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A phytohaemagglutinin challenge test to assess immune responsiveness of European tree frog *Hyla arborea*

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Abstract. Immune responsiveness, one measure of individual quality, can be used as a sensitive, non-lethal variable that may be negatively affected in animals exposed to degraded, contaminated or otherwise disturbed areas. One frequently used technique to measure immune responsiveness is the phytohaemagglutinin (PHA) challenge test. Swelling occurring at the injection sites are measured before and 24 h after PHA injection. The immune response is considered to be the difference between the two measures. Although this method is easily performed with wild animals, it has been rarely used on small amphibians. Here, we test the possibility of using a PHA test with the European tree frog, *Hyla arborea*, and we identify the optimal procedure for measuring immune responsiveness in this species. The results allowed us to simplify the procedure in eliminating phosphate-buffered saline (PBS) injection and reducing the duration of the experiment. Injection of PHA into the leg of *H. arborea* triggered an immune response with a peak of swelling 14 h after injection. A second injection of PHA into the same animal induced more intense leg swelling. In addition, haematological responses showed that the total number of leucocytes increased after PHA injection. A link between the leg swelling and the total leucocytes count recorded in blood has been found. Consequently, this method may provide a useful tool for predicting the pro-inflammatory capacity of field populations of small amphibians.

Keywords: conservation, immune system, PHA, pro-inflammatory capacity, tree frog.

Introduction

The capacity of an individual to produce an appropriate immune response following exposure to a pathogen is a critical aspect of disease resistance and survival (Demas et al., 2011). Although habitat destruction, chemical pollution exposure, and introduction of predators/competitors are obvious causes of amphibian declines, there is now compelling evidence for mass deaths among amphibian populations in diverse geographic locations due to disease outbreaks (Carey, Cohen and Rollins-Smith, 1999). The amphibian immune system involves both innate and adaptive defence mechanisms. The innate immunity system provides rapid and non-specific protection. During this phase, resistance mechanisms recognise an external pathogen via broadly conserved molecular structures and then respond to it (Boughton, Joop and Armitage, 2011). In contrast, an adaptive immune response is characterised by specific memory that allows an individual to recognise and remember specific pathogens and will often provide lifetime immunity against reinfection with the same pathogen (Carey, Cohen and Rollins-Smith, 1999). The adaptive immune response includes humoral immunity through the production of highly specific antibodies and cell-mediated responses which involve T lymphocytes and then macrophages and polynuclear leucocytes (Gilbertson et al., 2003). Constitutive immunity provides a response that is always ready to act; it tends to be a rapid response and is unspecific in that it is elicited or effective against a variety of immune challenges (Boughton, Joop and Armitage, 2011). In contrast induced responses are only expressed after pathogen infection, wounding, cancer recognition, allergies and immunopathologies. These activities encompass both innate and adaptive immunity. Because of the complexity of the physiological reactions triggered by infection,

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Table 1. Literature overview of studies using PHA to measure immune responsiveness in amphibian species.

Species[(1)]	Stage	Method	Reference
Lithobates pipiens	Adult	Toe web injection, 2 mg/ml, swelling measure at 24, 48, and 72 h	Gilbertson et al., 2003
Lithobates spenocephalus	Tadpole	Tail injection, 1 mg/ml, swelling measure at 24 h	Venesky et al., 2012
Rana sylvatica	Froglet	Thigh injection, 1 mg/ml, swelling measure at 24 h	Gervasi and Foufopoulos, 2008
Rhinella marina	Adult	Toe web injection, 1, 2, and 5 mg/ml, swelling measure at 6, 12, 24, 48, and 72 h	Brown, Shilton and Shine, 2011

[(1)] Many studies not mentioned in the table use PHA with spleen cell culture of *Xenopus leavis* (e.g., Kinney and Cohen, 2005).

measuring and interpreting immune responsiveness in wildlife studies require standardised techniques.

Most of the techniques used to quantify vertebrate immune response require blood sampling. Such techniques include, for example, the measure of natural antibodies and complement effectiveness using a haemolysis-haemagglutination assay, the measure of the bactericidal capacity of plasma and the determination of the leucocyte profile (Matson et al., 2006; Pigeon et al., 2013). Few non-destructive methods have been developed. Among them, the phytohaemagglutinin (PHA) challenge test is the most popular because it causes no physiological stress responses in the animal other than those associated with capture and handling (Merino et al., 1999). The PHA challenge test has been used extensively in birds and reptiles (e.g., Meylan, Haussy and Voituron, 2010; Grasman et al., 2013) but more rarely in amphibians (table 1). It can be applied in natural contexts because of its short-term and localised effects and its feasibility in vivo under field conditions. This test consists of a PHA injection which produces temporary localised swelling. The use of PHA challenge test as a surrogate of T cell-mediated immunity only, as proposed in several studies (e.g., Tella et al., 2008), was questioned. Brown, Shilton and Shine (2011) showed that PHA injection initiated the rapid infiltration of neutrophils, eosinophils and macrophages at the injection site, following by an influx of lymphocytes 24 h after injection in cane toads. A study on cytokine expression profiles in grey partridges (Vinkler et al., 2014) showed no sign of any induction of T cell proliferation at the site where PHA was injected. Recent studies have suggested that PHA-induced swelling measured, at least in part, a rapid onset, non-specific inflammatory and innate immune response (Kennedy and Nager, 2006) that may provide a clue for predicting the pro-inflammatory capacity of the individual (Vinkler et al., 2010; Vinkler and Albrecht, 2011).

This study aimed to test the possibility of using a PHA challenge test with the European tree frog, *Hyla arborea* (Hylidae), and to identify the optimal procedure for measuring the immune responsiveness in this species and, more generally in small amphibian species. Methods requiring blood sampling cannot be applied in *H. arborea*; individuals are too small (3 to 7 g) to allow blood sampling by cardiac puncture without risking the death of this protected species. To validate the PHA response, we also examined the white blood cell types that proliferate during the inflammatory response after one and two PHA injections.

Materials and methods

Animal collection and rearing

Individuals were obtained from a population located near Lyon, France, on the Crémieu Plateau (5°21′7″E, 45°44′20″N). During the springs of 2012 (experiments 1 and 2) and 2014 (experiment 3), male tree frog adults calling

at a pond were captured individually by hand. Males were individually housed in terrariums (25 × 17 × 15 cm) with a water-filled basin and a tree branch placed in the housing room of our laboratory kept at temperature of 23.1 ± 1.5°C (mean ± sd). During the entire experiment, males were fed *ad libitum* with house crickets (*Acheta domesticus*) and exposed to a natural photoperiod.

Treatment inocula

PHA is a lectin derived from red kidney beans. PHA-P (L8754, Sigma-Aldrich) is a mixture of five isolectins that are tetramers composed of varying proportions of two PHA subunits, L and E (Vinkler, Bainova and Albrecht, 2010). In each experiment, PHA was dissolved in phosphate-buffered saline (PBS) (D5773, Sigma-Aldrich).

Experiment 1: PHA dose effect

This experiment allowed us to determine the capacity of different PHA doses for inducing an inflammatory response in *H. arborea*. Classical injection into a toe web and the standard measure of thickness (e.g., Gilbertson et al., 2003; Brown, Shilton and Shine, 2011) cannot be applied to a small species such as *H. arborea*. Hence, using a Hamilton syringe with a 26S-gauge needle (±0.5 μl accuracy), we injected 20 μl of PHA solution into the right leg muscle of *H. arborea*. In agreement with the ethics protocol, only nine frogs were used to test 0.25 mg, 0.5 mg or 1 mg of PHA per individual (3 individuals per dose). In order to verify the presumption of no swelling response after the PBS treatment in the tree frog, 20 μl of PBS was injected into the left leg of the same individual. Leg swelling was measured (mean of three consecutive measures) before and 2, 10, 12, 14, 24 and 48 h after injection using a spessimeter (Mitutoyo, Absolute, 547-301, Japan) with an accuracy of 0.01 mm. The reaction to PHA was expressed as the difference between the change in thickness of the right PHA-inoculated leg and the change in thickness of the left PBS-inoculated leg. Data from experiment 1 was analysed using repeated measures ANOVA (*R 2.12.1*; R Development Core Team, 2010).

Experiment 2: measure of the immune challenge response

The goal of experiment 2 was to develop and validate the dynamic of the response after a PHA challenge test. Eighty-nine males were sampled in the field and reared in the laboratory according to the protocol described above. Twenty μl of PBS ($n = 22$) or PHA (0.5 mg/20 μl; $n = 67$) was injected into the right leg of each frog (see results of experiment 1). The thickness of the leg was measured before injection and 12, 14, 16, 18, 20, 22 and 24 h after injection. A linear mixed model (LMM) allowed us to assess the magnitude of individual changes between leg thickness before and after PHA injection. In addition, we used variance-covariance matrix with compound symmetry because we detected heteroscedasticity before and after ($F = 0.15$, df = 23, $P < 0.001$). Computations were carried out with *R 2.12.1* (R Development Core Team, 2010).

Experiment 3: identification of white blood cells implicated in the PHA response

To verify the involvement of immune cells in the swelling response, 11 males were divided into two groups: the first group (control) wherein males were injected only once (C-males, $n = 6$), and the second group (experimental), wherein males where injected twice (E-males, $n = 5$). According to the results of experiment 1 and 2, 1 mg of PHA diluted in 20 μl of PBS was injected into the right leg of all males (C- and E-males). Male leg thickness was measured 16 h after PHA injection. After this first inflammatory measure, C-males were euthanized for blood sampling according to the ethics protocol (see below) and E-males were housed for 10 more days. Then, E-males received a second PHA injection under the same conditions, and leg thickness was measured 16 h after injection. These individuals were then also euthanized for blood sampling.

One drop of blood was smeared on a glass slide and air-dried. Blood smears were stained by immersion in May Grünwald-Giemsa (320-07-1 and 320-31-1, RAL Reactive, France). The identification and counts of different types of leucocytes (white blood cell types, WBCs) were obtained with a light microscope oil immersion lens (1000×) in a monolayer-sector of the smear. Five types of leucocytes were considered: basophils, monocytes, neutrophils, eosinophils and lymphocytes. A differential count based on 100 leucocytes was performed for each individual (Davis, Maney and Maerz, 2008; Fokidis, Greiner and Deviche, 2008). The leucocytes number per 10 000 erythrocytes was obtained by counting the number of all erythrocytes in one microscopic visual field (no difference in erythrocytes concentration was found, $U = 12$; $P = 0.58$) and multiplying it by the number of microscopic visual fields that were scanned to obtain 100 leucocytes (e.g., Lobato et al., 2005; Dehnhard, Quillfeldt and Hennicke, 2011). We examined the ratio of neutrophils to lymphocytes (N/L ratio). These two cell types covary with the glucocorticoid levels, and this ratio is used as a rough index of haematological stress (Gross and Siegel, 1986; Vleck et al., 2000; Davis, Maney and Maerz, 2008). All slides were examined by a single observer (O.G.) without knowledge of the treatment of the collected samples. Data from experiment 3 were analysed using the Wilcoxon matched-pairs test to compare leg swelling between the first and second injections and to compare the N/L ratio and TLC after one or two PHA injections. The correlation between the leg swelling and the number of leucocyte was tested with a Pearson test. We applied a log transformation to normalise the "WBCs" (*R 2.12.1*; R Development Core Team, 2010).

Ethical note

Researchers obtained the European certificate that legally allows us to design and conduct experimental research work using live animals. Our study was conducted under the approval of the ethics committee of Lyon 1 University (approval number BH2012-15). The frogs housing structure were located at the EcoAquatron of the University of Lyon and approved by the Veterinary Services (approval number

692661201). In agreement with capture authorization (prefectural approval number DDPP01-13-197), one week after manipulation, frogs were released *in natura* close to their original pound. All released frogs were in good health at the end of the experiment.

Results

PHA dose effect (experiment 1)

The results of the different doses of PHA injection, as shown by the mean increase in leg thickness over 48 h, are illustrated in fig. 1. The change in leg thickness of 9 frog legs injected with 20 μl of PBS exhibited no variation in swelling during 48 h (repeated measures ANOVA, $F_{5,40} = 1.87$, $P = 0.1$), although a slight increase occurred 2 h after injection (fig. 1). This first peak of leg swelling also occurred with PHA, regardless of the dose used. The injection of 0.25 mg of PHA per individual induced an increase in leg thickness similar to that recorded for the PBS injection (fig. 1). In contrast, the injection of 0.5 mg of PHA per individual produced a second increase in thickness, reaching a maximum value of 0.34 mm (increase of 20% on average) 12 h after injection. The amount of swelling induced by 1 mg of PHA did not differ from the results observed with 0.5 mg of this compound and remained stable until 24 h after injection. Forty-eight hours after injection, the thickness of the leg was similar to the thickness of the leg before PHA injection (repeated measures ANOVA, $F_{10,30} = 10.29$, $P < 0.01$). Because the two higher doses (0.5 and 1 mg) produced similar response times, we conducted all further trials using 0.5 mg/20 μl.

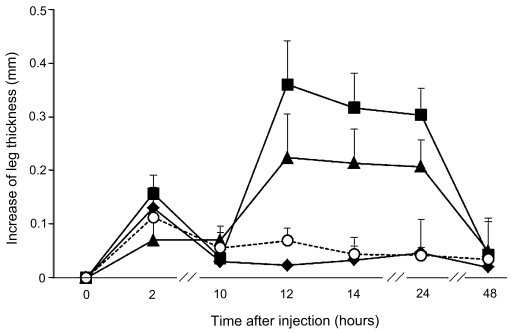

Figure 1. Increase (mean ± SE) in leg thickness (mm) of *H. arborea* over 48 h following injection of 0.25 (black diamond), 0.5 (black square) and 1 (black triangle) mg of PHA or 20 μl of PBS (white circle).

Immune challenge response (experiment 2)

The goal of this experiment was to verify that an injection of PHA under the experimental conditions determined in experiment 1 triggered a differential individual response in frogs. Injection of PHA induced an inflammatory response in *H. arborea* that peaked 14 h after injection (+0.21 ± 0.18 mm; fig. 2) and remained stable afterwards. The results obtained in males injected with PBS ($n = 22$) and with PHA ($n = 67$) showed that the change in leg thickness in response to PHA injection differed among individuals and that the thickness of the frog legs injected with PHA increased significantly compared to frog legs injected with PBS (parameter estimate = 0.21, $t = 3.64$, df = 85; $P < 0.01$). We can note that although the frog mass had a significant effect on the thickness leg before injection (Pearson, $t = 8.03$, df = 65, $P < 0.001$, fig. 3A), the increase in leg thickness 14 hours after PHA injection was not correlated with the

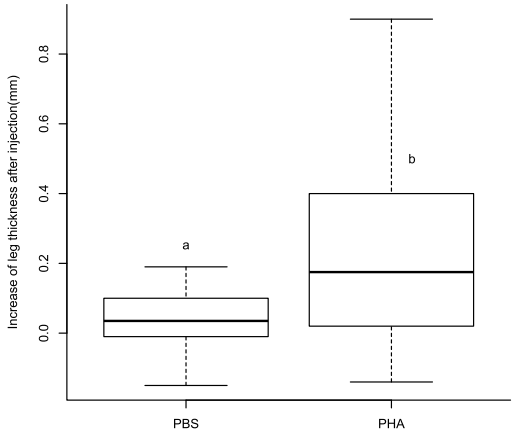

Figure 2. Boxplot representing the increase (mean ± SE) in leg thickness (mm) 14 h after injection of PBS or PHA ($n = 89$) (horizontal line: median value, box ends: upper and lower quartiles, whiskers: maximum and minimum values). Different letters (a and b) represent a significant difference between treatments.

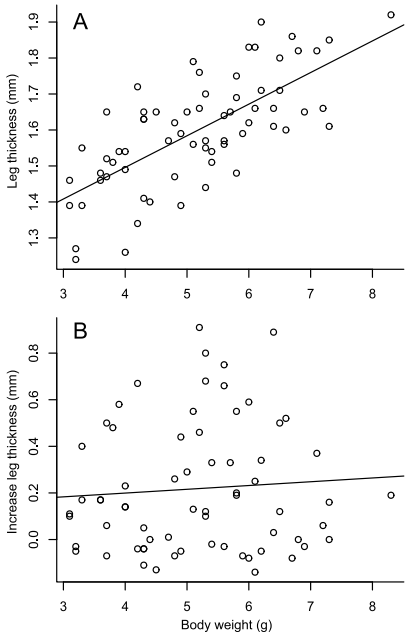

Figure 3. Leg thickness (mm) regressed on male mass before injection (A) and increase in leg thickness (mm) 14 hours after PHA injection regressed on male mass (B).

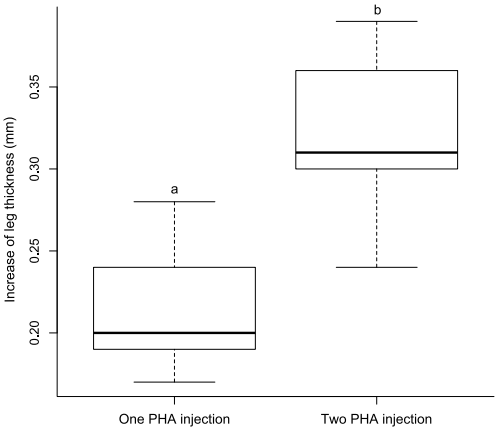

Figure 4. Boxplot representing the increase in male leg thickness (mean ± SE) swelling (mm) after one or two PHA injection (horizontal line: median value, box ends: upper and lower quartiles, whiskers: maximum and minimum values). Different letters (a and b) represent significant a difference between treatments.

initial body mass (Pearson, $t = 0.59$, df $= 65$, $P = 0.56$; fig. 3B).

Cellular immune response to PHA injection (experiment 3)

Before treatment leg thickness did not differ between groups ($W = 13$; $P = 0.78$). For both groups the first PHA injection induced significant swelling (all $P < 0.05$), and the swelling intensity did not differ between groups ($W = 19$, $P = 0.925$). Ten days after the first injection, the leg thickness of E-males returned to baseline ($W = 9.5$, $P = 0.6$). After a second PHA injection, the swelling response was significantly more prominent in comparison with the skin swellings observed after the first injection ($W = 2$, $P = 0.03$; fig. 4) in E-males.

The haematological values presented in table 2 showed that using two PHA injections led to a higher leucocyte number per 10 000 erythrocytes ($W = 2$, $P = 0.04$; table 2) compared to using only one injection. A similar increase of lymphocytes was observed ($W = 4$, $P = 0.05$). Our results also show a higher N/L ratio for C-males compared to E-males ($W = 18$, $P = 0.06$; table 2). We can note that the leg swelling after PHA injection was linked to the WBCs (Pearson, $t = 2.56$, df $= 9$, $P < 0.05$, fig. 5). Eosinophils are very scarce (<1% of all WBCs) and monocytes represented 8% of all WBCs in both C- and E-males.

Discussion

Our results clearly showed that the PHA challenge test is a suitable non-destructive method that can provide a measure of immune responsiveness in *H. arborea*. Although an initial peak of swelling, representing a 15% increase in thickness, rapidly occurred after the injection of PHA or PBS, a more intense second peak (a 34% increase in leg thickness) occurred between 12 and 14 h with only a 0.5 and 1 mg/20 μl PHA injection. This leg swelling remained stable until 24 h after injection. Because we quickly measured the first increase in leg thickness after injecting PHA or PBS, the initial swelling could have resulted from the injected fluid that remained unabsorbed. This hypothesis was consistent with the decrease in leg thick-

Table 2. Differential white blood cell counts expressed per 10 000 red blood cells (erythrocytes) and the ratio of neutrophils to lymphocytes (N/L) recorded in blood sampled in *H. arborea* injected once (C-males) or twice (E-males) with PHA. *P*-values less than $\alpha = 5\%$ are indicated with an asterisk.

Cell type (/10 000 erythrocytes)	C-males	E-males	*P*-value
	Mean ± SD	Mean ± SD	
N/L ratio	1.17 ± 0.24	0.72 ± 0.32	0.06
Leucocytes	63.35 ± 21.07	108.54 ± 36.03	0.04*
Lymphocytes	21.45 ± 4.72	55.44 ± 30.91	0.05*
Neutrophils	25.08 ± 12.66	32.75 ± 6.41	0.18

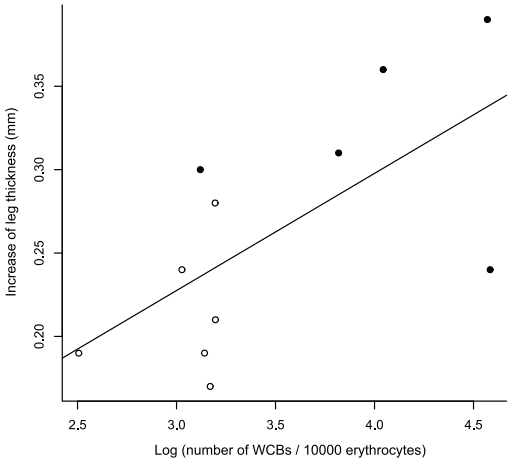

Figure 5. Increase in leg thickness (mm) regressed on WBCs/10000 erythrocytes expressed in logarithm after the first PHA injection (white circle) or the second PHA injection (black circle).

ness observed between 2 and 10 h suggesting slow absorption of the injected fluid. In contrast, the second swelling peak was triggered only by PHA. In this case, cytokines released from cells act as beacons for white blood cells (Kennedy and Nager, 2006; Martin et al., 2006), resulting in the rapid recruitment of white blood cells at the PHA injection site (Martin et al., 2006; Brown, Shilton and Shine, 2011) but not at the PBS injection site. Even if the temporal evolution of white blood cells in the same animal after a PHA injection was not be measured in our study, the pattern of increase in *H. arborea* legs in response to PHA injection seemed similar to the pattern observed with the cane toad *Rhinella marina* and blood analyses suggest the same processes (Brown, Shilton and Shine, 2011).

One approach for clarifying the nature of the immune system responses that underlie changes in leg thickness after a PHA injection is to examine the accumulation of each blood cell type at the injection site after one and two injections using histologic methods (e.g., Martin et al., 2006). This study measured the number of circulating leucocytes after two PHA injections separated by ten days. The results showed that the WBCs observed for E-males (2 injections) is higher than for C-males (1 injection). Although we cannot exclude the possibility that the lymphocyte levels might be elevated after the first injection and simply did not have time to decrease before the second injection was applied, we can note that the number of lymphocytes involved in a variety of immunological functions, such as immunoglobulin production and modulation of immune defence (Davis, Maney and Maerz, 2008), was higher after two injections than after one injection. Although the number of neutrophils did not vary between the C- and E-males, the N/L ratio is higher for males that were injected only once; the lymphocyte proportion is thus more important after a second injection. Due to the protection regime of *H. arborea*, the initial blood parameters of animals captured in the field without PHA injection were not determined. However, it seems that the N/L ratio usually observed in amphibian species varies between 0.3-0.5 (reviewed in Davis, Maney and Maerz, 2008). Thus, we can hypothesise that, in this study, the ratios increase approximately 3-fold when the frogs are injected once and remain higher than normal after the second injection. Taken together,

these results suggest that the primary response to PHA injection is a classical non-specific innate inflammation and that the secondary response observed in E-males is adaptive (Tella et al., 2008; Vinkler, Bainova and Albrecht, 2010). Indeed, after the first contact with an antigen the cells of adaptive immunity (lymphocytes) can be activated (Akira et al., 2006), but this activation takes time (>24 h). PHA is a tetrameric structure with two subunits. Only the PHA-L subunit stimulates T-cell mitosis (Vinkler, Bainova and Albrecht, 2010). In contrast, the PHA-E subunit activates erythroagglutination but does not stimulate T-cells (Vinkler, Bainova and Albrecht, 2010). It has been shown that the PHA-E isolectin stimulates a stronger immune response than PHA-L (Vinkler, Bainova and Albrecht, 2010). In addition, the results of experiment 2 in this study, using a substantial number of individuals, clearly showed that individuals respond with different amplitudes, confirming that the PHA responses can provide a robust measure of anuran immune responsiveness. In addition, we have also shown that a second PHA injection induces a larger tissue swelling (49.7% on average) than the first one. Moreover we found a significant positive relationship between leg swelling and the total number of circulating leucocytes number. In a bird study, Martin et al. (2006) showed significant correlations between the numbers of immune cell types and the degree of wing-web swelling. Particularly, they suggest that the link between swelling only 6 h after PHA injection represents a high degree of cytokine secretion by these cell types at this time, leading to increased infiltration and subsequent degranulation of other effector cells (e.g., heterophils, macrophages and basophils) shortly thereafter (Martin et al., 2006).

Although *H. arborea* was too small for toe web injection, we successfully injected PHA into *H. arborea* legs and we reduced the number of measure by eliminating the PBS control injection, as suggested by Smits et al. (1999) in birds and Brown, Shilton and Shine (2011) in amphibians. By injecting only one leg and measuring leg thickness only once between 14 to 24 h after injection, we reduced handling time, which causes an increase of stress hormones that affect immunosuppression (Martin et al., 2006). We also reduced the possibility of trauma during field manipulation. The results of PHA test have become a widespread measure of immune responsiveness, especially in wild birds (Martin et al., 2006). However, even if recent studies have improved our understanding of PHA assays (Salabarria et al., 2013; Vinkler et al., 2014), the immunological background of the test is highly complex and the results of the swelling should be interpreted with caution (Kennedy and Nager, 2006; Martin et al., 2006; Owen and Clayton, 2007; Vinkler, 2010). Further investigations are particularly needed to verify whether the test reflects resistance to any particular type of parasite (Owen and Clayton, 2007).

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