



## Does testosterone affect foraging behavior in male frogs?



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### ABSTRACT

During the breeding season, males often produce costly and extravagant displays or physical ornaments to attract females. Numerous studies have established that testosterone could directly influence the expression of certain sexual signals. However, few of these studies have focused on the indirect role that testosterone could play in modulating prey detection and visual performance to improve the foraging ability of males and hence their acquisition of nutritional resource. In the present study, we experimentally modified the testosterone levels of European tree frog males (*Hyla arborea*), staying in the natural range previously measured in the field, and we investigated the effect of testosterone on the foraging ability of individuals. Foraging capacities were measured on males placed in an arena with a virtual cricket moving on a computer screen. Our results demonstrated a significant effect of testosterone on the hunting behavior of *H. arborea*. We observed that testosterone reduced the orientation latency to virtual prey for supplemented males compared to controls. In addition, testosterone significantly increased the attack promptness of male frogs. Finally, our experiment did not demonstrate any impact of testosterone on male attack success.

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### 1. Introduction

In animals, energy acquired by the individual through feeding or provided by reserves is a key factor in many ecological and behavioral processes. The amount of energy available to the individual could be explained mostly individual foraging ability and metabolic efficiency of transforming nutrients to energy and in specific cases by territory quality (in terms of nutritive resources). The organism uses a large portion of this energy for survival, growth and reproduction (Allen and Gillooly, 2009; Boretto et al., 2015). Reproductive behaviors are particularly costly in energy (e.g., McLister, 2003). In vertebrates, when females invest in large gametes and parental care, their breeding success links to the energy available for reproduction. With respect to males, one major cost is from sexual signaling; females estimate male quality through signals that honestly reflect their quality. Indeed, through the hypothesis that only the best males can support the expression and maintenance of costly signals (Zahavi, 1977), sexual selection leads to an exaggerated expression of male sexual signals to differentiate an individual as the most attractive to females. For example, acoustic signals are particularly costly for males to produce with rates of oxygen consumption 10–22-fold that of resting individuals in amphibians (Taigen and Wells, 1985; Prestwich et al., 1989; Grafe and Thein, 2001), and Voituron et al. (2012) have shown that energetic cost is linked to male quality. The costs of visual signals are not always energetic but can be due to the

acquisition and sequestration of particular compounds. For example, blackbirds (*Turdus merula*) signal their health state through orange beak coloration due to carotenoid compounds (Faivre et al., 2003). These pigments, which are only acquired by alimentation, are involved in the up-regulation of various aspects of the immune system, and only good-quality individuals would allocate large amounts of carotenoids to ornaments (Alonso-Alvarez et al., 2004; Chew and Park, 2004).

Several studies highlighted that these extravagant sexual signals were testosterone-dependent. Males with higher testosterone levels often presented the most attractive signals to females (Hill et al., 1999; Cox et al., 2008; Desprat et al., 2015). In a pioneer study, Blas et al. (2006) demonstrated that testosterone increases the bioavailability of carotenoid pigments in the plasma and liver of the red partridge (*Alectoris rufa*). Testosterone also plays a role in the calling behavior of birds, acting in particular on the medial preoptic nucleus in the brain to increase singing (Alward et al., 2013). In addition to these direct effects on signal quality, testosterone may also act indirectly via behaviors such as territorial behavior (Mougeot et al., 2005) and foraging behavior (Chavez-Zichinelli et al., 2014). Because testosterone is known to increase cognition capacity (Lacreuse et al., 2012) and muscular performance (Huyghe et al., 2010), we could hypothesize a relationship between this hormone and foraging ability. Indeed, sexual steroid hormones have been found to have diverse effects on perception, attention and visuospatial processes involved during foraging behavior (Neave, 2008). Hence, we could expect that males with higher testosterone levels have the most attractive signals. By manipulating testosterone levels within the natural range, the present study aimed to determine the relationship between this hormone and foraging ability.

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To test this relationship, we conducted an experimental study on the European tree frog (*Hyla arborea*). This species breeds in leks, and previous experiments clearly established that females choose their mate based on male acoustic signals and vocal sac coloration (Gomez et al., 2009; Richardson and Lengagne, 2010). Moreover, the expressions of both acoustic and visual sexual signals are testosterone-dependent (Desprat et al., 2015); males having the most attractive signals are those with the highest testosterone levels. *H. arborea* is an income breeder. Because there is no resource on the pond, this area is exclusively used by frogs for the reproduction. Acquisition of resource has to be done before coming to the pond to former the lek. Hence in *H. arborea* species, males present a succession of foraging periods outside the pond and calling periods to attract females that probably correspond to fasting periods (Friedl and Klump, 2005). The shorter the foraging period is, the longer calling period is. In the present study, we experimentally increase within the natural range the testosterone levels of *H. arborea* males, and we investigated the effect of testosterone on the foraging ability of individuals. For predators, foraging ability could be viewed as the result of the interaction of several behavioral components such as prey detection (the orientation latency to the prey), the motivation to pursue the prey (the promptness of the attack), and the attack efficiency (the attack success rate). Hence, a positive testosterone effect on one of these items could improve the foraging ability of an individual and, *in fine*, its investment in sexual signal expression. For example, even if the attack success is not improved by testosterone supplementation but the orientation latency is shorter, the individual will improve its foraging ability. These three components of foraging ability were measured on males placed in an arena with a virtual cricket moving on a computer screen. We hypothesized that testosterone may affect the different components of hunting behavior by 1) reducing the orientation latency to the prey, 2) increasing the attack promptness, and 3) improving attack success.

## 2. Materials and methods

### 2.1. Animals capture

Fifty-nine sexually mature *H. arborea* males were collected during five consecutive nights in mid-April 2015, corresponding to the beginning of the breeding season. The males came from a French population located around Lyon called 'Mepieu' (N 45° 43'47", E 5° 27'18"). The pond is part of a network of wetlands located in a protected regional natural reserve and is surrounding with dry lawns, mowing meadows and forests of oaks. The individuals were housed in the laboratory (EcoAquatron University of Lyon, approved by the Veterinary Services; approval number 692661201) in individual terrariums (25 × 17 × 15 cm) with a basin containing approximately 25 cl of dechlorinated water and a tree branch. The terrariums were placed under a neon light (Exo Terra-Neon reptile Glo 5.0; 40 W; 30% UVA; 5% UVB) with timers set to guarantee 12 h of light per day. The housing room of our laboratory was maintained at 23 ± 1.5 °C (mean ± sd).

### 2.2. Experimental design and hormone manipulation

Upon their arrival in the laboratory, the body masses of the males were measured (balance DL-501, 0.01 g, Denver Instrument, Sartorius). Males were distributed in 2 groups: C-males (control males,  $n = 29$ ), and T-males (testosterone-supplemented males,  $n = 30$ ) with care taken to ensure equal mass distribution in the two groups (5.29 g ± 0.21 and 5.20 g ± 0.22 for C-males and T-males respectively (mean ± sem); Student's *t*-test,  $t = -0.30$ ,  $df = 30.85$ ,  $p = 0.77$ ). Testosterone was delivered transdermally, by topic application on the frog back, to each T-male following the method used by Desprat et al. (2015). Briefly, the testosterone (number 86500, FLUKA analytical, Sigma-Aldrich) was diluted in commercial-grade sesame oil to obtain a 3 mg/ml hormone solution. T-males received 4.5 μl of hormone

solution dorsally each day during the experiment, whereas C-males received an identical amount of sesame oil. This method permits increases the testosterone levels in saliva of male frog within the natural range observed in the field. Indeed, during the breeding season, males express a significant and non-permanent elevation of their testosterone levels (Desprat et al., 2015). In the present study, although we obtained a 9 fold increase of the testosterone level for T-males (from 4.34 ± 0.59 pg of testosterone/mg saliva at the day of their capture to 39.67 ± 3.84 pg/mg after 20 days of supplementation, with a maximal measure at 57.71 pg/mg for one individual, see the Results section below), this level clearly remained below the maximal dose measured in the field (66.0 ± 18.0 pg of testosterone/mg saliva in the middle of the breeding season of *H. arborea*, Desprat et al., 2015).

Before being tested, all males experienced a succession of two contrasted diet periods. During the first ten days of the experiment, C-males and T-males were each fed with 2 crickets (*Acheta domesticus*) to guarantee a similar energetic state for all males. The following ten days males were fasted in order to increase their hunting motivation. On the 20th day after the beginning of the experiment, the foraging behavior test was performed on each male as described below.

After the experiments, all males were provided with 5 crickets over 3 days before being released to their pond of origin.

### 2.3. Hormone analysis

A saliva sample was obtained to determine the testosterone levels of each male upon their arrival and after 20 days of hormonal manipulation before the behavioral test. A cotton ball was put into a frog's mouth for 20 s. Cotton balls were formed from biological agricultural cotton and were small enough to be put inside the frog mouth (~5 mm<sup>3</sup>). They were weighed before and after sampling, to deduce the amount of saliva. Cotton balls with saliva were kept at -20 °C in tube with double bottoms drilled (forensic microspin, ref. 88918, Dutcher). Next, 120 μl of a phosphate buffer (1 M phosphate solution containing 1% BSA, 4 M sodium chloride, 10 mM EDTA and 0.1% sodium aside) was add on the cotton ball. Saliva was extracted from each cotton ball by centrifugation. Testosterone analysis was performed in duplicate with a colorimetric 96-well testosterone Enzyo-Immuno Assay (EIA) kit (number 582701, Cayman Chemical). The coloration was evaluated using a spectrophotometer (Absorbance Microplate Reader ELx808, Biotek, France) at 405 nm wavelength. The concentration of testosterone in pg/mg of saliva was calculated using a standard curve for each plate. The EIA used to measure testosterone in the saliva was validated for use with *H. arborea* saliva (Desprat et al., 2015).

### 2.4. Foraging behavior test

Each male was tested individually in the arena (70 × 70 × 35 cm) with a floor that was covered with a moist burlap cloth. We placed a computer screen (31 × 17.4 cm) on one side of the arena. The background was plain green and a virtual cricket moved on it. The program was coded in JavaScript and HTML5. The cricket's path was made in vector format (SVG) which was subsequently transformed into an array used in JavaScript. Speed of the virtual cricket was stable at 2 cm per second. The path of the virtual cricket was determined by a random trajectory with restraint: the initial point and ending points had identical coordinates, one loop lasted 20 s and was repeated, no linear trajectory was allowed and the virtual cricket had to move in the inferior half part of the screen (see Supplementary Material Video). At the beginning of the test, a male was placed into a half opaque box (10 cm diameter × 4 cm height). The box was placed 30 cm from the front of the screen in the middle of the arena.

The test occurred after 6 pm in a room with reduced lighting mimicking dusk ambience (0.1 photon s<sup>-1</sup>, ULM-500, Walz, Germany) and the virtual cricket program running on the screen. Each male was kept for 2 min inside the box for habituation before the beginning of the

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