



universität
wien

DISSERTATION / DOCTORAL THESIS

Titel der Dissertation / Title of the Doctoral Thesis

„Examining the mechanisms underlying territorial trade-offs in a Neotropical poison frog: behavioural flexibility and sexual hormones“

verfasst von / submitted by

Camilo Rodríguez López

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

Wien, 2021 / Vienna 2021

Studienkennzahl lt. Studienblatt /
degree programme code as it appears on the student record sheet:

UA 794 685 437

Dissertationsgebiet lt. Studienblatt /
field of study as it appears on the student record sheet:

Biologie

Betreut von / Supervisor:

ao. Univ.-Prof. i.R. Mag. Dr. Walter Hödl

Mitbetreut von / Co-Supervisor:

Mitbetreut von / Co-Supervisor:

This thesis is dedicated to my beloved family, Luz, Alvaro E., Alvaro, Greta & Max

Acknowledgements

Infinite thanks to my beloved family! Thank you for being my support and inspiration during this PhD.

I am grateful with my supervisor Prof. Walter Hödl for giving me the opportunity to do this PhD. Thank you for your outstanding supervision, friendship and support during this journey.

I also want to thank my co-supervisors Prof. Leonida Fusani and Dr. Virginie Canoine. Thank you so much for all the enriching discussions and for sharing your invaluable knowledge that inspired me to venture into the field of endocrinology.

Many thanks to all the CogBio family, administrative staff, PIs, friends and colleagues! You made my time at the department a fabulous and unforgettable experience. Special thanks to Sarah Deventer for her priceless support both in personal and PhD matters.

A huge thank you to my beloved friends, 'the Latin crew' and 'the froggers'. Thank you so much for the amazing moments that softened and brightened the difficult moments during this PhD.

My sincere gratitude to all the people that in one way or another contributed to build steps that allowed me to achieve this PhD. Huge thanks to my herpetologist friends, colleagues and my field assistants.

Finally, I would like to thank all the funding agencies that contributed to my PhD. Many thanks to the Austrian Science Fund (FWF), the Vienna Zoo (Schönbrunner Tiergarten Ges.m.b.H), the Austrian Herpetological Society (Österreichische Gesellschaft für Herpetologie), the Centre National de la Recherche Scientifique (CNRS) and the Company of Biologists.

Table of Contents

<i>General introduction.....</i>	7
Territorial behaviour: an overview	7
The role of acoustic communication and sexual hormones in territoriality	8
Neotropical poison frogs: a good model to study territoriality.....	11
Trade-offs associated to territorial behaviour in Neotropical poison frogs	12
Aim of the thesis and chapters overview.....	14
References	16
<i>Chapter 1. Sound radiation pattern of the advertisement call of the highly territorial poison frog</i> <i>Allobates femoralis</i>	21
Introduction	24
Methods	25
Results and discussion	28
Acknowledgements	30
References	31
<i>Chapter 2. Calling amplitude flexibility and acoustic spacing in the territorial frog Allobates femoralis</i>	35
Introduction	38
Methods	40
Results.....	45
Discussion.....	49
Funding	52
Acknowledgements	53
References	54
<i>Chapter 3. Behavioural responses to hot and dry weather during the breeding season in a Neotropical</i> <i>poison frog.....</i>	60
Introduction	63
Methods	65
Results.....	70
Discussion.....	76
Conclusions	79
Funding.....	79
Acknowledgements	79
References	80

<i>Chapter 4. Androgen responsiveness to simulated territorial intrusions in Allobates femoralis males: evidence supporting the challenge hypothesis in a territorial frog</i>	85
Introduction	88
Methods	91
Results.....	98
Discussion.....	101
Conclusions	105
Acknowledgements	105
References	106
<i>General discussion.....</i>	113
Methodological considerations and future research	116
Concluding remarks	117
References	119
<i>Appendix.....</i>	121
Supplementary material in Chapter 2.....	121
Supplementary material in Chapter 3.....	123
Supplementary material in Chapter 4.....	127
<i>Abstract</i>	130
<i>Zusammenfassung.....</i>	132

General introduction

Territorial behaviour: an overview

The definition of “territoriality” has been subject of controversy among animal behaviourists in the past decades due to its extensive utility for the study of life history traits related to communication, resource acquisition and defence, space use and mating (Kamath & Wesner, 2020). According to Kaufmann (1983) territoriality “... is only an abstraction that describes more or less accurately a certain range of behaviours that occurs often enough to merit a name”. Despite this disagreement, territoriality is certainly associated with the defence of a space which mediates primary access to resources that are essential for the fitness of the territory holder including food, shelter, mates and breeding places. Territoriality is widespread across the animal kingdom, from invertebrates to mammals.

The acquisition of territories and its attached benefits are shaped by competitive social interactions usually between conspecific males (Stamps, 1994). Such interactions occur mainly by means of communication signals between neighbouring individuals within a complex social network, where different combinations between signallers and receivers are possible (McGregor, 1993). Acoustic, visual and/or chemical cues are among the most used communication signals to advertise a territory (Brown and Orians, 1970). The effectiveness of communication in the defence of a territory depends on several factors, including the characteristics of the environment where the signal is transmitted, and the signal content in terms of information about location and individual identification (McGregor, 1993). These factors will determine how territory holders correctly perceive and interpret signals from conspecific neighbours and therefore, the decision of repel a potential territorial intrusion.

Beyond the already mentioned benefits accompanying the tenure of a territory, territorial behaviour often comes with associated costs in terms of time and energy expenditure, the risk of injury and predation during territorial contests (Davies, 1978; Kaufmann, 1983). That is, territorial males keep intruders at bay using advertisement displays, such as sounds, bright colours, movements or odours, which are energetically expensive and increase conspicuousness towards

predators (Bradbury and Vehrencamp, 2011). However, when signalling does not persuade trespassers, territory holders initiate an agonistic response to chase and kick-out the intruder, which can result in injuries or even death (Brown and Orians, 1970; Davies, 1978). In this context, natural selection favours territoriality when the benefits in resources acquisition outweigh the costs of territorial defence.

The role of acoustic communication and sexual hormones in territoriality

One of the functions of communication in territorial species is to maintain conspecific competitors distanced enough from territorial boundaries and therefore, mediate territorial agonistic encounters (Bradbury and Vehrencamp, 2011). In this sense, large territories would require larger inter-individual distances between neighbours and therefore, the communication signal must also travel larger distances. Among the signals used by territorial species acoustic communication appears to be highlighted, because of its capacity to propagate over long distances (McGregor, 1993; Forrest, 1994). Sound can travel farther distances and even reach areas out of the visual field of the signaller. Moreover, because sound travels in form of pressure waves through the air and the water, several species (from insects to mammals) use acoustic signals as means of communication in both terrestrial and aquatic habitats (Owings and Morton, 1998). The effect of sound propagation on the socio-spatial organization has been broadly studied in insects (Michelsen, 1992; Römer, 1992), amphibians (Duellman and Pyles, 1983; Narins et al., 2006) and birds (Wiley and Richards, 1982; Morton, 1986).

The effectiveness of acoustic communication between territorial competitors depends on three sequential factors: 1) the emitted sound must contain information about the identity and location of the sender, 2) then it must propagate and finally, 3) the receiver must decode the information and react (Wells and Schwartz, 2007). However, the propagation of the acoustic signal can be affected by extrinsic factors related to sound attenuation and/or degradation through the environment (Bro-Jørgensen, 2010; Gerhardt, 1994; Narins, Hodl, & Grabul, 2003; Wiley & Richards, 1982). For instance, sound amplitude decreases -6 dB as the traveled distance increases twice (i.e., inverse square law of spherical spreading; Bradbury and Vehrencamp, 2011). In

addition, acoustic signals can be attenuated due to reflection, refraction and/or diffraction by structures in the environment e.g., by forest vegetation (Wiley and Richards, 1982). However, some species use sound degradation as a reliable cue to estimate neighbour's distance (Morton, 1986; Wiley and Richards, 1982; Naguib and Wiley, 2001). That is, the more degraded the signal is perceived, the farther the sender might be. This ability allows animals to identify their neighbours and assess their location relative to a shared boundary, before making the decision to approach and therefore minimize the associated risks of injury (McGregor, 1993).

In addition to environmental constraints, acoustic signals also undergo limitations related to morphological, physiological and energetical constraints of the signaler (Bradbury and Vehrencamp, 2011). For instance, extensive evidence suggests that sex steroid hormones play an important role in the acoustic communication of vertebrates (Yamaguchi and Kelley, 2003). Sex steroids are systemic messengers that bind to specific receptors found in both the central nervous system and peripheral structures. Consequently, the influence of steroid hormones on the vocal behaviour can be approached at different levels. From an organizational level, steroid hormones have permanent and irreversible effects during the development of structures related to vocal production. For instance, estrogen promotes the sexual differentiation of the song system in songbirds (Konishi and Akutagawa, 1988). On the other hand, steroids can also have an activational effect by inducing temporary changes on such structures during adulthood, which promote typical patterns of acoustic behaviour in males and females (Bradbury and Vehrencamp, 2011). For instance, steroids have a graded effect on both the contractile properties of trunk muscles and cells of the inner ear, which are responsible of the production of vocalizations and sound perception, respectively (Girgenrath and Marsh, 2003; Marsh, 1999; Miranda and Wilczynski, 2009).

Androgens are the main sex steroid in male vertebrates and one of their main functions is to modulate male aggressive behaviour over territories and reproductive behaviour (Adkins-Regan, 2005). In most territorial species, territories are established and defended during the breeding season, where androgen levels are higher compared to other times of the year. This increase above homeostatic levels contributes to the display of mating and territorial behaviours such as calling and aggressiveness (Adkins-Regan, 2005). Interestingly, androgens can rise facultatively above breeding levels when territory holders face a territorial intrusion. Thirty years

ago, an explanation for the facultative increase of males' androgen levels in response to social challenges was named as the "Challenge Hypothesis" (Wingfield et al., 1990). The function of this increase in androgen levels is expected to enhance the aggressive behaviour of the territory holder in order to counteract a potential territorial intrusion. Originally proposed for birds (Wingfield et al., 1990), the Challenge Hypothesis aimed to explain the fluctuation in males' androgen levels product of social interactions during the breeding season. In fact, early experimental studies using simulated territorial intrusions (STIs) showed that males increased androgen levels in response to the territorial challenge (Wingfield and Wada, 1989; Wingfield et al., 1990). However, later studies in fish, amphibians, non-avian reptiles, birds and mammals have shown mixed outcomes that no longer supported the Challenge Hypothesis (reviewed by Moore et al., 2019).

One important aspect that explain the exceptions to the Challenge Hypothesis in diverse taxa, is that the androgen responsiveness to territorial intrusions is strongly dependent on the social mating system and the degree of male parental care of the tested species (Goymann et al., 2019). For instance, in monogamous and polyandrous species with male only parental care, males should show an increase in androgen levels when establishing territories and when females are sexually receptive. Otherwise, when males are taking care of the offspring, androgen levels must remain low and only rise briefly when challenged by a conspecific intruder. The reason is that elevated androgen levels have an antagonistic effect over parental care (Wingfield et al., 1997). On the other hand, males of species with a polygynous mating system without parental care should have elevated androgens only if females are fertile. However, recently Goyman et al., (2019) have proposed an 'alternative Challenge Hypothesis', where social interactions between males and females could explain better the sudden fluctuations in androgen levels than male-male interactions do, especially during parental care. Even so, behavioural endocrinologists have urged for the test of the Challenge Hypothesis in animal taxa with diverse social mating systems and parental care modes, in order to further understand how changes in androgen levels facilitate behavioural decision-making in territorial species (Bell et al., 2019; Moore et al., 2019).

Neotropical poison frogs: a good model to study territoriality

Neotropical poison frogs (Dendrobatidae and Aromobatidae) are a diverse group of predominantly terrestrial and diurnal species (but see *Aromobates nocturnus*; Myers et al., 1991), with more than 300 species distributed through tropical South and Central America. With their multitude of shapes, colours, and calls, Neotropical poison frogs are unmistakable icons of tropical ecosystems. Constant high temperature and humidity, and the diversity of reproductive resources have favoured the evolution of behaviours which elsewhere are seldom found in amphibians, such as territoriality, terrestrial breeding and parental care (Lotters et al., 2007). This diversity in life-histories have made Neotropical poison frogs a good model to answer a multitude of questions in behavioural ecology (Wells, 2007).

Although territorial behaviour in Neotropical poison frogs is present at least in one of the two sexes, usually males establish territories which they vocally advertise and actively defend (Pröhl, 2005). These territories serve mainly for reproductive purposes, but also for sheltering and feeding. Territory acquisition in Neotropical poison frogs usually starts shortly before the onset of the reproductive season, which typically matches with the local rainy season. During this time, males strongly compete through vocal contests and agonistic encounters (Pröhl, 2005). Even though the word *dendrobates* means "tree-climber", many dendrobatid species establish territories at the ground level and use trees for feeding or breeding purposes. Once the territory is established, males use advertising calls to announce the ownership of the territory towards conspecific males and to attract females. The calling activity in Neotropical poison frogs happens throughout the day, usually starting very early in the morning and concluding with the sunset. Most vocalizing males present site attachment and are usually found perching on elevated structures on the forest ground (e.g., fallen logs, branches or rocks). The reproductive behaviour in dendrobatids usually begins when the female approaches to the calling male. Then, the male leads the female to the oviposition site using acoustic, visual and/or tactile cues, where they later mate. Parental care is present in different forms including egg-guarding, tadpole-transportation and egg-feeding, and is provided by either the male or the female, depending on the species (Schulte et al., 2020).

Numerous aspects of the natural history of Neotropical poison frogs make them ideal models to study the mechanisms underlying territorial behaviour. First, as mentioned earlier, most dendrobatid males are strongly territorial and present stereotyped responses towards conspecific calls, such as phonotaxis and antiphonal calling. This characteristic has led several studies to successfully induce territorial responses in free-living males, by presenting a playback stimulus featuring the call of a conspecific male within their territories (Narins et al., 2006; Ursprung et al., 2009). In addition, most males present site attachment to their advertising perches (Pröhl, 2009, Ringler et al., 2009), which not only make them relatively easy to localise in the vast and dense forest ground, but also the researcher can perform tests on the same individual in a repeated way. Also, most dendrobatid males present a long calling period, which in combination with the site fidelity make them an ideal model to study bioacoustics and its relation to other life histories. Finally, although Neotropical poison frogs are small-sized amphibians and therefore challenging to obtain hormones from blood samples (Narayan, 2013), a recently developed non-invasive technique to obtain hormones from water samples can facilitate the investigation of the endocrine mechanisms underlying territorial behaviour in dendrobatids (Baugh et al., 2018; Gabor et al., 2013).

Trade-offs associated to territorial behaviour in Neotropical poison frogs

According to Garland (2014) a trade-off situation in biology involves an inverse interaction between two traits, where one increases while the other decreases. Trade-offs are strongly driven by selective pressures and therefore they allow to determine how organisms adapt to conflicting situations and how behaviours evolve. Traditionally, amphibians' behaviour has been considered as 'stereotyped' (Nice and Timbergen, 1954) with trade-offs implemented at the evolutionary level and not through individual behavioral flexibility. However, recent studies on Neotropical poison frogs are defying this view by showing learning and behavioral flexibility comparable to that seen in other vertebrates (Liu et al., 2016; Pašukonis et al., 2014; Pašukonis et al., 2016; Ringler et al., 2013; Ringler et al., 2015).

Calling behaviour in Neotropical poison frogs poses different ecological trade-offs related to energy expenditure and predation risk. For instance, calling at higher rates and from elevated structures has been shown to be advantageous for male frogs' mating success (Marten and Marler, 1997; Brenowitz et al., 1984; Kime et al., 2000; Greer and wells, 1980; Wells and Schwartz, 1982; Pröhl, 2003). However, calling activity demands high energetic investment, which perhaps constitutes the most energetically expensive behaviour in male frogs' lifetime (Taigen and Wells, 1985; Pough et al., 1992). In addition to calling energetic constraints, acoustic signals are highly conspicuous and even more if they are broadcasted from elevated structures (Magnhagen, 1991), which increases the risk of being detected by potential predators. Most dendrobatids counteract this predation risk by the presence of toxic or noxious substances in their skin obtained from their diet, in combination with bright warning colorations (i.e., aposematism; Summers and Clough, 2011; Darst et al., 2006; Santos and Canatella, 2011).

Beyond predation and energetic costs related to the calling behaviour, the diurnal activity in Neotropical poison frogs make them also susceptible to desiccation through evaporative water loss. That is, dendrobatids are exposed to high temperatures for prolonged periods of time during the day while advertising, so the risk of dehydration increases. Some Neotropical anurans have developed adaptations to counteract the evaporative water loss during the day, including the secretion of lipidic substances through the skin or behavioural adaptations that increases the water uptake from the substrate (reviewed by Lillywhite, 2006). However, very little is known about such adaptations in Neotropical poison frogs (Navas, 1996).

One of the most common trade-offs in the animal kingdom occurs between investing into current vs. future offspring (Stearns, 1989). While the former often requires parental care, the later typically involves acquiring new mates through advertisement displays and defence of resources such as territories. Fitness-related trade-offs between parental investment and territoriality are likely shaping life histories of many tropical frogs, but few studies have investigated them (see Summers, 1990; Brown et al., 2009). For instance, Neotropical poison frogs are ideal candidates to investigate this trade-off, because they often end up in a situation where they should invest time and energy to take their offspring to a secure hatching location, and at the same time defend/advertise their territory against an intruder. By testing the Challenge Hypothesis, one can

investigate whether the regulatory mechanisms behind territoriality involve a trade-off between resource defence and reproductive success.

Aim of the thesis and chapters overview

In this thesis I explore the mechanisms underlying territorial trade-offs in a Neotropical poison frog. To do so, I combined different established as well as novel experimental procedures such as acoustic playbacks and recordings, sound propagation experiments, simulated territorial intrusions, tracking and a non-invasive technique to measure androgens from water samples. I conducted detailed behavioral experiments on the brilliant-thighed poison frog, *Allobates femoralis*, a Neotropical poison frog commonly found in South American rainforests. The brilliant-thighed poison frog is a particularly well-studied species, in which males are known both to provide obligate parental care through tadpole transport as well as actively defend and vocally advertise their territories.

In chapter 1 and 2, I explore how *A. femoralis* males optimize the effectiveness acoustic communication in order to increase the likelihood to reach conspecific neighbours and mates, and therefore to mitigate the effects of sound scattering by environmental factors. Specifically, in chapter 1 I described the nearly omnidirectional sound-radiation pattern of the advertisement call of *A. femoralis*. There, I discussed the advantages of having a uniform sound radiation pattern in terms of attracting females from and repel rivals in surrounding territories in all directions.

In chapter 2, I investigated how the propagation of the advertisement call in *A. femoralis* is affected by environmental constraints associated with the calling-perch height, and how males adjust their calling behaviour in the context of acoustic spacing. Using a correlative approach and signal propagation experiments, my colleagues and I demonstrate that *A. femoralis* males are capable to vary the calling amplitude according to the calling-perch height. I then discussed how this adjustment offers an alternative to the general hypothesis of a positive effect of calling-perch height on signal propagation, which allows males to reduce energy expenditure without affecting the effectiveness signal propagation.

In chapter 3 my colleagues and I investigated how *A. femoralis* adjust its territorial behaviour during hot and dry conditions during the reproductive season, in order to maintain the territorial advertisement without experience a thermal shock and desiccation. I later discussed how substrate selection, the reduction of activity and a behaviour allegedly linked to the water absorption response account for much of the thermal and water balance in *A. femoralis* during hot and dry conditions.

Finally, in chapter 4, my colleagues and I explored the endocrine mechanism of territorial behaviour in *A. femoralis* males, by testing the Challenge Hypothesis. For this, we confronted males to simulated territorial intrusions in form of acoustic playbacks. We further used a novel and non-invasive technique to measure androgens from water samples, which allowed us to measure androgen concentrations repeatedly. There, we could show that *A. femoralis* males present a behavioural response to simulated territorial intrusions, followed by an androgenic response. We discussed how an increase of androgens may co-opt to promote both territorial behaviours and parental care in *A. femoralis* males, favouring social interactions and reproductive success.

I believe that my PhD thesis brings new and valuable perspectives for understanding the factors that mediate territorial trade-offs in Neotropical poison frogs. Furthermore, the insights gained from this thesis set ground for a better understanding of the mechanisms and selective forces that shape behavioral flexibility and territoriality in tropical amphibians.

References

- Adkins-Regan, E., 2005. Hormones and animal social behavior, Monographs in behavior and ecology. Princeton University Press, Princeton.
- Baugh, A.T., Bastien, B., Still, M.B., Stowell, N., 2018. Validation of water-borne steroid hormones in a tropical frog (*Physalaemus pustulosus*). *Gen. Comp. Endocrinol.* 261, 67–80.
- Bell, A.M., 2019. Individual variation and the challenge hypothesis. *Horm. Behav.* 104549.
- Bradbury, J.W., Vehrencamp, S.L., 2011. Principles of animal communication, 2nd ed. ed. Sinauer Associates, Sunderland, Mass.
- Brenowitz, E.A., Wilczynski, W., Zakon, H.H., 1984. Acoustic communication in spring peepers. Environmental and behavioral aspects. *J. Comp. Physiol. A* 155, 585–592.
- Bro-Jørgensen, J., 2010. Dynamics of multiple signalling systems: animal communication in a world in flux. *Trends Ecol. Evol.* 25, 292–300.
- Brown, J.L., Morales, V., Summers, K., 2009. Home range size and location in relation to reproductive resources in poison frogs (*Dendrobatidae*): a Monte Carlo approach using GIS data. *Anim. Behav.* 77, 547–554.
- Brown, J.L., Orians, G.H., 1970. Spacing Patterns in Mobile Animals. *Annu. Rev. Ecol. Syst.* 1, 239–262.
- Davies, N.B., 1978. Ecological questions about territorial behaviour, in: (Eds.) Krebs J. R. & Davies N. B. *Behavioural Ecology*, pp. 317–350. Oxford: Blackwell.
- Darst, C.R., Cummings, M.E., Cannatella, D.C., 2006. A mechanism for diversity in warning signals: Conspicuousness versus toxicity in poison frogs. *Proc. Natl. Acad. Sci.* 103, 5852–5857.
- Duellman, W.E., Pyles, R. a, 1983. Acoustic Resource Partitioning in Anuran Communities. *Copeia* 1983, 639–649.
- Forrest, T.G., 1994. From sender to receiver: propagation and environmental effects on acoustic signals. *Am. Zool.* 34, 644–654.
- Gabor, C.R., Gabor, C.R., Bosch, J., Gabor, C.R., Bosch, J., Fries, J.N., Gabor, C.R., Bosch, J., Fries, J.N., Davis, D.R., 2013. A non-invasive water-borne hormone assay for amphibians. *Amphib.-Reptil.* 34, 151–162.
- Garland, T., 2014. Trade-offs. *Curr. Biol.* 24, R60–R61.
- Gerhardt, H.C., 1994. The Evolution of Vocalization in Frogs and Toads. *Annu. Rev. Ecol. Syst.* 25, 293–324.
- Girgenrath, M., Marsh, R.L., 2003. Season and testosterone affect contractile properties of fast calling muscles in the gray tree frog *Hyla chrysoscelis*. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* 284, R1513–R1520.
- Goymann, W., Moore, I.T., Oliveira, R.F., 2019. Challenge Hypothesis 2.0: A Fresh Look at an Established Idea. *BioScience* 69, 432–442.
- Greer, B.J., Wells, K.D., 1980. Territorial and reproductive behavior of the tropical american frog *Centrolenella fleischmanni*. *Herpetologica* 36, 318–326.
- Kamath, A., Wesner, A.B., 2020. Animal territoriality, property and access: a collaborative exchange between animal behaviour and the social sciences. *Anim. Behav.* 164, 233–239.
- Kaufmann, J.H., 1983. On the definitions and functions of dominance and territoriality. *Biol. Rev.* 58, 1–20.
- Kime, N.M., Turner, W.R., Ryan, M.J., 2000. The transmission of advertisement calls in Central American frogs. *Behav. Ecol.* 11, 71–83.
- Konishi, M., Akutagawa, E., 1988. A critical

- period for estrogen action on neurons of the song control system in the zebra finch. *Proc. Natl. Acad. Sci.* 85, 7006–7007.
- Lillywhite, H.B., 2006. Water relations of tetrapod integument. *J. Exp. Biol.* 209, 202–226.
- Liu, Y., Day, L.B., Summers, K., Burmeister, S.S., 2016. Learning to learn: advanced behavioural flexibility in a poison frog. *Anim. Behav.* 111, 167–172.
- Lötters, S., Jungfer, K.-H., Henkel, F., Schmidt, W., 2007. Poison frogs: biology, species and captive husbandry. Edition Chimaira, Frankfurt am Main, Germany.
- Magnhagen, C., 1991. Predation risk as a cost of reproduction. *Trends Ecol. Evol.* 6, 183–186.
- Marsh, R.L., 1999. Contractile properties of tree frog muscles. *J. Exp. Biol.* 202, 3215–3223.
- Marten, K., Marler, P., 1977. Sound transmission and its significance for animal vocalization. *Behav. Ecol. Sociobiol.* 2, 271–290.
- McGregor, P., 1993. Signalling in territorial systems: a context for individual identification, ranging and eavesdropping. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 340, 237–244.
- Michelsen, A., 1992. Hearing and Sound Communication in Small Animals: Evolutionary Adaptations to the Laws of Physics, in: Webster, D.B., Popper, A.N., Fay, R.R. (Eds.), *The Evolutionary Biology of Hearing*. Springer New York, New York, NY, pp. 61–77.
- Miranda, J.A., Wilczynski, W., 2009. Sex differences and androgen influences on midbrain auditory thresholds in the green treefrog, *Hyla cinerea*. *Hear. Res.* 252, 79–88.
- Moore, I.T., Hernandez, J., Goymann, W., 2019. Who rises to the challenge? Testing the Challenge Hypothesis in fish, amphibians, reptiles, and mammals. *Horm. Behav.* S0018506X19301916.
- Morton, E.S., 1986. Predictions From the Ranging Hypothesis for the Evolution of Long Distance Signals in Birds. *Behaviour* 99, 65–86.
- Myers, C.W., Paolillo, A., Daly, J.W., 1991. Discovery of a Defensively Malodorous and Nocturnal Frog in the Family Dendrobatidae: Phylogenetic Significance of a New Genus and Species from the Venezuelan Andes. *Am. Mus. Novit.* 36.
- Naguib, M., Wiley, R.H., 2001. Estimating the distance to a source of sound: mechanisms and adaptations for long-range communication. *Anim. Behav.* 62, 825–837.
- Narayan, E.J., 2013. Non-invasive reproductive and stress endocrinology in amphibian conservation physiology. *Conserv. Physiol.* 1, cot011–cot011.
- Narins, P.M., Feng, A.S., Fay, R.R., 2006. Hearing and Sound Communication in Amphibians, *Springer Handbook of Auditory Research*. Springer New York.
- Narins, P.M., Hodl, W., Grabul, D.S., 2003. Bimodal signal requisite for agonistic behavior in a dart-poison frog, *Epipedobates femoralis*. *Proc. Natl. Acad. Sci.* 100, 577–580.
- Navas, C.A., 1996. Implications of microhabitat selection and patterns of activity on the thermal ecology of high elevation neotropical anurans. *Oecologia* 108, 617–626.
- Nice, M.M., Tinbergen, N., 1954. Social Behaviour in Animals. With Special Reference to Vertebrates. *Bird-Band.* 25, 84
- Owings, D.H., Morton, E.S., 1998. *Animal Vocal Communication: A New Approach*. Cambridge University Press.
- Pašukonis, A., Loretto, M.-C., Landler, L., Ringler, M., Hödl, W., 2014. Homing trajectories and initial orientation in a Neotropical territorial frog, *Allobates femoralis* (Dendrobatidae). *Front. Zool.* 11, 29.

- Pašukonis, A., Trenkwalder, K., Ringler, M., Ringler, E., Mangione, R., Steininger, J., Warrington, I., Hödl, W., 2016. The significance of spatial memory for water finding in a tadpole-transporting frog. *Anim. Behav.* 116, 89–98.
- Pough, F., Magnusson, W., Ryan, M., Wells, K., Taigen, T., 1992. Behavioral energetics. *Environmental Physiology of the Amphibia*.
- Pröhl, H., 2005. Territorial Behavior in Dendrobatid Frogs. *J. Herpetol.* 39, 354–365.
- Pröhl, H., 2003. Variation in Male Calling Behaviour and Relation to Male Mating Success in the Strawberry Poison Frog (*Dendrobates pumilio*). *Ethology* 109, 273–290.
- Ringler, E., Pašukonis, A., Hödl, W., Ringler, M., 2013. Tadpole transport logistics in a Neotropical poison frog: indications for strategic planning and adaptive plasticity in anuran parental care. *Front. Zool.* 10, 67.
- Ringler, M., Hödl, W., Ringler, E., 2015. Populations, pools, and peccaries: simulating the impact of ecosystem engineers on rainforest frogs. *Behav. Ecol.* 26, 340–349.
- Ringler, M., Hödl, W., Ursprung, E., 2009a. Phonotactic approach pattern in the neotropical frog *Allobates femoralis*: A spatial and temporal analysis. *Behaviour* 146, 153–170.
- Ringler, M., Ursprung, E., Hödl, W., 2009b. Site fidelity and patterns of short-and long-term movement in the brilliant-thighed poison frog *Allobates femoralis* (Aromobatidae). *Behav. Ecol. Sociobiol.* 63, 1281–1293.
- Römer, H., 1992. Ecological Constraints for the Evolution of Hearing and Sound Communication in Insects, in: Webster, D.B., Popper, A.N., Fay, R.R. (Eds.), *The Evolutionary Biology of Hearing*. Springer New York, New York, NY, pp. 79–93.
- Santos, J.C., Cannatella, D.C., 2011. Phenotypic integration emerges from aposematism and scale in poison frogs. *Proc. Natl. Acad. Sci.* 108, 6175–6180.
<https://doi.org/10.1073/pnas.1010952108>
- Schulte, L.M., Ringler, E., Rojas, B., Stynoski, J.L., 2020. Developments in Amphibian Parental Care Research: History, Present Advances, and Future Perspectives. *Herpetol. Monogr.* 34, 71.
- Stamps, J., 1994. Territorial behavior: testing the assumptions. *Adv. Study Behav.* 23, 232.
- Stearns, S.C., 1989. Trade-offs in life-history evolution. *Funct. Ecol.* 3, 259–268.
- Summers, K., 1990. Paternal care and the cost of polygyny in the green dart-poison frog. *Behav. Ecol. Sociobiol.* 27, 307–313.
- Summers, K., Clough, M.E., 2001. The evolution of coloration and toxicity in the poison frog family (Dendrobatidae). *Proc. Natl. Acad. Sci.* 98, 6227–6232.
- Taigen, T.L., Wells, K.D., 1985. Energetics of vocalization by an anuran amphibian (*Hyla versicolor*). *J. Comp. Physiol. B* 155, 163–170.
- Wells, K.D., 2007. *The Ecology and Behavior of Amphibians*. University of Chicago Press
- Wells, K.D., Schwartz, J.J., 2006. The Behavioral Ecology of Anuran Communication, in: Narins, P.M., Feng, A.S., Fay, R.R., Popper, A.N. (Eds.), *Hearing and Sound Communication in Amphibians*, Springer Handbook of Auditory Research. Springer, New York, NY, pp. 44–86.
- Wells, K.D., Schwartz, J.J., 1982. The Effect of Vegetation on the Propagation of Calls in the Neotropical Frog *Centrolenella fleischmanni*. *Herpetologica* 38, 449–455.
- Wiley, R.H., Richards, D.G., 1982.

- Adaptations for acoustic communication in birds: sound transmission and signal detection, Acoustic communication in birds.
- Wingfield, J.C., Hegner, R.E., Dufty, A.M., Ball, G.F., 1990. The "Challenge Hypothesis": Theoretical Implications for Patterns of Testosterone Secretion, Mating Systems, and Breeding Strategies. *Am. Nat.* 136, 829–846.
- Wingfield, J.C., Jacobs, J., Hillgarth, N., 1997. Ecological Constraints and the Evolution of Hormone-Behavior Interrelationships. *Ann. N. Y. Acad. Sci.* 807, 22–41.
- Wingfield, John C., Wada, M., 1989. Changes in plasma levels of testosterone during male-male interactions in the song sparrow, *Melospiza melodia*: time course and specificity of response. *J. Comp. Physiol. A* 166.
- Yamaguchi, A., Kelley, D.B., 2003. Hormonal Mechanisms in Acoustic Communication, in: Simmons, A.M., Fay, R.R., Popper, A.N. (Eds.), *Acoustic Communication*, Springer Handbook of Auditory Research. Springer-Verlag, New York, pp. 275–323.

Chapter 1. Sound radiation pattern of the advertisement call of the highly territorial poison frog *Allobates femoralis*

This chapter has been published in the peer-review journal *Behavioural Processes*.

Citation:

Rodríguez, C., Hödl, W., 2020. Sound radiation pattern of the advertisement call of the highly territorial poison frog *Allobates femoralis*. *Behavioral Processes* 170, 103996.
<https://doi.org/10.1016/j.beproc.2019.103996>

Sound radiation pattern of the advertisement call of the highly territorial poison frog *Allobates femoralis*

Camilo Rodríguez ^{a,†} and Walter Hödl ^b

^a Department of Cognitive Biology, University of Vienna, Vienna, Austria

^b Department of Integrative Zoology, University of Vienna, Vienna, Austria

[†] Corresponding author. E-mail: camilo.rodriguez@univie.ac.at

Highlights

- We measured the sound radiation pattern in a poison frog for the first time.
- *Allobates femoralis* presents a nearly omnidirectional sound radiation pattern.
- Directionality does not affect the peak frequency of the call in *A. femoralis*.
- Territorial males orient towards the sound source during antiphonal calling.

Abstract

Sound radiation patterns have ecological implications in the effective communication between conspecifics, like optimization of the sound propagation, increase the likelihood to reach mates and to mitigate effects of sound scattering by environmental factors like vegetation. The territorial frog *Allobates femoralis* advertises its territory against conspecific males and attract females with advertisement calls. Here we report the nearly omnidirectional sound-radiation pattern of the advertisement call of *A. femoralis*. This sound spreading pattern allows the males to attract mates and repel rivals in all directions. Furthermore, *A. femoralis* males direct the advertisement call to conspecific neighbours after phonotactic orientation.

Keywords: Sound radiation pattern, Omnidirectional, Advertisement call, Dendrobatidae.

Introduction

In acoustically communicating species sound production and signal detection is critical for species-specific recognition, where selective forces (e.g. predators, sexual selection) and constraints (e.g. Physiological, morphological, environmental constraints) may affect the evolution of the signal (Amézquita et al., 2006; Andersson & McGregor, 1999; Forrest, 1994; Narins & Zelick, 1988). Acoustic communication in frogs is an important factor in reproductive biology, where males produce calls to attract females. In territorial frogs (e.g. Dendrobatidae) the calling behavior also allows the males to advertise a territory (Pröhl, 2005).

The effectiveness of frog communication depends on sound-propagation patterns, the sound-pressure level at the caller position, and the sensory system of the receiver (Bernal et al., 2009). The sound-propagation pattern of the acoustic signals has ecological implications for both, the sender and the receiver in a heterogeneous environment (Hunter et al., 1986). On the one hand, calling with spatially uniform radiation allows information to be transmitted equally into all directions (Gerhardt, 1975; Narins & Hurley, 1982). On the other hand, directionally emitted calls could optimize energy expenditure, reduce predation or unwanted intraspecific aggression by addressing only receivers in specific directions, and improve the orientation of the receiver to the sender (Narins & Hurley, 1982; Hunter et al., 1985). Sound- radiation patterns have been studied in frog species with lek mating system (Bernal et al., 2009; Brenowitz et al., 1984; Gerhardt, 1975; Greer & Wells, 1980; Narins & Hurley, 1982; Parris, 2002; Table 1), but have been largely ignored in territorial species where sound propagation is critical for territory advertisement.

Most of the neotropical poison frogs are well known to be highly territorial (Lötters et al., 2007; Wells, 2007). For instance, males of the brilliant-thighed poison frog, *Allobates femoralis* Boulenger, 1883 (Dendrobatidae: Aromobatinae; AmphibiaWeb, 2018; Pyron & Wiens, 2011; Grant et al., 2017) announce territory ownership against intruders with advertisement calls and defend it with physical attacks (Narins et al., 2003; Ringler et al., 2011; Roithmair, 1992). Usually, males rotate phonotactically on the same spot towards calling neighbours and produce antiphonal calling when the amplitude of the received signal is between 56 and 68 dB. Signals above ~68 dB evoke an aggressive response (Hödl, 1982, 1983, 1987). The behavioural significance of the head-body turning behaviour might be to direct the call towards specific targets, as has been shown in several species of songbird (Breitwisch & Whitesides 1987; Glutz

von Blotzheim & Bauer 1997; Brumm, 2002). However, when calling alone, males usually call without rotating, suggesting that the signal spreads uniformly in all directions.

Table 1. Sound radiation patterns of the advertisement call of frog species.

Family	Species	Sound radiation pattern	Δ 0°-180°	Reference
Hylidae	<i>Pseudacris (Hyla) crucifer</i>	Omnidirectional	~0.5dB	(Gerhardt, 1975)
	<i>Hyla chrysoscelis</i>	Directional	~6dB	
Bufonidae	<i>Anaxyrus (Bufo) americanus</i>	Directional	~3dB	(Bernal, 2009)
Leptodactylidae	<i>Engystomops (Physalaemus) pustulosus</i>	Directional	~6dB*	
Dendrobatidae	<i>Allobates femoralis</i>	Nearly omnidirectional	~2dB	This study

*Highest radiated SPL above the frog at ~90° from the horizontal direction.

If the main functions of the advertisement call of *A. femoralis* are to announce the ownership of a territory to neighbouring males and to attract females, then we expect that an omnidirectional sound radiation pattern would be advantageous to attract females from and repel intruders in surrounding territories in all directions, within highly structured environments. We thus aimed to test whether territorial frogs are more likely to have an omnidirectional sound propagation pattern. Since frogs could also address the spectral parameters of the call as a function of sound direction and position of the caller (Reviewed in Bee & Christensen-Dalsgaard, 2016), we additionally tested how spectral features of the advertisement call could change as a function of the direction of the sound source.

Methods

Study species and study area

We conducted our study with the *A. femoralis* population in the field camp 'Saut Pararé', located in the Natural Reserve 'Les Nouragues' in French Guiana (4°02' N - 52°41' W; Bongers et al., 2013; see Ringler et al., 2015 for a description of the population characteristics), during the early reproductive season (February to April) in 2018 and 2019. The measurements were taken between 1500–1800h, which corresponds to the peak calling activity of this species (Kaefer et al., 2012). The advertisement call of *A. femoralis* usually consists of four notes with a small ascending

frequency sweep and a mean-call peak frequency of 3582 ± 213.5 Hz (mean $\pm$ SD, $n=12$; Figure 1).

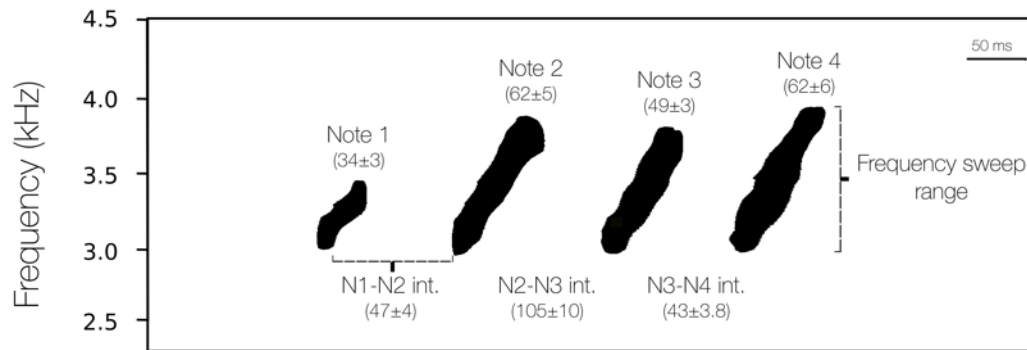


Figure 1. Temporal and spectral properties of the advertisement call of *A. femoralis*. Numbers in brackets are time in milliseconds (mean $\pm$ SD).

Sound radiation pattern

To determine the sound-radiation pattern of the advertisement call of *A. femoralis*, we measured the Sound-Pressure Levels (SPL) of the advertisement call of 10 males from the four cardinal directions ($0/360^\circ$ = front, 90° =right side, 180° =back and 270° =left side) with a SPL-meter (Votcraft 329, Conrad Electronic SE, Wenberg Köblitz, Germany). Since the sound can be attenuated due to scattering by vegetation (Forrest, 1994), we carefully cleaned the path from the groundcover vegetation with garden clippers, between the frog and the SPL-meter to avoid variation in the SPL measurements due to differential vegetation effects. We used low-intensity playbacks from the four cardinal directions to orientate the focal individual phonotactically into the direction of the speaker at its calling perch. Upon playback cessation the individual started to call without changing its calling site and position (Hödl, 1982). The SPL-meter was mounted on a full-size tripod, aligned parallel to the ground, and pointing towards the calling frog. We adjusted the height of the SPL-meter according to the calling-perch height of the focal frog (average $\pm$ SD of the calling-perch height of the 10 measured frogs = 9.7 ± 3 cm). All SPL measurements were taken at a fixed distance of 1 m from the frog using the peak detector of the SPL-meter in the C-weighting curve and on its fast setting. The frogs generally start calling with “warm-up calls”, defined as suboptimal advertisement calls of less than steady-state SPL (Jameson, 1954; Toledo et al., 2014); therefore, we measured the SPL from the 5th four-note call in a call series consisting

of at least 15 consecutive four-note call and we took the average of three SPL measurements after a linear conversion to Pascals ($\text{Pa} = 20\mu\text{Pa} \cdot 10^{\text{dB}/20}$). SPL values were standardized within the z-distribution (with mean = 0 and SD = 1) and the z-scores were used in subsequent analyses.

Call recordings and analysis

Acoustic recordings of the advertisement call of 12 males of *A. femoralis* were taken with directional microphones (Sennheiser ME64 or ME66, Sennheiser, Wedemark, Germany) on digital recorders (H4n, Zoom, Tokyo, Japan) in three non-consecutive days. We recorded between 10 and 29 consecutive four-note calls with the microphone positioned at a distance of 0.5 m to 1.0 m from each male, parallel to the ground, and pointing towards the calling frog. We calculated the signal's peak frequency using Praat (Boersma et al., 2019) by averaging the measurements from three calls on every recording. We also registered the cardinal position of each frog in reference to the microphone, being 0/360° the position of the microphone in the cardinal plane. To allow individual body-size measurements (snout-urostyle length mean = 2.94 ± 0.05 , $n=12$) and identification, we captured and photographed males after each call recording. Additionally, we measured the air temperature at the exact spot where the males were calling to control the effect of temperature-dependent variation in the call frequency.

Statistical analysis

To establish the sound radiation pattern, we first transformed the four directions into a circular variable by using the "circular" function within the *circular* package in R (R Core Team, 2017). Then, we predicted the SPL from a simulated vector of 300 angle values from 0° to 360° by splitting the circular predictor in the sine and cosine components of the circular variable and running a simple linear model (Pewsey et al., 2013).

To determine differences in the SPL between the four directions, we performed a general linear mixed model (LMM) using the function "lmer" within the *lme4* package (Bates et al., 2015) using SPL as the response variable, the angles as the fixed factor and the individual as the random factor. In the same way, to evaluate the relationship between the calling direction and the peak frequency, we performed a LMM using the peak frequency as the response variable, the sine and cosine components of the calling angle as fixed factors and the individual as the random effect.

Results and discussion

At a distance of 1 m the advertisement call of *A. femoralis* showed a nearly omnidirectional radiation pattern, decreasing by ~ 1.5 dB towards the sides (LMM: $t_{90^\circ}=2.04$, $P=0.05$; $t_{270^\circ}=2.84$, $P=0.008$; $N=10$; Figure 2A & B) and ~ 2 dB towards the back (LMM: $t_{180^\circ}= 8.44$, $P<0.001$; $N=10$; Figure 2A & B) of the calling frog, compared to the front (0°). Linear regression analyses showed that neither the temperature ($R^2=0$, $F= 0.016$, $P=0.89$), nor the body size ($R^2=0$, $F= 0.75$, $P= 0.39$) had an effect on the peak frequency of the advertisement call in *A. femoralis*. The peak frequency of the signal did not change in function to the directionality of the call (LMM: $\cos(\theta)=-98.49$, $P=0.06$; $\sin(\theta)=-21.71$, $P=0.67$; $N=12$; Figure 2C).

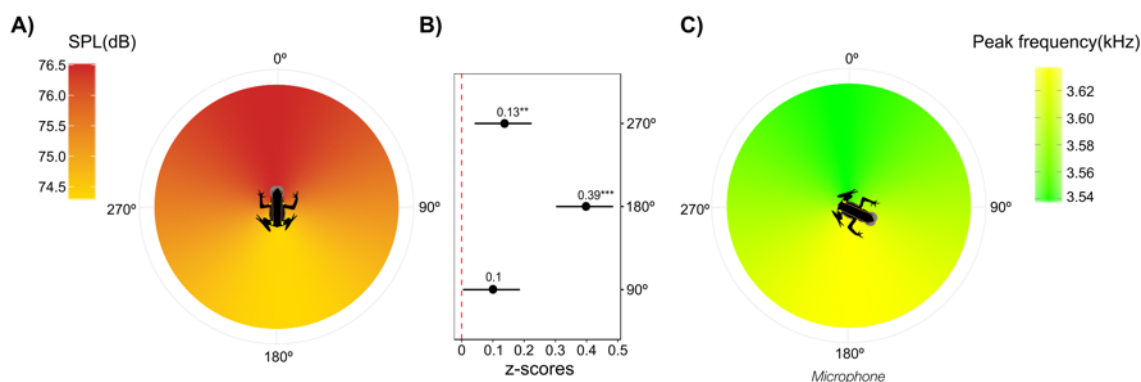


Figure 2. Radiation pattern of the advertisement call of *Allobates femoralis*. A) Polar heatmap showing the predicted SPLs spreading at four different angles as cardinal points; B) Linear Mixed Model plot showing z-scores values (x-axis) and significance of the effect size (numbers over mean-points) of LMM models. Solid lines represent 95% confidence intervals in each angle; C) Polar heatmap showing the predicted peak frequencies of the advertisement call at four different angles as cardinal points.

Many animal communication systems present directionality in the radiation of the acoustic signals, allowing them to optimize energy expenditure by focusing the signal to a specific target (e.g. Cicadas (Michelsen & Fonseca, 2000), frogs (Bernal et al., 2009), birds (Hunter Jr et al., 1986) and dogs (Frommolt & Gebler, 2004)). However, omnidirectional emitted sounds could improve the effective communication in complex frogs' assemblages, attracting females from and advertising to neighbouring males in all directions (Gerhardt, 1975; Narins & Hurley, 1982). Previous studies in other anuran species showed that frogs are able to discriminate sound-

pressure levels differences of 2 to 6dB (Fay & Simmons, 1999). Our study found that the differences in SPL for *A. femoralis* in the four cardinal directions are equal to or below 2dB (see Figure 2A & B). Frogs might be using the small differences in SPL to accurately orientate phonotactically towards a conspecific caller (Gerhardt & Rheinlander, 1980). In fact, *A. femoralis* often turn on the same axis when responding towards an acoustically active neighbour (Hödl, 1982, 1983, 1987). The same rotating behaviour has been found in *Dendrobates granuliferus*, another neotropical poison frog, suggesting the same effective vocal transmission in all directions (Goodman, 1971). Furthermore, directing the advertisement call to specific targets (i.e. conspecific neighbouring males or females) from elevated perches also increase the visibility of the calling male using the vocal sac as a multimodal signalling tool (Narins et al., 2003). Thus, we suggest that the rotating behavior in *A. femoralis* toward the calling rival could be driven by both, the perception of small differences in the SPL of neighbouring males and the display of the vocal sac as a visual signal.

Although the peak frequency of the advertisement call of *A. femoralis* did not change in function of the direction of the call, a slight difference of about 0.1 kHz can be noticed in azimuth to the microphone within 110°-130° (Figure 2C). Previous studies in the green tree frog (*Hyla chrysoscelis*) showed that females can localize with better accuracy sound sources consisting of call components of only 0.9 kHz (Rheinlaender et al., 1979). At present, we can only speculate that the small differences in peak frequency related to the direction of the call in *A. femoralis* are meaningless for the accuracy of localization by females or neighbouring males. Further research on the tympanum sensitivity in *A. femoralis* is needed to give insight on how call frequencies could provide information about acoustic localization of neighbouring frogs.

Overall, our results suggest that *A. femoralis* males present a nearly omnidirectional radiation pattern, which grants the males to advertise the territory and attract females from all directions. However, antiphonal calling neighbours elicit body rotation towards each other, suggesting directivity of the call and the visual field to specific targets.

Acknowledgements

We thank the Nouragues research field station (managed by CNRS) which benefits from “Investissement d'Avenir” grants managed by Agence Nationale de la Recherche (AnaEE France ANR-11-INBS-0001; Labex CEBA ANR-10-LABX-25-01). This work was funded by the Austrian Science Fund (FWF): W1262-B29. For providing field assistance we are grateful to A. Lembens and V. Canoine. For providing help in call recordings and posterior analysis we especially thank G. Raboisson.

References

- Amézquita, A., Hödl, W., Lima, A. P., Castellanos, L., Erdtmann, L., & de Araújo, M. C. (2006). Masking interference and the evolution of the acoustic communication system in the Amazonian dendrobatid frog *Allobates femoralis*. *Evolution*, 60(9), 1874–1887.
- AmphibiaWeb (2018). AmphibiaWeb: Information on amphibian biology and conservation. University of California. Berkeley, CA, USA. www.amphibiaweb.org, last checked 07.11.2018.
- Andersson, S., & McGregor, P. K. (1999). Animal communication: what is the signal to noise ratio? *Trends in Ecology & Evolution*, 14(5), 174–175.
- Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, 67(1), 1–48.
- Bernal, X. E., Page, R. A., Ryan, M. J., Argo, T. F., & Wilson, P. S. (2009). Acoustic radiation patterns of mating calls of the túngara frog (*Physalaemus pustulosus*): Implications for multiple receivers. *The Journal of the Acoustical Society of America*, 126(5), 2757–2767.
- Boersma, P. & Weenink, D. (2019). Praat: doing phonetics by computer [Computer program]. Version 6.1.03, retrieved 1 September 2019 from <http://www.praat.org/>
- Bongers, F., Charles-Dominique, P., Forget, P.-M., & Théry, M. (2013). *Nouragues: dynamics and plant-animal interactions in a neotropical rainforest* (Vol. 80). Springer Science & Business Media.
- Boulenger, G. A. (1883). On a collection of frogs from Yurimaguas, Huallaga River, northern Peru. In *Proceedings of the Zoological Society of London* (Vol. 51, pp. 635–638).
- Breitwisch, R. & Whitesides, G. H. (1987): Directionality of singing and non-singing behaviour of mated and unmated northern mockingbirds, *Mimus polyglottos*. *Animal Behaviour* 35(2), 311–339.
- Brenowitz, E. A., Wilczynski, W., & Zakon, H. H. (1984). Acoustic communication in spring peepers. Environmental and behavioral aspects. *Journal of Comparative Physiology A*, 155(5), 585–592.
- Brumm, H. (2002). Sound radiation patterns in nightingale (*Luscinia megarhynchos*) songs. *Journal of Ornithology*. 143(4), 468–471.
- Bee, M.A., & Christensen-Dalsgaard, J. (2016). Sound source localization and segregation with internally coupled ears: the treefrog model. *Biological Cybernetics*. 110(4–5), 271–290.
- Fay, R. R., & Simmons, A. M. (1999). The sense of hearing in fishes and amphibians. In R. R. Fay & A. N. Popper (Eds.), *Comparative Hearing: Fish and Amphibians* (pp. 269–318). New York: Springer.
- Forrest, T. G. (1994). From sender to receiver: propagation and environmental effects on acoustic signals. *American Zoologist*, 34(6), 644–654.
- Frommolt, K.-H., & Gebler, A. (2004). Directionality of dog vocalizations. *The Journal of the Acoustical Society of America*, 116(1), 561–565.
- Gerhardt, H. C. (1975). Sound pressure levels and radiation patterns of the vocalisations of some North American frogs and toads. *Journal of Comparative Physiology*, 102(1), 1–12.
- Gerhardt, H.C., & Rheinlaender, J. (1980). Accuracy of sound localization in a miniature dendrobatid frog. *Naturwissenschaften*, 67, 362–363.

- Glutz von Blotzheim, U. N. & Bauer, K. M. (1997). Handbuch der Vögel Mitteleuropas. Wiesbaden.
- Goodman, D. E. (1971). Territorial behavior in a Neotropical frog, *Dendrobates granuliferus*. *Copeia* 1971(2), 365–370.
- Grant, T., Rada, M., Anganoy-Criollo, M., Batista, A., Dias, P. H., Jeckel, A. M., et al. (2017). Phylogenetic systematics of dart-poison frogs and their relatives revisited (Anura: Dendrobatoidea). *South American Journal of Herpetology*, 12(s1), S1–S90.
- Greer, B. J., & Wells, K. D. (1980). Territorial and reproductive behavior of the tropical american frog *Centrolenella fleischmanni*. *Herpetologica*, 36(4), 318–326.
- Hödl, W. (1982). *Phyllobates femoralis* (Dendrobatidae): Rufverhalten und akustische Orientierung der Männchen (Freilandaufnahmen). Scientific Film Ctf 1788 BHWK.
- Hödl, W. (1983). *Phyllobates femoralis* (Dendrobatidae): Rufverhalten und akustische Orientierung der Männchen (Freilandaufnahmen). Wissenschaftlicher Film 30, 12-19.
- Hödl, W. (1987). *Dendrobates femoralis* (Dendrobatidae): a handy fellow for frog bioacoustics. In *Proceedings of the 4th Ordinary General Meeting of the Societas Europaea Herpetologica* (pp. 201–204).
- Hunter Jr, M. L., Kacelnik, A., Roberts, J., & Vuillermoz, M. (1986). Directionality of avian vocalizations: a laboratory study. *Condor*, 371–375.
- Jameson, D. L. (1954). Social Patterns in the Leptodactylid Frogs *Syrhophus* and *Eleutherodactylus*. *Copeia*, 1954(1), 36–38.
- Kaefer, I. L., Montanarin, A., Da Costa, R. S., & Lima, A. P. (2012). Temporal patterns of reproductive activity and site attachment of the brilliant-thighed frog *Allobates femoralis* from central Amazonia. *Journal of Herpetology*, 46(4), 549–554.
- Lötters, S., & Mutschmann, F. (2007). *Poison Frogs: Biology, Species and Captive Care*. Edition Chimaira.
- Michelsen, A., & Fonseca, P. (2000). Spherical sound radiation patterns of singing grass cicadas, *Tympanistalna gastrica*. *Journal of Comparative Physiology A*, 186(2), 163–168.
- Narins, P. M., & Hurley, D. D. (1982). The Relationship between Call Intensity and Function in the Puerto Rican Coqui (Anura: Leptodactylidae). *Herpetologica*, 38(2), 287–295.
- Narins, P. M., & Zelick, R. (1988). The effects of noise on auditory processing and behavior in amphibians. *The Evolution of the Amphibian Auditory System*. Wiley, New York, 511–536.
- Narins, P. M., Hödl, W., & Grabul, D. S. (2003). Bimodal signal requisite for agonistic behavior in a dart-poison frog, *Epipedobates femoralis*. *Proceedings of the National Academy of Sciences*, 100(2), 577–580.
- Parris, K. M. (2002). More bang for your buck: The effect of caller position, habitat and chorus noise on the efficiency of calling in the spring peeper. *Ecological Modelling*, 156(2–3), 213–224.
- Pewsey, A., Neuhauser, M., & Ruxton, G. D. (2013). *Circular statistics in R*. Oxford University Press.
- Pröhl, H. (2005). Territorial Behavior in Dendrobatid Frogs. *Journal of Herpetology*, 39(3), 354–365.
- Pyron, R. A., & Wiens, J. J. (2011). A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. *Molecular Phylogenetics and Evolution*, 61(2), 543–583.
- R Core Team. (2017). *R: A Language and Environment for Statistical Computing*. Vienna, Austria. Retrieved from

- <https://www.r-project.org/>
- Rheinlaender, J., Gerhardt, H. C., Yager, D. D., & Capranica, R. R. (1979). Accuracy of phonotaxis by the green treefrog (*Hyla cinerea*). *Journal of Comparative Physiology*, 133(4), 247–255.
- Ringler, M., Ringler, E., Mendoza, D. M., & Hödl, W. (2011). Intrusion experiments to measure territory size: development of the method, tests through simulations, and application in the frog *Allobates femoralis*. *PLoS One*, 6(10), e25844.
- Ringler, M., Hödl, W., & Ringler, E. (2015). Populations, pools, and peccaries: simulating the impact of ecosystem engineers on rainforest frogs. *Behavioral Ecology*, 26(2), 340–349.
- Roithmair, M. E. (1992). Territoriality and Male Mating Success in the Dart-poison Frog, *Epipedobates femoralis* (Dendrobatidae, Anura). *Ethology*, 92(4), 331–343.
- Toledo, L. F., Martins, I. A., Bruschi, D. P., Passos, M. A., Alexandre, C., & Haddad, C. F. B. (2014). The anuran calling repertoire in the light of social context. *Acta Ethologica*, 18(2), 87–99.
- Wells, K. D. (2007). *The Ecology and Behavior of Amphibians*. University of Chicago Press.

Chapter 2. Calling amplitude flexibility and acoustic spacing in the territorial frog

Allobates femoralis

This chapter has been published in the peer-review journal *Behavioural Ecology and Sociobiology*.

Citation:

Rodríguez, C., Amézquita, A., Ringler, M., Pašukonis, A., Hödl, W., 2020. Calling amplitude flexibility and acoustic spacing in the territorial frog *Allobates femoralis*. *Behavioral Ecology and Sociobiology* 74, 76. <https://doi.org/10.1007/s00265-020-02857-6>

Calling amplitude flexibility and acoustic spacing in the territorial frog *Allobates femoralis*

Camilo Rodríguez ^{a,b,†}, Adolfo Amézquita ^b, Max Ringler ^{a,c,d}, Andrius Pašukonis ^e and Walter Hödl ^d

^a Department of Behavioural and Cognitive Biology, University of Vienna, Vienna, Austria

^b Department of Biological Sciences, University of Los Andes, Bogotá, Colombia

^c Department of Ecology and Evolutionary Biology, University of California, Los Angeles, USA

^d Department of Evolutionary Biology, University of Vienna, Vienna, Austria

^e Department of Biology, Stanford University, Stanford, USA

[†] Corresponding author. E-mail: camilo.rodriguez@univie.ac.at

Abstract

Most male frogs produce calls to attract females and repel rivals. The transmission of these calls can be affected by many acoustic and environmental characteristics, which can influence the detection and decoding of the signal by the receiver. Calling-perch height has a strong influence on sound propagation and acoustic spacing with neighbouring males, but how frogs optimize their calling behaviour in this context is poorly understood. In this study, we investigated if and how frogs can adjust the calling energy in the context of acoustic spacing. Our aim was to evaluate the relationship between calling-perch height, nearest-neighbour distance, and sound pressure level in the Brilliant-Thighed Poison frog *Allobates femoralis*. We found that frogs flexibly adjust the calling amplitude according to the calling-perch height without affecting the effectiveness signal-propagation. Accompanying signal propagation experiments demonstrated that calls produced with lower amplitude from higher perches propagate similar to louder calls from the ground. Our results suggest an adjustment to the hypothesis of a general positive effect of calling-perch height on signal effectiveness, where highly territorial frogs adjust their calling behaviour to reduce energy expenditure and optimize acoustic communication with conspecifics.

Key words: Calling amplitude, Calling-perch height, Nearest-neighbour distance, Dendrobatidae, amplitude flexibility, acoustic spacing.

Introduction

In acoustically communicating species sound production and signal detection are critical for social interactions, such as species recognition and mating (Gerhardt 1994; Owings & Morton 1998). In anuran amphibians, acoustic communication determines the reproductive behavior where usually males produce calls to attract females (Gerhardt 1994). In territorial frogs, acoustic communication also mediates the acquisition and defence of territories by males (Gerhardt 1994; Pröhl 2005). For effective communication, the emitted signal containing certain information (e.g. species identity, position, reproductive motivation) must propagate, be received, and decoded by the receiver to influence its behavior (Wells & Schwartz 2007).

The efficiency of information transfer is shaped by the sender's intrinsic morphological and energetic constraints on signal production (Hödl et al. 2004; Amézquita et al. 2006; Göd et al. 2007). Extrinsic environmental conditions (e.g. distance from the ground, vegetation structure, temperature; Wiley & Richards 1982; Gerhardt 1994; Narins et al. 2006; Bro-Jørgensen 2010) can additionally limit the propagation by sound attenuation and degradation. In frogs, sound attenuation (Brenowitz et al. 1984; Robertson 1984; Wilczynski & Brenowitz 1988; Brenowitz 1989; Gerhardt et al. 1989) and degradation (Ringler et al. 2017) have been shown to affect the phonotactic response of both males and females, and the calling responses of neighbouring males, resulting in modified mate choice and male spacing patterns. Excess attenuation, i.e. the reduction of sound-pressure level (SPL) beyond loss through spherical spreading, can provide additional information on habitat characteristics and the distance of the sender, and thus further influence the response of a signal's receiver (Marten & Marler 1977; Wiley & Richards 1982).

In complex acoustic environments, where acoustically communicating species face acoustic attenuation and degradation, abiotic acoustic interference, and the competition for acoustic space, callers should adjust their calling behavior to maximise signal effectiveness (Hödl 1977; Duellman & Pyles 1983; Gerhardt & Huber 2002). Calling at higher perches allows the signal to propagate over larger distances and thereby increases the likelihood of reaching intended receivers (e.g. females, conspecific intruders). This positive relationship between calling-perch height and sound propagation has been shown in insects (Walker & Forrest 1989), birds (Sorjonen 1986) and amphibians (Greer & Wells 1980; Wells & Schwartz 1982; Brenowitz et al. 1984; Kime

et al. 2000; Parris 2002; Kats et al. 2012; Schwartz et al. 2015). However, animals can also adjust the amplitude of the signal depending on the level of masking abiotic and/or inter- and intraspecific background noise (Lombard 1911; Brumm & Todt 2002; 2004). For instance, signals with higher amplitude may counteract masking effects and travel farther and so be more easily detected by receivers (Lombard 1911; Forrest 1994). This noise-dependent vocal plasticity has been described in few species of amphibians (Halfwerk et al. 2016; Shen & Xu 2016; Zhao et al. 2018). However, to our knowledge amplitude adjustment independent of masking background noise has only been demonstrated in the White-Lipped Frog, *Leptodactylus albilabris* (Lopez et al. 1988). This species presents plasticity of the advertisement call amplitude in response to increasing-amplitude stimuli, suggesting a “shouting match” between directly competing males (Love & Bee 2010).

Among amphibians, Neotropical poison frogs (Dendrobatidae) present some of the most complex social behaviors, including a high degree of territoriality, different types of parental care, and acoustic communication (Lötters et al. 2007; Wells 2007). Males of the Brilliant-Thighed Poison Frog, *Allobates femoralis*, are strongly territorial, a behavior associated with prominent advertisement calls (Hödl 1983). Males therefore may benefit from producing calls that propagate as far as possible to better repel conspecific males and reach more females. Whereas specific temporal and spectral properties, sound-radiation patterns, behavioral aspects, evolutionary patterns, and adaptive consequences related to the advertisement call have been extensively studied in this species (Narins et al. 2003; Hödl et al. 2004; Amézquita et al. 2005; Amézquita et al. 2006; Göd et al. 2007; Gasser et al. 2009; de Luna et al. 2010; Rodríguez & Hödl 2020), little information exists on how males adapt their calling behavior to interfering habitat-induced factors to optimize signal effectiveness.

In a pilot experiment, we found that the calling-perch height in *A. femoralis* males was not positively related to sound propagation. This observation is quite surprising giving the strong evidence of such a positive effect in other anuran species (Greer & Wells 1980; Wells & Schwartz 1982; Brenowitz et al. 1984; Parris 2002), suggesting that the call amplitude and/or the calling-perch height can be adjusted by *A. femoralis* males in the context of territoriality and acoustic spacing. In this study, we addressed this issue with two different approaches: first, by relating natural calling-perch heights with the produced SPL and the inter-individual distances of calling

A. femoralis males in two geographically distant populations; and second, by conducting signal propagation experiments to test the effect of calling-perch height on the SPL at the location of the receiver. Our specific aims were to test (1) whether males that call at higher perches deliver calls with higher amplitude at the receivers and therefore have larger nearest-neighbour distances (NND) than males that call at lower perches; (2) whether the calling amplitude and/or the calling-perch height are being flexibly adjusted by *A. femoralis* males; and (3) whether the calling-perch height has an effect on sound propagation under natural conditions.

Methods

Study species and study area

We conducted our study on the Brilliant-Thighed Poison Frog, *Allobates femoralis* BOULENGER (1883; Dendrobatidae: Aromobatinae; AmphibiaWeb 2020). This species is terrestrial and diurnally active. The reproductive behavior consists of prolonged courtships involving auditory, visual, and tactile signals (Montanarin et al. 2011; Stückler et al. 2019). Males usually call from slightly elevated vegetation-structures on the forest ground, like fallen trees, logs, lianas and leaves (Roithmair 1992). Parental care is usually performed by males, who transport tadpoles to small water bodies (Roithmair 1992; Ringler et al. 2013). We conducted our study in two geographically distant populations of *A. femoralis* and in different years. First, we used 15 frogs from a population in the Tanimboca Natural Reserve near the city of Leticia in the Colombian Amazon (4°07' S - 69°57' W; www.tanimboca.org) in July and August 2011 (from now on, Let11) and April 2014 (from now on, Let14). Later we replicated the experiments using 34 frogs from a population near the "Pararé field camp", located in the Les Nouragues Natural Reserve in French Guiana (4°02' N - 52°41' W; Bongers et al. 2013; see Ringler et al. (2015) for a description of the population characteristics), during the early reproductive season between February and April in 2018 (from now on, FG18) and in 2019 (from now on, FG19). Both study sites present structural and floristic heterogeneity. Detailed information about the structure and floristic composition is given in Bongers et al. (2001) for the "Pararé field camp", and CORPOAMAZONIA (2006) for Tanimboca.

We performed the experiments at the two sites in the same way (except for NND measurements; see below) and between 0700–1000 and 1500–1800, which corresponds to the peak calling activity of this species (Kaefer et al. 2012). We captured frogs using transparent plastic bags and measured their snout-urostyle length (SUL). We used digital images of the individually unique ventral patterns of *A. femoralis* for individual identification.

Calling intensity, perch height and nearest-neighbour distance

We measured the sound-pressure level (SPL) of the advertisement call of *A. femoralis* males with a SPL-meter (Votcraft 329, Conrad Electronic SE, Wenberg Köblitz, Germany), using the peak detector in the C-weighting curve and in fast mode. The SPL-meter was mounted either on a small table-top or a full-size tripod, aligned parallel to the forest floor, at the same height relative to the frog and pointing towards the frog with no vegetation in between. The frogs generally start calling with suboptimal advertisement calls of less than steady-state SPL, defined as “warm-up calls”, (Jameson 1954; Toledo et al. 2014); therefore, we measured the SPL from the 5th four-note call in a call series and we took the average of three SPL measurements after a linear conversion to Pascals ($\text{Pa} = 20\mu\text{Pa} \cdot 10^{\text{dB}/20}$). Because SPL measurements were taken at different distances (in a range of 0.24–3.1 m) from the calling frog, we fitted an exponential regression using the distance as a predictor and the logarithm of SPL as the response variable and used the residuals as the new distance-independent SPL (Residuals (SPL)) in the subsequent statistical analysis. Snout to urostyle length (SUL) was not related to the SPL ($F_{1,13}=2.13$; $P=0.16$; $R^2=0.07$; $N=15$). Since *A. femoralis* males have a nearly omnidirectional sound radiation pattern (Rodríguez & Hödl 2020), the cardinal position of the frogs in reference to the SPL-meter was registered, being 0/360° the position of the SPL-meter in the cardinal plane, in order to know whether the frogs’ orientation affect the SPL measured in relation to the calling-perch height.

Subsequently, we measured the calling-perch height of the males from the exact spot where they were calling to the surface of the compacted leaf litter on the ground. Subsequently, we measured the NND as follows: In “Tanimboca” we measured the inter-individual distances from the exact spot where the frogs were calling by using measuring tapes. At the “Pararé field camp” we first performed a series of capture-recapture surveys only when the males were calling and recorded every capture point on a digital map of the study area (distance error estimate ~10 cm; Ringler et al. 2014) using pocket PCs (MobileMapper 10, Spectra Precision, Sunnyvale, CA,

USA) with the mobile GIS software ArcPad (ESRI, Redlands, CA, USA). We used the traversing function in ArcPad to map the 2-D position of the frogs using laser rangefinders (DLE 50, Bosch, Gerlingen Germany) and precision compasses (Tandem (magnetic inclination zone 2), Suunto, Vantaa, Finland) for distance and direction measurements, respectively. Finally, we calculated the capture-point centroid for each male and measured the Euclidean distance between centroids to obtain NNDs in R (R Core Team, 2017), using the "centroid" function of the R-package *geosphere* (Hijmans et al. 2017).

Effect of perch height on sound propagation

This experiment was conducted between February and April 2018 at the "Pararé field camp". To test the effect of calling-perch height on sound propagation we used an artificial call of *A. femoralis* featuring the average call properties from a nearby population (with a call consisting of four notes), approximately 30 km downstream on the Arataye river (For details on temporal and spectral parameters see Gasser et al. 2009; Narins et al. 2003). We calibrated the loudspeaker with an integrated music player (Creative MUVO 2c, Creative, Singapore) at SPLs of 73, 76 and 81 dB using an SPL-meter (Voltcraft 329) at 1 m distance by adjusting the volume setting. Then, we repeatedly broadcast playback signal three times at each SPL with the loudspeaker placed on logs at heights of 0, 10 and 20 cm. We replicated 20 times the playback experiments using broadcast locations and directions within the natural habitat of the local *A. femoralis* population and, selected opportunistically to let unobstructed playback paths between the speaker and the SPL-meter. Both, the SPLs and heights were chosen to match the natural range of variation of the "Pararé field camp" population of *A. femoralis* (Table 1). SPL measurements of broadcast signals were taken at a horizontal distance of 6 m from the loudspeaker, which corresponds to the average territory radius of the "Pararé field camp" population of *A. femoralis* (Ringler et al. 2011; Tumulty et al. 2018), with the SPL meter on the ground pointing towards the speaker.

Table 1. Descriptive statistics of calling behavior traits at “Tanimboca natural reserve” and “Pararé field camp”. SPL = sound pressure level; NND = nearest-neighbour distance

Site	SPL (dB)		Calling-perch height (cm)		NND (m)	
	$\bar{x} \pm SD$	Range (n)	$\bar{x} \pm SD$	Range (n)	$\bar{x} \pm SD$	Range (n)
Tanimboca	71.60 $\pm$ 2.94	65–77.6 (15)	26.88 $\pm$ 18.41	2.78–72.6 (15)	13 $\pm$ 6.9	1.4–27 (15)
Pararé	74.38 $\pm$ 4.17	64.23–81.5 (34)	13.22 $\pm$ 6.23	3–31.6 (34)	8.79 $\pm$ 3.87	4–17.41 (30)

Repeatability of call amplitude and calling-perch height

This experiment was conducted between February and April 2019 in the *A. femoralis* population near the “Pararé field camp”. For each calling *A. femoralis* male we measured the SPL and the height from their calling-perch to the ground on three different occasions with minimum intervals of three days. To measure repeatability, we estimated the proportion of the total variance that is assigned to between-individual variation in the SPL and calling -perch height across the three different occasions (Nakagawa & Schielzeth 2010).

Statistical analyses

To estimate the relationship between the calling-perch height, call amplitude and NND we fitted linear mixed-effects models by maximum likelihood using the “lmer” function within the *lme4* package (Bates et al. 2015) in R (R core Team, 2017). As p-values for mixed effects models are not as easy to interpret as for linear models (Johansson 2011), we compared the likelihood of several models by using the “lrttest” function within the package *lmttest* (Zeileis & Hothorn 2002) in R. Prior to all comparisons, we evaluated whether the fixed effects were inter-dependent on each other or not (i.e. if the interaction matters), by comparing models with and without the interaction effect. Subsequently, in all cases we compared a *full* model (including all the fixed effects) against a *reduced* model (excluding the fixed effect of interest in each comparison) as follows: (1) To evaluate the effect of calling-perch height on the call amplitude, we fitted a *full* model using calling-perch height and data sets (i.e. Let11, Let14, FG18 and FG19) as fixed factors, the individual intercepts as random factor and Residuals (SPL) as the response variable. The *reduced* model was identical but excluded the calling-perch height; (2) To evaluate the effect of the call amplitude on the NND, we fitted a *full* model using residuals (SPL) and data sets as fixed factors, the individual intercepts as random factor and NDD as the response variable. The *reduced*

model was identical but excluded the residuals (SPL); and, (3) To evaluate the effect of the calling-perch height on the NND, we fitted a *full* model using calling-perch height and data sets as fixed factors, the individual intercepts as random factor and the NND as the response variable. The *reduced* model was identical but excluded the calling-perch height. In all cases, we log-transformed the calling-perch height and squared root-transformed the NND to fit a normal distribution. In each comparison, we assumed that the fixed effect had a significant effect when the difference between the likelihood of the *full* and *reduced* models was significant. Additionally, we performed simple linear regressions for every comparison in Let11, Let14 and FG18 data sets, and linear-mixed regression in FG19. Furthermore, we estimated the effect of the frogs' orientation on the relationship between calling amplitude and calling-perch height by fitting a linear mixed-effects model. We used the residuals (SPL) as dependent variable, the interaction between calling-perch height and the orientation of the frog as fixed factor and, the ID of the frog as random factor. Likelihood ratio test was also performed between a *full* model (including the interaction) versus a *null* model (without the interaction).

To estimate the effect of the calling-perch height on signal propagation, we performed a linear mixed-effects model using the received SPLs as response variable, the broadcast heights and SPLs as fixed effects and repetitions and trials as nested random factors. Then, we used planned least-squares means contrasts using the "*lsmeans*" function within the *lsmeans* package (Lenth 2016). Post-hoc comparisons were adjusted using Tukey's method for multiple comparisons. Significant statistical differences between the mean values would be indicated by non-overlapping 99.9% confidence intervals.

To estimate repeatability (R), we used the "*rpt*" function of the package *rptR* (Nakagawa & Schielzeth 2010) in R. In our fitted model SPL or calling-perch height were used as the response variable, the data sets as the fixed factor and the individual ID of the frog as the random factor. Statistical significance against the *null* hypothesis (Repeatability = 0) was estimated by likelihood ratio test.

Ethical note

All experiments were conducted in strict accordance with current French, European Union and Colombian laws and were approved by the Animal Ethics and Experimentation Board of the

University of Vienna (No. 2018-009). Our study was approved by the scientific committees of the “Nouragues Ecological Research Station” and “Tanimboca Natural Reserve” where field work was conducted. We adhered 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.

Results

We found that *A. femoralis* males that perch higher produced calls at significant lower amplitudes ($X^2 = 4.14$, $df = 1$, $P = 0.04$; **Fig. 1a**). While this effect was highly significant for the FG18 data set ($F = 15.35$, $R^2 = 0.4$, $P < 0.01$; **Fig. 1b**), for the Let11, Let14 and FG19 data sets it was not statistically significant (Let11: $F = 0.52$, $R^2 = 0.07$, $P = 0.5$; Let14: $F = 0.74$, $R^2 = 0.04$, $P = 0.42$; FG19: $t = -0.08$, $P = 0.93$; **Fig. 1b**).

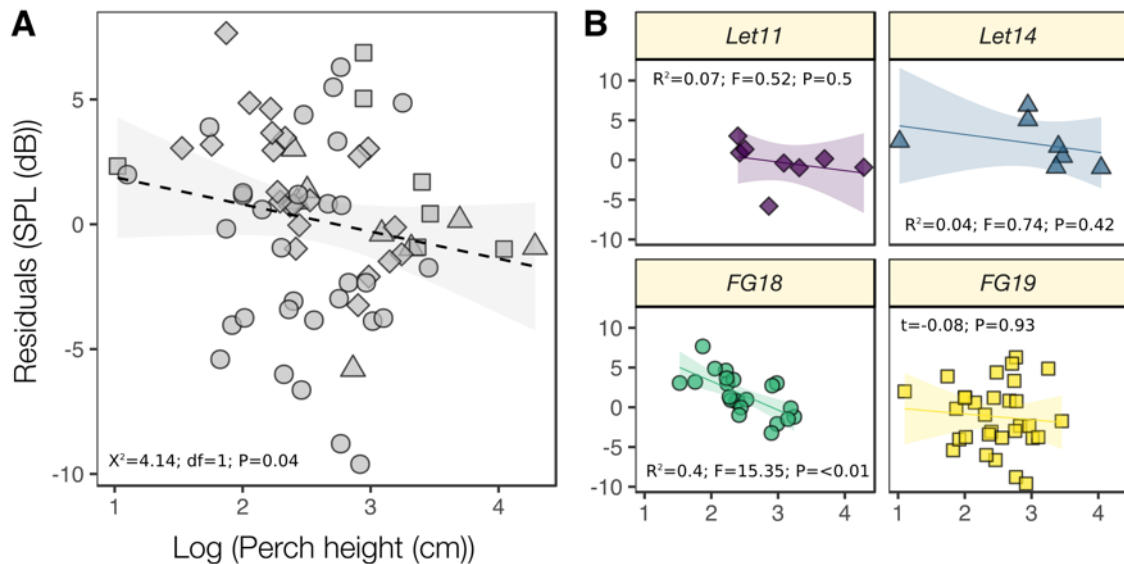


Figure 1. Relationship between Sound Pressure Levels (SPL) and Perch height in *A. femoralis*. **(A)** fitted regression line from a Linear regression mixed model with “data set” as a co-variable (diamonds: Let11 (N=8); triangles: Let14 (N=7); circles: FG18 (N=22); squares: FG19 (N=12)). **(B)** Simple linear regression models for every “data set”. Shaded regions indicate 95% confidence intervals.

Males producing higher amplitude calls did not have larger inter-individual distances to the nearest males ($X^2 = 0.02$, $df = 4$, $P = 0.99$, **Fig. 2a**). Interestingly, in the FG18 data set, males had significant larger inter-individual distances when calling at lower amplitudes ($t = -2.3$, $R^2 = 0.2$, $P = 0.03$; **Fig. 2b**), while this effect was not significant for the other data sets (Let11: $F = 0.2$, $R^2 = 0.12$, $P = 0.67$; Let14: $F = 0.56$, $R^2 = 0.07$, $P = 0.48$; FG19: $t=0$, $P=1$; **Fig. 2b**).

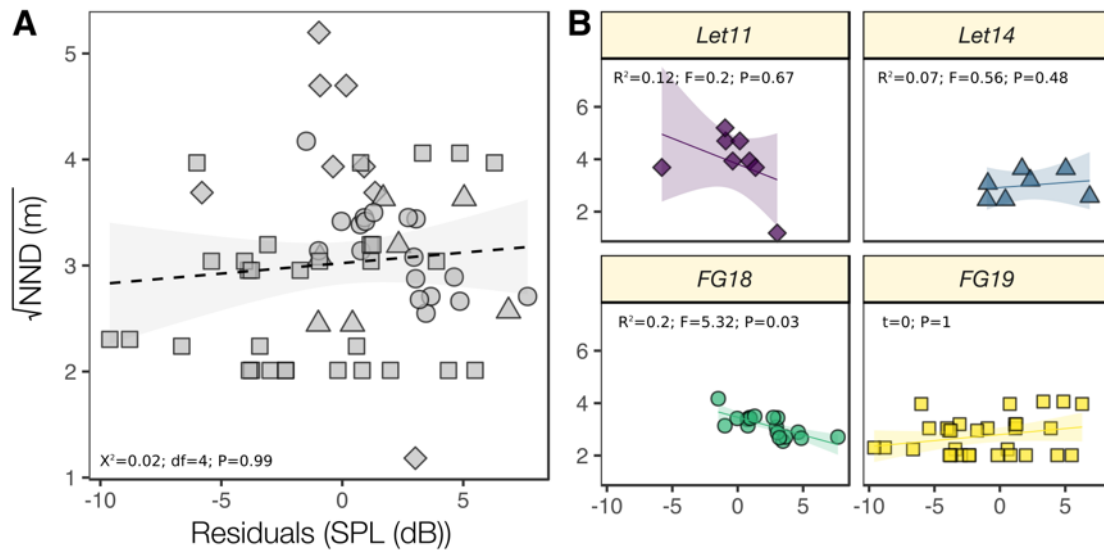


Figure 2. Relationship between nearest-neighbour distance (NND) and sound-pressure levels (SPL) in *A. femoralis*. (A) fitted regression line from a Linear regression mixed model with “data set” as a co-variable (diamonds: Let11 (N=8); triangles: Let14 (N=7); circles: FG18 (N=18); squares: FG19 (N=12)). (B) Simple linear regression models for every “data set”. Shaded regions indicate 95% confidence intervals.

Moreover, higher perched males had larger inter-individual distances to the nearest males ($X^2 = 17.16$, $df = 4$, $P = 0.001$; **Fig. 3a**). Nevertheless, while this effect was significant for the FG18 data set ($F = 4.42$, $R^2 = 0.16$, $P = 0.05$; **Fig. 3b**) being not statistically significant for the Let11, Let14 and FG19 data sets (Let11: $F = 4.5$, $R^2 = 0.33$, $P = 0.07$; Let14: $F = 0.54$, $R^2 = 0.08$, $P = 0.5$; FG19: $t=0$, $P=1$; **Fig. 3b**).

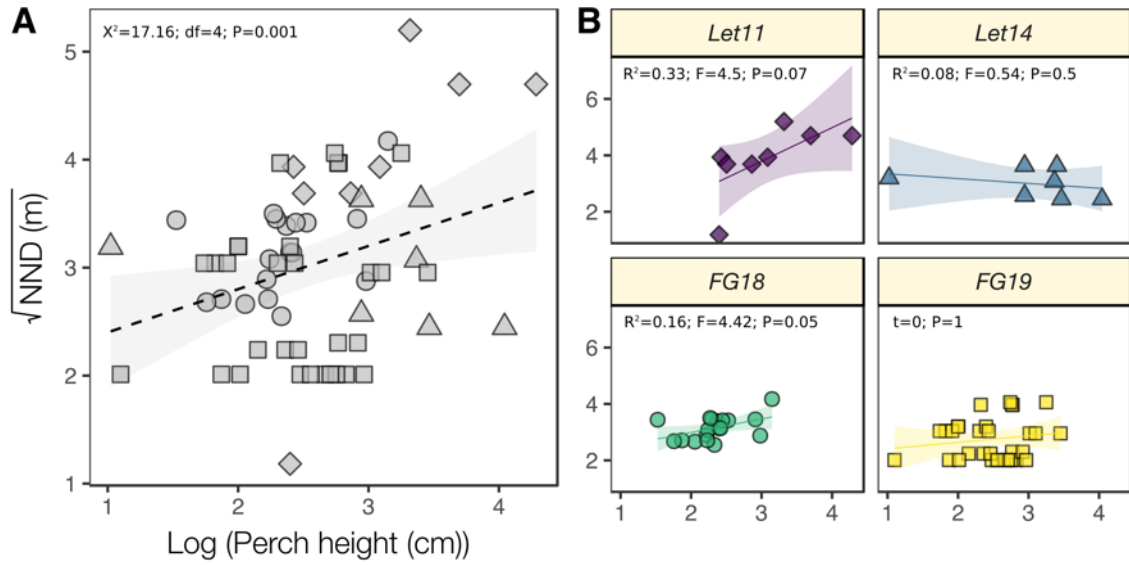


Figure 3. Relationship between Nearest-neighbour distance (NND) and calling-perch height in *A. femoralis*. (A) fitted regression line from a Linear regression mixed model with “data set” as a co-variable (diamonds: Let11 (N=8); triangles: Let14 (N=7); circles: FG18 (N=18); squares: FG19 (N=12)). (B) Simple linear regression models for every “data set”. Shaded regions indicate 95% confidence intervals.

The relationship between calling-perch height and calling amplitude was not interdependent on the frog’s orientation when measured the SPL ($X^2 = 4.9$, $df = 3$, $P = 0.17$). SPLs of the advertisement call of *A. femoralis* males were statistically not repeatable ($R = 0.18$, $p = 0.14$; **Fig. 4**). Conversely, calling-perch height was statistically repeatable across the three trials ($R = 0.36$, $p = 0.02$; **Fig. 4**).

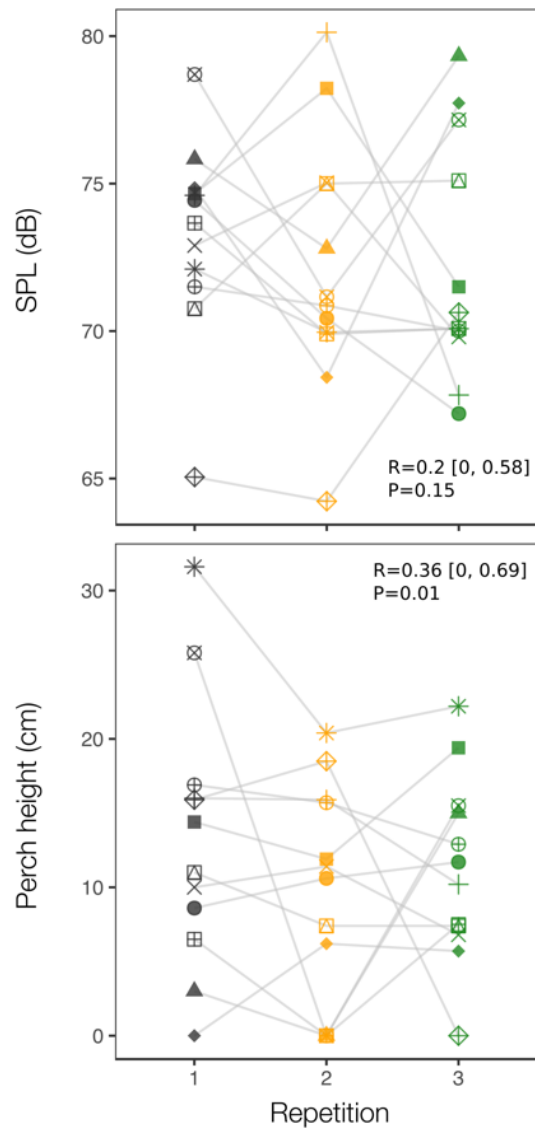


Figure 4. Plot showing individual measurements in *A. femoralis* of sound-pressure levels (SPL) of the advertisement call (upper plot) and calling-perch height (lower plot) over three trials. Symbols represent each individual. We estimated repeatability (R) and 95% confidence intervals (in square brackets).

In our propagation experiments, signals broadcast at 76 dB from 0 cm height propagated equally as signals at 73 dB from 20 cm height (planned lsmeans contrast: $t = -2.51$, $P = 0.22$; **Fig. 5**; Supplementary Material Table **S1**) while signals broadcast at 81 dB from 0 cm height propagate farther than signals at 76 dB from 20 cm height (planned lsmeans contrast: $t = 10.48$, $P < 0.001$; **Fig. 5**; Supplementary Material Table **S1**). We found no significant differences between 0 cm and 10 cm or between 10 cm and 20 cm (**Fig. 5**; Supplementary Material Table **S1**) of perch height with test signals broadcast at most of the three different intensities (73, 76 and 81 dB; **Fig. 5**). However, perch height differences between 0 cm and 20 cm significantly increase the sound propagation (planned lsmeans contrast: all $P < 0.001$; **Fig. 5**; Supplementary Material Table **S1**).

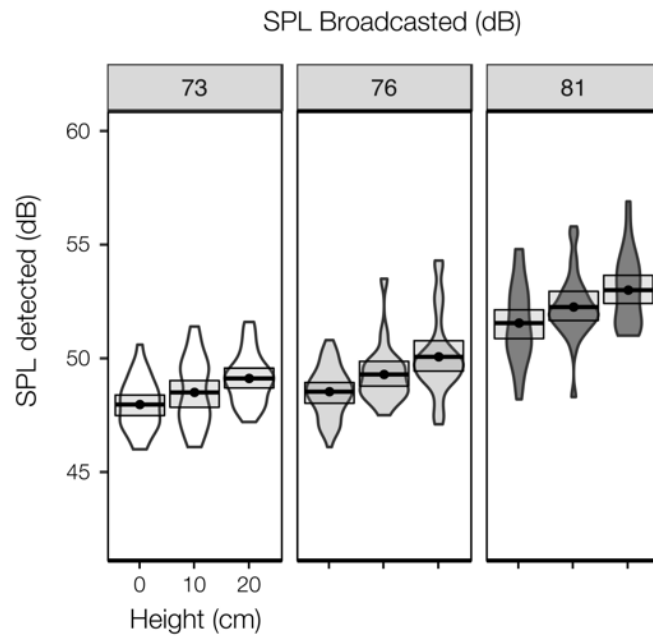


Figure 5. Violin plots showing the effect of the calling-perch height on the detected SPL at three different broadcasted SPLs and at 6 m of horizontal distance from the loudspeaker. Black dots and horizontal bars denote means and shaded square boxes denote 99.9% confidence intervals after Bonferroni's correction

Discussion

Numerous studies have shown the positive effect of calling-perch height on sound propagation on long- and short-distance communication systems. Sound is attenuated when closer to the ground because of the dense vegetation and ground effect (Marten & Marler 1977; Richard & Wiley 1980; Wells & Schwartz 1982; Forrest 1994; Schwartz et al. 2015). In the case of *A. femoralis*, our results suggest an adjustment to the hypothesis of a general positive effect of calling-perch height on signal effectiveness, where males take advantage of the signal-enhancing effects of calling from high perches without affecting the effectiveness of acoustic spacing with conspecifics.

As acoustic communication mediates reproductive and territorial behaviors (e.g. territorial advertising, aggressive encounters, mating), evolution exerts selective pressures on the signals

and/or on behavioral adaptations (Gerhardt 1994; Owings & Morton 1998). Two selective pressures might be driving the behavioral plasticity regarding calling amplitude and the choice of calling-perch heights in *A. femoralis*. First, because calling with higher amplitude is energetically costly (Prestwich 1994; Ryan 1988), calling at higher perches and thereby reducing attenuation (Morton 1975; Marten & Marler 1977) allows the frogs to save energy by producing calls with lower amplitude but still sufficient propagation. Second, calling at elevated sites above the ground level enhance acoustic and visual conspicuousness to females and conspecific males, as has been shown in the glass frog *Centrolenella fleischmanni* (Greer & Wells 1980) and in the strawberry poison frog *Oophaga pumilio* (Kats et al. 2012). However, possible costs include an increased visual and acoustic conspicuousness also towards predators (Haff & Magrath 2011; Dugas et al. 2015), which could be counteracted by either reducing the amplitude of the signal (Tuttle & Ryan 1982) or developing visual antipredatory mechanisms (e.g. cryptic appearance, Batesian or Müllerian mimicry; Ruxton et al. 2004; Amézquita et al. 2017).

We present evidence of both antipredator mechanisms in *A. femoralis*. First, males had a significant intra-individual variation in advertisement call amplitude, but they maintained relatively small variation in the calling-perch height (**Fig. 4**), indicating that they can actively vary the calling amplitude. Plasticity in the call amplitude has been shown in some anuran species in response to ambient noise (Lopez et al. 1988; Penna et al. 2005; Cunningham & Fahrig 2010; Halfwerk et al. 2016), and also by exploiting signal-enhancing structures (Lardner & bin Lakim 2002). To our knowledge, this is the first study that shows evidence of call amplitude adjustment in a territorial frog. Second, in our two study populations *A. femoralis* co-occurs with their toxic "co-mimic" *Ameerega hahneli*. The two species have similar brightly coloured femoral patches and dorsolateral lines, with reflectance spectra that do not differ significantly at the Tanimboca natural reserve near Leticia (see Online Resource ESM1).

Several studies have shown that acoustic communicating species use the amplitude of the signal as a reliable cue to assess neighbours distances, allowing acoustic spacing between neighbours (Naguib 1997; Nelson 2000). However, if neighbouring males are able to modulate their call amplitude, it is unreliable to use only SPL of the signal to infer neighbours proximity (Richards 1981; Morton 1982). In our study, SPL did not predict the inter-individual distances between calling *A. femoralis* males. Considering that *A. femoralis* can vary the calling amplitude,

acoustic ranging and territory spacing may therefore depend on other factors than SPL alone. Indeed, another study in the “Pararé field camp” population suggested that males use also other cues independent from SPL, like spectral degradation or reverberation, for assessing neighbour distance (Ringler et al. 2017). However, SPL had a negative effect on the NND in the FG18 data set (**Fig. 2b**). One possible explanation for this unexpected result is that signals close to the ground are more prone to degradation and likely they sound as if they would originate from further away than signals produced from higher perches, even when they are at a lower SPL. Therefore, frogs assessing their neighbour’s distance (also) from signal degradation (Ringler et al. 2017) consequently may settle closer to more degraded signals, assuming they come from further away. This third effect might add to the concomitant effect of signal source height and the amplitude of the signal on inter-individual spacing in *A. femoralis*.

In line with the hypothesis that male frogs benefit from calling at elevated perches to increase inter-individual spacing, by improving the sound propagation (Greer & Wells 1980; Wells & Schwartz 1982; Gerhardt et al. 1989), we found that higher perched *A. femoralis* males had larger NND. Several studies have shown that the source height of the signal affects the propagation distance, with a pronounced effect on sound attenuation below 1 m above the ground (Morton 1975; Marten & Marler 1977; Wiley & Richards 1982; Forrest 1994; Kime et al. 2000; Parris 2002). In fact, under realistic field conditions, our propagation experiments showed that calling-perch heights of just 20 cm significantly increase the signal propagation compared to calling-perches on the ground. Therefore, males calling close to the ground could suffer disadvantages from unwanted signal reflection and/or excess attenuation due to ground cover. Interestingly, our experimental data also show that softer signals produced at higher calling perches propagate as well as louder signals from the ground. That is, frogs might spend less metabolic energy in calling (i.e. low rate of oxygen consumption to produce low amplitude calls) by exploiting the enhancing effects on sound propagation of calling from elevated positions (Ryan 1988; Forrest 1994). Then, our results suggest that reducing the calling amplitude when perching higher might have concomitant benefits associated with energy saving, antipredator strategies and acoustic communication optimization. Further propagation experiments are needed with the speaker and the SPL-meter positioned on the average of typical calling-perch height in order to mimic the natural position of both, senders and receivers.

It is noteworthy that the relationships between calling amplitude, calling-perch height and NND did not follow the same pattern across the different data sets. This suggests that the acoustic behavior in *A. femoralis* is more complex and may depend on additional ecological factors like weather conditions, motivational state, or population density. Also, we cannot discard that the inverse relationship between call amplitude and calling-perch height could be an epiphenomenon of multimodal signaling, where males perch higher to improve the sound propagation but also increase the visibility of the vocal sac towards neighbouring males and females (Narins 2003; Halfwerk et al. 2017). Further research is needed to clarify the reasons of such variation in the vocal behavior across different locations and time periods. Likewise, we present evidence for elevation-related changes in call amplitude in *A. femoralis* males for just one location and year (FG19). However, to claim a behavioural adaptation across the *A. femoralis* species complex (Amézquita et al. 2019; Grant et al. 2017), further experimental studies are needed to assess the flexibility of territorial frogs' calling amplitude in different populations, for instance, by measuring SPLs after presenting calling-perch structures with different heights within male's territories.

Behavioral adjustments may grant benefits when selected or developed in a specific context (Sol et al. 2013). In the acoustic communication context, animals must interact with the environment and adjust their calling behavior to improve their communication. Overall, our study suggests that highly territorial frog males can adjust their calling behaviour by exploiting the signal-enhancing effects of elevated calling positions and producing calls with lower amplitude. We propose that such behavioral adjustment can be advantageous to enhance the effectiveness of communication through improving call propagation, energy saving and minimizing predation risk.

Funding

This work was supported by the Austrian Science Fund (FWF): W1262-B29 and by an Erwin Schrödinger Fellowship (FWF J3868) to MR.

Acknowledgements

We are grateful to S. Chaloupka for help in the propagation experiments. We thank A. Lembens, V. Canoine and G. Raboisson for providing field assistance and, two anonymous reviewers for helpful comments this article. We thank the Nouragues research field station (managed by CNRS) which benefits from "Investissement d'Avenir" grants managed by Agence Nationale de la Recherche (AnaEE France ANR-11-INBS-0001; Labex CEBA ANR-10-LABX-25-01) and Tanimboca Natural Reserve for the logistic support. Additional support for the study at Leticia was provided by grants to CR from the Research Committee of the Faculty of Sciences at the Universidad de Los Andes, Bogotá.

References

- Amézquita A, Castellanos L, Hödl W (2005) Auditory matching of male *Epipedobates femoralis* (Anura: Dendrobatidae) under field conditions. *Anim Behav* 70: 1377–1386.
- Amézquita A, Hödl W, Lima AP, Castellanos L, Erdtmann L, de Araújo MC (2006) Masking interference and the evolution of the acoustic communication system in the Amazonian dendrobatid frog *Allobates femoralis*. *Evolution* 60: 1874–1887.
- Amézquita A, Lima AP, Jehle R, Castellanos L, Ramos Ó, Crawford AJ, Gasser H, Hödl W (2009) Calls, colours, shape, and genes: a multi-trait approach to the study of geographic variation in the Amazonian frog *Allobates femoralis*. *Biol J Linn Soc* 98: 826–838.
- Amézquita A, Ramos Ó, González MC, Rodríguez C, Medina I, Simões PI, Lima AP (2017) Conspicuousness, color resemblance, and toxicity in geographically diverging mimicry: The pan-Amazonian frog *Allobates femoralis*. *Evolution* 71: 1039–1050.
- AmphibiaWeb (2018) AmphibiaWeb: Information on amphibian biology and conservation. University of California. Berkeley, CA, USA. www.amphibiaweb.org, last checked 07.11.2018.
- Bongers F, Charles-Dominique P, Forget PM, Théry M (2013) *Nouragues: dynamics and plant-animal interactions in a neotropical rainforest*. Springer Science & Business Media.
- Boulenger GA (1883) On a collection of frogs from Yurimaguas, Huallaga River, northern Peru. *Proc Zool Soc London* 51: 635–638.
- Brenowitz EA (1989) Neighbor Call Amplitude Influences Aggressive Behavior and Intermale Spacing in Choruses of the Pacific Treefrog (*Hyla regilla*). *Ethology* 83: 69–79.
- Brenowitz EA, Wilczynski W, Zakon HH (1984) Acoustic communication in spring peepers. Environmental and behavioral aspects. *J Comp Physiol A* 155: 585–592.
- Bro-Jørgensen J (2010) Dynamics of multiple signaling systems: animal communication in a world in flux. *Trends Ecol Evol* 25: 292–300.
- Brumm H, Todt D (2002) Noise-dependent song amplitude regulation in a territorial songbird. *Anim Behav* 63: 891–897.
- Brumm H, Todt D (2004) Male-male vocal interactions and the adjustment of song amplitude in a territorial bird. *Anim Behav* 67: 281–286.
- Corporación para el Desarrollo sostenible del sur de la Amazonía - CORPOAMAZONIA (2006) Plan de ordenación y manejo de la microcuenca de la quebrada Yahuaraca. CORPOAMAZONIA. Leticia, Colombia.
- Cunnington GM, Fahrig L (2010) Plasticity in the vocalizations of anurans in response to traffic noise. *Acta Oecol* 36: 463–470.
- de Luna AG, Hödl W, Amézquita A (2010) Colour, size and movement as visual subcomponents in multimodal communication by the frog *Allobates femoralis*. *Anim Behav* 79: 739–745.
- Duellman WE, Pyles RA (1983) Acoustic Resource Partitioning in Anuran Communities. *Copeia* 1983: 639–649.
- Dugas MB, Halbrook SR, Killius AM, del Sol JF, Richards-Zawacki CL (2015) Colour and Escape Behaviour in Polymorphic Populations of an Aposematic Poison Frog. *Ethology* 121: 813–822.
- Forrest TG (1994) From sender to receiver: propagation and environmental effects on acoustic signals. *Am Zool* 34: 644–

- 654.
- Gasser H, Amézquita A, Hödl W (2009) Who is Calling? Intraspecific Call Variation in the Aromobatid Frog *Allobates femoralis*. *Ethology* 115: 596–607.
- Gerhardt HC (1994) The Evolution of Vocalization in Frogs and Toads. *Annu Rev Ecol Syst* 25: 293–324.
- Gerhardt HC, Diekamp B, Ptacek M (1989) Inter-male spacing in choruses of the spring peeper, *Pseudacris (Hyla) crucifer*. *Anim Behav* 38: 1012–1024.
- Gerhardt HC, Huber F (2002) Acoustic Communication in Insects and Anurans: Common Problems and Diverse Solutions. University of Chicago Press.
- Göd M, Franz A, Hödl W (2007) The influence of internote-interval variation of the advertisement call on the phonotactic behaviour in male *Allobates femoralis* (Dendrobatidae). *Amphibia-Reptilia* 28: 227–234.
- Grant T, Rada M, Anganoy-Criollo M, Batista A, Dias PH, Jeckel AM, Machado DJ, Rueda-Almonacid JV (2017) Phylogenetic systematics of dart-poison frogs and their relatives revisited (Anura: Dendrobatoidea). *S Am J Herpetol* 12: S1–S90.
- Greer BJ, Wells KD (1980) Territorial and reproductive behavior of the tropical american frog *Centrolenella fleischmanni*. *Herpetologica* 36: 318–326.
- Haff TM, Magrath RD (2011) Calling at a cost: elevated nestling calling attracts predators to active nests. *Biol Lett* 7: 493–495.
- Halfwerk W, Lea AM, Guerra MA, Page R, Ryan M (2016) Vocal responses to noise reveal the precense of the Lombard effect in a frog. *Behav Ecol* 27: 669–676.
- Hijmans RJ, Williams E, Vennes C, Hijmans, MR (2017) Package “geosphere.” Retrieved from <https://cran.r-project.org/web/packages/geosphere/>
- Hödl W (1977) Call differences and calling site segregation in anuran species from Central Amazonian floating meadows. *Oecologia* 28: 351–363.
- Hödl W, Amézquita A, Narins PM (2004) The rôle of call frequency and the auditory papillae in phonotactic behavior in male Dart-poison frogs *Epipedobates femoralis* (Dendrobatidae). *J Comp Physiol A* 190: 823–829.
- Hödl W, Amézquita A, Narins P, Lima A (2012) The brilliant-thighed poison frog *Allobates femoralis* (Aromobatidae): a handy fellow for anuran field bioacoustics. *Bioacoustics* 21: 37–38.
- Jameson DL (1954) Social Patterns in the Leptodactylid Frogs *Syrrhophus* and *Eleutherodactylus*. *Copeia* 1954: 36–38.
- Johansson T (2011) Hail the impossible: p-values, evidence and likelihood. *Scand* 52: 113–125.
- Kaefer IL, Montanarin A, Da Costa RS, Lima AP (2012) Temporal patterns of reproductive activity and site attachment of the brilliant-thighed frog *Allobates femoralis* from central Amazonia. *J Herpetol* 46: 549–554.
- Kats LB, Bucciarelli GM, Schlais DE, Blaustein AR, Han BA (2012) Ultraviolet Radiation Influences Perch Selection by a Neotropical Poison-Dart Frog. *PLoS ONE* 7: 3–7.
- Kime NM, Turner WR, Ryan MJ (2000) The transmission of advertisement calls in Central American frogs. *Behav Ecol* 11: 71–83.
- Lardner B, bin Lakim M (2002) Tree-hole frog exploit resonance effects. *Nature* 420: 475–475.
- Lenth R (2016) Least-squares means: the R package lsmeans. *J Stat Softw* 69: 1–33.
- Lombard E (1911) Le signe de l’élévation de la voix. *Ann Maladies l’Oreille et du*

- Larynx 37: 101-119.
- Lopez PT, Narins P, Lewis ER, Moore SW (1988) Acoustically induced call modification in the white-lipped frog, *Leptodactylus albilabris*. *Anim Behav* 36: 1295-1308.
- Lötters S, Jungfer KH, Henkel F, Schmidt W (2007) Poison frogs: biology, species and captive husbandry. Frankfurt am Main, Germany: Edition Chimaira.
- Love EK, Bee MA (2010) An experimental test of noise-dependent voice amplitude regulation in Cope's grey treefrog, *Hyla chrysoscelis*. *Anim Behav* 80: 509-515.
- Maan ME, Cummings ME (2012) Poison frog colors are honest signals of toxicity, particularly for bird predators. *Am Nat* 179: E1-E14.
- Marten K, Marler P (1977) Sound transmission and its significance for animal vocalization. *Behav Ecol Sociobiol* 2: 271-290.
- Magrath RD, Haff TM, Horn AG, Leonard ML (2010) Calling in the face of danger: predation risk and acoustic communication by parent birds and their offspring. *Adv Study Behav* 41: 187-253.
- Master TL (1998) *Dendrobates auratus* (Black-and-Green Poison Dart Frog). Predation. *Dendrobates auratus* (rana venenosa negra y verde). Depredación. *Herpetol Rev* 29: 164-165.
- Montanarin A, Kaefer IL, Lima AP (2011) Courtship and mating behaviour of the brilliant-thighed frog *Allobates femoralis* from Central Amazonia: implications for the study of a species complex. *Ethol Ecol Evol* 23: 141-150.
- Morton ES (1975) Ecological sources of selection on avian sounds. *Am Nat* 109: 17-34.
- Morton ES (1982) Grading, discreteness, redundancy, and motivation-structural rules. In: Kroodsma DE, Miller EH, Ouellet H, editors. *Acoustic communication in birds: production, perception, and design features of sound*. New York, Academic Press P. 183-212.
- Naguib M (1997) Use of song amplitude for ranging in Carolina wrens, *Thryothorus ludovicianus*. *Ethology* 103: 723-731.
- Nakagawa S, Schielzeth H (2010) Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. *Biol Rev* 85: 935-956.
- Narins PM, Feng AS, Fay RR (2006) *Hearing and Sound Communication in Amphibians*. New York: Springer.
- Narins PM, Hödl W, Grabul DS (2003) Bimodal signal requisite for agonistic behavior in a dart-poison frog, *Epipedobates femoralis*. *Proc Natl Acad Sci USA* 100: 577-580.
- Nelson BS (2000) Avian dependence on sound pressure level as an auditory distance cue. *Anim Behav* 59: 57-67.
- Owings DH, Morton ES (1998) *Animal Vocal Communication: A New Approach*. Cambridge University Press.
- Parris KM (2002) More bang for your buck: The effect of caller position, habitat and chorus noise on the efficiency of calling in the spring peeper. *Ecol Model* 156: 213-224.
- Prestwich KN (1994) The energetics of acoustic signaling in anurans and insects. *Am Zool* 34: 625-643.
- Pröhl H (2003) Variation in Male Calling Behaviour and Relation to Male Mating Success in the Strawberry Poison Frog (*Dendrobates pumilio*). *Ethology* 109: 273-290.
- Pröhl H (2005) Territorial Behavior in Dendrobatid Frogs. *J Herpetol* 39: 354-365.
- Pyron RA, Wiens JJ (2011) A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. *Mol Phylogenet Evol* 61: 543-583.

- R Core Team (2017) R: A Language and Environment for Statistical Computing. Vienna, Austria. Retrieved from <https://www.r-project.org/>
- Richards DG (1981) Estimation of distance of singing conspecifics by the Carolina wren. *Auk* 98: 127–133.
- Ringler M, Hödl W, Ringler E (2015) Populations, pools, and peccaries: simulating the impact of ecosystem engineers on rainforest frogs. *Behav Ecol* 26: 340–349.
- Ringler E, Pašukonis A, Hödl W, Ringler M (2013) Tadpole transport logistics in a Neotropical poison frog: indications for strategic planning and adaptive plasticity in anuran parental care. *Front Zool* 10: 67.
- Ringler M, Mangione R, Pašukonis A, Rainer G, Gyimesi K, Felling J, Kronaus H, Réjou-Méchain M, Chave J, Reiter K, Ringler E (2014) High-resolution forest mapping for behavioural studies in the Nature Reserve “Les Nouragues”, French Guiana. *J Maps* 12: 26–32.
- Ringler M, Ringler E, Mendoza DM, Hödl W (2011) Intrusion experiments to measure territory size: development of the method, tests through simulations, and application in the frog *Allobates femoralis*. *PLoS One* 6: 10.
- Ringler M, Szípl G, Hödl W, Khil L, Kofler B, Lonauer M, Provin C, Ringler E (2017) Acoustic ranging in poison frogs—it is not about signal amplitude alone. *Behav Ecol Sociobiol* 71: 114.
- Ringler M, Ursprung E, Hödl W (2009) Site fidelity and patterns of short-and long-term movement in the brilliant-thighed poison frog *Allobates femoralis* (Aromobatidae). *Behav Ecol Sociobiol* 63: 1281–1293.
- Robertson JG (1984) Acoustic Spacing by Breeding Males of *Uperoleia rugosa* (Anura: Leptodactylidae). *Zeitschrift Für Tierpsychologie* 64: 283–297.
- Rodríguez C, Hödl W (2020) Sound radiation patter of the advertisement call of the highly territorial poison frog *Allobates femoralis*. *Behav Process* 170: 103996.
- Roithmair ME (1992) Territoriality and Male Mating Success in the Dart-poison Frog, *Epipedobates femoralis* (Dendrobatidae, Anura). *Ethology* 92: 331–343.
- Ruxton GD, Sherratt TN, Speed MP (2004) Avoiding attack: the evolutionary ecology of crypsis, warning signals and mimicry. Oxford University Press.
- Ryan MJ (1988) Energy, calling, and selection. *Am Zool* 28: 885–898.
- Saporito RA, Zuercher R, Roberts M, Gerow KG, Donnelly MA (2007) Experimental evidence for aposematism in the dendrobatid poison frog *Oophaga pumilio*. *Copeia* 2007: 1006–1011.
- Shen JX, Xu ZM (2016) The Lombard effect in male ultrasonic frogs: regulating antiphonal signal frequency and amplitude in noise. *Sci Rep* 6: 27103.
- Sol D, Lapiedra O, Gonzáles-Lagos C (2013) Behavioural adjustments for a life in the city. *Anim Behav* 85: 1101–1112.
- Sorjonen J (1986) Factors Affecting the Structure of Song and the Singing Behaviour of Some Northern European Passerine Birds. *Behaviour* 98: 286–304.
- Schwartz JJ, Hunce R, Brandon L, Powers K (2015) Calling site choice and its impact on call degradation and call attractiveness in the gray tree frog, *Hyla versicolor*. *Behav Ecol Sociobiol* 70: 1–19.
- Stückler S, Ringler M, Pašukonis A, Weinlein S, Hödl W, Ringler E (2019) Spatio-Temporal Characteristics of the Prolonged Courtship in Brilliant-Thighed Poison Frogs, *Allobates femoralis*. *Herpetologica* 75: 268–279.
- Toledo LF, Martins IA, Bruschi DP, Passos MA, Alexandre C, Haddad CF (2014) The anuran calling repertoire in the light of social context. *Acta Ethol* 18: 87–99.

- Tumulty JP, Pašukonis A, Ringler M, Forester JD, Hödl W, Bee MA (2018) Brilliant-thighed poison frogs do not use acoustic identity information to treat territorial neighbours as dear enemies. *Anim Behav* 141: 203–220.
- Tuttle MD, Ryan MJ (1981) Bat predation and the evolution of frog vocalizations in the Neotropics. *Science* 214: 677–678.
- Walker TJ, Forrest TG (1989) Mole cricket phonotaxis: effects of intensity of synthetic calling song (Orthoptera, Gryllotalpidae, *Scapteriscus acletus*). *Fla Entomol* 72: 655–659.
- Wells KD (2007) *The Ecology and Behavior of Amphibians*. University of Chicago Press.
- Wells KD, Schwartz JJ (1982) The effect of vegetation on the propagation of calls in the Neotropical frog *Centrolenella fleischmanni*. *Herpetologica* 38: 449–55.
- Wells KD, Schwartz JJ (2007) The behavioral ecology of anuran communication. In: *Hearing and sound communication in amphibians*. Springer.
- Wilczynski W, Brenowitz EA (1988) Acoustic cues mediate inter-male spacing in a neotropical frog. *Anim Behav* 36: 1054–1063.
- Wiley RH, Richards, DG (1982) Adaptations for acoustic communication in birds. In: Kroodsma DE, Miller EH (ed) *Sound transmission and signal detection*, Academic Press, New York pp 131–181.
- Zeileis A, Hothorn T (2002) Diagnostic checking in regression relationships. *R News* 2: 7–10.
- Zhao L, Sun X, Chen Q, Yang Y, Wang J, Ran J, Brauth S, Tang Y, Cui J (2018) Males increase call frequency, not intensity, in response to noise, revealing no Lombard effect in the little torrent frog. *Ecol Evol* 8 : 11733–11741.

Chapter 3. Behavioural responses to hot and dry weather during the breeding season in a Neotropical poison frog

This chapter has been submitted in the peer-reviewed *Journal of Thermal Biology*

Citation:

Rodríguez, C., Arcila-Pérez, L.F., Deventer, S.A., Fischer, M-T., Hödl, W., Raboisson, G., Serrano-Rojas, S.J., and Pašukonis, A. (submitted). Behavioural responses to hot and dry weather during the breeding season in a Neotropical poison frog.

Behavioural responses to hot and dry weather during the breeding season in a Neotropical poison frog

Camilo Rodríguez^{1*}, Luisa F. Arcila-Pérez², Sarah A. Deventer¹, Marie-Therese Fischer³, Walter Hödl¹, Gaëlle Raboisson¹, Shirley Jennifer Serrano-Rojas³, and Andrius Pašukonis^{3, 4}

¹ Department of Behavioral and Cognitive Biology, University of Vienna, Djerassiplatz 1, 1030, Vienna – Austria

² Programa de Biología, Universidad del Quindío, Colombia.

³ Department of Biology, Stanford University.

⁴ CEFE, Univ Montpellier, CNRS, EPHE, IRD, Montpellier, France.

* Corresponding author. Email: camilo.rodriguez@univie.ac.at

Abstract

Currently, global warming is perhaps one of the biggest anthropogenic threats to the maintenance of life on earth. Diurnal amphibians appear to be particularly susceptible to changes in environmental temperature as they must balance the trade-off between activity in hot conditions and associated water loss through their permeable skin. By using a passive telemetry technique, we studied the behavioural responses of *Allobates femoralis*, a small territorial and diurnal rainforest frog, to a natural hot and dry interruption during its normally rainy breeding season. Our results suggest that *A. femoralis* mitigate the water loss and high temperatures by actively choosing the substrates for temperature conduction from the substrate to the body (i.e., thigmothermy) and the reduction of mating and parental activities. In addition, we describe a new behaviour only observed during dry and hot conditions and allegedly linked to water absorption. We discuss our results in relation to the costs of these behavioural adaptations on fitness.

Keywords: Thigmothermy. Behavioural thermoregulation. *Allobates femoralis*. Water absorption response. Short dry period.

Introduction

Global ambient temperatures have been increasing dramatically over the last decades, having a tremendous negative impact on ecosystems' function and therefore, on the maintenance of life on earth (Colwell et al., 2008; Moritz and Agudo, 2013). Particularly, the thermal ecology of Neotropical organisms has drawn the attention of ecologists, because temperatures in lowland tropics are currently close to lethal levels for some species (Deutsch et al., 2008; Foden et al., 2013; von May et al., 2019). Ectothermic organisms are mostly affected by changes in ambient temperatures because they use external heat sources to regulate their body temperature for its metabolic functions (Beltrán et al., 2021; Kingsolver et al., 2013). In response to climatic changes, ectotherms have developed physiological adaptations or thermoregulatory behaviours allowing to maintain body temperature within a narrower range than ambient temperature (Hutchison and Dupré, 1992). For instance, small amphibians and reptiles bask in the sun to gain heat and increase their metabolic rate. Once the temperature is above the optimal, they seek for shady areas to cool down (Lillywhite, 1971; Wells, 2007). Other species take advantage of a broader range of temperatures, offered by different substrates in its microhabitat, to gain or lose body temperature by conduction (i.e., thermophilic or thermophobic thigmothermy; Hillman et al., 2009).

Because amphibian skin is sensitive to water loss due to high temperatures, thermoregulation is often compromised by needing to maintain water balance (Lillywhite, 2006). While a few species have developed physiological mechanisms, including arginine-vasotocin regulation, skin resistance and secretion of lipids on the skin, many others use behavioural adaptations as means to prevent dehydration while thermoregulating (Reviewed by Toledo and Jared, 1993). For instance, many amphibians are associated to aquatic habitats such as creeks or swamps, so they have available water to quickly rehydrate when it is too hot (Wells, 2007). Other species from terrestrial and/or arid habitats can absorb water from wet soils by increasing the contact area of the highly vascularized ventral skin (i.e., the water absorption response; Brekke et al., 1991; Dole, 1967; Hillyard et al., 1998; Johnson, 1969; Lillywhite et al., 1997; Lillywhite and Licht, 1974; McClanahan and Baldwin, 1969; Seymour, 1972; Stille, 1958).

Both, physiological and behavioural adaptations, have allowed amphibians to successfully colonize diverse ecosystems regardless of its environmental conditions (Navas, 2006). However,

the interplay between thermo- and hydro-regulation appears to be particularly important in diurnal species, as they are active during periods of peak ambient temperatures (Tracy et al., 2013). For instance, Neotropical poison frogs (Dendrobatidae and Aromobatidae) are diverse families of mostly diurnal frogs that show some of the most complex social behaviors known among amphibians (Wells, 2007). Most territorial males seek for open (i.e., with less groundcover vegetation) and elevated places in the forest understory (e.g., fallen branches, logs, piles of leaf-litter), from which they perform visual and acoustic displays to repel conspecific rivals and to attract females during the day (Pröhl, 2005). Therefore, dendrobatids can get exposed to hot conditions when advertising for territoriality and reproduction. Despite the evident trade-off between thermal and water-balance in Neotropical poison frogs, only a few studies have described their thermal biology and most of them have been conducted during the rainy season when temperatures are lower and the humidity is higher (Navas, 1997, 1996a, 1996b; Rivera-Ordonez et al., 2019).

Adaptations to thermal environments in Neotropical poison frogs vary according to the temporal changes in ambient temperature and the altitudinal distribution of the species (Navas, 1996a, 1996b). For instance, high-elevation *Colostethus* experience dramatic changes of ambient temperature between day and nighttime, which cannot be compensated simply with behavioural adjustments. Consequently, they increase their aerobic capacity to enhance activity at cold temperatures (Navas, 1997). On the other hand, lowland *Colostethus* undergo small changes in daily temperatures that can be balanced with behavioural modifications, such as the reduction of vocal behaviour when ambient temperature is above the optimum (Navas, 1996b). However, lowland Neotropical frogs can experience severe intraseasonal variations in climatic conditions as product of the dynamics of the global wind systems (Marengo et al., 1997). For instance, in equatorial forests at the Northern-hemisphere, the rainy season is interrupted by a short hot and dry period produced by the oscillations in the Intertropical Convergence Zone (Grimaldi and Riéra, 2011). This period varies in length and strength among years, with increments in ambient temperature and rainfall cessation, which causes the decrease of reproductive activity in many amphibian species (Grimaldi and Riéra, 2011; Born & Gaucher, 2001). Even so, to the best of our knowledge, no studies have evaluated how Neotropical poison frogs respond to this short climatic disruption during the core rainy season, during which reproduction occurs.

Here we describe the behavioural responses of the Brilliant-thighed poison frog, *Allobates femoralis* (Boulenger, 1883), to a short dry interruption in its reproductive season. As in other dendrobatids, the reproductive season of *A. femoralis* matches with the local rainy season (Kaefer et al., 2012), when males are strongly territorial and display multiple behaviours to attract females and increase their reproductive success (Roithmair, 1992; 1994; Ringler et al., 2009a; Ursprung et al., 2011). To investigate how *A. femoralis* respond to a hot and dry interruption in the reproductive season, we monitored body temperatures, microhabitat use and behaviours during the short dry period, by using a passive telemetry technique. We further compared the effect of climate (ambient temperature and precipitation) on movements and calling activity between the short dry period in 2020 and data collected previously in the rainy seasons of 2016, 2018 and 2019, in the nature reserve 'Les Nouragues' - French Guiana. Finally, we describe a new behaviour only observed during dry periods and discussed its relationship with the "water absorption response".

Methods

Study species and site

We conducted our research on the Brilliant-thighed poison frog, *Allobates femoralis* (Boulenger, 1883), in a population located at the Nouragues Ecological Research Station 'Pararé' (NERSP) in French Guiana (4°02' N – 52°41' W; Bongers et al., 2013). The breeding season of *A. femoralis* in French Guiana lasts from mid-December to mid-June, matching with the local rainy season. Mean ambient temperature during the rainy season oscillates around 25 °C, with up to 407 mm of monthly mean rainfall in May. As we mention before, the rainy season in French Guiana is interrupted by a short-dry period between February and April, as a result of oscillations in the Inter-tropical convergence zone while the main dry season takes place between August and November. Mean ambient temperatures for the dry season oscillates around 27 °C, with 89 mm of monthly mean rainfall in September (Grimaldi and Riéra, 2011).

The daily activity time of *A. femoralis* occurs between 06h30 and 19h00. During the reproductive season, males are strongly territorial and spend most of the day perching on elevated forest-floor structures, calling and remaining conspicuous to repel conspecific males and

to attract females (Roithmair, 1992). Although females are not territorial, they also remain visible for calling males (Ringler et al., 2009b). Acoustic interactions between males occur mainly in the afternoon and after rainfall (Kaefer et al. 2012). When males face a territorial intrusion, they quickly jump towards the intruder to expel it from the territory (Ringler et al., 2011). Courtship behaviour involves a dynamic locomotor performance, in which the male conducts the female towards the oviposition site (i.e., Courtship march, sensu Montanarin et al., 2011; Stücker et al., 2019). Parental care is provided mainly by males (but see Ringler et al., 2015) in form of tadpole transport. This usually occurs early in the morning and after rainfall, when males “pick-up” the tadpoles on their backs and shuttle them to pools outside their territory (Ringler et al., 2013) where tadpoles develop. Males can travel over a hundred meters when shuttling tadpoles to the pools and they tend to move more in lower temperatures (Beck et al. 2017). *Allobates femoralis* is a ‘sit-and-wait’ predator and eats preys throughout the day, without moving long distances (Toft, 1980).

Thermal ecology of A. femoralis during the short dry period

During the short dry period between the 2nd and the 14th of March of 2020, a total of 12 adults (6 males and 6 females) of *A. femoralis* were followed for behavioural observations and thermal measurements. In order to easily localize the focal frogs, we used harmonic direction finding, a passive telemetry technique successfully used in previous ecological studies in *A. femoralis* (Beck et al., 2017; Fischer et al., 2020; Pašukonis et al., 2014). Each captured frog was photographed for identification, measured, weighted, and tagged with a miniature transporter (~0.1 g and <5% of frog body weight), which consists of a silicone-sealed diode soldered to a thin flexible ~12 cm antenna, which reflects the radio signal sent by a hand-held harmonic direction finder (RECCO R8, Recco AB, Lindigö, Sweden) giving a directional signal when in proximity of the frog. The transponders were fitted with a thin silicone tubing (~1 mm) around the waist with an additional strap between the legs to prevent tag rotation. Handling and tagging usually took less than 5 minutes and every frog was released at its capture position after tagging. Frogs were tracked, observed and measured during the entire study period, every day from 06h30 to 19h30 and at intervals of 2h, for a total of 7 observations per frog, per day. Once localized, frogs’ position was recorded on a high-resolution digital GIS map of the study area (Ringler et al., 2016), using tablets (Vanquisher SV-86 rugged Windows tablet, Sinicvision Technology Co., Ltd., Shenzhen, China) and the software ArcPad (ESRI, Redlands, CA, USA). For a better accuracy, distance and direction measurements from a fixed reference point were taken using laser range finders (GLM

40, Bosch, Gerlingen Germany) and precision compasses (Suunto KB20, Vantaa, Finland), respectively. To estimate the activity pattern of *A. femoralis*, we observed every focal frog for five minutes from about 2 m distance and recorded 5 behavioural variables, namely # of head-body orientations (HBO), # of jumps, # of calls (advertisement and courtship) and # feeding events. Then, we recorded body temperatures using an infrared thermometer (Votcraft IR-SCAN-350RH/2, Wollerau, Switzerland; Basic accuracy: $\pm 1.5\%$ + $2\text{ }^{\circ}\text{C}$), cautiously pointed at the back of the frog from $\sim 10\text{ cm}$. We also measured the temperature of the substrate where the frog was perching and the temperature of the adjacent common substrates. For this, we distinguished 4 types of substrates where the frogs were commonly perching, namely "earth", "logs", "leaves" and "under leaves". We also recorded ambient temperature and relative humidity at $\sim 20\text{ cm}$ height from the forest understory with the built-in temperature sensor of the infrared thermometer. All temperatures were measured three times and averaged for subsequent analyses. We used the frog locations recorded at each temperature and behaviour sampling (seven points per day) to calculate cumulative daily distances moved. If there was any movement observed between the last point of one day and the first point on the next day, this movement was attributed to the next day. We continued tracking some individuals during the short dry period at an irregular sampling rate for several days after detailed behavioural observations. We filtered these data for days where at least four locations of the frog were recorded and included these data in the analysis of the ambient temperature and rainfall influence on daily cumulative distance.

5-year climate at NERSP and *A. femoralis* activity patterns

In order to describe the climate at NERSP between 2016 and 2020, we extracted daily-mean ambient temperature and precipitation from meteorological data provided by NERSP from an above-canopy weather station (Nouraflex: rainfall sensor Campbell ARG100, Campbell Scientific Europe, Loughborough, UK; temperature sensor Vaisala HUMICAP® HMP155, Vaisala Oyj, Vantaa, Finland). Because data from the 18th of March to the 30th of June of 2020 was missing from the weather station at NERSP, we complemented this missing data from another meteorological station at the Nouragues Ecological Station "Inselberg", approximately at 5 km away from the study site. We further compared the influence of ambient temperature and precipitation on movement and calling activity of *A. femoralis*, between the short dry period of 2020 and the rainy season from previous years. For this, we extracted data of the daily cumulative

distance moved by *A. femoralis* from two previous studies conducted during the rainy season in 2016 (Fischer et al., 2020) and 2018 (A. Pašukonis & M. -T. Fischer, unpublished data), and of males' calling activity during the rainy season in 2019 from Rodríguez et al. (2020b). To make the movement data comparable, we down sampled the previously collected data to fit the spatial sampling rate of the current study (~ 4 - 6 point per frog per day). In the same way, we extracted ambient temperature from temperature-data loggers (HOBO U23 Pro v2 Temperature/Relative Humidity Data Logger, Bourne, MA, USA), that were attached to ~20 cm long plastic sticks and placed at the forest understory, during every study.

Statistical analyses

We log-transformed behavioural and climate variables to fit normality, when necessary. In order to compare ambient temperature above-canopy and precipitation during the core reproductive period of *A. femoralis* in NERSP (from January to June) over the last 5 years (2016-2020), we performed two separate ANOVA using either the average of daily ambient temperatures above-canopy or precipitation as the response, and years as independent factor with 5 levels (2016-2020). We used planned least-squares means contrasts between years. Post hoc comparisons were adjusted by Tukey's method for multiple comparisons. In order to compare temperatures between substrates, we first calculated the difference between understory and substrate temperature (delta temperature). This value indicates by how much substrate is cooler than understory temperature. Then, we performed an ANOVA with substrate type as predictor with 4 levels (earth, log, leaves and under leaves) and delta temperature as the response. We used planned least-squares means contrasts between substrates. Post hoc comparisons were adjusted using Tukey's method for multiple comparisons. In order to investigate the substrate used by *A. femoralis*, throughout the day and during the short dry period, we fitted a Generalized Linear Mixed-Effects Model (GLMER) using the "glmer" function within the R package lme4 (Bates et al., 2015). For this, we used substrate type as response variable (represented by a dummy ordinal variable), understory temperature and time of the day (in radians) as fixed factors, and the ID of the frog as random factor. In order to determine association between substrate use, activity patterns and climatic conditions in *A. femoralis*, during the short dry period, we performed a Hierarchical Clustering for Principal Components (HCPC) based on a Multiple Correspondence Analysis (MCA). MCA is a principal component method that explores associations between qualitative variables, based on a similarity matrix from an Euclidean space, creating continuous

principal components (Lê et al., 2008). These components are finally clustered using HCPC based on a distance and agglomeration criterion (Husson et al., 2011). For this analysis we used four variables as input: behaviours performed during the observation, substrate, understory temperature and relative humidity. In order to reduce redundancy between behaviours, we merged # of HBO and Jumps to create a new variable describing "short-movements" and, also, we merged advertisement calls and courtship calls in one variable describing "calling behaviour". In order to account for non-linear relationships between the variables (Husson et al., 2011), we cut ambient temperature and relative humidity into intervals, with equal width (4 categories each). For the analyses we used the functions "MCA" and "HCPC" within the R package FactoMineR (Lê et al., 2008). We performed the MCA keeping the first 7 components, because they explained more than the 80% of cumulative percentage of variance. For the HCPC we kept 5 clusters (by default). In order to compare the influence of ambient temperature and precipitation on the activity patterns of *A. femoralis* between the rainy season and the short dry period, we performed two linear mixed-effects models (LMMs) using the "lmer" function within the R package lme4 (Bates et al., 2015), using daily cumulative distance or calling activity as response variable, respectively. For both models, we used ambient temperature and precipitation as fixed predictors, sex as a fixed factor, and the ID of the frogs as random factor. Finally, we compared daily cumulative distance between sexes and among years using an ANOVA followed by post hoc comparisons between years and sexes with planned least-square means.

Permits and ethical Statement

The experiments were conducted in strict accordance with the European, French and USA laws and following the 'Guidelines for use of live amphibians and reptiles in the field and laboratory research' by the Herpetological Animal Care and Use Committee (HACC) of the American Society of Ichthyologists and Herpetologists (Beaupre et al., 2004) and the Association for the Study of Animal Behaviour (ASAB) "Guidelines for the treatment of animals in behavioral research and teaching" (Vitale et al., 2018). The experimental procedures were approved by the scientific committee of the Nouragues Ecological Research Station and the Animal Ethics and Experimentation Board of the University of Vienna (No. 2020-001).

Results

5-year climate in NERSP, French Guiana

From 2016 to 2020, mean daily ambient temperatures above-canopy ranged from 22.34 °C to 29.51 °C (Figure 1A) at NERSP. On average, the daily ambient temperature ranged from 22.34 to 29.51 °C during the main dry season (~July-November), with 4.01 mm of cumulative precipitation on average/day/month (Figure 1A). The match in the ranges of mean daily temperatures above-canopy, between the years (2016-2020) and the dry seasons, indicates that the coldest and hottest days of the year occurred in the dry season. On the other hand, during the rainy season (~December-June), mean-daily ambient temperature above-canopy ranged from 22.47 to 27.32 °C, with 9.1 mm of cumulative precipitation on average/day/month (Figure 1A). During the first half of the year, the warmest month was March (where the short dry period takes place), with mean-daily ambient temperatures ranging from 23.52 °C to 26.94 and 5.13 mm of cumulative precipitation on average/day/month (Figure 1B; C). While ambient temperature was significantly higher during the first half of 2016, during 2018 it was significantly lower among the compared years (ANOVA: $F_{4,902}=26.99$, $p<0.001$; Figure 1B; Table A.1). Precipitation during the first half of 2019 was significantly lower compared to that of 2017 (ANOVA: $F_{4,902}=2.35$, $p=0.05$; Figure 1C; Table A.2).

Weather and body temperatures during the short dry period

For the tracking period in the short dry season, precipitation was almost absent, with just 2.42 mm of rainfall. Understory mean temperatures ranged through the day from 22.5 °C to 30.9 °C, peaking between 12.00 h and 16.00 h (Table A.3; Figure 2A). Understory relative humidity was negatively related with understory temperature ($F=367.9$, $R^2=0.53$, $P<0.001$), with the lowest percentages (<80%) also between 12.00 and 16.00 h (Table A.3; Figure 2A). Understory temperature was significantly higher compared to substrate (planned lsmeans contrast: $\beta = 2.49$, $t = 21.04$, $P < 0.001$; Fig. 2B) and body temperature (planned lsmeans contrast: $\beta = 2.58$, $t = 21.79$, $P < 0.001$; Fig. 2B). Substrate temperature better explained the variation in frogs' body temperature ($F=258.3$, $R^2=0.44$, $P<0.001$; Figure 2C), compared to ambient temperature ($F=278.9$, $R^2=0.46$, $P<0.001$; Figure 2D).

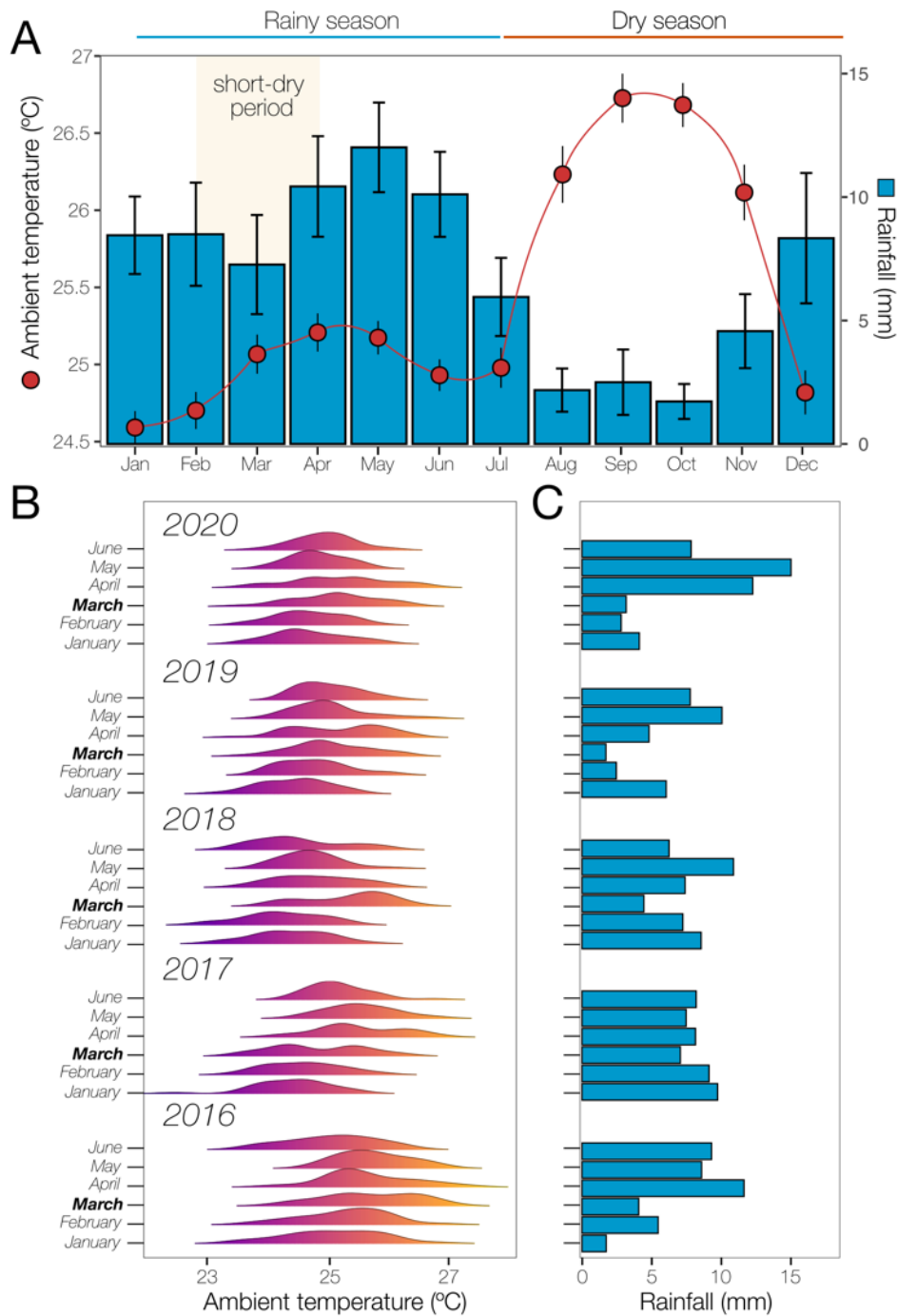


Figure 1. Temperature and precipitation trends at NERSP over 5 years (2016-2020). **A-** Cumulative temperatures and precipitation on average/day/month, from January to December, indicating a bimodal weather regime. **B-** Variation of Cumulative temperatures and **C-** precipitation on average/day/month, during the core breeding season of *Allobates femoralis*. March is highlighted as the month of the “short dry period”.

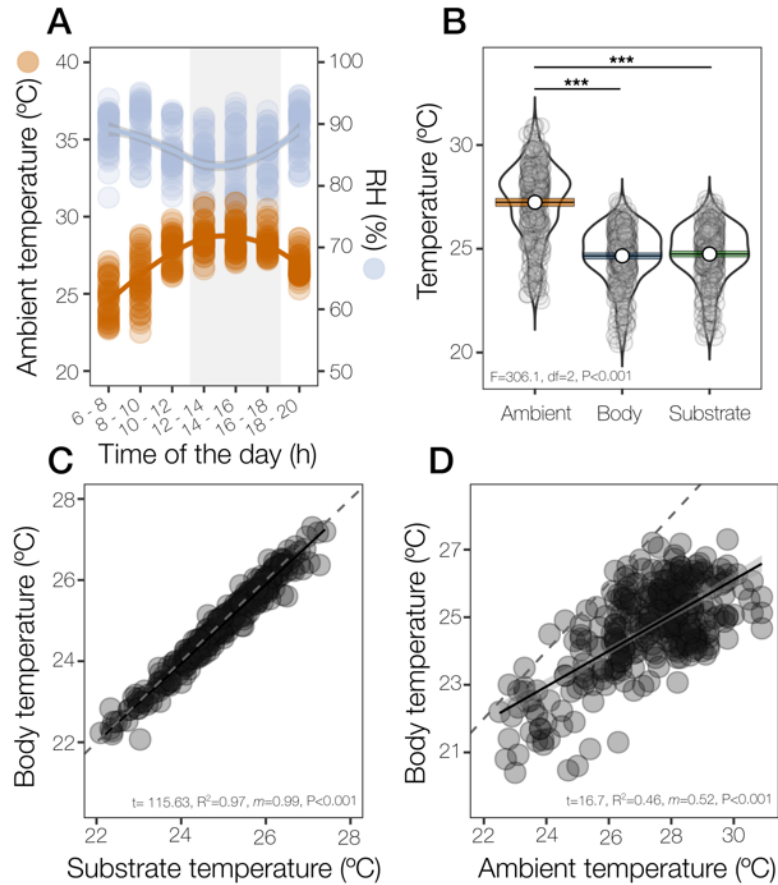


Figure 2. Thermal features of the short dry period, during the breeding season of *A. femoralis* at NERSP. **A-** Daily variation of ambient temperature and relative humidity. Grey-shaded vertical bar indicates the hottest and driest moment of the day. **B-** Violin plot showing differences between ambient, body and substrate temperatures. White dots denote means and horizontal coloured boxes 95% confidence intervals. Asterisks denote significance after least-square means comparison between substrates. **C-** Effect of ambient temperature on *A. femoralis* body temperature. **D-** Effect of substrate temperature on *A. femoralis* body temperature. Dashed lines indicates isotherm. *** $P < 0.001$.

Thermal patterns of commonly used substrates

Compared to understory temperature, earth was the coolest substrate followed by logs, leaves and under leaves (Table A.4; Figure 3A). Substrate use was negatively related with understory temperature (GLMER: $\beta = -0.09$, $z = -4.32$, $P < 0.001$; Figure 3B) but positively related with time of the day (GLMER: $\beta = 0.09$, $z = 2.52$, $P = 0.01$; Figure 3B). In other words, while in the hottest time of the day *A. femoralis* perched mostly on earth and logs (28.5 °C on average and between 12.00h and 16.00h), in the coolest time of the day they perched mostly on leaves or

were hiding under them (26.31 °C on average between 06.00h-10.00h and between 18.00-20.00h; Figure 3C).

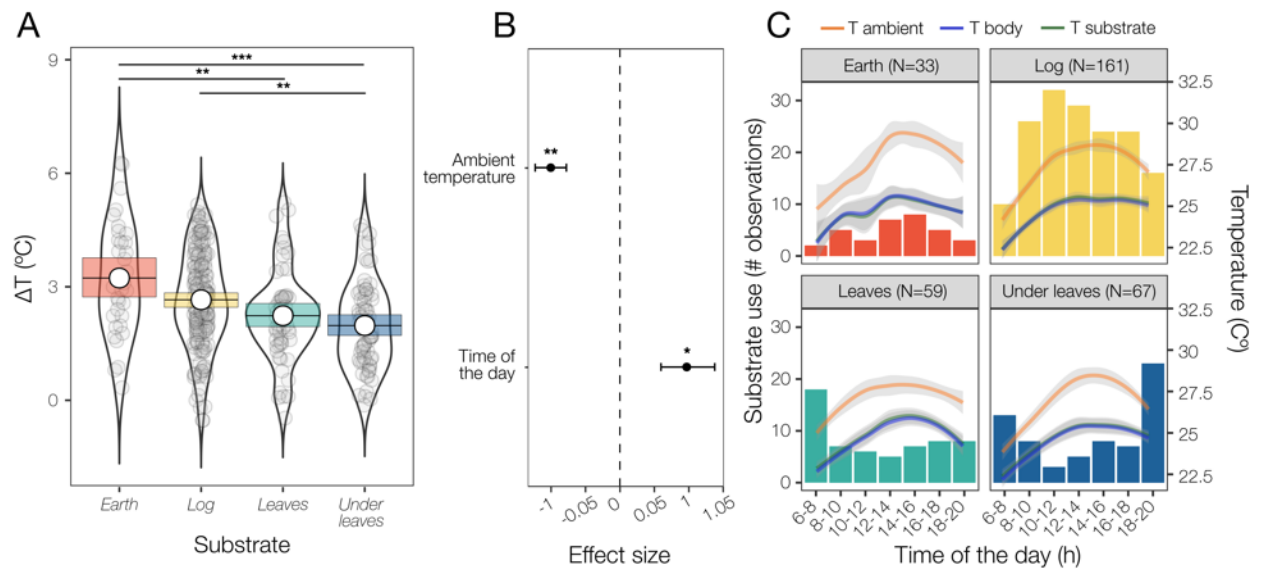


Figure 3. Effect of ambient temperature and time of the day on substrate use by *A. femoralis* in the short dry period at NERSP. **A-** Violin plots showing differences in delta T (ambient temperature - substrate temperature) between substrates. White dots denote means and horizontal coloured boxes 95% confidence intervals. Asterisks denote significance after least-square means comparison between substrates: * <0.05 , ** <0.01 . **B-** Effect sizes of ambient temperature and time of the day on substrate use. Black dots denote estimate value and black horizontal lines denote standard error. **C-** Frequency of substrates selected by frogs along the day, according to ambient, substrate and body temperature. Note that substrate and body lines are overlapped.

Substrate use and body temperatures

Besides the three categories of behaviours defined initially (i.e., calling, feeding and short movements), we here describe a new behaviour observed in *A. femoralis* during the short dry period. This behaviour consists of a repeated abduction of the hind limbs, accompanied by a rubbing of the ventral skin against the substrate (Figure 4, Video A.1). Every 'rubbing' event lasted in average for $1.26 \text{ sec} \pm 0.47 \text{ SD}$ (N=5). We thus also included this behaviour in the HCPC analysis.

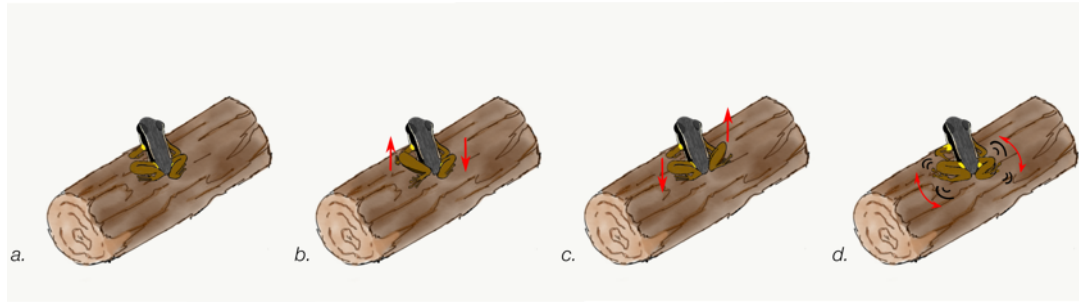


Figure 4. Drawing of the sequence of the “Rubbing” behaviour of *A. femoralis* on a log. **a-** Frog sitting on a log. **b-** Abduction & **c-** adduction of the hind limbs. **d-** Wiping of the pelvic patch surface.

Four clusters originated from the hierarchical clustering: Cluster 1 was characterized by individuals that hide under leaves, almost motionless, at the lowest range of understory temperature and between 83.2 and 90.5 % of humidity (Table A.5). Cluster 2 represented mainly males that perched on leaves for calling, when the understory temperature ranged between 26.2 and 27.6 °C, and relative humidity between 83.2 and 90.5 % (Table A.5). Cluster 3 was mainly grouping males and females, in the same proportion, that perched on logs for short movements, at the lowest range of understory temperature, and at the highest range of relative humidity (Table A.5). Similarly, Cluster 4 was characterized mainly by males that perched on logs and ‘rubbed’ their ventral-pelvic area against the log, when understory temperature ranged between 28.3 and 30.6 °C, and relative humidity between 83.2 and 90.5 % (Table A.5). Finally, in the cluster 5 individuals mainly perched on logs and on earth for feeding, when understory temperature ranged between 27.6 and 30.6 °C, and relative humidity between 72.1 and 83.2 % (Table A.5). Daily distance moved by *A. femoralis* in the short dry season ranged between 0 and 31 m (overall mean of daily distance moved $\pm$ SD = 7.53 m $\pm$ 6.74). Neither understory temperature (LMM: β = -0.97, t = -0.49, P = 0.62) nor precipitation (LMM: β = 0.38, t = 0.87, P = 0.38) was related to daily distance moved by *A. femoralis*, during the short dry period.

Activity patterns in the rainy season vs. the short dry period

Overall, *A. femoralis* individuals moved larger distances when understory temperature was low (LMM: β = -3.73, t = -1.97, P = 0.05; Figure 5A), regardless of rainfall (LMM: β = 0.05, t = 0.45, P = 0.65; Figure 5A). Particularly, males moved more during the rainy season than males during the short dry period (LMM: β = 8.36, t = 2.96, P < 0.01; Figure 5A; B; Table A.6). Interestingly, daily cumulative distance was not different in females between the rainy and the short dry period

(planned lsmeans contrast: $\beta = -1.16$, t -ratio = -1.16, $P = 0.22$; Fig. 5B; Table A.6). On the other hand, males had higher calling activity in rainy days compared to the short dry period (LMM: $\beta=0.37$, $t=2.31$, $P=0.03$ Figure 5C), regardless of the understory temperature (LMM: $\beta=0.33$, $t=1.96$, $P=0.06$ Figure 5C).

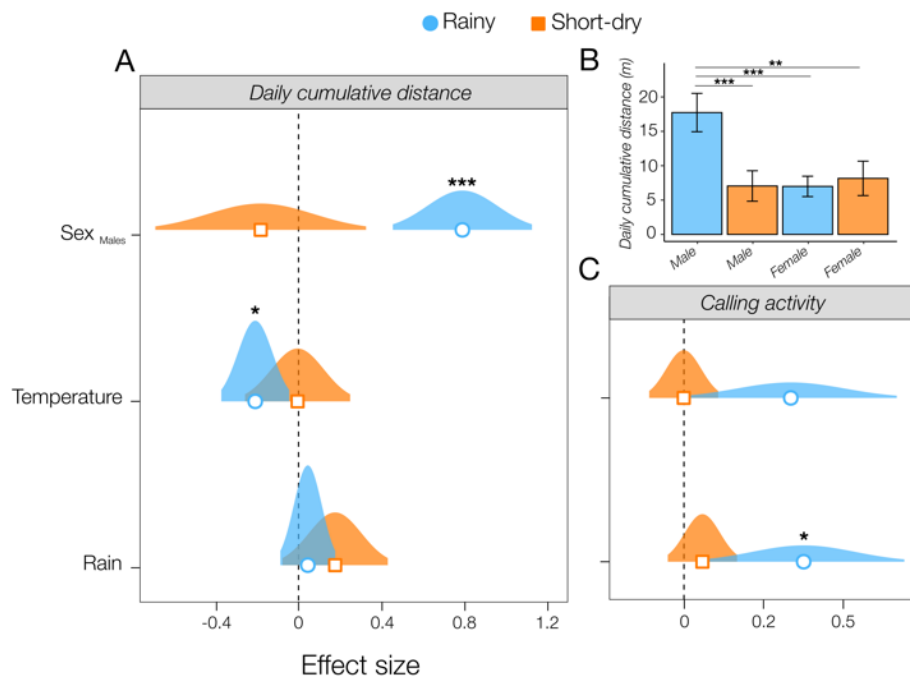


Figure 5. Activity patterns in the rainy season vs. the short dry period in *A. femoralis*. **A-** Effect sizes of sex, ambient temperature and rain on daily cumulative distance. **B-** Barplot showing differences in daily cumulative distance between sexes and years. Whiskers denote 95% confidence intervals. **C-** Effect sizes of ambient temperature and rain on calling activity. Normal distributions are shown representing the width of each estimate. Asterisks denote significance: * <0.05 , ** <0.01 , *** <0.001 .

Discussion

During the short dry period, body temperatures in *Allobates femoralis* resembled those of the substrates where they perched (Figure 2). In addition, they also buffered changes in ambient temperature during the day, by shifting substrates across a natural thermal gradient (Figure 3). This pattern may suggest that *A. femoralis* present thigmothermy (i.e., temperature conduction by the substrate) as a behavioural response to avoid thermal stress. Our results are in line with the general idea that lowland amphibians present behavioural rather than physiological adaptations to thermal stress (Feder, 1982; Navas, 1996a). According to the Bogert effect (Huey et al., 2003), a species ability for behavioural thermoregulation is likely to limit local physiological adaptations to tolerate high temperatures such as the Critical thermal maximum - CTMax (Rivera-Ordonez et al., 2019). However, previous evidence showed that *A. femoralis* present one of the highest CTMax (~40 °C), compared to that of other 50 sympatric anurans (von May et al., 2019). In addition, under a hypothetical 3°C increase in ambient temperature in lowland Amazon forests, *A. femoralis* would not experience temperatures close to critical limits (von May et al., 2019). Thus, the flexibility of moving along a thigmothermal gradient combined with a high CTMax might allow *A. femoralis* to cope with hot environmental conditions.

In our study, substrate use in *A. femoralis* was mainly influenced by ambient temperature and the time of the day. While individuals perched on earth and logs when ambient temperature peaked in the afternoon, they preferred to perch on leaves or under them when ambient temperature was low. However, beyond thigmothermy, substrate use can be also influenced by other aspects of the natural history of *A. femoralis*. For instance, in 50% of the observations in our study, *A. femoralis* perched on logs throughout the day even when ambient temperature was high. Although logs were the second coolest substrate, they are also among the highest perches on the forest ground and males take advantage of its height to improve the communication with conspecifics and reduce related energetic costs (Rodríguez et al., 2020). Likewise, females remain visible on small perches to look for potential mates (Fischer et al., 2020; Ringler et al., 2009b). Thus, *A. femoralis* might prioritize the use of logs for mating rather than thermoregulatory behaviours, even if logs are warmer than other available substrates. On the other hand, *A. femoralis* was frequently found on leaves early in the mornings. This substrate use might be rather linked to the 'sit-and-wait' foraging behaviour of *A. femoralis*, because they eat

prey on the leaf litter usually in the mornings (Toft, 1980). Indeed, we found that short movements related to foraging behaviour (e.g., head-body orientations, jumps) were mainly performed on the leaf litter. We also found *A. femoralis* individuals under leaves at the start and end of our sampling period (06h30 – 19h30), when ambient temperatures were low. Probably, these occurrences are likely to be product of the end of the activity and sleeping time of *A. femoralis*, an exclusively diurnal species. In sum, environmental conditions and life-history traits interact resulting in trade-off for the optimal substrate use in *A. femoralis*.

Besides its thermoregulatory function, the use of substrates in diurnal species has been also associated with the compromise between water and thermal balance (Navas, 1996a; Tracy et al., 2013). That is, substrate use could be also linked to the amount of moisture available for the frogs to uptake water, when compensating for evaporative cooling during hot days (Shoemaker et al., 1992; Wells, 2007). For instance, it is possible that the use of 'earth', the coolest substrate type in our study, could be determined by the need of the frogs for rehydration. The soil at our study site is of a clay type and retains water inside its porous structure (Bongers et al., 2013), so its water potential might be preferred by frogs among the other substrates available. Indeed, *A. femoralis* happen to perch mostly on 'earth' in the hottest and driest moments of the day (see Figure 3C). Moreover, we hypothesize that the 'rubbing' behaviour described earlier in our study, is putatively linked to the 'water absorption response', a behaviour associated with cutaneous water absorption in anurans (Stille, 1958). The movements of the 'rubbing' behaviour are very similar to those of the water absorption response described in other anurans (Brekke et al., 1991; Hillyard et al., 1998; Seymour, 1972; Stille, 1958), which consist in the abduction of the hind limbs followed by a wiping of the ventral surface of the pelvic patch against the substrate (Video A.1). The function of this behaviour is to expose the most contact area of the ventral pelvic region against the substrate, because the pelvic region is highly vascularized and specialized for water uptake from substrates (Shoemaker et al., 1992; Tracy et al., 2010). This behaviour may mediate the tradeoff between thermal and water stress in *A. femoralis* resulting from prolonged exposure on logs during hot and dry conditions. In fact, the 'rubbing' behaviour mainly occurred when *A. femoralis* was perching on logs. Experimental evidence of substrate selection in the laboratory combining a thigmothermal - with a moisture gradient is needed in order to further understand hydro- and thermoregulatory mechanisms used by *A. femoralis* during hot and dry conditions.

Beyond the use of substrates to maintain water and thermal balance during hot and dry conditions, amphibians can also limit the activity to moments when weather conditions are favorable (Wells, 2007). Indeed, we found that *A. femoralis* reduce its reproductive activity during hot and dry conditions. These results generally agree with those in Beck et al., (2017) and Fischer et al., (2020), who showed a negative effect of high temperatures on movements in males and females of the same population of *A. femoralis*. Kaefer et al., (2012) also showed that calling activity and reproductive behaviours are dependent on rainfall at a seasonal scale in a population of *A. femoralis* from central Amazonia. At least two physiological constraints can explain the reduction of reproductive activities in *A. femoralis* during hot and dry conditions. First, ambient temperatures above optimal body temperatures have a negative effect on the contractile capacity of hind-limb and trunk muscles, which are responsible for locomotion and calling behaviours, respectively (Rome et al., 1992). Second, small-sized frogs (<10 g) are likely to be affected by higher evaporative water-loss rates due to small skin resistant (Shoemaker et al., 1992; Tracy et al., 2010), thus the risk of dehydration might increase in *A. femoralis* with energetically expensive activities, like calling or moving long distances when rainfall is insufficient. However, when it comes to movement, the main difference between rainy and dry conditions in *A. femoralis* might be due to lack of tadpole transport (for males) and lack of courtship (for females) when it is dry (Beck et al., 2017; Fischer et al., 2020; Stücker et al., 2019). Thus, despite its benefits on water- and thermal balance, the reduction of movements and calling activity in the middle of the breeding season is likely trade-off with mating and parenting behaviours in *A. femoralis*. It is noteworthy that the first half of 2016 was the warmest period in the global earth surface during the last 137 years (NOAA, 2016). This anomaly in the global temperature might explain why daily movements in females were almost the same during rainy conditions in 2016 and dry conditions in 2020 (Figure 5B). Therefore, increases in the global temperature during the rainy season in tropical regions can significantly influence the reproductive behaviour of amphibians and have negative influence on fitness despite the presence of thermo- and hydroregulatory behaviours.

Conclusions

Overall, our results suggest that for *A. femoralis*, substrate selection, the reduction of activity and a behaviour allegedly linked to the water absorption response mediates thermal and water balance during hot and dry conditions. However, behavioural adaptations to extreme environmental conditions may conflict with life-history traits, such as mating, parenting or advertising, which might result in trade-off decisions. Further experimental studies on the operative temperatures to estimate the thermal environment of Neotropical poison frogs would contribute to the understanding on thermal and water relationships in obligate diurnal amphibian species.

Funding

This project was funded by the Austrian Science Fund (FWF), W1262-B29. A.P. was funded by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 835530. A.P., M.T.F. and S.J.S.R. were also supported by Lauren A. O'Connell with Stanford University and the National Science Foundation (IOS-1845651) funds. The meteorological data and logistic support were provided by the Nouragues Ecological Research Station (managed by CNRS), which benefits from the Investissement d'Avenir funds of the ANR (AnaEE France ANR-11-INBS-0001; Labex CEBA ANR-10-LABX-25-01).

Acknowledgements

This work is part of a partnership agreement between A. P. and the Nouragues Nature Reserve aimed at improving and spreading the knowledge about amphibians. We thank the staff of the Nouragues Nature Reserve for their commitment to preserving our natural world. We are grateful to the staff of the CNRS Nouragues Ecological Research Station for the logistic support and providing the meteorological data and to Max Ringler for the logistic support in the field.

References

- Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* 67, 1–48.
<https://doi.org/10.18637/jss.v067.i01>
- Beck, K.B., Loretto, M.-C., Ringler, M., Hödl, W., Pašukonis, A., 2017. Relying on known or exploring for new? Movement patterns and reproductive resource use in a tadpole-transporting frog. *PeerJ* 5, e3745.
<https://doi.org/10.7717/peerj.3745>
- Beltrán, I., Herculano-Houzel, S., Sinervo, B., Whiting, M.J., 2021. Are ectotherm brains vulnerable to global warming? *Trends Ecol. Evol.* 36, 691–699.
<https://doi.org/10.1016/j.tree.2021.04.009>
- Bongers, F., Charles-Dominique, P., Forget, P.-M., Théry, M., 2013. Nouragues: dynamics and plant-animal interactions in a neotropical rainforest. Springer Science & Business Media.
- Boulenger, G.A., 1883. On a collection of frogs from Yurimaguas, Huallaga River, northern Peru, in: *Proceedings of the Zoological Society of London*. Wiley Online Library, pp. 635–638.
- Brekke, D.R., Hillyard, S.D., Winokur, R.M., 1991. Behavior Associated with the Water Absorption Response by the Toad, *Bufo punctatus*. *Copeia* 1991, 393. <https://doi.org/10.2307/1446588>
- Colwell, R.K., Brehm, G., Cardelus, C.L., Gilman, A.C., Longino, J.T., 2008. Global Warming, Elevational Range Shifts, and Lowland Biotic Attrition in the Wet Tropics. *Science* 322, 258–261.
<https://doi.org/10.1126/science.1162547>
- Deutsch, C.A., Tewksbury, J.J., Huey, R.B., Sheldon, K.S., Ghalambor, C.K., Haak, D.C., Martin, P.R., 2008. Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci.* 105, 6668.
<https://doi.org/10.1073/pnas.0709472105>
- Dole, J.W., 1967. The Role of Substrate Moisture and Dew in the Water Economy of Leopard Frogs, *Rana pipiens*. *Copeia* 1967, 141.
<https://doi.org/10.2307/1442187>
- Feder, M.E., 1982. Environmental variability and thermal acclimation of metabolism in tropical anurans. *J. Therm. Biol.* 7, 23–28. [https://doi.org/10.1016/0306-4565\(82\)90015-8](https://doi.org/10.1016/0306-4565(82)90015-8)
- Fischer, M.-T., Ringler, M., Ringler, E., Pašukonis, A., 2020. Reproductive behavior drives female space use in a sedentary Neotropical frog. *PeerJ* 8, e8920.
<https://doi.org/10.7717/peerj.8920>
- Foden, W.B., Butchart, S.H.M., Stuart, S.N., Vié, J.-C., Akçakaya, H.R., Angulo, A., DeVantier, L.M., Gutsche, A., Turak, E., Cao, L., Donner, S.D., Katariya, V., Bernard, R., Holland, R.A., Hughes, A.F., O'Hanlon, S.E., Garnett, S.T., Şekercioğlu, Ç.H., Mace, G.M., 2013. Identifying the World's Most Climate Change Vulnerable Species: A Systematic Trait-Based Assessment of all Birds, Amphibians and Corals. *PLoS ONE* 8, e65427.
<https://doi.org/10.1371/journal.pone.0065427>
- Gottsberger, G., 2017. Canopy Operation Permanent Access System: a novel tool for working in the canopy of tropical forests: history, development, technology and perspectives. *Trees* 31, 791–812.
<https://doi.org/10.1007/s00468-016-1515-1>
- Grimaldi, M., Riéra, B., 2001. Geography and climate. In: Bongers F, Charles-Dominique P, Forget P, Théry M (eds)

- Nouragues. Dynamics and plant–animal interactions in a neotropical rainforest. Kluwer Academic, Dordrecht, pp 9–18
- Hillman, S.S., Withers, P., Drewes, R.C., Hillyard, S.D., 2009. Ecological and environmental physiology of amphibians. Oxford University Press.
- Hillyard, S.D., Hoff, K. von S., Propper, C., 1998. The Water Absorption Response: A Behavioral Assay for Physiological Processes in Terrestrial Amphibians. *Physiol. Zool.* 71, 127–138. <https://doi.org/10.1086/515900>
- Huey, R.B., Hertz, P.E., Sinervo, B., 2003. Behavioral Drive versus Behavioral Inertia in Evolution: A Null Model Approach. *Am. Nat.* 161, 357–366. <https://doi.org/10.1086/346135>
- Husson, F., Lê, S., Pagès, J., 2011. Exploratory multivariate analysis by example using R, Chapman & Hall/CRC computer science and data analysis. CRC Press, Boca Raton.
- Hutchison, V.H., Dupré, R.K., 1992. Thermoregulation. *Environ. Physiol. Amphib.* 206–249.
- Johnson, C.R., 1969. Water Absorption Response of Some Australian Anurans. *Herpetologica* 25, 171–172.
- Kaefer, I.L., Montanarin, A., Da Costa, R.S., Lima, A.P., 2012. Temporal patterns of reproductive activity and site attachment of the brilliant-thighed frog *Allobates femoralis* from central Amazonia. *J. Herpetol.* 46, 549–554.
- Kingsolver, J.G., Diamond, S.E., Buckley, L.B., 2013. Heat stress and the fitness consequences of climate change for terrestrial ectotherms. *Funct. Ecol.* 27, 1415–1423. <https://doi.org/10.1111/1365-2435.12145>
- Lê, S., Josse, J., Husson, F., 2008. FactoMineR : An R Package for Multivariate Analysis. *J. Stat. Softw.* 25. <https://doi.org/10.18637/jss.v025.i01>
- Lillywhite, H.B., 2006. Water relations of tetrapod integument. *J. Exp. Biol.* 209, 202–226. <https://doi.org/10.1242/jeb.02007>
- Lillywhite, H.B., 1971. Temperature selection by the bullfrog, *Rana catesbeiana*. *Comp. Biochem. Physiol. A Physiol.* 40, 213–227. [https://doi.org/10.1016/0300-9629\(71\)90162-9](https://doi.org/10.1016/0300-9629(71)90162-9)
- Lillywhite, H.B., Licht, P., 1974. Movement of Water over Toad Skin: Functional Role of Epidermal Sculpturing. *Copeia* 1974, 165. <https://doi.org/10.2307/1443019>
- Lillywhite, H.B., Mittal, A.K., Garg, T.K., Agrawal, N., 1997. Wiping Behavior and Its Ecophysiological Significance in the Indian Tree Frog *Polypedates maculatus*. *Copeia* 1997, 88. <https://doi.org/10.2307/1447843>
- Marengo, J.A., Nobre, C.A., Culf, A.D., 1997. Climatic Impacts of “Friagens” in Forested and Deforested Areas of the Amazon Basin. *J. Appl. Meteorol.* 36, 14.
- Born, M., Gaucher, P., 2001. Amphibian and reptile species at the Nouragues Nature Reserve. In: Bongers F, Charles-Dominique P, Forget P, Théry M (eds) Nouragues. Dynamics and plant–animal interactions in a neotropical rainforest. Kluwer Academic, Dordrecht, pp 9–18
- McClanahan, L., Baldwin, R., 1969. Rate of water uptake through the integument of the desert toad, *Bufo punctatus*. *Comp. Biochem. Physiol.* 28, 381–389. [https://doi.org/10.1016/0010-406X\(69\)91351-6](https://doi.org/10.1016/0010-406X(69)91351-6)
- Montanarin, A., Kaefer, I.L., Lima, A.P., 2011. Courtship and mating behaviour of the brilliant-thighed frog *Allobates femoralis* from Central Amazonia: implications for the study of a species complex. *Ethol. Ecol. Evol.* 23, 141–150.
- Moritz, C., Agudo, R., 2013. The Future of Species Under Climate Change:

- Resilience or Decline? *Science* 341, 504–508.
<https://doi.org/10.1126/science.1237190>
- Myers, C.W., Paolillo, A., Daly, J.W., n.d. Discovery of a Defensively Malodorous and Nocturnal Frog in the Family Dendrobatidae: Phylogenetic Significance of a New Genus and Species from the Venezuelan Andes. *Am. Mus. Novit.* 36.
- Navas, C.A., 2006. Patterns of distribution of anurans in high Andean tropical elevations: Insights from integrating biogeography and evolutionary physiology. *Integr. Comp. Biol.* 46, 82–91. <https://doi.org/10.1093/icb/icj001>
- Navas, C.A., 1997. Thermal extremes at high elevations in the Andes: Physiological ecology of frogs. *J. Therm. Biol.* 22, 467–477.
[https://doi.org/10.1016/S0306-4565\(97\)00065-X](https://doi.org/10.1016/S0306-4565(97)00065-X)
- Navas, C.A., 1996a. Implications of microhabitat selection and patterns of activity on the thermal ecology of high elevation neotropical anurans. *Oecologia* 108, 617–626.
<https://doi.org/10.1007/BF00329034>
- Navas, C.A., 1996b. The Effect of Temperature on the Vocal Activity of Tropical Anurans: A Comparison of High and Low-Elevation Species. *J. Herpetol.* 30, 488.
<https://doi.org/10.2307/1565691>
- NOAA National Centers for Environmental Information, State of the Climate: Global Climate Report for June 2016, published online July 2016, retrieved on June 12, 2021
 from <https://www.ncdc.noaa.gov/sotc/global/201606>.
- Pašukonis, A., Loretto, M.-C., Landler, L., Ringler, M., Hödl, W., 2014. Homing trajectories and initial orientation in a Neotropical territorial frog, *Allobates femoralis* (Dendrobatidae). *Front. Zool.* 11, 29. <https://doi.org/10.1186/1742-9994-11-29>
- Pröhl, H., 2005. Territorial Behavior in Dendrobatid Frogs. *J. Herpetol.* 39, 354–365.
- Ringler, E., Pašukonis, A., Fitch, W.T., Huber, L., Hödl, W., Ringler, M., 2015. Flexible compensation of uniparental care: female poison frogs take over when males disappear. *Behav. Ecol.* 26, 1219–1225.
<https://doi.org/10.1093/beheco/arv069>
- Ringler, E., Pašukonis, A., Hödl, W., Ringler, M., 2013. Tadpole transport logistics in a Neotropical poison frog: indications for strategic planning and adaptive plasticity in anuran parental care. *Front. Zool.* 10, 67.
- Ringler, M., Hödl, W., Ursprung, E., 2009a. Phonotactic approach pattern in the neotropical frog *Allobates femoralis*: A spatial and temporal analysis. *Behaviour* 146, 153–170.
<https://doi.org/10.1163/156853909X410711>
- Ringler, M., Mangione, R., Pašukonis, A., Rainer, G., Gyimesi, K., Felling, J., Kronaus, H., Réjou-Méchain, M., Chave, J., Reiter, K., Others, 2016. High-resolution forest mapping for behavioural studies in the Nature Reserve “Les Nouragues”, French Guiana. *J. Maps* 12, 26–32.
- Ringler, M., Ringler, E., Mendoza, D.M., Hödl, W., 2011. Intrusion experiments to measure territory size: development of the method, tests through simulations, and application in the frog *Allobates femoralis*. *PLoS One* 6, e25844.
- Ringler, M., Ursprung, E., Hödl, W., 2009b. Site fidelity and patterns of short-and long-term movement in the brilliant-thighed poison frog *Allobates femoralis* (Aromobatidae). *Behav. Ecol. Sociobiol.* 63, 1281–1293.
- Rivera-Ordóñez, J.M., Justin Nowakowski, A., Manansala, A., Thompson, M.E.,

- Todd, B.D., 2019. Thermal niche variation among individuals of the poison frog, *Oophaga pumilio*, in forest and converted habitats. *Biotropica* 51, 747–756. <https://doi.org/10.1111/btp.12691>
- Roithmair, M. E., 1992. Territoriality and male mating success in the dart-poison frog, *Epipedobates femoralis* (Dendrobatidae, Anura). *Ethology* 92, 331–343.
- Roithmair, M. E., 1994. Field studies on reproductive behaviour in two dart-poison frog species (*Epipedobates femoralis*, *Epipedobates trivittatus*) in Amazonian Peru. *Herpetol. J.* 4, 77–85.
- Rome, L.C., Stevens, E.D., John-Alder, H.B., 1992. The influence of temperature and thermal acclimation on physiological function. *Environ. Physiol. Amphib.* 183–205.
- Seymour, R.S., 1972. Behavioral Thermoregulation by Juvenile Green Toads, *Bufo debilis*. *Copeia* 1972, 572. <https://doi.org/10.2307/1442931>
- Stille, W.T., 1958. The Water Absorption Response of an Anuran. *Copeia* 1958, 217. <https://doi.org/10.2307/1440591>
- Stückler, S., Ringler, M., Pašukonis, A., Weinlein, S., Hödl, W., Ringler, E., 2019. Spatio-Temporal Characteristics of the Prolonged Courtship in Brilliant-Thighed Poison Frogs, *Allobates femoralis*. *Herpetologica* 75, 268–279. <https://doi.org/10.1655/Herpetologica-D-19-00010.1>
- Toft, C.A., 1980. Feeding ecology of thirteen syntopic species of anurans in a seasonal tropical environment. *Oecologia* 45, 131–141. <https://doi.org/10.1007/BF00346717>
- Toledo, R.C., Jared, C., 1993. Cutaneous adaptations to water balance in amphibians. *Comp. Biochem. Physiol. A Physiol.* 105, 593–608. [https://doi.org/10.1016/0300-9629\(93\)90259-7](https://doi.org/10.1016/0300-9629(93)90259-7)
- Tracy, Christopher R., Christian, K.A., Tracy, C. Richard, 2010. Not just small, wet, and cold: effects of body size and skin resistance on thermoregulation and arboreality of frogs. *Ecology* 91, 1477–1484. <https://doi.org/10.1890/09-0839.1>
- TRACY, C.R., CHRISTIAN, K.A., BURNIP, N., AUSTIN, B.J., CORNALL, A., IGLESIAS, S., REYNOLDS, S.J., TIXIER, T., LE NOËNE, C., 2013. Thermal and hydric implications of diurnal activity by a small tropical frog during the dry season. *Austral Ecol.* 38, 476–483. <https://doi.org/10.1111/j.1442-9993.2012.02416.x>
- Ursprung, E., Ringler, M., Jehle, R., Hödl, W., 2011. Strong male/male competition allows for nonchoosy females: high levels of polygyny in a territorial frog with paternal care: POLYGYNANDRY IN *A. FEMORALIS*. *Mol. Ecol.* 20, 1759–1771. <https://doi.org/10.1111/j.1365-294X.2011.05056.x>
- von May, R., Catenazzi, A., Santa-Cruz, R., Gutierrez, A.S., Moritz, C., Rabosky, D.L., 2019. Thermal physiological traits in tropical lowland amphibians: Vulnerability to climate warming and cooling. *PLOS ONE* 14, e0219759. <https://doi.org/10.1371/journal.pone.0219759>
- Wells, K.D., 2007. *The Ecology and Behavior of Amphibians*. University of Chicago Press.

Chapter 4. Androgen responsiveness to simulated territorial intrusions in *Allobates femoralis* males: evidence supporting the challenge hypothesis in a territorial frog

This chapter has been submitted in the peer-reviewed Journal *General and Comparative Endocrinology*

Citation:

Rodríguez, C., Fusani, L., Raboisson, G., Hödl, W., Ringler, E., and Canoine, V. (submitted). Androgen responsiveness to simulated territorial intrusions in *Allobates femoralis* males: evidence supporting the challenge hypothesis in a territorial frog.

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

Camilo Rodríguez^{a, 1}, Leonida Fusani^{a,b}, Gaëlle Raboisson^a, Walter Hödl^a, Eva Ringler^{a,c,d} and Virginie Canoine^a

^a Department of Behavioral and Cognitive Biology, University of Vienna, Djerassiplatz 1, 1030, Vienna – Austria.

^b Konrad Lorenz Institute of Ethology, University of Veterinary Medicine, Vienna, Austria.

^c Messerli Research Institute, University of Veterinary Medicine Vienna, Medical University Vienna, University of Vienna, Vienna, Austria.

^d Division of Behavioural Ecology, Institute of Ecology and Evolution, University of Bern, Bern, Switzerland.

¹ Corresponding author. E-mail: camilo.rodriguez@univie.ac.at

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.

Key words: Water-borne androgen, territorial response, Neotropical poison frog, androgens, Challenge Hypothesis.

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 breeding places. In many species, only males engage in competitive interactions and contests with conspecifics for the acquisition of territories (Davies, 1991). 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 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). However, while high androgen levels favour courtship and aggressive behaviour, they simultaneously inhibit parental activity (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 males’ androgen levels in response to social interactions, in different life history stages of various breeding systems. For instance, in monogamous species with a high degree of paternal care, androgen levels should rise when territorial males are challenged by an intruder male but return to low 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 including other vertebrate groups found conflicting results that no longer supported the Challenge Hypothesis (reviewed by Moore et al., 2019). 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; reviewed by 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; Townsend and Moger, 1987; Mendoça et al., 1985). 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, 2005; 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 close-by 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, 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.

Methods

Study system

The brilliant-thighed poison frog, *Allobates femoralis*, is a diurnal and terrestrial species belonging to the family Dendrobatidae ("AmphibiaWeb," 2020; Boulenger, 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). During territorial interactions or courtship displays, males produce acoustic signals from elevated structures on the forest ground. Courtship behaviour is accompanied by a locomotor display called "courtship march", which consists in the conduction performed by the male to the female towards the oviposition site (sensu Montanarin et al., 2011) and usually starts in the afternoon and ends on the next morning (Hödl, 1983; Ringler et al., 2013; Roithmair, 1992; Stücker et al., 2019). 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).

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). It is noteworthy that anti-androgen immunoreactive substances in water samples may include some androgenic conjugate forms (Baugh and Gray-Gaillard, 2020). Therefore, 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 μ m 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 N₂ at 37°C, resuspended with 250 μ L of the assay buffer (provided in the ELISA kit, see below) and incubated overnight at 4°C for further analysis.

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) 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.

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

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 60min (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 $\pm$ SD: males=2.74 $\pm$ 0.03 cm, N=7; females = 2.79 $\pm$ 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.

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 $\pm$ SD = 2.8 $\pm$ 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; 2000xg) to separate the plasma. Plasma volume was recorded, and samples were transferred into 1.5 mL eppendorf tubes prefilled with 750 μ L 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 N₂ at 37°C. Dried pellets were resuspended in 4 mL of dichloromethane and 100 μ L of distilled water and, then incubated at 4°C overnight for equilibration. The following day, samples were shaken for 1h and then centrifuged at 4000rpm 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 N₂ stream and then resuspended in the assay buffer supplied by the ELISA manufacturer and incubated overnight at 4°C.

Simulated territorial intrusion-tests (STIs)

Field site and playback stimuli

Between February and April of 2018 and 2019, seventeen free-living adult *A. femoralis* males (SUL mean $\pm$ SD = 2.95 $\pm$ 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; 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), 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 under rainless conditions and in the afternoon (14:00h - 17:00h), 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.

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). 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 series of three consecutive water baths of 60 min/each (1h, 2h and 3h). This sequence of water baths 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 less than 10 min. Water samples were collected individually after every 60 min 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 20mL 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 1h 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.

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: $\beta_{\text{morning}} = 1.18$, $t = 1.22$, $P = 0.22$; $\beta_{\text{afternoon}} = 1.68$, $t = 1.61$, $P = 0.11$), 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 (0h-morning/afternoon baselines-, 1h, 2h and 3h 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 ($R_{\text{male-male}}$; 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., 2007). 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 ($R_{\text{male-male}}$) as the fixed effect predictor and the male ID as the random effect.

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).

Results

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 (water-borne androgen mean $\pm$ 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**). Water-borne androgen concentration was positively correlated with plasma testosterone concentration ($t=4.82$, $r=0.76$, $P<0.001$; **Figure S3**).

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 represented positively vocal behaviour variables (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.

Variable	Principal components		
	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: $\beta=0.31$, $t=2.55$, $P=0.01$; **Figure 1A**; PC1: $\beta=0.56$, $t=3.62$, $P<0.001$; **Figure 1B**). However, vocal behaviour was not dependent on water-borne androgen concentrations (PC1: $\beta=0.07$, $t=0.42$, $P=0.67$; **Figure 1B**). Courtship behaviour (PC2) and foraging behaviour (PC3) were not significantly different over the day (PC2: $\beta=0.28$, $t=1.52$, $P=0.13$; **Figure 1C**; PC3: $\beta=-0.02$, $t=-0.1$, $P=0.92$; **Figure 1D**) and/or dependent on water-borne androgen concentrations (PC2: $\beta=0.1$, $t=0.55$, $P=0.58$; **Figure 1C**; PC3: $\beta=0.19$, $t=1$, $P=0.32$; **Figure 1D**).

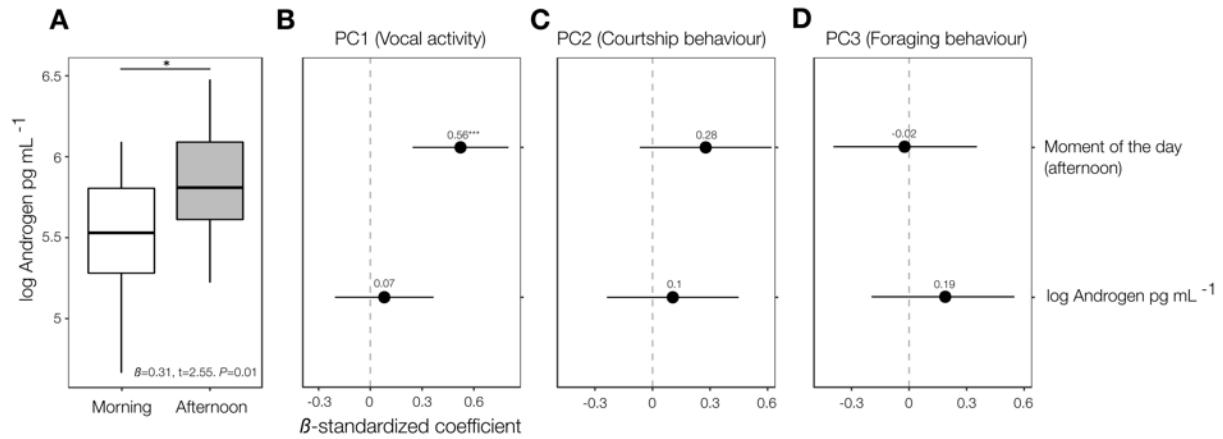


Figure 1. Water-borne androgen levels, vocal, courtship and foraging behaviour across the day. **(A)** Boxplot showing the difference of water-borne androgen concentration between morning and afternoon; Linear Mixed Model plots showing z-scores values (x-axis) and the effect size (numbers over mean-points)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

Effect of STI on water-borne androgen levels (Rmale-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 1h after the STI compared to the pre-challenge water-borne androgen levels both, in the morning (LMM: $\beta=0.40, t=3.49, P=0.001$; **Figure 2A**) and in the afternoon (LMM: $\beta=0.21, t=0.10, P=0.04$; **Figure 2A**). Subsequently, androgen concentration dropped nearly to the morning levels in the 2h sampling point, and under both morning and afternoon levels in the 3h sampling point (**Figure 2A**).

Responsive males to the playback had a positive effect size and 95% confidence intervals crossed zero (Cohen's $d = 0.85 \pm 0.94$; **Figure 2B**), 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 did not cross zero (Cohen's $d = -0.06 \pm 1.15$; **Figure 2B**), and thus suggesting a null effect of STIs on androgen levels.

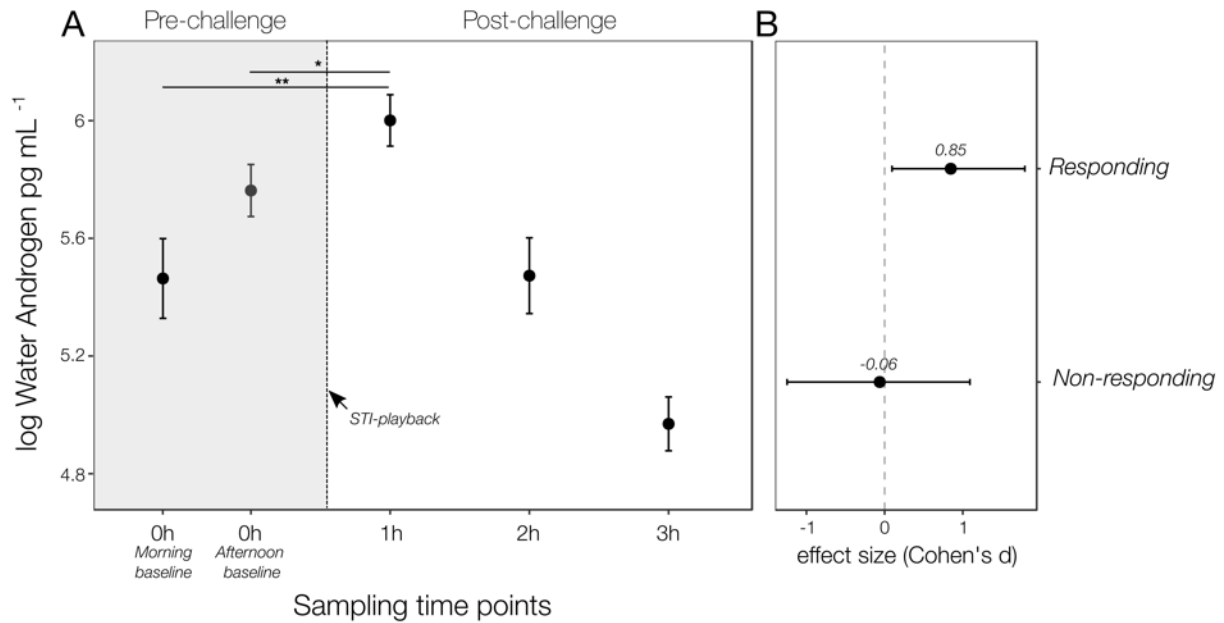


Figure 2. Androgen responsiveness after STI in *A. femoralis* males. **(A)** Comparison of baselines of water-borne androgen concentration (morning/afternoon; pre-STI) between concentrations over three sampling times (1h, 2h and 3h; post-STI). * $P < 0.05$, ** $P < 0.001$. **(B)** Differences in effect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

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 S1A**). This component represented positively 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: $\beta=0.58$, $t=0.55$, $P=0.58$).

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*), a territorial species with iteroparous breeding and high paternal care during the breeding season. Our results demonstrate that water-borne androgen concentration was elevated after presenting a conspecific 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 androgen concentration significantly increased 1h 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 polyandric 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; Figure 1) and afternoon (when androgen concentration and vocal interactions were high; Figure 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 male *A. femoralis* fitness (Ursprung et al., 2011), this "shot" of 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., 2019). 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. This result suggests that androgens are necessary to trigger an aggressive response to a territorial intrusion in *A. femoralis* males. But what could explain the absence of such a response to the STI? 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 consider unlikely that non-responding males became habituated to the repeated STIs and recognized the playback as a non-threatening stimulus, because in most of the cases non-responding males presented positive phonotaxis to STIs conducted before and after the negative response. Third, because foraging activity occurred through the day and was not dependent on androgen levels in *A. femoralis* (Figure 1D), we also discard the possibility of an energetic constraint on the phonotaxis caused by a low food intake (Marler and Ryan, 1996). 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., 2007).

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). 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 impairment 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 hours, as a result of the inhibitory interlinkage between the hypothalamo-pituitary-gonadal and the hypothalamo-pituitary-interrenal axis (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.

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.

Acknowledgements

We thank the Nouragues research field station (managed by CNRS) which benefits from “Investissement d'Avenir” grants managed by Agence Nationale de la Recherche (AnaEE France ANR-11-INBS-0001; Labex CEBA ANR-10-LABX-25-01). This work was funded by the Austrian Science Fund (FWF): W1262-B29, and also supported by the Austrian Science Fund (FWF): P31518 grant to ER. We specially thank the Vienna Zoo (Schönbrunner Tiergarten Ges.m.b.H), the Austrian Herpetological Society (Österreichische Gesellschaft für Herpetologie) and the Centre National de la Recherche Scientifique (CNRS) for additional funding provided to CR. We are especially grateful with M. Ringler, P. Gaucher and N. Marchand for the invaluable logistic support in the field. For providing field assistance we are grateful with A. Lembens, K. Dellefont, C. Leeb and M. Peignier.

References

- Adkins-Regan, E., 2005. Hormones and animal social behavior, Monographs in behavior and ecology. Princeton University Press, Princeton.
- Amézquita, A., Castellanos, L., Hödl, W., 2005. Auditory matching of male *Epipedobates femoralis* (Anura: Dendrobatidae) under field conditions. *Anim. Behav.* 70, 1377–1386. <https://doi.org/10.1016/j.anbehav.2005.03.012>
- Amézquita, A., Hödl, W., Lima, A.P., Castellanos, L., Erdtmann, L., de Araújo, M.C., 2006. Masking interference and the evolution of the acoustic communication system in the Amazonian dendrobatid frog *Allobates femoralis*. *Evol. Int. J. Org. Evol.* 60, 1874–1887. <https://doi.org/10.1111/j.0014-3820.2006.tb00531.x>
- AmphibiaWeb [WWW Document], 2020. . AmphibiaWeb. URL <https://amphibiaweb.org>
- Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* 67, 1–48. <https://doi.org/10.18637/jss.v067.i01>
- Baugh, A.T., Bastien, B., Still, M.B., Stowell, N., 2018. Validation of water-borne steroid hormones in a tropical frog (*Physalaemus pustulosus*). *Gen. Comp. Endocrinol.* 261, 67–80. <https://doi.org/10.1016/j.ygcen.2018.01.025>
- Baugh, A.T., Gray-Gaillard, S.L., 2020. Excreted testosterone and male sexual proceptivity: a hormone validation and proof-of-concept experiment in túngara frogs. *Gen. Comp. Endocrinol.* 113638. <https://doi.org/10.1016/j.ygcen.2020.113638>
- Bell, A.M., 2019. Individual variation and the challenge hypothesis. *Horm. Behav.* 104549. <https://doi.org/10.1016/j.yhbeh.2019.06.013>
- Bongers, F., Charles-Dominique, P., Forget, P.-M., Théry, M., 2013. Nouragues: dynamics and plant-animal interactions in a neotropical rainforest. Springer Science & Business Media.
- Boulenger, G.A., 1883. On a collection of frogs from Yurimaguas, Huallaga River, northern Peru, in: Proceedings of the Zoological Society of London. Wiley Online Library, pp. 635–638.
- Brzoska, J., Obert, H.J., 1980. Acoustic signals influencing the hormone production of testes in the grass frogs. *J. Comp. Physiol.* 140, 25–29.
- Boyd, S.K., 1994. Gonadal Steroid Modulation of Vasotocin Concentrations in the Bullfrog Brain. *Neuroendocrinology* 60, 150–156. <https://doi.org/10.1159/000126745>
- Burmeister, S.S., Wilczynski, W., 2001. Social Context Influences Androgenic Effects on Calling in the Green Treefrog (*Hyla cinerea*). *Horm. Behav.* 40, 550–558. <https://doi.org/10.1006/hbeh.2001.1723>
- de Assis, V.R., Navas, C.A., Mendonça, M.T., Gomes, F.R., 2012. Vocal and territorial behavior in the Smith frog (*Hypsiboas faber*): Relationships with plasma levels of corticosterone and testosterone. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* 163, 265–271. <https://doi.org/10.1016/j.cbpa.2012.08.002>
- Davies, N.B., 1991. Mating Systems, in: Krebs, J.R., Davies, N.B. (Eds.), Behavioural Ecology: An Evolutionary Approach. Blackwell Scientific Publications, Oxford, pp. 263–299.
- Delrio, G., D’istria, M., Iela, L., Chieffi, G.,

1979. The possible significance of testosterone in the female green frog *Rana esculenta*. *Bolletino Zool.* 46, 1–9.
<https://doi.org/10.1080/11250007909440271>
- Emerson, S.B., Hess, D.L., 1996. The Role of Androgens in Opportunistic Breeding, Tropical Frogs. *Gen. Comp. Endocrinol.* 103, 220–230.
<https://doi.org/10.1006/gcen.1996.0113>
- Fischer, E.K., O’Connell, L.A., 2020. Hormonal and neural correlates of care in active versus observing poison frog parents. *Horm. Behav.* 120, 104696.
<https://doi.org/10.1016/j.yhbeh.2020.104696>
- Folstad, I., Karter, A.J., 1992. Parasites, Bright Males, and the Immunocompetence Handicap. *Am. Nat.* 139, 603–622.
<https://doi.org/10.1086/285346>
- Fusani, L., Canoine, V., Goymann, W., Wikelski, M., Hau, M., 2005. Difficulties and special issues associated with field research in behavioral neuroendocrinology. *Horm. Behav.* 48, 484–491.
<https://doi.org/10.1016/j.yhbeh.2005.05.005>
- Gabor, C.R., Bosch, J., Fries, J.N., Davis, D.R., 2013. A non-invasive water-borne hormone assay for amphibians. *Amphib.-Reptil.* 34, 151–162.
<https://doi.org/10.1163/15685381-00002877>
- Gabor, C.R., Zabierek, K.C., Kim, D.S., da Barbiano, L.A., Mondelli, M.J., Bendik, N.F., Davis, D.R., 2016. A Non-Invasive Water-Borne Assay of Stress Hormones in Aquatic Salamanders. *Copeia* 104, 172–181. <https://doi.org/10.1643/OT-14-207>
- Gasser, H., Amézquita, A., Hödl, W., 2009. Who is Calling? Intraspecific Call Variation in the Aromobatid Frog *Allobates femoralis*. *Ethology* 115, 596–607.
<https://doi.org/10.1111/j.1439-0310.2009.01639.x>
- Goymann, W., Dávila, P.F. 2017. Acute peaks of testosterone suppress paternal care: evidence from individual hormonal reaction norms. *Proc. R. Soc. B Biol. Sci.* 284, 20170632.
- Goymann, W., Landys, M.M., Wingfield, J.C., 2007. Distinguishing seasonal androgen responses from male–male androgen responsiveness—Revisiting the Challenge Hypothesis. *Horm. Behav.* 51, 463–476.
<https://doi.org/10.1016/j.yhbeh.2007.01.007>
- Goymann, W., Schwabl, I., Trappschuh, M., Hau, M., 2007. Use of ethanol for preserving steroid and indoleamine hormones in bird plasma. *Gen. Comp. Endocrinol.* 150, 191–195.
<https://doi.org/10.1016/j.ygcen.2006.09.014>
- Hau, M., Goymann, W., 2015. Endocrine mechanisms, behavioral phenotypes and plasticity: known relationships and open questions. *Front. Zool.* 12, S7.
<https://doi.org/10.1186/1742-9994-12-S1-S7>
- Hödl, W., 1983. *Phyllobates femoralis* (Dendrobatidae): Rufverhalten und akustische Orientierung der Männchen (Freilandaufnahmen). *Wiss. Film* 30, 12–19.
- Jameson, D.L., 1954. Social Patterns in the Leptodactylid Frogs *Syrrhophus* and *Eleutherodactylus*. *Copeia* 1954, 36–38.
- Kaefer, I.L., Montanarin, A., Da Costa, R.S., Lima, A.P., 2012. Temporal patterns of reproductive activity and site attachment of the brilliant-thighed frog *Allobates femoralis* from central Amazonia. *J. Herpetol.* 46, 549–554.
<https://doi.org/10.1670/10-224>
- Kidd, C.E., Kidd, M.R., Hofmann, H.A., 2010. Measuring multiple hormones from a single water sample using

- enzyme immunoassays. *Gen. Comp. Endocrinol.* 165, 277–285.
<https://doi.org/10.1016/j.ygcen.2009.07.008>
- Leary, C.J., 2009. Hormones and acoustic communication in anuran amphibians. *Integr. Comp. Biol.* 49, 452–470.
<https://doi.org/10.1093/icb/icp027>
- Licht, P., McCreery, B.R., Barnes, R., Pang, R., 1983. Seasonal and stress related changes in plasma gonadotropins, sex steroids, and corticosterone in the bullfrog, *Rana catesbeiana*. *Gen. Comp. Endocrinol.* 50, 124–145.
[https://doi.org/10.1016/0016-6480\(83\)90249-6](https://doi.org/10.1016/0016-6480(83)90249-6)
- Lynn, S.E, Wingfield, J.C. 2008. Dissociation of testosterone and aggressive behavior during the breeding season in male chestnut-collared long- spurs, *Calcarius ornatus*. *Gen. Comp. Endocrinol.* 156, 181–189.
<https://doi.org/10.1016/j.ygcen.2008.01.004>
- Miles, D.B., Sinervo, B., Hazard, L.C., Svensson, E.I., Costa, D., 2007. Relating endocrinology, physiology and behaviour using species with alternative mating strategies. *Funct. Ecol.* 21, 653–665.
<https://doi.org/10.1111/j.1365-2435.2007.01304.x>
- Millikin, A.R., Woodley, S.K., Davis, D.R., Moore, I.T., Anderson, J.T., 2019. Water-borne and plasma corticosterone are not correlated in spotted salamanders. *Ecol. Evol.* 9, 13942–13953.
<https://doi.org/10.1002/ece3.5831>
- Montanarin, A., Kaefer, I.L., Lima, A.P., 2011. Courtship and mating behaviour of the brilliant-thighed frog *Allobates femoralis* from Central Amazonia: implications for the study of a species complex. *Ethol. Ecol. Evol.* 23, 141–150.
<https://doi.org/10.1080/03949370.2011.554884>
- Moore, F.L., Boyd, S.K., Kelley, D.B., 2005. Historical perspective: Hormonal regulation of behaviors in amphibians. *Horm. Behav.* 48, 373–383.
<https://doi.org/10.1016/j.yhbeh.2005.05.011>
- Moore, I.T., Hernandez, J., Goymann, W., 2019. Who rises to the challenge? Testing the Challenge Hypothesis in fish, amphibians, reptiles, and mammals. *Horm. Behav.* S0018506X19301916.
<https://doi.org/10.1016/j.yhbeh.2019.06.001>
- Narayan, E.J., 2013. Non-invasive reproductive and stress endocrinology in amphibian conservation physiology. *Conserv. Physiol.* 1, cot011–cot011.
<https://doi.org/10.1093/conphys/cot011>
- Narins, P.M., Feng, A.S., Fay, R.R., 2006. Hearing and Sound Communication in Amphibians, Springer Handbook of Auditory Research. Springer New York.
- Narins, P.M., Hödl, W., Grabul, D.S., 2003. Bimodal signal requisite for agonistic behavior in a dart-poison frog, *Epipedobates femoralis*. *Proc. Natl. Acad. Sci.* 100, 577–580.
<https://doi.org/10.1073/pnas.0237165100>
- Oliveira, R. F., 2004. Social Modulation of Androgens in Vertebrates: Mechanisms and Function, in: Slater, P. J. B., Rosenblatt, J. S., Roper, T. J., Snowdon, C. T., Brockmann, H. J., Naguib, M. (Eds.), *Advances in The study of behavior*. Elsevier Academic Press, pp. 165–239. [https://doi.org/10.1016/S0065-3454\(04\)34005-2](https://doi.org/10.1016/S0065-3454(04)34005-2)
- Pašukonis, A., Loretto, M.-C., Landler, L., Ringler, M., Hödl, W., 2014a. Homing trajectories and initial orientation in a Neotropical territorial frog, *Allobates femoralis* (Dendrobatidae). *Front. Zool.* 11, 29. <https://doi.org/10.1186/1742-9994-11-29>

- Pašukonis, A., Ringler, M., Brandl, H.B., Mangione, R., Ringler, E., Hödl, W., 2013. The Homing Frog: High Homing Performance in a Territorial Dendrobatid Frog *Allobates femoralis* (Dendrobatidae). *Ethology* 119, 762–768.
<https://doi.org/10.1111/eth.12116>
- Pašukonis, A., Trenkwalder, K., Ringler, M., Ringler, E., Mangione, R., Steininger, J., Warrington, I., Hödl, W., 2016. The significance of spatial memory for water finding in a tadpole-transporting frog. *Anim. Behav.* 116, 89–98.
<https://doi.org/10.1016/j.anbehav.2016.02.023>
- Pašukonis, A., Warrington, I., Ringler, M., Hödl, W., 2014b. Poison frogs rely on experience to find the way home in the rainforest. *Biol. Lett.* 10, 20140642.
<https://doi.org/10.1098/rsbl.2014.0642>
- Pough, F.H., Taigen, T.L., 1990. Metabolic correlates of the foraging and social behaviour of dart-poison frogs. *Anim. Behav.* 39, 145–155.
[https://doi.org/10.1016/S0003-3472\(05\)80734-1](https://doi.org/10.1016/S0003-3472(05)80734-1)
- Pröhl, H., 2005. Territorial Behavior in Dendrobatid Frogs. *J. Herpetol.* 39, 354–365.
<https://www.jstor.org/stable/4092919>
- Propper, C.R., Dixon, T.B., 1997. Differential Effects of Arginine Vasotocin and Gonadotropin-Releasing Hormone on Sexual Behaviors in an Anuran Amphibian. *Horm. Behav.* 32, 99–104.
<https://doi.org/10.1006/hbeh.1997.1408>
- R Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
- Revelle, W., 2019. psych: Procedures for Psychological, Psychometric, and Personality Research. Northwestern University, Evanston, Illinois.
- Ringler, E., Pašukonis, A., Hödl, W., Ringler, M., 2013. Tadpole transport logistics in a Neotropical poison frog: indications for strategic planning and adaptive plasticity in anuran parental care. *Front. Zool.* 10, 67.
<https://doi.org/10.1186/1742-9994-10-67>
- Ringler, E., Pašukonis, A., Fitch, W.T., Huber, L., Hödl, W., Ringler, M., 2015. Flexible compensation of uniparental care: female poison frogs take over when males disappear. *Behav. Ecol.* 26, 219–225.
<https://doi.org/10.1093/beheco/arv069>
- Ringler, E., Beck, K.B., Weinlein, S., Huber, L., Ringler, M., 2017. Adopt, ignore, or kill? Male poison frogs adjust parental decisions according to their territorial status. *Sci Rep.* 7, 43544.
<https://doi.org/10.1038/srep43544>
- Ringler, E., Szípl, G., Harrigan, R.J., Bartl-Binder, P., Mangione, R., Ringler, M., 2018. Hierarchical decision-making balances current and future reproductive success. *Mol. Ecol.* 27, 2289–2301.
<https://doi.org/10.1111/mec.14583>
- Ringler, M., Ringler, E., Mendoza, D.M., Hödl, W., 2011. Intrusion experiments to measure territory size: development of the method, tests through simulations, and application in the frog *Allobates femoralis*. *PLoS One* 6, e25844.
- Ringler, M., Szípl, G., Hödl, W., Khil, L., Kofler, B., Lonauer, M., Provin, C., Ringler, E., 2017. Acoustic ranging in poison frogs—it is not about signal amplitude alone. *Behav. Ecol. Sociobiol.* 71, 114.
<https://doi.org/10.1007/s00265-017-2340-2>
- Rodríguez, C., Amézquita, A., Ringler, M., Pašukonis, A., Hödl, W., 2020. Calling amplitude flexibility and acoustic spacing in the territorial frog *Allobates femoralis*. *Behav. Ecol. Sociobiol.* 74, 76. <https://doi.org/10.1007/s00265->

- 020-02857-6
- Rodríguez, C., Hödl, W., 2020. Sound radiation pattern of the advertisement call of the highly territorial poison frog *Allobates femoralis*. *Behav. Processes* 170, 103996. <https://doi.org/10.1016/j.beproc.2019.103996>
- Roithmair, M.E., 1992. Territoriality and Male Mating Success in the Dart-poison Frog, *Epipedobates femoralis* (Dendrobatidae, Anura). *Ethology* 92, 331–343. <https://doi.org/10.1111/j.1439-0310.1992.tb00970.x>
- Romero, L.M., Reed, J.M., 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* 140, 73–79. <https://doi.org/10.1016/j.cbpb.2004.11.004>
- Scott, A.P., Hirschenhauser, K., Bender, N., Oliveira, R., Earley, R.L., Sebire, M., Ellis, T., Pavlidis, M., Hubbard, P.C., Huertas, M., Canario, A., 2008. Non-Invasive Measurement of Steroids in Fish-Holding Water: Important Considerations When Applying the Procedure to Behaviour Studies. *Behaviour* 145, 1307–1328. <https://www.jstor.org/stable/40296047>
- Schulte, L., Ringler, E., Rojas, B., Stynoski, J., 2020. Developments in amphibian parental care research: history, present advances and future perspectives. *Herpetol. Monogr.* 34, 71–97. <https://doi.org/10.1655/HERPMONOG-RAPHS-D-19-00002.1>
- Simon, N.G., Lu, S., 2006. Androgens and Aggression, in: Nelson, R.J. (Ed.), *Biology of Aggression*. Oxford University Press, New York, pp. 211–230.
- Spring, S., Lehner, M., Huber, L., Ringler, E., 2019. Oviposition and father presence reduce clutch cannibalism by female poison frogs. *Front. Zool.* 16, 8. <https://doi.org/10.1186/s12983-019-0304-2>
- Still, M.B., Lea, A.M., Hofmann, H.A., Ryan, M.J., 2019. Multimodal stimuli regulate reproductive behavior and physiology in male túngara frogs. *Horm. Behav.* 115, 104546. <https://doi.org/10.1016/j.yhbeh.2019.06.010>
- Stückler, S., Ringler, M., Pašukonis, A., Weinlein, S., Hödl, W., Ringler, E., 2019. Spatio-Temporal Characteristics of the Prolonged Courtship in Brilliant-Thighed Poison Frogs, *Allobates femoralis*. *Herpetologica* 75, 268–279. <https://doi.org/10.1655/Herpetologica-D-19-00010.1>
- Solís, R., Penna, M., 1997. Testosterone levels and evoked vocal responses in a natural population of the frog *Batrachyla taeniata*. *Hormon. Behav.* 31, 101–109. <https://doi.org.uaccess.univie.ac.at/10.1006/hbeh.1997.1366>
- Sonnleitner, R., Ringler, M., Loretto, M. C., Ringler, E., 2020. Experience shapes accuracy in territorial decision-making in a poison frog. *Biol. Lett.* 16, 20200094. <https://doi.org/10.1098/rsbl.2020.0094>
- Toft, C.A., 1980. Feeding ecology of thirteen syntopic species of anurans in a seasonal tropical environment. *Oecologia* 45, 131–141. <https://doi.org/10.1007/BF00346717>
- Toledo, L.F., Martins, I.A., Bruschi, D.P., Passos, M.A., Alexandre, C., Haddad, C.F.B., 2014. The anuran calling repertoire in the light of social context. *Acta Ethologica* 18, 87–99. <https://doi.org/10.1007/s10211-014-0194-4>
- Tumulty, J.P., Pašukonis, A., Ringler, M., Forester, J.D., Hödl, W., Bee, M.A., 2018. Brilliant-thighed poison frogs do not use acoustic identity information to treat territorial neighbours as dear enemies. *Anim. Behav.* 141, 203–220.

- <https://doi.org/10.1016/j.anbehav.2018.05.008>
- Ursprung, E., Ringler, M., Hödl, W., 2009. Phonotactic approach pattern in the neotropical frog *Allobates femoralis*: A spatial and temporal analysis. *Behaviour* 146, 153–170. <https://doi.org/10.1163/156853909X410711>
- Ursprung, E., Ringler, M., Jehle, R., Hödl, W., 2011. Strong male/male competition allows for nonchoosy females: high levels of polygynandry in a territorial frog with paternal care: polygynandry in *A. femoralis*. *Mol. Ecol.* 20, 1759–1771. <https://doi.org/10.1111/j.1365-294X.2011.05056.x>
- Wada, M., 1986. Circadian rhythms of testosterone-dependent behaviors, crowing and locomotor activity, in male Japanese quail. *J. Comp. Physiol. A* 158, 17–25. <https://doi.org/10.1007/BF00614516>
- Wetzel, D. M., and Kelley, D. B., 1983. Androgen and gonadotropin effects on male mate calls in South African clawed frogs, *Xenopus laevis*. *Horm. Behav.* 17, 388–404. [https://doi.org/10.1016/0018-506X\(83\)90048-X](https://doi.org/10.1016/0018-506X(83)90048-X)
- Weygoldt, P., 1980. Zur Fortpflanzungsbiologie von *Phylllobates femoralis* (Boulenger) im Terrarium. *Salamandra*. 16, 215–226.
- Wingfield, J.C., 2005. A continuing saga: The role of testosterone in aggression. *Hormon. Behav.* 48, 253–255. <https://doi.org/10.1016/j.yhbeh.2005.05.009>
- Wingfield, J.C., Hegner, R.E., Dufty, A.M., Ball, G.F., 1990. The “Challenge Hypothesis”: Theoretical Implications for Patterns of Testosterone Secretion, Mating Systems, and Breeding Strategies. *Am. Nat.* 136, 829–846.
- Wingfield, J.C., Marler, P.R., 1988. Endocrine basis of communication in reproduction and aggression, in: Knobil, E., Neil, J.D. (Ed.), *The physiology of reproduction*. Raven Press, New York, p.p. 1647–77.
- Wingfield, J.C., Moore, I.T., Goymann, W., Wacker, D.W., Sperry, T., 2006. Contexts and ethology of vertebrate aggression: implications for the evolution of hormone–behavior interactions, in: Nelson, R.J. (Ed.), *Biology of Aggression*. Oxford University Press, New York, pp. 179–210.
- Wingfield, J.C., Wada, M., 1989. Changes in plasma levels of testosterone during male-male interactions in the song sparrow, *Melospiza melodia*: time course and specificity of response. *J. Comp. Physiol. A* 166. <https://doi.org/10.1007/BF00193463>
- Yanagida, T., 2020. misty: Miscellaneous Functions “T. Yanagida.”
- Yao, M., Denver, R.J., 2007. Regulation of vertebrate corticotropin-releasing factor genes. *General and Comparative Endocrinology* 153, 200–216. <https://doi.org/10.1016/j.ygcen.2007.01.046>
- Yao, M., Hu, F., Denver, R.J., 2008. Distribution and corticosteroid regulation of glucocorticoid receptor in the brain of *Xenopus laevis*. *J. Comp. Neurol.* 508, 967–982. <https://doi.org/10.1002/cne.21716>

General discussion

Along this thesis I studied the mechanisms that allow a territorial frog to compensate the fitness-related costs of territoriality. To do so, I used as a model *Allobates femoralis*, a highly territorial Neotropical poison frog. Particularly, I approached and discussed 1) how males adjust their vocal behaviour to optimize the sound propagation and its implication in territoriality and fitness, 2) how *A. femoralis* adjust its behaviour to challenging climatic conditions while advertising a territory, and 3) how changes in androgen levels facilitate males' behavioural decision-making when facing a territorial challenge. I used methods ranging from behavioural observations and playback experiments to passive telemetry and hormonal measurements. With this approach, I could show that *A. femoralis* has the ability of subtle adjustments of individual behaviour to overcome challenging situations inherent to territorial behaviour.

Acoustic communication is perhaps one of the most widespread communication signals among terrestrial vertebrates and it is critical in species recognition, reproductive success and resource acquisition. Consequently, the machinery behind the production of acoustic signals, its propagation patterns and the perception mechanisms are strongly driven by selective pressures (Owings and Morton, 1998; Gerhardt, 1994). As in many acoustically communicating species, the production of sounds in anuran amphibians demands high energy expenditure and elevates the risk of predation. Thus, natural selection should promote adaptive mechanisms that improve the effectiveness of acoustic communication with intended receivers but without energy spill outs (Wells and Schwartz, 2006). In this thesis, I showed correlative and experimental evidence on the mechanisms that allows a territorial frog to reduce energy expenditure and optimize acoustic communication with conspecifics.

Several studies have focused on investigating signal adaptations to environmental constraints that affect sound propagation (Wilczynski and Brenowitz 1988; Brenowitz 1989; Gerhardt et al. 1989; Wiley and Richards 1982; Gerhardt 1994). However, very little attention has been paid to the function of sound radiation patterns on the evolution of the acoustic signal. Importantly, acoustic radiation patterns are strongly driven by selective pressures related to energy use, mating and predation (Bernal et al., 2019). In Chapter 1, I described the nearly

omnidirectional radiation pattern of the advertisement call in *A. femoralis*. I further suggested that males probably reinforce the directionality of the signal by directing the call towards antiphonal calling neighbours. There are at least three reasons to suggest that having the advantages of both the directional and omnidirectional radiation pattern might be favoured by natural selection: 1) because it allows males to advertise the tenure of a territory to multiple neighbouring competitors and attract females from all directions; 2) because the energy of the signal can be focalized to increase the active space with neighbours and, 3) because it reduces the attraction of unintended receivers such as predators or parasites. Thus, the sound radiation pattern of the advertisement call of *A. femoralis* is probably adaptive, as it optimizes acoustic communication and territorial defence while reducing fitness-related costs.

In the evolution of acoustic communication, natural selection not only acts on the structure of the acoustic signal and the biophysics of the communication system (e.g., sound radiation patterns), but also on the calling behaviour of the signaler (Ryan and Kime, 2003). In chapter 2, I could show that *A. femoralis* males can vary the amplitude of the advertisement call as function of the calling-perch height, where males produce soft signals when perching high. With sound propagation experiments I showed that low amplitude calls emitted from high perches do not constraint sound propagation but present a comparable propagation pattern to louder calls produced from the forest ground. Also, I showed experimental evidence for one of our study populations in which males can possibly counteract the risk of predation when calling at high perches with Müllerian or Batesian mimicry (ESM1), as has been previously shown in other populations of *A. femoralis* (Amézquita et al., 2017). Furthermore, this mimicry defence might also ease the predation-related costs of the nearly omnidirectional radiation pattern of the advertisement call in *A. femoralis*. Thus, these adjustments in the calling behaviour have the potential to be favoured by natural selection, because they might serve as an energy saving mechanism without concomitant costs in the propagation of the acoustic signal and the risk of being predated.

Because amphibians lack the ability of self-regulation of body temperature and have highly permeable skins, most of their life histories are dependent on ambient temperature and the availability of water in the habitat (Wells, 2007). In chapter 3, I showed how the climatic conditions influence the territorial behaviour in *A. femoralis*. I found that *A. femoralis* use the

available substrates in its habitat to thermoregulate (i.e., thigmothermy) and an alleged water absorption response to regulate the water balance. It seems reasonable to think that these behavioural adaptations should be selected, because they mediate the responses to keep metabolic functions during hot and dry conditions. However, I also found that *A. femoralis* reduce its mating and parental activities during hot and dry environmental conditions. In this sense, despite the presence of behavioural mechanisms to thermo- and hydoregulate, the impact of elevations in ambient temperature has a deleterious effect on fitness. These results highlight the importance of investigating the thermal ecology of ectotherms in the neotropics, in order to know which mechanisms they use to deal with increments in global temperatures due to global warming.

Beyond the costs of territorial advertising on fitness, the regulatory mechanisms behind territorial behaviours can also conflict with other life histories in male vertebrates. Extensive evidence has shown that androgens are important in modulating both acoustic communication and territorial aggression in amphibians (Adkins-Regan, 2005; Moore et al., 2005). That is, androgens contribute to the development and activation of structures related to the production and perception of acoustic signals, but also enhance males' territorial aggression. While the first seems to be a conserved mechanism across vertebrate taxa, the latter varies and might produce detrimental effects on other life histories (Wingfield, 2001). For instance, among the negative effects of elevated levels of androgens the reduction on paternal care activities appears to be critical as it decreases reproductive success. In chapter 4, my results suggest that androgen-mediated territorial responses seem not to conflict with parental care in *A. femoralis* males. This result suggests novel support for the Challenge Hypothesis in an amphibian species, where elevated androgens might favour both territorial defence and reproductive success. It is worth noting that this result is far from being conclusive for Neotropical poison frogs, as this group present different combinations in sex roles during parental care and territorial defence (Wells, 2007). Nevertheless, my results in chapter 4 provide groundwork to understanding how territorial trade-offs may promote the evolution of hormone-behaviour interactions in Neotropical poison frogs.

Methodological considerations and future research

Overall, studies on the ecology and evolution of endocrine mechanisms in dendrobatids are scarce and are limited to manipulative (Schulte and Summers, 2017) or correlative (Fischer and O'Connell, 2020) experiments in the lab. Part of this limitation is due to the difficulty in obtaining hormone samples, because blood sampling is not feasible (in most cases) in such tiny frogs without excessive manipulation or even euthanasia. However, growing evidence on the effectiveness of water-borne sampling is challenging this view, because one can measure hormones in small amphibians, in a non-invasive and repeated way (Baugh and Gray-Gaillard, 2020; Bell, 2020). To the best of my knowledge, my study in chapter 3 is the first using water-borne hormones to measure androgens in a Neotropical poison frog. This is to highlight, not because is another species in the list of water-borne hormone studies (see below), but because it offers an alternative to measure hormones in a non-invasive way in frogs with a diverse suite of life history traits. With this method, researchers can evaluate, the regulatory mechanisms underlying different endocrine status (Adkins-Regan, 2005; Leary, 2009). Also, one can compare hormonal responses in a pre-post experimental design, as I did in chapter 4 by comparing water-borne androgens before and after the territorial challenge.

It is noteworthy, however, that one of the main limitations of this technique is that water-borne hormones do not mirror the amount of hormone that was available for binding to specific receptors, because water-borne hormones are product of the breakdown of plasma hormones into multiple molecules after being metabolized (Palme, 2005). Goymann in 2009 and 2012 suggested that studies using these hormone metabolites as means of circulating hormones must carry a biological or physiological validation. Indeed, we validated water borne-androgens in *A. femoralis* by conducting a correlation between water-borne androgen levels and testosterone concentration in plasma. We could demonstrate that androgen metabolites levels released in water are reliable indicators of those levels of testosterone in plasma. With this validation we extend the water-borne hormones sampling to Neotropical poison frogs (see Gabor et al., 2013; Baugh et al., 2018; Still et al., 2019; Baugh and Gray-Gaillard, 2020), but again most importantly, it offers a powerful tool to understanding the endocrine mechanisms underlying the social behaviour in amphibians.

One question that remains unsolved is whether tadpole transport would reduce the likelihood of an androgenic and aggressive response to a territorial intrusion in *A. femoralis*. Although tadpole transport is present in males, we did not test STIs in males when they were found transporting. One experimental way to answer this question would be to test STIs in males with induced tadpole-transport (i.e., transference of tadpoles from a developed clutch to the back of a territorial male; Pašukonis et al., 2017), and then measure androgen levels, after they start transporting. However, one limitation of such experimental design could be the negative effect of the manipulation on males' androgen levels while adding the tadpoles, because androgens are likely to be suppressed by the effect of restraint-related stress (McCreery and Licht, 1984). In this sense, this experiment may profit from water-borne hormones to measure the stress-related glucocorticoid response in males before adding tadpoles and in control situations (e.g., brushing the males' back without adding tadpoles). Overall, the results of such study could contribute to understand whether an androgen response to a territorial intrusion might depend on the type of parental care in Neotropical poison frogs.

Finally, as I mentioned earlier, it is important to highlight the complexity of sex roles in parental care and territoriality within Neotropical poison frogs. Although egg guarding and territorial aggressive behaviour are present in males of at least 25 species (Wells, 2007), in the rest of species either the male and/or the female present parental care and/or territorial aggression. For instance, females of the Panama rocket frog (*Colostethus panamensis*) present both parental care and territorial aggression, while males only present territorial aggression. Otherwise, in the blue poison dart frog (*Dendrobates azureus*), only males provide parental care and only females show territorial aggression. It would be interesting to investigate which endocrine mechanisms are in action when solving territorial trade-offs in those species with such complex sex roles in parental care and territoriality. With this approach one may contribute to broaden the predictions of the Challenge Hypothesis.

Concluding remarks

The study of trade-offs shaping life histories offers a powerful approach to understand how individuals optimize resource allocation and how behaviours evolve. In this thesis, I took

advantage of some of the most effective and novel techniques in the study of behavioural ecology and endocrinology, to investigate the trade-offs inherent to territorial defence in *Allobates femoralis*. I showed that males can adjust its calling behaviour to optimise communication and reduce fitness costs. That androgens mediate the trade-off between territorial defence and parental care. I showed that behavioural mechanisms allow a territorial frog to survive under extreme climatic conditions while keeping the territory. I demonstrate that water-borne hormones are an effective method to investigate hormone behaviour-relationships in Neotropical poison frogs, encouraging further research to study the endocrine mechanisms underlying life history trade-offs. Taken together, my results along this thesis provide insights into the mechanisms at work in a frog species facing the dilemma of having to choose between conflicting behavioral strategies.

References

- Adkins-Regan, E., 2005. Hormones and animal social behavior, Monographs in behavior and ecology. Princeton University Press, Princeton.
- Amézquita, A., Ramos, Ó., González, M.C., Rodríguez, C., Medina, I., Simões, P.I., Lima, A.P., 2017. Conspicuousness, color resemblance, and toxicity in geographically diverging mimicry: The pan-Amazonian frog *Allobates femoralis*. *Evolution* 71, 1039–1050. <https://doi.org/10.1111/evo.13170>
- Baugh, A.T., Bastien, B., Still, M.B., Stowell, N., 2018. Validation of water-borne steroid hormones in a tropical frog (*Physalaemus pustulosus*). *General and Comparative Endocrinology* 261, 67–80. <https://doi.org/10.1016/j.ygcen.2018.01.025>
- Baugh, A.T., Gray-Gaillard, S.L., 2020. Excreted testosterone and male sexual proceptivity: a hormone validation and proof-of-concept experiment in túngara frogs. *General and Comparative Endocrinology* 113638. <https://doi.org/10.1016/j.ygcen.2020.113638>
- Bell, A.M., 2019. Individual variation and the challenge hypothesis. *Hormones and Behavior* 104549. <https://doi.org/10.1016/j.yhbeh.2019.06.013>
- Bernal, X.E., Page, R.A., Ryan, M.J., Argo, T.F., Wilson, P.S., 2009. Acoustic radiation patterns of mating calls of the túngara frog (*Physalaemus pustulosus*): Implications for multiple receivers. *The Journal of the Acoustical Society of America* 126, 2757–2767. <https://doi.org/10.1121/1.3212929>
- Brenowitz, E.A., 1989. Neighbor Call Amplitude Influences Aggressive Behavior and Intermale Spacing in Choruses of the Pacific Treefrog (*Hyla regilla*). *Ethology* 83, 69–79. <https://doi.org/10.1111/j.1439-0310.1989.tb00520.x>
- Fischer, E.K., O'Connell, L.A., 2020. Hormonal and neural correlates of care in active versus observing poison frog parents. *Hormones and Behavior* 120, 104696. <https://doi.org/10.1016/j.yhbeh.2020.104696>
- Gabor, C.R., Gabor, C.R., Bosch, J., Gabor, C.R., Bosch, J., Fries, J.N., Gabor, C.R., Bosch, J., Fries, J.N., Davis, D.R., 2013. A non-invasive water-borne hormone assay for amphibians. *Amphib Reptilia* 34, 151–162. <https://doi.org/10.1163/15685381-00002877>
- Gerhardt, H.C., 1994. The Evolution of Vocalization in Frogs and Toads. *Annual Review of Ecology and Systematics* 25, 293–324. <https://doi.org/10.1146/annurev.es.25.110194.001453>
- Gerhardt, H.C., Diekamp, B., Ptacek, M., 1989. Inter-male spacing in choruses of the spring peeper, *Pseudacris* (*Hyla*) *crucifer*. *Animal Behaviour* 38, 1012–1024. [https://doi.org/10.1016/S0003-3472\(89\)80140-X](https://doi.org/10.1016/S0003-3472(89)80140-X)
- Goymann, W., 2012. On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. *Methods in Ecology and Evolution* 3, 757–765. <https://doi.org/10.1111/j.2041-210X.2012.00203.x>
- Goymann, W., 2009. Social modulation of androgens in male birds. *General and Comparative Endocrinology* 163, 149–157. <https://doi.org/10.1016/j.ygcen.2008.11.027>
- Leary, C.J., 2009. Hormones and acoustic

- communication in anuran amphibians. *Integrative and Comparative Biology* 49, 452–470.
<https://doi.org/10.1093/icb/icp027>
- McCreery, B.R., Licht, P., 1984. Effects of gonadectomy and sex steroids on pituitary gonadotrophin release and response to gonadotrophin-releasing hormone (GnRH) agonist in the bullfrog, *Rana catesbeiana*. *General and Comparative Endocrinology* 54, 283–296.
[https://doi.org/10.1016/0016-6480\(84\)90183-7](https://doi.org/10.1016/0016-6480(84)90183-7)
- Moore, F.L., Boyd, S.K., Kelley, D.B., 2005. Historical perspective: Hormonal regulation of behaviors in amphibians. *Hormones and Behavior* 48, 373–383.
<https://doi.org/10.1016/j.yhbeh.2005.05.011>
- Owings, D.H., Morton, E.S., 1998. *Animal Vocal Communication: A New Approach*. Cambridge University Press.
- Palme, R., 2005. Measuring Fecal Steroids: Guidelines for Practical Application. *Annals of the New York Academy of Sciences* 1046, 75–80.
<https://doi.org/10.1196/annals.1343.007>
- Pašukonis, A., Beck, K.B., Fischer, M.-T., Weinlein, S., Stücker, S., Ringler, E., 2017. Induced parental care in a poison frog: a tadpole cross-fostering experiment. *Journal of Experimental Biology jeb*—165126.
- Ryan, M.J., Kime, N.M., 2003. Selection on Long-Distance Acoustic Signals, in: Simmons, A.M., Fay, R.R., Popper, A.N. (Eds.), *Acoustic Communication*, Springer Handbook of Auditory Research. Springer-Verlag, New York, pp. 225–274.
https://doi.org/10.1007/0-387-22762-8_5
- Schulte, L.M., Summers, K., 2017. Searching for hormonal facilitators: Are vasotocin and mesotocin involved in parental care behaviors in poison frogs? *Physiology & Behavior* 174, 74–82.
<https://doi.org/10.1016/j.physbeh.2017.03.005>
- Still, M.B., Lea, A.M., Hofmann, H.A., Ryan, M.J., 2019. Multimodal stimuli regulate reproductive behavior and physiology in male túngara frogs. *Hormones and Behavior* 115, 104546.
<https://doi.org/10.1016/j.yhbeh.2019.06.010>
- Wells, K.D., 2007. *The Ecology and Behavior of Amphibians*. University of Chicago Press.
- Wells, K.D., Schwartz, J.J., 2006. The Behavioral Ecology of Anuran Communication, in: Narins, P.M., Feng, A.S., Fay, R.R., Popper, A.N. (Eds.), *Hearing and Sound Communication in Amphibians*, Springer Handbook of Auditory Research. Springer, New York, NY, pp. 44–86. https://doi.org/10.1007/978-0-387-47796-1_3
- Wilczynski, W., Brenowitz, E.A., 1988. Acoustic cues mediate inter-male spacing in a neotropical frog. *Animal Behaviour* 36, 1054–1063.
[https://doi.org/10.1016/S0003-3472\(88\)80065-4](https://doi.org/10.1016/S0003-3472(88)80065-4)
- Wiley, R.H., Richards, D.G., 1982. Adaptations for acoustic communication in birds: sound transmission and signal detection, *Acoustic communication in birds*. <https://doi.org/10.1016/B978-0-08-092416-8.50014-0>
- Wingfield, J.C., Lynn, S.E., Soma, K.K., 2001. Avoiding the ‘Costs’ of Testosterone: Ecological Bases of Hormone-Behavior Interactions. *Brain Behav Evol* 57, 239–251.
<https://doi.org/10.1159/000047243>

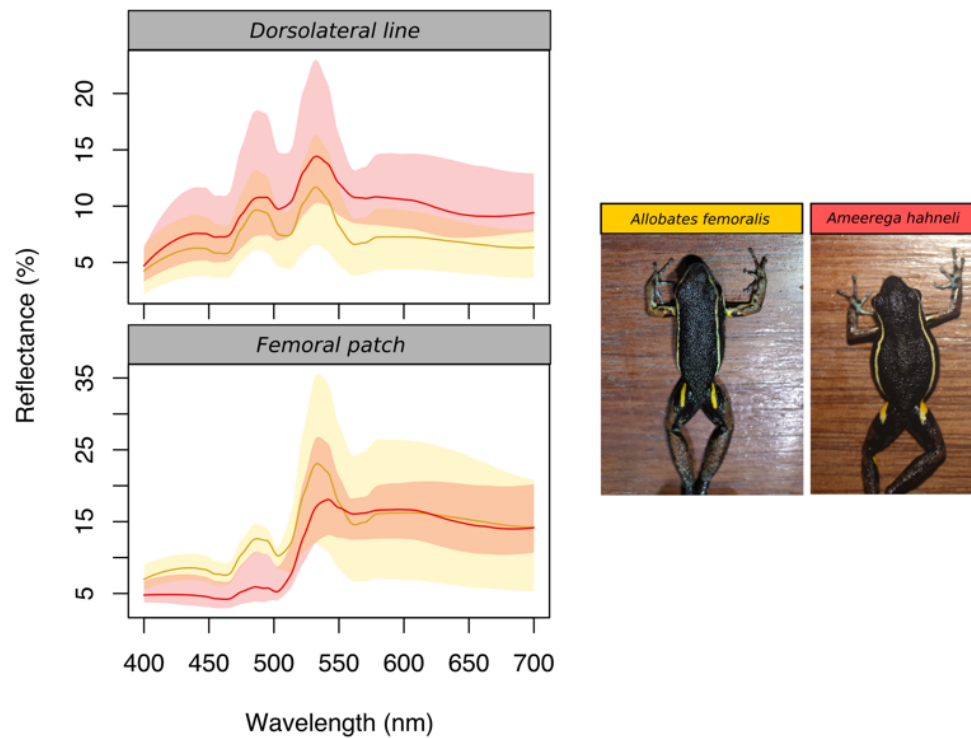
Appendix

Supplementary material in Chapter 2.

Table S1. Summarized results of planned least-squares means contrast for multiple comparisons between heights and SPL broadcasted in the signal propagation experiments. P-values were adjusted using Tukey's method.

Height (cm)		0			10			20	
SPL (dB)		73	76	81	73	76	81	73	76
Average (+/-SD)		47.96 (1.12)	-	-	-	-	-	-	-
0	76	48.5 (1.1)	0.26	-	-	-	-	-	-
	81	51 (2)	<0.0001	<0.001	-	-	-	-	-
	73	48.5 (1.5)	0.34	1	<0.001	-	-	-	-
10	76	49.3 (1.2)	<0.0001	0.03	<0.001	0.02	-	-	-
	81	52.3 (1.4)	<0.001	<0.001	0.06	<0.001	<0.001	-	-
	73	49.11 (1.1)	<0.001	0.22	<0.001	0.17	0.99	<0.001	-
20	76	50.2 (1.7)	<0.001	<0.001	<0.001	0.02	<0.001	<0.05	-
	81	53 (1.6)	<0.001	<0.001	<0.001	<0.001	0.03	<0.001	<0.001

ESM1. Reflectance light spectra of femoral patches and dorsolateral lines in *A. femoralis* (n=6) and *A. hahneli* (n=6) at the locality of Leticia. Shaded regions indicate 95% confidence intervals. Overlapping confidence intervals between the compared distributions indicate that the average values are statistically not different ($\alpha=0.05$). To quantify body coloration in frogs, we measured the colour reflectance of the frog's skin with a spectrophotometer (Ocean Optics USB4000/SpectraSuite software) of the two femoral patches and the two dorsolateral lines. Reflectance light spectra were obtained using the “procspec” and “aggplot” functions within the R package pavo (Maia et al., 2013).



Supplementary material in Chapter 3.

Table A.1. Estimates of least-squares means contrasts in above-canopy ambient temperature between years during the first third of the year from 2016 to 2020, at the field camp 'Pararé'.

Contrast	Estimate	SE	df	t-ratio	p-value
2019-2020	-0.01	0.07	902	-0.07	1
2018-2019	-0.23	0.07	902	-3.07	0.02
2018-2020	-0.23	0.07	902	-3.15	0.01
2017-2018	0.34	0.07	902	4.56	0.00
2017-2019	0.11	0.07	902	1.49	0.57
2017-2020	0.11	0.07	902	1.42	0.62
2016-2017	0.41	0.07	902	5.45	<.0001
2016-2018	0.75	0.07	902	10.01	<.0001
2016-2019	0.52	0.07	902	6.94	<.0001
2016-2020	0.51	0.07	902	6.87	<.0001

Table A.2. Estimates of least-squares means contrasts in above-canopy precipitation between years during the first third of the year from 2016 to 2020, at the field camp 'Pararé'.

Contrast	Estimate	SE	df	t-ratio	p-value
2019-2020	-2.60	1.25	902	-2.08	0.23
2018-2019	2.56	1.25	902	2.05	0.24
2018-2020	-0.04	1.25	902	-0.03	1
2017-2018	1.06	1.25	902	0.85	0.92
2017-2019	3.62	1.25	902	2.89	0.03
2017-2020	1.02	1.25	902	0.82	0.93
2016-2017	-1.96	1.25	902	-1.57	0.52
2016-2018	-0.90	1.25	902	-0.72	0.95
2016-2019	1.66	1.25	902	1.33	0.67
2016-2020	-0.94	1.25	902	-0.75	0.94

Table A.3. Weather features of *A. femoralis*'s habitat during the short dry season, at the field camp 'Pararé'. Mean, standard deviation (SD) and range of 320 measurements for Rainfall (mm), relative humidity (RH), mean understory temperature (Tambient), mean body temperature (Tbody) and mean substrate temperature (Tsubstrate).

Temperature	6h-8h	8h-10h	10h-12h	12h-14h	14h-16h	16h-18h	18h-20h	Total
Body								
Mean (SD)	22.5 (1.15)	23.8 (1.11)	25.0 (1.02)	25.4 (0.837)	25.6 (0.811)	25.4 (0.720)	24.8 (0.725)	24.7 (1.35)
Range	[20.7, 24.5]	[21.1, 25.6]	[22.3, 27.3]	[23.8, 26.9]	[24.1, 27.2]	[24.0, 27.4]	[23.7, 26.4]	[20.7, 27.4]
Substrate								
Mean (SD)	22.7 (1.12)	23.8 (1.11)	25.1 (1.10)	25.5 (0.885)	25.6 (0.798)	25.5 (0.722)	24.9 (0.710)	24.7 (1.35)
Range	[20.7, 24.5]	[21.1, 25.6]	[22.3, 27.3]	[23.8, 26.9]	[24.1, 27.2]	[24.0, 27.4]	[23.7, 26.4]	[20.7, 27.4]
Understory								
Mean (SD)	24.6 (1.27)	25.8 (1.56)	27.8 (0.981)	28.6 (0.900)	28.6 (1.00)	28.3 (0.754)	26.8 (0.726)	27.2 (1.76)
Range	[22.7, 27.1]	[22.5, 28.8]	[26.1, 29.8]	[26.6, 30.9]	[27.0, 30.9]	[27.2, 30.5]	[25.3, 28.6]	[22.5, 30.9]
Humidity								
Mean (SD)	88.7 (3.17)	89.2 (3.92)	85.6 (3.64)	83.0 (5.53)	83.9 (5.33)	85.2 (3.57)	89.2 (3.60)	86.4 (4.83)
Range	[78.1, 94.1]	[81.4, 95.1]	[78.2, 92.2]	[69.0, 91.0]	[74.7, 93.4]	[78.1, 92.1]	[81.5, 94.7]	[69.0, 95.1]

Table A.4. Estimates of least-squares means contrasts between temperatures of the most used substrates by *A. femoralis* during the short dry period, at the field camp 'Pararé'.

Contrast	Estimate	SE	df	t-ratio	p-value
Earth-Leaves	1	0.28	316	3.61	0
Earth-Log	0.58	0.24	316	2.37	0.08
Earth-Under_leaves	1.26	0.27	316	4.65	<.0001
Leaves-Log	-0.42	0.19	316	-2.18	0.13
Leaves-Under_leaves	0.26	0.23	316	1.15	0.66
Log-Under_leaves	0.68	0.19	316	3.69	0

Table A.5. Global, Across- and within-cluster distribution of categories in behaviours, substrates, understory temperature and relative humidity. Only categories with a p-value < 0.05 and positive v.test are included, as this shows over-expressed categories that are significantly linked to the other categories.

Variable	Cluster 1			Cluster 2			Cluster 3			Cluster 4			Cluster 5		
	Across cluster (%)	Within cluster (%)	Global (%)	Across cluster (%)	Within cluster (%)	Global (%)	Across cluster (%)	Within cluster (%)	Global (%)	Across cluster (%)	Within cluster (%)	Global (%)	Across cluster (%)	Within cluster (%)	Global (%)
T (°C)															
23-26.2	4.22	53.84	26.56	2.40	2.28	26.56	79.51	88	26.56	-	-	-	8.48	10.40	43.36
26.2-27.6	-	-	-	82.08	81.14	27.68	7.51	8.66	27.68	-	-	-	0	0	27.68
27.6-28.3	0	0	21.92	21.16	16.57	21.92	2.18	2	21.92	5.10	10.60	21.92	71.53	44.34	21.92
28.3-30.6	0	0	23.84	0	0	23.84	1.34	1.33	23.84	16.10	36.36	23.84	82.55	55.65	23.84
RH (%)															
72.1-83.2	0	0	33.60	0	0	33.6	0.47	0.66	33.60	5.23	16.66	33.60	94.28	89.59	33.6
83.2-90.5	4.05	84.61	43.36	59.4	92	43.36	12.54	22.66	43.36	15.5	63.63	43.36	8.48	10.4	43.36
90.5-95.1	-	-	-	9.72	8	23.04	79.86	76.66	23.04	-	-	-	0	0	23.04
Substrate															
Earth	-	-	-	8.19	2.85	9.76	1.63	0.66	9.76	1.63	1.51	9.76	88.52	24.43	9.76
Leaves	0	0	40.48	60.07	86.85	40.48	28.45	48	40.48	-	-	-	2.37	2.71	40.48
Log	0	0	47.68	6.04	10.28	47.68	-	-	-	14.09	63.63	47.68	54.02	72.85	47.68
Under leaves	100	100	2.08	0	0	2.08	0	0	2.08	-	-	-	0	0	2.08
Behaviour															
Calling	-	-	-	83.33	14.28	4.80	-	-	-	0	0	4.80	0	0	4.8
Feeding	-	-	-	8.33	1.14	3.84	-	-	-	-	-	-	75	8.14	3.84
Short movements	-	-	-	-	-	-	23.06	92.8	80.48	0	0	33.60	40.15	91.40	80.48
'Rubbing'	-	-	-	0	0	10.88	0	0	10.88	97.05	100	10.88	1.47	0.45	10.88
Sex															
Female	-	-	-	12.65	11.42	25.28	46.83	49.33	25.28	16.45	39.39	25.28	20.88	14.93	25.28
Male	-	-	-	33.19	88.57	74.72	16.27	50.66	74.72	8.56	60.60	74.72	40.25	85.06	74.72

Table A.6. Estimates of least-squares means contrasts between long-range distances of males and females of *A. femoralis*, during the rainy and short dry period, at the field camp 'Pararé'.

Contrast	Estimate	SE	df	t-ratio	p-value
Frainy-Mrainy	-10.74	2.36	21	-4.56	0
Frainy-Fshort_dry	-1.17	3.47	59.80	-0.34	1
Frainy-Mshort_dry	-0.06	3.21	44.2	-0.02	1
Mrainy-Fshort_dry	9.58	3.05	66	3.14	0.01
Mrainy-Mshort_dry	10.68	2.75	44.5	3.89	0
Fshort_dry-Mshort_dry	1.11	3.75	85.3	0.3	0.99

Video A.1. Video sequence of the alleged water absorption response (Rubbing behaviour) in *Allobates femoralis*.

Supplementary material in Chapter 4.

Table S1A. Principal Component Analysis showing the loadings matrix of three variables related to responsiveness latencies.

Variable	Principal components		
	PC1	PC2	PC3
Latency to the 1 st head-body orientation (sec)	0.99	-0.01	-0.16
Latency to the 1 st 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

Figure S1A. Validation of the extraction method for water-borne androgen. (A) Recovery percentages of testosterone standards of 0 (Blank), 200 and 1000 pg mL⁻¹; (B) Correlation between expected and obtained testosterone concentrations; (C) Release rates of water-borne androgen in 60-min, 30-min and 15-min. Release rates are also shown for females in 60-min water baths. Solid black-points and bars represent the mean and 95% confidence intervals, respectively. *P<0.05.

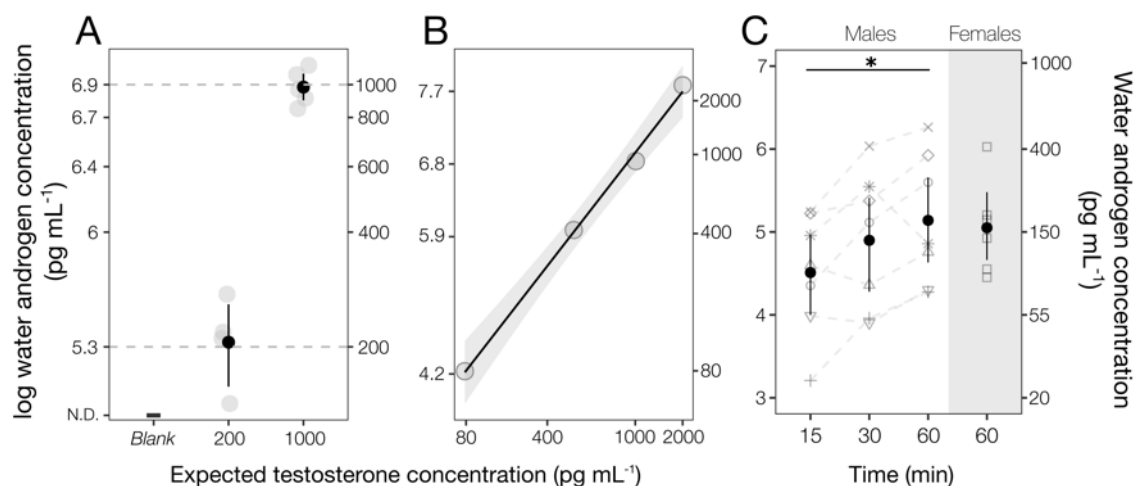


Figure S2A. Comparison between males and females in water-borne androgen concentration. Both, males and females were placed in individual water baths for 60 min. **P<0.01.

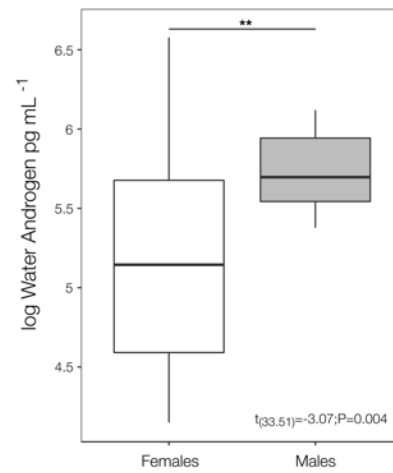
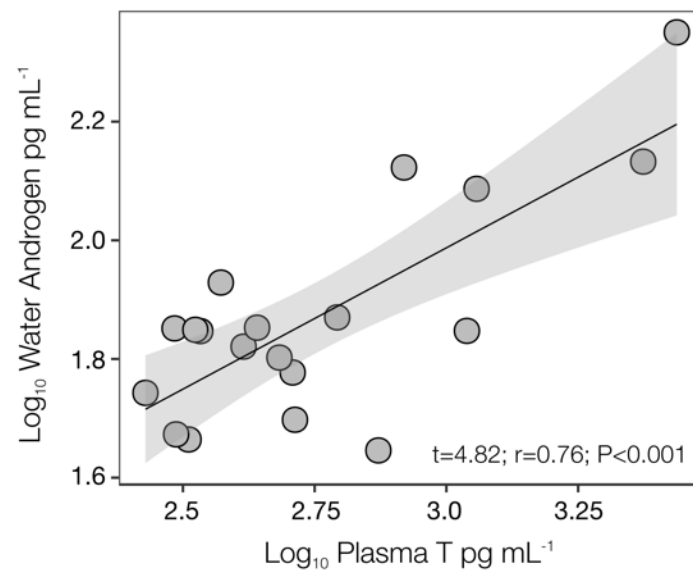


Figure S3A. Parametric correlation (Pearson) between plasma testosterone and water-borne androgen concentrations. Shaded grey region represents 95% confidence intervals.



Abstract

Territoriality is the defence of an area containing critical resources for the reproductive success and survival of the territory holder. The defence of a territory involves a multitude of behavioural mechanisms such as ritualized displays and aggressiveness towards competitors. However, territorial defence also entails costs related to energy expenditure and parental investment, among others, which often result in trade-off decisions. The study of such trade-offs offers a powerful method to understand how territorial animals optimize resource allocation and how behaviours evolve. In this PhD thesis I explored the mechanisms underlying territorial trade-offs in the Brilliant-thighed poison frog, *Allobates femoralis*, a highly territorial species. To do so, I combined behavioural and hormonal methods to investigate how *A. femoralis* respond to natural conflicting situations while defending a territory. In chapter 1 and 2, I used a correlative approach together with sound propagation experiments and acoustic playbacks to show that males adjust their vocal behaviour to optimize energy use and sound propagation. In chapter 3, I confronted males to territorial challenges, in form of acoustic playbacks, to show that males present an androgen and behavioural response to territorial intrusions, without interfering with egg attendance. Finally, in chapter 4, I used behavioural and thermal measurements combined with passive telemetry to show that *A. femoralis* present behavioural modifications in response to hot and dry climatic interruptions during the breeding season, while keeping the territory. Altogether, my results demonstrate that *A. femoralis* can flexibly adjust its behaviour to optimize territoriality when facing trade-off situations. This contributes to our understanding on the selective pressures underlying evolutionary changes in territorial behaviours.

Zusammenfassung

Territorialität im Tierreich ist die Verteidigung eines Gebiets, welches wichtige Ressourcen für den Fortpflanzungserfolg sowie für das Überleben beinhaltet. Zur Verteidigung eines solchen Gebiets, bzw. Reviers werden eine Vielzahl von Mechanismen wie ritualisierte Verhaltensweisen und Aggressivität gegenüber Konkurrenten und Eindringlinge ausgeübt. Die Revierverteidigung ist jedoch auch mit Kosten – wie zusätzlicher Energieaufwand und elterliche Investitionen in die Brutpflege – verbunden, was häufig zu Kompromissentscheidungen führt. Die Untersuchung solcher Kompromisse bietet eine wirkungsvolle Methode, um zu verstehen, wie territoriale Tiere die Ressourcenverteilung optimieren und wie sich ihr Verhalten entwickelt. In der vorliegenden Dissertation untersuchte ich die Mechanismen, die solch territorialen Kompromissen zugrunde liegen anhand einer äußerst territorialen Pfeilgiftfroschart, *Allobates femoralis*. Um zu zeigen wie *A. femoralis* Männchen auf natürliche Konfliktsituationen während der Revierverteidigung reagieren, wurden verhaltensbiologische und hormonelle Methoden kombiniert. In den Kapiteln 1 und 2 wurde mittels eines korrelativen Ansatzes in Verbindung mit Schallausbreitungsexperimenten und akustischen Playbacks gezeigt, dass die Männchen ihr Rufverhalten so anpassen, dass der Energieverbrauch sowie die Schallausbreitung optimiert werden. In Kapitel 3 wird beschrieben, dass die Männchen bei territorialen Herausforderungen (akustische Playbacks simulierter Eindringlinge) eine hormonelle sowie auch eine Verhaltensreaktion zeigen, um territoriale Eindringlinge abzuwehren, ohne dabei die Eiablage zu beeinträchtigen. Schließlich wird im Kapitel 4 gezeigt, wie *A. femoralis* sein Verhalten ändert, um auf heiße und trockene klimatische Bedingungen während der Brutzeit zu reagieren und gleichzeitig sein Territorium zu behalten. Dazu wurden Verhaltens- und Wärmemessungen in Kombination mit passiver Telemetrie verwendet. Insgesamt zeigen meine Ergebnisse, dass der territoriale Pfeilgiftfrosch *A. femoralis* sein Verhalten in Kompromissituationen zum Zwecke der Territorialität flexibel anpassen kann. Daraus können Schlüsse über Selektionsdruck und die zugrundeliegenden evolutionären Veränderungen der Territorialität gezogen werden.

