

Immune challenges and visual signalling in tree frogs

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Abstract In animals, mate-choice is often based on sexual signals that carry information and help the receiver make the best choice to improve the receiver's fitness. Orange visual sexual signals have been hypothesised to carry immune information because they are often due to carotenoid pigments which are also involved in immunity response. Although many studies have focused on the direct relationships between coloration and immunocompetence, few studies have simultaneously studied immunocompetent response and coloration variation after an immune challenge. We tested this hypothesis on starved and ad libitum-fed males of the European tree frog *Hyla arborea*. Our results show that male coloration is not a reliable indicator of its immune response capacity in this species. However, after an immune challenge induced by a PHA (*Phaseolus vulgaris* phytohaemagglutinin) injection, starved males presented a significant coloration loss and this alteration was related to the immune response intensity. Taken together, these results suggest that the brighter (lighter) coloration may be used as a cue by female to exclude males with a recent immune challenge, due to diseases or parasites for example.

Keywords Amphibian · Coloration · *Hyla arborea* · Immunocompetence · Phytohaemagglutinin

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Introduction

In many species, females use honest sexual signals to assess the quality of males (Andersson 1994). An honest signal must be costly to produce and/or to maintain, so that lower quality individuals cannot afford to cheat (Zahavi 1975, 1977). Hamilton and Zuk (1982) proposed that animals choose mates with high genetic resistance to parasites. Only the best quality mates in prime condition should be able to invest more resources into sexual signals. Such healthy mates would be capable of higher paternal investment and/or would transmit fewer parasites during physical contact. Moreover, if resistance to parasites is heritable, which has been demonstrated in numerous bird species, (*Parus major*, Brinkhof et al. 1999; *Sturnus vulgaris*, Ardia 2005; *Ficedula albicollis*, Cichon et al. 2006), females should also gain resistant genes for their offspring (review in Andersson 1994).

A large body of literature (Saks et al. 2003; Alonso-Alvarez et al. 2004; McGraw and Ardia 2003; Mougeot 2008) has shown that carotenoid-based orange ornaments are an indicator of male immunocompetence, i.e. an individual's capacity to mount an appropriate immune response following exposure to a pathogen (Vinkler and Albrecht 2011). Several hypotheses have been suggested to explain the honesty of carotenoid-based signals. First, carotenoid pigments are not synthesised de novo by vertebrates (Olson and Owens 1998) but have to be ingested in the diet. The coloration may hence reflect the foraging capacity of individuals (Endler 1980; Møller et al. 2000). Second, ingested carotenoids must often undergo costly metabolic transformation before their incorporation in teguments (Møller et al. 2000). Only the best quality males in prime condition should be able to allocate energy to this biosynthesis (Hill 2000). Third, carotenoids play an important role in immunoregulation and immunostimulation, leucocyte proliferation and especially in free radical scavenging and

detoxification (Britton 1995; Møller et al. 2000, Chew and Park 2004; Yeum et al. 2009). According to this view, carotenoids are irreversibly destroyed in the antioxidative processes, and hence, there should be a trade-off between their physiological utilisation and their allocation into ornamentation (Møller et al. 2000). As a consequence, the use of carotenoids in sexual traits diverts carotenoids away from the immune function and antioxidant capacity creating a handicap. However, several studies suggest more complex interactions and propose that under certain circumstances, carotenoids may be dangerous for the organism because they easily transform into toxic cleavage products (Britton 1995; Yeum et al. 2009). When reserves of other protective antioxidants are insufficient, physiological trade-offs may exist between maintenance of carotenoids for ornament expression and their removal from the body (Vinkler and Albrecht 2010).

During breeding period, when sexual signal expression is maximal, males could experience food restriction if their reproduction area is separated from the foraging area. This constraint is particularly high in ‘income’ breeding species (sensu Jönsson 1997) because they do not have enough energy supply to face the energetic demand of breeding. For them, when sexual signalling is particularly costly, there is a trade-off between time spent to produce sexual signals and time spent to forage. The decrease of food intake in quality and quantity is known to affect individual’s immune capacities (Lochmiller et al. 1993), and we suggest that it could indirectly impact the carotenoid-based signalling. Immune responses are costly. These costs depend on the type and amount of immunostimulants presented to the host immune system, but also on the amount of resources available to fuel host immune responses (Bize et al. 2010). Several studies have demonstrated that nutrition influences both the quality and quantity of immunological defences in herbivorous insects (Singer et al. 2014; Graham et al. 2015). Experiments conducted both in captivity and in the wild showed a stronger immunocompetence in animals whose diet was supplemented with proteins (Bounous et al. 1983; Smith et al. 2007) or antioxidants (Szuroczi et al. 2016). Food restriction and fasting have also been tested with contrasting results: in captive yellow-legged gulls (*Larus cachinnans*), a decrease in T cell-mediated immune response was observed in individuals that undergo a food restriction (Alonso-Alvarez and Tella 2001), while in gerbils (*Meriones unguiculatus*), no modification of cellular and humoral immunity was found when food was restricted at 80% of baseline food intake (Xu et al. 2011).

Many studies have found correlations between colours and immune response; however, few focused on the effect of an immune challenge on the coloured signal expression (Faivre et al. 2003; Perez-Rodriguez et al. 2008; Toomey et al. 2010; Ibáñez et al. 2014). While periods of food restriction may be common in the wild, the relationship among coloration, nutritional condition and immune response is, to our knowledge, a

rather unexplored aspect. We chose to address this topic using males of *Hyla arborea*, the European tree frog. As in many anuran species, assessment of mate quality by females is based on costly acoustic signals emitted by males (Richardson et al. 2010a; Voituron et al. 2012; Brepson et al. 2013). In addition, Gomez et al. (2009, 2010, 2011a) showed experimentally that female *H. arborea* take into account male coloration, preferring those with a more intense vocal sac coloration (darker and more chromatic) compared to a pale vocal sac coloration (lighter and less chromatic). The orange red coloration of the vocal sacs is based on several carotenoid pigments (Richardson et al. 2009). *H. arborea* is an income breeder alternating foraging periods outside the pond and caloric restriction periods inside the pond during calling periods when attracting females (Friedl and Klump 2005). In the present study, by manipulating the nutritional condition (starvation or ad libitum) and experimentally provoking an immune challenge, we hypothesised that the vocal sac coloration characteristics in male *H. arborea* should be (i) directly related to the immune responsiveness and (ii) dependent on the current health state of males. In addition, we predict that the coloration variation after an immune challenge should be related to the nutritional condition of males.

Material and methods

Male capture and housing conditions

Forty-four *H. arborea* calling males (i.e. sexually mature males) were collected by hand during nightly choruses in mid-April from La Saulsaie, near Lyon, France (45° 54' 19.97" N, 5° 0' 49.27" E). After their capture, individuals were housed in individual terrariums (25 × 17 × 15 cm) with a water-filled basin and a tree branch in the EcoAquatron animal rearing facility (University of Lyon). The terrariums were placed under a neon light (Repti Glo 5.0; 40 W; 30% UVA; 5% UVB, Exo Terra, Combs la Ville, France) with timers set to guarantee 12 h of light per day.

Experimental design

The experiment consisted in a 2 × 2 factorial design. Using a random selection, males were distributed into two groups. The draw was redone until groups presented a similar mean of the body mass (4.92 ± 1.10 g, mean \pm s.e.m., and 5.47 ± 0.85 g, student test $t = 1.86$, $df = 39.45$, $P = 0.07$) and an equal distribution of variances (Bartlett test $K^2 = 1.36$, $df = 1$, $P = 0.24$). One group ($N = 22$) was fed ad libitum with living crickets (*Acheta domesticus*), whereas the other half were starved (no feeding) during the entire experiment (10 days). In our experiment, males did not have access to an external source of carotenoids because domestic crickets are carotenoid-free. We checked the amounts of

cricket carotenoids by ultra-high performance liquid chromatography (UHPLC) coupled with atmospheric pressure chemical ionization mass spectrometry (ACPI-MS) (data not shown). After 3 days in the laboratory, half of the males in each group received an injection of *Phaseolus vulgaris* phytohaemagglutinin (PHA) solution to provoke an immune challenge, whereas the other half (controls) received a phosphate-buffered saline (PBS) injection. Male body mass was measured the day of arrival and the day of injection. The male vocal sac coloration properties were measured the day of arrival, the day of injection and 3 and 6 days after injection.

Coloration measurements

The male vocal sac coloration was measured using an Ocean optics JAZ spectrophotometer (AvaSpec-3648-SPU2, Avantes, Apeldoorn, The Netherlands) with a deuterium-tungsten halogen lamp light source (AvaLight DHS, Avantes, Apeldoorn, The Netherlands) emitting in a wavelength range of 200–1500 nm and a coaxial optic fibre (FCR-7UV200-2-45-ME, Avantes, Apeldoorn, the Netherlands) reflective of white reference and dark noise. The coaxial optic fibre (FCR-7UV200-2-45) had a terminal area (2×3 mm) cut at 45° to avoid specular reflectance and was protected by a silica window that guaranteed a constant distance between the detector and the measured patch. For each individual, three measurements were taken, removing the probe between each measurement. The obtained average of the reflectance spectrum was analysed using the software AVICOL®, version 5 (Gomez 2006) to extract the brightness (the mean reflectance within the range 350–700 nm) and the chroma, also referred to as colour saturation, (the difference between the minimal and maximal reflectance over the mean reflectance within the range 350–700 nm) to characterize the coloration (Gomez et al. 2009). A more intense coloration is darker and more chromatic, and a brighter coloration is the same as a lighter coloration. In this study, we did not use the hue characteristic of coloration because Gomez et al. (2011a) have observed that this characteristic was not involved in female mate choice.

Immunity test

The immune response was measured after an injection in the right leg of 20 μ l of PBS (phosphate buffer saline, EC, number 231.791.2, Sigma Aldrich, Saint-Louis, Missouri, USA) with 0.5 mg of *P. vulgaris phytohaemagglutinin* (PHA-P, L8754, Sigma-Aldrich, Saint-Louis, Missouri, USA) for half the males using a Hamilton syringe with a 26S-gauge needle (± 0.5 μ l accuracy). The phytohaemagglutinin (PHA) response, tested with a subcutaneous injection of PHA that induces local T cell stimulation and proliferation, resulting in swelling (Davis et al. 2008), has been proved to be a reliable tool for assessing amphibian cell-mediated immunity

(Josserand et al. 2015). Swelling was measured before and 18 h after injection (according to Josserand et al. 2015) with a spessimeter (C112, Mitutoyo, Kanagawa, Japan) with an accuracy of 0.01 mm by the same person (N.M.). A larger inflammatory response to injection indicates a more robust immune response (Vinkler et al. 2010; Josserand et al. 2015). To verify the presumption of no swelling response after the PBS treatment in the tree frog, 20 μ l of PBS was injected into the left leg of the other half of the males.

Statistics

At least 11 males per group were tested. It was impossible to use a larger sample size in our experiments, as *H. arborea* is a protected species, and the number of animals captured was limited by the ethics committee.

A Pearson's test was used to check the correlation between mass and the properties of vocal sac coloration. To determine the dietary effect on the variation of male body mass and vocal sac coloration properties after 3 days, the data were analysed using ANOVA.

The reaction to the injection of PHA or PBS was expressed as the difference between the change in thickness of the right leg before injection and 18 h after injection. Correlations between leg swelling and other variables—the male body mass and vocal sac coloration properties on the injection day—were tested using Pearson's test. We used a two-way ANOVA test to examine whether the dependent variable (the swelling response after injection) was influenced by diet and injection treatment and their interaction. We then used a Tukey's test to assess the differences between each group. Because of the non-normality of the data, we used Spearman's test to evaluate if the brightness coloration variation was correlated to the immune response intensity in starved males who received a PHA injection.

We used a generalized estimating equation (GEE) statistical method to analyse the effects of injection (two modalities: PBS and PHA), diet (fed or fasted) and time (injection day, 3 and 6 days after injection) on the different vocal sac coloration properties (chroma and brightness). In our models, individual identity was defined as a random effect to take into account the multiple measurements (three dates) that were performed on each male. An autoregressive first-order working correlation structure was used. The Wald statistic, based on the asymptotic normality of estimators, was used to estimate levels of significance. For the linear models, the residual normality, independence and variance homogeneity were checked. For all of the statistical models, all of the main effects and the interaction terms for each data set were fitted. If interactions were not significant, they are deleted from the model.

The data were analysed using the statistical software package lme4 in R 3.2.1 (R Core Team 2013). The R package 'geepack' and the function geeglm were used for the GEE analyses.

Results

Impact of fasting on body mass and vocal sac coloration properties

Before the experiments, groups were randomly formed, with a low variance of body mass and coloration. Starved males lost on average 4.6% of their body mass in 3 days (-0.25 ± 0.17 g; mean \pm s.e.m.) whereas males fed ad libitum presented a significant gain of 10.5% mass ($+0.52 \pm 0.17$ g; ANOVA, $F_{1, 42} = 9.54$, $P = 0.004$, Fig. 1a). On the contrary, 3 days of fasting did not impact significantly the vocal sac coloration properties (brightness ANOVA, $F_{1, 42} = 0.39$, $P = 0.54$; Fig. 1b and chroma $F_{1, 42} = 2.75$, $P = 0.10$; Fig. 1c).

Coloration and immune response

In accordance with previous results, regardless of the diet, injection of PHA provoked a leg swelling on average 88.6% thicker than PBS injection (glm, injection effect, $F_{1, 39} = 38.42$, $P < 0.001$, Fig. 2). However, the immune responses, expressed in millimeters of swelling after PHA injection, of males experiencing fasting were not significantly different compared to males fed ad libitum (glm, diet \times injection, $F_{1, 39} = 2.47$, $P = 0.12$, Tukey's test, $P = 0.16$).

The swelling response did not correlate with the body mass of frog (Pearson, $t_{40} = -0.92$, $P = 0.36$) nor with vocal sac coloration properties before injection (Pearson, $t_{40} = 0.33$, $P = 0.74$, and Pearson, $t_{40} = 0.68$, $P = 0.50$, for brightness and chroma, respectively, Fig. 3).

Coloration variation after an immune challenge

The coloration of males fed ad libitum, measured before the injection and 3 and 6 days after, was not modified by the immunological challenge with PHA nor by the PBS injection (brightness, geeglm, fed males, injection effect, $\chi^2 = 0.02$, $P = 0.89$, Fig. 4a and chroma, geeglm, fed males, injection effect, $\chi^2 = 0$, $P = 1$, Fig. 4b). In contrast, the brightness of the vocal sac of starved males significantly increased 3 days after the injection of PHA while the brightness of vocal sacs of males injected with PBS did not change (geeglm, injection treatment effect, $\chi^2 = 5.43$, $P = 0.02$, Fig. 4c). Indeed, the vocal sacs of starved males who experienced an immune challenge were 1.2-fold brighter (i.e. less coloured and lighter) than starved males injected with PBS after 3 days.

The increase in brightness after the PHA injection was even stronger when the immune response was high (Spearman's rank correlation, $r^2 = 0.7$, $S = 36$, $P = 0.04$, Fig. 5). Six days after the injection, the brightness of the vocal sac came back to the initial level (Fig. 4c). However, there was no significant effect of the injection treatment on chroma between groups (geeglm, injection treatment effect, $\chi^2_{1, 40} = 0.05$, $P = 0.83$,

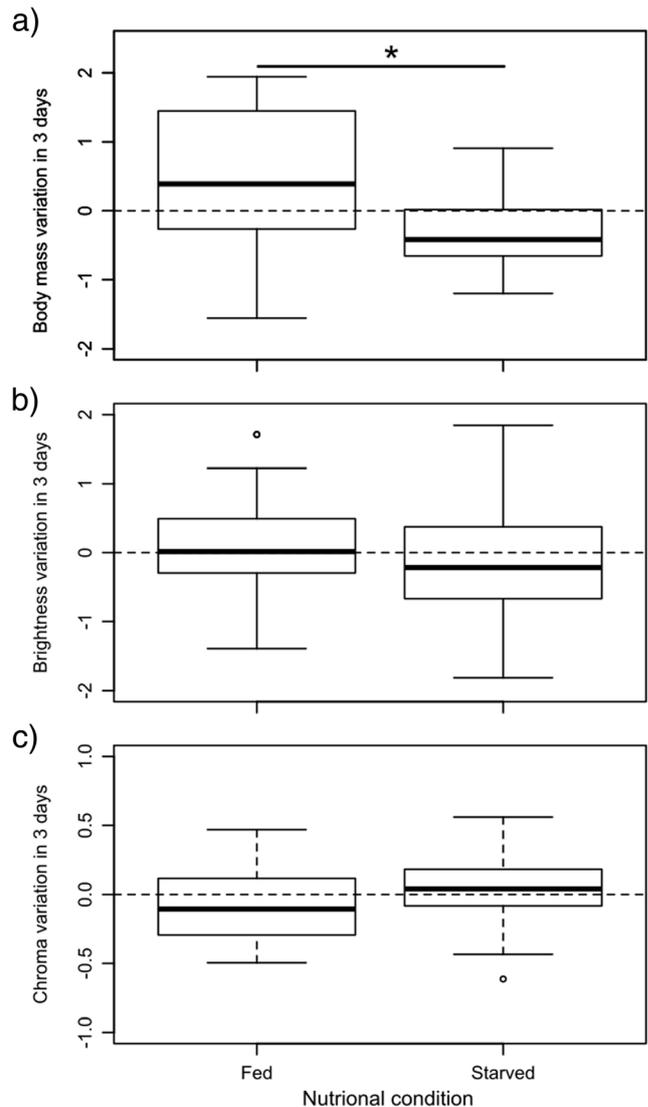


Fig. 1 Effect of the nutritional treatment on male body mass in grams (a) and vocal sac coloration properties (b: brightness, c: chroma). Variations are expressed as the difference between day of capture and 3 days after. The nutritional treatment impacted the body mass but not the coloration properties in 3 days. Horizontal line median value, box ends upper and lower quartiles, whiskers variation range of values. The dotted line represents the zero value. * $P < 0.05$. $N = 44$

Fig. 4d) and no significant variation between days (geeglm, day effect, $\chi^2_{2, 40} = 1.58$, $P = 0.45$, Fig. 4d).

Discussion

Contrary to what we expected, the vocal sac coloration of *H. arborea* males was not a reliable indicator of the immune response capacity of males in our experimental conditions. However, depending on their nutritional condition, we found that coloration could be used as a cue of a recent immune challenge in males. Indeed, starved males presented a

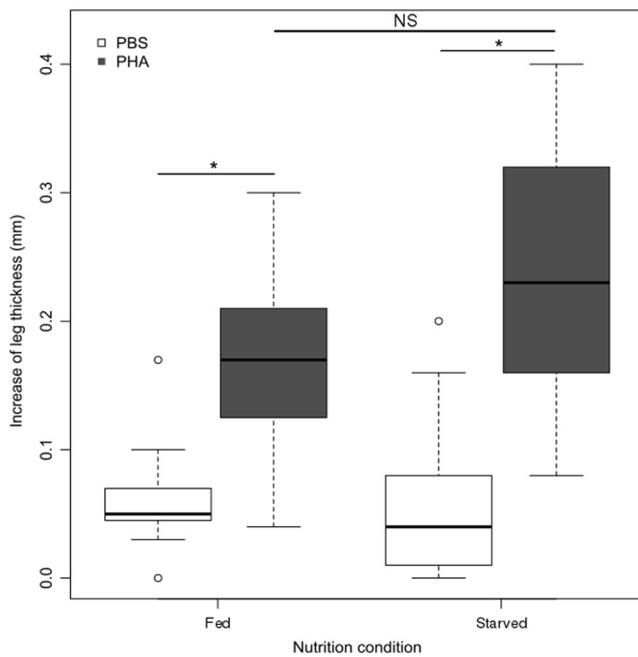


Fig. 2 Effect of the nutritional treatment on the increase in leg thickness (millimeters) 18 h after injection of PBS (in white) or PHA (in grey). Whatever the nutritional treatment, the PHA injection induced a significant increase in leg thickness. Horizontal line median value, box ends upper and lower quartiles, whiskers variation range of values. $N = 44$. * $P < 0.05$. NS means non-significant

reversible lightening of the vocal sac after an immune challenge with PHA injection.

Vocal sac coloration did not signal immunocompetence and do not vary with fasting

In our study, contrary to our first hypothesis about the immunity role of carotenoids pigments, coloration properties of the male vocal sac coloration of *H. arborea* were not related to the immune response measured by the PHA test. This finding is unexpected because of the large amounts of studies that found

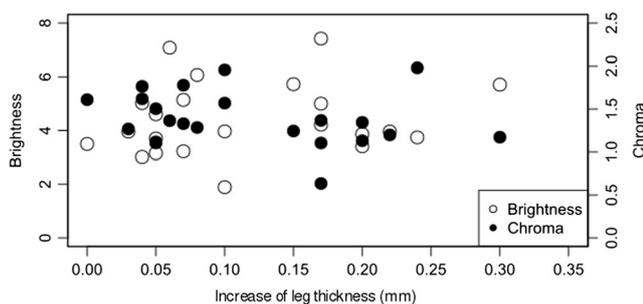
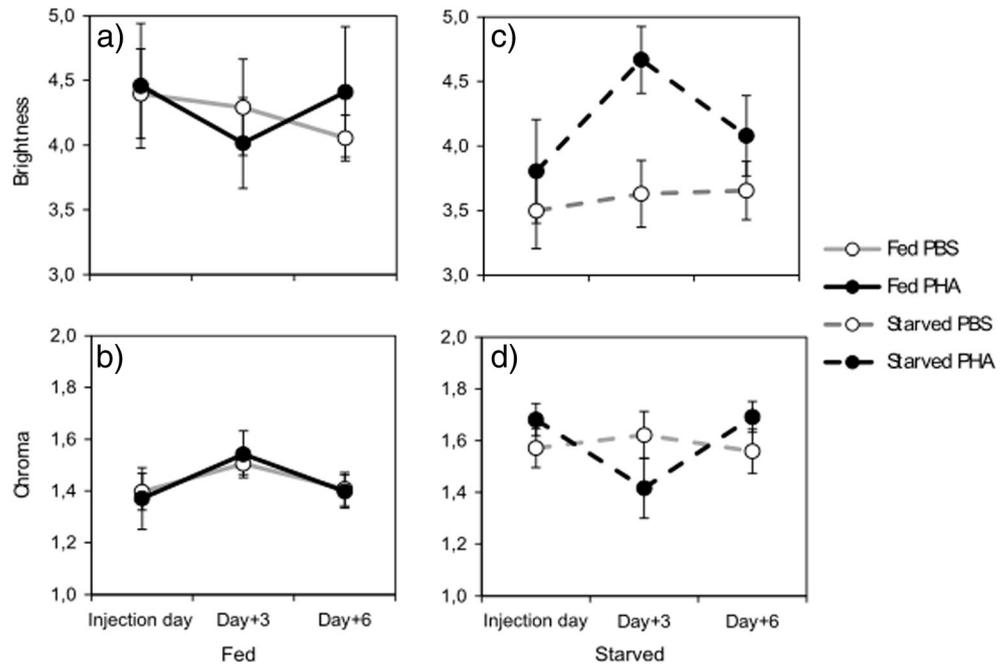


Fig. 3 Male vocal sac coloration properties on the day of the injection (brightness in white and chroma in black) regressed on male swelling response 18 h after a PHA injection. Coloration properties were not correlated with immune response. Individuals are represented twice (once for brightness and once for chroma). $N = 22$

links between coloration levels and immune response capacity in birds (review in Simons et al. 2012) but also in amphibians (Szuroczi et al. 2016). Similar correlation has been found in a lizard species, *Crotaphytus dickersonae*: males with darker collars showed a greater PHA response and had smaller parasite loads (Plasman et al. 2015). However, the predicted negative relationship between the expression of carotenoid-based signals and parasite loads is not universal: in male red-winged blackbirds, there was no relationship (Weatherhead et al. 1993), and an inverse correlation was observed in male greenfinches (Saks et al. 2003). Other kinds of (non-carotenoid) pigments like melanin have been related to ectoparasite load (Roulin et al. 2001) or mating success (Parker et al. 2003), suggesting that melanin-based traits can also be considered as honest signals (McGraw 2007). In the same way, immunocompetence could be tied to characteristics of coloration caused by nano-scale reflective tissues producing ultraviolet coloration in other species (mostly studied in birds Mougeot 2008; Griggio et al. 2010; Ibanez et al. 2013). The PHA challenge test has been shown to be a useful tool for predicting the pro-inflammatory capacity of animals (Vinkler et al. 2010) and especially on *H. arborea* (Josserand et al. 2015; Desprat et al. 2015). In *H. arborea*, a link between the leg swelling and the total leucocyte count recorded in blood has been found (Josserand et al. 2015). Nevertheless, several authors used the PHA test combined with other tests such as the measure of natural antibodies or bacteria killing assays to quantify vertebrate immune response. In our case, measurements other than PHA use would require blood samples that are technically complex to obtain due to the high risk of animal death during sampling. As a consequence, we cannot exclude the existence of a functional link between coloration and immunity due to humoral immune response. However, Simons et al. (2012) in a meta-analysis showed that carotenoids and colour signal were both associated with a greater swelling in response to PHA injection, and our results could be confirmed in the future with other techniques.

The 3-day fasting period before PHA injection did not allow us to find any direct effect of starvation on male vocal sac coloration properties. Our results show that coloration was similar between starved males and males fed ad libitum when chroma and brightness were measured before and after 3 days of fasting. This result is in accordance with a previous study in *H. arborea* that found no relationship between male condition and coloration (Gomez et al. 2011a). In the absence of immunity challenge and intake of carotenoids, the pigments present in the vocal sac of *H. arborea* were not modified or reallocated to other functions during fasting, at least within few days. In the same way, while 3-day fasting decreased male body mass, we found that starved males and ad libitum-fed males presented a similar immune response. Along with Josserand et al. (2015), this result confirms that the immunocompetent response of *H. arborea* males is not mass-dependent.

Fig. 4 Nutritional treatments' effect on male vocal sac coloration after an immune challenge. Fed males (**a** and **b**, for brightness and chroma, respectively) are represented with *full lines*, and starved males (**c** and **d**, for brightness and chroma, respectively) are represented with *dotted lines*. Males which received a PBS injection are represented with *white circles* and those which received a PHA injection are represented with *black circles*. Measures were repeated on the same male the day of the injection and 3 and 6 days after the injection. *Error bars* indicate s.e.m. values. $N = 44$



Coloration as a cue to recent immune challenge

Experimentally induced immune challenges have been shown to reduce carotenoid-based coloration in a number of bird studies (Saino et al. 2000; Faivre et al. 2003; McGraw and Ardia 2003; Peters et al. 2004; Toomey et al. 2010). Nevertheless, in our study, such relationship was found in starved males only. The brightness of the vocal sac of males fed ad libitum did not

present any variation after PHA injection, while the brightness of vocal sac of starved males increases 3 days after PHA injection compared to the brightness measured the day of the injection. In addition, the stronger the immune response was, the greater the brightness of the vocal sac increased in starved males was. Thus, the coloration change—an increase in brightness corresponding to a decrease in vocal sac coloration—may reflect the intensity of the response to the immune challenge experienced by the male.

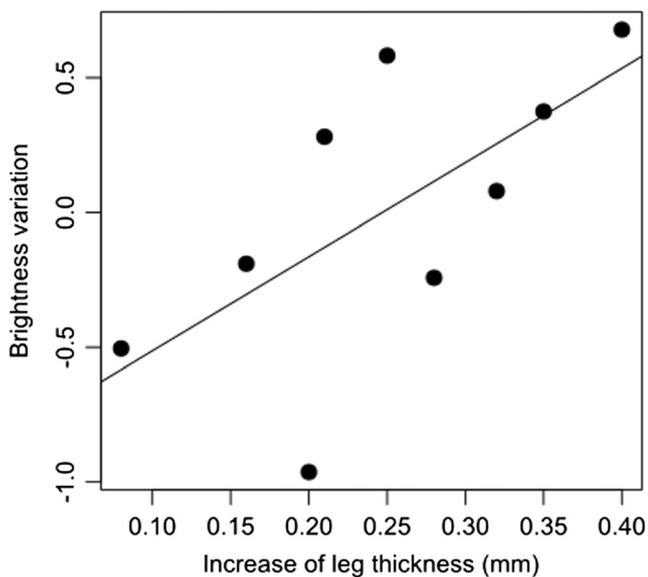


Fig. 5 Brightness variation regressed on immune response after a PHA injection in fasted males. Brightness variation is expressed as the difference between the injection day and 3 days after. The coloration loss was even greater when the increase of leg thickness was large. The *line* represents the positive and significant Spearman regression, $y = 3.5 \times -0.9$

From a mechanistic point of view, the decrease in male coloration could be explained by two non-exclusive mechanisms: (i) a degradation of pigments fixed in the tegument and/or (ii) an active reallocation of carotenoids from the vocal sac tegument to the plasma. Once in plasma, carotenoids could modulate the immune function by stimulating T cell and antibody production, facilitating gap-junction communication, influencing gene expression, and also protecting vulnerable cells and tissues from reactive oxygen species generated during immune response (Møller et al. 2000; Blount et al. 2003; Chew and Park 2004; Svensson and Wong 2011). One hypothesis to explain why only starved males experienced a loss in coloration during an immunity challenge is the energetic cost of carotenoid utilisation in the generation of colourful teguments. It is possible that the maintenance of the vocal sac coloration needs a constant supply of carotenoids. This supply is dependent on the physiological mechanisms by which carotenoids move from the storage organ to the vocal sac tegument and by which carotenoids are structurally modified before being deposited in tegument. During an immunity challenge, starved males may not meet the energy demand necessary for the carotenoid metabolism. Such costs

incurred by starved organisms have been shown in finches during moult (Hill 2000). This hypothesis is consistent with the fact that in our study, the vocal sac coloration of the starved males returned to its initial value after 6 days. Without the contribution of external carotenoids through food intake, and once the immune challenge had passed, carotenoids might have been remobilized from the liver (storage organ) to the vocal sac. Another explanation to why only the starved males experienced a loss in coloration during an immunity challenge could be that the carotenoid cleavage products may be harmful to the organism under certain conditions (Vinkler and Albrecht 2010). Hence, only male in good nutrition condition, with enough resources, will deal with the immune challenge without impacted other functions.

Furthermore, as in many nocturnal species, *H. arborea* males also express acoustic sexual signals. Relationships between call characteristics and parasitism had been found in many birds (e.g. *Acrocephalus schoenobaenus*, Buchanan et al. 1999; *Strix aluco*, Redpath et al. 2000). So the use of two communication channels (both acoustic and visual) could enhance female mate choice efficiency when the quality of two potential mates is closed (Gomez et al. 2011b). Moreover, in case of conflicted acoustic and visual cues, females sometimes show no consensual preference (Richardson et al. 2010b). It is now necessary to determine if some acoustic cues support or not information about emitter health and how these cues interact with information brings by carotenoid-based coloration (our present results).

Conclusion

From an ecological point of view, our results did not confirm that male vocal sac coloration is a reliable indicator of male immunocompetent response. Female mate choice based on male coloration may not allow them to choose a male that will potentially transmit its good immune response capacity to their offspring. However, the carotenoid-based coloration might be an indicator of the immune state of a male, particularly when males are under nutritional constraint, as is usually the case during the breeding period. Moreover, we showed that coloration was a reliable indicator of the intensity of the immune challenge. Therefore, female choice excluding brighter males would exclude males in poor nutritional condition with a recent infection or disease. Females choosing a male with a darker coloration would not totally exclude the risk the male is subjected to an infection or disease but would improve the choice to a male with sufficient energetic reserves to deal with infections. This finding brings new insight concerning sexual selection both for the cost of immune function and the evolution of secondary sexual traits. Moreover, contrary to what was expected in species breeding in leks, our study emphasized the possibility for females to gain direct

benefits concerning transmission disease during pairing. Further studies on lekking species are now necessary to validate such gain.

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Compliance with ethical standards

Ethical notes We obtained the European certificate that legally allows us to design and conduct experimental research work using live animals, and all work was performed with the approval of the ethics committee of University of Lyon 1 (BH2012–15). Frogs were housed in the EcoAquatron of University of Lyon, which is a facility approved by Veterinary Services (approval number 692661201). All males were released to the original pond the night after the end of the experiments.

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Conflict of interest The authors declare that they have no conflict of interest.

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