

Effect of an immune challenge on the anti-predator response of the green Iberian frog (*Pelophylax perezi*): the influence of urban habitats

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Previous studies suggest that immune-challenged individuals need to allocate resources to the immune system to combat infection, reducing escape ability and increasing the vulnerability of infected individuals to predators. Such behavioural responses might change in anthropogenic habitats where the balance between predation risk and countering infections can differ from that in natural habitats. We experimentally explored whether fighting an infection has an effect on hiding behaviour in a frog (*Pelophylax perezi*), and whether frogs from urban ponds respond differently from those from natural forested ponds. Independently of habitat type, we found no evidence for changes in hiding behaviour of male frogs before and after they were immune challenged (injected with lipopolysaccharide) or not (injected with phosphate-buffered saline). We also explored whether immune response (phytohaemagglutinin assay) or morphological traits (head width, body condition, body size and length) were related to variation in hiding time, but we did not find a relationship between the measured traits and behavioural response for males from either habitat. In conclusion, we show that frog anti-predator behaviour is not always affected by an immune challenge and the effect of urban habitats can be weaker than expected, at least in species that could be pre-adapted to the novel environmental conditions.

ADDITIONAL KEYWORDS: immunity – life-history traits – *Pelophylax perezi* – predation – urban habitats.

INTRODUCTION

Predation is a major selective force driving the evolution of morphological and behavioural characteristics of animals (Lima & Dill, 1990). Time spent avoiding predators takes time away from other fitness-enhancing activities, so animals need to balance anti-predator responses with other life history requirements (Lima & Dill, 1990; Sih, 1992). An overreaction may incur undue costs. For example, prolonged use of refuges in response to the presence of a predator can reduce the time available for activities such as mating and foraging (Martín & López, 2003; Blumstein & Pelletier, 2005). Consequently, selection should favour prey that accurately assess the level of risk posed by predators (Helfman, 1989), and

adjust their anti-predator responses to the prevailing conditions (Lima & Dill, 1990).

Individuals may vary in the way they allocate time and resources to different activities when the costs and benefits of each activity differ between individuals (McGlothlin, Jawor & Ketterson, 2007). Both an individual's phenotype (French, DeNardo & Moore, 2007a), and the local environment might alter the balance between the costs and benefits of exhibiting a specific response. For instance, escape decisions are affected by an individual's traits (e.g. conspicuousness, body size) and hence by their perceived vulnerability (Cabido *et al.*, 2009). For instance, predation risk is usually size-dependent (Tejedo, 1993; Mänd, Tammara & Mappes, 2007), so different sized individuals might alter their anti-predator responses due to differences in perceived vulnerability (Schmidt, Philipp & Abele, 2008). Escape decisions can also be affected by

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environmental factors, such as predator abundance (Cooper, Pérez-Mellado & Hawlena, 2007). Consequently, any modification of the habitat that influences the phenotype of individuals such as body size or condition, or biotic/abiotic factors of the environment such as predation risk, might alter the optimal anti-predator responses of individuals.

There is an interaction between anti-predator responses and many other behavioural, morphological and physiological traits, including those associated with immune responses. Immune activation is costly (Uller, Isaksson & Olsson, 2006), and allocation of energetic resources to the immune system reduces the resources available to invest in other life-history traits (Norris & Evans, 2000). For example, immune challenges can reduce the reproductive rate and general activity of individuals (French, Johnston & Moore, 2007b), as well as influence feeding rates (Llewellyn *et al.*, 2011) and limit individual escape ability (Zamora-Camacho *et al.*, 2015) by reducing their performance. In such cases, individuals may increase their hiding time to compensate for their increased vulnerability (Cabido *et al.*, 2009), and hence reduce time feeding, mating or defending territories (Martín & López, 2003; Martín, López & Cooper, 2003).

Here we tested how activating the immune system in male Iberian green frogs (*Pelophylax perezi*) influences their response to a simulated predator attack. We conducted our experiment on frogs from four populations, two from natural ponds and two from urban ponds, because Iberian green frogs inhabit both natural and urban areas. Because animals that inhabit urban areas are often exposed to different conditions, such as lower or higher predation risk (Sorace, 2002; Ditchkoff, Saalfeld & Gibson, 2006), and often diverge in traits relative to their counterparts in natural habitats (Evans *et al.*, 2009), the optimal anti-predator responses could be expected to differ between habitats (Iglesias-Carrasco, Head & Cabido, 2016b). We predicted that immune-challenged males would have greater hiding times after potential predator attacks than non-infected males to compensate for reduced escape ability. We also predicted that this response may be disrupted in urban habitats. For example, urban frogs could have longer hiding times than their natural counterparts because they are in better condition, or they could have shorter hiding times because they are predator-naïve (if the presence of predators is lower in urban areas) and have not learnt to exhibit appropriate responses to predatory attacks.

MATERIAL AND METHODS

COLLECTION AND MAINTENANCE OF INDIVIDUALS

We captured 52 adult male *P. perezi* from two urban parklands in the middle of the city of Donostia/San

Sebastián in northern Spain (population 1 $N = 24$, population 2 $N = 28$) and 34 adult males from nearby natural habitats (population 3 $N = 15$, population 4 $N = 19$) in Hernani. All males were captured in May 2014 during the breeding season. The urban populations tended to have fewer predators than natural populations, and they differed in pond structure and human disturbance from the natural populations (see Supporting Information for descriptions of the species and the habitats). The number of populations was limited to two for each habitat type due to the lack of suitable ponds in surrounding cities. Although it would be interesting to conduct our experiment with both males and females to look at sex-specific responses, we opted to only test males in order to avoid manipulation of females during the breeding season.

Frogs were individually housed in 87 indoor 30-L PVC aquaria (36 × 42 cm) containing a small pond with 4 L of mineral water and a terrestrial area with moss for refuge. Aquaria were maintained at 25 °C during the day and 18 °C during the night, mirroring the average temperature in the field. We allowed natural daylight to enter the laboratory to mimic the photoperiod of the surrounding region. Food (mealworm larvae and crickets dusted with multivitamin powder) was provided *ad libitum*. Placement of aquaria within the laboratory was randomized to avoid potential environmental gradients within the laboratory from confounding our results. The day after the collection of frogs, we measured several morphological traits (head width, body size, leg length and body condition) and immune response that can correlate with anti-predator behaviour in frogs (Blouin & Brown, 2000) and that could vary between natural and urban habitats (details below). Subsequently, frogs were housed for 5 days prior to the behavioural trials to allow them to acclimatize to laboratory conditions and to allow recovery from the immune response assay. This paper follows the order of the hypotheses being tested, instead of the chronology of the experiments. We first explain the main question: What is the effect of an immune challenge on male anti-predatory responses? We then explain the test of the effect that morphological traits might have on this behaviour in each type of habitat. All the animals were healthy during the trials and were returned to their capture sites at the end of the trials, 10 days after being captured.

ANTI-PREDATOR BEHAVIOUR AND EXPERIMENTAL IMMUNE CHALLENGE

We studied the anti-predator behaviour of males within 6 days of capture, between 11.00 and 15.00 h, when the animals were fully active. We moved frogs to a smaller glass container (13 cm diameter × 16 cm depth) filled with 0.5 L of mineral water 1 h before each



Figure 1. Illustration of the experimental design. In these small aquaria (0.5 L of water), animals could hide by sinking under water (B), but the water column was shallow enough to allow individuals to breathe outside the water while leaning on the floor (A). This design ensured that animals had enough water to hide in, without the need to constantly swim. Illustration by Carlos Cabido.

trial (Fig. 1). Containers were separated by opaque plastic which prevented individuals from seeing each other and potentially copying the behaviour of others (Martín, Luque-Larena & López, 2006). In each trial, we simulated a predatory attack and video recorded male behaviour until they stopped hiding (measured as 'hiding time', in seconds). We rapidly approached the aquaria and tapped the male once near his head and at a standard distance of 3 cm to simulate a predator attacking from above. All the trials were made by the same person, M.I.C., to avoid potential bias due to the experimenter behaviour. After the simulation of the predatory attack, we rapidly retreated to another room to avoid being seen by the animals. Tapping the males near the head elicited an anti-predator response (i.e. submersion underwater) in all animals similar to that observed in the field (M.I.C., personal observation, Fig. 1), so we are confident that we successfully simulated a predatory attack. In the field, individuals jump into the water when faced by a predation risk. When there is no aquatic vegetation (as in our laboratory trials) individuals stay under water until the danger disappears. They usually then swim back to the pond edge to feed and bask. As we could not replicate this entire process in the laboratory, we considered the moment when the male left the protection of the water as the cessation of hiding.

For each frog, we conducted two trials before the experimental immune activation (one per day over consecutive days), and two trials after the experimental

immune activation. The only identification of individuals was a random number from 1 to 86, randomly assigned with respect to treatment and site of origin, so all the data were recorded blind. After the simulated attack, we noted the moment when the male's head reached the surface of the water as a measure of 'hiding time'.

We challenged the immune system of males with a lipopolysaccharide (LPS) from the cell wall of *Escherichia coli* (serotype 0111-B4; Sigma-Aldrich, St. Louis, MO, USA). LPS elicits an inflammatory response and induces both an innate and an adaptive immune response by the activation of B and T lymphocytes (Janeway *et al.*, 2001). After the initial recording of anti-predator behaviour, half of the frogs in each population were injected with LPS diluted at a dose of 2 µg/g of body weight into their dorsal lymph sac. LPS solutions were prepared by diluting 1 mg of LPS in 1 mL of phosphate-buffered saline serum (PBS). This concentration has previously been used to stimulate the immune system of other amphibian species (Llewellyn *et al.*, 2012). The remaining control males were injected with the same volume of PBS, to account for any effects of handling and injection.

HABITAT-DEPENDENT MORPHOLOGICAL TRAITS

To better understand the potential changes in behaviour after an immune challenge in relation to habitat, we also measured several functionally important

morphological traits that can correlate with anti-predator behaviour in frogs (Blouin & Brown, 2000): snout–vent length (SVL: from the tip of the snout to the cloacal overture), head width, body condition [calculated as the residuals from the regression of log-transformed body mass (g) on log SVL] and leg length (measured in millimetres from the cloacal overture to the end of the foot – without including the fingers). All individuals were anaesthetized by immersion in Tricaine methane sulphonate (0.15 g MS-222 per litre dechlorinated water) for 5–10 min. We then photographed the frogs face up with the leg completely extended so that we could later measure morphological traits using Image J (Abràmoff, Magalhães & Ram, 2004).

We also estimated the immunological condition of the males by using the phytohaemagglutinin injection assay (PHA test). All individuals behaved normally and fed after recovering from the anaesthesia. We measured the immune response just after capturing the individuals to ensure that the response was not influenced by being brought into captivity. The PHA test measures T-cell-dependent immunocompetence *in vivo* (Lochmiller, Vestey & Boren, 1993), and has been used in many vertebrate species including amphibians (Brown, Shilton & Shine, 2011; Iglesias-Carrasco, Martín & Cabido, 2017). 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, López & Martín, 2014). After frogs were photographed, but before they had recovered from the anaesthesia, we measured the thickness of the left hind footpad with a pressure-sensitive spessimeter (accuracy: 0.01 mm; we use the average of five measurements made on each frog). Immediately thereafter, we injected 0.02 mg of PHA dissolved in 0.01 mL PBS. The effect of the PHA injection is a slight swelling of the skin, caused by the immune response, which disappears after around 72 h (Brown *et al.*, 2011). Frogs were then placed back in their individual aquaria. After 24 h, we anaesthetized the frogs again and measured the thickness of the footpad at the same point to calculate the difference between pre- and post-injection measures (inflammation).

STATISTICAL ANALYSES

To test for the effects of immune challenge on hiding time we ran a generalized linear mixed model (GLMM) with the package lme4 (Bates *et al.*, 2015). We treated the mean hiding time (pre- or post-injection) as the response variable. We also wanted to explore whether

the relationship between the immune challenge and the hiding time differed between urban and natural habitats, so treatment (LPS or PBS), habitat (natural or urban) and time (pre-, post-injection) and all the three- and two-way interactions involving these variables were included as fixed factors in the model. We included body condition as a covariate to control for potential variation related to this trait, and population and 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 a randomly assigned number (from 1 to N) as a random effect (Harrison, 2014). Pairwise comparisons were planned using Tukey's honestly significant difference tests.

To further explore our data we tested whether males captured in different habitat types differed in morphological traits (head width, body size, body condition and leg length) or immune response using linear mixed models (LMMs) with habitat as a fixed factor and population as a random effect. As SVL was highly correlated with the PHA response, head width and leg length (all $R^2 > 0.418$, $P < 0.001$), we calculated the residuals from the regression of these three traits on SVL to account for variation due to body size. When necessary, data were transformed (powerTransform function, car package). All tests were two tailed.

There could be an inverse association between male hiding time and morphological traits associated with their ability to escape from predators (i.e. frogs with longer legs would emerge from hiding sooner) and traits related to their susceptibility to predation (i.e. larger frogs would emerge from hiding sooner). This association might differ in urban and natural habitats. To explore this, we ran an LMM to determine whether male morphological traits and immune response were associated with variation in hiding time prior to the experimental immune challenge. We also explored whether these relationships differed depending on the habitat from which a male came. We used the mean of the two measured hiding time trials before the immune challenge as the dependent variable. We included habitat type, five covariates (SVL, PHA response residuals, body condition, head width residuals, leg length residuals) and the two-way interactions between each covariate and habitat type as terms in our model. We included population as a random effect. We checked the residuals of our model to ensure that it met the assumption of normality and homoscedasticity. We also ran our model including the two measured hiding times as a random effect (instead of using the mean) and we obtained statistically similar results.

All statistical tests were conducted using R 3.2.2 (R Core Team, 2015).

RESULTS

Here we report test statistics (i.e. chi-square values) and P -values from models (GLMM and LMM) (see Table 1 and Supporting Information Tables S1, S2 for model estimates).

EXPERIMENTAL IMMUNE CHALLENGE

Overall, we found no significant change in hiding time before and after the experimental infection. Independent of treatment, all individuals spent a similar amount of time hiding (treatment \times pre/post: $\chi^2 = 1.622$, d.f. = 1, $P = 0.203$, Table S1, Fig. 2). Likewise, there was no significant habitat difference in the change in hiding time before and after the experimental immune challenge (treatment \times pre/post \times habitat: $\chi^2 = 2.646$, d.f. = 1, $P = 0.104$, Fig. 2). However, there was a significant two-way interaction between the habitat animals came from and the experimental treatment (treatment \times habitat: $\chi^2 = 8.565$, d.f. = 1, $P = 0.003$). Interestingly, in the natural habitat males injected with the PBS control had shorter hiding times than control males from the urban habitat (Tukey's test $P < 0.001$), as well as shorter hiding times than immune-challenged males from the natural habitat (Tukey's pairwise comparisons $P = 0.020$). This result is probably driven by the significant reduction in hiding time of the natural control group after injection (Tukey's test $P = 0.004$).

HABITAT-DEPENDENT MORPHOLOGICAL TRAITS

Urban male frogs had significantly wider heads for their body size than those captured in the natural populations (estimate \pm SE = -0.056 ± 0.018 , $t_{(84)} = -3.033$, $P = 0.003$, Table 1). There was no difference between habitats in male SVL, body condition, PHA immune response or leg length (Table 1, total $N = 86$). Moreover, we did not find any influence of the morphological and immune traits measured on the hiding behaviour of frogs in relation to the type of habitat (all P -values > 0.518 , Table S2).

Table 1. Habitat differences in male morphological traits and immune response prior to immune challenge treatment (results from LMM; significant values are in bold)

Factor	Estimate	SE	d.f.	t	P
SVL	0.821	0.691	2.073	1.188	0.355
PHA response (residuals)	-0.080	0.659	84.000	-1.217	0.326
Head width (residuals)	-0.022	0.007	84.000	-3.149	0.002
Leg length (residuals)	0.006	0.007	84.000	0.949	0.345
Body condition	0.101	0.046	1.963	2.204	0.161

DISCUSSION

ANTI-PREDATOR BEHAVIOUR AND IMMUNE CHALLENGE

An immune challenge is energetically costly, and can affect the allocation of resources to different traits, compromising the escape ability of individuals. Reduced locomotor performance might, therefore, elicit compensatory behavioural changes to avoid predation. If this is the case, we would expect that immune-challenged individuals would have compromised anti-predator responses (i.e. spend more time hiding than non-challenged individuals). Previous studies in different taxa have found that immune activation favours reallocation of resources to fight infection, which reduces locomotor performance (Zamora-Camacho *et al.*, 2015), which in turn can lead to increased hiding times (Aubert, 1999; Otti *et al.*, 2012). However, our data provide no support for this hypothesis in *P. perezi*.

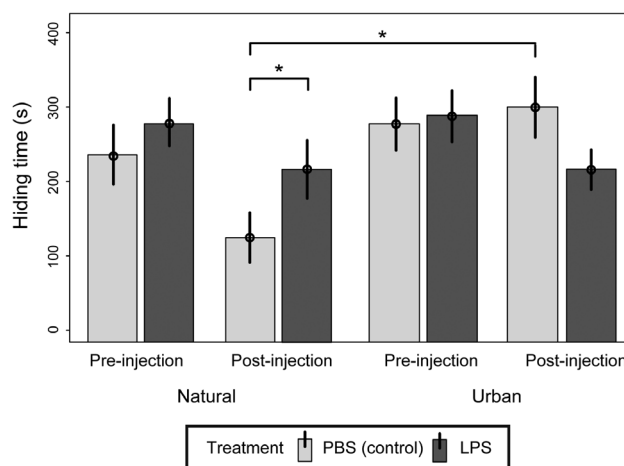


Figure 2. Male hiding time (mean \pm SE) in relation to habitat type, time and treatment. PBS males from the natural habitat after the treatment differed significantly (horizontal bars) from: (1) LPS males from the natural habitat after the treatment (Tukey's test $P = 0.020$), and (2) PBS males from the urban habitat after the treatment (Tukey's test $P = 0.004$).

We based our predictions on the premise that an immune challenge is energetically costly and will lead to reduced performance and changes in behaviour. Several studies have shown that facing an immune challenge requires energetic expenditure, so immunity often trades-off with other fitness-enhancing traits, such as secondary sexual traits (Faivre *et al.*, 2003). However, to evaluate the impact of the immune activation, we have to take into account the energetic state of each individual (Smith *et al.*, 2017), as well as the environment and season (French, Moore & Demas, 2009). For individuals with high availability of resources, the energetic demands of the activation of the immune system might not be enough to cause behavioural changes (French *et al.*, 2007a). Interestingly, we found that body condition did not affect hiding behaviour of males (Table S2). However, the need to invest in male–male competition and calling effort during the breeding season, when we carried out our experiment, might constrain the ability of males to mount an immune response (Nordling *et al.*, 1998). The lack of behavioural responses to an immune challenge in our study could be related to the reproductive status of males or other factors that influence the energetic state of individuals. Testing how an immune challenge affects the anti-predator behaviour of males at different times of the year (e.g. outside the breeding season) could help to understand the mechanisms by which individuals regulate the allocation of resources to different life-history traits, and how this is reflected in behaviour.

Physiological responses related to stress and the use of LPS as a treatment to activate the immune system might also explain the lack of behavioural responses to the immune challenge. Frogs are exposed to many stressors in the wild such as the constant presence of predators. This baseline stress, combined with the stress related to the capture of animals, could increase the production of corticosterone, which can reduce the strength and duration of anti-predator responses (e.g. the duration of the unken reflex response, Neuman-lee *et al.*, 2015). Similarly, the consequences of an immune challenge induced with LPS can differ depending on the species and traits measured. For instance, LPS immune-challenged cane toads (*Rhinella marina*) show reduced levels of activity and feeding rates (Llewellyn *et al.*, 2011). In contrast, male palmate newts (*Lissotriton helveticus*) did not alter their mating behaviour or their investment in secondary sexual traits after being injected with LPS (Cornuau *et al.*, 2014). Thus, the strength of the effect of LPS appears to be species-specific, such that the immune reaction to this lipopolysaccharide does not always lead to measurable behavioural changes.

HABITAT-DEPENDENT MORPHOLOGICAL TRAITS AND BEHAVIOURAL RESPONSES

We found a general lack of morphological differences between frogs from urban and natural ponds. The only trait that differed between the urban and natural populations was relative head width. Larger head sizes in males from urban areas might be related to higher rates of cannibalism (Walls, Belanger & Blaustein, 1993), stronger male–male competition (Byrne & Roberts, 2004) or a stochastic founder effect. Exploration of how urbanization affects female and juvenile morphological traits, as well as common garden experiments could help us to understand the causes of habitat-dependent differences in head size and whether these changes are adaptive.

Contrary to expectation, we found no habitat differences in male body size, leg length immune response or body condition. Neither did we find a habitat-dependent relationship between male traits and hiding time. Previous research exploring the effects of urban habitats on morphology has found mixed results. While some studies find both larger (Liker *et al.*, 2008) and smaller (Murphy *et al.*, 2016) body sizes in urban populations in different animal taxa, others do not find any effect of urbanization on this trait (Iglesias-Carrasco *et al.*, 2017). Similarly, previous research has found that colonizers of anthropogenic habitats show either enhanced (French *et al.*, 2007b; Audet, Ducatez & Lefebvre, 2016) or diminished immunocompetence (Lewis *et al.*, 2013; Iglesias-Carrasco *et al.*, 2016a) compared to their counterparts in natural habitats. These contrasting findings highlight the difficulty of making predictions about the potential effects of urbanization on morphological and physiological traits. Given that urban habitats can differ from natural habitats in many ways, it is perhaps not surprising to expect responses to be species-specific. *Pelophylax perezii* occurs in a wide range of habitats and is tolerant of a variety of chemicals and pollutants (Llorente *et al.*, 2002; Egea-Serrano, Tejado & Torralva, 2008), and as such this species may be robust to changes resulting from urbanization. Also, the lack of a relationship between hiding time and morphological traits suggests that the association between specific traits and vulnerability to predation is not a pattern observed in all amphibians. Therefore, more studies exploring multiple species in a variety of urban areas that vary in different ways are critical to understand what makes some species more sensitive to habitat transformation than others.

We predicted that individuals originating from urban and natural ponds might respond differently to the need to avoid predators. However, the difference in hiding time before and after the treatment was similar for immune-challenged individuals from both natural

and urban habitats, suggesting that an immune challenge has little effect on anti-predator behaviour in *P. perezi*, regardless of their habitat of origin. If changes in the anti-predator behaviour in response to an immune challenge are related to morphology, the lack of morphological differences in traits between the habitats could explain the lack of differences in the pattern observed. Interestingly, we found that the natural control group decreased their hiding time after the infection. This suggests that healthy males in the natural habitat are able to adjust their anti-predator behaviour and discriminate the current level of risk in the face of a simulated predator. One potential explanation for this behavioural adjustment is that unchallenged males from the natural habitat were able to learn the lack of real predation risk. Learning is expected to be costly (e.g. Mery & Kawecki, 2003), so any stressful situation, such as an immune challenge, or the continuous exposure to noise in urban areas, could constrain the learning ability of individuals.

In some urban areas the presence of natural predators is lower than in the natural habitats. In such cases, one might expect individuals from different habitats to respond differently to potential predation attacks. Lack of experience with predators could lead to an increase in hiding time due to an inability to assess real risk, or it could lead to a decrease in hiding time because they have not learned to be wary. However, our results suggest that these differences in ecological and evolutionary pressures, if present, are not strong enough to alter frog anti-predator behaviour. Due to the difficulties of performing extensive field work to check whether the actual predation risk differs between habitats, we cannot be certain that predation pressures are different. However, transects performed to survey predators found no cats or natural predators near the urban ponds, suggesting that even if these predators are present their abundance is likely to be low. Exploring the effect of predators and people in the urban habitats further could be critical to understanding how different predation pressures in these novel environments affect the behaviour of individuals.

CONCLUSIONS

Unlike in other species, we found no alteration in anti-predator behaviour in *P. perezi* after an immune challenge, suggesting that changes in the allocation of resources in the face of an infection do not always affect the performance and escape decisions of individuals. Moreover, our findings highlight that the effects of anthropogenic habitats are hard to predict and that distinct ecological pressures in altered environments are not always enough to promote strong changes in behaviour or resource allocation of individuals.

Further studies comparing additional urban and natural habitats and with ecologically more restricted frog species are needed to determine the effect of urbanization on behavioural responses of individuals.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Effects of habitat (natural or urban), treatment (LPS or PBS control), time (pre- or post-injection) and body condition on hiding time (results from GLMM). Significant values are in bold.

Table S2. Effects of habitat and male traits on hiding time prior to immune challenge (results from LMM).