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Androgen responsiveness to simulated territorial intrusions in *Allobates femoralis* males: Evidence supporting the challenge hypothesis in a territorial frog

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ABSTRACT

Territoriality has been widely described across many animal taxa, where the acquisition and defence of a territory are critical for the fitness of an individual. Extensive evidence suggests that androgens are involved in the modulation of territorial behaviours in male vertebrates. Short-term increase of androgen following a territorial encounter appears to favour the outcome of a challenge. The "Challenge Hypothesis" proposed by Wingfield and colleagues outlines the existence of a positive feedback relationship between androgen and social challenges (e.g., territorial intrusions) in male vertebrates. Here we tested the challenge hypothesis in the highly territorial poison frog, *Allobates femoralis*, in its natural habitat by exposing males to simulated territorial intrusions in the form of acoustic playbacks. We quantified repeatedly androgen concentrations of individual males via a non-invasive water-borne sampling approach. Our results show that *A. femoralis* males exhibited a positive behavioural and androgenic response after being confronted to simulated territorial intrusions, providing support for the Challenge Hypothesis in a territorial frog.

1. Introduction

Territoriality is widespread across many animal taxa and provides the territory holder with primary access to critical resources for individual fitness including food and shelter, but most importantly mating opportunities and nesting places. In many species, males and females engage in competitive interactions and contests with conspecifics for the acquisition of territories, although far more research is done in males (Davies, 1991; Rosvall et al., 2020). There is extensive evidence that androgens are involved in the modulation of territorial behaviours such as advertisement signalling and agonistic encounters in male vertebrates (Adkins-Regan, 2005). Furthermore, because androgens are also essential for reproductive physiology and behaviour, it is not surprising that the androgenic regulation of aggressive behaviour is generally more pronounced within a reproductive context (Wingfield et al., 1997).

Indeed, in most species of temperate zones with a seasonal breeding pattern, androgen levels also undergo a seasonal fluctuation being higher during territory establishment and during the reproductive season, when the competition between males is high (Wingfield et al., 2006). Instead, tropical species with prolonged breeding and year-round territoriality present typical low androgen-baseline concentrations along the year but can facultatively rise during heightened male-male competition (Wingfield et al., 2006; Eikenaar et al., 2012). However, while high androgen levels favour courtship and aggressive behaviour, they simultaneously inhibit parental activity (Goymann and Dávila, 2017; Wingfield et al., 2001). These contrasting effects lead to a strong trade-off between keeping androgen levels high to favour resource defence and mating, or low to optimise reproductive success. Consequently, seasonal fluctuation in androgens is defined by the breeding system of the species and the level of male-parental investment. The "Challenge Hypothesis" proposed by Wingfield et al. (1990) outlines the dynamics of male androgen levels in response to social interactions, in different life history stages of various breeding systems. For instance, in males of monogamous species, androgen levels should rise when

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territorial males are challenged by an intruder male but drop down below breeding baseline during parental care. Thus, increase in androgen levels should be brief to allow onset of parenting duties, but strong enough to promote aggressiveness towards intruders, favouring resource defence and reproductive success.

The Challenge Hypothesis has been tested in many different species with various classical mating systems such as monogamy or polygamy, by comparing androgen levels in a non-stimulated condition (baseline) and after a simulated territorial intrusion tests (STI). These tests typically consist in presenting to a territorial male a stuffed or alive conspecific male decoy, a male conspecific acoustic stimulus, or both. Although early studies in birds confirmed males' androgenic response to STIs, later studies also in birds and including other vertebrate groups found conflicting results that no longer supported the original predictions of the Challenge Hypothesis (Dijkstra et al., 2011; Hay and Pankhurst, 2005; Landys et al., 2007; Ross et al., 2004; Thompson and Moore, 1992). These mixed outcomes have led to rethink the predictions of the Challenge Hypothesis (see Goymann et al., 2019), and prompted wider research across vertebrate taxa with a diverse suite of life-histories regulated by androgens (Goymann et al., 2019; Moore et al., 2019). Curiously, the Challenge Hypothesis has been extensively tested in fish and birds but has been largely ignored in amphibians (Moore et al., 2019). Probably but not certainly, hormone sampling was a limiting factor as most frog species are too small to yield enough plasma in a repeated pre- and post-treatment experimental design. Also, because the mating systems, reproductive modes and parental care types in amphibians are more complex (Wells, 2007), and in many cases less "clear cut" compared to birds, for instance, which have precise breeding and non-breeding territories.

Like in birds, tropical amphibians exhibit a multitude of traits allegedly modulated by androgens, such as advertisement calling, parental care and reproductive behaviours (Moore et al., 2005; Mendonça et al., 1985; Townsend and Moger, 1987). For instance, previous evidence has shown that conspecific acoustic signals promote the production of androgens and evoke calling activity in male frogs with a lek mating system (Brzoska and Obert, 1980; Burmeister and Wilczynski, 2000, 2001; Chu and Wilczynski, 2001; Still et al., 2019; but see: Leary, 2014; Muñoz et al., 2020; Solís and Penna, 1997). Also, Emerson and Hess (1996), suggested that testosterone levels can predict intermale aggression in opportunistic breeding tropical frogs. Furthermore, de Assis et al., (2012), showed that males of the gladiator frog (*Hypsiboas faber*) did not show a behavioural or androgenic response after a STI-test. Yet, experimental evidence on androgens shifts in response to STIs in territorial males that also care for offspring is still missing. Almost all male Neotropical poison frogs (Dendrobatidae and Aromobatidae) have been found to exhibit both aggressive territorial defence towards conspecifics and parental care in form of egg-guarding and/or tadpole-transport (Pröhl, 2005; Schulte et al., 2020). Males defend multi-purpose territories that serve for foraging, oviposition and egg-attendance (Pröhl, 2005). In most cases, males are the caregivers for eggs and tadpoles (Schulte et al., 2020). Furthermore, Fischer and O'Connell (2020) recently showed evidence in the laboratory that male caregivers of the dying poison frog (*Dendrobates tinctorius*) present lower plasma testosterone levels when caring tadpoles, compared to males caring for eggs or non-caring males. This evidence suggests that changes in androgen levels influence parental care in Neotropical poison frogs. Thus, Neotropical poison frogs remain a good but unexplored model to study the trade-off between androgen-mediated territorial defence and paternal care.

In this study, we tested the effects of territorial challenges on the behavioural and androgenic response of the Brilliant-thighed poison frog, *Allobates femoralis*, during the breeding season. This species has a polygynandrous mating system, in which both males and females have multiple reproductive partners along the breeding season (Ursprung et al., 2011). Previous studies have shown that males present a strong territorial aggression towards intruders and/or acoustic playbacks (i.e., positive phonotaxis; Narins et al., 2006; Ursprung et al., 2009). Parental care is mainly provided by males in form of clutch attendance and obligate transport of tadpoles (Ringler et al., 2015). While egg-attendance occurs inside the territory, males shuttle tadpoles to nearby water pools that are usually outside their territory and quickly return to resume territorial advertisement (Beck et al., 2017). Therefore, in *A. femoralis* territoriality contributes only partially to parental phase and is dissociated from full development of the offspring. Based on these life-history traits, according to the Challenge Hypothesis one may predict that *A. femoralis* males should show a brief increase of androgen levels, above the breeding baseline, when challenged by a conspecific male. To test this prediction, we challenged territorial males by performing STI-tests in the form of conspecific acoustic playbacks. Additionally, we measured males' pre- and post-challenge androgen concentrations from water samples. Measurement of water-borne hormones has become an advantageous and non-invasive technique that minimizes the stress to the animals and allows the researcher to repeatedly measure hormone levels in the same individuals (Still et al., 2019; Baugh and Gray-Gaillard, 2020; Baugh et al., 2018; Gabor et al., 2016, Gabor et al., 2013). Prior to analysis, we carried out a series of laboratory and field tests to validate the efficiency of the technique, and to investigate if hormone concentrations in the holding water correlate to those in the blood. Since there might be effects of time of the day and spontaneous behaviours on androgens concentration (Wada, 1986), we measured calling, locomotor, courtship and foraging activity across the day in addition to water-borne androgen levels prior to the territorial challenge. We further examined whether the intensity of the behavioural response was coupled with the androgen response to STIs.

2. Material and methods

2.1. Study system

The brilliant-thighed poison frog, *Allobates femoralis*, is a diurnal and terrestrial species belonging to the family Dendrobatidae (AmphibiaWeb, 2020; Boulenqer, 1883). Males exhibit strong territoriality within the prolonged breeding season, which usually begins with the onset of the rainy season (Kaefer et al., 2012; Montanarin et al., 2011). Territories are abandoned during the dry season and re-negotiated by males at the beginning of the next rainy season (Ringler et al., 2009). During territorial interactions or courtship displays, males produce acoustic signals from elevated structures on the forest ground to attract females and repel rivals from surrounding areas. Vocal and territorial behaviours of *A. femoralis* males are more frequent in the afternoon than in the morning (Roithmair, 1992). Males' territorial displays consist, first in antiphonal calling to warn neighbouring males of the ownership of a territory and second, in a phonotactic or agonistic response towards intruder males (Hödl, 1983; Narins et al., 2003; Ursprung et al., 2009). Males' phonotactic approach consists in an 'all-or-none' behavioural response towards conspecific calls. That is, once they perceive a conspecific territorial intrusion, they approach towards the sound source to repel the intruder (Hödl et al., 2004; Ringler et al., 2011; Ursprung et al., 2009). Courtship behaviour usually starts in the afternoon and ends on the next morning (Hödl, 1983; Ringler et al., 2013; Roithmair, 1992; Stückler et al., 2019), where the male leads the female towards the oviposition site through a locomotor display called "courtship march" (sensu Montanarin et al., 2011). After oviposition, the female leaves the territory and the male takes care of his offspring until they are developed as tadpoles. At that stage, the male will transport the tadpoles on its back and deposit them into a nearby water-pool, where the finalization of the development will take place.

2.2. Sample collection

For water-borne hormone sampling and extraction we followed the methodology published elsewhere (Gabor et al., 2013; Baugh et al.,

2018; Baugh and Gray-Gaillard, 2020). Methods to measure water-borne steroids were originally developed in fish, where free steroids are mostly diffused into the water through the gills (Scott et al., 2008). In amphibians, alternative releasing pathways of free steroids include the skin and mucous membranes whereas conjugated forms or metabolites are released through the urine and faeces (Baugh et al., 2018; Narayan et al., 2019). It is noteworthy that anti-androgen immunoreactive substances in water samples may include some androgenic conjugate forms that cross-react with the antiserum in the assay, therefore, the assay is likely to measure more androgens than water-borne testosterone alone (Baugh and Gray-Gaillard, 2020). Thus, throughout this manuscript we refer to "water-borne androgen" as to androgens and metabolic products present in the holding water, as mentioned in similar publications (Scott et al., 2008). Every water bath consisted of a glass container (14cmx9cmx5cm) filled with 40 mL of distilled water. Androgens were extracted by collecting each water sample with 20 mL sterile syringes coupled to an individual C18 cartridge (SPE, Sep-Pak C18 Plus, 360 mg Sorbent, 55–105 μm particle size, #WAT020515, Waters corp., Milford, MA) with a flow rate of ca. 10 mL/min. Later, cartridges were eluted with 4 mL of 96% EtOH into 8 mL borosilicate vials and stored at 4 °C until further hormonal analysis in laboratory. Samples were dried down with N_2 at 37 °C, resuspended with 250 μL of the assay buffer (provided in the ELISA kit, see below) and incubated overnight at 4 °C for further analysis.

2.3. Hormone assays

In order to estimate androgen concentration, we used a commercial enzymatic immunoassay for testosterone (ADI-900–065; Enzo Life Sciences, Farmingdale, NY, USA). Reconstituted samples were brought to room temperature and shaken at 500 rpm for 1 h prior the assay. Samples were analysed in duplicate, and assays were performed following the manufacturer's protocol. Plates were read at 405 nm, with correction between 570 and 590 nm, using a microplate reader (Multiskan Go, Thermo Fisher Scientific Oy, Finland) and androgen concentrations were calculated using the Thermo Scientific SkanIt Software (version 4.1). The detection limit for the assay was 5.67 pg mL^{-1}. The cross reactivity of the testosterone antibody with other androgens was below 15% (see manufacturers manual). Intra-assay coefficient of variation (CV) of duplicates was calculated for every plate by averaging the CVs of all the experimental samples. Inter-assay CV was calculated by including one pooled standard sample (125 pg mL^{-1} of T) in duplicate, in every plate and then averaging the CVs for that standard. Pooled samples were prepared in 100% ethanol and dried together with our unknown samples. The average intra- and inter-assay coefficient of variation were 3.38% and 11.05%, respectively.

2.4. Validation of water-borne androgens in the laboratory and the field

2.4.1. Recovery efficiency and androgens release rate in the lab

In order to calculate recovery efficiency of testosterone after SPE extraction, we spiked two pools with 2 different testosterone concentrations, using commercial standards of the ELISA kit (see above). Water samples "without frog" were also processed as blank controls to evaluate any possible contamination of the holding water. Samples were extracted and processed as described above and stored at 4 °C until proceeding with the assay. Although in previous studies the duration of keeping frogs into the water bath to collect water borne hormone samples was usually 60 min (Gabor et al., 2013; Baugh et al., 2018; Baugh and Gray-Gaillard, 2020), we additionally assessed water-borne androgen release rate in order to optimize sampling duration within the first hour. For this we used thirteen adult *A. femoralis* (Body-size mean ± SD: males = 2.74 ± 0.03 cm, N = 7; females = 2.79 ± 0.02 cm, N = 6) from a laboratory population kept at the animal care facilities at the University of Vienna. Briefly, we manually placed each frog in consecutive water baths of sampling periods of 15, 30 and 60 min. We compared samples collected from females and males as an additional control for methodology to quantify water borne androgen concentration. All samples were collected between 08:00 and 10:00 A.M., then extracted and processed as described above and stored at 4 °C until the assay. All frogs were fed at libitum with wingless fruit flies every second day.

2.4.2. Correlation between plasma testosterone and water-borne androgens in the field

In order to test whether water-borne androgen reflected the actual levels of circulating testosterone in plasma at the time of sampling, we collected eighteen free-living adult *A. femoralis* males (snout-urostyle length (SUL) mean ± SD = 2.8 ± 0.1 cm) in April 2019, from a population in the vicinity of Roura, French Guiana (4°43′ N – 52°18′ W). Frogs were attracted using playbacks, captured using plastic bags and transferred into individual water baths for 60 min. Water samples were processed as described above. After completion of the water baths, frogs were immediately euthanized with an overdose of 20% benzocaine gel and rapidly decapitated. Trunk blood was collected into 1.5 mL Eppendorf tubes and centrifuged at 6000 rpm for 5 min (6-position rotor; 2000×*g*) to separate the plasma. Plasma volume was recorded, and samples were transferred into 1.5 mL eppendorf tubes prefilled with 750 μL of 96% ethanol. In the laboratory, testosterone was extracted from ethanol samples three times with freeze-decanting following the methodology in Goymann et al., (2007). Briefly, samples were dried down with N_2 at 37 °C. Dried pellets were resuspended in 4 mL of dichloromethane and 100 μL of distilled water and, then incubated at 4 °C overnight for equilibration. The following day, samples were shaken for 1 h and then centrifuged at 4000 rpm for 10 min to separate the aqueous and organic phase, which was transferred into a new tube by freeze-decanting. This process was repeated twice, and the organic phase was then dried down at 37 °C under N_2 stream and then resuspended in the assay buffer supplied by the ELISA manufacturer and incubated overnight at 4 °C.

2.5. Simulated territorial intrusion-tests (STIs)

2.5.1. Field site and playback stimuli

Between February and April of 2018 and 2019, seventeen free-living adult *A. femoralis* males (SUL mean ± SD = 2.95 ± 0.06 cm) from a population located in the field station "Pararé" at Les Nouragues nature reserve in French Guiana (4°02′ N – 52°41′ W, 40 m a.s.l.; Bongers et al., 2013) were used for the STI-tests. STI-tests consisted in presenting the playback of an artificial advertisement call featuring the spectral and temporal parameters of a nearby population within the nature reserve Les Nouragues (Gasser et al., 2009; Narins et al., 2003). To avoid pseudoreplication, we created 11 different playback stimuli (16-bit, 44.1-kHz WAV-file) of ~4 min duration, which varied in the inter-note interval and the inter-call interval. Playbacks were broadcast using a loudspeaker (Creative MUVO 2c, Creative, Singapore) connected to a music player (G-Flash 512, Maxfield, Düsseldorf, Germany; company liquidated). Sound-pressure levels (SPLs) of every playback stimulus were calibrated at 75 – 80 dB (threshold range of phonotactic response; Hödl, 1983) using an SPL-meter (Voltcraft 329) at 1 m distance by adjusting the volume setting of the music player. All playbacks were conducted during the core breeding season of *A. femoralis* at the study site and all males tested were reproductively active adults. Furthermore, playbacks were performed under rainless conditions and in the afternoon (14:00 h – 17:00 h), because 1) male-male acoustic interactions are highest at that moment of the day, so individuals are more likely to respond to an STI and, 2) to avoid interference with males' tadpole transport, which usually occurs in the mornings (Ringler et al., 2013).

Finally, it is worth noting that we did not present an acoustic control stimulus (e.g. heterospecific calls, pure tone burst, frequency modulated notes, broad band noises, pulsed white noise or sine tones) to *A. femoralis* males, because previous evidence has shown that

A. femoralis males do not present phonotactic response to non-specific acoustic stimulus unless they have the correct species-specific temporal pattern and energy within the natural call note-frequency range (Amézquita et al., 2005; Göd et al., 2007; Hödl et al., 2004). Intraspecific acoustic communication is driven by selective pressures on the recognition mechanisms of species-specific signals (Gerhardt, 1994). Because a phonotactic and androgenic response is energetically expensive and carry concomitant costs (e.g., suppression of parental care, predation, risk of losing the territory), reacting to non-specific stimulus would be detrimental. Thus, it is unlikely that non-specific acoustic stimulus would elicit an agonistic response followed by an increase in androgen levels in *A. femoralis* males.

2.5.2. Experimental design

Focal males were tested using a pre-post experimental design which consists in comparing a hormonal and behavioural baseline with a post-social stimulation phase. During the pre-challenge phase (baseline), we measured water-borne androgens and spontaneous behaviours both in the morning and the afternoon. For this, we observed every focal male for 1 h from about 1.5 – 2 m distance and recorded the following behaviours: (1) duration of advertisement calls in seconds (four-note long-range call; Weygoldt, 1980), (2) duration of "warm-up" calls in seconds (suboptimal advertisement calls of less than steady-state SPL; Jameson, 1954; Toledo et al., 2014), (3) duration of courtship calls in seconds (close contact call directed to females; Weygoldt, 1980), (4) # of feeding events, (5) # of head-body orientations (HBO) and (6) # of jumps. Observations were made between 08:00 and 18:00 h. They were repeated three or four times at different moments of the day (i.e., morning and/or afternoon), in non-consecutive days and with a minimum of three days in between observations. After every behavioural observation, each frog was gently captured with a plastic bag and immediately transferred into a water bath for 60 min to assess the pre-challenge water-borne androgen concentration (breeding baseline levels). Every frog in a water bath was covered with a dark shell to avoid visual stimulation. Recaptured frogs were identified using digital images of their distinctive ventral pattern. As an additional control group, 24 females from the same population were additionally placed into individual water baths for 60 min in order to compare pre-challenge water-borne androgen levels between sexes. Because androgens are the main class of sex steroid hormones in male vertebrates and circulating androgens are typically lower in females (Adkins-Regan, 2005), we predicted to find higher water-borne androgen levels in males compared to females.

In the challenge phase, we presented STIs to focal males exclusively when they were found calling, in order to confirm the territorial advertisement stage. Once a focal male was located, we placed the loudspeaker on the forest ground at 1 – 1.5 m distance from the focal male. We considered a positive response (responding) when males approached the playback and reached a plastic-circular perimeter around the loudspeaker of 30 cm diameter (Amézquita et al., 2005). A neutral response was recorded when males did not approach the loudspeaker and/or did not cross the perimeter before the playback was finished (non-responding). In order to determine the behavioural responsiveness of the frogs to the territorial challenge, we repeated the STIs trials three times per individual. Every trial was audio recorded and we measured the following behavioural parameters: (1) latency to the first head-body orientation towards the speaker, (2) latency to the first jump and, (3) latency until the frog reached the perimeter. Frogs were not handled or manipulated at least three days before any further STI. After the STIs (regardless of whether the males responded or not) males were caught and immediately transferred to a water bath from which we collected three samples reflecting the water-borne androgens released in the water in each consecutive 60 min (1 h, 2 h and 3 h). This sequence of water bath samples allowed us to determine whether androgens increase within the next hour after the STI and whether they return to baseline after such increase as predicted by the Challenge Hypothesis (Wingfield et al., 1990). Time elapsed between the end of the STI and the beginning of the water baths was always <10 min. Water samples were collected individually after every 60 min from the water bath without manipulating the frog to avoid stress. For this, we used two flexible polymer tubes with one end attached to the glass box and the other end attached to a 20 mL syringe. One tubing was used to pump the water into the glass box and the other was used to suck out the sample after every 1 h water-bath. Samples were processed and extracted as explained in the water-borne extraction section. We repeated STIs three times per focal male with at least three days between trials.

2.6. Statistical analysis

Prior to analysis, hormone data were log-transformed to fit normality. In order to know whether water-borne androgen concentration was dependent on the frogs' body size and/or body area, we first calculated the body area-SUL ratio for every frog by dividing the body area with the SUL. Later, we performed separate linear mixed models (LMM) for afternoon and morning baselines as response variables, body area-SUL ratio as fixed factor and the ID of the frogs as random factor. Because androgen baseline concentrations in the afternoon or the morning were not dependent on body area-SUL ratio (LMM: $\beta_{morning} = 1.18$, $t = 1.22$, $P = 0.22$; $\beta_{afternoon} = 1.68$, $t = 1.61$, $P = 0.11$), water-borne androgen levels were not corrected for body size or area. In order to determine the release rate of androgens in water, we compared the time series water baths (15, 30 and 60 min) by performing a LMM to account for the repeated measurements, using the "lmer" function within the *lme4* package (Bates et al., 2015) in R (R Core Team, 2017). We used androgen concentration as the response variable, the time series of water baths as the fixed factor and the frog ID as the random factor.

To determine the correlation between hormone concentration in blood and holding water, we performed a parametric correlation between the plasma and water-borne androgen concentrations using the Pearson's product moment correlation coefficient. In order to compare water-borne androgen levels between males and females, we performed a two-sample *t*-test. Since time of the day, vocal and locomotor activity might be interdependent with androgen concentrations (Wada, 1986), we asked whether baseline androgen levels were dependent on natural behaviours and varied across the day. For this, we first performed a LMM with water-borne androgen levels as response variable, time of the day (morning/afternoon) as fixed factor and frog ID as the random factor. Then, we performed a Varimax normalized principal component analysis (PCA) in order to minimize redundancy among the behavioural variables by using the function "principal" within the R package *psych* (Revelle, 2019). Further, we performed a series of independent LMMs with the scores of the principal components obtained as response variables, time of the day (morning/afternoon) and water-borne androgen levels as fixed factors and frog ID as the random factor.

In order to investigate whether *A. femoralis* males respond to territorial challenges (STIs) with an increase in androgen levels, we first performed a LMM with androgen concentration as dependent variable, and the sampling time points (0 h-morning/afternoon baselines-, 1 h, 2 h and 3 h water bath sampling after STIs) as fixed effects. We used frog ID as the random factor to account for repeated measurements. Further, to compare the androgen responsiveness to STIs between responding and non-responding males, we estimated the androgen responsiveness to male-male interactions ($R_{male\text{-}male}$; Goymann et al., 2007). To do so, we computed the within-subjects standardized effect size (Cohen's d) of the ratio between the water-borne androgen concentration of every male after the STI and the baseline levels. Cohen's d allows us to directly compare the magnitude of the androgen response by estimating the difference between pre (baseline) and post (STI-challenged frogs) water-borne androgen concentrations on a standardized scale (Goymann et al., 2007a,b). For this, we used the function "cohens.d" within the R package *misty* (Yanagida, 2020).

Finally, to test if the phonotactic approach of *A. femoralis* males is proportional with the androgen responsiveness, we first minimized redundancy among the three responsiveness latencies (latency to the first head-body orientation towards the speaker, latency to the first jump and, latency until the frog reached the perimeter) by using a varimax normalized principal component analysis (PCA). Then, we performed a LMM with the principal component scores as the response variable, the androgen responsiveness ($R_{male\text{-}male}$) as the fixed effect predictor and the male ID as the random effect.

2.7. Ethics approval

All experiments were conducted in strict accordance with current Austrian, French and European Union laws and were approved by the Animal Ethics and Experimentation Board of the University of Vienna (No. 2018–010; 2019–002). Our study was approved by the technical director of the "Nouragues Ecological Research Station" where field work was conducted. We adhere to the "Guidelines for the use of live amphibians and reptiles in field and laboratory research" by the Herpetological Animal Care and Use Committee (HACC) of the American Society of Ichthyologists and Herpetologists. Collection permits were provided by the *Ministère de la transition écologique et solidaire, République Française* (No. TREL1902817S/152).

3. Results

3.1. Validation and sex differences of water-borne androgens

Recoveries of low and high standards were 98.21% and 105.98%, respectively. "Blank" water samples were below the detection limit of the assay (**Figure S1A**). The correlation between expected and obtained androgen concentrations was highly significant ($r = 0.99$, $P = 0.006$; **Figure S1B**), with concentrations falling within the range of detectability of the assay. Androgens released in 60 min water baths were significantly higher than 15 min (LMM: $\beta = -112.43$, $t = -2.33$, $P = 0.03$; **Figure S1C**), but not than 30 min water baths (LMM: $\beta = -46.05$, $t = -0.95$, $P = 0.35$; **Figure S1C**). Males had higher water-borne androgen levels than females after a 60-min water baths in the wild population (water-borne androgen mean ± SD: males = 317.30 ± 78.67 pg/mL; females = 243.64 ± 170.70 pg/mL; two sample *t*-test: $t_{(33.51)} = -3.07$, $P = 0.004$; **Figure S2**), but not in the laboratory population (LMM: $\beta = 41.77$, $t = 0.33$, $P = 0.74$; **Figure S1C**). Water-borne androgen concentration was positively correlated with plasma testosterone concentration (t = 4.82, r = 0.76, P < 0.001; **Figure S3**).

3.2. Daily variation of behaviours and water-borne androgen levels

Three components were generated with eigenvalues greater than 1 (Table 1): the first component (PC1) held 39% of the explained variance and was positively related to vocal behaviour (advertisement and warm-up call durations). The second component (PC2) accounted for 31% of the source of variation and represented positively courtship behaviour variables (number of HBOs, jumps and duration of courtship calls). The third component (PC3) explained 30% of the variance and represented positively variables related to foraging behaviour (number of HBOs and feeding events).

Table 1
Principal Component Analysis showing the loadings matrix of the behavioural variables in principal components with eigen values greater than 1.

	Principal components		
Variable	PC1	PC2	PC3
Advertisement call duration (sec)	**0.97**	0.09	0.03
"warm-up" call duration (sec)	**0.97**	−0.11	0.04
Courtship call duration (sec)	−0.01	**0.75**	−0.20
# of feeding events	0.06	−0.28	**0.86**
# of HBO	0.01	**0.45**	**0.77**
# of jumps	0	**0.78**	0.19
Proportion of explained variance	0.39	0.31	0.30
Eigenvalues	1.91	1.55	1.30

Pre-challenge water-borne androgen concentrations and vocal behaviour (PC1) were significantly higher in the afternoon than in the morning (water-borne androgens: ß=0.31, t = 2.55, $P = 0.01$; Fig. 1**A;** PC1: ß=0.56, t = 3.62, $P < 0.001$; Fig. 1**B**). However, vocal behaviour was not related to water-borne androgen concentrations (PC1: ß=0.07, t = 0.42, $P = 0.67$; Fig. 1**B**). Courtship behaviour (PC2) and foraging behaviour (PC3) were not significantly different over the day (PC2: ß=0.28, t = 1.52, $P = 0.13$; Fig. 1**C;** PC3: ß=−0.02, t = -0.1, $P = 0.92$; Fig. 1**D**) and/or dependent on water-borne androgen concentrations (PC2: ß=0.1, t = 0.55, $P = 0.58$; Fig. 1**C;** PC3: ß=0.19, t = 1, $P = 0.32$; Fig. 1**D**).

3.3. Effect of STI on water-borne androgen levels ($R_{male\text{-}male}$)

While 16 out of 17 males responded positively to the playback presentation in two of the three STI-tests repetitions, six males did not respond to the STIs in one of the repetitions. When frogs responded approaching towards the playback (i.e. positive phonotaxis), water-borne androgen levels significantly increased 1 h after the STI compared to the pre-challenge water-borne androgen levels both, in the morning (LMM: $\beta = 0.40$, $t = 3.49$, $P = 0.001$; Fig. 2**A**) and in the afternoon (LMM: $\beta = 0.21$, $t = 0.10$, $P = 0.04$; Fig. 2**A**). Subsequently, androgen concentration dropped nearly to the morning levels in the 2 h sampling point (LMM: $\beta = -0.06$, $t = -0.56$, $P = 0.57$; Fig. 2**A**), and under both morning and afternoon levels in the 3 h sampling point (LMM: $\beta = -0.58$, $t = -5.1$, $P = <0.001$; Fig. 2**A**).

On the other hand, when frogs did not react to the playback (i.e. neutral response), water-borne androgen levels weakly increased 1 h after the STI compared to the morning pre-challenge levels (LMM: $\beta = 0.35$, $t = 2.17$, $P = 0.05$; Fig. 2**A**) and did not change compared to the afternoon pre-challenge levels (LMM: $\beta = -0.02$, $t = -0.17$, $P = 0.86$; Fig. 2**A**). Later, androgen levels significantly decreased under both morning and afternoon pre-challenge levels in the 2 h sampling point (LMM: $\beta = -0.77$, $t = -6.07$, $P < 0.001$; Fig. 2**A**) and in the 3 h sampling point (LMM: $\beta = -1.44$, $t = -11.31$, $P < 0.001$; Fig. 2**A**).

Responsive males to the playback had a positive effect size and 95% confidence intervals did not cross zero (Cohen's d = 0.85 ± 0.94; Fig. 2**B**), suggesting a positive effect of STI tests on water-borne androgen levels. On the other hand, non-responsive males had a negative (and close to zero) effect size and 95% confidence intervals crossed zero (Cohen's d = −0.06 ± 1.15; Fig. 2**B**), and thus suggesting a null effect of STIs on androgen levels.

3.4. Effect of STI on the phonotactic behaviour

Three principal components were generated, but just one component had an eigen value greater than 1, which explained the 72% of the total variance (Table 2). This component was positively related to the three responsiveness latencies (latency to the first head-body orientation towards the speaker, latency to the first jump and, latency until the frog reached the perimeter). The phonotactic approach of *A. femoralis* males towards the playback was not related to the androgen responsiveness (LMM: $\beta = 0.58$, $t = 0.55$, $P = 0.58$).

4. Discussion

In this study we tested the ability of an androgenic response to a playback presentation, simulating a territorial intrusion in males of the brilliant-thighed poison frog (*Allobates femoralis*). Males of this species are territorial with multiple iteroparous breeding and high parental care during the breeding season. Our results demonstrate that water-borne androgen concentration was elevated after presenting a conspecific

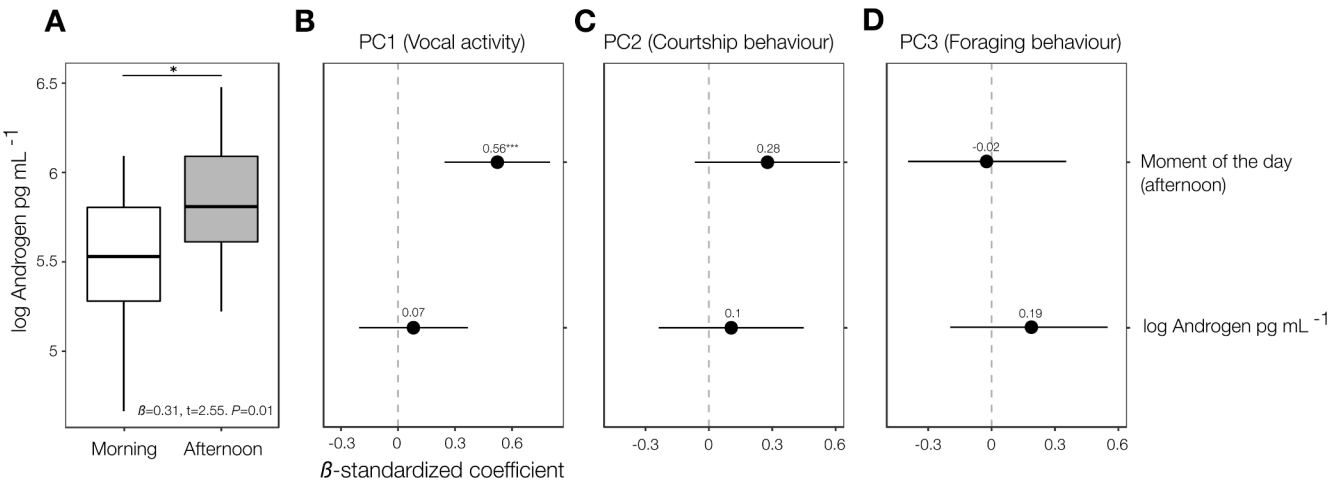

Fig. 1. Water-borne androgen levels, vocal, courtship and foraging behaviour across the day. (A) Boxplot showing the difference of water-borne androgen concentration between morning and afternoon; Linear Mixed Model plots showing z-scores values (x-axis) and the effect size (numbers over mean-points) of time of the day and androgens over vocal activity (B), courtship behaviour (C) and foraging behaviour (D). Solid lines represent 95% confidence intervals. *P < 0.05, **P < 0.001.

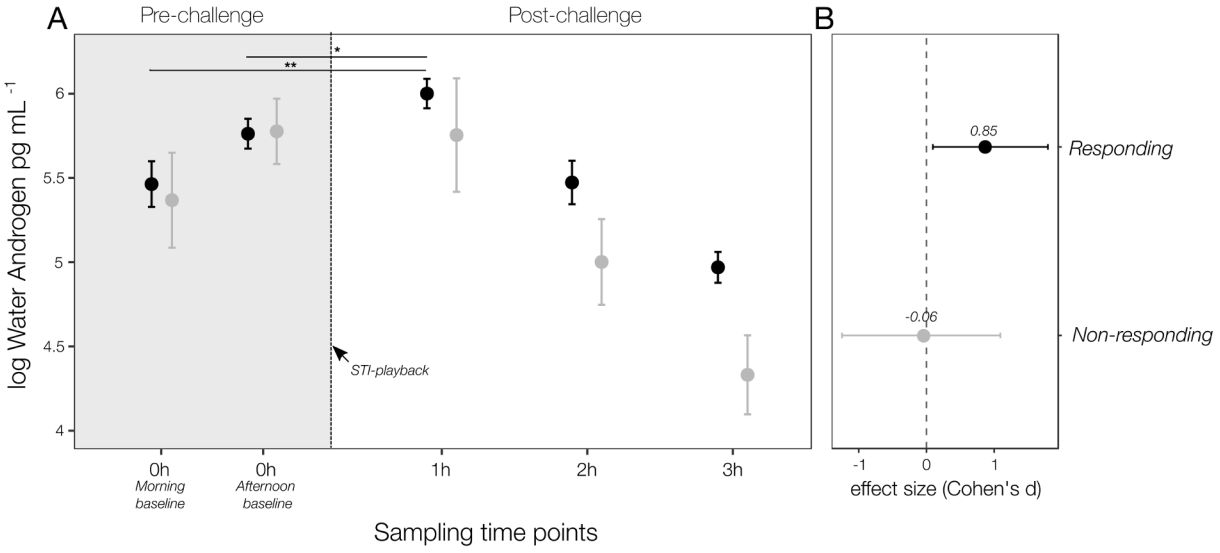

Fig. 2. Androgen responsiveness after STI in *A. femoralis* males. (A) Comparison of baselines of water-borne androgen concentration (morning/afternoon; pre-STI) between responding (black) and non-responding (grey) males over three sampling times (1 h, 2 h and 3 h; post-STI). *P < 0.05, **P < 0.001. (B) Differences in effect size (±95% confidence intervals for both variables) of the male-male androgen responsiveness ($dR_{male\text{-}male}$) between responding (N = 16) and non-responding (N = 6) males.

Table 2
Principal Component Analysis showing the loadings matrix of three variables related to responsiveness latencies.

	Principal components		
Variable	PC1	PC2	PC3
Latency to the 1st head-body orientation (sec)	**0.99**	−0.01	−0.16
Latency to the 1st jump (sec)	**0.99**	0	0.17
Latency to the perimeter (sec)	**0.48**	**0.88**	0
Proportion of explained variance	**0.72**	0.26	0.02
Eigenvalues	**2.28**	0.66	0.05

playback in *A. femoralis* males compared to a control situation, but only when males approached the playback loudspeaker and not in males which did not show a behavioural response to the playback. Our results suggest novel support for the Challenge Hypothesis in a Neotropical poison frog. Interestingly, the intensity of the phonotaxis to the playback was not related to males' androgen responsiveness to STIs.

Water-borne androgens concentration significantly increased 1 h after confronting *A. femoralis* males to STIs. This result supports the original prediction of the Challenge Hypothesis (Wingfield and Wada, 1989; Wingfield et al., 1990), which states that polyandrous species with male-only parental care should show an immediate and short increase of circulating androgens following a territorial encounter. Our results also suggest that such increase in androgen levels in response to the STI was facultative and independent from the natural endocrine status and spontaneous behaviours in both the morning (when androgen concentration and vocal interactions were low; Fig. 1) and afternoon (when androgen concentration and vocal interactions were high; Fig. 1). The general idea of the functional significance of the increase of androgens is that of preparing males for a potential agonistic contest, such as by increasing its muscular contractile capacity and locomotor performance (Miles et al., 2007). Because territorial defence is critical for the fitness of *A. femoralis* males (Ursprung et al., 2011), STI-induced androgens

would increase the chances of maintain territorial intruders at bay (Wingfield, 2005).

However, the androgen responsiveness to a conspecific territorial challenge might depend on the type of care that is provided by *A. femoralis* males when confronting the challenge. On the one hand, clutch defence in *A. femoralis* is a by-product of territorial defence, because males repel intruders that can overtake the territory and cannibalize the clutches in it (Ringler et al., 2017). In this sense, facultative elevations in androgen levels are likely to favour clutch and territorial defence, without concomitant costs in paternal duties (Moore et al., 2019). On the other hand, *A. femoralis* males have also obligatory tadpole transport for offspring survival (Weygoldt, 1980; Ringler et al., 2013). Experimental evidence suggests that males do not attack playbacks when parental care is induced (i.e., via artificial tadpole adding; Rodríguez et al., unpublished data) and that natural tadpole-carriers have significant lower water-borne androgen levels compared to non-carriers (Fischer and O'Connell, 2020; Rodríguez et al., unpublished data). Together, this evidence could hint towards the existence of a trade-off between an aggressive territorial response and tadpole transport. However, experimental evidence on the androgenic and behavioural response to STIs in tadpole carriers is needed to further develop this idea.

In this study, six out of seventeen males did not respond to one of the playback repetitions. Also, post-challenge water-borne androgen concentrations in non-responsive males were not statistically different compared to the pre-challenge levels in the afternoon. The absence of such a response to the STI in some *A. femoralis* males suggests several alternative explanations. First, if there is a trade-off between tadpole transport and a territorial aggressive response in *A. femoralis*, one may think that non-responsive males could have been close to pick-up their tadpoles if there were clutches ready-to-transport inside their territory. Although we were not able to confirm the presence of clutches ready-to-transport inside males' territories, tadpole transport predominantly occurs in the mornings (Ringler et al., 2013) and we tested the STIs in the afternoons when male-male territorial interactions mostly occur (Roithmair, 1992; this study). In this sense, the advantages of tadpole-transport logistics in *A. femoralis* may ease the temporal overlapping between paternal care and social interactions, therefore favouring reproductive success, territorial defence and mating.

Second, we cannot discard that the behavioural and androgenic response could be constrained by a high energetic demand due to increased calling activity (Emerson, 2001; Leary et al., 2006). That is, vocal effort is mediated by changes in circulating androgens and is energetically costly. In turn, glucocorticoids increase to meet the energetic requirements but at the same time reduce androgen production and calling activity. It is possible that non-responding males of *A. femoralis* were actively calling before the STI and therefore, the high energetic demand could have limited an androgen-mediated response. However, further evidence is needed to confirm this hypothesis.

Third, extensive evidence suggests that repeated territorial intrusions can reduce the probability of an increase in androgen concentrations due to a habituation in the physiological response (Aires et al., 2015; Hsu and Wolf, 1999; Kempenaers et al., 2008). However, we consider unlikely that non-responding males became habituated to the repeated STIs, because in most of the cases non-responding males presented positive phonotaxis to STIs conducted before and after the negative response. This is supported by the lack of differences in androgen levels between repetitions in any of the sampling time points in non-responding males (Figure S4). Finally, other factors like the synergistic effect of other hormones, climatic conditions and the density of the acoustic environment could have also contributed to the absence of response to the playback in some *A. femoralis* males. Yet, further experimental evidence is needed to understand the factors that affect the decision-making in *A. femoralis* males during a territorial challenge.

We did not find a relationship between androgen responsiveness and the latency of approach. In other words, males with higher androgen levels did not approach the loudspeaker faster. This may depend on the experimental setup of the STI and the nature of the STI stimuli (i.e. duration of the playback, live vs. synthetic decoy; reviewed by Goymann et al., 2007a,b). Although *A. femoralis* males are strongly territorial and usually jump towards the sound source in playback experiments (Hödl, 1983), they require to be confronted by bimodal signals (acoustic and visual) in order to display physical attacks (Narins et al., 2003). Thus, in *A. femoralis* males, playbacks alone may be enough to provoke an androgenic and phonotactic response, but the intensity of phonotactic approach may depend on the combination of acoustic and visual signals (see also Sonnleitner et al. 2020). Further experiments on the hormonal and behavioural response to territorial intrusions in territorial frogs may profit from the combination of playbacks and robotic frog models in order to create more realistic situations.

Water-borne sampling has enormous advantages for estimating hormonal concentrations with little manipulation of the research animals (Narayan, 2013). We found a positive correlation between plasma and water-borne androgens in *A. femoralis*, which allowed us to obtain physiologically relevant information on its territorial behaviour (evidence for the Challenge Hypothesis). While circulating hormones offer a snapshot of the current endocrine status of the individual, water-borne hormones are an integrated measure that reflects the endocrine status over a longer time frame (Baugh et al., 2018). As such, water-borne hormones are a retrospective measure of the endocrine state of the individual before the water bath (Narayan et al., 2019). Our results are in line with those found in other species e.g., fishes and amphibians (Baugh et al., 2018; Baugh and Gray-Gaillard, 2020; Gabor et al., 2016, 2013; Kidd et al., 2010). Water-borne androgen dropped down to pre-challenge levels (i.e., baseline) or even below two hours after the STI in *A. femoralis* males, which suggests a few alternative explanations. First, there will be costs associated with maintaining high androgen levels for a prolonged period of time such as the suppression of immune function, increase of parasitic infections (Folstad and Karter, 1992) and impairement of parental care (Wingfield et al., 1990). Thus, the return of androgens to pre-challenge levels after a short-term increase may ease to resume activities ongoing before the intrusion, as predicted by the Challenge Hypothesis. Second, we cannot exclude any immediate inhibition caused by the stress of being restraint in the glass box for 3 h, as a result of the inhibitory interlinkage between the hypothalamo-pituitary–gonadal and the hypothalamo-pituitary-interrenal (McCreery and Licht, 1984). However, additional research is necessary to further corroborate these alternatives. In sum, water-borne sampling is a non-invasive technique, which can give biologically informative means on how to study the interplay between social behaviour and hormones in small frogs.

5. Conclusions

Our study supports the Challenge Hypothesis in a territorial frog with male-parental care, by using STIs and a non-invasive technique to characterize androgen levels. Elevated androgen levels may promote both territorial behaviours and egg-attendance in *Allobates femoralis* males, favouring social interactions and reproductive success. Further experimental studies on the variability in androgen levels in amphibian species with diverse and complex life-history traits (e.g., biparental or female-only care, essential egg-attendance, multimodal communication) are needed, in order to broaden the conclusions on the Challenge Hypothesis across vertebrate animal taxa. Since water-borne hormones provide biologically and physiologically relevant information by strongly correlating with hormone levels in plasma, the integration of behavioural experiments and non-invasive hormone sampling in amphibians may open new research perspectives.

CRediT authorship contribution statement

Camilo Rodríguez: Conceptualization, Methodology, Investigation,

Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Resources, Funding acquisition, Visualization. **Leonida Fusani:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Supervision. **Gaëlle Raboisson:** Methodology, Data curation. **Walter Hödl:** Conceptualization, Methodology, Writing – review & editing, Resources, Funding acquisition, Supervision. **Eva Ringler:** Methodology, Writing – review & editing, Resources, Funding aquisition. **Virginie Canoine:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Resources, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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