
High Seasonal Variation of Plasma Testosterone Levels for a Tropical Grassland Bird Resembles Patterns of Temperate Birds

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ABSTRACT

Testosterone (T) is a sexual hormone capable of modulating several traits in birds, including aggressiveness and reproductive behavior. Although variation in T-related traits is well-known for temperate zone birds, this variation has not been extensively studied in tropical species. The campo miner (*Geositta poeilopectera*) is a threatened bird endemic to the grasslands of the South American Cerrado. We investigated the seasonal variation in plasma T levels and associated behavior in the campo miner, addressing the following questions: (1) Does the species exhibit seasonal variation in T profile? (2) Do males have higher plasma T levels than females, irrespective of season? (3) Are males with higher plasma T levels more aggressive than males with lower T levels? (4) Do males' plasma T levels decrease after females lay eggs? We found that T levels are higher during the breeding season than during the nonbreeding season and that males present higher T levels than females throughout the year. Such high T levels are associated with a higher probability to engage in aggressive behavior; however,

T levels decline toward the egg-laying date and keep decreasing afterward. Higher T levels before egg laying are apparently related to territorial defense against invaders and extrapair copulations. With the beginning of parental care, T levels decrease, which is in line with previous observations that the species becomes less aggressive after egg laying. This study contributes to the understanding of environmental endocrinology of tropical birds, filling some knowledge gaps about the diverse Neotropical avifauna.

Keywords: aggressiveness, androgens, challenge hypothesis, *Geositta poeilopectera*, parental care, seasonal habitat.

Introduction

Androgen hormones are well-known mediators of reproductive behavior and territorial defense in vertebrates (Wingfield et al. 1990; Hau 2007; Moore et al. 2020). Testosterone (T) largely mediates the individual balance between behavior related to competition and maintenance (Wingfield et al. 1987; Hau et al. 2010), influencing, for example, aggressive behavior, attractiveness, immune function, and paternal care (Wingfield et al. 1990; Roberts et al. 2004; Ketterson et al. 2005; Hau 2007).

The challenge hypothesis provides a model to explain individual variation in plasma T levels in birds and other vertebrates. It predicts that high plasma T levels are directly involved with aggression associated with reproduction, especially male-male interactions (Wingfield et al. 1990). This hypothesis has recently been modified by Goymann et al. (2019), who proposed the challenge hypothesis 2.0, which predicts that variations in T are mainly a function of interactions with reproductively active females not with males. These two hypotheses (hereafter, CH 1.0 and CH 2.0, respectively) assume that high plasma T levels inhibit paternal behavior (Wingfield et al. 1990; Goymann et al. 2019).

To accommodate the costs and benefits of T, animals may modulate their circulating levels of T in response to environmental and social cues (Hau 2007; Addis et al. 2013). Marked seasonal variations in plasma T levels are well-known to occur in birds of the highly seasonal northern temperate zones, where breeding seasons are shorter and breeding synchrony is higher than for tropical birds, resulting in intense competition for territories and mates (Goymann et al. 2004; Hau 2007; Stutchbury and Morton 2008). In these temperate zone species, several studies report large seasonal differences in plasma T levels (Wingfield et al. 1987; Goymann et al. 2004; Ketterson et al. 2005). Conversely, for birds living in tropical lowland rain forests, there are lower and seasonally less

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variable levels of circulating T (Hau et al. 2000, 2004; Fedy and Stutchbury 2006; Gill et al. 2007; Busch et al. 2008; Chiver et al. 2014). One hypothesis for this pattern is that because most rain forest birds hold year-round territories and/or exhibit rather long (>6 mo), poorly synchronized breeding seasons, competition for territories and/or mates is not as intense as in the temperate zones (Goymann et al. 2004), and therefore there are lower levels of T.

The dichotomy of temperate/tropical T level patterns in birds as described above is, however, oversimplistic. Not all tropical habitats are benign and constant throughout the year; many tropical birds live in markedly seasonal habitats because of patterns of rainfall and food availability rather than temperature variations (Goymann and Helm 2014). Birds living in such habitats often experience low social stability, as territories and/or mates are not usually held year-round and male-male disputes are common in the early breeding season during territorial establishment and mate recruitment. Also the short breeding season of birds living in highly seasonal habitats promotes high breeding synchrony (Lopes and Marini 2005; Paiva and Marini 2013), which is likely to increase the chances of extrapair copulations (Stutchbury 1998). In other words, some challenges faced by tropical birds living in highly seasonal habitats are similar to those that affect birds living in temperate zones (Goymann and Helm 2014).

To the best of our knowledge, no study to date has investigated the seasonal variation in plasma T levels of birds inhabiting tropical grasslands. Such habitats exhibit two interesting features to help understand the seasonal variation of T in tropical birds. First, these habitats are quite seasonal, and as we stated above, birds living in highly seasonal habitats usually exhibit short breeding seasons and high breeding synchrony. Second, tropical grasslands are fire-prone habitats, with fires quickly consuming patches of the habitat in an unpredictable way, often producing a pyrodiverse mosaic of habitats (Cochrane 2009; Murphy and Bowman 2012). Consequently, the more open forms of tropical grasslands are also dynamic, which may force some bird species to compete every year to establish territories and recruit mates, causing significant temporary social instability (Wingfield et al. 2014). For both reasons, we expect that birds inhabiting tropical grassland habitats would exhibit a marked seasonal variation in plasma T levels, similar to birds inhabiting highly seasonal temperate zones.

We studied the seasonal variation in plasma T levels of the campo miner, *Geositta poeciloptera* (Scleruridae), a Neotropical passerine that inhabits fire-prone open grasslands (Ridgely and Tudor 1994). This is a socially monogamous species with biparental care, with males showing strong territorial behavior, often exhibiting hovering display flights and intense vocalization during territorial establishment, courtship, and nest construction (Machado et al. 2017). Campo miners, however, show a considerable decrease in territoriality during incubation and especially during nestling care (Machado et al. 2017).

We asked four questions related to the effect of plasma T levels on the biology and behavior of the campo miner. First, does the species exhibit seasonal variation in T profile? Second, do males have higher plasma T levels than females, irrespective of season? Third, are males with higher plasma T levels more aggressive (as measured by display behavior) than males with lower plasma T levels? Fourth, do

male plasma T levels decrease after females lay eggs? We predicted that plasma T levels of both sexes would be higher during the breeding season than during the nonbreeding season but that plasma T levels in males would be higher than in females all the time. We also expected that males with higher plasma T levels would be more likely to exhibit aggressive territorial behavior. Finally, we expected that males' plasma T levels would be higher before egg laying, decreasing thereafter with the onset of incubation and nestling care.

Methods

Study Area

We studied a population of the campo miner found in the upper Rio Grande grasslands, an extensive mountainous region with altitudes ranging mainly from 900 to 1,600 m in the southern part of the state of Minas Gerais, southeastern Brazil. Our study area was within the municipalities of São João del-Rei and Tiradentes (centered at 21°12'S, 44°10'W; ~1,000–1,100 m asl), covering an area of ~15,000 ha. The region has a humid subtropical climate, classified as Cwb type according to Köppen's climate classification system (Sá Júnior et al. 2012). The mean temperature is 14.3°C during winter and 17.0°C during summer (Sá Júnior et al. 2012). Mean annual rainfall is ~1,500 mm, with marked dry (May to August) and wet (September to April) seasons (Sá Júnior et al. 2012).

Study Species

The campo miner is a threatened passerine (MMA 2014; BirdLife International 2019) endemic to the open grasslands of the South American Cerrado biogeographic province (Silva and Bates 2002). It is a small terrestrial and insectivorous bird that nests within cavities dug on steep soil banks, with a breeding season extending from early August to early December (Machado et al. 2017; CEMAVE 2018; Meireles et al. 2018). Campo miners favor areas with low and sparse grass cover (see <https://youtu.be/EM6SRlyiHVA>) and often use recently burnt areas, shifting the location of their territories depending on fire and/or grazing regimes (Machado et al. 2017; CEMAVE 2018). This is a socially monogamous bird, with both parents incubating and feeding the nestlings; the mean incubation period is 17.5 d, and the mean nestling period is 15.5 d (Machado et al. 2017). The breeding season length is about 4.5 mo, and making multiple breeding attempts in a single season is a common strategy, with up to three nesting attempts recorded (Machado et al. 2017). Even though the species does not show sexual dimorphism, we can use bird behavior as a proxy for sex determination because males are more active in territorial defense, frequently performing hovering display flights (for a picture of a displaying male, see <https://www.wikiaves.com.br/1813784>). Notably, females seldom sing (Machado et al. 2017). No information is available about the rate of extrapair paternity in the species or, to the best of our knowledge, in any other species of Scleruridae.

Territorial Aggressiveness

During previous studies with this species (Machado et al. 2017; Meireles et al. 2018), we noticed that while incubating, males rarely

performed their hovering display flights or even sang in response to the playback of the typical song of the species. Even though some males silently approached the observer in response to a recorded sound of a territorial male, they did so by walking amid the grass cover and not in a defensive manner, being easily overlooked. Therefore, as a proxy for aggressiveness, we used a binary evaluation scheme. Males that responded to the playback by performing a hovering display flight were considered aggressive, whereas males that did not respond in that way were considered nonaggressive. We conducted all behavioral observations while conducting the playbacks to capture birds for collecting blood samples.

We captured birds in the morning (0700–0900 hours) during the breeding (August to December 2015) and nonbreeding (January 2016) seasons using up to three 12-m-long mist nets and playback of the bird songs and calls. Given that aggressive events, such as territorial intrusions, can lead to peaks of T (Wingfield et al. 1990; Gill et al. 2007), we used a standardized playback track comprising 1 min of bird songs followed by 30 s of bird calls with 15 s of silence between songs and calls and 1 min of silence between repetitions. We played the track until we captured the bird, which usually occurred in less than 20 min.

Blood Sampling

After birds were captured, we immediately removed them from the net (Vernasco et al. 2019) and restrained them in a cloth bag for 5 min before we took a blood sample from the brachial vein (maximum of 100 μ L; Chiver et al. 2014) in heparinized capillary tubes. After that, the birds were color banded and released in the same place that they were captured. We stored blood samples in ambient conditions for a maximum of 3 h, and in the lab, we centrifuged the blood (10,000 rpm for 10 min) and stored the plasma and erythrocyte pellet at -20°C (Kristal et al. 2005). We used plasma samples for the T assay, and we used erythrocyte pellets to determine the sex of each individual. Sexing was conducted by a private laboratory (BioTech Ciência e Tecnologia Pelo DNA, Goiânia, Brazil).

Testosterone Assay

To measure plasma T levels, we used a competitive enzyme immunoassay kit from Enzo Life (catalog no. ADI-900-065; Farmingdale, NY). The sensitivity of the assay was 5.67 pg/mL, and the average was 2.37 ng/mL. The assay was performed following the instructions of the manufacturer. Briefly, plasma samples were diluted 1:20 with 0.5% steroid displacement buffer, and 50 μ L were pipetted into a microtiter plate coated with anti-T antibodies in duplicate. Then a T bound to alkaline phosphatase was added and competitively bound to the plate. After washing the plate, we measured the bound hormone using the reaction of the phosphatase with p-nitrophenyl phosphate. Finally, we read the optical density on a standard plate reader at 405 nm. T levels were expressed in nanograms per milliliter. The intra-assay coefficient of variation was 4.45%.

Data Analysis

To compare whether the mean levels of T varied between seasons and sexes, we performed a generalized linear model (GLM) ANOVA with Gaussian distribution. We used plasma T levels (ng/mL) as a response variable, log transformed to achieve an adequate model fit. We used the identity of seasons (breeding vs. nonbreeding), the identity of sexes (male vs. female), and the interaction between both variables as explanatory variables. Given that the campo miner is a threatened species, we did not try to recapture the same individuals in both seasons, so we used the mean T levels to test the hypothesis that the plasma T levels would be higher during the breeding season. All assumptions on model fit, Gaussian distribution of errors, and homoscedasticity were checked through residual analyses (Crawley 2012).

To test the probability of aggressive behavior of males (response variable; 0 = no hovering display, 1 = hovering display) increasing with rising plasma T levels (explanatory variable), we performed a logistic regression using GLM and binomial distribution of errors. Finally, we used a linear regression (Hastie and Pregibon 1992) to test whether plasma T levels in males (response variable) decrease in relation to the first egg-laying date (explanatory variable), which was assigned zero value. This measure was possible only for those nests for which we knew the exact date of egg laying or for which we could backdate the laying date using the mean incubation period. Similarly to the first analyses, we log transformed plasma T levels to achieve an adequate model fit. Again, all assumptions regarding model fit and distribution adequacy were checked through residual analyses. All tests were performed in the R software (R Development Core Team 2016) with an α of 5%.

Results

We obtained blood samples from 42 birds: 16 females, 23 males, and 3 unsexed. We captured 27 birds during the breeding season and 15 during the nonbreeding season. We could calculate egg-laying date for 15 nests, with all other nests that were monitored excluded from the analysis. Plasma T levels were significantly higher during the breeding season than during the nonbreeding season (fig. 1; table 1). Plasma T levels were significantly higher in males than in females (fig. 1; table 1); however, the interaction term was not significant ($F_{1,35} = 2.56$, $P = 0.11$).

The probability of males engaging in aggressive behavior significantly increased with higher plasma T levels (fig. 2; deviance = 18.37, $df = 1$, $n = 23$, $P < 0.001$). We also found a steady decline in plasma T levels in males with the approach of the egg-laying date. These levels kept declining after the onset of incubation (fig. 3; $F_{1,13} = 6.84$, $P = 0.02$).

Discussion

Although assumptions of CH 2.0 regarding T responsiveness to social interactions changed the focus from male-male interactions to male-female interactions (see table 1a in Goymann et al. 2019), specific predictions can easily be confounded with those of CH 1.0 if one is not running a controlled experiment. For example, a male

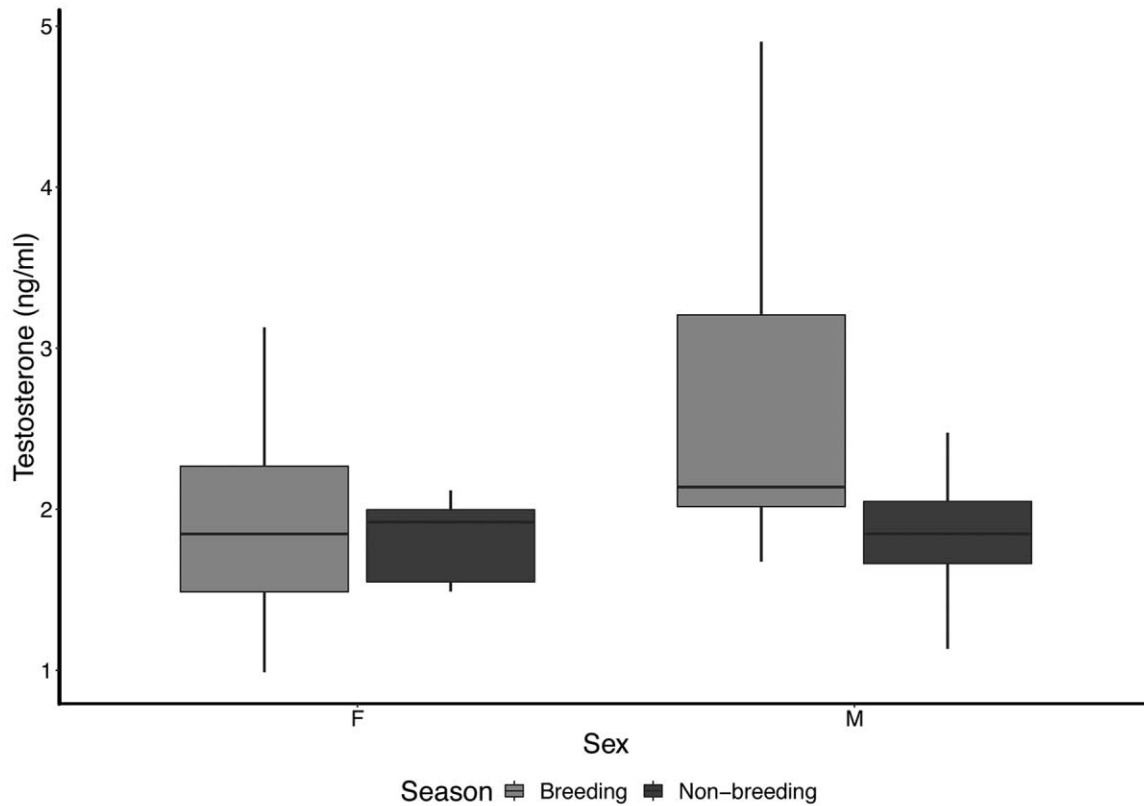


Figure 1. Boxplot representing plasma testosterone levels of females and males of the campo miner (*Geositta poeciloptera*), comparing breeding ($n = 27$) and nonbreeding ($n = 15$) seasons. The bottom and top of the box are the lower and upper quartiles, respectively, the horizontal band is the median, and the ends of the whiskers represent extreme values excluding outliers.

of a socially monogamous species with biparental care that exhibits a peak in T just before egg laying may be responding to the territorial intrusion by another male, possibly seeking extrapair copulation (CH 1.0) or responding to copulation solicitation from his receptive female (CH 2.0). Therefore, throughout the discussion, we will interpret our results considering both hypotheses. It is also important to highlight that because this was a field study conducted with a rare and threatened species, the sample size obtained was relatively low yet representative of the small population

size. Therefore, readers are encouraged to consider with care the generalities of our results, including negative ones.

Seasonal Variation in Testosterone

Males of the campo miner presented comparatively high mean values of T compared with the average values for tropical birds, reaching levels comparable to the averages found for north temperate birds (Goymann et al. 2004; Hau et al. 2008; Goymann and

Table 1: Plasma testosterone levels of the campo miner (*Geositta poeciloptera*)

Response variable	Explanatory variable		df	F	P
	Breeding	Nonbreeding			
Testosterone level	2.68 \pm 1.83, $n = 27$	1.81 \pm .35, $n = 15$	36	4.34	.044
Testosterone level	Males	Females	36	4.16	.048
	2.72 \pm 1.95, $n = 23$	1.89 \pm .56, $n = 16$			

Note. Results of a generalized linear model ANOVA with Gaussian distribution fitted with identity of seasons (breeding vs. nonbreeding), identity of sexes (male vs. female), and the interaction between both variables as explanatory variables and plasma testosterone levels (ng/mL) as the response variable. Values are presented as means \pm standard deviations, followed by sample sizes. The interaction term was not significant ($F_{1,35} = 2.56$, $P = 0.11$).

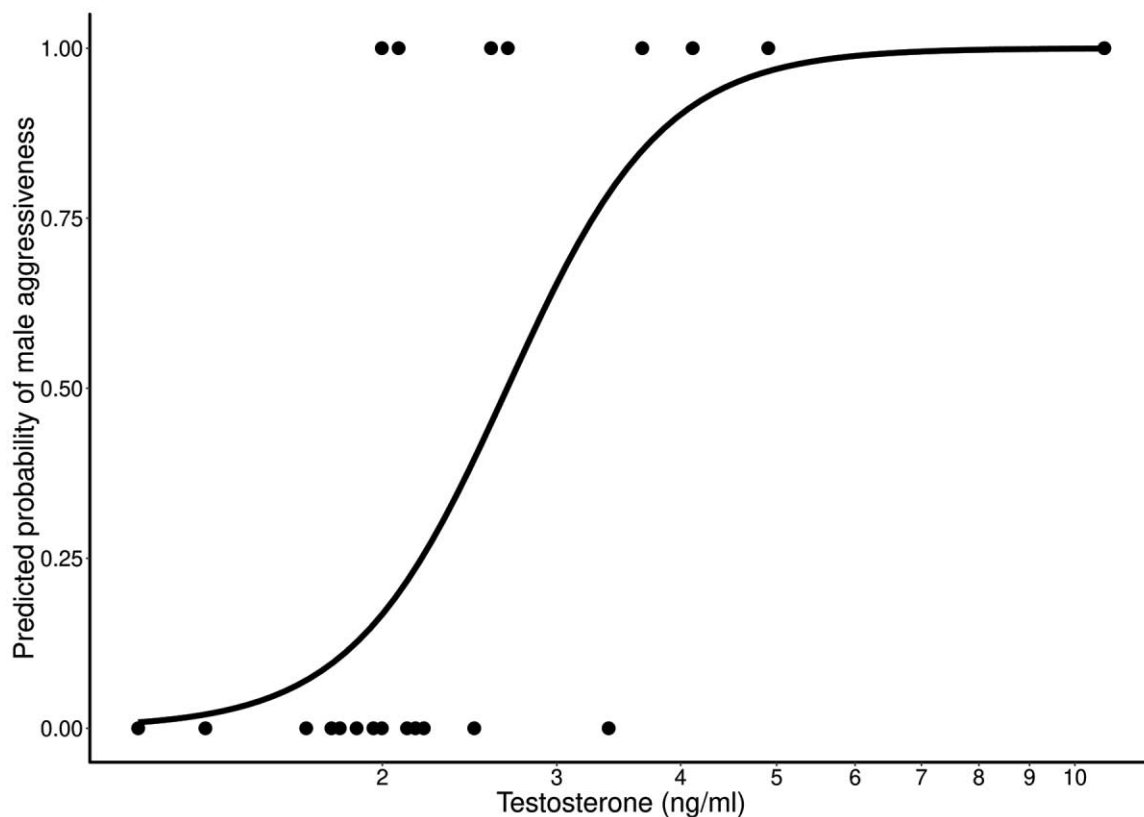


Figure 2. Predicted probability of aggressive behavior of males of the campo miner (*Geositta poecilopectera*) related to the increase in concentration in plasma testosterone levels ($n = 23$). Response variable: 0 = no hovering display, 1 = hovering display.

Landys 2011). Most studies made to date in the tropics have been conducted in habitats with low seasonality, leading to a bias in the overall perception of hormonal traits in tropical regions (Hau et al. 2008). Prolonged high plasma T levels might reduce lifetime fitness because, for example, they may interfere with parental care, inhibit immune function, augment susceptibility to parasites, and increase risk of injury and predation (Ketterson and Nolan 1992; Wingfield et al. 2001). Therefore, species with a comparatively short breeding season, such as most northern and southern temperate birds, as well as tropical birds living in highly seasonal habitats are expected to present a marked increase in plasma T levels during the early breeding season (Goymann and Landys 2011; Addis et al. 2013). This transient increase in circulating T levels would enhance performance of the intense and frequent aggressive displays and contests during territory establishment and defense and during mate guarding, without unnecessarily prolonging high T levels. On the other hand, species with a comparatively long breeding season, such as most resident tropical birds studied to date, can avoid the costs of elevating T because frequency and intensity of aggressive interactions never spike the way they do in species with short, relatively synchronous breeding seasons (Wingfield et al. 2001; Hau et al. 2010). The campo miner, which has a comparatively short breeding season of ~4 mo, seems to follow the pattern predicted by both versions of the challenge hypothesis because males apparently show a marked increase in T during the breeding

season. We did not find a significant interaction of sex and season in our analyses, perhaps because of the small sample size obtained.

More interesting than the differences in mean values of males' plasma T levels between breeding and nonbreeding seasons are the differences in variance. During the breeding season, variance of plasma T values in males was much larger (5.209) than during the nonbreeding season (0.190). This was caused by some marked peaks in T when breeding. There are some explanations, not mutually exclusive, for this pattern. First, fires are frequent in the study area, but they usually burn only comparatively small areas (some affecting only a few hectares) because the riparian forests along the valleys as well as the dense network of dirt roads act as firebreaks. This results in a dynamic and heterogenous landscape mosaic of grassland patches of different ages and vegetation structure (Lopes and Peixoto 2018). As a result, campo miners, which favor low and sparse vegetation cover (CEMAVE 2018; Lopes and Peixoto 2018), are frequently forced to establish new territories at the beginning of the breeding season (which coincides with the end of the dry season). This could lead to frequent male-male aggressive encounters (CH 1.0). Second, the species has the type of comparatively short breeding season that is often associated with synchronous breeding and the consequent competition for extrapair fertilizations (Goymann et al. 2004). This can promote an increase in plasma T levels due to aggressive male-male encounters (CH 1.0) or to the availability of sexually receptive females (CH 2.0). Therefore, both

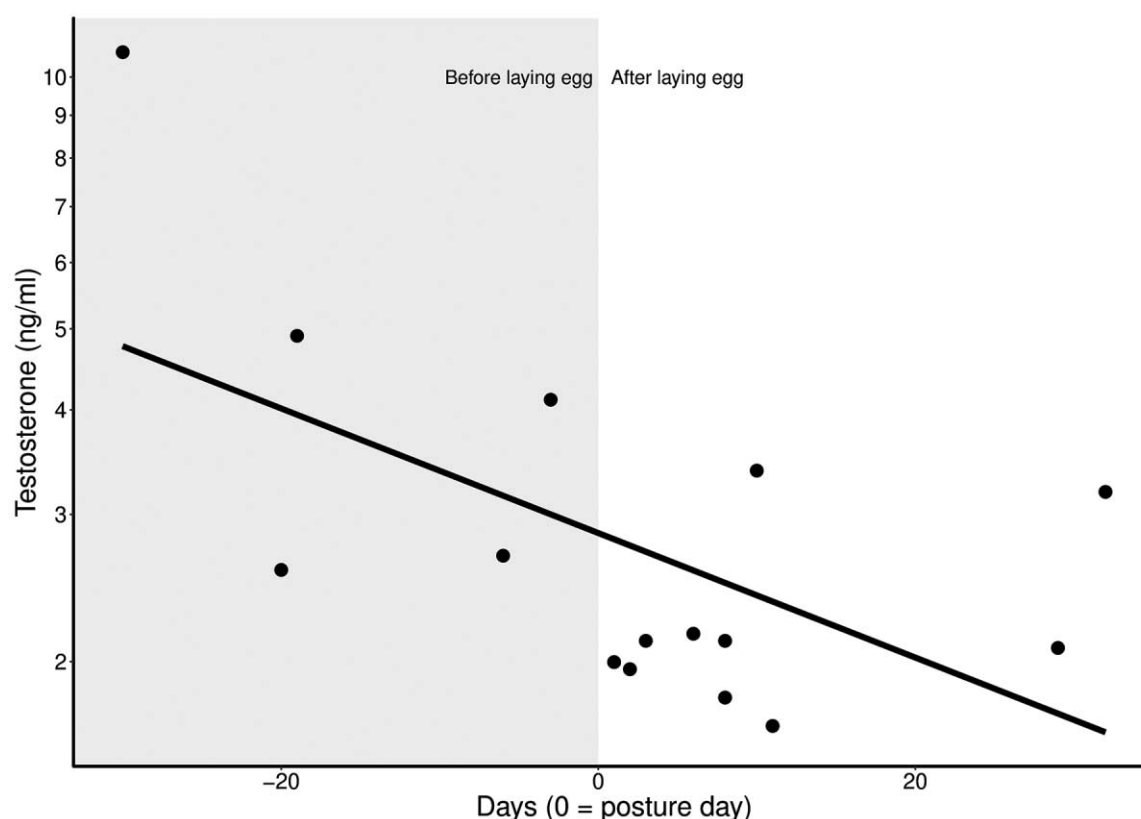


Figure 3. Testosterone levels in relation to egg-laying date (standardized at 0) in 15 males of the campo miner (*Geositta poeciloptera*). Note that plasma testosterone levels tend to decrease after egg laying.

versions of the challenge hypothesis may explain the peaks in T leading to the high variance observed during the breeding season.

All in all, the campo miner, despite being a tropical resident species, exhibits a comparatively short breeding season (and therefore experiences high breeding synchrony) with intense intrasexual competition during territorial establishment (i.e., seasonal territoriality). These life-history traits, which are commonly associated with temperate migratory birds, may explain the latitude effects on T identified by previous researchers (see Garamszegi et al. 2008).

Testosterone in Females

Although male campo miners have higher plasma T levels than females do, females presented comparatively high levels of T (mean: 1.93 ng/mL) during the breeding season, with one female showing a very high level (3.12 ng/mL). These values corroborate the idea that females of monomorphic species with a socially monogamous mating system express higher plasma T levels than females of dimorphic species with other mating systems (Ketterson et al. 2005). For comparison, plasma T levels of female birds (belonging to 44 species, 9 orders) measured throughout the annual cycle were seldom above 0.60 ng/mL (Ketterson et al. 2005). Possible explanations for the high levels of T reported in females in this study include aggressive encounters (see below) or a temporary peak related to egg production and ovulation (Ketterson et al. 2005; Johnson 2014).

Testosterone and Aggressiveness

T levels correlate roughly with expression of territorial aggression, especially in birds that are not territorial throughout the year (Wingfield et al. 2007). Although this is a general trend in the temperate region with many exceptions, plasma T levels are not related to aggressive behavior in males of most tropical birds (Moore et al. 2019). In this sense, the campo miner represents a notable exception because males with higher plasma T levels were more prone to exhibit an aggressive behavior, performing the hovering display flight after the playback of its song. The hovering display flight is also exhibited by the male birds when defending the nest against potential predators, such as the black-tufted marmoset (*Callithrix penicillata*), and nest usurpers, such as the tawny-headed swallow (*Alopochelidon fucata*; Machado et al. 2017). A question that deserves further investigation is whether campo miners exhibit seasonal territorial behavior, which would be divergent from behavior noted in other tropical birds that present year-round territorial behavior (Goymann and Landys 2011).

Testosterone and Parental Care

Campo miners aggressively responded when we played back their songs before egg laying, but afterward, birds showed less aggressiveness, as demonstrated by the unresponsiveness to the playback by most males during the incubation or nestling stages of

reproduction. This evidence is in line with Machado et al. (2017), who observed that campo miners become markedly less aggressive after egg laying. A decrease in male aggressiveness can be related to the decrease of T with the beginning of paternal effort, as demonstrated by previous experiments that supplemented T and that resulted in a decrease in paternal effort in both temperate and tropical birds (Hegner and Wingfield 1987; Peters 2002; Lynn et al. 2009).

Campo miners perform multiple breeding attempts in a single season, with a maximum of three attempts recorded (Machado et al. 2017). Therefore, even though there is a tendency for plasma T levels to decrease after egg laying, some isolated high T levels in the middle of the breeding season were observed, probably triggered by subsequent nesting attempts that caused a decrease in breeding synchrony at the population level. These peaks may result from male-male aggressive encounters (CH 1.0) or the availability of sexually receptive females (CH 2.0). Secondary peaks in plasma T levels between nesting attempts is a possible explanation for the low percentage of variance explained by the model ($r^2 = 0.489$), but this hypothesis needs to be tested with a much larger sample size.

Conclusion

The seasonal and comparatively high plasma T levels found for the campo miner resemble the pattern usually observed in temperate zone birds, probably due to the comparatively short breeding season and seasonal defense of territory in the species. This finding is in line with Hau et al. (2008), who proposed that, with more studies in markedly seasonal environments, more species of tropical birds would be shown to have plasma T changes similar to temperate ones. Our results support the interpretation that habitat seasonality and life-history traits may be more important features influencing hormones and behavior than latitude per se (see Hau et al. 2008; Moore et al. 2019); future studies of environmental endocrinology should not assume the tropical/temperate T dichotomy, as it may well be incorrect.

Our findings make an important contribution to the understanding of environmental endocrinology of tropical birds and fill some gaps of knowledge about the diverse Neotropical avifauna (Moore et al. 2019). This article is the first to evaluate seasonal variations in plasma T levels of a member of the family Scleruridae. Additionally, the campo miner is unusual among previously studied tropical bird species because of its unique habitats (highly seasonal tropical grasslands) and cavity-nesting habits. Finally, further studies with the species using detailed behavioral observations in association with other complementary techniques, such as simulated territorial intrusion experiments and investigation of extrapair fertilizations, will allow a more comprehensive test of the predictions of the two versions of the challenge hypothesis.

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