



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 ($5.29 \text{ g} \pm 0.21$ and $5.20 \text{ g} \pm 0.22$ 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 mm³). 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 Enzy-mo-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⁻¹, 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

test. Frogs were free of moving inside the box. The box door facing the screen was opened by pulling a long line to avoid any interaction of the experimenter with the tested frog. Males were free to move in the arena. The behavioral sequence of attack is described as follows. The behavioral sequence of attack is described as follows. Males first point their heads toward the prey. Then, if near enough, they close in, keeping their head pointed toward the moving prey. Finally, they jump on the prey, sticking their tongues out for capture. Attack success was defined by the tongue touching the virtual prey on the computer screen. We stopped the test after 10 min from the opening of the box if no attack. The test was video filmed and watched with Windows Live Media Player to determine the time of each item in the behavioral sequence. We measured 1) the orientation latency (in s.) – i.e., the time from the opening of the box and the first head orientation toward the virtual prey, 2) the attack promptness – i.e., duration in s from the head orientation to the tongue sticking out and 3) the attack success by screening videos image per image to measure precisely whether the tongue touched the virtual cricket surface or not.

Data from males who did not go out of the central box during the 10 min were discarded from the experiment. Seventeen C-males and 16 T-males were successfully scored.

2.5. Statistics

The comparison of the body mass at the beginning of the experiment and testosterone levels between groups (T-males and C-males) was performed using a Student's *t*-test.

Because of the non-normality of the data distribution for detection time and attack duration, these two variables were log-transformed. After transformation, the comparison between groups (T-males and C-males) was performed using a Student's *t*-test.

Correlations among behavioral variables were tested using a Pearson test. Because we performed several correlations, we adjusted the *p*-value with Bonferroni correction.

We used logistic model to investigate the variation in attack success according to the testosterone supplementary treatment. More specifically, we constructed a logistic linear model using the individual binary response as the dependent variable.

Cohen's *d* effect size estimates were used to represent the amount of differences between two sample means. Commonly Cohen's *d* is categorized in 3 broad categories: 0.2–0.3 represents a small effect, ~0.5 a medium effect and over 0.8 to infinity represents a large effect.

Data were analyzed using packages “ade4” and “effsize” with the statistical software R 3.2.1 (R Development Core Team 2013).

3. Results

When males were captured on the pond at the beginning of the breeding season, both C- and T-males had the same testosterone levels in their saliva (Student's *t*-test, day 0, $t = -0.93$, $df = 16.29$, $p = 0.37$; Fig. 1). After 20 days of daily hormonal manipulation, T-males had testosterone levels in saliva 8.7 times higher than C-males (Student's *t*-test, day 20, $t = -8.71$, $df = 14.94$, $d = 3.28$ (large), $p < 0.001$; Fig. 1). Hence, when males performed the hunting behavior test, T-males and C-males had significantly different testosterone levels.

There were no significant correlations between pairs of any of the measured variables: the orientation latency, the attack promptness and the attack success score (Pearson test with Bonferroni correction, all $p > 0.05$, maximum $r^2 = 0.31$). Concerning foraging behavior, we found a significant effect of testosterone on the orientation latency. Indeed, the first attack of T-males was sooner than those for C-males ($81.84 \text{ s} \pm 36.25$ (mean \pm sem) and $108.06 \text{ s} \pm 28.06$ for T-males and

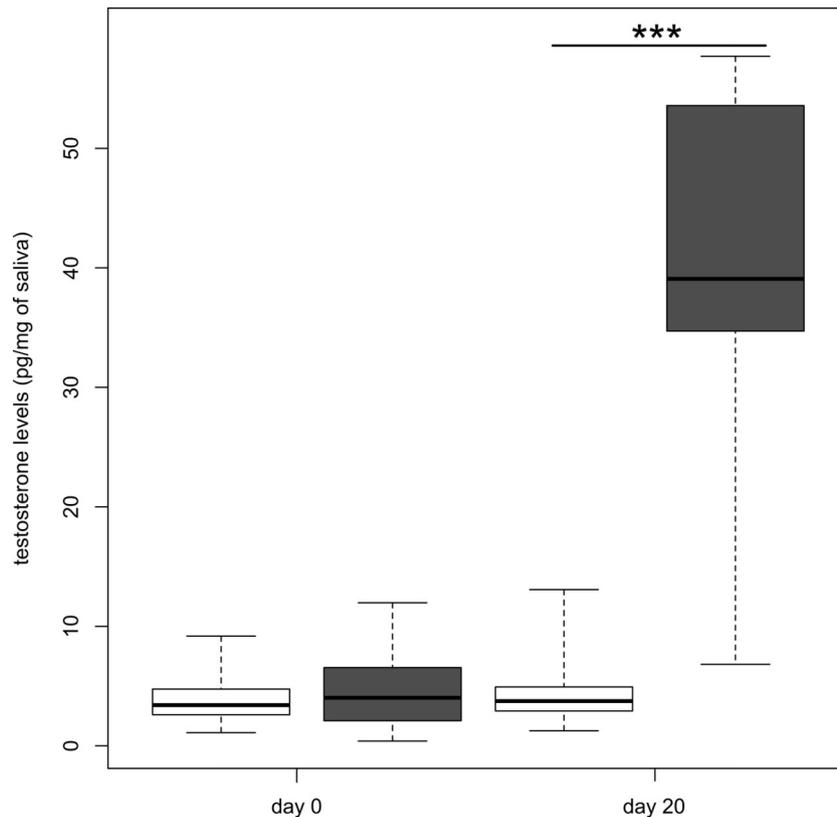


Fig. 1. Testosterone levels in saliva in pg/mg before and after 20 days of male testosterone level manipulation by daily application of $13.5 \mu\text{g}$ of testosterone on the backs of *H. arborea* ($n = 33$). C-males are represented in white, T-males in grey. Horizontal line: median value; box ends: upper and lower quartiles; whiskers: variation range of values. *** $p < 0.001$ on log-transformed values. The increase of testosterone observed in T-males after 20 days of supplementation is within the natural range of testosterone levels observed on the field during the breeding season (Desprat et al., 2015).

C-males, respectively; Student's *t*-test, log-transformed values, $t = 2.15$, $df = 25.85$, $d = 0.78$ (medium), $p = 0.04$; Fig. 2A). In addition, there was a significant difference between the attack promptness of T-males and C-males, defined as the time from the moment frog orients its head to its tongue touching the screen ($1.51 \text{ s} \pm 0.36$ and $3.23 \text{ s} \pm 0.88$ for T-males and C-males, respectively; Student's *t*-test, log-transformed values, $t = 2.20$, $df = 29.99$, $d = 0.78$ (medium), $p = 0.035$; Fig. 2B). At last, the success attack of T-males and C-males was similar (7/16 and 6/17 for T-males and C-males, respectively; family binomial, $F_{1,31} = 0.23$, $p = 0.63$; Fig. 2C).

4. Discussion

We demonstrated that an increase of the testosterone levels modified the hunting behavior of the male European tree frog *H. arborea*. We found that T-males first detected its prey more quickly and were more prompt to attack it than C-males. Nevertheless testosterone did not improve the male attack success. The testosterone dose used in our study permitted to increase by approximately 9 fold the testosterone levels between T and C-males. In previous exogenous supplementation studies, testosterone elevation rate is variable, often higher than in the present study (16.3 fold in the grey tree frog *Hyla chrysoscelis*, Girgenrath and Marsh, 2003; 15 fold in human Cueva et al., 2017), in the same range (7 fold in zebra finch *Taeniopygia guttata* Ardia et al., 2010) or sometimes lower (2 fold in lizards Pollock et al., 2012). In the present study, we choose to investigate the relationship between testosterone and foraging behavior using the method published previously

on the same species (Desprat et al., 2015). Indeed, a daily supplementation of $4.5 \mu\text{l}$ of hormone allowed increasing testosterone level of frogs while remaining within the natural range measured in the field for this species.

4.1. Testosterone improves prey detection

T-males were more watchful than C-males. The first detection time of the target was 20 s sooner for T-males than C-males. In our study, the hunting test began when the frog was released. We found that the duration of time between opening the box and the first attack was testosterone dependent. To the best of our knowledge, there is no study that has investigated the testosterone effect on hunting amphibian behavior. In a larger framework, if we consider the effect of testosterone on cognitive tasks, our results are in agreement with those published by Lacreuse et al. (2012) on the primate *Macaca mulatta*. Their study showed that testosterone modulates visual recognition memory independently of the delay duration of the stimuli, suggesting that testosterone increases prey detection. However, the neural mechanism that underlies this effect is unknown and likely complex. It has been proposed that testosterone increases the social vigilance in humans by acting on oxytocin peptide circuits in the central amygdala situated toward the hippocampus (Huber et al., 2005; Bos et al., 2010; Johnson and Breedlove, 2010). A meta-analysis (Pfannkuche et al., 2009) concluded that such an effect of testosterone on cognitive function could be observed in other vertebrates groups. It is now interesting to investigate the relationship between testosterone and cognitive abilities involved

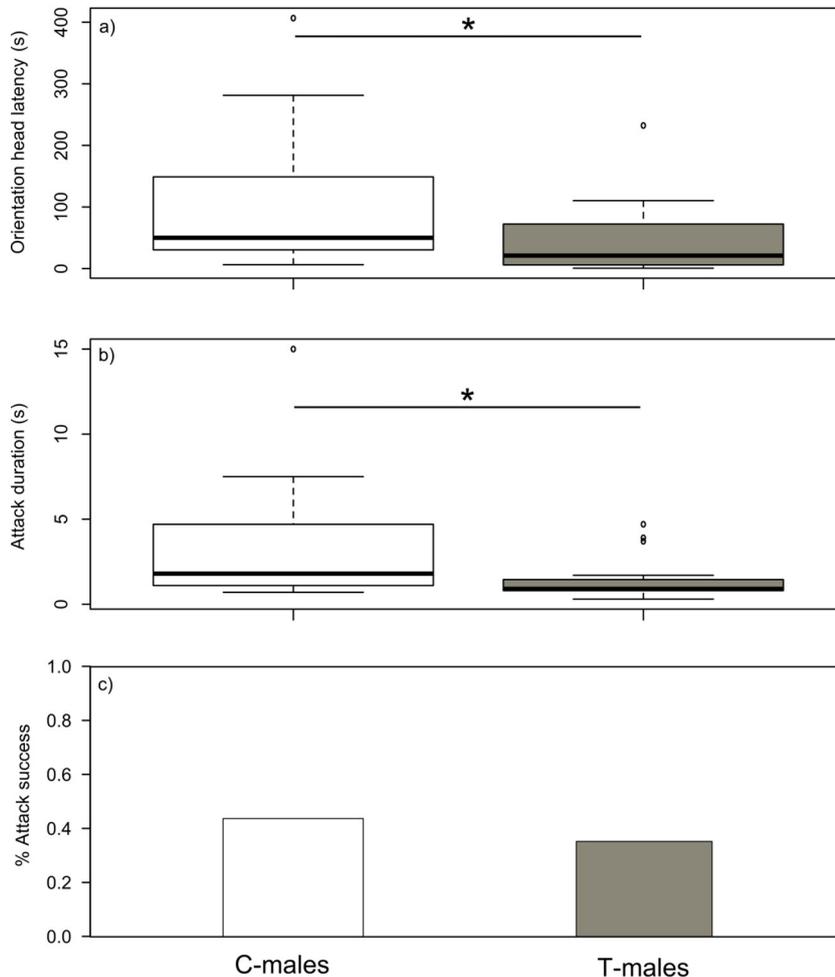


Fig. 2. measured variables during the hunting behavior test. Whisker plot represent the orientation latency (a) and the attack duration (b) and histogram represents the percentage of males which have success their attack (c). C-males are in white and T-males in grey. $n = 33$. (Horizontal line: median value, box ends: upper and lower quartiles, whiskers: maximum and minimum values). * $p < 0.05$ on log transformed values.

in hunting tasks because energy availability is a key point in almost all behavioral processes.

4.2. Testosterone increase attack promptness

We found that T-males' attack promptness was 2 fold higher than in C-males. Our experimental design was thought to assure a similar motivation to hunt for each male group. Indeed, during the breeding season in the field, males alternate fasting periods when calling from the pond and foraging periods once reserves are critically depleted (Friedl and Klump, 2005). We chose the fasting period duration (10 days) in our experiment to decrease the body mass of frogs and increase their hunting motivation during the test in accordance with field observations (Friedl and Klump, 2005).

In a physiological and mechanistic point of view, a higher promptness attack suggests a testosterone effect on integration and interpretation of information in the central neural system (CNS) and/or an effect on motor command reactivity. In mammalian, it is known that testosterone exerts activation effects on the CNS (review in Bos et al., 2012) and these effects can be observed within a few minutes (Heinlein and Chang, 2002; Michels and Hope, 2008). In amphibian, androgen receptors have been observed in the CNS (Perez et al., 1996) and we could make the assumption that information treatment by CNS in T-males frog brain is faster than in C-males thus reducing the delay to take the decision of attack. Furthermore, testosterone could also increase the motor command reactivity. Brennan and Henderson's (1995) study in *Xenopus* suggests that androgen could directly alter ACh-mediated synaptic transmission by altering the calcitonin gene-related peptide (CGRP) content in innervating motor neurons. Even if these testosterone effects have been mostly studied in dimorphic skeletal muscles (see citations in Brennan and Henderson, 1995), we cannot exclude testosterone effects on neuromuscular performance of the hindlimb and tongue muscles activated during frog hunting attack.

4.3. Testosterone and attack success

Contrary to what was expected, testosterone supplementation did not affect the attack success of male frogs. According to the results of Aho et al. (1993) on another anuran, indicating that toads are usually best hunters in low lighting conditions due to the speed retinal of their ganglion cell responses, we chose a low light intensity during the behavioral test. In order to acquire precise information about attack success, we chose to use an experimental setup that ensured all frogs were submitted to the same conditions. Video tracking permitted us to measure precisely whether the tongue touched the virtual cricket surface. In our study, males presented on average <40% successful attacks against the virtual cricket. Very few studies have experimentally measured the success of attacks in anurans. Because a virtual prey could not be eaten and continue to move after attack attempt, virtual attack success is difficult to determine. That is the reason why Gonzalez-Bernal et al. (2011) measured only the feeding rate as the number of attempted insect capture by the cane toad (*Rhinella marina*). To our knowledge, one study focused specifically on the link between testosterone and attack success. In humans (Tsimane hunters), although testosterone levels increased when killing and remained high for successful hunters, there is no evidence that testosterone levels were directly responsible for the hunt success, particularly as humans share prey with family and benefits from social recognition (Worthman and Konner, 1987; Trumble et al., 2013).

4.4. Foraging ability and sexual selection

In *H. arborea*, 20 days of hormonal supplementation highlighted testosterone implications in foraging behavior. Indeed, testosterone

enhanced prey detection and attack promptness of male frogs in a virtual hunting behavior test. Conversely, in our experimental conditions, there were no differences between the attack successes of T-males and C-males. Our experimental setup ensured the same motivation for all tested males, and the levels of testosterone due to exogenous supplementation were in the natural range of field hormone levels measured during the breeding season (Desprat et al., 2015). Nevertheless, this experimental setup was far from the natural hunting conditions. Tree frogs are "sit and wait" predators: they remain motionless in a bush or tree branch and wait for prey to come within ambush distance before pouncing. In nature, the attack success is the result of complex behaviors that require a synchrony and visual coordination: detect and keep eyes on the prey, estimate its trajectory, jump with precision and pull out the tongue to capture the prey. In dense vegetation such as *Rubus fruticosus* where frogs are often observed hunting, it is probably a key advantage to detect prey more quickly and take rapid decision to attack because prey moving in such vegetation disappears quickly out of the reach of the frog. Hence, an increase in prey detection associated to a decrease in delay for attack decision strongly suggests a better foraging efficiency for T-males compared to C-males. Foraging theory's central premise assumes that behavioral actions that affect the acquisition of food ultimately affect fitness (Blanckenhorn, 1991). In the guppy (*Poecilia reticulata*), Karino et al. (2005) showed that males bearing the most conspicuous orange coloration, which is a characteristic selected by females, exhibit the highest foraging ability during a test in experimental conditions. In *H. arborea* a previous study emphasized the relationship between sexual signals and testosterone levels (Desprat et al., 2015). The next step is to test in the field that males with the higher testosterone levels are the most efficient hunters and have the most attractive sexual signals.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yhbeh.2017.02.003>.

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