

## Leaf extracts from an exotic tree affect responses to chemical cues in the palmate newt, *Lissotriton helveticus*



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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 has rarely been investigated. We experimentally tested how eucalypt tree chemicals influenced three key aspects of chemical communication in adult male palmate newts. We tested for effects of both exposure to eucalypt water (i.e. extracts obtained by soaking leaves in mineral water) and 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 or conspecific alarm cues and to find food. We found that eucalypt leachates had different effects on each behavioural trait. Fewer males detected female chemicals when exposed to the eucalypt than the oak water treatment, independent of the males' habitat of origin. Newts from oak forest were less able to detect conspecific alarm cues signalling predatory events when exposed to eucalypt water than when exposed to oak water, or than 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 predators or detect mates.

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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. Ferrari, Wisenden, & Chivers, 2010; Johansson & Jones, 2007; Todd, Atmea, & Bardach, 1967). 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. Chemical cues are particularly important in aquatic habitats 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 hear very low-frequency calls and detect their prey, túngara frogs, *Engystomops pustulosus*, by their mating calls, but their ability to locate frogs decreases with increased complexity of the environment and greater background

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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), but 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 increase the perception and consequent response of the receiver (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 them or, more generally, perceive informative cues (Fabian, Albright, Gerlach, Fisher, & Rosenthal, 2007; Wolf & Moore, 2002).

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 (Gill & Raine, 2014; Polo-Cavia, Burraco, & Gomez-Mestre, 2016; Wolf & Moore, 2002). 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 signalling: Candolin, 1999; Hunt, Brooks, & Jennions, 2005). Human-induced disruption of chemical communication can have drastic evolutionary and conservation effects for species that use chemical cues to discriminate between potential mates. 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). 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, have not, however, 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 the 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, *Ambystoma tigrinum*: 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 addressed 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, 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 natural oak 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.

## METHODS

The palmate newt has an aquatic reproductive period lasting from January to June. In February 2014, we captured, by dip netting, 135 adult males from native oak, *Quercus robur*, forests and 135 males from eucalypt, *Eucalyptus globulus*, plantations over 4 consecutive days (1 day per experiment 1 and 2; 2 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 from 0.6 km<sup>2</sup> to 1.2 km<sup>2</sup>. 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 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 experiments 1 and 2 we captured the males in the morning and maintained them in laboratory conditions in groups of 10 individuals/10 litres of original pond water during the afternoon until the experiment started. All experimental trials were conducted after dark at 2100 hours to ensure that males were active (Montori & Herrero, 2004). The three experiments were conducted in darkness at a constant temperature of 12 °C, 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 h 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<sup>2</sup> areas inside each forest), which were transferred to, and dried immediately in, a heated room (at 20 °C) 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 analysed 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 1 h 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. Hickman & Watling, 2014; Maerz et al., 2005). 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 searching for females and for new ponds. We collected males in February and conducted our experiments over a 4-day period to eliminate any effects of seasonal variation in male newt behaviour.

### Experiment 1: Male's Ability to Locate Ponds with Females

We conducted a 2 × 2 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 × 42 cm and 36 cm high, with two 1-litre plastic pools at opposite ends 15 cm apart. 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 s 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 each pool was covered with steps (1 mm high, 5 mm 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 (M. Iglesias-Carrasco, 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 (12 h 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 *Lissotriton 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: 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 anaesthetized 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 2 × 2 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: 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 2 × 2 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 an aquarium, 40 × 32 cm and 22 cm high, 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 are a common prey item (Fontanet, 1992), and are readily consumed by newts in captivity (M. Iglesias-Carrasco, 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 moment of 'capture' of the prey is very conspicuous since newts perform a sudden, distinctive lunge forwards 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 handling licence 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 72 h (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 (The R Foundation for Statistical Computing, Vienna, Austria, <http://www.r-project.org>) with  $\alpha = 0.05$ . All tests were two tailed.

## RESULTS

#### Experiment 1: Male's Ability to Locate Ponds with Females

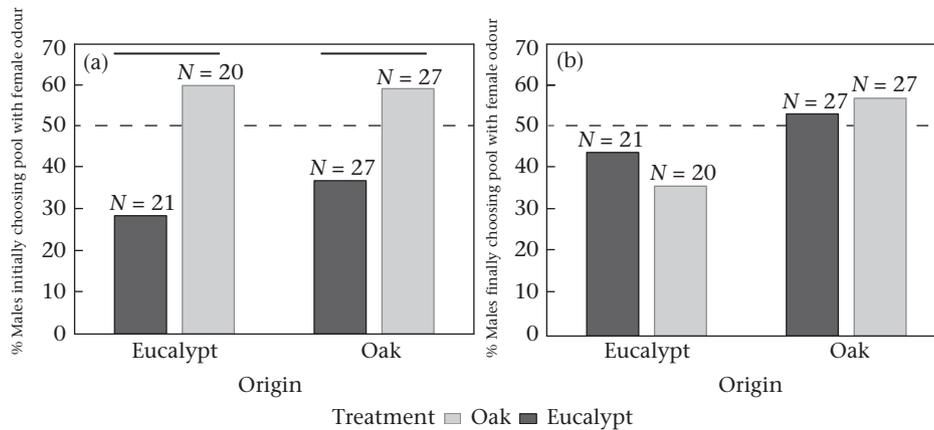
When males were tested in water with oak extract they initially chose the pool that contained female odour significantly more often than when they were tested in water with eucalypt extract (Table 1, Fig. 1a; Tukey's test:  $P = 0.011$ ). This effect did not depend on the male's collection habitat (Tukey's 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 analysed 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

**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)

Cues of female presence					
Trait	Predictors	Estimate	SE	z	P
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	<b>0.046</b>
	Collection Oak* Treatment 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* Treatment Oak	0.48	0.84	0.56	0.569

The intercept represents the estimate for males from the eucalypt collection site tested in the eucalypt water. Bold type indicates a significant effect.



**Figure 1.** Percentage of newts from different collection habitats and water treatments 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. *N* = number of males.

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  $P > 0.50$ ).

#### Experiment 2: 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 we combined 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 nonsignificant tendency to detect pools with alarm chemicals in water with either oak (binomial test:  $P = 0.13$ ) or eucalypt extract (binomial test:  $P = 0.50$ ). When pooled 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 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)

Cues of predator presence					
Trait	Predictors	Estimate	SE	<i>z</i>	<i>P</i>
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	0.370
	Collection	-1.09	1.05	-1.04	0.296
	Oak*Treatment Oak				
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	-2.28	1.13	-2.00	<b>0.045</b>
	Oak*Treatment Oak				

The intercept represents the estimate for males from the eucalypt collection site tested in the eucalypt water. Bold type indicates a significant effect.

#### Experiment 3: Male's Ability to Find Food

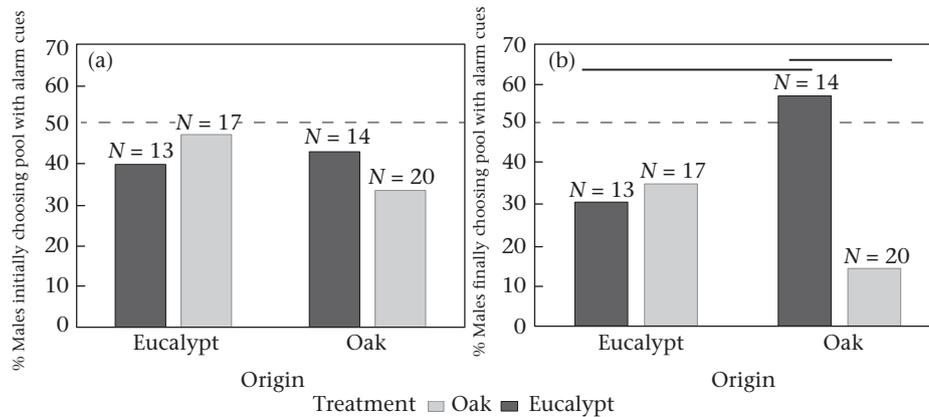
The time that newts took to locate a food item was not statistically affected by the male's habitat of collection, the water extract type or their interaction (Table 3, Fig. 3; all pairwise tests:  $P > 0.873$ ).

## 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 contained 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 had a greater tendency than males from eucalypt plantations to choose pools without predator cues when the test water contained 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 was 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 that 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 h of the experiment males



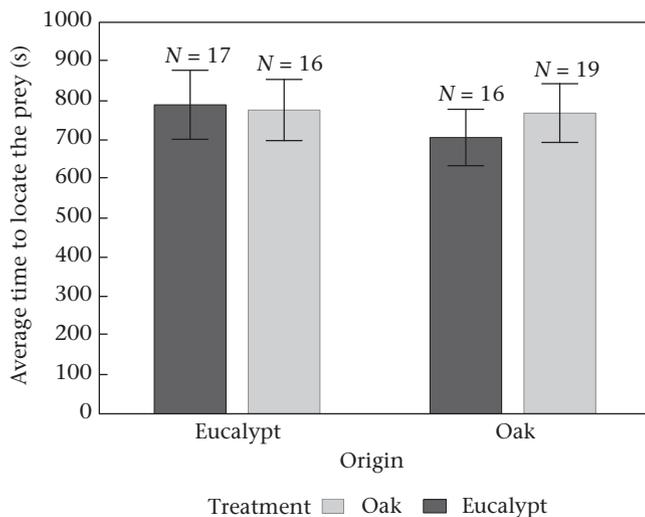
**Figure 2.** Percentage of newts from different collection habitats and water treatments that chose the pool with conspecific alarm cues as their (a) initial choice and (b) final choice. Dashed line indicates no choice (i.e. 50:50). Horizontal bars show statistically significant differences. *N* = number of males.

**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)

Predictors	Estimate	SE	<i>t</i>	<i>P</i>
Intercept	6.67	0.09	68.87	<b>&lt;0.001</b>
Collection Oak	-0.11	0.14	-0.75	0.451
Treatment Oak	-0.01	0.14	-0.11	0.905
Collection Oak* <i>Treatment Oak</i>	0.10	0.20	0.48	0.628

Bold type indicates a significant effect.



**Figure 3.** Average time (mean  $\pm$  SE) that newts from different collection habitats and water treatments took to find the prey. *N* = number of males.

changed pools several times (M. Iglesias-Carrasco, 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 not all males 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 h, 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 signalling 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, Head, Jennions, & Cabido, in press).

#### 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 for both adult male survival and that of any offspring they sire. 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 (Dixon, Munday, & Jones, 2010), have increased predation rates (Ortiz-Santaliestra, Fernández-Benítez, Marco, & Lizana, 2010) and have impaired predator detection (Lüring & Scheffer, 2007). Males from oak forests in our study might have failed to identify pools with alarm cues because leachates released by eucalypt leaves disrupted 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 of males from eucalypt plantations to detect the pool with alarm cues 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, Head, Jennions 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 induce 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, Dunham, & Harvey, 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 et al., 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 chemolocate prey.

If we extrapolate from our laboratory findings, it appears that newts in eucalypt plantations do not have poorer 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, 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 antipredator 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 programmes to fully understand how the transformation of habitats affects endangered species. We have shown that large-scale habitat transformation can affect amphibian behaviours that 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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