

Journal Pre-proofs

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PII: S0016-6480(23)00221-6
DOI: <https://doi.org/10.1016/j.ygcen.2023.114416>
Reference: YGCEN 114416

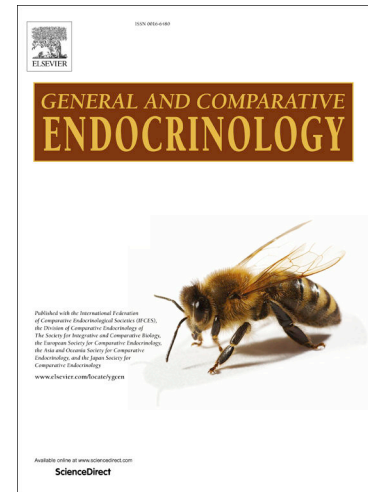
To appear in: *General and Comparative Endocrinology*

Received Date: 27 June 2023
Revised Date: 22 October 2023
Accepted Date: 20 November 2023

Please cite this article as: Ringler, E., Dellefont, K., Peignier, M., Canoine, V., Water-borne testosterone levels predict exploratory tendency in male poison frogs, *General and Comparative Endocrinology* (2023), doi: <https://doi.org/10.1016/j.ygcen.2023.114416>

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1 **Water-borne testosterone levels predict exploratory tendency in male poison frogs**

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21

22 Abstract

23 Hormones play a fundamental role in mediating social behaviors of animals. However, it is less well
24 understood to what extent behavioral variation between individuals can be attributed to variation in
25 underlying hormonal profiles. The goal of the present study was to infer if individual androgen levels,
26 and/or the modulation thereof, can explain among-individual variation in aggressiveness, boldness and
27 exploration. We used as a model the dart-poison frog *Allobates femoralis* and took repeated non-invasive
28 water-borne hormonal samples of individual males before (baseline) and after (experimental) a series of
29 behavioral tests for assessing aggression, boldness, and exploratory tendency. Our results show that
30 androgen levels in *A. femoralis* are quite stable across the reproductive season. Repeatability in $_{wb}T$ baseline
31 levels was high, while time of day, age of the frog, and trial order did not show any significant impact on
32 measured $_{wb}T$ levels. In general, experimental $_{wb}T$ levels after behavioral tests were lower compared to the
33 respective baseline levels. However, we identified two different patterns with regard to androgen
34 modulation in response to behavioral testing: individuals with low baseline $_{wb}T$ tended to have increased
35 $_{wb}T$ levels after the behavioral testing, while individuals with comparatively high baseline $_{wb}T$ levels rather
36 showed a decrease in hormonal levels after testing. Our results also suggest that baseline $_{wb}T$ levels are
37 linked to the personality trait exploration, and that androgen modulation is linked to boldness in *A. femoralis*
38 males. These results show that differences in hormonal profiles and/or hormonal modulation in response to
39 social challenges can indeed explain among-individual differences in behavioral traits.

40

41 **Keywords:** Testosterone, animal personality, behavioral challenge

42

43 1. Introduction

44 Several studies have demonstrated high within-individual consistency and between-individual variation of
45 behavior across time and contexts in several animal taxa (i.e. termed *animal personality*; Araya-Ajoy and
46 Dingemanse, 2014; Réale et al., 2007). Empirical and theoretical approaches have demonstrated how such
47 differences in behavior ultimately affect an animal's prospects of survival, competitive ability, mating
48 success and other fitness relevant traits (Dingemanse et al., 2004; Sih and Bell, 2008). One key question in
49 the study of animal personality is to what extent differences in behavioral phenotypes can be attributed to
50 constraints imposed by underlying physiology, such as hormonal profiles (Groothuis and Carere, 2005). In
51 turn, behavioral phenotypes might also arise from differences across individuals in their physiological
52 response to challenges in their social/natural environment (e.g. Biro and Stamps, 2010; Fürtbauer et al.,
53 2015; Réale et al., 2010; Sih et al., 2015).

54 Hormones play a fundamental role in the expression of morphological and behavioral traits
55 (Adkins-Regan, 2005). The causal relationship between hormones and behavior is bidirectional, as
56 hormones regulate the expression of social behaviors, but at the same time being exposed to behavior of
57 con- or heterospecifics can also induce a hormonal response in the focal individual (Adkins-Regan, 2005;
58 Gabor and Grober, 2010; Vitousek et al., 2014; Wingfield et al., 1990). The precise interplay between
59 hormones and behavior may differ between individuals of a population, as it depends on various factors,
60 such as experience during early development, an animal's physiological condition, environment and
61 adaptation to specific life-history stages – all of which may lead to a variation in behavioral phenotypes.

62 Animal personality is typically measured along five main axes: aggressive-docile, exploration
63 affine-averse, sociable-solitary, bold-shy and active-passive (Réale et al., 2007). Several behaviors across
64 these five axes are known to be modulated by sex steroids, precisely androgens and especially during the

65 reproductive season (Hau, 2007; Nelson, 2005). In male vertebrates, androgens play a key role in the
66 development and maintenance of primary and secondary sexual traits but also regarding the modulation of
67 different behaviors related to reproduction, such as courtship, mating behavior or territorial aggression
68 (Burmeister and Wilczynski, 2001; Fusani, 2008; Hirschenhauser et al., 2003; Hunt et al., 2019; Rosvall et
69 al., 2020; Rosvall et al., 2012). However, persistent high levels of testosterone (T) are expected to bear
70 considerable costs (Wingfield et al., 2001), such as reduced immune function (Dufty, 1989; McGlothlin
71 and Ketterson, 2008), increased risk-taking and resulting elevated predation risk (Marler and Moore, 1988;
72 Raynaud and Schradin, 2014), interference with parental and other social behavior (Fürtbauer et al., 2020;
73 Hegner and Wingfield, 1987; McGlothlin et al., 2007), and metabolic expenses (Buchanan et al., 2001;
74 Tobler et al., 2007). To minimize these negative impacts, androgens undergo seasonal fluctuations, with
75 the highest concentration during the breeding season and lowest during the non-reproductive period
76 (Goymann et al., 2019; Hau, 2007; Husak et al., 2021). High among- and within-species differences in the
77 levels of circulating T have been shown for many vertebrate taxa, and these differences have been linked
78 to respective social and environmental factors, such as breeding season length, type of mating system, and
79 latitude (cf. Husak et al., 2021). Also, on smaller timescales, fine-tuned temporal fluctuations in T
80 modulating behavioral and physiological responses to sudden environmental challenges may reflect the
81 trade-off associated with high and low levels of circulating T in males (Hunt et al., 1995; Rodríguez et al.,
82 2022; Romero et al., 1998; Wingfield et al., 1990). Identifying the ecological and physiological factors that
83 shape behavior at the species, population, but also individual level will advance our understanding about
84 the mechanisms that underline behavioral variation across these different levels of biological organization.
85 Previous research identified a prominent role of glucocorticoids (e.g. cortisol) for explaining differences in
86 behavioral profiles (Baugh et al., 2017; Baugh et al., 2012; Dosmann et al., 2015; Grace and Anderson,
87 2014), due to their prominent role in stress-axis-programming. Only few studies have looked how
88 androgens shape individual personalities (but see Hau and Goymann, 2015; Kraus et al., 2020; Mutzel et
89 al., 2011).

90 The aim of the present study was to infer if individual baseline androgen levels are repeatable (i.e.
91 if hormone levels show low within-individual and high between-individual variation) and can explain
92 among-individual variation in the personality traits aggressiveness, boldness and exploration. Furthermore,
93 we asked if also the modulation of androgen levels after a suite of behavioral challenges is repeatable across
94 individuals. We used as a model the Neotropical poison frog *Allobates femoralis*, a territorial species that
95 shows highly aggressive behavioral response towards acoustic playbacks, simulating calling intruders
96 (Hödl, 1983; Narins et al., 2003; Ringler et al., 2011; Rodríguez et al., 2022). We took repeated samples of
97 individual 'baseline' water borne testosterone (w_bT) levels (i.e., without any prior behavioral manipulation)
98 to assess repeatability in individual hormonal profiles over the course of the study period. Furthermore, we
99 took hormonal samples directly after a series of behavioral tests (hereafter 'experimental' w_bT), which
100 assessed territorial aggression, boldness and explorative behavior of individual males. For the hormonal
101 sampling, we used a non-invasive water bath method (Baugh et al., 2018; Gabor et al., 2013; Rodríguez et
102 al., 2022), which enabled us to take repeated samples of the same individuals in the field. We assessed
103 among- and within-individual consistency of w_bT to gain information about the consistency of individual
104 hormonal profiles over the course of several weeks during the breeding season. Additionally, we identified
105 if and how preceding behavioral testing will alter obtained hormonal measurements. The combination of
106 repeated hormonal sampling and behavioral testing allowed us to link the endocrine profile and modulation
107 of each individual to its personality.

108 We expected w_bT levels to be highly repeatable, especially when sampled without any preceding
109 behavioral manipulation. We also expected w_bT to be positively linked to levels of territorial aggression,
110 boldness, and/or exploration in the behavioral tests, given that previous studies in several species, including
111 *A. femoralis*, suggested a link between androgen modulation and spatial behavior in the context of territory
112 defense or homing (cf. Herman and Wallen, 2007; Hodgson et al., 2008; Pašukonis et al., 2022; Rodríguez
113 et al., 2022).

114

115 2. Materials and methods

116 2.1 Study site and study species

117 This study was conducted in a free-ranging population of *A. femoralis* on a river island of approx. 5ha,
118 close to the field camp ‘Saut Pararé’ (4°02’ N, 52°41’ W) in the nature reserve ‘Les Nouragues’, in French
119 Guiana (Bongers et al., 2001; Ringler et al., 2016). The island population of *A. femoralis* was established
120 in 2012 by introducing tadpoles from the nearby mainland population and has been stable ever since with
121 approximately 150 individuals (Ringler et al., 2015). We conducted fieldwork during the rainy season, from
122 the beginning of February 2019 until the end of April 2019, which coincides with the reproductive season
123 of the focal species (Gottsberger and Gruber, 2004).

124 *Allobates femoralis* (Boulenger 1883) is a small, diurnal Neotropical poison frog (Dendrobatidae
125 *sensu* AmphibiaWeb, 2023), which is distributed throughout the Amazon Basin and Guiana Shield. During
126 the reproductive season, males are highly territorial and advertise territory occupancy to male competitors
127 and potential female mating partners by producing loud advertisement calls from exposed, elevated
128 positions (Hödl, 1983; Ringler et al., 2011; Rodríguez López and Hödl, 2020; Roithmair, 1992). Males
129 vigorously defend their territory against conspecific intruders (Narins et al., 2003). Females exhibit site
130 fidelity but are typically not aggressive towards either sex (Ringler et al., 2012; Ringler et al., 2009), and
131 actively approach neighboring calling males for courtship and mating (Montanarin et al., 2011; Stückler et
132 al., 2019). Egg deposition takes place in the male’s territory and both sexes mate multiple times with
133 multiple partners (Ringler et al., 2012; Ursprung et al., 2011). After hatching, tadpoles are typically
134 transported by the male to medium sized water bodies located up to 200m outside the territory (Beck et al.,
135 2017; Ringler et al., 2018; Ringler et al., 2013).

136

137 2.2 Population monitoring

138 We surveyed the entire population every day from 0900 to 1800 h. We identified all frogs on site via digital
139 pictures of their unique ventral patterns and later verified their identity with the pattern matching software
140 Wild-ID (Bolger et al., 2012). Frogs were sexed by the presence (males) or absence (females) of a vocal
141 sac. We recorded the precise location of the frogs on a digital map, using a tablet PC (WinTab 9, Odys,
142 Willich, Germany) equipped with the mobile GIS software ArcPad 10.2 (ESRI, Redlands, CA, U.S.A.).
143 We determined body size (snout urostyle length) from dorsal photographs taken on top of a measurement
144 grid using the software Image J 1.52a (Rasband, 1997-2021). Information on the age of individuals was
145 available from a concurrent long-term monitoring on the island population since its origin in 2012.

146

147 2.3 Experimental design

148 To gain information about the among- and within-individual variation of individual w_bT levels and further
149 investigate the effect of preceding behavioral tests on their T response, we repeatedly sampled w_bT under
150 two following conditions: First we collected ‘baseline’ levels by capturing a focal frog without the use of
151 any acoustic stimuli (e.g. playback) and immediately transferred it to the water bath (for details see
152 ‘Hormonal sampling’ and Figure 1). Second, we also measured ‘experimental’ w_bT levels immediately after
153 the focal individual had completed a consecutively deployed suite of behavioral tests to assess personality
154 traits (for details see ‘Behavioral experiments’ and Figure 1). In every trial we noted the date and time of

155 day (am or pm) when the measurement was taken, as well as individual parameters such as body size (in
156 mm) and age, measured as a binomial variable (first reproducer vs. recapture from previous years). We
157 aimed for obtaining three replicates in each condition per individual, summing up to a total of six
158 measurements per frog. Half of the tested individuals started with ‘baseline’, while the other half started
159 with the ‘experimental’ sampling. Consecutive samples were always taken more than 24 hours apart. After
160 every second trial we added a break of at least 3 days to minimize any confounding effects of the procedure
161 on the measurements.

162

163 2.4 Behavioral tests

164 All individuals underwent a set of behavioral tests to quantify the following behavioral traits: territorial
165 aggression, boldness and explorative tendency. The procedure of these combined tests lasted for a total of
166 about 30 min and to facilitate reading, we will from now on define both tests with ‘behavioral test’ unless
167 we specifically refer to one of these tests only.

168 *Territorial aggression:* We assessed within- and between-individual variation in the levels of
169 territorial aggression in individual males by simulating a calling intruder inside a male’s territory. To do
170 so, we used a simulated territorial intrusion test to induced territorial defense behavior of the territorial male
171 by broadcasting /presenting synthetic conspecific call by a loudspeaker (for details see Peignier et al., 2022).
172 These conspecific male calls elicit aggressive responses of a territorial male (Rodríguez et al., 2022;
173 Sonnleitner et al., 2020; Ursprung et al., 2009) which can be categorized/quantified in following behavioral
174 parameters: a) latency until the first head-body orientation and b) until the first jump, c) the likelihood to
175 jump in moments when the speaker was silent (i.e., between bouts of calls), and d) the speed to approach
176 the speaker (cf. Chaloupka et al., 2022; Peignier et al., 2022).

177 *Boldness and Exploration:* Immediately after the previous test, we caught the frog and assessed
178 exploration- and boldness-related behaviors using a Novel Environment Setup (cf. Peignier et al., 2022).
179 The setup consisted of a cooler box (hereafter “Novel Environment”), with a PVC tube attached on one
180 side of the box (hereafter “shelter”). We first put the frog in the dark shelter for five minutes, to allow the
181 individual to acclimatize to the setup. Afterwards we opened the shelter and allowed the focal frog to
182 explore the Novel Environment for 15 minutes. We measured the a) latency and b) probability to leave the
183 shelter as well as c) the distance travelled, d) the number of jumps performed, and e) the area covered in
184 the novel environment (for more details see Peignier et al., 2022).

185 To assess within- and between-individual variation in behavior we repeated those tests several
186 times: we conducted 163 territorial defense tests with 51 males (mean \pm SD = 3.20 ± 1.31 repetitions per
187 individual) and 156 Novel Environment Tests with 50 males (mean \pm SD = 3.31 ± 1.50 repetitions per
188 individual). In a previous study, using the same behavioral dataset, it was shown that the behaviors
189 measured during the both tests are repeatable and that the latency until the first jump, the distance travelled,
190 and the time spent in the shelter best represented aggression, exploration, and boldness, respectively
191 (Peignier et al., 2022). In the present study, we use these measures as proxies for the personality traits
192 aggression, exploration and boldness to investigate the link between personality and w_bT levels.

193

194 2.5 Hormonal sampling and analysis

195 We used a non-invasive water-bath method (Baugh et al., 2018; Baugh and Gray-Gaillard, 2021; Gabor et
196 al., 2013) with variations following the protocol described in Rodríguez et al. (2022) to collect repeated w_bT

197 measurements of male *A. femoralis*. In brief, after capture, we put the frogs in a small glass box (14cm x
 198 9cm x 5cm), filled with 40 mL of distilled water and left them in this box for one hour (Figure 1). Resulting
 199 concentrations (pg/mL) thus represent androgen release rates of one individual over one hour. The
 200 dimensions of the box and the water volume were chosen so that the frogs' body was covered with water,
 201 water levels did not constrain breathing (nose was outside water), and frogs were not able to climb out of
 202 the water. An opaque cover was placed over the box to minimize any disturbances from outside and to
 203 minimize stress of the focal individual. After one hour the frog was gently released at the original capture
 204 location. Non-polar hormones were extracted by processing each water sample through 20 mL sterile
 205 syringes coupled to an individual C18 cartridge (SPE, Sep-Pak C18 Plus, 360 mg Sorbent, 55–105 μ m
 206 particle size, #WAT020515, Waters corp., Milford, MA) with a flow rate of ca. 10 mL/min. Afterwards,
 207 cartridges were eluted with 4 mL of 96% EtOH into 8 mL borosilicate vials and stored at 4 °C until further
 208 processing in the endocrinological lab at the University of Vienna. Between water-bath samplings, water
 209 bowls were thoroughly rinsed with ethanol and distilled water, and fully dried before subsequent use.
 210 Researchers were wearing nitrile gloves at all times, which were changed between each sample, to avoid
 211 contamination.

212 In order to quantify $_{wb}T$ (in pg/mL), we used a commercially available ELISA kit (Enzo Life science
 213 #ADI-900 065). Beforehand, 1mL out of the 4mL of 96% EtOH eluted samples were pipetted into a glass
 214 tube and dried down under a N_2 -stream and then re-suspended in 250ul Assay buffer provided by the
 215 manufacturer. Preliminary tests have shown that 1 mL of 96% EtOH was sufficient to quantify reliably
 216 testosterone concentration. Because the antibody has a very low cross-reaction with other androgens (19-
 217 hydroxytestosterone <15%, androstendione <7.2%, Estradiol < 0.4, all others < 0,001%) we dare to assume
 218 to have mostly measured testosterone. Final concentration of the samples was corrected for dilution factor.
 219 The detection limit for the assay was 5.67 pg/mL. The intra-assay CV% of all duplicates was below 5.3%.
 220 The inter-assay CV% was calculated using a control sample and was below 11% ($n=8$).

221

222 2.6 Statistical analysis

223 The statistical analyses were conducted in RStudio (RStudio Team, 2019). We log transformed the $_{wb}T$
 224 measurements as it deviated from normality. Where possible, we report results as $p > 0.1$ no evidence, 0.1
 225 $< p < 0.05$ weak evidence, $0.05 < p < 0.01$ moderate evidence, $0.01 < p < 0.001$ strong evidence, $p < 0.001$
 226 very strong evidence (Muff et al., 2022).

227 To investigate factors that affect the overall androgen levels, we fitted a linear mixed model using
 228 the function 'lmer', in the package 'lme4', with condition ('baseline'/'experimental'), time of day (am/pm),
 229 age (new encounters/survivors from a previous reproductive season), body size, whether the frog was
 230 calling or not before the hormonal measurement, and trial order as fixed effects. We included ID as random
 231 effect, and $_{wb}T$ concentration (log transformed) as response variable. We assured that model assumptions
 232 of residual normality were met by visually inspecting qq-plots. The condition was the only factor
 233 influencing overall $_{wb}T$ level. We further investigated the consistency of $_{wb}T$ levels within and between
 234 individuals, using both reduced ('baseline' only or 'experimental' only) datasets, with the 'rpt' function in
 235 the rptR package (Stoffel et al., 2017). To identify if $_{wb}T$ levels at 'baseline' itself had an influence on
 236 androgen modulation during/after the behavioral tests, we calculated $\Delta_{wb}T$ by subtracting individual
 237 average 'baseline' levels from the respective average 'experimental' levels from all individual males. We
 238 then tested for a possible correlation between 'baseline' $_{wb}T$ and $\Delta_{wb}T$ using a Pearson correlation test.

239 We also studied how aggression, exploration and boldness covary with $_{wb}T$ levels and modulation
 240 at the among- and within-individual level using a Markov chain Monte Carlo method. We built two
 241 Bayesian linear mixed effect models (Hadfield, 2010) with the three personality scores as response variables

242 and ID as random factor. In addition, we added as response variables the log transformed ‘baseline’ $_{wb}T$ in
 243 the first model and the $\Delta_{wb}T$ in the second model. We scaled each response variable by centering to their
 244 mean value and standardizing to units of 1 phenotypic standard deviation. We estimated the among- and
 245 within-individual correlations and covariances between each of the personality score and the $_{wb}T$ using the
 246 posterior distributions. We used an uninformative prior and ran 2,000,000 iterations with a burn-in of
 247 80,000, and selected every 750th posterior parameter sample after the initial burn-in. We assumed statistical
 248 significance if the 95% credible intervals did not overlap 0. We assured that model assumptions were met
 249 by verifying the absence of autocorrelation (correlation between lags <0.1 ; Hadfield, 2010), sufficient
 250 mixing (plots of Markov-Chain-Monte-Carlo chains), and performing a Heidelberg and Welch diagnostic
 251 test.

252

253 3. Results

254 In total we collected 252 hormonal samples from 40 individual males. We obtained samples for baseline
 255 $_{wb}T$ from 37 males (‘baseline’: 1–6 samples per male, mean \pm SD = 3.51 ± 1.19 samples per male), and
 256 samples after the behavioral manipulations from 39 males (‘experimental’: 1–5 samples per male; mean \pm
 257 SD = 3.13 ± 1.08 samples per male).

258 We did not find any evidence that hormonal measurements were influenced by the time of day
 259 when samples were collected, the age or body size of the individual, the activity (i.e., calling or not) of the
 260 male, or the trial order (all $p > 0.05$; Table 1). However, we found very strong evidence that $_{wb}T$ levels were
 261 lower ($\beta = -0.3$, $p < 0.001$, Table 1) when measured after behavioral experiments (mean average $_{wb}T \pm$ SD
 262 = 306.15 ± 107.5 pg/mL) compared to the respective baseline samples (mean average $_{wb}T \pm$ SD = $387.43 \pm$
 263 171.1 pg/mL). Repeatability was quite high for the baseline samples (‘baseline’: $R = 0.45$; 95%CI =
 264 $[0.24;0.61]$), but much lower when hormones were collected after behavioral manipulations
 265 (‘experimental’: $R = 0.24$; 95%CI = $[0.03;0.43]$).

266 Not all individuals responded to the behavioral manipulations with a reduction in $_{wb}T$. Interestingly,
 267 we found a very strong evidence for a negative correlation between average baseline $_{wb}T$ and $\Delta_{wb}T$ in males
 268 (Pearson correlation test: $r = -0.76$, $t = -6.81$, $df = 33$, $p < 0.001$, Figure 2A). This means, that individuals
 269 with a relatively low baseline $_{wb}T$ tended to increase hormone levels after the behavioral tests, while
 270 individuals with a comparatively high baseline level showed a decrease in their androgen levels after the
 271 behavioral manipulation (Figure 2B).

272 Our results show a clear trend for a positive covariation between exploration and baseline $_{wb}T$ levels
 273 at the among- and at the within-individual level, as confidence intervals only slightly overlapped zero
 274 (among-individual level: estimate = 0.18, 95% CI = $[-0.01, 0.45]$; within-individual level: estimate = -0.14,
 275 95% CI = $[-0.31, 0.03]$; Table 2). Males showed increased levels of exploration when their baseline $_{wb}T$
 276 levels were high. We also found a clear trend for a covariation between boldness and androgen modulation
 277 during/after the behavioral tests at the within-individual level (estimate = -0.18, 95% CI = $[-0.39, 0.03]$;
 278 Table 2). Males that showed the highest increase in $_{wb}T$ levels after the behavioral tests were also very bold.

279

280 4. Discussion

281 4.1 Repeatability in androgen levels

282 Our results show that male *A. femoralis* have relatively consistent $_{wb}T$ levels throughout the breeding
283 season. The repeatability of hormonal measurements that were obtained from non-invasive water bath
284 samples was quite high, especially for the ‘baseline’ samples ($R = 0.45$). These values were considerably
285 higher than repeatability scores of glucocorticoid hormones across several vertebrates (Schoenemann and
286 Bonier, 2018). This indicates that T levels are relatively constant over the course of several weeks within
287 the reproductive season in *A. femoralis* and that there are consistent differences between individuals in their
288 baseline androgen levels. As a consequence, even a low number of repeated measurements allowed reliable
289 estimations of individual baseline hormonal profiles in male poison frogs.

290 Further, we did not find a significant difference between hormonal samples collected in the morning
291 and in the afternoon. This was contrary to what we expected, as in a previous study androgen levels were
292 found to be increased in the afternoon compared to morning hours, which was linked to general calling
293 activity in a nearby *A. femoralis* population (Rodríguez et al., 2022). In vertebrates, steroid concentrations
294 commonly undergo a circadian rhythm; they increase during early morning hours and drop in the afternoon
295 (Nelson, 2005). Several studies have documented the existence of diurnal cycles of circulating T (fish:
296 Lorenzi et al., 2008; monkeys: Schlatt et al., 2008; humans: Diver et al., 2003; but see also Licht et al.,
297 1985 for green sea turtles). In several animal taxa, T concentrations are positively related to latitude and
298 negatively to the length of the breeding season (Eikenaar et al., 2012; Husak et al., 2021), which suggests
299 that tropical animals usually exhibit lower T levels with very low seasonal fluctuation during the
300 reproductive season compared to temperate-zone species (see also Canoine et al., 2007; Hau et al., 2008).
301 However, it is possible that other environmental factors (e.g. predators, temperature, climatic conditions,
302 calling activity), might have contributed to the differences in diurnal T variation found in this and the study
303 of Rodríguez et al., 2022.

304 The factor ‘age’ did not show a significant relationship with androgen levels. There is evidence for
305 an age-related change of T levels in various animal taxa (Groothuis and Carere, 2005; Schlatt et al., 2008;
306 Těšický et al., 2022), however those taxa typically show a greater lifespan than our studied species. In *A.*
307 *femoralis* the majority of the population only survives one reproductive season (cf. Ringler et al., 2015),
308 and therefore age likely is not a relevant factor for the variation in androgen levels in this short-lived species.

309

310 4.2 Influence of behavioral tests on androgen levels

311 An increase in T has been observed in many species following social challenges (Goymann et al., 2019;
312 Wingfield et al., 2020; see also Assis et al., 2012; Leary, 2014; Moore et al., 2020). In our study, $_{wb}T$ levels
313 generally dropped after the behavioral tests. This is in contrast with a recent study which found a positive
314 androgenic response to simulated territorial intrusions in *A. femoralis* males, providing support for the
315 Challenge Hypothesis (Rodríguez et al., 2022). In this previous study, water-borne androgen levels were
316 elevated after presenting a conspecific playback compared to baseline conditions, but only in males that
317 actually approached the loudspeaker and not in males which did not react to the playback. Moreover, while
318 an increase of $_{wb}T$ was observed within the first hour of water sampling after playback presentation,
319 androgen levels clearly dropped in the following hours, even below baseline levels. Curiously, the ‘non-
320 responders’ showed a much stronger decline of $_{wb}T$ three hours after the playback test compared to males
321 who actively approached the loudspeaker. In this previous study, the decline of $_{wb}T$ levels was observed
322 only after more than 2 hours following exposure to a behavioral test/or stressor, and might have been due
323 to the activation of the negative feedback system of the hypothalamic–pituitary–gonadal (HPG) axis, or due
324 to other hormones, such as glucocorticoids, via an antagonistic effect on the HPG axis (Moore and Jessop
325 2003; see also “Energetics-Hormone Vocalization hypothesis” by Emerson and Hess 2001). We cannot
326 fully exclude similar effects may have impacted on our measurements, but since all hormonal samples were
327 collected in the same standardized procedure, we assume that such effect might have impacted all samples

328 equally. Because in the present study we collected the hormonal samples not immediately after the
329 territorial aggression test, but after a suite of behavioral tests which lasted in total about 30 min, the resulting
330 androgen levels actually represent a combined/integrated hormonal response to the entire test sequence.
331 Further studies are needed to clarify the link between the hypothalamic–pituitary–adrenal (HPA) and the
332 HPG axis in *A. femoralis*.

333 Most interestingly, not all individuals responded to the behavioral tests in the same way. We
334 observed two different patterns when comparing ‘baseline’ and ‘experimental’ w_bT levels: individuals with
335 low baseline w_bT tended to show an increase in hormonal levels, while individuals with comparatively high
336 baseline w_bT rather showed a decrease in hormonal levels following the behavioral manipulation (Figure
337 2). This phenomenon could happen if the physiological maximum of individuals’ high baseline w_bT was
338 already reached before the start of the behavioral tests, e.g. due to a stressful interaction. These individuals
339 might not be able to further increase their androgen levels when faced with a new challenge, while
340 individuals with low baseline w_bT could (Goymann et al., 2007; Wingfield et al., 1990). However, because
341 the repeatability of baseline w_bT was so high, it is unlikely that these individuals were caught each time just
342 prior a stressful event. Alternatively, our results could potentially be caused by differential personality types
343 being linked to differential physiological (i.e. hormonal) responses to stress and/or social challenges.
344 Previous studies have shown a link between animal personality and differential physiological response to
345 social challenges (i.e. “stress coping styles”; Baugh et al., 2017; Baugh et al., 2012; Carere and Maestripieri,
346 2013; Cockrem, 2013; Coppens et al., 2010). For example, proactive and reactive rats and mice differed in
347 the levels of serotonin release or receptor expression in the prefrontal cortex (Caramaschi et al., 2007;
348 Ferrari et al., 2003; van Erp and Miczek, 2000). Although we did not find a link between hormonal
349 modulation and any personality trait we measured, we cannot rule out that another underlying trait, such as
350 activity, is responsible for the observed pattern.

351

352 4.3 Link between personality traits and androgen levels and modulation

353 Interestingly, we found no evidence that ‘baseline’ androgen levels were associated with territorial
354 aggression in *A. femoralis* males. Similar results were reported in a previous study where the intensity of
355 the phonotactic approach towards a playback was not related to androgen responsiveness in *A. femoralis*
356 males (Rodríguez et al., 2022). However, our results suggest that w_bT levels are positively linked to the
357 personality trait ‘exploration’. Individuals with a high exploratory tendency in the Novel Environment Test
358 also had high baseline w_bT levels. These findings are in line with a recent study that found androgens to be
359 associated with navigation-associated behaviors in three species of poison frogs (Pašukonis et al., 2022).
360 Higher baseline androgen levels were found in individuals that also showed more exploration after
361 translocation in *D. tinctorius*. The amount of exploration during the navigation task was associated with an
362 increase in androgen levels in *A. femoralis*, while successful homing was found to result in a significant
363 decrease in androgen compared to baseline levels (Pašukonis et al., 2022). These results together with the
364 findings of our present study highlight a prominent role of androgens for among-individual variation in
365 exploration-related spatial behaviors in males.

366 Our results further suggest a link between T modulation and boldness. Males that showed the
367 highest increase in w_bT levels after the behavioral tests were also very bold. These findings are in line with
368 the Challenge Hypothesis, which states that the temporary increase of T is to facilitate potentially
369 challenging or threatening encounters (Wingfield et al., 1990). As our experimental design does not allow
370 to disentangle cause and consequence of these correlations, future studies using hormonal manipulation
371 experiments in combination with behavioral experiments are needed to precisely disentangle the causal
372 relationship between T modulation and exploration behavior and boldness.

373

374 *4.4 Methodological implications*

375 One key question in animal personality research is why there are consistent individual differences in
376 behavior. One hypothesis is that heritable traits leading to genetically determined physiological differences
377 among individuals could give rise to consistent behavioral differences (Baugh et al., 2012; Drent et al.,
378 2003; Stamps and Groothuis, 2010). The present study highlights the importance of the exact time point
379 when the hormonal sample is collected, for trying to link hormonal with behavioral profiles. To minimize
380 handling time and experimental effort, it would be ideal to measure hormonal levels directly before and/or
381 after behavioral tests, however this procedure could affect the hormonal and behavioral experiments,
382 respectively. Whether hormonal measurements should be taken independently from behavioral tests or
383 immediately thereafter, ultimately depends on the research question. To gain information about long-term
384 differences in hormonal profiles, measurements should be taken without any preceding behavioral
385 experiments. However, when determining differences in physiological responses after behavioral
386 challenges, it is important to carry out hormonal sampling completely independently from other
387 experimental manipulations as well as directly after behavioral testing.

388

389 *4.5 Conclusions*

390 Our findings show that androgen levels in *A. femoralis* are quite stable across the reproductive season, as
391 repeatability in w_bT baseline levels was high. Time of day, age and calling activity had no influence on
392 hormone levels. As expected, preceding behavioral experiments had a strong influence on the variation in
393 w_bT levels, but the direction of the hormonal response appears to be linked to individual baseline levels.
394 Individuals with low baseline levels showed an increase in androgen, while individuals with high baseline
395 levels showed a reduction of androgen levels after the behavioral tests. In addition, we show that in *A.*
396 *femoralis*, androgens are linked to the personality trait exploration. Taken together, these results suggest
397 that differences in hormonal profiles and/or responses to social challenges can – at least partially – explain
398 among-individual differences in behavioral traits.

399

400 **Ethics**

401 This study was approved by the scientific committee of the ‘Nouragues Ecological Research Station’ and
402 the ethics and animal welfare committee of the University of Veterinary Medicine Vienna. The hormonal
403 such as the behavioral sampling was conducted in strict accordance with current French and EU law,
404 according to the Study of Animal Behaviour (ASAB) guidelines.

405

406 **Funding**

407 This study was funded by the Austrian Science Fund (FWF) via the project P 31518 (PI: Eva Ringler).

408

409 **CRedit authorship contribution statement**

410 VC and ER designed the study. KD and MP collected the data. MP performed the statistical analysis and
411 did the data curation. KD wrote the first draft of the manuscript. MP, VC and ER reviewed, edited and
412 wrote sections of the manuscript. VC and ER supervised the study. All authors contributed to manuscript
413 revision, and have read, and approved the submitted version.

414

415 **Declaration of competing interest**

416 The authors declare no conflict of interest.

417

418 **Acknowledgements**

419 We thank the Nouragues research field station (managed by CNRS), which benefits from ‘Investissement
420 d’Avenir’ grants managed by the Agence Nationale de la Recherche (AnaEE France ANR-11-INBS-0001;
421 Labex CEBA ANR-10-LABX-25-01). We are grateful to the staff of CNRS Guyane for logistic support in
422 French Guiana. Many thanks to Camilo Rodriguez Lopez for providing help and advice regarding the
423 hormonal sampling, to Sarah Chaloupka and Christoph Leeb for help with fieldwork, and to Birgit Szabo
424 for valuable comments on the manuscript.

425

426 **Data availability**

427 The datasets generated during and/or analyzed during the current study are available in the Open Science
428 Framework repository: https://osf.io/67wvj/?view_only=48daf77111894781bd89af222c26be3e

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706 **Table 1.** Results of the linear mixed effect model looking at how personality and abiotic factors influence
 707 w_bT release rates (N = 244 for 39 individuals). Sample size (N) is presented. Results indicating at least weak
 708 evidence (*sensu* Muff et al., 2022) are written in bold.

Fixed effects	Estimate β	Standard-Error	p-value
Intercept	5.68	1.45	< 0.001
Time of the day (am/pm)	0.06	0.10	0.540
Trial order	-0.00	0.01	0.999
Condition	-0.30	0.08	< 0.001
Age (0/1)	-0.11	0.13	0.383
Body size	0.04	0.50	0.934
Calling (yes/no)	0.10	0.11	0.358
Random effects	Variance	Standard-Deviation	
ID	0.11	0.33	
Residual	0.20	0.45	

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714 **Table 2.** Covariance and 95% credible intervals between proxies for aggressiveness (agg), exploration (exp)
 715 and boldness (bol), and variation in w_bT level. Estimates were calculated based on a MCMCglmm model
 716 investigating the correlations between the four behaviors measured. An extended version of the table is
 717 presented in the Supplementary Table S1.

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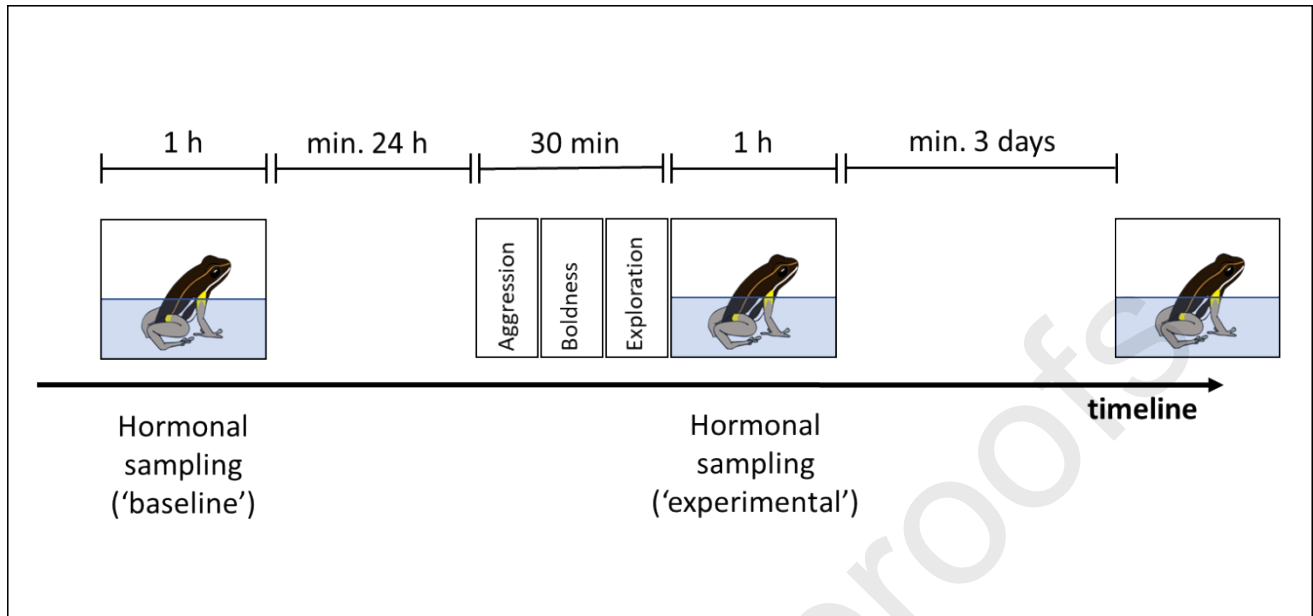
	among-individual covariance	within-individual covariance
baseline $_{wb}T - \text{agg}$	-0.01 (-0.21, 0.19)	0.08 (-0.09, 0.26)
baseline $_{wb}T - \text{bol}$	-0.05 (-0.28, 0.17)	0.09 (-0.08, 0.27)
baseline $_{wb}T - \text{exp}$	0.18 (-0.01, 0.45)	-0.14 (-0.31, 0.03)
$\Delta_{wb}T - \text{agg}$	-0.02 (-0.14; 0.09)	0.01 (-0.19; 0.22)
$\Delta_{wb}T - \text{bol}$	0.02 (-0.14; 0.16)	-0.18 (-0.39; 0.03)
$\Delta_{wb}T - \text{exp}$	-0.05 (-0.21; 0.1)	0.11 (-0.1; 0.32)

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722 **Figure 1.** Experimental design. We repeatedly sampled $_{wb}T$ under two following conditions: without the
723 use of any acoustic stimuli ('baseline') and immediately after a consecutively deployed suite of behavioral
724 tests ('experimental'). We aimed for obtaining three replicates of both 'baseline' and 'experimental'
725 hormonal samples per individual (a total of six measurements per frog). Half of the tested individuals started
726 with the 'baseline' condition (as visualized in the figure), while the other half started with the 'experimental'
727 condition. Consecutive samples were always taken more than 24 hours apart. After every second trial we
728 added a break of at least 3 days to minimize any confounding effects of the procedure on the measurements.



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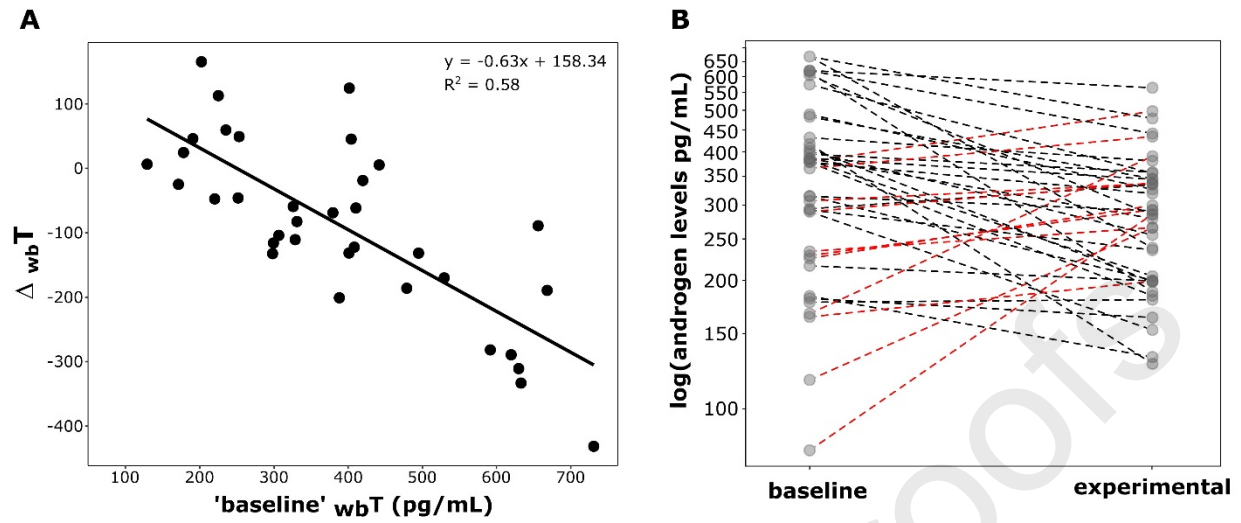
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739 **Figure 2.** Comparison of average individual w_bT levels from baseline samples and samples taken
 740 immediately after behavioral experiments. A) Correlation between average baseline w_bT and the relative
 741 change Δ_{w_bT} after behavioral testing; B) Pairwise comparisons of individual males. Red lines indicate
 742 individuals for which average w_bT measured in the 'experimental' condition is increased compared to
 743 'baseline'. Black lines indicate individuals for which the average w_bT level is decreased in the
 744 'experimental' condition compared to 'baseline'.



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746 Highlights

- 747 - Hormones play a key role in modulating social behavior in animals
- 748 - Baseline water-borne testosterone levels were highly repeatable among males
- 749 - Testosterone levels predicted individual exploratory tendency

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