

# Natural toxins leached from *Eucalyptus globulus* plantations affect the development and life-history of anuran tadpoles

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## Abstract

1. Rapid human-induced transformation of habitats is presenting organisms with challenging novel environmental conditions. Nowadays, extensive areas of the world are covered by exotic plantations, and among these, eucalypt plantations stand out for their worldwide occurrence. These novel environments probably threaten the persistence of native species, with several studies reporting biodiversity losses in these transformed areas. However, the effects of such habitat alteration on life-history traits that potentially constrain the ecology of individuals have been poorly explored.
2. In a mesocosm experiment, we exposed tadpoles of three anuran species (*Rana temporaria*, *Alytes obstetricans*, and *Pelophylax perezi*) to the leachates of natural oak trees and exotic eucalypt trees to explore how the transformation of natural forests into eucalypt plantation affects the expression of behavioural and morphological traits and immune response.
3. While anti-predatory responses appeared not to be impacted by leachate type, tadpoles exposed to eucalypt leachates differed in morphology and growth, and exhibited weaker immune responses and smaller size at metamorphosis than those exposed to oak leachates. Some of the responses were also species-specific, suggesting that some species are likely to be more sensitive than others to novel chemicals.
4. We conclude that the poor quality of eucalypt leaf litter (e.g., its low nutrient content), low amount of food (e.g., periphyton), and the presence of toxic detritus and leachates can have important stress-related consequences for the critical early stages of development in anurans.

## KEYWORDS

*Alytes obstetricans*, anthropic habitats, *Pelophylax perezi*, PHA assay, *Rana temporaria*

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## 1 | INTRODUCTION

Vast areas of the world's native forests have been converted to exotic tree plantations. Plantations have a higher density, but lower diversity of trees than natural forests (Lindenmayer & Hobbs, 2004), which has in turn been associated with reductions in the number of species of several animal groups, such as arthropods, reptiles, amphibians, and birds (Faruk et al., 2013; Fork et al., 2015; Glor et al., 2001; Zurita et al., 2006). However, research on the replacement of natural forests with exotic tree plantations has focused primarily on patterns of species abundance and diversity of different taxonomic groups. There still is only a limited understanding of the impact of such habitat transformations on the ecology of resident fauna (but see Iglesias-Carrasco et al., 2016, 2017; Remeš, 2003).

One way in which exotic plantations can affect the ecology of native fauna is by modifying the chemistry of terrestrial and the aquatic environments via the release of chemical compounds (Cohen et al., 2012; Maerz et al., 2005). Plant secondary compounds can be toxic to the native fauna (Canhoto & Laranjeira, 2007), especially when the chemical composition of introduced plants differs from that of native plants (Cohen et al., 2012), and can have important consequences on the life-history of species that occupy those habitats. While some research effort has started to focus on the impact of secondary compounds from invasive plant species on native fauna (e.g., effects on growth, Maerz et al., 2005; survival, Earl et al., 2012; predator-prey interactions, Hickman & Watling, 2014), our general understanding of how animal life-history traits are affected by these chemicals remains poor, especially in the context of the widespread habitat transformations that occur with the establishment of plantations.

Native wildlife remaining in plantations also can be affected by the altered quantity and quality of resources available, which can in turn vary the phenotypic responses (e.g., development of morphological traits) of resident animals, particularly those in pools and other water bodies where litter can tend to accumulate (e.g., Stoler & Relyea, 2013). For instance, the quality of the detritus as a food resource for detritivore animals in exotic pine plantations has been shown to be lower than in the natural deciduous forests (Martínez et al., 2016). This is because the litter produced in some of these plantations is mechanically harder to break down, offers poorer nutrient content, and contains a higher concentration of toxic compounds than native litter. In metazoans, the expression of several life-history traits and behaviours is widely understood to depend on the condition of individuals (*sensu* Rowe & Houle, 1996; e.g., Tomkins et al., 2004), which to some extent depends on the quality of the environment (e.g., Greenfield & Rodriguez, 2004). For example, poor environmental conditions can detrimentally affect the energetic state or condition of individuals that, in turn, dictate the strength of anti-predator behaviours (Fraker, 2008). Therefore, low-quality food resources in water bodies within plantations might impact the expression of life-history traits (e.g., morphological structures, physiological maintenance, immune response) or other fitness-related behaviours (e.g., anti-predator responses), compromising the survival and fitness of individuals in these exotic environments.

Eucalypt plantations stand out for their worldwide occurrence. Previous studies have found that eucalypt-infused water disrupts intra- and inter-specific communication, and reduces the immune response in adults of a species of newt that has come into contact with eucalyptus plant leachates within the last century (Iglesias-Carrasco et al., 2017a). Interestingly, even species that naturally occur in eucalypt forests are affected detrimentally by the toxic leachates found in these habitats (Morrongiello et al., 2013). Yet, despite the extent of eucalypt plantations worldwide and evidence that eucalypt leachate has adverse effects on resident fauna, there have been few experimental studies of how these toxins impact traits directly linked to survival of individuals (e.g., anti-predatory responses or immune response; but see Burraco et al., 2018). The area covered by eucalypt plantations is increasing all around the world. This increase is especially worrying since populations of several species are likely to have their first exposure to eucalypts in the coming years. Therefore, exploring the effects of novel eucalypt leachates is critical for understanding the short-term responses of amphibians to such environmental change.

Amphibians have been drastically affected by habitat loss globally. The chemical environment in forest ephemeral ponds, which support a high diversity and abundance of amphibians, depends in part on the leaf litter released by the surrounding forest, and usually differs from ponds found in exotic plantations. Small changes in the chemical characteristics of aquatic habitats, as well as surrounding terrestrial areas (depending on the life-cycle phase) can have major effects on amphibian survival and breeding success (Watling, Hickman, Lee, et al., 2011; Watling, Hickman, Orrock, 2011). As a consequence of their reduced potential for migration, amphibians are likely to rely on behavioural shifts or physiological adjustments to cope with adverse environmental change, making this animal group well-suited for the study of environmental alterations on metazoan fauna.

In this study, we examined the impact of the leaf litter of common exotic eucalypt trees (*Eucalyptus globulus* Labill., 1800) on amphibian life history and development. Under experimental conditions, we compared the strength of anti-predatory responses, morphological responses, growth, immune response, and size at metamorphosis of tadpoles reared in water containing leachates and leaf litter from native oak trees (*Quercus robur* L.) to those reared in the presence of toxic leachates and leaf litter from exotic eucalypt trees. In order to understand the generality and ecological significance of any effects recorded, we performed the experiment on three native pond-dwelling anuran species with different ecological requirements: the forest common frog, *Rana temporaria* (Linnaeus, 1758), the common midwife toad, *Alytes obstetricans* (Laurenti, 1768), and the Iberian green frog, *Pelophylax perezi* (Lopez-Seoane, 1885). All three species are found in native oak forests, with at least two also occurring in eucalypt plantations (see Appendix S1). We predicted that toxic leachates and the low food quality (e.g., low nutrient, hardness; Graça et al., 2002) of eucalypt detritus in ponds will lead to weaker anti-predatory responses, altered body morphology, reduced growth, a constrained immune response, and smaller metamorphic size of anurans compared to those residing in ponds littered with native oak.

We also predicted that the strength of these effects would differ between the three species studied because of their differences in general ecology and presence in eucalypt plantations (Appendix S1).

## 2 | METHODS

### 2.1 | Study species, capture, and maintenance of animals

In the study area, oak forests are the main type of forest habitat where the three species are found. Despite oak leaves having higher concentrations of lignin than eucalypts, eucalypts have a higher susceptibility of leaching this compound, and the amount of water-soluble compounds more generally is greater than those found in deciduous leaves that these amphibians encounter natively (Canhoto & Laranjeira, 2007). Eucalypt leaves are high in phenolic and tannin content, but also in essential oils (Canhoto & Graça, 1999). These characteristics of the leaf litter from eucalypts have been suggested to be detrimental for the native fauna in places where eucalypts have been established recently (Iglesias-Carrasco et al., 2016; Larrañaga et al., 2009), and to be responsible for altering the quality and quantity of aquatic resources (Molinero & Pozo, 2004). *Eucalyptus globulus* plantations were established in the study area approximately 50 years ago, and the surface covered by eucalypt plantations is increasing rapidly in the area.

During the breeding season of each species (January for *R. temporaria*, May for *P. perezi* and *A. obstetricans*) we collected three clutches from ponds in a native deciduous oak forest in Basque Country, North Spain. Consequently, tadpoles of all three species were naïve to pond conditions experienced in eucalypt plantations. This was deliberate because we were specifically interested in understanding how the future expansion of eucalypt monocultures in the area would impact these species. We placed each clutch into 4-L plastic tanks (one clutch/tank) filled with dechlorinated tap water (c. 12 h:12 h, light:dark photoperiod and 8°C for *R. temporaria* and 14 h:10 h, light:dark photoperiod and 15°C for *P. perezi* and *A. obstetricans*—mimicking the temperature in the ponds when collected). We moved hatchlings in groups of 20 individuals to new tanks filled with 4 L clean 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 water until they reached Gosner stage 25.

In order to simulate natural temperature and photoperiod we used outdoor 470-L mesocosms containing 200 L aged tap water. A fixed weight (100 g) of dried leaves (see Iglesias-Carrasco et al., 2016; Iglesias-Carrasco, Head, Jennions, Martín, et al., 2017 for details on doses) of either oak (*Q. robur*) or eucalypt (*E. globulus*) then were placed in each of the six outdoor mesocosms for each species (three mesocosms per leaf and anuran species). We used the litter of a single individual tree species (oak or eucalypt) instead of mixed litter (e.g., Martín et al., 2015) because of the ecological relevance of this

manipulation for the study system. Specifically, eucalypt plantations are monocultures where almost no other tree species are present, so the litter in ponds within these monocultures usually are composed of eucalypt leaves only. Likewise, the litter in the pond where the clutches were collected was composed predominantly of oak leaves with very low amounts of herbaceous species (personal observation; NB: in other locations outside the tadpole collection sites, the forest is a mix of oak, beech, and some shrubs such as hawthorn).

We collected both oak and eucalypt leaves that recently had fallen to the ground and hence corresponded to the top-most layer of litter in small 20 m<sup>2</sup> patches within each forest. Eucalypt trees drop leaves all year around and leach secondary compounds into surrounding water bodies. Leaves were dried immediately at room temperature (20°C) for 72 hr. We also added 5 L natural pond water to each mesocosm, collected from the field locations, in order to seed mesocosms with zooplankton and algae at the beginning of the experiment to provide a food source for tadpoles. The mesocosms were covered with mesh to prevent the entry of predators including dragonfly nymphs, which later were used to test anti-predator responses (see below). After 48 hr we introduced 80 tadpoles to each mesocosm. Tadpoles were supplementarily fed with a small amount of rabbit chow every third day (3 g), since we were unsure that the eucalypt detritus and seeded pond water was sufficiently nutrient-rich to fully sustain the tadpoles. The amount supplemented was based on the lowest supplement of food used in a previous study (0.001 g/day per individual; Maerz et al., 2005). As the amount was intentionally very low, it is unlikely that the food provided had any effects on our results, and was otherwise consistent across all mesocosms and treatments. Tadpoles also were observed to feed primarily on the leaves and algae found in the mesocosms, which subsequently provided the main resource of food during the experiment. Twenty-one days after the start of the experiment, we measured the pH, turbidity, and temperature of mesocosms, the values of which were very similar between treatments (see Table S1).

The 80 tadpoles placed in each replicate mesocosm from each litter treatment and for each species were later allocated to the following trait measurements (in chronological order): 12 for anti-predatory behaviour, 20 for morphology at Day (D)22, 15 for immune response, morphology, and growth at Gosner stage 31 (same individuals for the three traits), and the rest (*R. temporaria*: 10–21 per replicate; *P. perezi*: 12–32 per replicate) for metamorphic size. Tadpoles were not returned to the mesocosms, and after the data collection for each trait the pool of tadpoles was euthanized in MS-222.

### 2.2 | Effect of leachate treatment on tadpole anti-predatory responses

We used the odour of a natural predator, the nymph of *Aeshna* sp. dragonflies, to test anti-predator responses in tadpoles raised in the presence of oak and eucalypt leachates (following Relyea, 2001). We obtained the odour for our predator stimulus by maintaining six dragonfly nymphs in an aquaria containing 3 L water (dilution 0.5 L

per individual, mirroring those concentrations of predators found in the ponds where we collected the clutches; Iglesias-Carrasco et al. unpublished) for 48 hr. Dragonflies were not fed during this period of time since anti-predatory responses are known to differ between tadpoles exposed to predator kairomones alone, conspecific alarm cues, or a mix of predator kairomones and conspecific alarm cues (e.g., Hettyey et al., 2015). We confirmed that dragonfly faeces were not present. Responses to predators alone are usually weaker than those to other cues or mixes (Hettyey et al., 2015). Therefore, our design is conservative in that we can assume that if tadpoles reared in eucalypt-infused water responded to predators alone, they would probably do so when exposed to the other cues as well. Inside the aquarium, dragonfly nymphs were individually housed in fish farrows to avoid cannibalism, while allowing water and odour flow.

On D21 of the experiment, we randomly selected 12 tadpoles from each mesocosm and placed them in individual 1-L round tanks (13 cm diameter × 16 cm depth) filled with water. This water had been maintained for 4 days in an unused outdoor mesocosm covered with mesh (to exclude predators) to ensure that tests were conducted under the same temperature conditions in which tadpoles were maintained (i.e., to avoid thermal shocks resulting from a difference in water temperature between the mesocosms and test tanks that might alter behaviour). Behavioural trials were performed in untreated water to ensure that any effects measured were due to long-term (21 day) leachate exposure, rather than current leachate presence. The test tanks used were opaque in order to prevent adjacent individuals from seeing and influencing each other's behaviour. After a 2 hr acclimation period, we tested the response of tadpoles to a predation threat by measuring activity levels in a 10-min period before and after exposure to the predator stimulus. The stimulus—consisting of a mix of 5 ml predator cue + 5 ml water—was injected slowly down the side wall of the aquarium to minimize disturbance. All trials then were filmed, with each recording lasting 22 min (11 min pre-stimulus and 11 min post-stimulus). In the subsequent analysis of videos, we discarded the first minute after switching on the camera to allow tadpole behaviour to return to normal following our presence and the first minute after the injection of the stimulus to allow for diffusion of the cue through the tank water. From the video we recorded two variables related to activity level: (1) the time spent moving (s) and (2) the distance covered. The tank was divided into four equal size sections by two perpendicular lines. The distance covered was estimated in terms of number of line crosses made by the tadpole (Rohr & Madison, 2001). We considered that a tadpole crossed a line when its entire body moved completely over the line to the other side.

### 2.3 | Effect of leachate treatment on tadpole morphology and growth

Morphological traits of tadpoles reared in the presence of oak and eucalypt leaf litters were measured at two separate time points within the experiment. On D22, and again just before metamorphosis when tadpoles reached Gosner stage 31 (Gosner, 1960), 15–20 tadpoles

were selected randomly from each mesocosm, laterally photographed, and weighed. From these photographs, we obtained measures of larval tail fin length, tail fin depth, and tadpole total length using image analysis software (ImageJ v1.47, Abramoff et al., 2004). We measured these morphological traits at two separate time points in order to be able to determine whether early morphological differences persisted (or potentially increased) over exposure time. However, the analysis of growth (measured as total length) was performed only in the later Gosner stage 31 in order to explore for the long-term effect of leachate exposure. Characterization of tadpole morphology was based on linear measurements of individual body parts as opposed to the alternative of landmark-based geometric measurements (following Stoler & Relyea, 2013).

Since the relative shape of the tail fin is known to be a sign of environmental stress in tadpoles (Maher et al., 2013; Relyea & Hoverman, 2003; Richter-Boix et al., 2006), we examined the effect of leachate treatment on two specific morphological ratios likely to have been influenced by environmental stress: (1) tail fin depth as a proportion of total body length and (2) the ratio of tail length to total body length.

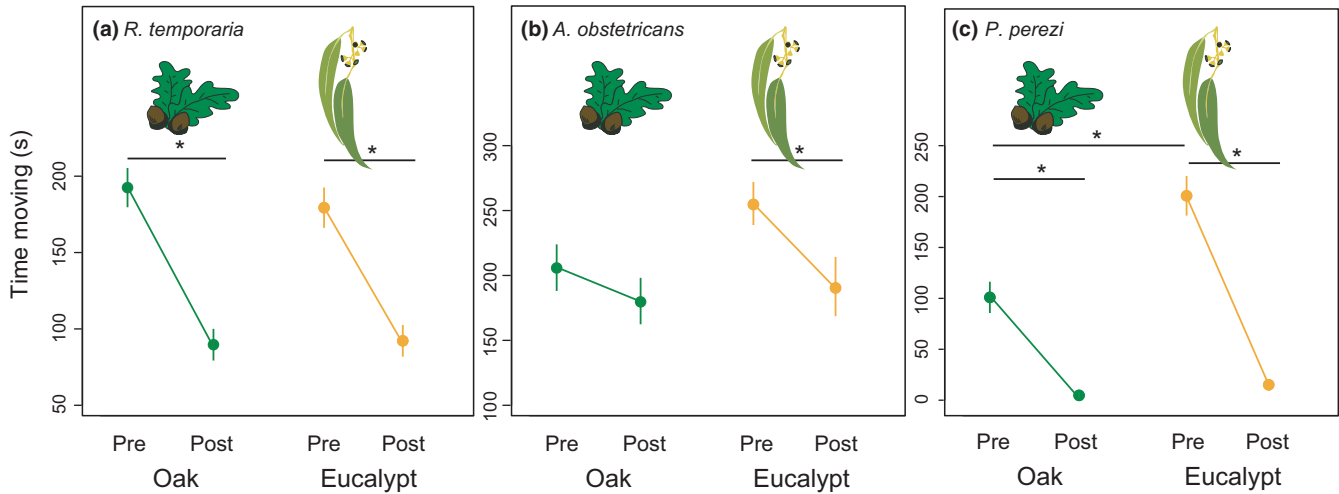
### 2.4 | Effect of leachate treatment on tadpole immune response

We measured the immune response of tadpoles as a downstream measure of stress, since chronically stressed individuals are known to show reduced immune responses (Iglesias-Carrasco et al., 2019; Martin, 2009). When tadpoles reached Gosner stage 31 (Gosner, 1960), 15 individuals were selected at random from each mesocosm and placed in individual opaque aquaria containing 1 L water (these same individuals also were used to obtain the pre-metamorphosis morphological data—see above). To estimate the physiological condition of the tadpoles we used the phytohaemagglutinin injection assay (PHA test). This assay measures T-cell-dependent immunocompetence in vivo (Lochmiller et al., 1993) and has been used successfully in many vertebrate species, including amphibians (Brown et al., 2011; Iglesias-Carrasco et al., 2016; see Appendix S1 for details on the assay).

### 2.5 | Effect of leachate treatment on the size at metamorphosis

After measuring the immune response, mesocosms were checked daily and all of the tadpoles with four visible legs (a total of 10–21 per mesocosm replicate for *R. temporaria* and 12–32 for *P. perezii*) were moved from the mesocosms to individual plastic containers with a small amount of water. Following the day of complete resorption of the tail, froglets were anaesthetized in MS-222 and photographed with the abdomen facing upwards against a millimetric paper to measure the snout-vent-length (SVL) with ImageJ.

All data were recorded blind to the water treatment of tadpoles.



**FIGURE 1** Behavioural response (measured as time spent moving) of tadpoles of (a) *R. temporaria*, (b) *A. obstetricans*, and (c) *P. perezii* raised in water containing either natural oak or non-native eucalypt leaf litter. Time spent moving is shown as mean  $\pm$  SE and presented pre- and post-exposure to predator odour cue. Asterisks indicate significant differences (all  $p < 0.001$ ) based on Tukey's pairwise comparison tests

## 2.6 | Data analysis

In order to test for the effect of leachate treatment on the anti-predatory response of tadpoles, we ran separate linear mixed models (LMM) for each of the three species. Since the time moving and the number of lines crossed (distance) were strongly correlated in all of the species (*R. temporaria*:  $R^2 = 82.7$ ; *A. obstetricans*:  $R^2 = 81.2$ ; *P. perezii*:  $R^2 = 88.4$ ; all  $p < 0.001$ ) and the results of the models were similar, we focused on the results for the differences in time spent moving before and after the exposure to the predator odour (hereafter "activity level"; see Tables S2 and S3 for results based on distance moved). In our models, we included the water treatment, the time (pre-, post-stimulus) and the two-way interaction between them as fixed factors. We included mesocosm ID and individual ID as random effects to control for group and individual variation in the activity level, respectively.

We ran three separate LMMs for each species, one for each of the morphological scores (i.e., relative tail fin depth and tail length), and another for the total body size as proxy of growth as the response variables, leaf litter treatment as a fixed factor and mesocosm ID as a random effect.

In order to test for the effect of leachate treatment on the immune response of tadpoles, we again ran a LMM for each of the three study species. In these models leachate treatment was included as a fixed factor and mesocosm ID as a random effect.

Often *A. obstetricans* tadpoles overwinter in the water, so the number of froglets obtained for this species in the year of the experiment was low ( $N = 28$ , only seven on eucalypt). For this reason, we discarded this species from the analysis of size at metamorphosis. For the other two species, we ran LMMs again, including SVL as response variable, leaf litter treatment as a fixed factor, and mesocosm ID as a random effect. We did not include the time to metamorphosis as covariate since there was no effect of the leachate type on that trait (all  $p$ -values  $> 0.133$ ).

For all models, where interactions were not significant we re-ran the model without interactions. If removal of interactions did not significantly reduce the model fit, we interpreted the main effects from the reduced model. We checked the residuals of all models to ensure that they met the assumption of normality, and when necessary data were log-transformed (tail depth in *R. temporaria*, PHA in *A. obstetricans* and growth in *P. perezii*) or power-transformed (growth and PHA in *R. temporaria*, and tail length in *P. perezii*). All models were run in R3.2.2 (R Core Team, 2015) using the lme4 package (Bates et al., 2015).

## 3 | RESULTS

### 3.1 | Effect of leachate treatment on tadpole anti-predator responses

Tadpoles of *R. temporaria* and *A. obstetricans* showed similar anti-predator responses when presented with the odour of a predator. For both of these species, there was no difference in the activity level between tadpoles reared in the presence of oak leaf litter and those reared in eucalypt leaf litter (treatment: *R. temporaria*,  $\chi^2_1 = 0.190$ ,  $p = 0.663$ , Figure 1a; *A. obstetricans*,  $\chi^2_1 = 0.176$ ,  $p = 0.185$ , Figure 1b). Independent of the leachate treatment, tadpoles of both species significantly reduced their activity level after exposure to the predator odour (time: *R. temporaria*,  $\chi^2_1 = 143.035$ ,  $p < 0.001$ ; *A. obstetricans*,  $\chi^2_1 = 12.524$ ,  $p < 0.001$ ). There was no two-way interaction between leachate treatment and time (treatment\*time: *R. temporaria*,  $\chi^2_1 = 0.971$ ,  $p = 0.325$ ; *A. obstetricans*,  $\chi^2_1 = 2.250$ ,  $p = 0.134$ ), meaning that both species of tadpole showed a similar reduction in activity levels post-stimulus when reared in the presence of oak or eucalypt leachates. Parameter estimates for these models are given in Table S2.



By contrast, for *P. perezii* there was a significant effect of leachate treatment on activity level (treatment:  $\chi^2_1 = 8.823$ ,  $p = 0.003$ , Figure 1c). However, this was not due to a different response to the predator odour (Tukey's pairwise comparison  $p = 0.158$ ), but rather, to a higher basal activity (time spent moving before exposure to the predator cue) of tadpoles reared in eucalypt leachates compared to those reared in oak (pairwise comparison test  $p = 0.036$ ). Just as for *R. temporaria* and *A. obstetricans*, we found that *P. perezii* tadpoles (independent of water treatment) reduced their activity level after exposure to the predator odour (time:  $\chi^2_1 = 263.411$ ,  $p < 0.001$ ). Once again we found no interaction between leachate treatment and time (pre- or post-predator stimulus), suggesting that the reduction in activity level was similar for tadpoles from mesocosms containing both oak or eucalypt leaf litter (treatment\*time:  $\chi^2_1 = 0.293$ ,  $p = 0.588$ ).

### 3.2 | Effect of leachate treatment on tadpole growth and morphology

At D21 after first exposure, *A. obstetricans* tadpoles exposed to eucalypt leachates had significantly shorter tails (relative to body size) than those exposed to oak leachates (tail length/total length:  $\chi^2_1 = 5.392$ ,  $p = 0.020$ , Figure 2g). However, these differences were no longer statistically significant at the pre-metamorphosis stage (Gosner stage 31 tail length/total length:  $\chi^2_1 = 2.797$ ,  $p = 0.094$ , Figure 2h) and no effect of leachate treatment was observed in the relative fin depths of these tadpoles either at D21 exposure (fin depth/total length:  $\chi^2_1 = 0.172$ ,  $p = 0.678$ , Figure 2e) or at pre-metamorphosis (Gosner stage 31 fin depth/total length:  $\chi^2_1 = 1.817$ ,  $p = 0.178$ , Figure 2f). *Rana temporaria* tadpoles showed no effect of leachate treatment on relative tail size (size corrected tail length and relative fin depth variables) either at D21 exposure (fin depth/total length:  $\chi^2_1 = 1.824$ ,  $p = 0.177$ ; tail length/total length:  $\chi^2_1 = 0.812$ ,  $p = 0.367$ , Figure 2a,c) or at Gosner stage 31 (fin depth/total length:  $\chi^2_1 = 3.357$ ,  $p = 0.067$ ; tail length/total length:  $\chi^2_1 = 0.631$ ,  $p = 0.427$ , Figure 2b,d Tables S4 and S5).

In the case of *P. perezii*, tadpoles at D21 after first exposure to eucalypt leachates had significantly larger tails (tail length/total length:  $\chi^2_1 = 41.773$ ,  $p < 0.001$ , Figure 2k) but narrower fins (fin depth/total length:  $\chi^2_1 = 14.902$ ,  $p < 0.001$ , Figure 2i) than those exposed to oak leachates. The same differences were observed in tadpoles at Gosner stage 31 (tail length/total length:  $\chi^2_1 = 10.326$ ,  $p = 0.001$ ; fin depth/total length:  $\chi^2_1 = 5.484$ ,  $p = 0.019$ , Figure 2j,l, Table S6).

We found that tadpoles of *A. obstetricans* exposed to eucalypt leachates were significantly smaller at the pre-metamorphosis stage (Gosner 31) than those exposed to oak leachates ( $\chi^2_1 = 44.443$ ,  $p < 0.001$ , Table S7, Figure 3c). We did not find any effect of the type of leachates in the body size of either *R. temporaria* ( $\chi^2_1 = 0.169$ ,  $p = 0.678$ , Figure 3a) or *P. perezii* ( $\chi^2_1 = 0.761$ ,  $p = 0.383$ , Figure 3b).

### 3.3 | Effect of leachate treatment on tadpole immune response

We found a strong reduction in the immune response of all three study species after the exposure to eucalypt leachates (Table S8). The average immune response of *R. temporaria* tadpoles exposed to eucalypt leachates was 71.6% lower than the response displayed by tadpoles exposed to oak leachates ( $\chi^2_1 = 35.91$ ,  $p < 0.001$ , Figure 4a), with a 73.5% lower response observed in *A. obstetricans* tadpoles ( $\chi^2_1 = 64.56$ ,  $p < 0.001$ , Figure 4b) and a 48.8% lower response observed in *P. perezii* ( $\chi^2_1 = 37.33$ ,  $p < 0.001$ , Figure 4c).

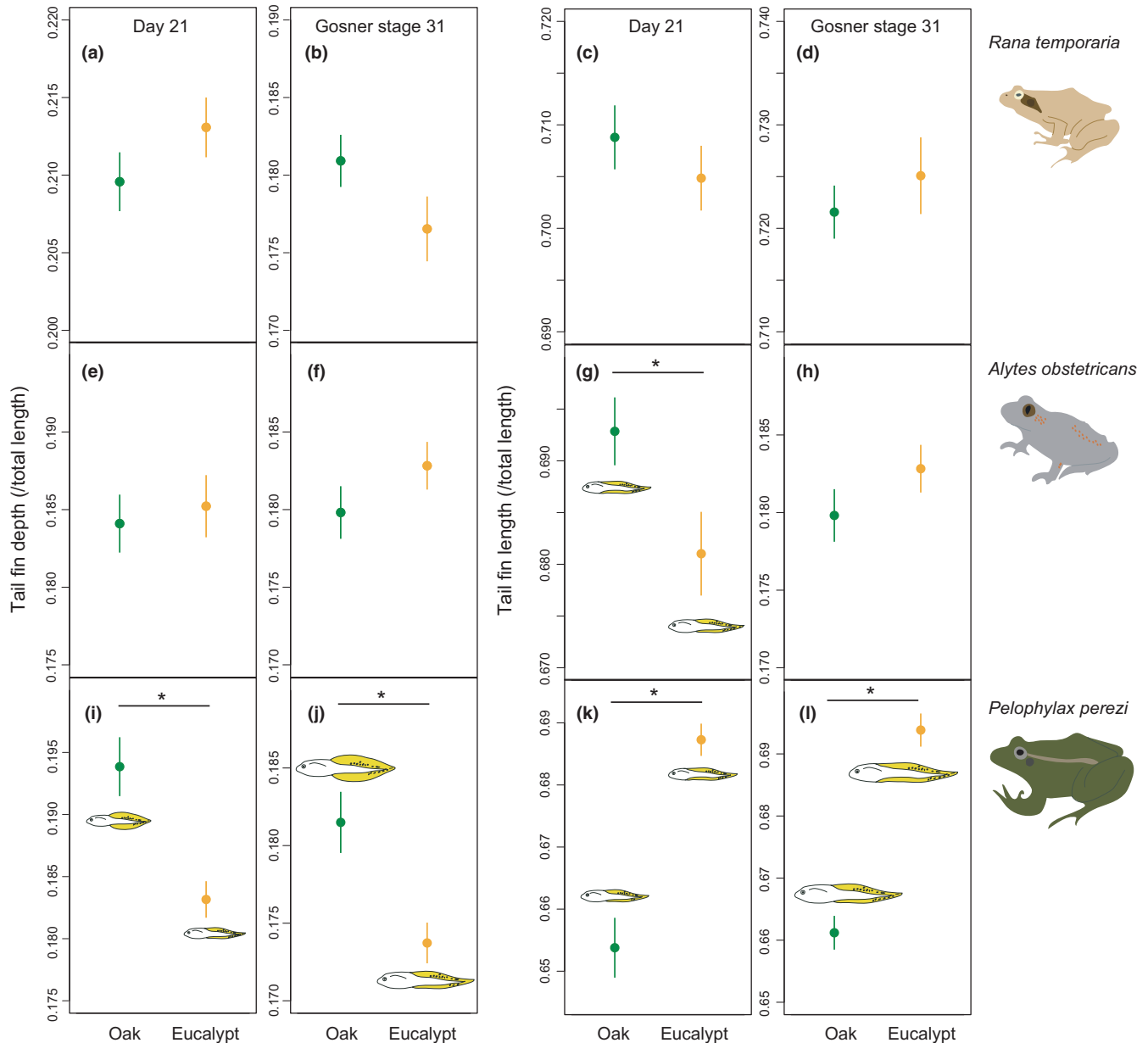
### 3.4 | Effect of leachate treatment on the size at metamorphosis

We found that individuals of *P. perezii* reared in water containing eucalypt leachates metamorphosed at significantly smaller size than those reared in water containing oak leachates ( $\chi^2_1 = 31.676$ ,  $p < 0.001$ , Figure 3e, Table S9). We did not find any effect of the leachate treatment in the size of metamorphosis of *R. temporaria* ( $\chi^2_1 = 0.002$ ,  $p = 0.961$ , Figure 3d).

## 4 | DISCUSSION

The effects of the presence of plant detritus and leachates not previously encountered during the evolutionary history of native species can have considerable consequences on the life history of those fauna. In our study, these compounds exhibited their effects through significant changes in the early life-history stages of amphibians, with these changes tending to be species-specific. Our data suggest that plantations of *E. globulus*, or at least their leachates, might provide a stressful environment for anurans, and one that induces changes in tadpole development and reduces immune response. These effects could have important cascading effects if they translate into loss of fitness in later stages.

Organisms exposed to chemically altered environments have previously been shown to fail to respond adaptively to predation cues (Dixon et al., 2010; Iglesias-Carrasco, Head, Jennions, Martin, et al., 2017). In the case of eucalypt leachates, previous research suggests that prolonged exposure to toxins has the potential to affect amphibian neurological pathways or sensory apparatus, and affect female mate preferences in newts (Iglesias-Carrasco, Head, Jennions, & Cabido, 2017). In addition, eucalypt leaf litter could reduce the condition of individuals, increasing the need to acquire more resources, and hence weakening their anti-predatory responses in order to obtain those resources. Consistent with this, we found that before the addition of the predator odour, tadpoles of *P. perezii* showed higher basal activity in eucalypt-infused water than in oak-infused water. This might indicate that tadpoles exposed to plantations of *E. globulus* need to maintain higher levels of activity to



**FIGURE 2** Effect of leachate treatment (native: oak vs. non-native: eucalypt) on the size-corrected tail fin depth and length of tadpoles (mean  $\pm$  SE) at Day 21 after first exposure and when tadpoles reached Gosner Stage 31. Asterisks indicate significant differences. Figure represents raw data, although some variables were transformed for the models

keep feeding and acquire resources in order to metamorphose with the largest size possible. However, we found that, independent of the water treatment, tadpoles of all three species responded to predator cues with a similar reduction in their activity level. Our findings therefore indicate two things. Firstly, in contrast to other pollutants (reviewed in Lüring & Scheffer, 2007), leachates of *E. globulus* do not act as information disruptors since tadpoles are able to react to predators. Secondly, at least in the mesocosm conditions where some extra food was added, the benefits of hiding outweigh the potential costs of losing foraging opportunities when a predator is present. Our experiment also shows that low predation risk—associated with the odour of predators alone (Hettley et al., 2015)—is sufficient to promote an adaptive anti-predatory response after the exposure

to water of exotic plantations. Our results contrast with a previous study that showed that tadpoles housed in eucalypt-infused water showed greater risk-prone behaviour than when housed in oak-infused water, even when predation risk was supposed to be highest as a result of dragonflies feeding on conspecifics (Burraco et al., 2018). Therefore, by only testing tadpoles in tap water with no presence of leachates during the trials, we might have underestimated the negative effect of eucalypt leachates on total tadpole anti-predator behaviour.

The effects of toxic leachates released by eucalypt plantations might be similar to those of other invasive plants on amphibians, which include changes in foraging behaviour, inability to recognize conspecifics, and increased mortality (Hickman & Watling, 2014;

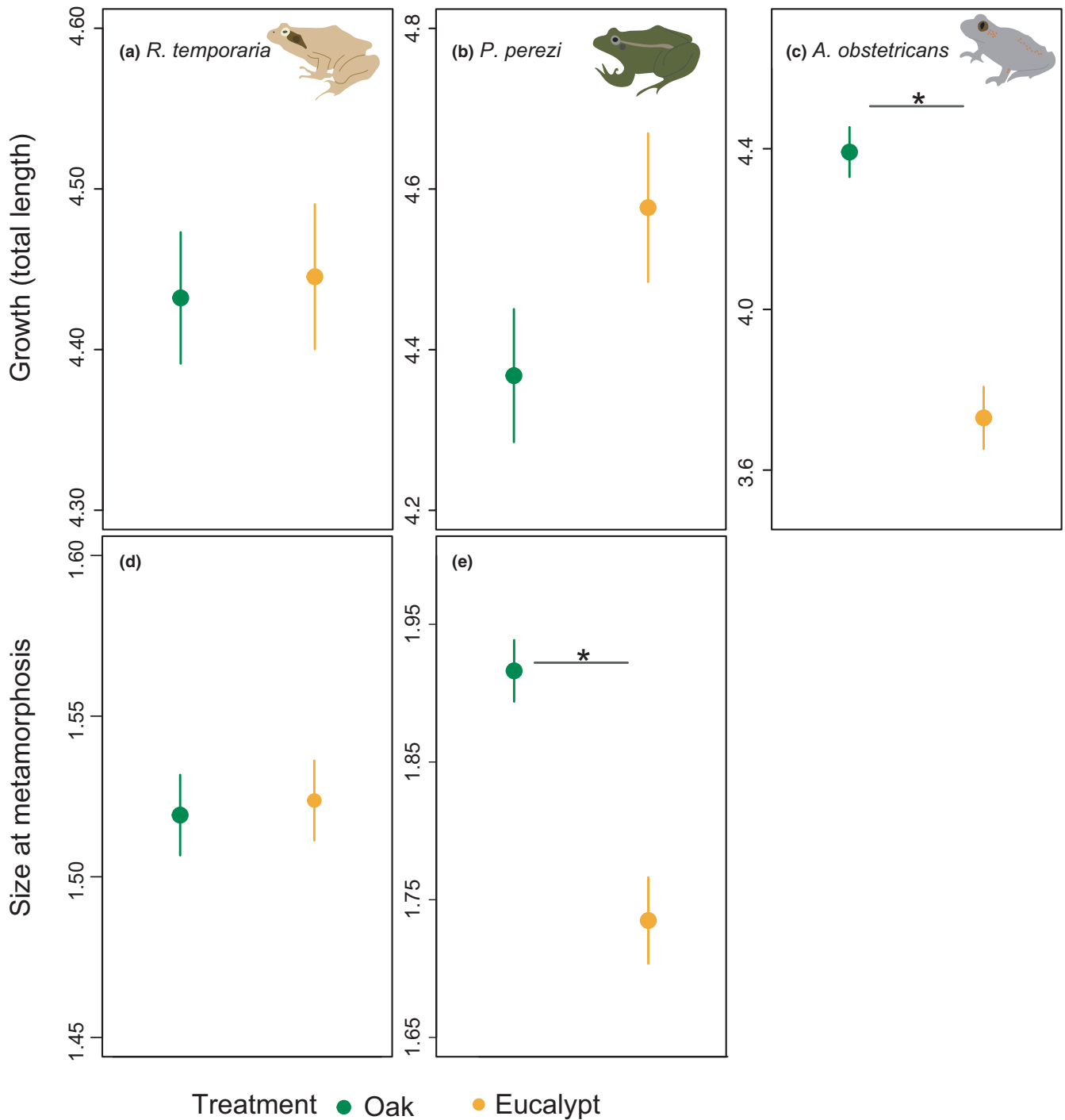
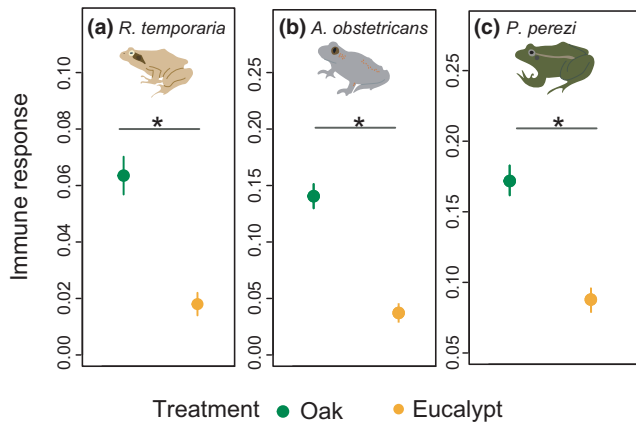


FIGURE 3 Effect of leachate treatment on growth of tadpoles of (a) *R. temporaria*, (b) *P. perezi*, and (c) *A. obstetricans*, and the size at metamorphosis (cm) of (d) *R. temporaria* and (e) *P. perezi*. Data shown are means  $\pm$  SE. Asterisks indicate significant differences. Figure represents raw data, although some variables were transformed for the models

Iglesias-Carrasco, Head, Jennions, Martín, et al., 2017; Watling, Hickman, Lee, et al., 2011). In tadpoles, the exposure to certain chemicals has some additional consequences, such as changes in morphology (Relyea, 2012). The morphological changes observed in *P. perezi* after the exposure to eucalypt leachates might be the result of chemical components shared with other types of litter (e.g., nitrogen, carbon, lignin content; Maerz et al., 2005; Martin

et al., 2015; Stoler & Relyea, 2013), but are otherwise different to those found in native oak litter. Alternatively, the morphology shown by tadpoles of *P. perezi* (long but narrow tails relative to their body size) could reflect the high toxicity of eucalypt water, which generally is consistent with the stress response of other amphibian species (Maher et al., 2013). Narrow tail fins have been associated with impaired swimming speed and ability to escape predators in





**FIGURE 4** Effect of leachate treatment on the immune response (PHA assay) of tadpoles of *R. temporaria*, *A. obstetricans*, and *P. perezi* measured before metamorphosis (Gosner stage 31). Data shown are means  $\pm$  SE. Figure represents raw data, although some variables were transformed for the models

the aquatic environment, and reduced jumping capacity of froglets after metamorphosis (e.g., Johansson et al., 2010). Although we did not measure jumping performance, one possibility is that *P. perezi* inhabiting eucalypt plantations might suffer detrimental fitness effects both during the aquatic larval and the terrestrial stages. However, neither *R. temporaria* nor *A. obstetricans* showed any morphological response to eucalypt-infused water. There are a variety of reasons for why this might be the case: (i) eucalypt is not stressful to these species (but see below), (ii) the morphological changes observed in *P. perezi* were a unique (species-specific) plastic response to the novel conditions experienced in eucalypt-infused water, and (iii) eucalypt is stressful to these species, but these anurans instead allocate resources to maintain their morphology at the expense of other traits. Given the strong effects of eucalypt water in the immune response of both species, and the effect on growth (see below) for *A. obstetricans*, the latter explanation of a potential trade-off seems like the most relevant. For example, *A. obstetricans* invests resources in maintaining a morphologically optimal shape for avoiding predators at the expense of gross body size and immunity. However, confirming that this is the case and the extent to which the allocation of resources to different traits has tangible fitness consequences, requires further research.

We found reduced growth of tadpoles of *A. obstetricans*, and a smaller size at metamorphosis of *P. perezi* exposed to leachates of *E. globulus*. These findings support the idea that eucalypt-infused water is a low-quality environment for these species. The reduced growth of eucalypt-exposed tadpoles might be the result of lower metabolic rates (Burraco et al., 2018), low-quality and scant nutrition (Canhoto & Graça, 1999; Molinero & Pozo, 2004), or a corticosterone-mediated stress response to the toxicity or potential oxygen ( $O_2$ ) reduction of the water (Denver, 2009). Since the size at metamorphosis largely determines survival of amphibians at later stages (Cabrera-Guzmán et al., 2013), our findings on the reduced size of froglets of *P. perezi* suggests that juvenile survival

might be lower in exotic plantations. However, as in the case of gross morphology, growth was not affected equally in all species. These differences might reflect variation in the respiratory capacity and ability to cope with potential reduction in oxygen levels in eucalypt-infused water (Canhoto & Laranjeira, 2007). For instance, *R. temporaria* is adapted to breed in small, ephemeral ponds that are likely to have lower levels of  $O_2$ , which might explain why this species seems to be the least affected by our treatment. In addition, the ecology and evolutionary history of each species can also affect their responses to novel conditions. For example, *P. perezi* and *A. obstetricans* usually breed in large ponds and have long larval stage periods. This might lead to tadpoles readily reacting to the environmental cues present in the pond, while species like *R. temporaria*, that breed in small ephemeral ponds threatened with rapid desiccation, invest less in potentially costly responses (i.e., altering the phenotype or growth) to avoid delaying the larval period. Alternatively, the forest specialist *R. temporaria* might be adapted to inhabit a wide variety of forests with different tree species that differ in their leaf litter quality and toxicity, so that conditions experienced in eucalypts are not all that novel or stressful. Future studies comparing more native and exotic tree species, and responses by more amphibian species, would help shed light on the physiological and life-history traits that allow some species to cope better with the altered environmental conditions experienced in exotic plantations.

The strong negative effect of eucalypt leachates on immune response also indicates that water infused with eucalypt leaf litter is likely to be a stressful environment for all three species examined. This is because impoverished immune responses often are one of the consequences of chronic stress (Breuner et al., 2013; Martin, 2009). Our findings are consistent with previous studies that have demonstrated lowered immune responses in individuals exposed to exotic eucalypt and pine plantations (Iglesias-Carrasco et al., 2016). One possible explanation for our result is that eucalypts provide a reduced quality (low-nutrient, high-toxicity) and amount of food, so that tadpoles have to trade-off the energy allocated to the immune response and other traits. Another possibility is that, because of the high toxicity of the eucalypt leachates (compared to the oak leachates), tadpoles invest as much available energy to combat the toxins as possible (increased oxidative stress, alteration in protein assimilation pathways; e.g., Morrongiello et al., 2011). However, in a previous study on *R. temporaria*, no effect of eucalypt leaf litter was found on tadpole oxidative stress (Burraco et al., 2018), suggesting that such physiological pathways are unlikely to have been altered. Future research should aim to understand the changes in the functioning of the neuroendocrine axis and variation of glucocorticoid levels linked to chronic stress in exotic plantations.

## 5 | CONCLUSIONS

We show that the presence of novel leachates, and a potential reduction in the quality and quantity of resources in exotic plantations, will

probably affect key traits of resident anurans. However, it is necessary to tease apart putative adaptive responses of those species from changes that actually reduce fitness and survival in the novel habitat. Novel environmental pressures in human-made habitats do not necessarily affect all life-history traits and all species that recently came into contact with exotic plantations, so understanding this variation and how it allows some wildlife to cope with novel environments is critical for preventing species loss in face of rapid environmental change. Exploring population-level responses also is important, such as examining populations that have had some contact with exotic plantations relative to those populations of the same species that are naïve to those plantations. Further studies would benefit from exploring potential effects of the terrestrial environment after the metamorphosis and adult stage, and hence the fitness consequences of exposure to exotic monocultures, to fully understand the effect of the replacement of natural forest with exotic plantations.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## AUTHOR CONTRIBUTIONS

MIC and CC designed the experiment and collected the data, and MIC and TJO analyzed the data and wrote the manuscript.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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