Immune responsiveness to phytohemagglutinin displays species but not sex differences in three anuran species

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Phytohemagglutinin (PHA)-induced skin swelling response is widely used as a rough surrogate of integrative cell-mediated and innate immunity across multiple vertebrate taxa due to its simplification and feasibility. However, little is known whether there are sex and interspecific differences of immune responsiveness to PHA in ectotherms, especially for anurans. Therefore, we studied sex and species differences of PHA response in three anurans, Asiatic toads (*Bufo gargarizans*), Dark-spotted frogs (*Pelophylax nigromaculatus*) and Mongolian toads (*Pseudepidalea raddei*), captured in northern regions of Anhui Province (China). Footpad thickness was measured prior to (0 h) and after (6, 12, 24, 48 and 72 h) a PHA injection and normalized against saline injection in the opposite footpad. Body mass was recorded at the beginning (0 h) and end of each assay (72 h). Results showed effects of PHA assay, sex and taxa on body mass. Relative maximum swelling response (PHA_{max}) ranged from 18.58 to 29.75 %, 9.77 to 20.56 % and 21.97 to 31.78 % and its occurrence over time was apparent 10.6 to 19.72 h , 7.74 to 14.01 h and 17.39 to 23.94 h postinjection for Asiatic toads, Dark-spotted frogs and Mongolian toads, respectively. Finally, the magnitude or timing of PHA_{max} in Dark-spotted frogs was significantly thinner and faster than in Mongolian toads, and Asiatic toads had an in-between value, not different from the other two species. The magnitude of PHA_{max} was significantly positively correlated with the timing of PHA_{max} considering individuals altogether, but not when analyzed within species. Our results indicate that male and female anuran species respond similarly to PHA antigen stimulation, but the magnitude and timing of PHA_{max} is species-specific. Briefly, we provide new evidence for the suitability of PHA assay in non-model anuran species with different body sizes, and exhort the need to further investigate the nature of PHA assay at the hematological and histological levels in order to extend its application in ecoimmunological studies of amphibians.

1 **Immune responsiveness to phytohemagglutinin displays species but not sex**

2 **differences in three anuran species**

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10 **Short title:** Sex and species changes of PHA response

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15 **ABSTRACT**

16 Phytohemagglutinin (PHA)-induced skin swelling response is widely used as a rough surrogate

17 of integrative cell-mediated and innate immunity across multiple vertebrate taxa due to its

18 simplification and feasibility. However, little is known whether there are sex and interspecific

19 differences of immune responsiveness to PHA in ectotherms, especially for anurans. Therefore,

20 we studied sex and species differences of PHA response in three anurans, Asiatic toads (*Bufo*

21 *gargarizans*), Dark-spotted frogs (*Pelophylax nigromaculatus*) and Mongolian toads

22 (*Pseudepidalea raddei*), captured in northern regions of Anhui Province (China). Footpad

23 thickness was measured prior to (0 h) and after (6, 12, 24, 48 and 72 h) a PHA injection and

24 normalized against saline injection in the opposite footpad. Body mass was recorded at the

25 beginning (0 h) and end of each assay (72 h). Results showed effects of PHA assay, sex and taxa

26 on body mass. Relative maximum swelling response (PHA_{max}) ranged from 18.58 to 29.75 %,

27 9.77 to 20.56 % and 21.97 to 31.78 % and its occurrence over time was apparent 10.6 to 19.72 h ,

28 7.74 to 14.01 h and 17.39 to 23.94 h postinjection for Asiatic toads, Dark-spotted frogs and

29 Mongolian toads, respectively. Finally, the magnitude or timing of PHA_{max} in Dark-spotted frogs

30 was significantly thinner and faster than in Mongolian toads, and Asiatic toads had an in-between

31 value, not different from the other two species. The magnitude of PHA_{max} was significantly

32 positively correlated with the timing of PHA_{max} considering individuals altogether, but not when

33 analyzed within species. Our results indicate that male and female anuran species respond

34 similarly to PHA antigen stimulation, but the magnitude and timing of PHA_{max} is species-specific.

35 Briefly, we provide new evidence for the suitability of PHA assay in non-model anuran species

36 with different body sizes, and exhort the need to further investigate the nature of PHA assay at

37 the hematological and histological levels in order to extend its application in ecoimmunological

38 studies of amphibians.

39 **INTRODUCTION**

40 Amphibians are experiencing a global biodiversity crisis derived from diseases, habitat

41 destruction and altered ecosystems (*Clulow, Harris & Mahony, 2015*). A wide range of

42 physiological traits including innate and adaptive immunity are important to cope with the

43 increasing number of stressors and pathogen threats faced in their lifetimes (*Carey, Cohen &*

44 *Rollins-Smith*, *1999*). As other physiological traits, immune defense is also energetically costly to

45 mount, and a trade-off relationship may occur within the immune system or between

46 immunological defenses and other nutrient-demanding processes such as growth, reproduction

47 and thermoregulation based on limited total energy resources (*Lochmiller & Deerenberg, 2000*).

48 Nowadays, it is still an open question what immune parameter measures are appropriate to reflect

49 an organism's defense capacity against pathogens (*Downs, Adelman & Demes*, *2014*). Among

50 several immunological assays, the phytohemagglutinin (PHA) skin-swelling test is one of the

51 most widely used techniques to evaluate the magnitude of vertebrate immune response (*Sheldon*

52 *& Verhulst, 1996*; *Boughton, Joop & Armitage, 2011*; *Vinkler & Albrecht, 2011*; *Brock, Murdock*

53 *& Martin*, *2014*). The assay consists of a subcutaneous injection of PHA that induces infiltration

54 of leukocytes and where swelling is interpreted as an integrative immune response (*Christe,*

55 *Arlettaz & Vogel*, *2000*; *Kennedy & Nager*, *2006*; *Martin et al.*, *2006*; *Demas et al.*, *2011*; *Brock,*

56 *Murdock & Martin*, *2014*) or as an inflammation in endotherms (*Vinkler et al.*, *2012*; *Bílková,*

57 *Vinklerová & Vinkler*, *2015*; *Bílková et al.*, *2016*). However, inferences using this assay should be

58 carefully evaluated because not always a greater immune response or inflammation means better

59 defenses against infections or higher survival probabilities (*Graham, Allen & Read*, *2005*; *Vinkler*

60 *et al.*, *2012*). To date, the histological base of this response has been demonstrated across

61 different vertebrate taxa [e.g., birds (*Martin et al.*, *2006*), bats (*Turmelle et al.*, *2010*), amphibians

62 (*Brown, Shilton & Shine*, *2011*) and crocodiles (*Finger et al.*, *2013*)], although no apparent

63 correlation between swelling response and leukocyte profiles was reported in the fossorial

64 mammal *Ctenomys talarum* (*Merlo, Cutrera & Zenuto*, *2014*). However, more recently PHA

65 swelling has been associated to leukocyte profiles and serum bactericidal activity in endotherms

66 (*Bílková, Vinklerová & Vinkler*, *2015*; *Zhang & Zhao*, *2015*), or parasite infection in rodents

67 (*Ctenomys talarum*, *Merlo, Cutrera & Zenuto*, *2015*). Despite all the merits of this assay, a caveat

68   must be warned because there is still some debate about its meaning. An objection is that the

69   response to PHA of an organism might not be representative of the actual response of the same

70   host to pathogens (reviewed by *Graham, Allen & Read*, *2005*; *Owen & Clayton*, *2007*; *Brock,*

71   *Murdock & Martin*, *2014*) or that energetic costs of mounting a response to PHA may vary in

72   small mammals (*Zhang, Qiu & Wang*, *2011*; *Merlo et al.*, *2014*) and birds (*Ots et al.*, *2001*;

73   *Martin, Scheuerlein & Wikelski*, *2003*; *Lee, Martin & Wikelski*, *2005*; *Nilsson, Granbom &*

74   *Råberg*, *2007*).

75   PHA assay was first used in ecoimmunological studies of amphibians in the northern leopard

76   frogs, *Rana pipiens*, showing noticeable alterations of the immune response after an exposure to

77   different pesticides (*Gilbertson et al.*, *200*3). The sites chosen to inject PHA are diverse for post-

78   metamorphic amphibians and none is applicable to all species, for example, toe webbing (*Brown,*

79   *Shilton & Shine*, *2011*), footpad (*Fites et al.*, *2014*), thigh (*Gervasi & Foufopoulos*, *2008*) and

80   lower leg below the knee (*Clulow, Harris & Mahony*, *2015*). The histological mechanism of the

81   inflammatory response induced by PHA antigen was examined in the toe webbing of cane toads,

82   *Rhinella marinus* (*Brown, Shilton & Shine*, *2011*), which displayed a rapid (<12 h) infiltration of

83   neutrophils, eosinophils and macrophages at the injection site, followed by an influx of

84   lymphocytes by 24 h postinjection. In addition, marked interspecific variations of leukocytes

85   were observed in the lower leg of green and golden bell frogs, *Litoria aurea* and striped marsh

86   frogs, *Limnodynastes Peronii* (*Clulow, Harris & Mahony*, *2015*). PHA assay has been also used

87   to assess the immune responsiveness under different ecologically relevant conditions, such as

88   when amphibians are forced to accelerate the metamorphosis by exposition to environment

89   desiccation (*Gervasi & Foufopoulos*, *2008*), treated with protein restricted diets *(Venesky et al.,*

90   *2012*), and exposed to density stress (*Clulow, Harris & Mahony*, *2015*). In different vertebrate

91   species, PHA response peaks in a wide time window, from 3 to 48 h (*de Bellocq et al.*, *2006*;

92   *Martin et al.*, *2006*; *Turmelle et al.*, *2010*; *Xu & Wang*, *2010*; *Brown, Shilton & Shine*, *2011*;

93   *Finger et al.*, *2013*; *Josserand et al.*, *2015*; *Zhang, Huang & Zhao*, *2015*; *Bílková et al.*, *2016*),

94   with no apparent sex differences in some small mammals (*Zhang, Qiu & Wang*, *2011*; *Merlo,*

95   *Cutrera & Zenuto*, *2014*) and amphibians (*Brown et al.*, *2015*; *Clulow, Harris & Mahony*, *2015*).

96   Most up-to-date information available on the time course and temporal dynamics of the PHA

97   responses within and among populations or species are for birds (*Navarro et al.*, *2003*) and small

98   mammals (*de Bellocq et al.*, *2006*; *Zhang, Huang & Zhao*, *2015*). Conversely, data on

99   amphibians are still scarce, especially for those anurans living in different environments (*Clulow,*

100 *Harris & Mahony*, *2015*). In a scenario of increasing disease and toxic threats, it is mandatory the

101 need to provide comparable information of individual health status induced by PHA antigen on

102 key amphibian species (*Carey, Cohen & Rollins-Smith*, *1999*; *Fites et al.*, *2014*; *Clulow, Harris*

103 *& Mahony*, *2015*).

104   In line with this situation, our aim was to test PHA assay response across different species with

105 different body masses to extend its use to other non-model anuran species with different

106 morphological and physiological adaptations to their habitats. Accordingly, we provide an

107 evaluation of the PHA-induced skin-swelling assay among three sympatric anuran species with

108 different habitat use in a range of body mass (30 to 90 g) of Central China (Northern Anhui

109 province), Dark-spotted frogs (*Pelophylax nigromaculatus*), Mongolian toads (*Pseudepidalea*

110 *raddei*) and Asiatic toads (*Bufo gargarizans*). In the three anuran species, both Asiatic and

111 Mongolian toads are terrestrial with slow movements, whereas Dark-spotted frogs dwell lentic

112 ponds or rice fields and move fast in their habitats (*Fei et al.*, *2009a, b*). In addition, Asiatic toads

113 start their reproductive activities during hibernation (*Ji et al.*, *1995*; *Zou et al.*, *1996*) and are

114 easily infected by multiple parasites (*Zhao et al.*, 2014). On the other side, while Mongolian toads

115 (*Zhou*, *1996*) and Dark-spotted frogs initiate their reproduction after hibernation (*Wu*, *1965*; *Cao*

116 *et al.*, *1996*; *Jin, Qu & Zhang*, *2014*; *Jin & Zhang*, *2014*), the former is not infected by multiple

117 digenetic trematodes (*Li & Gu*, *1978*), but the latter, displays a 90.38% helminth and parasite

118 infection rate (*Zhu et al.*, *2014*). We predicted that: (i) Within species, both sexes will exhibit

119 similar swelling responses. (ii) The temporal dynamics of the response to PHA will be species-

120 specific, as well as the magnitude of the maximum swelling at the injection point in the footpad

121 area. In addition, we tested the effect of the PHA assay on the body mass of the individuals and

122 its influence on the swelling response.

123 **MATERIALS AND METHODS**

124 **Animal husbandry**

125 All animal procedures were carried out under the guidelines of the Animal Care and Use

126 Committee of Anhui Agricultural University (Permit number: 20120410). During April and May,

127 2012, we captured nine and eight males and nine and ten females Asiatic and Mongolian toads,

128 respectively, in Mengcheng county (116.55°E 33.25°N Anhui province, China), and eight males

129 and eight females of Dark-spotted frogs in Feixi county (117.17°E 31.70°N Anhui province,

130 China). Immediately after capture, animals were transported to an animal indoor facility of the

131 Anhui Agricultural University in Hefei, and housed in aquarium tanks (40 × 50 × 90 cm). They

132 were kept under natural ambient temperature (17-21°C) and photoperiod (around 12L:12D).

133 Fresh dechlorinated tap water was provided *ad libitum* and mealworms were provided as their

134 food every 2-3 days during trials.

135 **PHA-induced skin-swelling test**

136 In all cases PHA-induced skin-swelling responses were always assayed two or three days after

137 capture. Animals were subcutaneously injected with a dose of 50 μl of the PHA solution (PHA-P,

138 Sigma L-8754, Sigma-Aldrich, St Louis, MO, USA, 1 mg of PHA-P dissolved in 0.3 mL of

139 saline solution) in the footpad area pointing to the junction of the second and third digits of the

140 right hind foot, and the opposite left footpad as an intragroup control was injected with an equal

141 volume of sterilized saline by using a 100 μl micro injector (Shanghai Anxiang micro injector

142 factory, China). Footpad thickness was measured with a micrometer (Tesa Shopcal, Renens,

143   Switzerland) to ± 0.01 mm, prior to injection (time = 0 h), and then at 6, 12, 24, 48 and 72 h

144   postinjection. Footpad thickness was measured six times at each time point by the same operator

145   at the point of injection and the average value was used for analysis (*Smits, Bortolotti & Tella*,

146   *1999*; *de Bellocq et al.*, *2006*). Body mass (± 0.001 g) was recorded at time 0 h and then, 72 h

147   after injection in each individual of each species. In order to minimize measurement biases due to

148   different initial footpad thickness, the expressed percentages of PHA-induced swelling responses

149   (%) were calculated as described by *Brown et al.* (*2015*) for cane toads (*Rhinella marina*).

$$100*\left[\left[\frac{\text{PHA thickn ess at x hour - PHA thickn ess at 0 hour}}{\text{PHA thickn ess at 0 hour}}\right] - \left[\frac{\text{Saline thickness at x hour - Saline thickness at 0 hour}}{\text{Saline thickness at 0 hour}}\right]\right]$$

150   **Statistics**

151   Data are presented as means ± 1 SEM. Each measurement of PHA swelling response was

152   replicated six times. A very high repeatability of these measurements was observed using

153   intraclass correlation coefficients ($r = 0.99$, $P < 0.001$). Therefore, hereafter the mean value of the

154   six measurements of the swelling response for each individual was used for analyses.

155   Differences among group means were analyzed using analyses of the variance and covariance.

156   Assumptions of ANOVAs and ANCOVAs were tested and when no met, the nonparametric

157   Kruskal-Wallis H test was used. Tukey's HSD test was carried out to evaluate pairwise

158   comparisons after ANOVAs, and Dunn's multiple comparisons test was performed after Kruskal-

159   Wallis H. Species, sex and PHA assay effects on body mass were evaluated using a mixed-design

160   ANOVA with an autoregressive (order 1) covariance structure (*Littell, Pendergast & Natarajan*,

161   *2000*) including pre and post-assay body masses, sex and species as fixed factors and subjects

162   nested in sex and species as random factors. A mixed-design ANOVA with an autoregressive

163 (order 1) covariance structure was performed to test differences within individuals of the swelling

164 response at different times (6, 12, 24, 48 and 72 h) after the PHA injection in the three species

165 studied, using time after injection and sex as fixed factors and subjects nested in sex as a random

166 factor. The maximum skin swelling response (PHA_{max}) after the PHA injection of an individual

167 was defined as the highest of the five values measured along trial time (i.e., 6, 12, 24, 48 and 72

168 h), and the time when PHA_{max} was reached, as the time of peak. PHA_{max} was contrasted among

169 the three species using an ANOVA, using full factorial design with taxa and sex as predictor

170 variables.

171   Likelihood comparisons of the Akaike Information Criterion (AIC_c) with a second-order bias

172 correction values produced by models using different parameters (i.e., time-after PHA injection

173 and sex as variables and mean body mass as a covariate) for the different species studied were

174 used as tool to select the predictors that described the best model for PHA swelling response

175 within species (*Burnham & Anderson, 2004*). Using this procedure, variables included in the

176 models were time-after PHA injection and sex. The same process was used to select the predictor

177 variables, taxa and sex, to analyze PHA_{max}. Since times of peak and transformed values did not

178 meet the normality assumption (D'Agostino's K^2 test, $P < 0.05$), a Kruskal-Wallis H test was used

179 to test differences among taxa and sex. The correlation between PHA_{max} and timing of the peak

180 was analyzed using the Spearman's rank correlation coefficient. In all tests, statistical

181 significance was accepted for $\alpha < 0.05$. Statistical analyses were performed using JMP Pro 12.2.1.

182 **RESULTS**

183 **Effects of PHA test, sex and species on body mass**

184   A mixed-design ANOVA was conducted to test PHA assay effect on body mass in male and

185 female individuals of the three species studied. Results showed significant body mass pre- and 72

186 h post- PHA injection changes within subjects (Table 1). Pairwise comparisons showed that all

187 groups, but Asiatic toad males lost body mass after the PHA assay (Fig. 1). Between subjects'

188 effects (i.e. sex and taxa) and all interactions were statistically significant (Table 1). Sex

189 differences of body mass before the assay were apparent for Asiatic toads, while Dark-spotted

190 frogs and Mongolian toads did not differ. After the assay, body mass differed significantly

191 between sexes in all species (Fig. 1). Body mass differences between taxa were also apparent,

192 Dark-spotted frogs were lighter than Mongolian toads and Asiatic toad males, and in turn, lighter

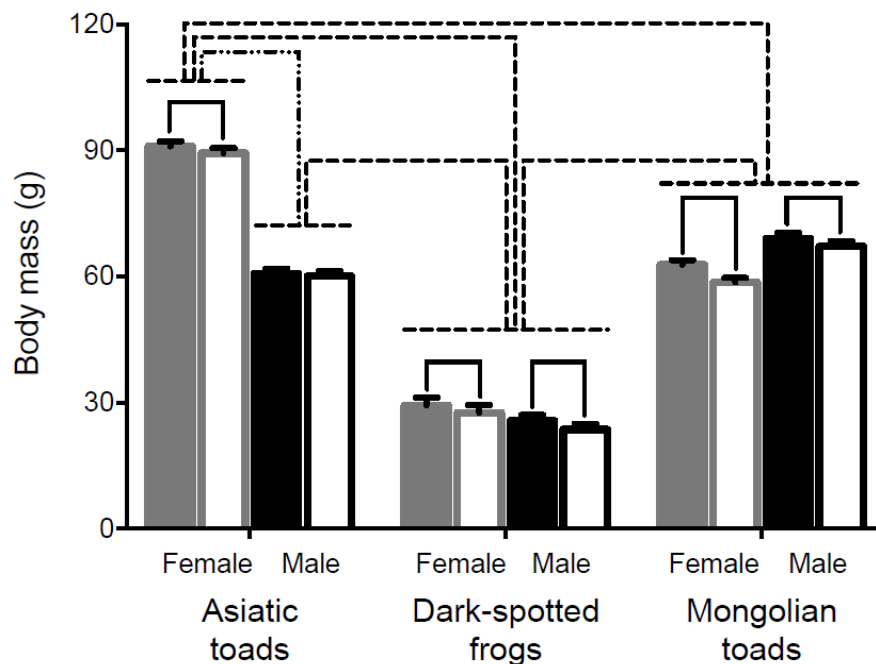
193 than the females of Asiatic toads (Fig. 1).

194 **Table 1.** Results of a full factorial mixed-design ANOVA with an autoregressive (order 1)

195 covariance structure (*Littell, Pendergast & Natarajan, 2000*) run to test changes within subjects

196 of the body mass before and after the PHA test using assay, sex and taxa as fixed factors and the

197 subjects nested in sex and taxa as random factors.

Source	d.f.	F	P
Assay	1, 43.3	146.952	<0.0001
Sex	1, 44	7.876	0.0074
Taxa	2, 43.9	83.365	<0.0001
Sex x assay	1, 43.6	8.767	0.0049
Sex x taxa	2, 43.9	13.683	<0.0001
Taxa x time	2, 43.4	13.107	<0.0001
Sex x taxa x assay	2, 43.5	4.988	0.0112

198   **Figure 1.** Effects of the phytohemagglutinin (PHA) assay, sex and taxa on body mass in the

199   three anuran species studied, Asiatic toads, Dark-spotted frogs and Mongolian toads. Bars

200   represent mean body mass ± 1 S.E.M. Body masses of female and male individuals of each

201   species before the PHA injections are represented by solid gray and black bars respectively, and

202   72 h postinjection body masses of females and males are represented by unfilled gray and black

203   bars, respectively. Bars or groups of bars connected by either solid (pre vs. post PHA injection),

204   dashed-double-dot (sex) or dashed (species) lines indicate significant differences of body masses

205   by Tukey's HSD test ($P < 0.05$).

206   **PHA-induced skin swelling response**

207   Skin swelling response after PHA injection showed significant differences within individuals of

208   the three species studied (Table 2, Ps <0.0001). All species increased the thickness of the footpad

209   area following the PHA injection reaching a maximum skin swelling response (Tukey's HSD test,

210    $P < 0.05$) and then the swelling significantly declined to a minimum at 72 h (Fig. 2). A closer

211   inspection of PHA_{max} and its occurrence over time (Fig. 3) showed that both parameters varied

212   within species: the 95% CI of PHA_{max} and times of peak ranged from 18.58 to 29.75 % and 10.6

213   to 19.72 h, 9.766 to 20.56% and 7.738 to 14.01 h, and 21.97 to 31.78 % and 17.39 to 23.94 h for

214   Asiatic toads, Dark-spotted frogs and Mongolian toads, respectively. Sex effect or interaction

215   time x sex were not significant in the three species (Table 2). Between species differences in the

216   magnitude of PHA_{max} were significant (ANOVA, taxa effect: $F_{2,49} = 4.1664$; $P < 0.022$, Fig. 3A),

217   but not for sex ($F_{1,49} = 0.4994$; $P = 0.484$) or taxa x sex interaction ($F_{1,49} = 0.6600$, $P = 0.522$).

218   Tukey's HSD test showed that maximum PHA-induced swelling response of Dark-spotted frogs

219   was significantly thinner than that of Mongolian toads, and Asiatic toads PHA_{max} was not

220   different from Mongolian and Dark-spotted frogs (Fig. 3A). Timing of PHA_{max} peaks between

221   species was also statistically significant (Kruskal-Wallis test: $H_2 = 12.2179$, $P = 0.0022$, Fig. 3B)

222   with score means of 23.33, 17.5 and 33.89 for Asiatic toads, Dark-spotted frogs and Mongolian

223   toads, respectively. Dunn's multiple comparisons test showed that Dark-spotted frogs reached

224   PHA_{max} significantly faster than Mongolian toads, and Asiatic toads had an in-between value, not

225   different from any of the other two species (Fig. 3B). The relationship between PHA_{max} and

226   timing of the peak between data of all individuals was significant (Spearman's rank correlation

227   coefficient, $\rho = 0.327$, $P < 0.05$), but we fail to find a significant correlation ($P > 0.05$) within

228   species with or without sex as a category.

229   **Table 2.** A full factorial mixed-design ANOVA with an autoregressive (order 1) covariance

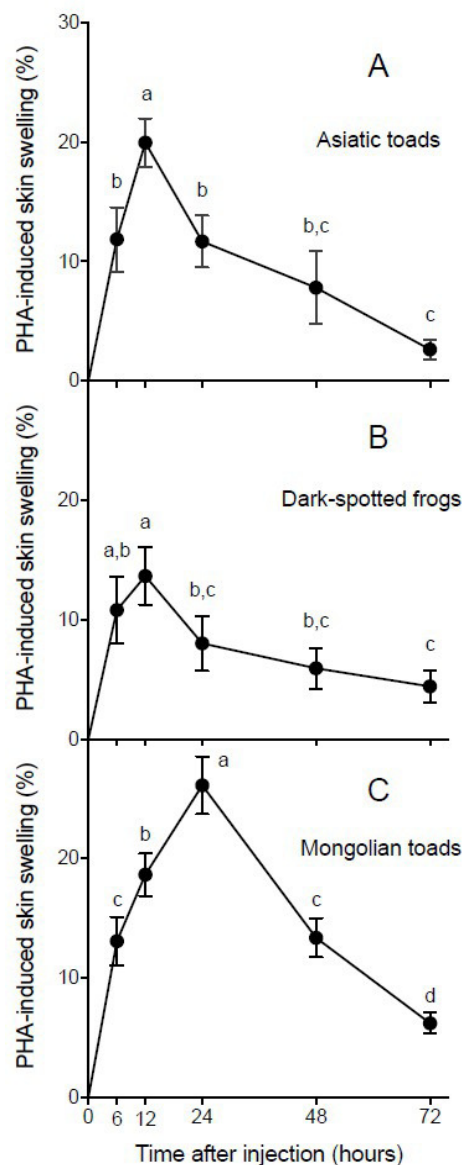
230   structure (*Littell, Pendergast & Natarajan, 2000*) was run to test changes of the swelling response

231   after the PHA injection using time after PHA injection, sex and taxa as fixed factors, and the

232   subjects nested in sex and taxa as random factors. Sex and taxa were included as predictor

233   variables and body mass excluded as a covariate based on likelihood comparisons of AICc of the

234   different models (*Burnham & Anderson, 2004*).

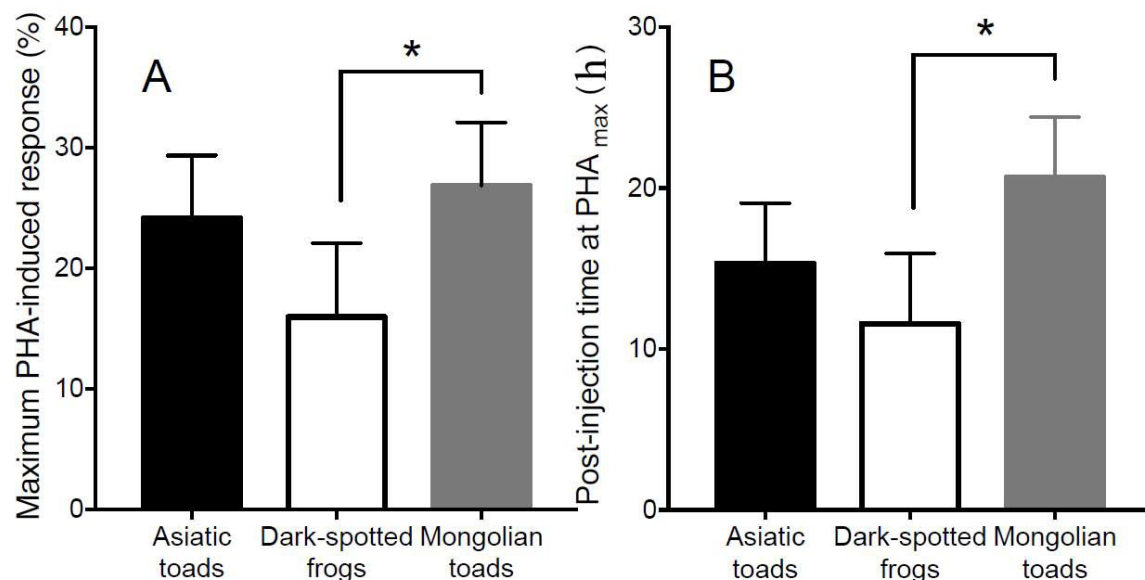
Species	time after PHA injection			sex			time x sex		
	d.f.	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*
Asiatic toads	4, 53.5	11.42	<0.0001	1, 54.4	1.07	0.306	4, 53.9	0.49	0.746
Dark-spotted frogs	4, 37.9	8.18	<0.0001	1, 14.7	1.69	0.214	4, 39	1.92	0.127
Mongolian toads	4, 46	31.80	<0.0001	1, 38.9	0.21	0.651	4, 46.5	1.41	0.246

235 **Figure 2.** Mean (± 1 S.E.M.) phytohemagglutinin (PHA)-induced skin swelling response,

236 expressed as the percentage of the initial footpad area thickness corrected by the swelling that

237 produce an injection of the same amount of saline solution in the left footpad (see Materials and

238 methods), at 6, 12, 24, 48 and 72 h after PHA injections in Asiatic toads (A), Dark-spotted frogs

239 (B) and Mongolian toads (C). Points not connected by the same lower case letter are significantly

240 different (Tukey's HSD test, $P < 0.05$).

241 **Figure 3.** Mean values ± 95% C.I. of (A) maximum phytohemagglutinin-induced skin swelling

242 response (PHA$_{max}$) and (B) the times of peak of Asiatic toads, Dark-spotted frogs and Mongolian

243 toads. Bars connected by brackets are significantly different (P <0.05) by Tukey's HSD test and

244 Dunn's multiple comparisons test in (A) and (B), respectively.

245 **DISCUSSION**

246 Consistent with our predictions, male and female anurans responded similarly to PHA (Table 2),

247 suggesting that PHA assay can be used as an effective index for measuring intra-population

248 variations in immune functions within either sex. In addition, PHA assay as an integrative

249 response is comparable in ecoimmunological studies of amphibians with different body sizes,

250 early or delayed primary PHA$_{max}$ may match with their reproductive activities and parasite

251 infections from outdoor environmental conditions.

252 Effects of the PHA assay, sex and taxa on body mass were marked in the three anuran species

253 (Table 1). All groups except for Asiatic toad males lost body mass after the PHA assay (Fig. 1),

254 suggesting that it is necessary to measure their body masses at least prior to injection (at 0 h) and

255 at 72 h postinjection. Compared with the toe webbing of large-bodied cane toads (*Brown, Shilton*

256 *& Shine, 2011*) and the lower leg below the knee of several small-bodied tree frogs (*Clulow,*

257 *Harris & Mahony, 2015*), the footpad site injected also displayed a measurable inflammation

258 response after PHA antigen injection, which can be further applied to other anuran species with

259 body sizes in a range of at least 30 to 90 g. As suggested from bird (*Smits, Bortolotti & Tella,*

260 *1999*) and later from amphibian (*Josserand et al., 2015*) studies on PHA measurement, a more

261 simplified protocol without saline injection should be considered in future research in

262 amphibians.

263  In the present study, no sex differences were found for immune responsiveness to PHA in the

264 three anuran species (Table 2, Fig. 2). Similar results had been recorded in chinstrap penguins,

265 *Pygoscelis antartica* (*Moreno et al., 1998*), wild rodent species (*Zhang, Qiu & Wang, 2011*;

266 *Merlo, Cutrera & Zenuto, 2014*), and several anuran species (*Brown et al., 2015*; *Clulow, Harris*

267 *& Mahony, 2015*). The PHA inflammation assay uses a lectin to elicit localized inflammation that

268 reflects an organism's capacity to mount a dynamic innate and cell-mediated immune response.

269 In a wild rodent, immune responsiveness to primary PHA was positively correlated with the

270 proportion of neutrophils and serum bactericidal capacity in circulating blood (*Zhang & Zhao,*

271 *2015*), and the energetic cost of mounting this response was low (*Zhang, Qiu & Wang, 2011*;

272 *Merlo et al., 2014*). In amphibians, the primary PHA injection may mainly involve innate

273 phagocytes and granulocytes, and a second PHA injection would strongly activate lymphocytes

274 (*Brown, Shilton & Shine, 2011*; *Fites et al., 2014*; *Josserand et al., 2015*). In the present study, the

275 activation of the innate immunity induced by a single dose of PHA antigen may represents male

276 and female anurans responses to cope with infectious diseases and changing environmental

277 conditions. During April and May, Asiatic toads almost terminated their reproductive activities

278 (*Ji et al., 1995*; *Zou et al., 1996*), whereas Mongolian toads (*Zhou, 1996*) and Dark-spotted frogs

279 just initiated their reproductive activities (*Wu, 1965*; *Cao et al., 1996*; *Jin, Qu & Zhang, 2014*; *Jin*

280 & Zhang, 2014). Male serum testosterone concentrations are higher during the reproductive than

281 in the non-reproductive period in Dark-spotted frogs (Hu, 1990), or before reproduction than after

282 reproduction in Asiatic toads (Hu, 1989). Based in life history theory, it can be hypothesized that

283 males may show a reduced immune response and an increased susceptibility to disease than

284 females due to the suppressive effects of androgens on the immune system (Zuk & McKean,

285 1996). Hence, a suppression or at least a reduction of male immune responses is a reasonable

286 prediction if a trade-off relationship exists between immunity and reproduction (Lochmiller &

287 Deerenberg, 2000). Moreover, male anurans might face a greater risk exposed to more infectious

288 diseases than females because they need croak and move to find a mate during reproduction,

289 which is energetically costly too (Hawley & Altize, 2011). Strikingly in this study, despite the

290 different reproductive status, vocal and locomotor activity, no apparent dissimilarities were found

291 between male and female anurans in the immune responsiveness pattern to PHA (Fig. 2). Sex

292 differences in immune functions may be associated to the immunological parameters (Boughton,

293 Joop & Armitage, 2011; Downs, Adelman & Demes, 2014) or vertebrate life history characters

294 involved (Stoehr & Kokko, 2006). For example, semi-terrestrial salamanders, Desmognathus

295 ochrophaeus females were reported to heal slower than males from an experimental cutaneous

296 wound, used as a measure of an integrative immune response, and the difference in healing time

297 was related to potential energetic constraints (Thomas & Woodley, 2015). However, it has also

298 described that Bufo americanus females and larger males display similar proportions of

299 heterophils acting as first-line defenses against bacterial infection in their leukocyte profiles

300 (Forbes, McRue & Shutler, 2006).

301 In a range from fast to slow life span rodents, empirical evidences show that different

302 components of the immune system might display similar or different changing tendencies

303 (Martin, Hasselquist & Wikelski, 2006; Martin, Weil & Nelson, 2007; Previtali et al., 2012). As

304 an integrative response, the temporal patterns of the PHA response might change in different

305   rodents, and individuals that responded faster had a lower maximum response than those with a

306   more delayed response (*de Bellocq et al., 2006*). In twelve Lark species, environmental proxies of

307   antigen exposure explained variation in immune investment better than proxies of pace of life

308   (*Horrocks et al., 2015*). In anurans, unlike endotherms and regardless of body size, the primary

309   response to PHA antigen injections is mainly attributed to innate immunity of immune system

310   and recognized as the first-line defense to outdoor pathogens (*Brown, Shilton & Shine, 2011*;

311   *Josserand et al., 2015*). The interspecific differences of the magnitude or timing of PHA_{max} were

312   observed in the three anuran species (Table 2, Fig. 3), suggesting that a trade-off relationship

313   between intensity and latency of the PHA response may hold among anurans. In adult anurans,

314   PHA_{max} may be reached at 12 h (*Clulow, Harris & Mahony, 2015*), 14 h (*Josserand et al., 2015*),

315   24 h (*Brown, Shilton & Shine, 2011*), or 48 h (*Clulow, Harris & Mahony, 2015*) after PHA

316   injection. For post-metamorphic amphibians, a range of ecologically meaningful environmental

317   stressors, such as pond desiccation (*Gervasi & Foufopoulos, 2008*), different protein

318   concentrations of diets (*Venesky et al., 2012*), and low and high population density (*Clulow,*

319   *Harris & Mahony, 2015*) have great influences on the inflammation response induced by PHA

320   antigen. Asiatic and Mongolian toads, and Dark-spotted frogs are sympatric common species, but

321   the two formers are terrestrial and disperse slowly, and the latter inhabit lentic ponds and disperse

322   fast by swimming or jumping (*Fei et al., 2009a, b*). It has been reported that the helminth and

323   parasite infection rates in Dark-spotted frogs and Asiatic toads are 90.38% and 100%,

324   respectively, and the average intensity of infection is 20.21 and 28.34, respectively in Shanghai

325   (*Zhu et al., 2014*; *Zhao et al., 2014*). However, Mongolian toads were not infected by multiple

326   digenetic trematodes from north China (*Li & Gu, 1978*). PHA-triggered inflammation was

327   similarly impaired by *Eimeria sp.* infection alone or co-occurring with a number of

328   gastrointestinal nematodes in the subterranean rodent, *Ctenomys talarum* (*Merlo, Cutrera &*

329   *Zenuto, 2015*). In the present study, Dark-spotted frogs with an early PHA response did show a

330 lower peak response than Mongolian toads with a late response (Fig. 3), suggesting that parasite

331 infections may evoke the changes of PHA response patterns. However, in *Xenopus laevis*,

332 swelling induced by single injection of PHA was not significantly affected by the fungus disease,

333 *Batrachochytrium dendrobatidis* supernatants, but swelling caused by a secondary injection of

334 PHA was significantly reduced by *B. dendrobatidis* supernatants (*Fites et al.*, *2014*). It is noted

335 that the magnitude of PHA$_{max}$ was significantly positively correlated with the timing of PHA$_{max}$

336 between data of all individuals from the three anuran species, but not for the data within species,

337 suggesting that the magnitude and timing of PHA$_{max}$ is species-specific.

338 Overall, we found that immune responsiveness to PHA did not differ between sexes for any

339 anuran species (Table 2, Fig. 2), but PHA$_{max}$ and its timing for toads and frogs varied among

340 species (Fig. 3). This scenario allows PHA assay to be applied to a broad range of amphibian

341 species under field conditions, as a simple, inexpensive and effective proxy to quantify and

342 evaluate a facet of immune response. For future ecoimmunological studies of amphibians, PHA

343 assay will play an important role by integrating the changes of other components of immune

344 system facing newly emerging pathogens or environmental factors. Lastly, we caution that it is

345 still necessary to test the validation of PHA assay in advance for a new species by using

346 appropriate internal controls before carrying out studies under specific circumstances.

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