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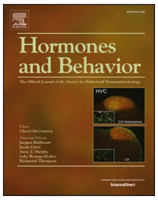

Does testosterone underly the interplay between male traits and territorial behavior in neotropical poison frogs?

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ABSTRACT

The ability of individual animals to defend a territory as well as various phenotypic and behavioral traits may be targets of sexual selection used by males to evaluate their competitors or by females to choose males. A frequent question in animal behavior is whether male traits and characteristics of their territory are correlated and what are the mechanisms that may mediate such associations when they exist. Because hormones link phenotype to behavior, by studying the role of testosterone in territoriality one may come closer to understanding the mechanisms mediating correlations or lack thereof between characteristics of territories and of males. We evaluated whether variation in characteristics of territories (size and quality) are correlated with variation in morphology, coloration, testosterone, heterozygosity, and calls in two species of poison frogs. The Amazonian frog *Allobates* aff. *trilineatus* exhibits male care and defends territories only during the breeding season, while the endangered frog *Oophaga lehmanni* displays maternal care and defends territories throughout the year. We found that morphological traits (body length, weight, thigh size), call activity, and testosterone levels correlated with size and various indicators of quality of the territory. However, the direction of these correlations (whether positive or negative) and which specific morphological, acoustic traits or testosterone level variables covaried depended on the species. Our findings highlight an endocrine pathway as part of the physiological machinery that may underlie the interplay between male traits and territorial behavior. We were able to identify some male traits related to territory attributes, but whether females choose males based on these traits requires further research.

1. Introduction

The quality of males is often defined by the fitness of their offspring (Harris and Uller, 2009; Möller and Alatalo, 1999). Various traits of the phenotype of individual animals are linked to their quality. Accordingly, traits such as body condition or body size (Blanckenhorn et al., 1998; Gleeson et al., 2005; O'dwyer et al., 2006), call activity or call parameters (Forsman and Hagman, 2006; Friedl and Klump, 2005; Galeotti et al., 1997; Pröhl and Hödl, 1999; Sirkiä and Laaksonen, 2009; Welch et al., 1998), coloration (Barber et al., 2001; Hill, 1991; Sheldon et al., 2003; Sirkiä and Laaksonen, 2009), courtship displays (Kotiaho et al., 1996, 2001), and ornaments (Wedekind et al., 2001) may be targets of sexual selection. Also, the territories of males and the ability of individuals to defend them may be honest indicators of male quality or social status (Ens et al., 1992; Högstedt, 1980; Price, 1984; Trivers, 1972; Yang and Richards-Zawacki, 2021). Thus, both traits intrinsic to males and traits of their territories may be used by other males to evaluate their competitive ability or by females to choose mates (Alatalo et al., 1986; Candolin and Voigt, 2001; Stamps, 1994; Wong and Candolin, 2005).

Hormones are phenotypic integrators which link phenotype to behavior (Emerson and Hess, 2001; Hau et al., 2016; Ketterson and Nolan, 1992; Lipshutz et al., 2019; Martin et al., 2011; Wilczynski et al., 2005). Some studies have evaluated interactions between hormones and behavior, particularly in terms of understanding associations among life-history traits, circulating androgens, and mating behaviors (Emerson and Hess, 1996; Fusani, 2008; Fuxjager et al., 2022; Leary, 2009; Wingfield et al., 1990; Woodley, 2010). However, although territorial behavior is widespread among animals and studying such behavior is essential in various fields of behavioral and evolutionary ecology, there

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are relatively few studies of the physiological mechanisms underlying territoriality in the wild. Physiological processes in which hormones (e.g., testosterone) and neuropeptides are involved may modulate the expression of traits such as body condition (Lind et al., 2018; Lind and Beaupre, 2015; Wittman et al., 2021), development of structures or muscle mass (Brantley et al., 1993; Emerson et al., 1999; Girgenrath and Marsh, 2003; Nagaya and Herrera, 1995), coloration (Sinervo et al., 2000; Wittman et al., 2021), locomotor performance (Miles et al., 2007), metabolic rate (Buchanan et al., 2001), or calling behavior (Emerson and Hess, 1996; Leary, 2009; Leary et al., 2004). Therefore, by studying the role of hormones in territoriality and their links with phenotypic traits of males, one may come closer to understanding the mechanisms by which correlations between male traits and those of their territories arise or not.

In species in which males establish breeding territories, characteristics of territories (e.g., size) and phenotypic traits of males associated with reproductive success are often correlated (Balmford et al., 1992; Bart and Earnst, 1999; Candolin and Voigt, 2001; Dale and Slagsvold, 1990; Price, 1984; Reid and Weatherhead, 1990; Roithmair, 1994a; Seddon et al., 2004; Vanpé et al., 2009). Such correlations may reflect that both characteristics of territories and intrinsic male traits are indicators of male quality (Searcy and Yasukawa, 1983; Verner, 1964; Yasukawa, 1981), yet the direction of the correlations may vary depending on various factors. First, correlations between traits can be positive if similar selection pressures act on such traits, if they depend on each other, or if traits act jointly as quality indicators (Alonso et al., 2010; Andersson et al., 2002; Johnstone, 1996; Searcy and Yasukawa, 1983). Alternatively, correlations may be negative if there are tradeoffs such that producing or maintaining traits while defending territories with particular features implies greater energy and survival costs (Anderson and Iwasa, 1996; Andersson et al., 2002; Kotiaho, 2001; Moller and de Lope, 1994; Roberts et al., 2004; Searcy and Yasukawa, 1983). In other cases, traits may exhibit no correlation if they are under different selection pressures or if any of them is not a reliable indicator of individual quality (Qvarnström and Forsgren, 1998; Wong and Candolin, 2005).

In territorial species, the interplay among territory attributes, male traits, and reproductive success does not adhere to a consistent pattern. For example, territory size correlates with male body mass and with female density in a species of mammal (*Capreolus capreolus*; Vanpé et al., 2009). In some bird species, females may choose mates according to their territory size, but territory size may or may not correlate with morphological traits or parameters of the song of males, and such attributes of males may or may not correlate with each other (Dale and Slagsvold, 1990; Reid and Weatherhead, 1990). Also in birds, genetic diversity can correlate with territory size, singing behavior, and reproductive success of males, indicating that territory size may be an honest indicator of genetic quality and male fitness (Seddon et al., 2004). Genetic heterozygosity may also be linked to the competitive ability of males and to the ownership of a territory in different taxa (Höglund et al., 2002; Lieutenant-Gosselin and Bernatchez, 2006; Tiira et al., 2006; Tiira et al., 2003). Although associations of morphological traits, song parameters and genetic diversity with territory size and reproductive success have been studied in a variety of territorial organisms, we are unaware of comprehensive studies jointly evaluating interactions among all such traits, territory quality, and hormones in animals.

Neotropical poison frogs (families Aromobatidae and Dendrobatidae; Grant et al., 2006) exhibit a wide diversity of mating and parental care strategies (Ah-King et al., 2004; Summers and McKeon, 2004; Summers and Tumulty, 2014; Wells, 2007; Wells, 1977), and males defend multipurpose areas with visual displays, advertisement calls, or physical attacks (Pröhl, 2005; Wells, 1977). Studies in this group have found contrasting evidence of the relationship between territory size, morphological and behavioral traits of males, and reproductive success (Marques Correia et al., 2018; Meuche et al., 2012; Roithmair, 1994b; Roithmair, 1992). For example, body mass, body size, call activity, reproductive success, and territory size of males of *Oophaga pumilio* are uncorrelated, but territory size and body condition of males of this species are negatively correlated (Meuche et al., 2012). In contrast, positive relationships between territory size, call activity and mating success have been documented in *Allobates femoralis*, *Ameerega trivittata*, and *Allobates paleovarzensis* (Marques Correia et al., 2018; Roithmair, 1994a; Roithmair, 1992). Considering these patterns, one may hypothesize that in species with paternal care, in which the male carries the tadpoles, vocal activity may be a signal used by females to gauge physical condition and competitive ability of males (Pröhl, 2005; Pröhl, 2003; Roithmair, 1994a), whereas in species in which tadpole survival depends on care provided by females, characteristics of the territory rather than those of the male are more likely chosen by females. However, because few studies have evaluated correlates of characteristics of the territory beyond its size, hypotheses about the interactions among traits of males, their territories, and mating success have not been amenable to evaluation.

Ecological characteristics of territories may be key factor for mating and for the survival of offspring (Pröhl, 2005; Yang and Richards-Zawacki, 2021). In poison frogs with paternal care, in which males transport tadpoles, females assess territories and males prior to mating (Roithmair, 1992, 1994a; but see Ringler et al., 2009). In these species, proximity to water bodies is likely a critical factor for successful tadpole development (Pašukonis et al., 2016). Accordingly, in *A. femoralis* the number of tadpoles transported correlates positively with distance from the water body (Ringler et al., 2013), although males may spread their offspring over several ponds (Pašukonis et al., 2019). In contrast, in species in which tadpole development occurs within the male's territory, usually in phytotelmata, the availability of such elements is critical (Donnelly, 1989a, 1989b; Pröhl, 2002). Likewise, the abundance of leaf litter is probably crucial for sheltering, oviposition, and maintaining the humidity and temperature of eggs. However, we do not know of any studies in anurans that concurrently evaluate whether ecological characteristics of the territory, beyond size, correlate with traits of males and their testosterone levels.

We sought to understand the relationship between traits of males and characteristics of their territories in the Neotropical poison frogs *Oophaga lehmanni* and *Allobates* aff. *trilineatus*, which represent distinct reproductive strategies (paternal vs maternal care) within dendrobatids. We evaluated whether the size and ecological features of territories correlate with phenotypic (body condition, coloration), physiological (testosterone levels), behavioral (vocal effort), and genetic (heterozygosity) traits of males of both species. To date, no study had jointly evaluated interactions among all such traits, and no previous analyses had considered links between testosterone concentrations and male traits and characteristics of territories, beyond its size. Because a greater number of signals indicating the quality of the male would likely facilitate mate choice by females and reduce the costs or errors associated with mate choice (Candolin and Reynolds, 2001; Johnstone, 1996), we hypothesized that males with better body conditions have higher testosterone levels and in turn exhibit greater vocal activity, allowing them to defend larger territories or territories with key resources for breeding. Also, because coloration may be under sexual selection (Maan and Cummings, 2009; Rojas and Endler, 2013) and given that genetic diversity correlates with territory size in different organisms (Höglund et al., 2002; Lieutenant-Gosselin and Bernatchez, 2006; Seddon et al., 2004), we predicted more colorful and more heterozygous males to have larger territories or to defend areas with greater abundance of key resources.

2. Materials and methods

2.1. Study species

The nurse frog *Allobates trilineatus* (Boulenger, 1884) is a diurnal and small dendrobatid (mean ± s.d. females = 17.09 ± 0.12 mm $N = 57$, males = 16.45 ± 0.01 mm $N = 48$; Grant and Rodríguez, 2001) from the Amazonian region of Peru, Ecuador and Colombia, which shows phenotypic and acoustic differentiation indicating it likely represents a species complex (Grant et al., 2006; Jaramillo et al., 2021). We studied a population of *Allobates* in the Tanimboca Nature Reserve in the Colombian Amazon (Km 11, Leticia, Amazonas). Because this population differs phenotypically and acoustically from *A. trilineatus* sensu stricto (Jaramillo et al., 2021), we refer to it as *Allobates* aff. *trilineatus*. This frog with dark brown dorsal coloration inhabits secondary forests, where males defend territories during the reproductive season (Betancourth-Cundar et al., in review). Males exhibit parental care that consists of wetting eggs and transporting larvae to bodies of water located outside male territories (Ringler et al., 2009). The reproductive season is from October-November to March-April and is tightly linked to the rainy season. We collected phenotypic, physiological, and behavioral data during two reproductive seasons between January and February 2017 and 2018.

Lehmann's Poison Frog *Oophaga lehmanni* (Myers and Daly, 1976) is a diurnal, aposematic, and relatively large dendrobatid (females 34.86 ± 1.85 mm $N = 34$, males = 35.56 ± 2.07 mm $N = 117$; Betancourth-Cundar and Palacios-Rodriguez, 2022) The species is endemic to Colombia, where it is narrowly restricted to its type locality (upper Río Anchicayá drainage, Valle del Cauca). Owing to massive commercial overexploitation for the pet trade and the destruction of its natural habitat, this species has declined drastically in the last 40 years (Betancourth-Cundar et al., 2020; Betancourth-Cundar and Palacios-Rodríguez, 2018; Castro-Herrera and Amézquita, 2004) and is currently categorized as Critically Endangered (IUCN SSC Amphibian Specialist Group, 2019). Males of *O. lehmanni* defend territories throughout the year and often occupy them for several years (M. Betancourth, unpubl. data). Egg deposition and larval development occur within the male's territory. Females exhibit maternal care, which consists of feeding tadpoles with unfertilized eggs until their development is complete (Bauer, 1994; Myers and Daly, 1976; Silverstone, 1973). We studied a population of *O. lehmanni* in March-May 2018 at 600 m elevation in primary forests in the Farallones de Cali National Park, nearby the Alto Anchicayá hydroelectric dam (Empresa de Energía del Pacífico-EPSA).

2.2. Territoriality data

We collected data for 18 males of *A.* aff. *trilineatus* during the 2017 and 2018 breeding seasons, and for 9 males of *O. lehmanni* in 2018. Data on territory size were taken from a previous study where estimates were obtained with intrusion experiments and analyzed using different area estimators: Generalized Additive Models (GAM), Kernel Density Estimators (KDE) using different smoothing parameters, and Minimum Convex Polygons (MCP; Betancourth-Cundar et al., in review). KDE use smoothing parameters to determine the width of isopleths connecting areas of equal use (i.e., with an equal probability of encountering a male; Barg et al., 2005; Calenge, 2006). Of three variations considered for the smoothing parameter (h_{ref}) in the previous study, for the present analyses we chose to employ estimates of territory size obtained using the smallest value for the smoothing parameter (KDE-h_{ref} minimum), which were similar to those calculated with MCP (Betancourth-Cundar et al., in review). In addition to estimates obtained using KDE-h_{ref} minimum, we also conducted analyses with MCP estimates because the two approaches provide different information about territorial behavior. MCP generates a minimum-area polygon (Mohr, 1947) where the focal male engaged in defensive behavior, whereas KDE allows one to identify areas of higher or lower use (Seaman and Powell, 1996).

To assess possibly defended resources within the territory of each male, we examined several potentially relevant variables based on the natural history of each species (Donnelly, 1989b; Ringler et al., 2013; Summers and McKeon, 2004). For *A.* aff. *trilineatus*, where oviposition takes place on the leaf litter and the tadpoles are transported by the males to bodies of water, we measured the distance to the nearest body of water, the coverage of leaf litter and low vegetation (<150 cm), and the depth of leaf litter (measured at three randomly selected points) in each territory. Considering that territories may be differentially affected by the temperature due to their spatial distribution, especially in Amazonia where vocal activity decreases considerably on sunny days (MBC, per. obs.), we measured the temperature of leaf litter at five randomly chosen points within each territory on three different days. On these same days, we also measured the temperature of the perch most frequently used by the males.

For *O. lehmanni*, in addition to leaf litter depth, we measured perch height, coverage of low vegetation (<150 cm), and the number of large (height from base to leaf tip >40 cm), medium (height between 20 and 40 cm) and small (height < 20 cm) bromeliads. For variables with more than one measurement, we used mean values for analyses. For each dataset, we did a Principal Component Analysis (PCA) with the 'psych' Package (Revelle, 2016) for R (R Core Team, 2013) and used the first two components to summarize ecological characteristics in two variables. To evaluate the relationship between size and ecological characteristics of the territory, we conducted a Pearson correlation analysis between the two principal components and the two area estimates (MCP and KDE).

2.3. Phenotypic data

At the end of the experiments to estimate territory size, we manually captured focal males, and then took morphological data, photographs, and hormone measurements. We measured the weight, body size, and thigh area of each focal male. We estimated weight using a digital scale to the nearest 0.01 g. From dorsal and ventral photographs taken with a digital camera (Canon PowerShot SX170) using size and color standards (Kodak Q-13), we estimated body size as snout-ventral length (SVL) to the nearest 0.1 mm. Using the photographs, we also calculated thigh area as the average between dorsal and ventral area of the right and left thighs measured using ImageJ software (Schneider et al., 2012). Based on body mass and SVL, we estimated body condition using a Scaled Mass Index (SMI), an indicator of energy reserves (Peig and Green, 2009) previously used in other studies in amphibians (Brodeur et al., 2020; MacCracken and Stebbings, 2012). Because for *A.* aff. *trilineatus* we did not find a significant relationship between body weight and SVL, we did not calculate the SMI; instead, we used the original variables as indicators of body condition in the subsequent analysis.

For *O. lehmanni*, in addition to morphological data, we quantified dorsal coloration traits. Conspicuousness in aposematic coloration could be subject to natural selection (Summers and Clough, 2001; Ruxton et al., 2004) may also be under sexual selection (Maan and Cummings, 2009; Reynolds and Fitzpatrick, 2007). We measured light reflectance on the frog's dorsum ($\lambda = 300$–700 nm) with a 600-μm bifurcated fiber-optic (QR600-7-UV/VIS) coupled to a spectrometer (USB4000) using a pulsed xenon light lamp (PX-2) as a light source. Before each measurement, the equipment was calibrated with a white standard (WS-1-SL) and a probe with a rubber back cover was used to exclude ambient light. Measurements were done at 2 mm and perpendicular to the surface. We described the coloration of frogs obtaining hue, chroma, and brightness data: H1-Peak wavelength, S8-Chroma, and B2-Mean brightness, respectively (Maia et al., 2013; Montgomerie, 2006). Reflectance data were analyzed with the PAVO package (Maia et al., 2013) for R (R Core Team, 2013). Conspicuousness in coloration was estimated as the chromatic contrast in Just-Noticeable-Differences (JNDs) relative to of a trunk substrate where males of *O. lehmanni* are found most frequently (Betancourth-Cundar and Palacios-Rodriguez, 2022). JND values equal to or >1 indicate that the observer can distinguish the object from its background; therefore, higher JND values indicate greater contrast (Maia et al., 2013). To estimate conspicuousness, we used two vision models: (1) birds with UV-vision (Endler and Mielke, 2005; Hart and Vorobyev, 2005) to evaluate perception by potential predators of *O. lehmanni*, and (2) a poison frog (*Oophaga pumilio*; Siddiqi et al., 2004) to evaluate how our study subjects may perceive each other in the context of sexual selection. For both models, we used measures of ambient light for forest included in the PAVO package (Maia et al., 2013).

2.4. Vocal activity

To estimate the vocal activity of each focal male, we installed automatic recorders (Song Meter SM3) 1 m from the perch where the male was observed most frequently. For *A.* aff. *trilineatus*, we recorded males between 4:00 and 19:00 h and estimated vocal effort as the total number of calls during the day and the average daily number of calls per male. For *O. lehmanni*, we recorded during two consecutive days between 5:00 and 19:00 h. Because of the uni-note call structure, we estimated vocal effort as the total time spent by the male calling during the day. To analyze automatic recordings, we used the Kaleidoscope Pro Analysis Software, version 5.4.2 (Wildlife Acoustics Inc.). We first separated vocalizations of focal species from those of other anurans using a cluster analysis. Then, to find vocalizations of focal males (i.e., to distinguish them from calls of other males), we built and trained advanced classifiers to identify recordings of each individual while reducing the false-positive rate (i.e., identifying calls of a conspecific male as if they were from the focal male). For this, we created manual identifications for individual vocalizations within clusters and then used those manual IDs to create and train the advanced classifier which we used to analyze the other recordings. The result was visually double-checked to avoid false clustering. To do this we used the default spectrograms of the software. We distinguished the calls of focal males because they always had greater amplitude (or energy) than calls from neighboring males.

2.5. Sample collection and hormone assays

Hormone levels were measured using a water-borne sampling method previously designed for fish (Kidd et al., 2010; Scott et al., 2008; Scott and Ellis, 2007) but used and validated in amphibians (Baugh et al., 2018; Baugh and Gray-Gaillard, 2021; Gabor et al., 2013; Rodríguez et al., 2022). This non-invasive technique allows one to sample the same individual multiple times, is less stressful than drawing blood, and enables non-lethal measurements on small animals (Narayan, 2013; Scott and Ellis, 2007). In anurans, water-borne steroid levels probably reflect the combined contributions of metabolites excreted by urine and feces and free steroids released from the skin and mucous membranes (Baugh et al., 2018; Narayan, 2013).

To collect samples from water, we placed each frog in a glass Buchner funnel and a glass funnel functioning as a cover. The glass funnel was coupled to a water inflow orifice which consisted of a 150 mL buretrol containing 70 mL of sterile water, and the Buchner funnel contained an outflow orifice to collect the water-borne hormones. Funnels were covered with a black box to minimize handling stress. Samples were collected in a sterile glass beaker, transferred to a 70 mL syringe, and manually filtered with a Sep-Pack Plus C18 cartridge (SPE, Sep-Pak C18 Plus, 360 mg Sorbent, 55 - 105 μm particle size, #WAT020515, Waters corp., Milford, MA), which retains non-polar compounds. Samples were then eluted with 4 mL of 100 % methanol in 4 mL borosilicate vials and stored at -20 °C until further analysis. Males of *A.* aff. *trilineatus* studied in 2017 were captured in the field and transferred to the Universidad de los Andes to standardize the protocol to measure hormones. Data for 2018 for both *A.* aff. *trilineatus* and *O. lehmanni* were obtained in the field. To reduce the effects of stress generated by capture, to monitor the conditions to which the frog is exposed before the experiment, and to induce habituation to captivity, the hormone sampling for animals in the field began no later than 12 h after capture and 12 h of fasting for captive frogs. Some studies in amphibians have found that hormone profiles, especially testosterone, cortisol and corticosterone, return to basal levels after 2 h of stimulation (Forsburg et al., 2019; Rodríguez et al., 2022). Additionally, a recent study revealed that basal testosterone levels within the same individual remain highly consistent throughout the reproductive season. As a result, even a limited number of repeated measurements offer reliable estimates of individual basal hormone profiles in male poison frog (Ringler et al., 2024). Because we wanted to quantify basal levels of testosterone, we took samples repeatedly in the same individual throughout the day (8:00, 12:00, 16:00, and 20:00 h). To get an estimate of circadian testosterone release at rest, for the first measurement (8:00 h) the frog was left in sterile water the night before (20:00 h). Therefore, the first measurement assayed 12 h of hormone release, whereas the subsequent three measurements assayed hormones released over periods of four hours.

Hormone concentrations were measured using a commercial Enzyme-linked immunosorbent assay (ELISA) with testosterone-specific antibodies (ADI-900-065; Enzo Life Sciences, Farmingdale, NY, 262 USA) and following the manufacturer's protocol with variations (see Baugh et al., 2018; Rodríguez et al., 2022). Samples were dried with N2 at 37 °C, then resuspended with 250 μL of the assay buffer, and incubated overnight at 4 °C. Samples were analyzed by duplicates and plates were read at 405 nm, with adjustments between 570 and 590 nm using a microplate reader (Multiskan Go, Thermo Fisher Scientific Oy, Finland). Based on readings, testosterone concentrations were estimated with the Thermo Scientific SkanIt Software (version 4.1). We calculated testosterone concentrations for each period by dividing the total concentration by the number of hours of sample collection (i.e., 12 or 4 h) and expressed it in pg/mL/h. The intra-assay coefficient of variation (CV) of duplicates was below 5.99 %. The average inter-assay coefficient of variation was 7.30 %. It was calculated by including one pooled standard sample (125 pg mL^{-1} of testosterone) in duplicate on each plate, followed by averaging the CV values for that standard. We used maximum, minimum, and mean testosterone values for subsequent correlation analyses. Testosterone levels are highly associated whit vocal activity patterns in amphibians (Emerson and Hess, 2001; Leary, 2009; Moore et al., 2005). Our focal species differ in their vocal activity pattern: whereas *O. lehmanni* is only active during the mornings (ca. for four hours), *A.* aff. *trilineatus* has two periods of activity, early morning (ca. three hours) and late afternoon (ca. two hours). Thus, maximum testosterone levels may be linked to high periods of vocal activity. Minimum testosterone levels refer to basal levels, i.e. the lowest testosterone levels recorded throughout the day for these males. A recent study (Ringler et al., 2024) found that, although intraindividual testosterone levels are quite stable across the reproductive season, the correlation between testosterone baseline levels and testosterone levels after behavioral tests is not linear. Males with low baseline testosterone tended to increase post-testing, while those with high baseline testosterone showed a decrease (Ringler et al., 2024). Thus, males with higher basal levels do not always have higher max testosterone levels. As a more conservative variable, we also included in the model mean testosterone levels. However, the mean may mask important differences between individuals and may not accurately represent hormonal variation throughout the day.

ELISA tests on samples of *A.* aff. *trilineatus* from 2017 were carried out at the Universidad de los Andes and those on samples of *A.* aff. *trilineatus* from 2018 and *O. lehmanni* were obtained at the endocrinology laboratory of the Department of Behavioral Biology, University of Vienna (Austria). Although we found no differences in mean testosterone concentrations between the two sampling years for *A. aff. trilineatus* (maximum: $F = 0.956$, $df = 1, 15$, $P = 0.344$; minimum: $F = 2.125$, $df = 1{,}14$, $P = 0.167$; mean: $F = 1.131$, $df = 1{,}15$, $P = 0.304$), we included the effect of year in subsequent analyses.

2.6. Genetic data

We collected tissue samples from nine focal males of *O. lehmanni* using mouth swabs (Betancourth-Cundar et al., 2020; Goldberg et al., 2003). DNA was obtained employing the DNeasy tissue extraction kit

(QIAGEN, Valencia, CA), following the manufacturer's protocol. We amplified nine microsatellite loci: Dpum 14, Dpum 63 and Dpum 44 (Wang and Summers, 2009), and Oop C11, Oop F1, Oop B9, Oop G5, Oop H5 and Oop E3 (Hauswaldt et al., 2009) following indications from previous studies for amplification and genotyping (Betancourth-Cundar et al., 2020) in *O. lehmanni* (Betancourth-Cundar et al., 2020; Medina et al., 2013). Fragments were sized with LIZ-500 size standard and run on an ABI3500 Genetic Analyzer (Applied Bio-systems) at the Universidad de los Andes. Genotypes were manually scored using GeneMapper V4.1 (Applied Biosystems) twice to verify that alleles were assigned correctly. We calculated the number of alleles per locus and the polymorphic information content (PIC) utilizing Cervus v.3.0.7 (Kalinowski et al., 2007). Genepop vs. 4.7.5 (Raymond and Rousset, 1995) was used to compute deviations from Hardy–Weinberg equilibrium (HWE) through a global test at each locus (Markov chain parameters: 1000 dememorizations, 100 batches, 1000 iterations per batch) and to check for linkage disequilibrium (LD) between each pair of loci through a log likelihood-ratio test (10,000 permutations). Using the GENHET function (Coulon, 2010) in R, we calculated five microsatellite-derived measures of multilocus heterozygosity: proportion of heterozygous loci (PHt), standardized heterozygosity based on the mean expected heterozygosity (Hs-exp), observed heterozygosity (Hs-obs), internal relatedness (IR), and homozygosity by locus (HL; Coulon, 2010).

2.7. Data analysis

For each predictor analyzed, we described its variation using measures of central tendency and dispersion (Fig. S1, Table S2). We used the function *mvn* in R package *MVN* (Korkmaz et al., 2014) to confirm that variables were normally distributed. To evaluate the relationship between male traits and territory characteristics in *A.* aff. *Trilineatus*, our predictors were morphological data (weight, SVL, and thigh size of males), testosterone concentration (minimum, maximum, and mean concentrations), and vocal activity data (mean number of calls). As response variables, we used the two estimates of territory size (MCP and KDE) and the two principal components resulting from the analysis of ecological characteristics. For *O. lehmanni*, we further used as predictors coloration data (chroma, hue, mean brightness, and JNDs), heterozygosity (expected and observed, IR, HL, PHt), and morphological data (body condition and arm length). We performed a model-selection analysis for each subset of variables to find which predictors best explained variation in territory characteristics. Then, with these best predictors, we built an overall model and again performed a model-selection analysis. Because for *A.* aff. *trilineatus* the data came from two different years, we employed linear mixed models (LMMs) using year as a random effect and the variables within each category (morphology, vocal activity, testosterone) as fixed effects using the function *lmer* in R package *lme4* (Bates et al., 2014). We used the *Anova* function of R to choose the best model based on the lowest AIC value. We selected the models with a difference of two AIC units that best explained variation in the territory's characteristics. For the *O. lehmanni* data, we used multiple linear regressions and model selection with the *stepAIC* function of the *MASS* package (Venables and Ripley, 2002) for R to choose the best predictors within each category and for the overall model. P values for the model or predictor were considered significant whether they were less than or equal to 0.05.

2.8. Ethical considerations

All applicable international, national, or institutional guidelines for the care and use of animals were followed. Procedures for capture, handling, and sample collection of live animals in the field were approved by Parques Nacionales Naturales de Colombia (Research permits 129-2020 and 024-2017 to MBC) and Autoridad Nacional de Licencias Ambientales (ANLA) under Permiso Marco to Universidad de los Andes (Resolution 1177 de 2014). To reduce disturbance to frogs, phenotypic and physiological data collection took <36 h, and animals were released at their capture sites. All males exhibited typical behavior during later observations.

3. Results

Variables with the highest variance for *A.* aff. *trilineatus* were those associated with the minimum, maximum, and mean testosterone levels (Coefficient of variation-CV = 152.34, 87.43, and 86.96, respectively; Table S2). The predictors with the lowest variance were those associated with morphological data (Table S2). In contrast, for *O. lehmanni* the predictors with the highest variance were internal relatedness-IR (CV = 148.99) and meantime calling during the day (CV = 109.50), and minimum testosterone levels (CV = 95.33). As in *A.* aff. *trilineatus*, the variables with the lowest variance were those associated with morphological data and additionally with coloration. Because the coefficients of variation we observed indicate that traits are indeed variable, we proceeded to assess whether variability in male traits could be associated with variation in territory characteristics.

Principal component analysis based on ecological data suggest that for *A.* aff. *trilineatus*, the first three principal components explained 87.4% of the variation in ecological characteristics of the territory. PC1 explained 50.1 % of the variation and was negatively associated with mean leaf litter temperature and mean perch temperature, and positively related with mean leaf litter depth and distance to water bodies. PC2 explained an additional 19.6 % of the variation and was positively associated with the coverage of low vegetation and negatively with the coverage of leaf litter. PC3 explained 17.7 % of the variation in ecological characteristics of the territory, but the variables associated with this PC were more closely related to PC1 and PC2 (Fig. 1a). For *O. lehmanni*, the first three principal components explained 83.8 % of the ecological variation. PC1 explained 40 % of the variation and was positively correlated with the number of large bromeliads and mean leaf litter depth, and negatively associated with the number of small bromeliads. PC2 accounted for an additional 26 % of the variation and was positively associated with coverage of low vegetation, the number of mid-sized bromeliads, and mean perch height. PC3 explained 17.9 % of the variation in ecological characteristics of the territory, but as in *A.* aff. *trilineatus*, the variables associated with this PC were more closely related to PC1 and PC2 (Fig. 1b).

Correlations between ecological characteristics and territory size were only significant in *A.* aff. *trilineatus*, such that larger territories had lower perches and lower leaf litter temperatures (PC1 and MCP: $t = 2.475$, df = 15, P-value = 0.025, $r = 0.54$). Larger territories also had greater coverage of leaf litter and were located at greater distances from water bodies. Other correlations between ecological characteristics and territory size in this species and in *O. lehmanni* were not significant.

Model-selection analyses indicated that the best predictors of variation in size and ecological characteristics of territories were mainly vocal activity, morphological variables, and testosterone levels (Tables 1 and 2). For *A.* aff. *trilineatus*, the best models explained 21 to 44 % of the variation in territory size (Table 1). Territory area (estimated with MCP or KDE H_{ref}-min) was negatively related to the mean number of calls in the day (Table 1, Fig. 2) indicating that males with smaller territories called more throughout the day (Fig. 2).

The best models explaining the variation in ecological characteristics of the territory (PC1 and PC2) included as predictors morphological variables (SVL; thigh size and weight) and maximum testosterone concentration (Table 1). These models explained 24 to 64 % of the variation in ecological characteristics of the territory. Because ecological PC1 was negatively associated with body length and positively with thigh size (Table 1, Fig. 2), frogs with greater body length had territories with higher perch temperatures and higher leaf litter temperatures, lower coverage of leaf litter, and were located closer to bodies of water. In addition, males with larger thighs had territories with lower perch temperatures and leaf litter temperatures and were located farther from

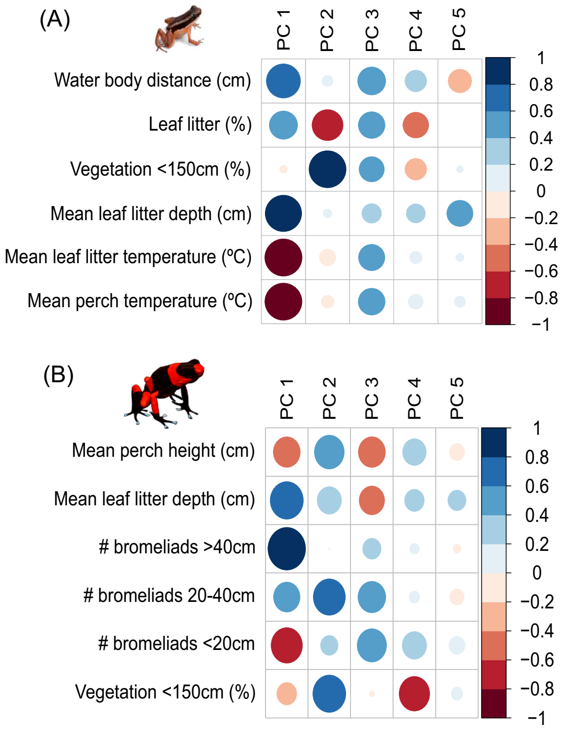

Fig. 1. Relative contribution of each ecological variable describing territories of *A.* aff. *trilineatus* and *O. lehmanni* to the first five principal components obtained in the PCA analysis. The absolute value of each contribution is represented according to the size of the circle, with blue and red colors show positive and negative contributions, respectively. (A). For *A.* aff. *trilineatus* PC1 and PC2 explained 69.7 % of the variation in ecological characteristics of the territory. PC1 was negatively associated with mean leaf litter temperature and mean perch temperature and positively related to mean leaf litter depth and water body distance. PC2 was positively correlated with the percentage of low vegetation (<150 cm) covering the territory and negatively with the percentage of leaf litter. (B). For *O. lehmanni* the two principal components explained 66 % of the variation in ecological characteristics of the territory. PC1 was positively correlated with the number of large bromeliads (height > 40 cm) and the mean leaf litter depth and negatively associated with the number of small bromeliads (height > 20 cm). PC2 was positively related to the percentage of low vegetation (<150 cm), the number of middle bromeliads (height 20-40 cm), and the mean perch height.

water bodies. Because ecological PC2 was negatively associated with body weight and maximum testosterone concentration (Table 1, Fig. 2), males with greater weight and higher testosterone concentration had territories with lower coverage of low vegetation and higher coverage of leaf litter.

For *O. lehmanni* the best models explained between 55 % and 64 % of the variation in territory size (Table 2). Body weight and mean testosterone levels were positively related to territory size estimated with MCP or minimum KDE-Href (Fig. 3). That is, males with larger territories had greater weight and higher testosterone levels (Fig. 3). Variables such as standardized heterozygosity based on the mean expected heterozygosity (Hs-exp), conspicuousness of coloration under the visual system of a poison frog, and total time called during the day were included in the models, but they did not have strong effects. Model-selection analysis of ecological characteristics of the territory (PC1 and PC2) indicated that the best models explained 38 to 44 % of the variation in ecological characteristics. These models included as predictors the vocal activity and mean testosterone concentration. PC1 was positively associated with total calling time during the day (Table 2, Fig. 3) suggesting that males with higher vocal activity have territories with more large bromeliads, fewer small bromeliads, and higher coverage of leaf litter. Given that ecological PC2 was negatively associated with mean testosterone level (Table 2, Fig. 3), males with higher testosterone levels have territories with lower coverage of low vegetation and few medium-sized bromeliads and use lower perches.

4. Discussion

Our study showed that traits of males such as body length, weight and thigh size, vocal activity, and mean water-borne testosterone levels correlate with territory characteristics in poison frogs, but which traits showed associations and whether correlations were positive or negative varied between the two species investigated here. A possible explanation for positive associations between traits of males and those of their territories is that similar sexual selection pressures involving male-male competition or female choice jointly act on the same traits. Having multiple signals act together to indicate male quality may facilitate mate choice by females (Candolin and Reynolds, 2001; Johnstone, 1996) and also avoid encounters or fights with other males. Conversely, negative associations between traits may reflect tradeoffs because producing or maintaining two or more costly traits together implies higher energy and survival costs for males (Buchanan et al., 2001; Roberts et al., 2004). Cases in which traits of males showed no correlation with those of territories (e.g., coloration or heterozygosity) suggest such traits are under different selection pressures or that they are not indicators of male quality in our study species.

Regarding territory size, we found that in a cryptically colored species with male parental care (Ringler et al., 2015; Ringler et al., 2013) and defending territories only during the breeding season (*A.* aff. *trilineatus*), males with higher vocal activity defended smaller territories. In contrast, other variables were associated with territory size in an aposematic species with maternal parental care (Bauer, 1994; Brust, 1993; Silverstone, 1973) and prolonged reproduction throughout the year (*O. lehmanni*), in which males with higher weight and testosterone levels had larger territories. Concerning ecological characteristics, in *A.* aff. *trilineatus*, we found a positive relationship between thigh size and PC1, a principal component axis which was negatively associated to leaf litter temperature and perch temperature and positively related to leaf litter depth and distance to water bodies. Also in this species, we found negative correlations between body size and PC1, and between testosterone levels and body weight with PC2, which was associated positively with coverage of low vegetation and negatively with leaf litter coverage in the territory. In *O. lehmanni*, we found a positive relationship between vocal activity and PC1 (positively correlated with the abundance of large bromeliads and leaf litter depth) and a negative relationship between testosterone levels and PC2 (positively related to the percentage of low vegetation, the number of mid-sized bromeliads, and perch height). Rather than seeking to explain in detail why might some traits be correlated or not with others and why might different patterns be observed in the two species, we begin by drawing attention to our overall finding that simple and consistent associations between traits of males and of their territories do not seem to exist. Also, territory characteristics, beyond territory size, must be considered to understand what territory resources are important to males and females when defending or choosing a territory.

Most studies documenting associations between territory size, male traits, and reproductive success in dendrobatids have estimated the defended area using the territory mapping or spot-mapping technique (Marques Correia et al., 2018; Meuche et al., 2012; Pröhl, 2005; Roithmair, 1994a; Roithmair, 1992). This approach consists of plotting locations of singing or calling animals on a map to estimate the territory area (Sutherland, 2006). This method can be effective in determining the area used by an individual but not the area defended (Anich et al., 2009; Ringler et al., 2011), which corresponds to the formal definition of territory separating it from the concept of the home range (Kaufmann, 1983). Therefore, when examining correlations between male traits and territory size one must bear in mind variation introduced by sampling

Table 1

Results of model-selection analysis using linear mixed-models that evaluated the effect of phenotypic, physiological, and behavioral variables on the territory size (MCP and KDE) and ecological characteristics of the territory (PC1 and PC2) of 19 territorial males of *A.* aff. *trilineatus.* The models with the lowest AIC values indicated that the territory area and its ecological characteristics are mainly explained by vocal activity, and morphological variables such as body length, thigh size, weight, and androgen levels. The number of parameters, AIC values, R-squared (R^2) of the general model, and R^2 of the fixed effects are indicated for each model. Estimates, standard errors-SE, t value and probability values (P), and standardized beta coefficient are shown for each predictor. Significant P values <0.05 are shown in bold and P values <0.1 are in gray.

Territory characteristics	Models					Fixed Effects						Random Effects		
	Models	**N par**	**AIC**	**R2 Total**	**R2 fixed effects**	**Predictor**	**Estimate**	**SE**	**t**	**P**	**Std. beta**	**Predictor**	**Variance**	**Std. Dev.**
Size: MCP	BM1	4	86.82	0.21	0.21	Mean of Calls Day	-0.082	0.039	-2.072	**0.059**	-0.450	Season	0.0	0.0
	BM2	5	87.86	0.26	0.16	Thigh size (mm2)	0.655	0.580	1.130	0.281	0.240	Season	0.714	0.845
						Mean of Calls Day	-0.067	0.043	-1.545	0.148	-0.370			
Size: KDE Href-minimum	BM1	4	78.68	0.30	0.30	Mean of Calls Day	-0.081	0.031	-2.599	**0.022**	-0.530	Season	0.0	0.0
	BM2	6	78.83	0.44	0.44	Weight (g)	-16.971	10.039	-1.691	0.119	-0.330	Season	0.0	0.0
						Body length - SVL (mm)	0.960	0.568	1.688	0.119	0.350			
						Mean of Calls Day	-0.093	0.030	-3.117	**0.010**	-62.000			
Ecological features - PC1	BM1	5	62.96	0.69	0.25	Body length - SVL (mm)	-0.825	0.359	-2.302	**0.040**	-0.390	Season	1.387	1.178
						Thigh size (mm2)	0.960	0.279	3.447	**0.005**	0.550			
	BM2	6	64.94	0.70	0.24	Body length - SVL (mm)	-0.825	0.358	-2.305	**0.042**	-0.390	Season	1.469	1.212
						Thigh size (mm2)	0.957	0.278	3.439	**0.006**	0.550			
						Mean of Calls Day	0.003	0.021	0.151	0.882	0.030			
Ecological features - PC2	BM1	6	45.59	0.64	0.64	Weight (g)	-21.800	4.653	-4.685	**< .001**	-0.920	Season	0.0	0.0
						Maximum testosterone levels (pg/mL/hour)	-0.002	0.001	-2.247	**0.046**	-0.440			
						Mean of Calls Day	-0.020	0.011	-1.916	**0.082**	-0.290			
	BM2	6	45.82	0.64	0.62	Weight (g)	-15.320	5.801	-2.640	**0.023**	-0.640	Season	0.021	0.145
						Thigh size (mm2)	-0.421	0.238	-1.764	0.105	-0.400			
						Maximum testosterone levels (pg/mL/hour)	-0.002	0.001	-2.326	**0.040**	-0.450			

Table 2

Results of model-selection analysis using multiple regressions assessing the effect of phenotypic, physiological, genetic, and behavioral variables on territory size (MCP and KDE) and ecological characteristics of the territory (PC1 and PC2) of nine territorial males of *O. lehmanni.* Models with the lowest AIC values indicated that territory area and its ecological characteristics were mainly explained by body weight, mean water-borne androgen level, and time calling during the day. The number of parameters, AIC values, R-squared (R^2), and probability values (P) are indicated for each model. Estimates, standard errors-SE, t value, and probability values (P) are shown for each predictor. Significant P values <0.05 are shown in bold and P values <0.1 are in gray.

Territory characteristics	**Models**	**N par**	**AIC**	**Adjusted R2**	**P model**	**Predictor**	**Estimate**	**SE**	**t**	**P**
Size: MCP	BM1	3	40.322	0.55	**0.038**	Weight (g)	30.274	10.591	2.858	**0.029**
						Mean testosterone levels (pg/mL/hour)	0.013	0.005	2.795	**0.031**
	BM2	4	40.714	0.55	0.077	Weight (g)	26.752	11.192	2.390	0.062
						Mean testosterone levels (pg/mL/hour)	0.010	0.005	1.828	0.127
						Hs-exp	-17.008	17.196	-0.989	0.368
Size: KDE Href-minimum	BM1	3	38.531	0.64	**0.019**	Weight (g)	28.129	9.588	2.934	**0.026**
						Mean testosterone levels (pg/mL/hour)	0.015	0.004	3.649	**0.011**
	BM2	4	38.941	0.64	**0.045**	Weight (g)	27.373	9.646	2.838	**0.036**
						Mean testosterone levels (pg/mL/hour)	0.017	0.005	3.740	**0.013**
						JND (dS -Poison Frog)	-6.347	6.457	-0.983	0.371
	BM3	5	38.959	0.64	0.086	Weight (g)	40.677	16.521	2.462	0.070
						Mean testosterone levels (pg/mL/hour)	0.023	0.007	3.053	**0.038**
						Time calling Day (s)	-0.014	0.015	-0.993	0.377
						JND (dS -Poison Frog)	-10.279	7.583	-1.355	0.247
Ecological features - PC1	BM1	2	5.93533	0.4105	**0.0374**	Time calling Day (s)	0.003	0.001	2.563	**0.037**
	BM2	3	6.05192	0.4421	0.0733	Weight (g)	-2.091	1.769	-1.182	0.282
						Time calling Day (s)	0.004	0.001	2.849	**0.029**
Ecological features - PC2	BM1	2	2.38701	0.387	**0.0435**	Mean testosterone levels (pg/mL/hour)	-0.001	0.001	-2.460	**0.044**
	BM2	3	2.42716	0.4248	0.0803	Mean testosterone levels (pg/mL/hour)	-0.001	0.001	-1.338	0.229
						Body length - SVL (mm)	0.430	0.356	1.208	0.272

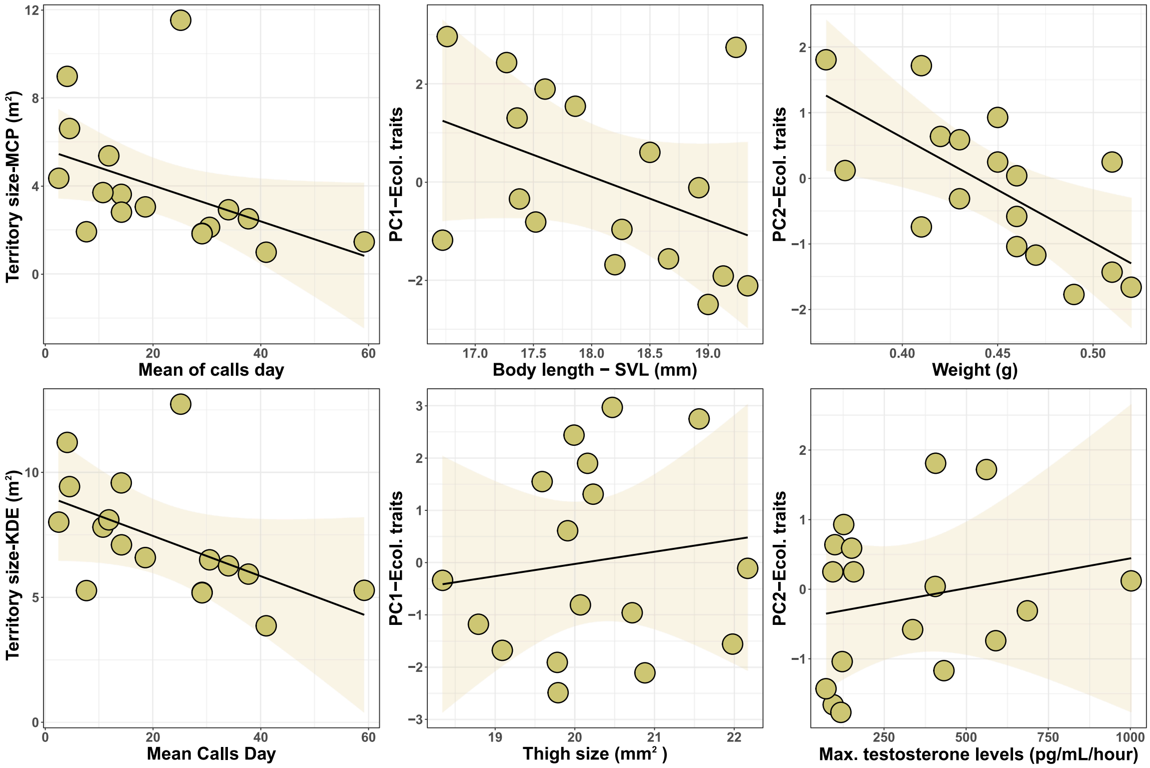

Fig. 2. Graphical summary of the effects of different predictors on territory size estimated with Minimum Convex Polygons (MCP) and Kernel Density Estimators (KDE) and ecological characteristics of territories summarized in two principal components (PC1 and PC2) for 19 males of *A.* aff. *trilineatus.* The mean number of calls during the day was negatively related to the territory area. PC1 was negatively associated with body length and positively correlated with thigh size. PC2 was negatively associated with weight and maximum testosterone concentration. Quantitative descriptions for these correlations are described in Table 1. Each data point corresponds to a focal male. Black lines indicate the trend line of the data based on a general linear model and the shaded areas describe the corresponding 95 % credible intervals.

techniques; thus, the below comparisons with previous studies of territorial behavior in poison frogs should be taken only as general guidelines.

In contrast to our results for *A.* aff. *trilineatus*, in which territory size and male vocal activity were negatively related, positive correlations between these variables exist in other dendrobatid species (Roithmair, 1994a; Roithmair, 1992). Previous studies have suggested that territory size reveals male quality because territory size likely depends on calling activity, which involves high energetic costs and thus may reflect the physical condition and competitive ability of the males (Pröhl, 2005; Pröhl, 2003; Roithmair, 1994a). Our finding of a negative correlation between territory size and vocal activity in *A.* aff. *trilineatus* may indicate that males compensate for having small territories with increased calling effort. An alternative but not exclusive explanation would be that vocal activity and territory size are associated with conspecific density (Adams, 2001; Emerson and Hess, 2001; Sells and Mitchell, 2020). At our study site, males are densely clustered in certain areas (e.g., 12 males in 150 m^2) and the presence of other males may limit the defended area size but may also increase male-male competition mediated by acoustic signals, resulting in greater vocal activity. In other anurans, male density may increase intrasexual competition and act as a driver of the spatial arrangement of territories (Adams, 2001; Meuche et al., 2012; Robertson, 1984; Wilczynski and Brenowitzt, 1988).

We found that in *O. lehmanni* territory size was positively correlated with weight and testosterone levels. In contrast, previous work in a congener (*O. pumilio*) documented a negative correlation between territory size and body condition, a pattern potentially reflecting that only males with high body conditions are able to establish territories in places with strong male-male competition and high female density (Meuche et al., 2012). Although we did not estimate female density in our study population of *O. lehmanni*, we noted that of the nine males analyzed, four had females in their territories and we did not observe females alone or living near the territories. Therefore, in this population, the operational sex ratio is likely biased towards males, so female density is not likely to influence territory setup. However, to evaluate whether female density is affecting our results, further studies comparing territory sizes and their relationship to body condition in areas with high and low female density would be necessary.

Also, in *O. lehmanni*, we observed that larger territories were occupied by heavier males, suggesting that individuals with more energy reserves or greater muscle mass reflecting better body condition (Brodeur et al., 2020) might be better able to defend larger areas. The positive correlation of territory size with testosterone levels we documented in this species may indicate direct effects of testosterone influencing levels of aggression to defend larger territories and further indirect effects of testosterone on male weight (Walkowski et al., 2019). Circulating levels of testosterone in males of many vertebrate species influence secondary sexual characteristics (Hau, 2007; Wingfield et al., 2001) such as vocalizations, courtship, or muscle mass development (Brantley et al., 1993; Girgenrath and Marsh, 2003), and aggressive behaviors like mate-guarding or territoriality (Goymann et al., 2004; Hau et al., 2010; Rodríguez et al., 2022; Wingfield, 1984). Accordingly, males with greater muscle mass are likely to defend larger territories, which in turn is associated with higher testosterone levels. These results support the hypothesis that testosterone may be part of the physiological machinery that underlies the interaction between male traits and territoriality. Although we did not find similar evidence in *A.* aff. *trilineatus*, testosterone levels were negatively associated with ecological traits of territories in both species. In *A.* aff. *trilineatus*, weight and testosterone levels explained 64 % of the variation in ecological PC2 (positively associated to the coverage of low vegetation and negatively to the coverage of leaf litter in the territory) and were negatively associated.

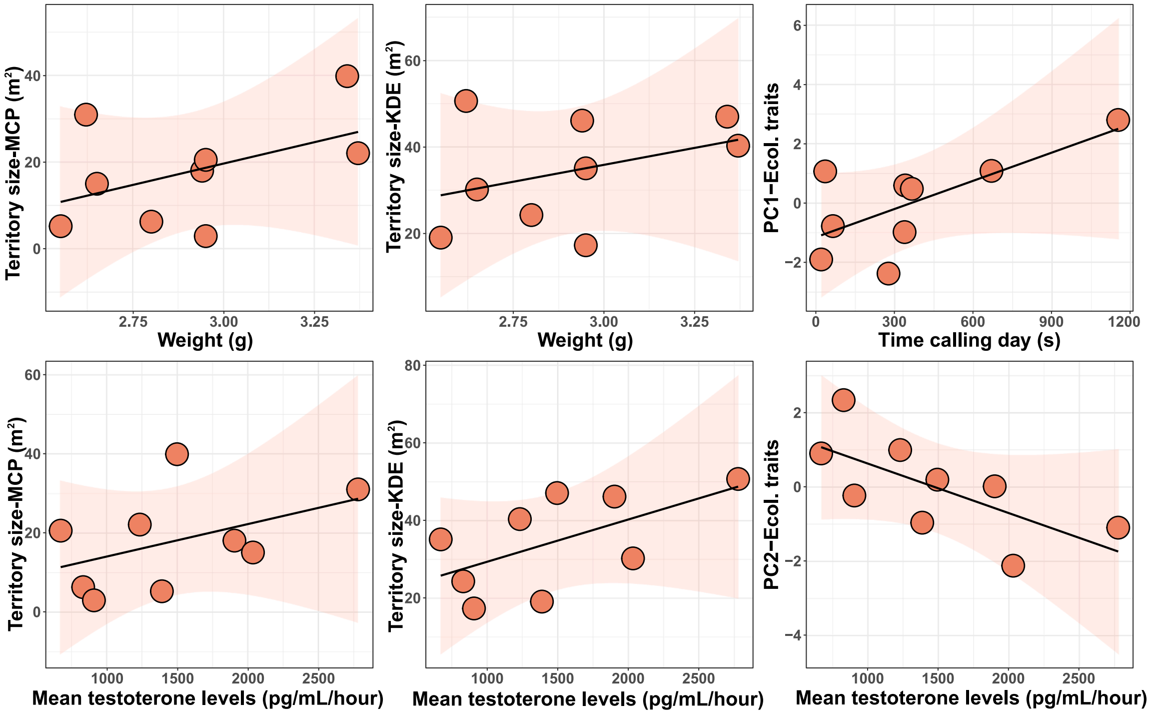

Fig. 3. Graphical summary of the effects of different predictors of territory size estimated with Minimum Convex Polygons (MCP) and Kernel Density Estimators (KDE) and ecological characteristics of territories summarized in two principal components (PC1 and PC2) for nine males of *O. lehmanni.* Weight and mean testosterone levels are positively associated with the territory area. Total time calling during the day is positively correlated with ecological characteristics of the territory (PC1) and mean testosterone levels are negatively related to PC2. Quantitative descriptions for these correlations are described in Table 2. Each point corresponds to a focal male. Black lines indicate the trend line based on a general linear model and the shaded areas describe the corresponding 95 % credible intervals.

This suggests that more aggressive males in better body condition defended territories with lower coverage of low vegetation and higher coverage of leaf litter. Therefore, leaf litter might be a relevant factor in establishing and defending a territory, and defense ability could be mediated by testosterone levels. In *O. lehmanni* we found a similar result, where testosterone levels were negatively associated with ecological PC2 (positively associated to the percentage of low vegetation, number of middle bromeliads, and mean perch height) and explained 39 to 42 % of its variation. These findings reveal links between parts of an endocrine pathway involving testosterone and phenotypic traits of males, and also ecological characteristics of their territories. Understanding in detail how testosterone may mediate the expression of phenotypic traits that may facilitate the defense of valuable resources is a challenge for further studies.

We found correlations between ecological characteristics and the size of territories only in *A.* aff. *trilineatus*, in which larger territories had lower surface temperatures, greater coverage of leaf litter, and were located farther from water bodies than smaller territories. Given that it is probably more economical to defend small territories (Brown, 1964; Kaufmann, 1983) close to water bodies than to incur the costs of defending large territories, the question arises of why males would defend large areas if they may have what they need in small areas. We suggest that addressing such question requires examining the influence of ecological characteristics beyond territory size on male breeding success. However, most studies focus on size as a descriptor of territory quality (Dale and Slagsvold, 1990; Marques Correia et al., 2018; Meuche et al., 2011; Price, 1984; Pröhl, 2005; Reid and Weatherhead, 1990; Roithmair, 1994a; Roithmair, 1992; Vanpé et al., 2009). Our data suggest that, although some ecological characteristics are related to territory size, others are not. Consequently, additional measures of quality, independent of their connection to territory size, need to be examined in order to gain a more precise understanding of the underlying mechanisms behind territorial behavior.

In *A.* aff. *trilineatus*, ecological characteristics such as leaf litter coverage and distance to water bodies appear to influence mating and survival of offspring (Pašukonis et al., 2016; Ringler et al., 2013). In this species, eggs are deposited on leaf litter within the territory, constantly moistened by the male (M. Betancourth-C, unpubl. data), and hatched tadpoles are carried by their fathers to suitable ponds to complete their development. Having a water body close to the territory could facilitate the transport of tadpoles yet work on other dendrobatids indicates that animals do not use the nearest water body (Pašukonis et al., 2019) and that distance to water bodies is unrelated to reproductive success (Marques Correia et al., 2018). However, even if water bodies are not used for larval deposition, they may influence local moisture gradients (Vargas-Salinas et al., 2014) potentially reducing evaporative water loss of embryos, especially in territories with lower leaf litter coverage. Given that dehydration is the main cause of embryonic mortality in frogs laying terrestrial eggs (Delia et al., 2013; Valencia and Delia, 2015), the mortality risk by dehydration of embryos may be greater in territories located far from water bodies although this effect might be compensated by having more leaf litter in the territory. Considering the dependence of amphibians on humidity (Vitt and Caldwell, 2014; Wells, 2007), it is plausible that selecting territories near water bodies could offer advantages to males. Such proximity might facilitate their calling behavior by allowing them to occupy more conspicuous or elevated perches and calling for prolonged periods. This preference could be attributed to the creation of local moisture gradients in these areas. Territories with greater coverage of leaf litter may also provide more refugia allowing escape from predators as shown in studies on fishes in which predation tended to be lower when structural complexity of territories increased (Candolin and Reynolds, 2001). Therefore, leaf litter coverage and distance to water bodies may not only be important for mating and survival of descendants, but also for other aspects of the natural history of *A.* aff.

trilineatus and species with similar behaviors.

In *O. lehmanni*, a greater number of large and medium-sized bromeliads likely indicate higher territory quality because these plants are used for larval deposition (Donnelly, 1989b; Donnelly, 1989a; Pröhl, 2002). Large bromeliads can store more water, which is a critical factor to avoid dehydration in tadpoles, especially in species that use phytotelmas for reproduction (Poelman et al., 2013; Summers and McKeon, 2004). Also, we found that males with a greater number of large bromeliads in their territory showed greater vocal activity. Our field observations suggest that males of *O. lehmanni* play an active role in parental care, vocalizing while females feed tadpoles with unfertilized eggs. This behavior has been observed in *Ranitomeya imitator*, the only species heretofore known to exhibit biparental care in dendrobatids (Brown et al., 2010). We believe it is likely that, during our study, males with more bromeliads of large size in their territory had already reproduced and were caring for tadpoles. Five of the males with higher vocal activity were observed with females in the territory. Of these, one of the three males with highest vocal activity had tadpoles in his territory and we observed the feeding behavior described above on two occasions. Therefore, increased vocal activity could be associated with parental care duties. Considering that the main function of the calls is associated with the defense of the territory, it is important to study their role in different aspects of the natural history of the species.

Heterozygosity may be an honest indicator of male fitness and has been suggested to influence the competitive ability and territorial behavior in various organisms (Höglund et al., 2002; Lieutenant-Gosselin and Bernatchez, 2006; Seddon et al., 2004; Tiira et al., 2006; Tiira et al., 2003). In our study, when we evaluated the correlation between heterozygosity and territory characteristics, we found that observed and expected heterozygosity were correlated with territory size and explained nearly 50 % of its variation (MCP; $F = 4.969$, $df = 2, 6$, $P = 0.053$, Adjusted-R^2: 0.498). However, when we conducted model selection with the best predictors, although expected heterozygosity was included in the second-best model, this model had not a significant effect on explaining variation in territory size. Although we did not find strong evidence for the role of heterozygosity in territoriality acting in conjunction with other traits in this species, we interpret our results as weak evidence for such a correlation when considering its effect alone. In addition, a caveat of our analysis is that the power to detect links between genetic diversity and life-history traits using microsatellite markers is admittedly limited (Selkoe and Toonen, 2006), partly because amphibians exhibit the largest genome sizes among terrestrial vertebrates (Portik et al., 2016) and such markers hardly sample genome-wide variation. Using next-generation sequencing tools would allow obtaining a wider genome coverage to assess the role of heterozygosity acting synergistically with other traits to modulate territory characteristics.

Taken together, our results suggest that morphological traits, vocal activity, and testosterone levels are phenotypic traits associated with attributes of the territories of males. However, we found that the nature of such associations depends on the species and traits involved. This suggests that links between territorial behavior and intrinsic traits of males are complex and likely context-dependent. Understanding which traits of males may determine the establishment and defense of “better” territories and which of these traits are being selected by females requires a comparison at different scales. For example, one could conduct further experimental studies in territorial species of the same genus with similar ecological characteristics, but also in territorial amphibians with different and complex life-history traits. A natural next step would be to define what is a “better” territory and a “better” male by quantifying the reproductive success of males and relating it to ecological characteristics of the territory as well as male traits such as morphology, vocal activity, and testosterone levels.

Finally, our findings support the role of testosterone as a phenotypic integrator which may be central to territorial behavior (Hau et al., 2016; Ketterson and Nolan, 1992; Lipshutz et al., 2019; Martin et al., 2011). Testosterone levels were correlated with phenotypic traits, territory size, and ecological characteristics of territory in both species, which differ in the mating system, parental care, and acoustic communication strategies. It is widely documented that testosterone levels increase during the breeding season (Wilczynski et al., 2005; Wingfield, 1984; Wingfield et al., 1990) and that maintaining high levels of testosterone for a prolonged period of time involves high costs for the animals (Hau, 2007), such as suppression of immune function (Olsson et al., 2000), reduction of parental care (Wingfield et al., 1990), increase of parasitic infections (Folstad and Karter, 2002; Salvador et al., 1996), and reduced survival (Sinervo et al., 2000). It would be interesting to monitor testosterone levels throughout the annual cycle, especially in species with prolonged reproduction and that defending territory for several years (i.e., *O. lehmanni*) in order to expand understanding of the role of circulating testosterone and territoriality at different lifecycle stages and the regulation of aggressive behaviors in anurans. Overall, these results highlight an endocrine pathway as part of physiological machinery that underlies the interplay between male traits and territorial behavior. An interesting future study would be to evaluate whether females choose males based on phenotypic traits or characteristics of the territory which our current work suggests might be under selection.

CRediT authorship contribution statement

Mileidy Betancourth-Cundar: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Virginie Canoine:** Resources, Supervision, Writing – review & editing. **Leonida Fusani:** Resources, Writing – review & editing. **Carlos Daniel Cadena:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

Data availability

Data are available in the following repository: https://github.com/cdanielcadena/frog_territoriality.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2024.105547.

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