

## Article

# Integrating time progression in ecoimmunology studies: beyond immune response intensity

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## Abstract

Habitat alterations in agroecosystems may damage amphibian immune capacity. As agroecosystem extension is increasing worldwide, broader-context knowledge on the effects of agroecosystem stressful conditions on amphibian immune capacity is crucial for understanding and management of amphibian global declines. However, most studies on ecoimmunology assume synchronal among-group immune-response peaks, and focus on immune response after standardized time lapses, neglecting its progression. Herein, I compared inflammatory response short-term progression of agroecosystem and natural-habitat female and male natterjack toads *Epidalea calamita*, by measuring swelling response, once per hour, 6 h following an artificial immune challenge with innocuous antigen phytohemagglutinin. I also compared maximum magnitude of swelling response, irrespective of the moment when it was reached. Habitat differences arose only 3 h after challenge, when natural-habitat toads showed greater swelling response. Maximum magnitude of swelling response was similar in toads from both habitats. However, agroecosystem toads showed a delayed swelling response as compared with natural-habitat conspecifics, probably as a consequence of agroecosystem stressful conditions. Such a delay suggests a weaker immune capacity, and consequently impaired anti-pathogen performance. Regarding sex, swelling response magnitude did not differ between males and females. Female swelling response peaked earlier, but that of males was more sustained in time, which contradicts general across-taxa findings that males show impaired immune response. Interestingly, results suggest that measuring swelling response only after some standardized period following stimulation may be a simplistic approach and produce unrealistic results. Consequently, studies on ecoimmunology should implement swelling response progression in order to produce unbiased science.

**Key words:** agroecosystem, amphibian, immunocompetence, natural habitat, immune response progression

Variation in prevalence of pathogens (Eizaguirre et al. 2012), as well as in natural (Gutiérrez et al. 2013) or anthropogenic environmental stressors (Pigeon et al. 2013a) that affect host energetic condition, may lead to geographic differences in disease dynamics (review in Bearley et al. 2013). Indeed, anthropogenic habitat alterations such as fragmentation (Berggren 2009) or pollution (Ménard et al. 2010; Pilosof et al. 2014) may exert a negative effect on animal immune

response (reviewed in Martin et al. 2010). Among human activities, habitat fragmentation and pollution are particularly intense in agriculture lands (Beebe 1983; Watson et al. 2000; Beja and Alcázar 2003). Also, fertilizers and pesticides used in agriculture contaminate terrestrial (Teodorescu and Cogălniceanu 2005; Mășilioniță and Maikštėnienė 2010) and water ecosystems (Addiscott and Thomas 2000), with negative effects on fauna health and population

dynamics (Green et al. 2005). Particularly, pollutants and pesticides in agroecosystems impair immune response of vertebrates (Pigeon et al. 2013b) and invertebrates (Smilanich and Dyer 2012). The forecasted expansion of agricultural land in the next years (Veldkamp and Lambin 2001), and its important role as fauna reservoir (Durán et al. 2014), makes the study of agroecosystem dynamics a priority in the context of global change.

Indeed, pathogens may severely compromise host's fitness and survival, as they consume a part of the host as a resource and jeopardize its health status (Schmid-Hempel 2011). Consequently, immune systems against pathogens have evolved and are widespread in nature (Litman et al. 2005). Vertebrate immune system is a diverse but highly conservative multi-layer defense of increasing specificity: from general physical barriers to pathogen infiltration, to leukocytes that recognize and combat foreign elements in an organized manner (Wakelin and Apanius 1997). Leukocytes may elicit a generic, innate (such as phagocytosis, lysozymes, or oxidant agents), or a highly specific, adaptive (such as antibodies) response (Medzhitov 2007). Due to its capital role as an anti-pathogen defense, immune capability increases survival (Møller and Saino 2004), and a faster immune response may better preclude pathogen spread in the host and thus provide a more efficient protection against disease (Rogers et al. 2000).

Nonetheless, mounting an immune response entails a number of costs (Schmid-Hempel 2011). Firstly, immune response increases metabolic rates (Sherman and Stephens 1998) and thus consumes energy (Derting and Compton 2003; Demas 2004), as well as metabolites such as amino acids (Klasing and Calvert 1999). Furthermore, one mechanism of immune defense is oxidative burst (Lambeth 2007), which, along with collateral reactive oxygen species production as a consequence of immune reactions (Sorci and Faivre 2009), may cause oxidative stress. Oxidative stress occurs when the production of pro-oxidant chemical species overwhelms anti-oxidant defenses, with negative effects on physiology homeostasis and health (reviewed in Halliwell and Gutteridge 2007). Moreover, strong immune responses may cause health issues, as fibrosis, if regenerative activity is excessive (Allen and Wynn 2011), or autoimmune diseases, if the immune system attacks components of the host itself (Bebo et al. 1998). Consequently, the immune response is under trade-offs with a number of traits (Lochmiller and Deerenberg 2000), such as dispersal (Brown and Shine 2014), escape performance (Zamora-Camacho et al. 2015), learning capacity (Schleich et al. 2015), social-status maintenance (Thomason et al. 2013), or reproduction (Nordling et al. 1998), in a number of taxa. Also, costs of immune-system deployment can differ between males and females, owing to different selective pressures on sexes (Zamora-Camacho et al. 2015). Moreover, a testosterone-mediated compromise of immune function could appear in males, due to reduced energy allocation to the immune system to the benefit of reproduction (Muehlenbein and Bribiescas 2005), according to the immunocompetence handicap hypothesis, proven in numerous taxa (Folstad and Karter 1992).

Among vertebrates, amphibians are particularly susceptible to the negative effects of agroecosystem stressful conditions for several reasons. Firstly, amphibians undergo complex life cycles, including an aquatic larva stage followed in most cases by a fairly terrestrial adult phase. Therefore, they require a high connectivity between both environments, which habitat fragmentation can damage. Moreover, complex life cycles imply that contamination in water, soil, and air may affect amphibians (Liu et al. 2011). Pollutants may also pass through amphibians' unshelled eggs (Clark and LaZerte

1985) and permeable skins (Brühl et al. 2011). Furthermore, amphibians feed mostly on invertebrates (Attademo et al. 2005), so they may ingest pollutants indirectly through diet. Actually, agrochemicals have proved negative effects on amphibian health (Cabagna et al. 2005), and, more specifically, immune capacity (Albert et al. 2007). Additional understanding of the factors affecting amphibian immune capacity is relevant, particularly in challenging environments such as agroecosystems, since disease plays a fundamental role in amphibian global declines (De Castro and Bolker 2005).

In this study, I compare immune response magnitude and short-term progression of female and male natterjack toads (*Epidalea calamita*; Laurenti 1768) from agroecosystems and natural habitats. Infections by fungus (May et al. 2011), protozoans (Harris et al. 2013), oomycetes (Fernández-Benítez et al. 2011), nematodes, and cestodes (García-París et al. 2004) have been reported for this species. Presumably, a faster response would prevent pathogen expansion more effectively, thus being more successful against disease, particularly taking into account the high reproduction rates of most pathogens (Pilyugin and Antia 2000). Nevertheless, most studies on immune response neglect its short-term progression, focusing instead on the response after a standardized period following immune system stimulation. This approach implicitly assumes synchronal activity of immune response in all individuals (e.g. Alonso-Álvarez and Tella 2001; Moreno-Rueda 2010; Carmona-Isunza et al. 2013; Zamora-Camacho et al. 2015; Zamora-Camacho et al. 2016). Other studies consider relatively long intervals of time (6, 12, or 24 h) that provide a mid-term view of immune-response time trends, often finding an effect only, or neatly more intensely, at early measurements (e.g., Navarro et al. 2003; Scharsack and Kalbe 2014; Zhiqiang et al. 2015).

Since the aforementioned agroecosystem conditions may deteriorate amphibian immune system as a consequence of physiological stress, I predict that immune response will be faster and more intense in natural-habitat than in agroecosystem toads. Regarding the impoverishing effect of testosterone on immune response (Muehlenbein and Bribiescas 2005), I expect females to elicit a faster and more intense immune response than males.

## Materials and Methods

### Study species

*Epidalea calamita* is a medium-size (50–75 mm of snout–vent length in this study system) Bufonid toad that occurs in diverse pristine and anthropized habitats across vast regions of Central and Southwestern Europe (Gómez-Mestre 2014). They are generalist predators of a variety of invertebrates (Boomsma and Arntzen 1985), though which they can ingest pesticides (Attademo et al. 2005). Reproduction takes place during late winter or spring, often in small temporary pools, and tadpoles can complete their metamorphosis in as little as 45 days (Gómez-Mestre 2014).

### Study sites

Field work was conducted in two adjacent habitats in Southwestern Spain: natural pine grove Pinares de Cartaya, and surrounding agroecosystems (37°20' N, 7°09' W). Capture sites were separated by 4.5–5 km. This distance is short enough to discard climate differences between them, but at once it is greater than maximum dispersal of this species (Miaud et al. 2000). On the other hand, this distance is long enough to ensure isolation of habitat characteristics. Indeed,

differences in age, body size, indicators of reproductive investment, and locomotor performance between *E. calamita* from these habitats (Zamora-Camacho and Comas, 2017; Zamora-Camacho, 2018) suggest different selective pressures in both. The natural pine grove was a *Pinus pinea* forest, with an undergrowth dominated by *Cistus ladanifer*, *Rosmarinus officinalis*, and *Pistacia lentiscus*. Although some controversy exists on the pristine or artificial origin of these trees in this region, they are known to be the dominant vegetal formation in this area for at least the last 4,000 years (Martínez and Montero 2004). As for the agroecosystem, it included traditional crop lands, which have lately turned into intensive orange, strawberry, and vegetable crops. While I obtained no permission to access private properties for water or soil analyses, land owners reported that manure and diverse synthetic NPK fertilizers are regularly added, as well as gibberellic acid, to enhance plant growth. Also, they claimed to use different kinds of insecticides, fungicides, and herbicides. The substances, the timing of their application, and the amounts spread depend on the type of crop and the discretion of each farmer, but the substances and the amounts of them that land owners declared to use were very similar. Since *E. calamita* can reproduce in very small rain pools, reproduction sites were widespread in both habitats. Due to warm climatic conditions in these locations, toads do not hibernate, but aestivate instead. Therefore, the breeding season spans from January to April.

### Toad capture and management

During the breeding season in 2015, I captured 69 toads (15 females and 18 males from agroecosystem, 18 females and 18 males from pine grove) by hand. Sexual dimorphism allows undoubted recognition of sexes: males have purple-to-pinkish vocal sacs in their throats, and blackish nuptial pads in the phalanges and tubercles of their forelimbs (Gómez-Mestre 2014). During the study, toads were kept in individual plastic terraria with wet peat as a substrate and an opaque plastic plaque as a shelter. Toad body mass was measured with a balance (model CDS-100, precision 0.01 g).

The day after capture, during the evening in order to avoid confounding effects of different moments of circadian rhythms, I measured left forelimb sole pad thickness with a pressure-sensitive micrometer (Mitutoyo; precision 0.01 mm), three times, and I considered sole pad thickness as the average of those three measurements. Immediately, I inoculated subcutaneously 0.1 mg of phytohemagglutinin (PHA; Sigma Aldrich, L-8754) diluted in 0.01 ml isotonic phosphate buffer in each toad's left forelimb sole pad (Brown et al. 2011). Phytohemagglutinin is a harmless protein that triggers a skin-swelling immune response, in which T-cells as well as other components of the immune system are involved (Kennedy and Nager 2006; Martin et al. 2006). The magnitude of the swelling is directly proportional to that of the cellular immune response (Parmentier et al. 1998; Vinkler et al. 2010). Following Smits et al. (1999), I did not use a phosphate buffer saline (PBS) control in the other forelimb sole pad of the same individuals. Smits et al. (1999) proved that PHA tests can be simplified by omitting PBS controls, with little impact on the results, and decreasing handling errors and the coefficient of variation owing to measurement inaccuracy. For this reason, PBS controls are commonly omitted in PHA tests (e.g., Martin 2005; Hale and Briskie 2007; Moreno-Rueda 2010; Moreno-Rueda and Redondo 2012), also in amphibians (Gervasi and Foufopoulos, 2008; Iglesias-Carrasco et al., 2017). The validity of this method has been assessed in amphibians (Brown et al. 2011; Clulow et al. 2015).

Habitat ( $F_{1,65} = 1.10$ ;  $P = 0.30$ ), sex ( $F_{1,65} = 0.72$ ;  $P = 0.40$ ), and their interaction ( $F_{1,65} = 0.02$ ;  $P = 0.90$ ) had no significant

effect on toad body mass, so I injected the same dose of PHA to all individuals. Afterwards, I measured sole pad thickness as described above, once per hour, for 6 h following injections. I chose this period because the objectives of this work focus on short-term immune response. Each time, I calculated sole pad swelling as the difference between each hourly post-inoculation sole pad thickness measure and sole pad thickness measure prior to inoculations. Therefore, I obtained sole pad swelling on an hourly basis. This way, I also obtained maximum sole pad swelling of each individual, irrespective of the moment when it was reached, as a measure of intensity of immune response. Also, in each case, I calculated area under the curve (AUC) of the six measurements of sole pad swelling, therefore in  $h \cdot mm$ , as an indicator of intensity of swelling response incorporating time (Fekedulegn et al. 2007), by applying the trapezoidal formula with software GraphPad Prism 7.0.

Once the experiments were finished, I released toads at their capture site. Besides sole pad swelling, toads suffered no detectable damage in the moment of their release as a consequence of this study. Toad capture and management was conducted according to Junta de Andalucía research permits (AWG/MGD/MGM/CB) and the bioethics committee of the University of Granada (permit number 18-CEEA-OH-2013); all applicable guidelines for the care and handling of animals were followed.

### Statistics

As data accomplished the criteria of residual homoscedasticity and normality, I used parametric statistics (Quinn and Keough 2002). I conducted Ordinary Least Squares Linear Models. I performed two-way ANOVAs to test the effects of habitat, sex, and their interaction on 1) hourly sole pad swelling, 2) maximum sole pad swelling, 3) area under the curve, and 4) progression of sole pad swelling (by means of a repeated measures ANOVA). Then, I repeated models removing non-significant habitat\*sex interactions. Statistical analyses were performed with software Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA).

## Results

### Hourly sole pad swelling

Within each hourly measurement, only at the third and the fourth hour after inoculation sole pad swelling differed significantly between habitats and between sexes, respectively (Table 1), being greater in pine grove than in agroecosystem toads 3 h after inoculations (Table 1; Figure 1), as predicted, and in males than in females four hours after inoculations (Table 1 and Figure 2), contrarily to expected. Note that habitat differences 2 h after inoculations were marginally non-significant (Table 1). Results remained similar when habitat\*sex interactions, non-significant in all cases (Table 1), were removed (data not shown).

### Maximum sole pad swelling

Effects of habitat ( $F_{1,65} = 0.579$ ;  $P = 0.450$ ), sex ( $F_{1,65} = 2.726$ ;  $P = 0.104$ ), and habitat  $\times$  sex interaction ( $F_{1,65} = 2.760$ ;  $P = 0.101$ ) on maximum sole pad swelling, regardless of the moment of the experiment when it was reached, were non-significant. When the interaction was removed from the model, both the effect of habitat ( $F_{1,66} = 0.454$ ;  $P = 0.503$ ) and sex ( $F_{1,66} = 2.412$ ;  $P = 0.125$ ) remained similar.

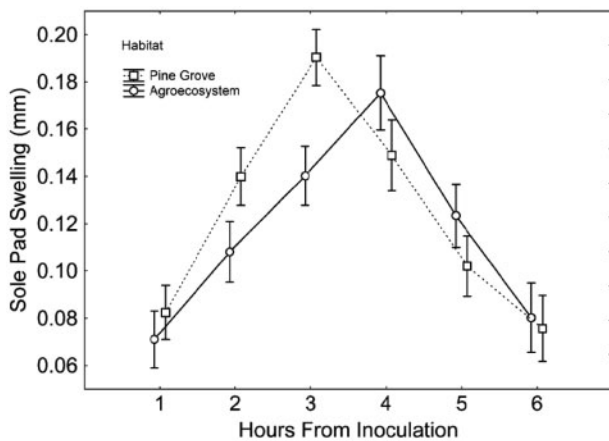
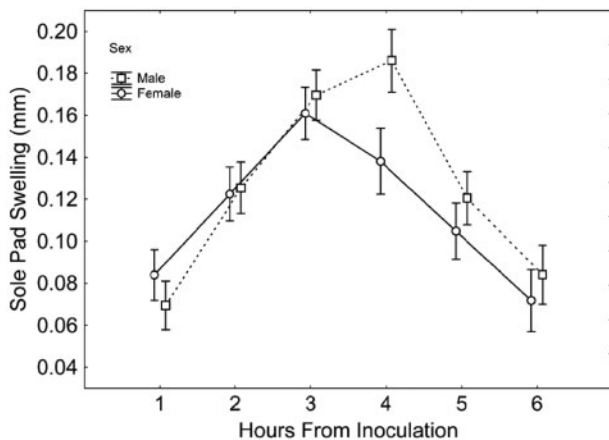
### Progression of sole pad swelling

Repeated measures ANOVAs showed that sole pad swelling peaked earlier in pine grove (the repeated measures ANOVA was significant

**Table 1.** Models testing the effects of habitat, gender, and their interaction on sole pad swelling hourly measurements

Hours from inoculation	Habitat $F_{1,65}$	Gender $F_{1,65}$	Habitat × Gender $F_{1,65}$
1	0.469 <sup>ns</sup>	0.759 <sup>ns</sup>	0.856 <sup>ns</sup>
2	3.225 <sup>§</sup>	0.028 <sup>ns</sup>	0.518 <sup>ns</sup>
3	<b>8.436**</b>	0.251 <sup>ns</sup>	1.568 <sup>ns</sup>
4	1.485 <sup>ns</sup>	<b>4.915*</b>	0.843 <sup>ns</sup>
5	1.337 <sup>ns</sup>	0.725 <sup>ns</sup>	0.185 <sup>ns</sup>
6	0.051 <sup>ns</sup>	0.367 <sup>ns</sup>	1.270 <sup>ns</sup>

Significant results are highlighted in bold. Symbols indicate: ns = non-significant; § = marginally non-significant; \* $P < 0.05$ ; \*\* $P < 0.01$ .

**Figure 1.** Sole pad swelling peaked earlier in pine grove than in agroecosystem toads. Note that habitat differences in maximum sole pad swelling were non-significant. Sample sizes were 33 toads from agroecosystem and 36 toads from pine grove. Vertical bars represent standard errors.**Figure 2.** Sole pad swelling peaked earlier in female than in male toads. However, gender differences were not significant 3 h after inoculations, while males showed greater immune response one hour later, suggesting more sustained immune response in males. Note that gender differences in maximum sole pad swelling were non-significant. Sample sizes were 33 females and 36 males. Vertical bars represent standard errors.

for habitat:  $F_{5,325} = 5.464$ ;  $P < 0.001$ ; Figure 1) and in female toads (the repeated measures ANOVA was significant for sex:  $F_{5,325} = 2.533$ ;  $P < 0.029$ ; Figure 2). Repeated measures ANOVA was not significant for habitat\*sex interaction ( $F_{5,325} = 0.599$ ;  $P < 0.701$ ).

## Area under the curve

Habitat ( $F_{1,65} = 0.213$ ;  $P = 0.646$ ), sex ( $F_{1,65} = 0.864$ ;  $P = 0.356$ ), and their interaction ( $F_{1,65} = 0.907$ ;  $P = 0.344$ ) showed no significant effect on AUC. Effects of habitat ( $F_{1,66} = 0.174$ ;  $P = 0.678$ ) and sex ( $F_{1,66} = 0.785$ ;  $P = 0.379$ ) remained non-significant when habitat\*sex interaction was removed from the model.

## Discussion

Pine grove toads deployed a more intense swelling response than agroecosystem toads, but only 3 h after inoculations. At all other times, swelling response was similar in toads from both habitats. Also, I detected no habitat differences in maximum sole pad swelling nor in AUC, which suggests a swelling response of similar intensity in toads from agroecosystems and natural habitats. Likewise, pristine habitat hellbender *Cryptobranchus alleganiensis* innate immune capacity is similar to that of conspecifics from human-disturbed habitat (Hopkins and DuRant 2011).

Those results suggest that, although animals in human-disturbed habitats can show impaired immune capacity (Brearley et al. 2013), compensation may occur under certain circumstances. In fact, urban tree lizards *Urosaurus ornatus* show reinforced innate immune capacity in comparison with individuals from natural sites, which could be triggered by more frequent immune challenges in a circumstance of non-limiting energy intake (French et al. 2008). Both habitats studied here provide toads with similar prey availability, which suggests that agroecosystem toads do not suffer reduced energy intake compared to pine grove toads (Zamora-Camacho and Comas 2017). Therefore, no reduction in energy intake could allow agroecosystem toads to compensate stressful conditions and elicit a swelling response whose magnitude is comparable to that of pine-grove toads.

However, habitat differences in toad swelling response are more accurately defined in a broader vision of short-term swelling response progression. As a whole, swelling response peaked earlier in pine-grove than in agroecosystem toads. Even though maximum pad swelling magnitude is similar in toads from both habitats, a faster swelling response could reflect a healthier immune system in pine grove toads, and hinder pathogen proliferation more efficiently (Rogers et al. 2000). In fact, agroecosystem conditions are known to reduce amphibian immune capacity (Christin et al. 2003, 2004) and increase infection prevalence as a consequence (Linzey et al. 2003; Rohr et al. 2008). Hence, the finding that agroecosystem stressful conditions impair amphibian immune capacity is not surprising. Nonetheless, results herein are novel to put forward that such impairment does not necessarily represent a reduced magnitude of the response, but may imply a delay of it instead. A delayed response as a consequence of physiological stress triggered by agroecosystem conditions could weaken toad protection against disease, and could thus be at least one of the causes behind agroecosystem toad shorter lifespan found in this system, as compared with pine grove toads (Zamora-Camacho and Comas 2017).

As an alternative explanation, although prey availability does not differ between both habitats (Zamora-Camacho and Comas 2017), energy budgets that toads allocate to different life-history traits could (Stearns 2000). Actually, agroecosystem toads seem to prioritize energy allocation to reproduction rather than to somatic preservation, as both male and female agroecosystem toads in this study system show greater indicators of reproductive investment, but shorter lifespans, than pine grove conspecifics (Zamora-Camacho and Comas 2017). Increased extrinsic mortality by

agroecosystem stressful conditions (see the introduction) could be reducing agroecosystem toad lifespan. Under those circumstances, short-lived agroecosystem toads may divert energy allocation to reproduction to the detriment of somatic preservation—which is indeed extrinsically constrained—thus compensating lesser reproductive events due to reduced lifespan with a higher reproductive investment in each, according to life-history theory (Cox et al. 2010). Consequently, reduced energy budget for somatic preservation, and thus for immune system, could result in a delayed response in agroecosystem toads. Similarly, trade-offs between immune response and reproduction have indeed been found in a number of taxa (Adamo et al. 2001; Ardia 2005; French et al. 2007).

In any case, the present results suggest that agroecosystem stressful conditions delay toad swelling response, which may impair protection against pathogens, compared with natural pine grove toads. In the light of these results, immune response impairment stands among the negative consequences of such conditions on amphibian health and populations (Alford et al. 2001; Hegde and Krishnamurthy 2014), which contributes to define the role of agroecosystems on amphibian population declines. Actually, amphibian populations are in particular decline in areas that have had pesticides applied historically (Davidson 2004). Agriculture land area is predicted to increase in the near future (Zabel et al. 2014), meaning that an increasing number of amphibian populations are bound to undergo agroecosystem conditions. Therefore, measures should be taken to milden such conditions, such as limitations in agrochemical use (Brodeur et al. 2014) or increased connectivity between amphibian habitats (Naito et al. 2012).

According to the immunocompetence handicap hypothesis, testosterone exerts suppressive effects on the immune system (Hillgarth and Wingfield 1997), which results in an across-taxa trend of males to elicit less intense immune responses than females (Klein 2000). Nevertheless, unexpectedly, the only moment when sex differences in swelling response were significant (4 h after inoculations), males deployed greater swelling response than females. However, at all other times, sex showed no effect on swelling response. Remarkably, maximum sole pad swelling, irrespective of the moment when it was reached, did not differ significantly between sexes. Similarly, lack of sex differences in AUC suggests similar intensity of swelling response in males and females. Therefore, hourly tests of sex effect on toad swelling response do not seem to support testosterone-induced immunosuppression.

Notably, a broader vision of the short-term progression of swelling response showed a significantly earlier swelling response peak in females than in males. However, sex differences 3 h after inoculations, when such female peak took place, were not significant, while 4 h after inoculations females had a significantly reduced swelling response as compared with males. Such progression pattern suggests that male swelling response was not actually slower, but more sustained in time, which should not represent a lower energy investment (Fox et al. 2005), and thus did not reflect impaired male immune response. As a whole, these results did not support male reduced immune capacity (Belliere et al. 2004). Similarly, response to PHA by testosterone-supplemented male European tree frog (*Hyla arborea*) was not reduced, but enhanced (Desprat et al. 2015). In fact, testosterone plays a dual effect on immune response: along with corticosterone, which often correlates positively with testosterone levels (Roberts et al. 2009) and is known to impair immune function (Berger et al. 2005), it can cause immunosuppression. But at the same time testosterone could improve immune capacity through increased competitive access to resources (Evans et al.

2000). Generalization of immunocompetence handicap hypothesis is indeed controversial (reviewed in Roberts et al. 2004). However, in this case, the physiological mechanisms underlying male toad capacity to deploy a more sustained swelling response than females remain obscure.

Results herein confirm impaired swelling response in agroecosystem toads, which is not surprising, as a number of studies on several taxa have reported similar negative effects on amphibian immune capacity (Christin et al. 2004; Albert et al. 2007). However, this study is novel to find evidence that such impairment does not necessarily involve a reduced magnitude of swelling response: in this case, it appears as a delay in swelling response. As for sex, swelling response magnitude did not differ between males and females. Female swelling response peaked earlier, but that of males was more sustained in time, which does not match previous across-taxa findings that male swelling response is impaired as compared to that of females.

In conclusion, most interestingly, these results show that measuring swelling response only after a standardized period following stimulation (Martin et al. 2006) might be simplistically assuming synchronal swelling response progression in all individuals. Consequently, results might be biased, or important and useful information could be lost. In fact, results in this work and their interpretation would have been completely different if I had measured swelling response only once, and the moment chosen for the measure would have caused dramatic differences. For instance, swelling response 3 h after inoculations was greater in pine grove than in agroecosystem toads, while it did not differ between sexes at that moment. However, the outlook was completely different 4 h after inoculations, with no habitat differences in swelling response, but males showing greater swelling than females. Finally, if I had made measures only at any other moment, I would have detected no effect of habitat or sex. Nevertheless, a broader view including AUC and maximum magnitude of swelling response, regardless of the moment when it was deployed, as well as progression of swelling response, provided an accurate panorama of the actual effects of habitat and sex on short-term toad swelling response. Therefore, I urge ecoimmunologists to implement immune response progression in their studies, or, at least, to assess its synchrony among the groups they are studying, in order to obtain realistic, unbiased results in a broader context.

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