

Original Article

Immunocompetence handicap hypothesis in tree frog: trade-off between sexual signals and immunity?

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The immunocompetence handicap hypothesis (ICHH) assumes that testosterone plays a dual role in mediating both sexual signal expression and immunosuppression. Although there is good evidence of the ICHH in birds, it was primarily tested using only one sexual signal approach. By focusing on the European tree frog *Hyla arborea*, a species for which mate choice is based on both acoustic and visual signals, we test for the first time the ICHH in a multimodal communication system. The ICHH may be viewed as a universal way to explain signal honesty no matter the sensory modality used by animals. After studying the effects of testosterone on the acoustic and visual sexual signals through a daily testosterone supplementation, the effects of androgen on the immune system response were measured through a phytohaemagglutinin injection. In *H. arborea*, we demonstrate that both the acoustic and visual sexual signals were testosterone dependent. However, the phytohaemagglutinin injection did not provoke immunosuppression for testosterone-supplemented males. Surprisingly, the testosterone-supplemented males with a high body mass presented an immunoenhancement. Therefore, it seems that ICHH is not an integrating mechanism that is able to explain signal honesty in the multimodal signaling system used by *H. arborea* in mate choice. On the contrary, our results support the idea that multiple mechanisms (e.g., cost-added and index signals) operating simultaneously may explain signal honesty of both acoustic and visual signals.

Key words: acoustic signal, communication, *Hyla arborea*, phytohaemagglutinin, sexual selection, visual signal.

INTRODUCTION

In many species, females invest more in reproduction than males and are the choosy sex. Usually, choice is not random and females expect to gain benefits from their choices. In species in which males contribute nothing beyond their sperm, females are expected to choose males based on “indirect benefits” (Andersson 1994). Because males differ in their quality, mate preference must confer genetic benefits to the offspring (the “good genes hypothesis” and/or “compatible gene hypothesis,” from Neff and Pitcher 2005). Whereas the assessment of male quality as a prospective mate is crucial, a direct assessment is often impossible. Therefore, females rely on males to honestly advertise their quality as potential mates

through honest sexual signals. Signal honesty can be achieved through several main types of signals, such as cost-added signals and index signals (Maynard Smith and Harper 1995).

Because the immune system competes for resources with sexually selected ornaments, the mechanism proposed by the immunocompetence handicap hypothesis (ICHH) is another way to consider signal honesty in the mate choice context (Folstad and Karter 1992). It suggests that testosterone serves a dual role in mediating both sexual signal expression and immunosuppression. The immunosuppressive effect of testosterone can be explained by the indirect action promoting the allocation of energy to reproduction—behavior and metabolism—rather than maintenance of the immune system (Norris and Evans 2000). As a consequence, high testosterone levels result not only in full sexual signal expression but also in a concomitant reduction of immunocompetence. Therefore, only high-quality males can afford to both fully express sexual traits and be able to resist or tolerate parasite/pathogen attack.

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One of the main assumptions of the ICHH is that elevated testosterone plays an important role in the expression of sexual signals (Folstad and Karter 1992). Such a hypothesis is supported by studies that show the regulation of sexual songs by testosterone (e.g., in birds, such as *Junco hyemalis carolinensis*, Enstrom et al. 1997; or mammals, such as *Scotinomys teguina* [singing mouse], Pasch et al. 2011). Similarly, testosterone influences the expression of colored sexual signals (e.g., in birds, such as *Carpodacus mexicanus*, Stoehr and Hill 2001; or reptiles, such as *Sceloporus jarrovi*, Cox et al. 2008). Although there is good evidence of a deleterious effect of elevated testosterone on humoral immunity (Peters 2000), as well as on cell-mediated immunity (Deviche and Cortez 2005), other studies have not found an effect on either (Buchanan et al. 2003; Roberts, Buchanan, Hasselquist, et al. 2007).

Most of the studies of ICHH focus on a single male sexual signal and, particularly, on males with a colored ornamented signal (Evans et al. 2000; Faivre et al. 2003). However, males often produce complex signals with different sensory modalities. For example, male wood-warblers are both brightly ornamented and perform an elaborate song (Taff et al. 2012). The association of several signals may increase the reliability of the information (Candolin 2003), whereas the use of 2 modalities increases the discrimination accuracy (Gomez et al. 2011). Although the communication process appears to be largely multimodal, the ICHH has never been investigated in a multimodal communication system. Nevertheless, the ICHH could be viewed as a universal way to explain signal honesty no matter the sensory modality used by animals.

In the present study, we experimentally investigated the ICHH as a way to understand signal honesty in both the acoustic and visual communications of the European tree frog (*Hyla arborea*). *H. arborea* is a lekking species and previous experiments clearly established that females choose their mate based on acoustic signals and vocal sac coloration. Indeed, females showed a significant preference for higher call rates and calls of short duration, as well as for higher call amplitudes (Richardson et al. 2010). They also favored lower peak frequencies. As the dominant frequency was negatively correlated with body weight, such a preference should lead to their selecting larger males (Richardson et al. 2010). Also, given equally attractive calls, females prefer males with a colorful and intense (darker and more chromatic) vocal sac over a pale (lighter and less chromatic) vocal sac (Gomez et al. 2009, 2010). Whereas acoustic signals are well studied and represent a short-term indicator of male quality based on their energetic state (Voituron et al. 2012; Brepson et al. 2013), few studies have investigated the role of visual signals as a male quality indicator in the tree frog. Concerning the ICHH, the demonstration of the relationship between the immune function and sexual signal production require experiments in which testosterone levels of *H. arborea* are manipulated. Here, we experimentally increased the male's testosterone levels and we predicted that testosterone-supplemented males would have both more colored vocal sac and more attractive calls at the end of the experiment. Simultaneously, we predicted that the immune response of testosterone-supplemented males would be less intense than control ones.

METHODS

Male capture and housing conditions

Ninety-six *H. arborea* calling males (i.e., sexually mature males) were collected during nightly choruses in mid-April 2013

from a population located near Lyon, France (45°56'33.34"N, 4°55'1.96"E).

After their capture, the individuals were housed in the laboratory in individual terrariums (25 × 17 × 15 cm) with a water-filled basin and a tree branch. In addition to the natural light of the room, the terrariums were placed under a neon light (Exo Terra-Neon reptile Glo 5.0; 40W; 30% UVA; 5% UVB) with timers set to guarantee 12 h of light minimum per days. Every night, the males were stimulated using a recording of the chorus playback of their population. In this way, we provided an identical stimulation over the captivity for all males. Because in natura the males *H. arborea* switch between calling and hunting periods, then during the entire experiment, the males were fed with 2 domestic crickets (*Acheta domesticus*) every 2 days.

Experimental design

The day before the beginning of the experiment (D0), each male was weighed and its call and vocal sac coloration were recorded. Simultaneously, a saliva sample was taken to estimate the testosterone levels. Then, males were randomly assigned to 2 groups of 48 individuals each: T-males (testosterone-supplemented males) and C-males (control males). The T-males' testosterone circulating levels were manipulated using a noninvasive method. Testosterone was delivered transdermally to each frog. Testosterone (Testosterone purum > 99.0%, number 86 500, FLUKA Analytical, Sigma-Aldrich) was diluted in commercial-grade sesame oil to obtain a 3.10⁹ pg/mL hormone solution. About 4.5 μL of hormone solution was applied with a micropipette on the back of T-males each day during the duration of the experiment, whereas C-males received an identical amount of sesame oil. In order to avoid the risk that testosterone could be flushed in the basin water, frogs were carefully put away from it for few minutes that insures a good transdermal infiltration. Ten (D10) and 20 days (D20) after the beginning of the experiment, the body mass, testosterone levels, and characteristics of the call and vocal sac coloration of each male were measured and recorded. At D20, a measure of immune reaction was performed by a phytohaemagglutinin injection on 30 randomly chosen males (14 T-males and 16 C-males). Because of an ethical protocol, we were not authorized to inject phytohaemagglutinin into all males.

In addition, the natural variation of testosterone levels during the entire breeding season (from 20 April 2013 to 22 May 2013) was recorded by collecting the saliva of randomly sampled males in the pond ($N = 40$). The breeding season of *H. arborea* is well known in our studied sites since first investigations in 2008. Frog activity was checked every night from the beginning of April to the end of May to be sure to take into account all the breeding season.

Recording and call analysis

We measured individual male call characteristics by recording their acoustic response to a chorus playback in a semianechoic chamber (40 × 40 × 40 cm) using a Sony ECM-T6 directional microphone connected to digital recorders (Roland R-44 or Fostex FR-2LE; Fe 22.05 kHz) during a 4-h period beginning at 8.00 PM on the nights D0, D10, and D20. In *H. arborea*, the mating calls are produced in series named bouts that usually contain an average of 26 calls (Richardson et al. 2010). Using Avisoft SASLab©, we extracted the main acoustic parameters: the average bout duration (in ms) and the within-bout call rate (WCR, in calls/s), which corresponds to the rhythm of call emission during each bout. In addition, the first

harmonic, which is usually the frequency with the highest energetic content of the call (named dominant frequency), was measured using a Fast Fourier Transform analysis (FFT windows size: 1024 pts; accuracy: 21.5 Hz).

Coloration measurements

The vocal sac coloration of males was measured using a JAZ-Ocean optics spectrophotometer (AvaSpec-3648-SPU2, Avantes) with a deuterium–tungsten halogen lamp light source (AvaLight DHS, Avantes) emitting in the range 200–1500 nm and a coaxial optic fiber (FCR-7UV200-2-45-ME, Avantes) reflective to white reference and dark noise. The obtained average of the reflectance spectrum was analyzed using the software AVICOL®, version 5 (Gomez 2006) to extract brightness (the mean reflectance within the range 350–700 nm) and the chroma, also named color saturation (the difference between the minimal and maximal reflectance divided by the mean reflectance within the range 350–700 nm) used to characterize coloration (Gomez et al. 2009). A more intense coloration is darker and more chromatic.

Hormone analysis

We investigated the testosterone levels in saliva, which is a noninvasive method used for wild species steroid detection that includes amphibians (Pearson et al. 2008; Janin et al. 2012). A cotton ball was directly introduced into a frog's mouth for 20 s and put in microtube equipped with a filter to retain the cotton fiber during centrifugation. Cotton balls were weighted before and after sampling saliva, to deduct the amount of saliva. Then saliva was extracted from the cotton ball with the addition of 120 μ L of a phosphate buffer (1 M phosphate solution containing 1% bovine serum albumin, 4 M sodium chloride, 10 mM ethylenediaminetetraacetic acid, and 0.1% sodium azide) and centrifugation.

Testosterone analysis was performed in duplicate with a colorimetric 96-well testosterone Enzyme-Immuno Assay (EIA) kit (number 582701, Cayman Chemical). EIA method is based on the competition between testosterone and a testosterone–acetylcholinesterase conjugate for a limited number of testosterone polyclonal antiserum binding sites. The color reaction was developed using Ellman's reagent containing acetylthiocholine and 5,5'-dithio-bis-(2-nitrobenzoic acid). The concentration of testosterone in saliva samples was calculated using a standard curve run in duplicate on each plate. The intensity of the color, evaluated using a spectrophotometer (Absorbance Microplate Reader ELx808, Biotek) at 405 nm wavelength, is proportional to the amount of testosterone–acetylcholinesterase conjugate bound to the well, which is inversely proportional to the amount of free testosterone present in the sample. The EIA used to measure testosterone in the saliva was validated for use with *H. arborea* saliva by demonstrating parallelism between serial dilutions of 2 saliva samples and the standard curve obtained with a calibrated solution of testosterone (Figure 1). The inter-assay variation and intra-assay variation was inferior to 5%. The assay detection limit was 1.5 pg/mL. In our experiment, there was no value under this detection limit guaranteeing analyses were done with enough accuracy.

Immunity test

The phytohaemagglutinin challenge test to assess immunocompetence of European tree frog *H. arborea* was finalized by Josserand et al. (forthcoming). Immune response was measured after an injection of 20 μ L of phosphate buffer saline (EC number 231.791.2,

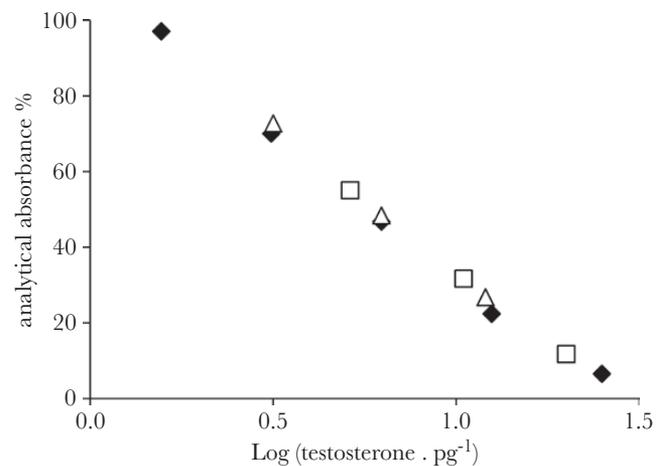


Figure 1

Analytical absorbance % of a standard curve of testosterone (black diamond) and dilutions from 2 saliva samples of *Hyla arborea* (sample 1: white square and sample 2: white triangle) after a logarithmic transformation of the testosterone quantity (in pg). Testosterone concentrations across unknown dilutions from 2 preliminary samples were parallel to the standard curve and between them. This result indicates that antibodies bound the hormone in the saliva sample at a similar rate than those in the standard.

Sigma-Aldrich) with 0.5 mg of *Phaseolus vulgaris phytohaemagglutinin* (PHA-P, L8754, Sigma-Aldrich) per animal in the right leg of 30 frogs (14 T-males and 16 C-males). Swelling was measured before and 18 h after injection with a spessimeter (ID-C112, Mitutoyo) with an accuracy of 0.01 mm.

Statistics

We used a generalized estimating equation (GEE) statistical method to analyze the effects of testosterone (2 modalities: T-males and C-males), body mass, and the day (D0, D10, and D20) on the different sexual parameters (chroma, brightness and WCR, dominant frequency, and the average bout duration). The GEE method accounts in generalized linear models for the correlation between observations (Liang and Zeger 1986). In our models, the identity of the individuals was defined as a random effect to account for several measures (3 dates) that were performed on each male. An autoregressive of first order working correlation structure was used (other working correlation structures yielded similar conclusions). A Gamma distribution of errors (inverse link function) was used except for frequencies that were analyzed by means of a Poisson distribution (log link function). Gamma is a continuous probability distribution that is frequently used to model positive variables because it has a positive value, and its 2 parameters (shape and scale) allow it to fit with large data distributions (McCullagh and Nelder 1989). The Wald statistic, based on the asymptotic normality of estimators, was used for estimating levels of significance.

In the field, males do not sing every night during the breeding season, they alternate singing nights with hunting nights (personal observation). In this context, we had difficulties recording the males on each recording date. Only 17 males were successfully recorded at D0, D10, and D20 (10 C-males and 7 T-males). These numbers drastically reduce the statistical power of our analysis. To avoid this pitfall, we analyzed 60 individuals (27 C-males and 33 T-males) who sang and were successfully recorded at least twice on the 3 recording days.

The effect of testosterone (i.e., testosterone-supplemented vs. control) on the leg swelling response after PHA injection of the

males was explored using linear models. The body mass, a morphological indicator, was added as another explanatory variable in the models after checking that immune response was not condition dependent (Pearson test between the leg swelling and the body mass; $t = 1.24$, $df = 28$, $P = 0.22$, $N = 30$). For the linear models, residual normality, independence, and variance homogeneity were checked. For all of the statistical models, all the main effects and the interaction terms for each data set were fitted. The comparison of testosterone levels between groups was performed using a Wilcoxon–Mann–Whitney test because of the non-normality of the data distribution. For all analyses, we followed a backwards procedure to remove the variables sequentially, thus allowing us to identify the most parsimonious model. Data were analyzed using the statistical software package R (R Development Core Team 2013). The R package “geepack” and the function `geeglm` were used for the GEE analyses.

RESULTS

Testosterone levels *in natura*

At the beginning of the breeding season, when males arrived at the pond (approximately 20 April 2013), the mean testosterone levels measured in their saliva was 9.3 ± 3.5 pg (mean \pm SEM) of testosterone/mg saliva (Figure 2). During the 2 following weeks, testosterone levels in the saliva increased until a peak was observed at the middle of the breeding season (66.0 ± 18.0 pg of testosterone/mg saliva on 3 May 2013). Then, the mean testosterone

levels decreased to 12.3 ± 2.4 pg of testosterone/mg saliva on 22 May 2013.

Evolution of testosterone during the experiment

At the beginning of our experiment, the mean testosterone levels in *H. arborea* saliva for both the T-males and C-males were similar (6.8 ± 0.5 and 5.4 ± 0.5 pg of testosterone/mg saliva, respectively; Mann–Whitney test; $U = 972.5$, $P = 0.14$, $N = 96$). After 10 days of the topical application of testosterone, the mean testosterone levels of the T-males was significantly higher than that recorded for the C-males (10.3 ± 0.8 and 5.9 ± 0.7 pg of testosterone/mg saliva, respectively; Mann–Whitney test; $U = 497.5$, $P < 0.01$, $N = 96$). After 20 days, the testosterone levels of the T-males were 8 times higher on average than the testosterone levels of the C-males (26.0 ± 2.6 and 1.9 ± 0.2 pg of testosterone/mg saliva, respectively; Mann–Whitney test; $U = 182$, $P < 0.01$, $N = 96$).

Testosterone effect on acoustic sexual signals

We observed a significant difference for all call parameters between the beginning and end of the experiment (day effect; Table 1, Figure 3A–C). However, the application of testosterone also induced a significant modification of 2 main acoustic parameters of the *H. arborea* call: the dominant frequency and the average bout duration (Table 1). Although the bout duration decreased during the experiment for both groups (day effect, Table 1, Figure 3C), the T-males sang longer than the C-males after 20 daily testosterone applications (treatment effect: T-males, D20, $N = 33$, 3.15 ± 1.14

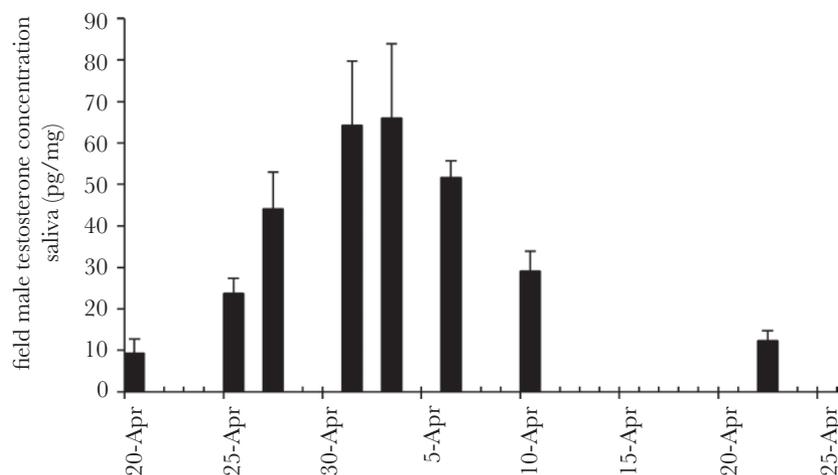


Figure 2

Evolution of male mean testosterone levels in the saliva (pg/mg) of *Hyla arborea* sampled at the pond during the 2013 breeding season ($N = 40$). Error bars indicate SEM values.

Table 1

Results from the GEE analyses of acoustic parameters (WCR, dominant frequency, and bout duration) in *Hyla arborea*

Variable	df	WCR		Dominant frequency		Bout duration	
		Wald statistic	<i>P</i> value	Wald statistic	<i>P</i> value	Wald statistic	<i>P</i> value
Body mass	1	0.15	0.69	58.30	<0.001	6.60	0.01
Day	2	13.86	<0.001	26.30	<0.001	35.40	<0.001
Treatment	1	0.38	0.54	4.90	0.03	5.50	0.02
Body mass \times Treatment	1	0.81	0.37	0.80	0.36	0.50	0.49
Day \times Treatment	2	2.22	0.33	1.80	0.42	0.40	0.82

The independent variables tested are: body mass, day, and treatment (2 modalities: C-males and T-males). The interactions with treatment and body mass or day are presented. $N = 60$. *P* values lower than 0.05 are indicated in bold. df, degrees of freedom.

s; C-males, D20, $N = 27$, 2.76 ± 0.82 s). Likewise, although the dominant frequency increased during the experiment for both groups (day effect, Table 1, Figure 3B), its increase was less for T-males (approximately +170 Hz) than for C-males (approximately +200 Hz) (treatment effect). Body mass, the day, and testosterone treatment all have an effect on call parameters independent of their interactions (Table 1). In agreement with previous studies (Richardson et al. 2010), frog body mass played an important role in the call's dominant frequency and bout duration; the heaviest males had a more attractive call than the smallest one.

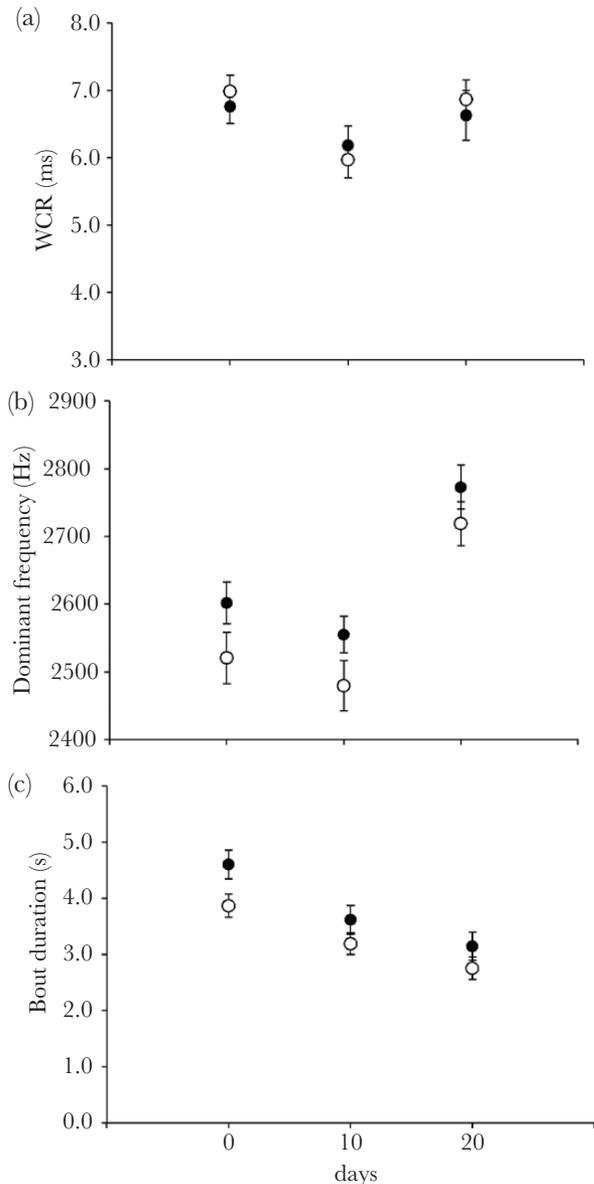


Figure 3
Means of WCR (a), dominant frequency (b), and bout duration (c) of testosterone-supplemented males (T-males, black circle, $N = 27$) or control males (C-males, white circle, $N = 33$) who sang and were successfully recorded at least twice on the 3 recording days during the entire experiment. Error bars indicate SEM values. Output of GEE models (see Table 1), taking account the male body mass, presents a significant effect of testosterone on the dominant frequency and the bout duration vocal parameters.

Testosterone effect on vocal sac coloration

GEE models indicate that the application of testosterone had a significant effect on the brightness but not on the chroma of the vocal sac (Table 2, Figure 4A,B). There was no significant difference between the brightness of C-males and T-males at D0 ($W = 1387$, $P = 0.08$, $N = 96$), and changes in brightness over time were linked to treatment (treatment effect; Figure 4A). Body mass had a significant effect on brightness the same way it affected the acoustic parameters (body mass effect). The heavier C-males were, the more their brightness decreased; whereas the brightness of T-males did not depend on their body mass (Body mass \times Treatment). A variation of vocal sac coloration between the initial capture date and the end of the experiment was also measured (day effect). In captivity, the vocal sac became brighter and less chromatic.

Testosterone effect on immune response

Contrary to our prediction, increasing testosterone levels in *H. arborea* did not trigger a decrease in male immunity even though the treatment and body mass interacted significantly in response to the PHA injection (Figure 5). T-males with less body mass and all C-males reacted to PHA in a similar manner. In contrast, the strength of the swelling response increased significantly for those T-males with a high body mass (linear mixed model, Body mass \times Treatment, $N = 30$, $df = 1$, 26; $F = 4.07$, $P = 0.05$). Qualitatively, the model's interpretation does not change if the outlier individual (Chi-squared = 7.34, $P = 0.007$, increased leg thickness: 0.49) is removed from the data set (linear mixed model, Body mass \times Treatment, $N = 29$, $df = 1$, 25; $F = 5.05$, $P = 0.03$).

We did not find that sexual signal parameters were correlated with immune response (all $P > 0.05$).

DISCUSSION

Our study, on *H. arborea*, a lekking species that uses both visual and acoustic signals in mate selection, tested the 2 main assumptions of the ICHH in a multimodal communication context. As predicted by the ICHH, several components of acoustic and visual sexual signals are testosterone dependent in *H. arborea*. Nevertheless, our results did not reveal an immunosuppression effect of testosterone and, thus, we did not validate the ICHH as a proximate mechanism explaining signal honesty in *H. arborea*.

Table 2

Results from GEE analyses of visual parameters (brightness and chroma) in *Hyla arborea*

Variable	df	Brightness		Chroma	
		Wald statistic	P value	Wald statistic	P value
Body mass	1	9.28	<0.01	0.01	0.97
Day	2	8.57	0.01	6.96	0.03
Treatment	1	7.13	<0.01	0.02	0.90
Body mass \times Treatment	1	4.48	0.03	0.01	0.91
Day \times Treatment	2	1.03	0.60	3.02	0.22

The independent variables tested are: body mass, day, and treatment (2 modalities: C-males and T-males). The interactions with treatment and body mass or day are presented. $N = 96$. P values lower than 0.05 are indicated in bold. df, degrees of freedom.

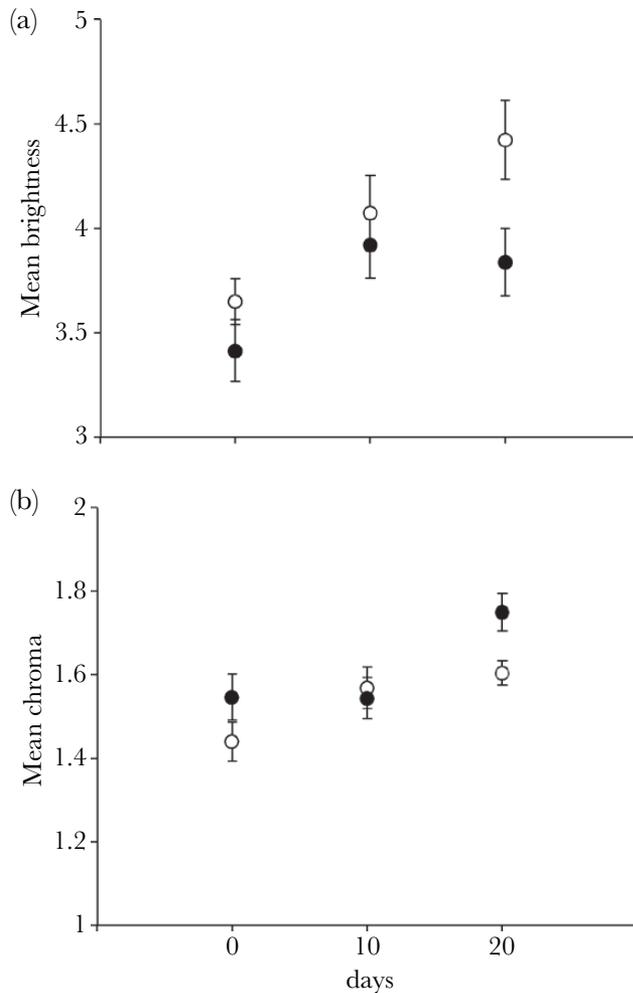


Figure 4
Means of male vocal sac brightness (a) and chroma (b) of testosterone-supplemented males (T-males, black circle) or control males (C-males, white circle) during the entire experiment for the 3 recording days. $N = 96$ for each recording day. Error bars indicate SEM values. Output of GEE models (see Table 2), taking account the male body mass, presents a significant effect of testosterone on the brightness color parameter.

Testosterone-dependent sexual signals

Gonadal testosterone secretion typically occurs at the beginning of the breeding season and seasonal changes in hormone levels correlate with seasonal changes in calling behavior (e.g., Maddison et al. 2012; and reviewed in anurans by Arch and Narins 2009) or in male coloration (e.g., Lindsay et al. 2009). To be realistic, we checked that testosterone levels of supplemented males remained within the limits that are observed in the wild.

Several studies have shown an improvement of male coloration within the breeding season (Salvador et al. 1996; Stoehr and Hill 2001); surprisingly, such an approach is scarce concerning acoustic signals (Enstrom et al. 1997). In our experimental conditions, although we observed a time effect that was likely a result of captivity conditions, we observed a modification of both the dominant frequency and bout duration for T-males (those who experienced an increase in testosterone levels) with regards to the call characteristics produced at the beginning of the experiment by the same males. Several acoustic parameters, such as dominant frequency, are often linked to the emitter morphology (review of 136 frog

species by Gingras et al. 2013). This is also the case in *H. arborea* with larger males calling at lower frequencies (Richardson et al. 2010; Gomez et al. 2011). However, because frog body mass did not increase during the entire experiment, we may dismiss this hypothesis to explain our results. The physiological action of testosterone on trunk muscles could be a second explanation of our results (Leary 2009).

To our knowledge, the present study is the first to emphasize a testosterone effect on the vocal sac coloration parameters in anurans. According to another study conducted on blue tits *Cyanistes caeruleus* (Remy et al. 2010), we failed to show that coloration was modified through a chroma effect. Our results clearly showed that testosterone treatment modified the vocal sac brightness. Indeed, T-males were more colorful than C-males and exhibited a darker vocal sac at the end of the experiment relative to their color at the beginning of the experiment. In *H. arborea*, the vocal sac coloration primarily depends on carotenoid pigments (Richardson et al. 2010) that are obtained exclusively in their diet. In our experiment, males did not have access to an external source of carotenoids because domestic crickets are free of carotenoids. During the first 10 days of the experiment, the brightness of both the T-males and C-males vocal sacs increased. Then, the brightness of the T-males vocal sac remained the same, whereas the brightness of the C-males vocal sac continued to increase. In a pioneer study, Blas et al. (2006) showed that carotenoid levels in the liver and plasma of testosterone-supplemented birds were higher than those of control birds, and the integument coloration was strongly associated with circulating carotenoids in the plasma. They concluded that testosterone increases the bioavailability of carotenoids, which impacts coloration. In the absence of an input of stream carotenoids, our results suggested 2 nonexclusive mechanisms to explain the role of testosterone in the bioavailability of carotenoids and the mechanism underlying the stabilization of brightness in T-males: 1) testosterone could prevent the degradation of vocal sac carotenoids and 2) testosterone could promote the redistribution of carotenoids from the liver to the vocal sac through plasma. Further investigations are necessary to test these hypotheses.

Hence, testosterone appears to affect the quality of visual and acoustic signals emitted by males. In both cases, signal modifications from the high testosterone levels observed in tree frogs promote more attractive traits in males (Gomez et al. 2009; Richardson et al. 2010).

Immune response and testosterone

Our results did not support one main assumption of the ICHH, which suggests that elevated testosterone has a deleterious effect on the immune system (Folstad and Karter 1992). In our experiment, the immune responsiveness of *H. arborea* was measured by using the PHA challenge test. Even if the immunological background of the test is highly complex, recent studies have improved our understanding and have suggested that PHA-induced swelling measured, at least in part, a rapid onset, nonspecific inflammatory and innate immune response (Kennedy and Nager 2006; Josserand et al. forthcoming) that may provide a clue for predicting the pro-inflammatory capacity of the individual (Vinkler et al. 2010; Vinkler and Albrecht 2011). Nevertheless, to quantify vertebrate immune response, several authors used PHA test combined with another test such as the measure of natural antibodies, bacteria killing assay, and white cell counts (e.g., Buchanan et al. 2003; Pigeon et al. 2013). Multiple immune measure methods could reveal different evidence for ICHH as shown by Buchanan et al. (2003) in *Passer domesticus*.

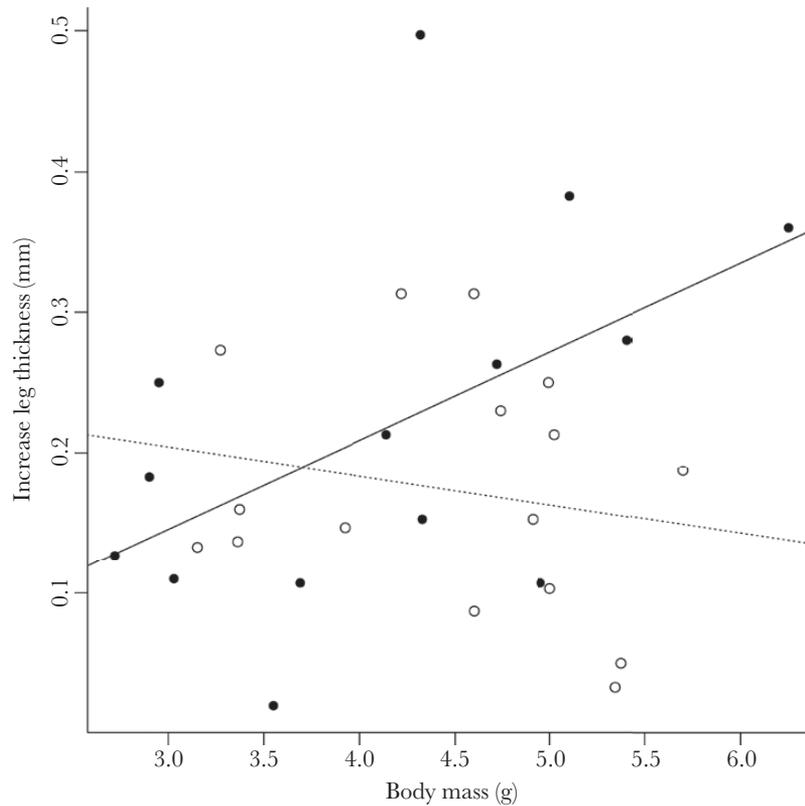


Figure 5

Effect of the interaction between treatment and body mass on leg thickness (in mm) of *Hyla arborea* after PHA injection ($N = 30$). The increase of leg thickness is a measure of immune response. Predictions of the linear model for the significant interaction between the 2 explanatory variables (body mass and testosterone treatment) are represented (full and dotted lines for T-males (black circle) and C-males (white circle), respectively).

In our case, other measurements than PHA measure would require blood samples that are technically very complex to obtain because of the high risk of death for *H. arborea*. It was also impossible to use a functional response such as survival like in insects (González-Tokman et al. 2012) because manipulating infection and measuring survival is not allowed on wild protected amphibian species (1976 French laws on biodiversity protection).

Nevertheless, the interaction between treatment and body mass revealed that the immune response was not the same for T-males and C-males: a positive trend was found only for T-males. Larger T-male (greater than 4.5 g) had a better immune response than small T-male. Similar effects have been found in birds (Buchanan et al. 2003; Roberts et al. 2009) without any clear explanation. The interaction of testosterone treatment and body mass effects could be an indirect consequence of energy reallocation from the immune system to other metabolic demands (e.g., Owen-Ashley et al. 2004; Alonso-Alvarez et al. 2007). Energy reallocation may be caused by leptin, a hormone primarily produced in lipogenic tissues, which regulates the amount of fat stored in the body (Brann et al. 2002). Only the individuals able to maintain large lipid reserves (with high leptin levels), whereas sustaining high testosterone levels, might be able to develop sexual displays without any impairment of their immune defenses (Alonso-Alvarez et al. 2007). This hypothesis does not entirely explain the immune-enhancement observed in those T-males with high body mass in *H. arborea*, but this should be considered.

Another possibility is the action of corticosterone, which is often correlated with testosterone levels (e.g., Evans et al. 2000; Roberts

et al. 2009) and is known to be an immunosuppressive (in review, Buchanan 2000; in iguana *Amblyrhynchus cristatus*, Berger et al. 2005). Evans et al. (2000) suggest that testosterone has a dual effect: it leads to immunosuppression through a mechanism involving corticosterone (see Buchanan et al. 2003; Roberts, Buchanan, Bennett, et al. 2007; Roberts et al. 2009); however, conversely, it leads to increased immunocompetence, likely via dominance behavior, influencing access to nutritional resources. Because in our experiment, we did not manipulate or observe corticosterone levels of *H. arborea* males, we cannot verify that it was not that hormone which played a role in the immunoenhancement observed in T-males.

Signal honesty in *H. arborea*

Whereas the expressions of both acoustic and visual signals are often linked to androgen, Blas et al. (2006) emphasize the lack of studies integrating both signaling systems in the same vision of signal honesty. However, the strength of sexual selection is strong in lekking species with a great variance in male breeding success. In this situation, mate selection by females is not a random phenomenon, although male density could decrease their ability to discriminate male quality (Richardson and Lengagne 2010). Females rely on signal characteristics emitted by males to infer their potential quality as a mate and the fitness benefits that such a choice can confer.

Several mechanisms dealing with signal honesty have been suggested in previous studies conducted on acoustic and visual signals in *H. arborea*. First, as suggested by the cost-added signals definition (Maynard Smith and Harper 1995), many signal

components used by females in their mate choice are costly to produce and to maintain. Such costs associated with signal productions may prevent cheating (Zahavi 1977), although some studies show that signal honesty can be maintained even with null or negative realized costs (Getty 1998; Szamado 1999). This is the case with the call rate, WCR, call amplitude or a preference for leader males (Richardson et al. 2008, 2010). Moreover, in some cases, this energetic cost differs for large and small males, a mechanism that could prevent cheating among low quality males (Voituron et al. 2012). Concerning visual sexual signals, as suggested by Gomez et al. (2009), a carotenoid-based coloration may be costly to maintain because these pigments are also involved in immune function (Faivre et al. 2003). Finally, according to the definition of index signal (Maynard Smith and Harper 1995), the low frequency calls preferred by females allow them to select large males. Indeed, such spectral call characteristics seem to be mechanically linked to the individual's body mass both at the inter- and intra-specific level, and the frequency can thus be viewed as an honest indicator of a male's body mass (Hoskin et al. 2009; Richardson et al. 2010).

The originality of our study relies on the measurements of multimodal signals in the context of ICHH. This study calls also for future research that will integrate both multimodal signals and several proxies of the immune response to test ICHH. This integrative approach is now necessary to improve our understanding of the link between immunocompetence and sexual selection.

Ethical note

Researchers 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 ethic's committee at Lyon 1 University (BH2012-15). Frog housing was developed in the EcoAquatron University of Lyon and approved by the Veterinary Services (approval number 692661201). All males were released to the original pond the night after the end of the experiment.

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