 2006). Species inhabiting both habitat types provide a unique opportunity to explore how trade-offs vary in different circumstances.

Predator avoidance usually trades-off with other fitness enhancing activities. For instance, in many species, individuals respond to predators by retreating to a refuge (Sih et al. 1992; Martín and López 1999a,b; Cooper and Blumstein 2015), but because refuges are suboptimal habitats prolonged use of a refuge can lower body condition (Martín and López 1999a) and reduce opportunities to mate or forage (Ydenberg and Dill 1986; Sih 1997; Martín and López 2003). In ectotherms, hiding in refuges (such as rock crevices which are often cold and sunless) can also impact their thermoregulation (Polo et al. 2005) with detrimental effects for their locomotion, growth, and reproduction (Stevenson et al. 1985; Deutsch et al. 2008). Individuals must therefore balance anti-predatory responses with other life history requirements (Lima and Dill 1990; Sih 1992), and selection should favor facultative adjustment of anti-predator behaviour to the prevailing conditions (Lima and Dill 1990). In support of this there is evidence that escape decisions are affected by perceived vulnerability (Cabido et al. 2009), predator abundance (Cooper et al. 2007), and the costs of refuge use (Cooper 1997; Martín and López 1999b; Cooper 2000; Martín and López 2000).

Optimal anti-predator behaviour can vary among individuals because behavioural, morphological and physiological traits influence the relative gains from other fitness-enhancing activities. For example, an elevated immune response induced by pathogens often reduces reproductive activity (French et al. 2007b), sexual coloration (López et al. 2009a), and other behaviours (Llewellyn et al. 2011), potentially affecting the optimal anti-predator response. Vertebrates usually respond to infection by elevating their body temperature and inducing fever (Llewellyn et al. 2011). In reptiles an immune challenge is expected to directly affect the use of a refuge as an anti-predator behaviour. Since reptiles are ectothermic, they need to elevate their temperature behaviourally by choosing microhabitats that increase net heat gain (Deen and Hutchison 2001), and/or by basking in the sun for longer periods (do Amaral et al. 2002). An immune challenge should therefore affect the optimal response to the inherent trade-off between escaping from a predator by retreating to a cool refuge and remaining in a warm, but exposed, site to fight infection (i.e. basking).

The Catalonian wall lizard (*Podarcis liolepis*) is a small lacertid distributed along the North-East Iberian Peninsula, from the Basque Country to Catalonia. It naturally occurs in rocky habitats, but is also found in urban habitats occupying artificial structures. Human modified environments have distinct biotic and abiotic characteristics, which can present different challenges for animals than are found in native habitat. Urban populations experience a variety of new conditions such as lower or higher predation risk (Gering and Blair 1999; e.g. pest species Sorace 2002), and unusual food sources (Ditchkoff et al. 2006) and breeding sites (Møller 2010). In some species that do well in urban areas, lower mortality and plentiful food improve physical condition (Contesse et al. 2004), and elevate reproductive rates and population densities (Møller 2009). However, higher population density, alongside pollutants, can also increase disease transmission, so the prevalence of diseases is sometimes greater in urban than natural habitats (Ditchkoff et al. 2006). This can result in urban populations being in worse health (Cabido et al. 2008). If being in an urban habitat affects immune system activity and predation risk, the optimal anti-predator response might differ between natural and urban habitats.

Here we test for a trade-off between the response to an immune challenge and anti-predator behaviour in male lizards (*P. liolepis*). We then test whether this trade-off is habitat-dependent. First, we examined whether males from an urban and a natural habitat differed in body condition, health status (immune response) and morphological features (indicative of social dominance). Then we investigated whether habitat type influenced the relationship between any of these traits and anti-predator behaviour. Finally we ran an experiment to test whether males alter their anti-predator behaviour after exposure to an immune challenge and to determine whether this response differed between urban and natural habitats. We predicted that:

1) There is a difference in body condition between males from urban and rural habitats. Better body condition might occur in urban areas because of higher food availability, or due to reduced predation pressure. On the other hand, poorer body condition might occur if predators, such as cats, are more abundant so that lizards have to invest more time hiding. We also predict a poorer immune response due to pollution and higher population densities that tend to increase disease transfer.

2) Morphological features, such as relative head size or colour badges, which are cues of social dominance, are associated with longer male hiding time.

3) Immune challenged individuals will leave a refuge sooner than healthy ones in order to bask to combat disease by elevating their body temperature.

MATERIAL AND METHODS

We captured (by harmless noosing) 30 adult male *P. liolepis* in May 2012 in urban parkland in the middle of the city of Donostia/San Sebastián in Northern Spain, and 30 males in nearby natural habitat in Donibane/Pasajes San Juan. The urban population is surrounded by the city and attracts large crowds of tourists. Lizards mainly occupy human constructions, like fortifications, and the occasional rocky sandstone outcrops. Due to the reduced appropriate habitat, lizards live in a limited space, so the population density is higher than in natural habitat (ICM, personal observation). The natural population is in a natural landscape along coastal cliffs visited by fewer people. Lizards occupy rocky sandstone outcrops interspersed with shrubs and grasses. Urban areas contain predators (e.g. domestic cats, jays (*Garrulus glandarius*) and magpies (*Pica pica*) (Aierbe et al. 2001; CC and ICM, personal observation;)), but there are fewer predatory species than occur in their natural habitat. To reduce individual variation attributable to age, sex and fitness effects due to tail loss, we only captured adult males with intact tails (e.g. Martín and López 2003).

Lizards were individually housed in 60 indoor 36 x 42 cm PVC terraria containing rocks for refuge heated by a UV-lamp. The lamp was situated at one end of the terrarium to ensure the presence of a heat gradient so that lizards could thermoregulate. The photoperiod mimicked that of the surrounding region. Water and food (mealworm larvae and crickets dusted with multivitamin powder) were provided *ad libitum*. Lizards were housed for at least a week to familiarize them with their new surroundings prior to testing. Placement of terraria within the laboratory was randomized to avoid potential environmental gradients within the laboratory confounding our treatments. All the animals were healthy during the trials and maintained their original body mass. They were returned to their capture sites at the end of the trials.

Morphological characteristics and immune response

We measured several morphological characteristics that correlate with anti-predator behaviour in lizards. To estimate body condition we used the residuals from the regression of log body mass on log snout vent length (SVL). The relationship between log transformed body mass (g) and log transformed snout vent length (mm) is linear ($p < 0.001$; $r^2 = 0.85$), and this index is expected to give an accurate estimate of relative amount of fat stored (review: Green 2001; see Garrido and Pérez-Mellado 2015).

We also measured three aspects of head size using digital calipers ($\underline{+}0.01$ mm): *depth* was the greatest vertical distance through the snout from the top of the head to the bottom of the lower jaw; *length* was the distance between the tip of the snout and the posterior side of the parietal scales and; *width* was the maximal distance between left and right parietal scales. Since head size is related to body size we calculated the residuals of each log transformed head measure from a regression on log transformed SVL to get head size measurements that were independent of body size. We then used Principal Component Analysis based on correlation matrix to reduce the head measurements into a single axis to describe the variation in these three measurements. PC1 (hereafter '*relative head size*') showed positive loading of depth (0.80), length (0.77) and width (0.84) and explained 65.7% of the total variance. Relative head size might affect antipredator behaviour because it is commonly associated with social status and increased aggression during male-male contests in lizards, including *P. liolepis* (Anderson and Vitt 1990; López et al. 2002). Social status has been suggested to influence anti-predator behaviour in lizards (Cooper and Wilson 2007). We also noted the number of ocelli (row of small distinctive blue spots along the outer margin of the belly) on each side of each male and calculated the mean number of ocelli per male. Ocelli might be correlated with anti-predator behaviour because they have previously been shown to play a role in intra-sexual social relationship (López et al. 2004), and they affect a male's perception of his risk of predation in related species (Cabido et al. 2009).

To assess a component of male immunocompetence we used a delayed-type hypersensitivity test: the phytohaemagglutinin injection assay (PHA test). This is a reliable measure of T-cell-dependent immunocompetence in vivo (Lochmiller et al. 1993) that has been used in many studies, including those on lizards (Svensson et al. 2001). The activation of the T-cells induces an inflammation at the place of injection of PHA, so we use the change in thickness as a proxy for the immune response.

We measured the thickness of the left hind footpad with a pressure-sensitive spessimeter (±0.01 mm) to standardize pressure. The measure was taken five times and we used the average measurement. Immediately after, we injected 0.02 mg of PHA dissolved in 0.01 ml of phosphate-buffered saline (PBS). Lizards were released into their terraria, and after 24 h we re-measured their footpad thickness. The immune response index was calculated as the difference between pre- and post- injection measurements (Lochmiller et al. 1993; Smits et al. 1999). As the thickness of the footpad might be related to body size, we calculated relative thickness, using the residuals from a linear regression of the log transformed immune response index on log SVL (hereafter '*PHA response*'). The only detectable effect of the injection was the skin swelling which disappeared after 48 h. None of the lizards showed any sign of stress or pain after the trials (all lizards behaved and fed normally after 15 minutes).

Anti-predator behaviour

We studied the anti-predator behaviour of males within 10 days of capture, from 11:00 to 15:00 h, when the captive lizards were fully active. We allowed lizards to thermoregulate for >1h before each trial. In each trial, we simulated a predatory attack and video recorded male behaviour for the following 30 min. We rapidly approached the terraria and tapped the male near his tail to simulate an avian predator attacking from above. We are confident that we simulated a predatory attack, because the flee response was similar to that observed in field experiments (Martín and López 1999a,b; CC, personal observation). We conducted two trials before the experimental immune activation (one per day over consecutive days), and two trials after the experimental immune activation (see below), for each lizard. The same person performed all predatory attacks. To minimize observer bias, blinded methods were used whenever behavioural data were recorded and/or analyzed. The only identification of individuals was a number from 1-60, randomly assigned with respect to treatment and site of origin.

For the 30 min after the simulated attack we noted the time spent in the refuge until the male's head appeared at the refuge entrance as a standard measure of 'hiding time'. Trials in which males did not entirely leave the refuge to bask (n= 3 of 240 trials) were discarded, because their response was ambiguous. When a lizard did not lean his head out of the refuge after 30 min (n = 9 of 240 trials), we assigned a hiding time of 1800s.

Experimental immune challenge

We challenged the immune system of males with a lipopolysaccharide (LPS) from the cell wall of the bacteria *E. coli* (serotype 0111-B4; Sigma-Aldrich, St. Louis, MO). LPS has no pathogenic effects (Janeway et al. 2001), but induces an inflammatory response by nonspecifically activating B and T lymphocytes, and producing specific anti-LPS antibodies. It induces immunopathological effects in reptiles (Deen and Hutchison 2001; do Amaral et al. 2002), and can reduce reproductive output and growth in female lizards (Uller et al. 2006), and sexual coloration and chemical signals of male lizards (López et al. 2009a,b). After the initial characterization of anti-predator behaviour, half the lizards captured in each habitat type ('LPS-males') were injected intraperitoneally with LPS (2.5 µg per g of body weight) diluted in 0.05 mL of phosphate-buffered saline serum (PBS). This concentration was previously used to stimulate the immune system of a closely related species (*P. hispanica*; López et al. 2009a,b). The remaining control males ('C-males') were injected with the same volume (0.05 mL) of PBS, to account for any effects of handling and being injected.

Statistical analyses

To test whether males from the different habitat types differed in morphology or immune response we used one-way ANOVAs with habitat as a fixed factor. We checked the residuals of all models to ensure that they met the assumption of normality.

To determine which male traits were associated with variation in hiding time prior to the experimental immune challenge, and whether these relationships differed depending on the habitat a male was captured in we ran a generalized lineal model (GLM) with quasi-poisson error distribution. Hiding time was significantly repeatable before the immune challenge ($r = 0.71$, $P < 0.01$). So, in our model we used the mean hiding time as the dependent variable. We included habitat type as a fixed factor, five covariates (body condition, PHA response, relative head size, SVL and ocelli number) and the two-way interactions between each covariate and habitat type.

To test for the effects of immune treatment and habitat type on hiding time we ran a generalized mixed model (GLMM) in package lme4 (Bates et al. 2015). We treated the mean hiding time as the response variable. Treatment (LPS or PBS), habitat (natural or urban) and time (pre-, post- injection) were included as fixed factors. Since males from the two habitats differed in relative head size, which was related to mean hiding time, we therefore included this trait as covariate in our model. We also included all two-way and three-way interactions involving habitat, treatment, time and relative head size. Although SVL differed between the two habitats, we did not include it as covariate because it was unrelated to mean hiding time in our prior analysis. We included individual as a random effect to control for individual variation in mean hiding time and we specified a Poisson error distribution. We corrected for overdispersion by including trial as a random effect (Harrison 2014). Following this correction our model was underdispersed (dispersion parameter = 0.1030) and our analysis should be considered conservative. Pairwise comparisons were planned using Tukey’s honestly significant difference tests. All statistical tests were conducted using R 3.2.2 (Team 2015).

RESULTS

Morphological characteristics and immune response.

Urban lizards were significantly bigger and had relatively larger heads than those from the natural habitat, but there was no habitat difference in PHA response, body condition or ocelli number ($n = 60$) (Table 1).

Table 1. Habitat differences in male morphology and immune response prior to immune-challenge treatment (results from one-way ANOVAs)

Trait	Habitat (mean ± SE)		F	P
	Urban (n=30)	Natural (n=30)		
Body condition	0.006 ± 0.037	-0.006 ± 0.047	1.210	0.270
SVL (mm)	73.430 ± 3.201	64.33 ± 2.770	138.530	**< 0.001**
Relative head size (PC1)	-0.269 ± 0.985	0.26 ± 0.955	4.610	**0.035**
Ocelli number	8.910 ± 4.620	7.33 ± 2.840	2.570	0.114
PHA response (residuals)	-0.010 ± 0.094	0.01 ± 0.101	0.668	0.416

Significant values are in bold

Anti-predator behaviour

We found no overall correlation of male traits with hiding time (all p-values > 0.07, see Table 2). However, the effect of relative head size differed significantly between the two habitats (habitat interaction with relative head size: estimate ± SE = 0.944 ± 0.349, $t_{(48)} = 2.707$, $P = 0.009$). We therefore looked at the individual correlations in each habitat type and found that males with relatively larger heads spent significantly less time hiding in the natural ($r^2 = 0.19$; $P = 0.015$), but not in the urban habitat ($r^2 = 0.07$; $P = 0.131$) (Fig. 1). There were no habitat differences in how the other traits were associated with hiding time (interactions: all p-values > 0.11, Table 2).

Experimental immune challenge

There was a significant habitat difference in the change in hiding time before and after the experimental immune challenge (pre-post*habitat*treatment: estimate ± SE = 1.302 ± 0.532, $z = 2.446$, $P = 0.014$) (Fig. 2). Before the treatment PBS treated males and LPS treated males from both habitats had similar hiding times (Tukey's tests: all $P > 0.36$). After the experimental treatment, however, LPS treated males in the natural habitat spent significantly less time hiding than did PBS treated males (Tukey's tests $P < 0.001$). This was not the case in the urban habitat (Tukey's test $P = 0.997$, Fig. 2). Finally, we found no significant effect of relative head size on this trade-off (estimate ± SE = 0.054 ± 0.790, $z = 069$, $P = 0.945$) (Table 3).

Table 2. Effects of habitat and male traits on hiding time (results from GLM)

Factors	Estimate	SE	t	P
Habitat	7.317	7.337	0.997	0.323
Body condition	8.739	5.901	1.481	0.145
SVL (mm)	0.161	0.089	1.806	0.077
PHA response	1.779	2.055	0.866	0.390
Relative head size	-0.430	0.283	-1.519	0.135
Ocelli	0.081	0.066	1.220	0.228
Habitat*body condition	-11.894	7.445	-1.598	0.116
Habitat*SVL	-0.102	0.108	-0.943	0.350
Habitat*PHA response	-3.291	2.677	-1.229	0.224
Habitat*Rel. head size	0.944	0.349	2.705	**0.009**
Habitat*ocelli	-0.107	0.078	-1.360	0.180

Significant values are in bold

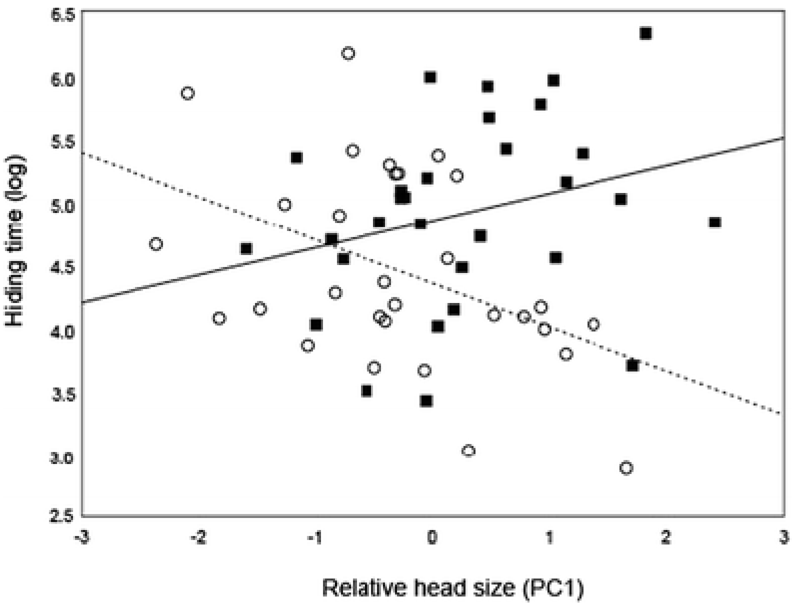

Figure 1. Relationship between hiding time and relative head size of males from natural (*circles, dashed line*) and urban (*squares, solid line*) habitats. Relative head size was significantly correlated with hiding time in the natural but not in the urban habitat. Graph shows the raw data and bivariate regression lines, not the results of the full GLM model

Table 3. Effects of treatment, habitat, time (pre- post- injection) and relative head size and their interactions on hiding time (results from GLMM).

Factors	Estimate	SE	z	P
Treatment	1.679	0.403	4.158	**< 0.001**
Habitat	1.814	0.407	4.452	**< 0.001**
Time (pre-post injection)	1.134	0.263	4.305	**< 0.001**
Relative head size	-0.719	0.387	-1.858	**0.063**
Treatment*habitat	-1.496	0.578	-2.586	**0.009**
Treatment*time	-1.501	0.372	-4.034	**< 0.001**
Habitat*time	-1.323	0.375	-3.521	**< 0.001**
Treatment*Relative head size	0.177	0.569	0.311	0.755
Habitat*Relative head size	0.792	0.636	1.245	0.213
Time*Relative head size	0.333	0.357	0.933	0.350
Treatment*habitat*time	1.302	0.532	2.446	**0.014**
Treatment*habitat*Rel. head size	-0.154	0.860	-0.180	0.857
Treatment*time*Rel. head size	-0.158	0.524	-0.302	0.762
Habitat*time*Rel. head size	-0.600	0.585	-0.103	0.918
Treat*habitat*time*Rel. head size	0.054	0.790	0.069	0.945

Significant values are in bold.

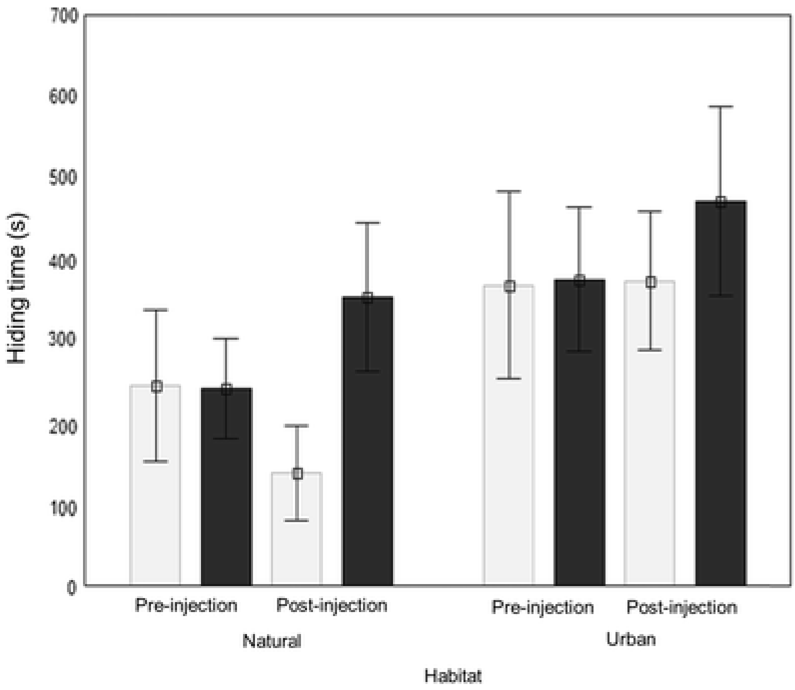

Figure 2. Male hiding time (mean ± SE) in relation to habitat type, time, and treatment. LPS treatment is shown in *light gray*. PBS is shown in *dark gray*. Only post-treatment LPS males from the natural habitat differed significantly from the other groups (all Tukey's test $P < 0.02$)

DISCUSSION

Our main experiment suggests that there is an evolutionary trade-off in *Podarcis liolepis* between hiding to evade predators and responding to an immune challenge in natural habitats (Fig. 2). After an immune challenge males from natural habitats decreased their hiding time in response to a simulated predatory attack. This was not the case for males from the urban habitat who showed no change in anti-predatory response after an immune challenge. In addition, we found that the correlation between male relative head size and hiding time differed between our urban and natural population. When combined our results suggest that the costs and benefits affecting the trade-off between hiding and basking (to counter an immune challenge) differ in our urban and natural habitat. Of course, future studies in other populations and/or lizard species are needed to test whether this will prove to be a general finding. When sufficient studies are published it will then be possible to conduct a formal meta-analysis looking at the effect of urbanization on the ecological trade-offs faced by individuals (e.g. Samia et al. 2015).

Habitat differences

Males from the urban population had larger bodies and larger heads (for their body size) than those from the natural population. These findings suggest that growth rates and/or survival (given indeterminate growth) might be greater in urban areas. It is possible that long-term selection has favored genotypes better suited to an urban environment. The city started to grow around this park in 1181 A.D. (Carrillo, 1979) and, although the extent to which this reduced gene flow is unknown, it is possible that the urban population has been isolated from nearby rural populations for 900 years. This is sufficient time for local adaptation to the new environment. It has been suggested that urban areas can impose similar selection to that seen on actual islands (see Marzluff 2005), since the conditions in both habitats can be quite similar. Indeed, lizard populations on islands usually tend to gigantism (Runemark et al. 2015), which might explain the between-population differences in body and head size that we observed in *P. liolepis*. Alternatively, the larger relative head size might be due to strong intra-sexual selection in the urban habitat. Relative head size is an indicator of social dominance in lizards (Braña 1996), and individuals in the urban population inhabited a limited area, so direct competition for access to females is likely to be greater (ICM pers. obs.). Finally, these morphometric differences may be due to greater survival in urban conditions of phenotypes that are linked to personality traits associated with urban tolerance (e.g. Miranda et al. 2013).

In contrast to our predictions, however, we found no habitat differences in mean male body condition or immune response to PHA. This contrasts with other studies that report that urban animals are in better body condition due to greater food availability (e.g. Contesse et al. 2004), but have weaker immune responses due to pollution and stress induced by the presence of people who increase the perceived predation risk (Amo et al. 2006; Cabido et al. 2008). The lack of differences in body condition also suggests that the predation pressure might be similar in both environments, so that animals spend similar time fleeing from predators. We also found no difference in blue ocelli number between the habitats. These ocelli appear to be sexually selected traits

(López et al. 2004). Previous studies in a range of taxa have reported both increased and reduced expression of sexual traits in urban areas (Yeh 2004; Ríos-Chelén et al. 2013). To date, however, there has been no formal meta-analysis of how urbanization affects sexually selected traits. More studies such as ours are needed to permit such an analysis.

Habitat type affected the relationship between male relative head size and anti-predatory response. Males with relatively large heads spent significantly less time hiding when they were from the natural habitat, but not when they were from the urban habitat. Relative head size is associated with social dominance so a plausible reason why larger headed males emerge sooner is the greater reward of being active and engaging in territorial defense (Cooper 1999; Díaz-Uriarte 1999). More generally, habitat differences in the strength of sexual selection could alter the trade-off between predation risk and social behaviour (Lima and Dill 1990; Martín and López 2003; Cabido et al. 2009). In the urban habitat the population density appears higher (ICM pers. obs.). Changes in density often affect mate choice, mate searching (Kokko and Rankin 2006) and the size of territories (Wood et al. 2012). This yields a testable prediction: territory ownership is less important in the urban habitat so larger headed males have less to gain from emerging sooner.

Immune challenge and hiding time

Our key finding is based on our experimental test of the relationship between an immune challenge and hiding time. Immune-challenged males from the natural habitat emerged significantly sooner than control males. No such difference was observed for males from the urban habitat. This suggests that the effect of immune activation on the trade-off between hiding in a cool, safe refuge and basking in a warm, but exposed site to fight an infection differs between the habitats. Individuals experiencing an immune challenge should leave their refuge sooner than healthy ones to achieve a higher body temperature to fight an infection (Otti et al. 2012). Unfortunately, the need to bask conflicts with increased exposure to predators. In contrast, however, immune activation might favor re-allocation of resources to fighting infections so that individuals then have reduced locomotory performance due to lower energy availability. This could make them more vulnerable to predators and thereby increase their hiding time (Aubert 1999; Otti et al. 2012).

The net effect of an immune challenge on male hiding time is hard to predict. Our results, at least from the natural habitat, suggest that the net benefits of basking outweigh the greater predation risk that follows when a male is subject to an immune challenge. Future studies should take advantage of habitat difference in hiding behaviour, such as the one reported here, in response to an immune challenge to try to pinpoint the associated costs and benefits of hiding. Specifically, it is important to understand in greater depth the effects of urban habitats on the perception of predation risk. One can also test whether fewer limitations on resource acquisition might explain the lack of change in the hiding-basking trade-off for immune challenged males in an urban habitat.

Summary

Our experimental study showed that an immune challenge affects the trade-off between anti-predator behaviour (hiding) and mounting an immune challenge (basking to elevate body temperature). Few studies to date have shown such a relationship (see Rigby and Jokela 2000). However, this trade-off differed between male lizards from urban and natural habitats. In addition, the correlation between male relative head size and hiding time differed between the two habitats. In conjunction these twin findings allow us to better understand the relative importance of maintaining body condition, fighting infection and avoiding predators. When evolutionary trade-offs change they affect the strength of natural selection on relevant traits. Unique ecological and social pressures in urban environments could promote changes in behaviour or resource allocation that lead to local adaptation. Further studies comparing additional urban and natural habitats and a wider range of species are needed to determine whether the results we have presented apply more generally to reptiles that hide in refuge but need to bask to counter infections.

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General conclusions

The massive replacement of natural forests by exotic tree plantations seems to have a detrimental effect on amphibian diversity and abundance. The mere presence of a species in a specific habitat is not a good indicator of the suitability of such habitat for the survival of populations (if, for example, some ecological traits with fitness consequences are detrimentally affected). It is important to explore whether exotic plantations may be acting as sinks, or even ecological traps by means of doing research on their effects at the level of individuals.

Leachates released by the leaves of exotic trees have to be considered one main driver of species diversity and ecology in exotic plantations. Anuran tadpoles exposed to eucalypt leaf leachates showed a reduced growth and lower immune responses, but the extent of the effect depended on the species. These results suggest that it is usually difficult to predict the exact direction of the effect on each species. Therefore, the specific ecological requirements of each species should be considered for future management plans to be successful.

Pine and eucalypt plantations seem to be detrimental for the ecology of amphibians, at least in the case of palmate newt, the species chosen as model. However, we may expect a stronger effect on species with more restricted ecological requirements. Novel ecological characteristics of these habitats in combination with changes in the acquisition and, hence, optimal allocation of resources to important life-history traits are driving selection for reduced investment into sexual traits and immune function. Again, I found that leave leachates have a causal effect on the poor ecology of amphibians. Nonetheless, novel habitats might generate long-term selection for new resource allocation strategies in native species.

Regarding mate choice, the environmental factors that influence the condition of choosy individuals might affect sexual selection. This is especially important in human-modified environments because they often differ dramatically from native habitats. Changes in female mate preferences after the exposure of females of palmate newt to eucalypt and oak water extracts are driven by changes in female physiology, rather than by the immediate inhibition of transmission or reception of pheromonal signals. In conclusion, human-modification of the habitat through the planting of eucalypts provides a large-scale ‘natural experiment’ to test how mating preferences evolve when the costs and benefits of mate choice change.

The presence of novel chemical substances released by exotic plantations may disrupt the chemical communication between organisms. Changes in the chemical environment may lead to a lack of recognition of potential mates, predators or prey. This can have ecological and conservation consequences if individuals inhabiting plantations have a reduced offspring quality and population survival.

The evolutionary pressures that differ between urban and natural areas, such as a reduction in predation risk, higher abundance of food or alteration in the environmental temperature, may alter the costs and benefits of a specific trait, driving the adaptation of populations in different habitats. Unique environmental pressures in urban areas seem to promote changes in the investment that each individual does toward different traits, but the direction of the changes is difficult to predict since it depends on each species’ ecology.

Organisms have to allocate resources and time to different activities depending on the trade-offs between those activities. In ectotherms, a potential trade-off exists between lowering predation risk and elevating body temperature to fight infection. Such trade-offs could be habitat dependent if habitats, such as urban areas, differ in evolutionary pressures, such as relative risk of predation. We conclude that when evolutionary trade-offs change they affect the strength of natural selection on relevant traits. Unique ecological and social pressures in urban environments could promote changes in behaviour or resource allocation that lead to local adaptation.

Finally, the replacement of natural habitats by anthropic areas may have both ecological and conservation implications for amphibians and reptiles. Our results suggest that the effects of novel pressures not previously encountered during the evolutionary history of species, potentially lower the fitness of individuals by altering their anti-predator behaviour, sexual behaviour and their investments in life-history traits. However, to fully understand how human-created habitats affect the conservation status of native fauna, it is critical to delve not only into the direct effects on species diversity, but also into the indirect effects on the ecology of individuals. It is, therefore, critical to integrate behavioural studies with conservation programs to fully understand how the habitat transformation affects endangered species.

Annex 1. Resumen general

Esta tesis doctoral examina cómo la rápida transformación del hábitat natural en ambientes antrópicos altera las respuestas comportamentales y los rasgos vitales de anfibios y reptiles. Se centra en dos de los medios antrópicos que más superficie del planeta ocupan: los hábitats urbanos y las plantaciones forestales exóticas. Los ambientes antrópicos son verdaderos nuevos hábitats con presiones selectivas distintas de las naturales, y las especies que los habitan necesitan adaptarse a ellas mediante rápidos cambios fenotípicos. Este tipo de hábitats ofrecen, por lo tanto, una oportunidad única para estudiar los cambios evolutivos de las especies que los ocupan, como corrobora el drástico incremento de publicaciones científicas referidas a ellos.

En el presente proyecto examinamos el efecto que las áreas urbanas y plantaciones exóticas de árboles ejercen sobre la ecología funcional de los individuos, siendo distintas especies de anfibios y reptiles las sujetas a estudio. La tesis incluye tanto análisis de datos de campo, como experimentos de laboratorio, combinando, en ocasiones, ambas técnicas con el objeto de interpretar en términos de causalidad lo observado en el campo.

Capítulo 1.

¿Qué efecto tienen las plantaciones exóticas en la riqueza de especies y en la ecología de los organismos nativos que las habitan? Algunos estudios muestran que las plantaciones reducen la diversidad y abundancia de la biota forestal, mientras que otros sugieren que pueden servir para preservar parte de esta. Las plantaciones de pinos y eucaliptos se encuentran entre las plantaciones exóticas más extendidas del planeta, influyendo de forma negativa en muchos taxones. Pero el grado en que estas plantaciones exóticas afectan a la riqueza de especies y a su densidad poblacional, o si condicionan rasgos relacionados con la supervivencia de los anfibios, apenas ha sido explorado. Para este estudio, en primer lugar se examinó el número de especies de anfibios y la abundancia relativa de cada una de ellas en charcas situadas en bosques autóctonos de roble o en plantaciones de pino y eucalipto. Los resultados mostraron que la cantidad de especies que habitaban ambos tipos de plantaciones era menor y que también las densidades eran más bajas (principalmente en las de eucaliptos) con respecto a los bosques nativos. El tipo de hábitat explicó la mayor parte de la varianza en ambas variables (57 y 36%, respectivamente), lo que sugiere que los factores que éste regula, como la acumulación de lixiviados en el agua, pueden ser responsables de las diferencias observadas. Así, en segundo lugar, para examinar cómo la exposición a los lixiviados de eucalipto pudiera explicar lo observado en el campo, elegimos tres especies de anuros con respuestas marcadamente diferentes por la influencia de plantaciones y mantuvimos sus renacuajos en mesocosmos hasta alcanzar la metamorfosis. Se midieron varios rasgos clave para su supervivencia: crecimiento y respuesta inmune de los renacuajos, y tamaño y capacidad de salto de los metamórficos. Las tres especies mostraron una reducción de la respuesta inmune tras su exposición a los lixiviados de eucalipto, pero el efecto sobre el crecimiento, el tamaño y la capacidad de salto resultó variable según las especies. Concluimos que los lixiviados sí que parecen ser un condicionante que afecta a los anfibios en las plantaciones, aunque su efecto varía dependiendo de las especie, pero no se deben descartar los efectos de otros factores asociados también al tipo de hábitat.

Capítulo 2.

En función de su condición, los individuos pueden modificar la asignación óptima de los recursos destinada a las señales sexuales y otros rasgos de la historia vital. La variación en la expresión de los rasgos sexuales entre diferentes hábitats también depende de los efectos combinados de la adaptación local, la condición corporal o las respuestas fenotípicas a señales ambientales específicas que afecten a la asignación de recursos. Es un fenómeno conocido que en algunas especies, los machos que habitan en ambientes antrópicos modifican sus señales sexuales. Sin embargo, apenas ha sido estudiado hasta qué punto esto afecta al coste de la señal y a la fiabilidad de ésta. El tritón palmeado es una especie que habita, durante la época reproductora, pequeñas charcas ubicadas tanto en bosques como en plantaciones de pino y eucalipto. Los machos desarrollan crestas y filamentos caudales y palmeaduras en las patas traseras durante el celo. Así, resulta una especie idónea para examinar si la relación de estas señales con la condición física o el estado de salud de los distintos individuos se ve afectada por los nuevos hábitats. Los resultados mostraron que el tamaño relativo de las señales sexuales y el nivel de la respuesta inmune eran significativamente más bajos para los machos de tritón palmeado (*Lissotriton helveticus)* capturados en plantaciones de pino y eucalipto, que para los capturados en los bosques autóctonos de roble. Sin embargo, no encontramos diferencias dependientes del hábitat en la condición corporal de los individuos (n =

382 machos) y la fiabilidad con la cual las señales sexuales reflejaban la condición corporal y la respuesta inmune fue la misma en los tres hábitats. A continuación se examinó esto experimentalmente manteniendo durante 21 días a machos en mesocosmos llenos de agua con hojas de pino, eucalipto o roble. Los machos expuestos a agua de pino o eucalipto mostraron respuestas inmunes significativamente más bajas, pero sin cambios en la condición corporal, lo cual coincide con lo observado en los machos capturados en el campo. Sin embargo, a diferencia de los machos capturados en el campo, en los mesocomos no se observó una relación entre el tipo de agua y el tamaño relativo de las señales sexuales. La reducida respuesta inmune y desarrollo de los rasgos sexuales sugieren que las plantaciones de pino y eucalipto son perjudiciales para el tritón palmeado. Esto podría deberse a que los aspectos ecológicos de estos nuevos hábitats, como la alta turbidez del agua o los cambios en la competencia entre machos, conducen a reducir la inversión en señales sexuales. Nuestro experimento de mesocosmos apoya la idea de que la toxicidad del agua es un factor causal. En general, los resultados sugieren que los nuevos hábitats pueden generar una selección a largo plazo en favor de nuevas estrategias de asignación de recursos.

Capítulo 3.

La selección puede favorecer la plasticidad fenotípica en la elección de pareja como respuesta a factores ambientales que alteran los costes y beneficios de ser exigente en la elección de compañeros específicos. El cambio ambiental inducido por el ser humano podría alterar la selección sexual al afectar a los costes relacionados con la elección de pareja o al perjudicar la capacidad de los individuos para distinguir a las mejores parejas. La selección de pareja por parte de un individuo puede verse condicionada por el ambiente al afectar éste a dos aspectos: 1) a la condición corporal del individuo, de manera que le resulten insostenibles los costes inherentes a la elección de pareja; 2) a la capacidad de detectar o discriminar las señales sexuales. En este trabajo separamos estas posibilidades de forma experimental, comparando la elección de pareja mediante señales químicas (feromonas) de las hembras del tritón palmeado (*Lissotriton helveticus*) tanto en un ambiente que imita el agua de las charcas de sus hábitats naturales (bosque autóctono de roble), o la de charcas situadas en plantaciones de eucalipto. En ensayos de laboratorio con agua limpia y donde a las hembras de tritón se les posibilitaba la elección entre dos machos, las hembras con exposición prolongada (21 días) a lixiviados derivados de hojas de roble optaron por el macho con una respuesta inmune más fuerte, sin embargo, no lo hicieron las hembras expuestas al agua con productos químicos de hojas de eucalipto. Es decir, la exposición previa al ambiente de un eucaliptal, afectaba a la elección de pareja, aunque esta se realizase en un medio que no condiciona la detección o eficacia de las señales sexuales. Por el contrario, la presencia inmediata o la ausencia de lixiviados de eucalipto durante la elección de pareja (usando sólo hembras previamente expuesta a agua tratada con roble), no afectaba al resultado; es decir, a la detección de las señales. Por lo tanto, nuestros resultados sugieren que el cambio observado en la elección de pareja entre hábitats es inducido o impuesto por los efectos que los lixiviados de eucalipto tienen en la fisiología de las hembras, y no por la inhibición inmediata de la transmisión de feromonas o el bloqueo en la recepción de las mismas.

Capítulo 4.

La comunicación química en especies acuáticas puede afectar muchos rasgos claves del ciclo vital, como son la detección de presas y depredadores, la búsqueda de pareja, la elección de esta o la agrupación social. Sin embargo, los cambios en el entorno pueden mermar la eficacia de las señales y la capacidad de los individuos para detectar las mismas. Muchos estudios han examinado el efecto que los compuestos secundarios de plantas exóticas ejercen sobre la ecología y la fisiología en un amplio rango de taxones. Sin embargo, rara vez se ha investigado si la trasformación de los bosques naturales por plantaciones exóticas influye en las respuestas comportamentales de los animales por modificación o interrupción de la comunicación química. En este estudio examinamos si la exposición a los lixiviados de eucalipto alteraba la capacidad quimiosensorial de machos adultos de tritón palmeado (*Lissotriton helveticus*) para detectar charcas con presencia de hembra, con señales de alarma de conespecíficos y para localizar el alimento. Se probaron los efectos de la exposición al agua de eucalipto (es decir, agua con extractos resultantes de la inmersión de hojas de eucalipto en agua mineral) discriminando a su vez la procedencias de los tritones (plantaciones de eucalipto o bosques naturales de roble). Se encontró que los lixiviados de eucalipto no afectaban por igual a todos los comportamientos estudiados. En comparación con los machos expuestos a agua de roble, los expuestos a la de eucalipto detectaron peor las charcas con olor de hembra, independientemente del hábitat de origen de los machos. Los tritones capturados en el bosque autóctono fueron menos capaces de detectar señales de alarma que señalaban eventos depredadores cuando fueron expuestos al agua de eucalipto que cuando fueron expuestos al tratamiento de roble. Por el contrario, la habilidad de los machos para encontrar alimento usando señales químicas fue similar en los tratamientos de roble y eucalipto. Nuestros resultados sugieren que compuestos químicos no encontrados previamente durante la historia evolutiva de la especie pueden influir algunos aspectos clave, como la capacidad de responder a los depredadores o de localizar pareja. Futuros estudios deberían comprobar cómo esto afecta a la eficacia biológica de los individuos.

Capítulo 5.

¿Afecta la urbanización a los rasgos vitales de los organismos nativos? Algunos estudios muestran que las áreas urbanas reducen la diversidad de ciertos taxones, sin embargo, poco sabemos sobre cómo estos ambientes afectan a rasgos fisiológicos y ecológicos de las especies que los habitan. Las áreas urbanas se caracterizan por presentar estructuras físicas y procesos ecológicos distintos a los hábitats originales y estos cambios ambientales pueden influir en los costes y beneficios de los diferentes rasgos y comportamientos de los organismos nativos. Algunos de estos efectos se han explorado en grupos como las aves; pero en animales con movilidad reducida, como los anfibios, los efectos podrían ser mayores. Además, los anfibios son el grupo de vertebrados más amenazado en el mundo, por lo que conocer el efecto de un fenómeno tan extendido como la urbanización de los hábitats es especialmente necesario. En este estudio comparamos tres rasgos vitales relacionados con la eficacia biológica de los anfibios en hábitats urbanos y naturales: tamaño corporal, condición corporal y respuesta inmune. Para probar la generalidad de nuestros resultados, se evaluaron estos tres rasgos en machos adultos de cuatro especies de anfibios: el tritón palmeado, el tritón jaspeado, la salamandra común y la rana verde ibérica. Se encontró que el tamaño corporal era mayor en ambientes urbanos en tres de cuatro especies estudiadas, mientras que la condición corporal era mejor en las poblaciones urbanas en las dos especies de tritones acuáticos. Finalmente, no encontramos ningún efecto de la urbanización en la respuesta inmune de los individuos. En conclusión, nuestros resultados muestran que diferentes especies de anfibios pueden verse afectadas de manera diferente por la alteración del hábitat dependiendo de su ecología específica.

Capítulo 6.

Los reptiles a menudo responden ante los depredadores ocultándose en refugios, con los costes asociados de reducir (o eliminar, en función de la duración del peligro) la posibilidad de termorregular de forma óptima, afectando al metabolismo, así como a su alimentación. Ocultarse puede, por lo tanto, afectar indirectamente a la condición corporal. Además, en animales ectodermos la termorregulación es importante para inducir "fiebre" y activar una respuesta inmunológica. Por lo tanto, existe un potencial compromiso entre reducir el riesgo de depredación y ocultarse, o exponerse y poder así mantener o elevar la temperatura corporal para combatir la infección. Este tipo de compromiso podría depender del hábitat si distintos hábitats difieren en el riesgo relativo de depredación. En este estudio comprobamos experimentalmente si el comportamiento termorregulador de las lagartijas está afectado por el compromiso entre evitar a un depredador y combatir una infección, y si el ambiente urbano cambia ese compromiso. Para ello cuantificamos el comportamiento antidepredador de machos de lagartija parda (*Podarcis liolepis*) tanto antes como después de provocarles una infección (inyectando LPS) o no (grupo control inyectado con PBS). El experimento se realizó con lagartijas procedentes de un hábitat urbano y de otro natural. Descubrimos que los machos, tras haber sido inoculados con LPS, permanecían menos tiempo escondidos tras el ataque simulado de un depredador. Sin embargo, esta reducción en el tiempo de espera en el refugio sólo fue significativa para los machos procedentes del hábitat natural. También cotejamos si los rasgos morfológicos, la condición corporal y el nivel de respuesta inmune explicaban la variación en el tiempo que los machos permanecían ocultos en el refugio. En el hábitat natural, pero no en el hábitat urbano, los machos con cabezas relativamente pequeñas (menos dominantes) permanecían ocultos durante mucho más tiempo. En conclusión, verificamos que el comportamiento antidepredador de las lagartijas se ve afectado por la activación del sistema inmune, y que la alteración de los factores que afectan al tiempo de permanencia en el refugio en función del hábitat, pueden explicar los diferentes resultados en ambas poblaciones.

Annex 2. Contributions

Relevant scientific publications

1. **Iglesias-Carrasco M**, Head M & Cabido C, 2016. Habitat dependent effects of experimental immune challenge on lizard anti-predator responses. *Behavioural Ecology and Sociobiology*, 70 (11), 1931-1939.

2. **Iglesias-Carrasco M**, Head M, Jennions MD & Cabido C. 2016. Condition-dependent trade-offs between sexual traits, body condition and immunity: the effect of novel habitats. *BMC Evolutionary Biology*, 16 (1) 135.

3. **Iglesias-Carrasco M**, Head M, Jennions MD, Martín J & Cabido C. 2017. Leaf extracts from an exotic tree affect responses to chemical cues in the palmate newt (*Lissotriton helveticus*). *Animal Behaviour*, 127: 243-251.

4. **Iglesias-Carrasco M**, Head M, Jennions MD & Cabido C. 2017. Secondary compounds from exotic tree plantations change female mating preferences. *Journal of Evolutionary Biology* (*in press*).

5. **Iglesias-Carrasco M**, Martín J, Cabido C. (*under review*). Urban habitats can affect body size and body condition, but not immune response in amphibians.

Selected conference contributions

1. **Iglesias-Carrasco, M**. & Cabido, C. 2012. Trade-off between immune and anti-predatory response in an urban and a natural population. A matter of personality? Oral communication. *TiBE 2012: Trends in biodiversity and evolution. Porto (Portugal).*

2. **Iglesias-Carrasco, M**. & Cabido, C. 2013. Are alien tree plantations ecological traps for amphibian? Effects on immune response and secondary sex characteristics of palmate newt. Poster communication. *17th European Congress of Herpetology. Veszprém (Hungary).*

3. Cabido, C.**, Iglesias-Carrasco, M** & Garin-Barrio, I. 2013. Besides how much, how: examination of the status of the herpetological biodiversity by monitoring their health. *IV Congreso Nacional de Biodiversidad y I Congreso Ibérico de Biodiversidad. Bilbao (Spain).*

4. **Iglesias-Carrasco, M**. 2013. Management plans of endangered fauna: rescue tools for biodiversity? Oral communication. *IV Congreso Nacional de Biodiversidad y I Congreso Ibérico de Biodiversidad. Bilbao (Spain).*

5. **Iglesias-Carrasco M**. & Cabido C. 2014. Immune response and antipredatory behaviour of common midwife toad across an urbanization gradient. Oral communication. *XIII Congreso Luso-Español de Herpetología. Aveiro (Portugal).*

6. Miqueleiz A, **Iglesias-Carrasco M** & Cabido C. 2014. Morph-specific correlations between immunity, sexual characteristics and personality in male *Podarcis muralis*. Poster communication. *XIII Congreso Luso-Español de Herpetología. Aveiro (Portugal).*

7. **Iglesias-Carrasco M** & Cabido C. 2014. Are exotic tree plantations ecological traps for amphibians? A field and experimental study with *Lissotriton helveticus.* Poster communication. *XIII Congreso Luso-Español de Herpetología. Aveiro (Portugal).*

8. **Iglesias-Carrasco M**, Miqueleiz A & Cabido C. 2014. Different reactions to chemical alarm cues and kairomones in larvae of the common midwife toad. Poster communication. *XIII Congreso Luso-Español de Herpetología. Aveiro (Portugal).*

9. **Iglesias-Carrasco, M**. & Cabido, C. 2014. Effect of exotic forest plantations on the immune response and sexual behaviour of the palmate newt *Lissotriton helveticus.* Oral communication. *XV Congreso Nacional y XII Iberoamericano de la Sociedad Española de Etología. Barcelona (Spain).*

10. **Iglesias-Carrasco M**. & Cabido C. 2015. Are alien tree plantations ecological traps for amphibians? Studying the impact of eucalyptus plantations on Iberian amphibian larvae. Poster communication. *18th European Congress of Herpetology. Wroclaw (Poland).*

11. **Iglesias-Carrasco M**., Berroneau M, Garin-Barrio I & Cabido C. 2015. The older, the better? Effect of pine plantations' age on the immune response and secondary sexual characters in the palmate newt (*Lissotriton helveticus*). Poster communication. *18th European Congress of Herpetology. Wroclaw (Poland).*

12. **Iglesias-Carrasco M**. & Cabido C. 2015. Who is better? Eucalyptus plantations affect sexual selection in the palmate newt (*Lissotriton helveticus*). Poster communication. *11th Conference Ecology and Behaviour. Toulouse (France).*

13. **Iglesias-Carrasco M**. & Cabido C. 2016. Effects of eucalyptus plantations, temperature and predation on amphibian ecology. Poster communication. *XIV Congreso Luso-Español de Herpetología.Lleida (Spain).*