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Social environment and anogenital distance length phenotype interact to explain testosterone levels in a communally rearing rodent: Part 2: The female side

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ABSTRACT

Testosterone is known as a "male" hormone; however, females also synthetize testosterone, which influences female sexual and aggressive behavior. In female vertebrates, as in males, testosterone levels can vary seasonally. However, female testosterone levels may also be related with female anogenital distance (AGD) length phenotype (a proxy of prenatal androgen exposure), and the social group environment. We used data from a long-term rodent study (2009–2019) in a natural population of degus (*Octodon degus)* to examine the potential associations between female serum testosterone levels, season, female AGD phenotype, and social group composition. We quantified female serum testosterone levels during the mating and offspring rearing seasons, and we determined the number of females and males in social groups, as well the composition of groups, in terms of the AGD of the female and male group mates. Our results indicate that female testosterone levels vary with season, being highest during the offspring rearing season. Additionally, female testosterone levels were associated with the number of male group-members and the AGD of male group-members but were not associated with female social environment and focal female AGD phenotype. Together, our results suggest that female testosterone levels are sensitive to intersexual interactions. Our results also reveal that female and male testosterone levels do not differ between the sexes, a finding previously reported only in rock hyraxes. We discuss how the complex social system of degus could be driving this physiological similarity between the sexes.

1. Introduction

Testosterone is the main sex hormone in males and modulates several morphological, reproductive, and behavioral traits in this sex (Wingfield et al., 1990; Ketterson and Nolan, 1992; Ketterson et al., 2005; MØller et al., 2005; Koren et al., 2006). However, females also produce testosterone and other androgens, which are important for the development and regulation of the female reproductive and central nervous systems, and for the normal functioning of several tissues such as muscle, bone, cartilage, adipose, and the epidermis (Staub and De Beer, 1997; Ketterson et al., 2005; Koren and Geffen, 2009; Rosvall et al., 2020). Additionally, androgens also regulate the aggressive and sexual behavior of females (Staub and De Beer, 1997; MØller et al., 2005; Drea, 2007; Rosvall et al., 2020). In females, testosterone and other androgens are synthetized by the ovaries, the adrenal glands, and the brain (Staub and De Beer, 1997; Ketterson et al., 2005; Rosvall, 2013). Studies in female birds and mammals have revealed cyclical variation in testosterone levels, with higher levels generally being measured during the reproductive season (Staub and De Beer, 1997; Ketterson et al., 2005; Lutermann et al., 2013; Rosvall, 2013; Rosvall et al., 2020; Drea et al., 2021). During the reproductive season, female ovaries are active because they are producing gametes and female sex hormones (Staub and De Beer, 1997). However, female adrenal glands are also active during the reproductive season, as the adrenals produce

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glucocorticoids to help match the increased rate of energy intake, storage, and mobilization during the reproductive period (Hau et al., 2010). Thus, the higher testosterone levels recorded during the reproductive season could be explained by heightened activity of the ovaries, adrenal glands, or both (Hau et al., 2010).

Ultimate explanations suggest that female testosterone levels could vary in relation to aspects of the social system including social organization, social structure, mating systems, and parental care systems. For example, in relation to social organization, a comparative analysis across 42 bird species indicates that females of colonial nesting bird species had higher testosterone levels relative to females of solitary nesting species (MØller et al., 2005; Goymann and Wingfield, 2014). Higher testosterone levels recorded in female birds of colonial species could be explained because females in these species play an important role defending breeding territories, and relatively more aggressive females could be more efficient in this context (Ketterson et al., 2005; MØller et al., 2005; Rosvall, 2013; Rosvall et al., 2020). In relation to mating systems, a comparative analysis across 42 bird species revealed that higher female testosterone levels tended to characterize species with monogamous mating systems, relative to species with polygynous and polyandrous mating systems (Ketterson et al., 2005; Rosvall, 2013). Thus, higher testosterone levels recorded in females of bird species with monogamous mating systems could be a consequence of the high frequency of inter-female conflict over mates (Ketterson et al., 2005; Rosvall, 2013). In relation to parental care systems, a meta-analysis that analyzed six bird species with sex-role reversals where females do not provide parental care, indicates that in these species, differences between male and female testosterone levels are subtle or nonexistent, while in birds with conventional sex-roles, males have higher testosterone than females (Lipshutz and Rosvall, 2020). However, compared to other social system components, social structure has been the most studied in relation to its effect on female testosterone. For example, the agonistic social interactions that occur between females affect female testosterone levels, especially in species where aggressive and dominant females monopolize reproduction. Specifically, in mole rats (Natal mole rat, *Cryptomys hottentotu natalensis;* Damaraland mole rat, *Fukomys damarensis*; naked mole rat, *Heterocephalus glaber*) (Clarke and Faulkes, 1997; Lutermann et al., 2013), and in meerkats (*Suricata suricata*) (Clutton-Brock et al., 2006; Young et al., 2006; Drea et al., 2021) females increase their testosterone levels during the reproductive season regardless of whether they breed or not. Likely, relatively high testosterone levels are needed to mediate heightened aggressive behavior of dominant females and suppress subordinate female reproduction (Clarke and Faulkes, 1997; Lutermann et al., 2013). Additionally, intersexual social interactions can also modulate female testosterone levels. For example, a comparative study in bonobos (*Pan paniscus*) and chimpanzees (*Pan troglodytes*) found that males exhibit significantly higher testosterone levels than females in both species, but that these sex differences in testosterone levels are much larger in chimpanzees than in bonobos (Sannen et al., 2003). This difference may underlie sex differences in social rank, as males are dominant over females in chimpanzees, while females are dominant over males in bonobos (Sannen et al., 2004; French et al., 2013). Similar findings have been reported in three hyena species; males of brown (*Hyaena brunnea*) and striped (*Hyaena hyaena*) hyenas exhibit one order of magnitude higher testosterone levels than females (Racey and Skinner, 1979), while spotted hyena (*Crocuta crocuta*) males, which are known to be socially subordinate to females (Drea, 2009; French et al., 2013), have testosterone levels that are only slightly higher than females (Racey and Skinner, 1979).

Female testosterone levels may also be influenced by the gradient of female masculinization that has been described in several species of litter bearing mammals (Clark and Galef, 1998; vom Saal et al., 1999; Ryan and Vandenbergh, 2002). The gradient of female phenotypical masculinization is the consequence of either maternal stress or the intrauterine position phenomenon (IUP). During maternal stress, adrenal glands may produce and release more androgens than normal, thus exposing female fetuses to high levels of maternal androgens and giving rise to female offspring with masculinized behavioral phenotypes (Bauer et al., 2019). In the context of IUP, female fetuses from the same litter are exposed to a gradient of androgens released from their male siblings. Thus, a female fetus that develops between two male siblings is exposed to higher concentrations of androgens, and therefore develops into a female with some masculine traits. In contrast, a female fetus that develops without contiguous males, or between two female siblings, would be exposed to lower concentrations of androgens, and therefore develop into a female with exacerbated feminine traits. A female fetus that develops between one male and one female fetus experiences an intermediate concentration of androgens and develops into a female with typical feminine traits (vom Saal, 1989; vom Saal et al., 1999; Ryan and Vandenbergh, 2002; Correa et al., 2021). Taken together, prenatal exposure to high or low concentrations of androgens may result in litter and population gradients of female offspring masculinization that irreversibly modify the phenotype of females through adulthood (Clark and Galef, 1998; vom Saal et al., 1999; Ryan and Vandenbergh, 2002). The specific morphometric trait that varies with female masculinization gradient is the length of the anogenital tissue, also known as anogenital distance (AGD). Given that prenatal exposure to androgens affects the development of perineal tissue, the distance between the genital papilla and anus is longer in females that were exposed to higher concentrations of androgens, and shorter in females that were exposed to lower concentrations of androgens (vom Saal, 1989; vom Saal et al., 1999; Ryan and Vandenbergh, 2002). Thus, AGD allows for noninvasive assessment of adult female masculinization levels (vom Saal et al., 1999; Vandenbergh, 2003).

Short AGD and long AGD females differ in their behavior, including aggressiveness and social dominance (vom Saal et al., 1999; Ryan and Vandenbergh, 2002). For instance, long AGD females of domestic mice (*Mus musculus domesticus*), domestic pigs (*Sus scrofa*), and degus (*Octodon degus*) are more aggressive than short AGD females (vom Saal and Bronson, 1978; Rhode-Parfet et al., 1990; Correa et al., 2013), and long AGD females of domestic mice, degus, and alpine marmots (*Marmota marmota*) are socially dominant over short AGD females (vom Saal, 1989; Hackländer and Arnold, 2012; Correa et al., 2013). Additionally, a few studies report that short and long AGD females exhibit species-specific differences in fertility. Short AGD female European rabbits (*Oryctolagus cuniculus*) and Mongolian gerbils (*Meriones unguiculatus*) produce larger litters than long AGD females (Clark and Galef, 1998; Bánszegi et al., 2012), but in degus, long AGD females produce larger litters than short AGD females (Correa et al., 2021). In contrast, litter size is not associated with AGD length in house mice (Vandenbergh and Huggett, 1994), mound-building mice (*Mus spicilegus*, Szenczi et al., 2013), Alpine marmots (Hackländer and Arnold, 2012), and yellow-bellied marmots (Monclús and Blumstein, 2012). Despite the evidence that the female masculinization gradient is a consequence of differential androgen exposure in utero, only two studies have examined the potential association between female AGD phenotype and female testosterone levels. These previous studies, conducted in captivity, found no significant associations between female AGD phenotype and female serum testosterone levels in domestic mice (vom Saal, 1989) or degus (Correa et al., 2013), therefore suggesting that the female AGD length gradient is the consequence of testosterone organizational effects (vom Saal, 1989; Correa et al., 2013; Bauer et al., 2019). This hypothesis is further supported by evidence from domestic mice and Wistar rats (*Rattus norvegicus domestica*), where long AGD females are more sensitive to exogenous testosterone than short AGD females (Gandelman et al., 1977; Houstmuller and Slob, 1990; Ryan and Vandenbergh, 2002).

The common degu is a highly social species where individuals live in social groups that vary in size and individual sex composition (Ebensperger et al., 2004; Ebensperger et al., 2019). Females can be found living in multimale-multifemale groups, unimale-multifemale groups, female pairs, and less frequently, as solitary individuals or in

multifemale groups (Correa et al., 2021). Male and female degus mate with multiple partners from the same or different social groups (Ebensperger et al., 2019), and do not exhibit territoriality (Ebensperger et al., 2016), nor sexual dimorphism in size. Degus mate during June (austral winter) and after a gestation period of 87 ± 3 days (Rojas et al., 1982), females give birth to 1–10 pups (average ± SD: 3.42 ± 2.71) (Ebensperger et al., 2019) and immediately undergo a postpartum estrus so they can mate for a second time (Ebensperger et al., 2013, 2019). During the offspring rearing season, females share their burrows and communally rear their offspring (Ebensperger et al., 2004). Previous studies in captive degus indicate that the nursing season is the most demanding period for females (Veloso and Bozinovic, 2000), as degu mothers care for large litters of precocial offspring (Correa et al., 2023). In accordance with this finding, studies from wild degus indicate that females attain their highest stress induced cortisol levels during the offspring rearing season (Kenagy et al., 1999; Bauer et al., 2014). Additionally, in female (and in male) wild degus, a gradient of AGD lengths has been described (Correa et al., 2016, 2018, 2021). In females, the AGD length gradient has been positively associated with female aggressive behavior, and negatively associated with submissive and affiliative behavior (Correa et al., 2013), while in male degus, no studies have been carried out relative to the behavior of different AGD phenotypes. However, in domestic mice, long AGD males are more aggressive and dominant than short AGD males (Drickamer et al., 1995; Drickamer, 1996). In degus, a previous study (Correa, 2012) suggests that AGD length represents the IUP in which each female (and male) was located, and that testosterone concentrations in amniotic fluid varies depending on IUP (Correa, 2012; Bauer et al., 2019). Thus, female degus that developed between two male fetuses have longer AGDs than females that developed between one male and one female fetus, with even shorter AGDs being observed in females that developed without contiguous male fetuses (or between two females Correa, 2012). More importantly, AGD length variability recorded at birth is accentuated and fixed during puberty, thus causing AGD length variability within a cohort to remain consistent through adulthood (Correa, 2012; Roff et al., 2017). Thus, in degus, AGD length variability can be utilized as a proxy of androgen exposure during prenatal development (Correa, 2012).

Female degus are homophilic by AGD phenotype, meaning that females organize themselves into social groups composed of females of similar AGD phenotype (Correa et al., 2021). Interestingly, offspring quantity and quality increases with the length of the mother's AGD. Under wild conditions, long AGD females wean more and heavier offspring (Correa et al., 2016) when in social groups with other long AGD females (Correa et al., 2021). Additionally, female reproductive success is negatively affected by the number of males in the social group (Hayes et al., 2019), and by increasing instability in social group composition (Ebensperger et al., 2016). These findings indicate that the number, composition, and stability in individual social groups are important factors influencing female reproductive success. In degus, only two previous studies have measured female testosterone, and both studies were performed in captivity (Ebensperger et al., 2010; Correa et al., 2013). In the first study, relative to hormonal correlates of female and male parental care, Ebensperger et al. (2010) measured testosterone in females that were mothers, in females that were not mothers but that accompanied mothers during lactation, and in the fathers. Results from this study indicate that testosterone levels were similar between females that were mothers, females that were not mothers, and fathers, but that mother testosterone levels were negatively associated with the frequency of offspring grooming. Thus, high testosterone mothers tend to groom their offspring at lower frequencies. In the second study, relative to female social dominance and female AGD length, Correa et al. (2013) measured testosterone in females of different AGD phenotypes and found no association between female AGD phenotype and female testosterone levels. More importantly, both studies found that female and male degu testosterone levels are low, so much so that many samples have concentrations below the detectability limit of standard hormone assay kits. However, female degu testosterone levels have not yet been measured under wild conditions.

Our aim was to examine the potential associations between focal female serum testosterone levels and (i) reproductive season (mating/offspring rearing), (ii) focal female AGD phenotype, and (iii) degu social organization, under natural conditions. Factors of degu social organization include the number of males and the number of females in the social group, and the composition of social group in terms of the mean group male and female AGDs (see below). Considering evidence from literature, and taking into account that degu ovaries are active in both the mating and offspring rearing seasons but the adrenal glands are more active during the offspring rearing season, we hypothesized that (1) focal female serum testosterone levels vary across the reproductive season, and we predicted that focal female testosterone levels (i) would be higher during offspring rearing relative to the mating season. Additionally, and considering previous studies in degus and domestic mice that found no or weak associations between female AGD and female testosterone levels, and considering that long AGD males and females are typically more aggressive, we hypothesized that (2) during the mating season, female testosterone levels are not associated with female AGD phenotype, but are associated with the female social environment. Specifically, we predicted that female testosterone levels (ii) would be similar in females of different AGD length phenotypes. Additionally, we predicted that female testosterone levels (iii) would be higher in females of any AGD length phenotype within a social group with few or several long AGD females, and would be lower in females of any AGD length phenotype within a social group with few or several short AGD females. We also hypothesized that (3) during the mating season female testosterone levels are not associated with female AGD phenotype, but are associated with the male social environment. Specifically, we predicted that female testosterone levels (iv) would be similar in females of different AGD length phenotypes. Additionally, we predicted that female testosterone levels (v) would be higher in females of any AGD phenotype within a social group with few or several long AGD males, and would be lower in females of any AGD length phenotype within a social group with few or several short AGD males. Considering that long AGD females demonstrate higher collective reproductive success during the offspring rearing season and have lower individual variance in their reproductive success within the social group than short AGD females, we further hypothesized that (4) female testosterone levels during the offspring rearing season are not associated with female AGD phenotype but are associated with the female social group environment. Specifically, we predicted that female testosterone levels (vi) would be similar between females of different AGD length phenotypes, and (vii) would be lower in females of any AGD length phenotype within a social group with several long AGD females, and would be higher in females of any AGD length phenotype within a social group with several short AGD females. Finally, we hypothesized that (5) female testosterone levels during the offspring rearing season are not associated with female AGD phenotype, but are associated with the male social group environment. Specifically, we predicted that female testosterone levels (viii) would be similar between females of different AGD length phenotypes, and (ix) would be higher in females of any AGD length phenotype within a social group with few or several long AGD males, and would be lower in females of any AGD length phenotype within a social group with few or several short AGD males.

2. Materials and methods

2.1. Study population

Data came from a long-term study conducted between 2009 and 2019 (11 generations) on a natural degu population located at Estación Experimental Germán Greve Silva (33°23′ S, 70°31′ W, altitude 495 m), a field station of the Universidad de Chile. This study area is characterized by a Mediterranean climate with cold, wet winters and warm,

dry summers (di Castri and Hajek, 1976). The sampling site consisted of open areas with scattered scrubs (*Proustia pungens, Vachellia caven*, and *Baccharis* spp.) and several herbaceous species (e.g., *Erodium* spp., *Senecio adenotrichius*; Root-Bernstein et al., 2014). Shrub cover, as assessed from nine 200 m linear transects, reached 14.5 ± 1.2 % (±SE). The total area examined was 2–3 ha and did not vary between years of the study.

2.2. Live trapping and telemetry

Every year we used degu capture, recapture, and radio-tracking to determine degu identity, phenotype based on AGD, and social group membership (winter and spring seasons). Winter live trapping and telemetry were conducted from the first week of May through the last week of July, despite that mating activity is synchronous and concentrated in the last two weeks of June (see the date of estrus and date of blood sampling in Table S1 of Supplementary material 1 - estimated estrus date). We carried out a long period of trapping and telemetry to habituate degus to traps, because the peak of mating activity can be advanced or delayed depending on the amount of autumnal rainfall, and because we needed to remove all radio-collars. Spring live trapping and telemetry were conducted from the last week of August to the first week of November. We chose this lengthy monitoring period because, despite that birth and second mating activity are synchronous and concentrated in the middle of September, we wanted extra time in case the population gave birth earlier or later than normal. Independent of the duration of the field sampling, females were captured and sampled during peak mating activity in the winter and in the first days after the birth, and thus generally coincided with when most females were in estrus (see Table S1, Fig. 1, Suppl. Mat. 1). Degus are diurnally active and remain in underground burrows overnight (Ebensperger et al., 2004). A burrow system was defined as a group of burrow openings surrounding a central location spanning 1–3 m in diameter where individuals were repeatedly found during night-time telemetry (Fulk, 1976; Hayes et al., 2007). Ten traps (Tomahawk model 201, Tomahawk Live Trap Company, Tomahawk, WI) were used at each burrow system daily. Traps were set prior to the emergence of adults during morning hours (06:00 h). After 1.5 h, traps were closed until the next trapping day. The identity, location, sex (degus were sexed morphologically for anogenital distance length, genital papilla size, and presence of a vaginal commissure), body weight (weighed to the nearest 0.1 g), and AGD (see below) were determined for all captured degus. At first capture, each degu received ID-coded tags on each ear (Monel 1005-1, National Band and Tag Co., Newport). Adults weighing >130 g were fitted with 6–7 g radio-collars (AVM Instrument Co., Colfax, CA) with unique pulse frequencies. Previous studies at our field site have confirmed that night-time locations represent underground nest sites (Ebensperger et al., 2004). Locations were determined once per night approximately 1 h before sunrise using LA 12-Q receivers (for radio collars tuned to 150.000–151.999 MHz frequency; AVM Instrument Co., Auburn, CA) and handheld, 3-element Yagi antennas (AVM Instrument Co., Auburn, CA). Given that degus do not leave their nest sites at night, we used radio-telemetry locations during the nighttime to determine group membership (Hayes et al., 2009). The number of burrow systems monitored for each year, the number of days that each burrow system was trapped per year, and the number of radio-collared degus per year are reported in Table S1 of Supplementary Material 2 - Social groups.

2.3. Social group determination

In this study, we utilized two complementary methodologies to define different social groups and to determine which individuals were members of each social group. Degus group naturally and individuals of the same social group share the same burrow at night. To determine which individuals shared the burrow at night, we employed 1) burrow trapping during early morning activity and 2) telemetry during the night-time. To determine group composition, we first compiled a symmetric similarity matrix of pairwise associations of burrow locations of all adult degus during trapping and telemetry (Whitehead, 2008). The association (overlap) between any two individuals was determined by dividing the number of early mornings that these individuals were

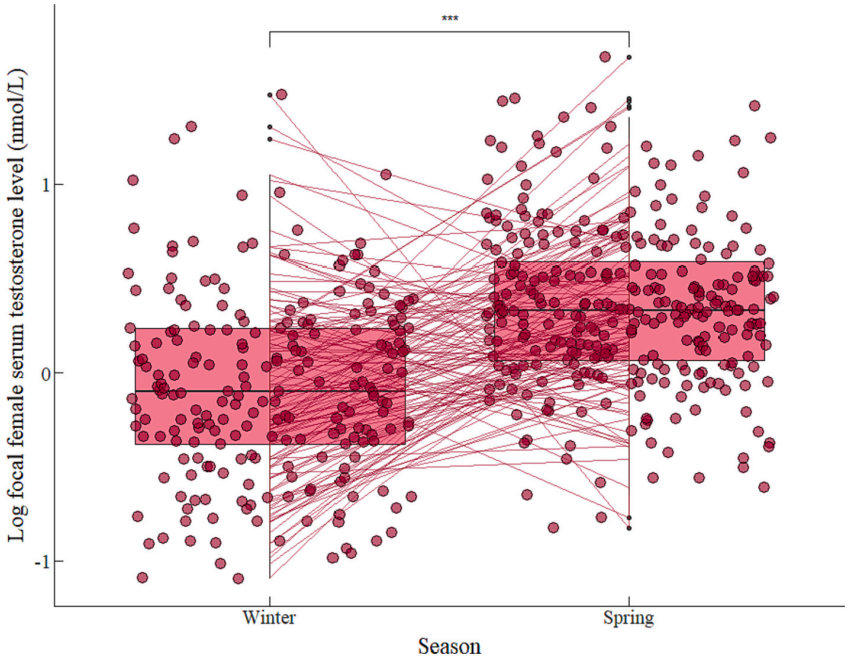

Fig. 1. Effects of season (mating or winter vs. offspring rearing or spring) on focal female serum testosterone levels. *** indicate statistically significant differences. The middle line in the boxplots represents median values, with their respective quartiles. Dots outside the error bars represent outlier values. Points represent the total 525 samples from the 334 females that served as replicates for this analysis. Lines between boxplots represent females whose samples were available for mating and offspring-rearing season.

captured at or tracked with radio-telemetry to the same burrow system, by the number of early mornings that both individuals were trapped or tracked with radio-telemetry on the same day (Ebensperger et al., 2004; Hayes et al., 2009). To determine social group composition, a hierarchical cluster analysis of the association matrix was conducted using SOCPROG software (Whitehead, 2009). The fit of the data was analyzed using cophenetic correlation coefficients, correlations between the actual association indices and the levels of clustering in the diagram. In this procedure, values above 0.8 indicate that hierarchical cluster analysis has provided an effective representation of the data (Whitehead, 2008). The maximum modularity criterion (Newman, 2004) was used to cut off the dendrogram and define social groups.

2.4. Sample size

In this study we include a total sample of 334 different adult females. From these females, 192 were captured only during one season of the study, and sampled once for testosterone; 110 females were captured during two consecutive seasons (i.e., mating and offspring rearing) of the same year, and were sampled twice; 18 females were captured during three different seasons, and sampled three times; 11 females were captured during four different seasons, and sampled four times; and 3 females were captured during five different seasons, and sampled five times. For the 142 females that were captured and sampled during two or more consecutive seasons, each capture and testosterone sample were considered an independent event, as social group and environmental conditions were different between seasons (Correa et al., 2021). Thus, our sample size was 525 samples of testosterone from 334 females. These 525 samples corresponded to 217 from the mating season and 308 from the offspring rearing season. From the 217 samples from the mating season, 13 of them were obtained from females that were alone, and 204 were obtained from females that were members of a social group. Of the 204 females that were in a social group, 31 of them were in social groups without other female members (i.e., the focal female was the unique female in the group), and 45 of them were in social groups without male members (i.e., the focal female did not have a male companion). The remaining females were in social groups with at least one male and one female group member. Of the 308 samples from the offspring rearing season, 22 of them were obtained from females that were alone, and 286 were obtained from females that were members of a social group. Of the 286 females that were in a social group, 43 of them were in social groups without other female members (i.e., the focal female was the unique female in the group), and 82 of them were in social groups without male members (i.e., the focal female did not have a male companion). The remaining females were in social groups with at least one male and one female group member.

2.5. Individual-level variables: female and male AGD

Individual phenotype in terms of masculinization was assessed through anogenital distance (AGD) length, the distance between the ventral anal commissure to the base of the genital papilla (females) or to the base of the penis (males) (Vandenbergh and Huggett, 1994). We measured the AGD (mm) of all adult individuals. At every capture event, we used a digital caliper (precision 0.1 mm) to measure AGD in females exhibiting a non-perforated vagina (a perforated vagina is an indicator of either estrus or recent parturition, as degu females have closed vaginas for all other stages). All AGD length measurements were taken by the same observer (LAC) across all eleven years. We calculated the average AGD from 14.63 ± 12.20 measurements per individual (range: 1–61, $n = 3892$ measurements, from 334 females), resulting in a single AGD estimate per individual (Correa et al., 2021). Intra-season repeatability of female AGD length was 0.90 (measurement error 0.09) from 192 females examined during 2009–2019 ($n = 801$ measurements). Inter-season repeatability of female AGD length was 0.92 (measurement error 0.07) from 110 females which were sampled in both seasons of same year ($n = 2068$ measurements). Inter-year repeatability of female AGD length was 0.94 (measurement error 0.06) from 32 females which were sampled in more than one year ($n = 1023$ measurements). These data suggest that female AGD length is a stable measurement within and between individuals and, therefore, an appropriate trait to estimate the masculinization level of individuals. In wild female degus (as in males), AGD length distribution follows a normal distribution (mean = 2.10 mm; SD = 0.54), with short and long AGD females being a rare phenotype, and intermediate AGD females being a frequent phenotype (Correa, 2012). Similar findings were reported in female fetuses of domestic mice, where vom Saal (1981) and Hotchkiss and Vandenbergh (2005) determined that the most common IUP (~60–65 % of individuals) is between one male and one female, and the other two IUPs (between two males, and without contiguous males or between two females), are less frequent, and are represented in same proportion (~15–20 % of individuals per IUP type, Vandenbergh, 2003, Hotchkiss and Vandenbergh, 2005). This representation of each AGD length phenotype mirrors that of a normal distribution of ±1SD. Thus, we identify short AGD females as those one standard deviation below the mean (≤ 1.55 mm AGD), long AGD females as those one standard deviation above the mean (≥ 2.65 mm), and intermediate AGD females as those within one standard deviation of the mean (between 1.56 and 2.64 mm) (Correa, 2012; Correa et al., 2013, 2016, 2021). This classification is only to describe different AGD phenotypes when discussing our results, as AGD was used as a continuous predictor for all statistical analyses. The mean AGD length of each female was included in our analyses as the *focal female AGD*.

Before statistical modeling, we verified that body weight and AGD length were correlated during the winter (mating season) ($r_s = 0.197$; *p value* < 0.05, $n = 242$), but not during the spring (offspring rearing season) ($r_p = 0.082$; *p value* > 0.05, $n = 325$). This seasonal variation in AGD-body weight correlations is likely explained by the fact that ~1/3 of the female population weigh <180 g in the winter, indicating they have not yet reached their adult weight (the same pattern is observed in male degus, Correa et al., 2024). In developing degus, AGD and body weight are correlated, but this correlation disappears when animals attain their adult body weight (Correa, unpublished data). While all male degus have longer AGDs than female degus, AGD length shows considerable variation in male degus and relates with the IUP in which males were located during gestation (Correa, 2012; Roff et al., 2017; Correa et al., 2018).

2.6. Social group variables

We considered four attributes of social groups, including (1) *the number of females,* (2) *the number of males*, (3) *the mean group female AGD,* the mean AGD of female group members after excluding the focal female AGD, and (4) *the mean group male AGD,* the mean AGD of male group members.

2.7. Individual-level variables: serum testosterone levels

Blood samples were obtained from all adult females one time per season (winter and spring) at their first capture. During the spring season, blood samples of females were collected when females were in early lactation. No blood samples were obtained for the winter season of 2009. Samples were obtained by venipuncture of the saphenous vein, which was punctured with a sterile 14 G needle, allowing ~700 μL of blood to drip into a 1.7 mL Eppendorf tube. Each individual was punctured only once, and after obtaining the sample hemostasis was performed to stop bleeding. All samples were obtained between 08:00–10:00 am. The total handling time from the initial restraint of an animal to the completion of the blood sample collection did not surpass 3 min. Blood samples were consistently taken by the same experienced veterinarians (CL, JRE). We centrifuged blood samples at 6000 rpm for 10 min. Serum was separated from blood cells and stored at −20 °C before subsequent analysis.

Testosterone levels were measured by radio-immunoassay (RIA) for samples from 2009 to 2015, and by enzyme-immunoassay (ELISA) for samples from 2016 to 2019. We used two techniques because starting in 2015, RIA equipment was replaced with ELISA equipment due to biosafety reasons. All samples were analyzed in the Endocrinology Laboratory at P. Universidad Católica de Chile. The RIA technique had a detection limit of 0.3 nmol/L, while the ELISA technique had a detection limit of 0.19 nmol/L. All samples were analyzed in duplicate, and the precision of the assay was evaluated by determining the coefficient of intra- and inter-assay variation. For RIA, intra- and inter-assay variation were 7.7 % and 9.9 %, respectively. For ELISA, intra- and inter-assay variation were 10.6 % and 6.39 %, respectively.

We compared mean estimates of testosterone measured via RIA and ELISA, and found that both techniques obtained statistically similar values. For this statistical comparison, we log-transformed testosterone values and used a Student's *t*-test for independent samples with technique as the main factor. We ran the first analysis using all adult male and female degus in our study population, and the second analysis using the subset of data included in this manuscript (i.e. adult males and females with known social groups). When analyzing the 1475 blood samples from 2009 to 2019, we found that mean testosterone levels did not significantly differ between samples measured using RIA ($n = 798$) and ELISA ($n = 677$, t value $= -0.823$, df $= 1473$, p value $= 0.410$). Similarly, when analyzing the 836 blood samples taken from degus with known social groups, we also found no significant difference between mean testosterone levels assayed via RIA ($n = 457$) and ELISA ($n = 379$, t value $= 0.661$, df $= 834$, p value $= 0.508$).

2.8. Statistical analyses

We used three different sets of models to test each of our predictions (Full models in Supplementary Material 3). To test the prediction (i) focal female serum testosterone levels vary with season, we examined model 1 (Table S1, Suppl. Mat. 3) where we included $n = 525$ females, all of whom were alone or in social groups of different size and composition. For our predictions (ii and iii) that female social environment, but not female AGD phenotype, significantly relates with focal female serum testosterone levels during the mating season, we examined model 2.1 that included all elements of the female social environment and focal female AGD (Table S2, Suppl. Mat. 3) and included $n = 173$ females that had at least one female group mate. For our predictions (iv and v) that focal female serum testosterone levels during the mating season significantly relate with the male social environment but not with female AGD phenotype, we examined model 2.2 that includes all elements of the male social environment and focal female AGD (Table S2, Suppl. Mat. 3) and included $n = 159$ females, all of whom had at least one male group mate. For our predictions (vi and vii) that focal female serum testosterone levels during the offspring rearing season are significantly related with the female social environment but not with female AGD phenotype, we examined model 3.1 that included all elements of the female social environment and focal female AGD (Table S3, Suppl. Mat. 3), and included $n = 243$ females, all of whom had at least one female group mate. For our predictions (viii and ix) that focal female serum testosterone levels during the offspring rearing season are significantly related with the male social environment but not with female AGD phenotype, we examined model 3.2 that included all elements of the male social environment and focal female AGD (Table S3, Suppl. Mat. 3) and included $n = 204$ females, all of whom had at least one male group mate.

All models were analyzed with linear mixed models (Zuur et al., 2009), with serum testosterone log-transformed. Specifically, model 1 included the year of study and degu identity (Degu ID) as random factors, while model 2 and model 3 included the year of study, Degu ID, and social group identity (SGID) as random factors. Model fits were assessed with quantile residual dispersion. Both in the case of the subset of models from model 2 and model 3, SGID did not explained any additional variance and model fit revealed singularity. Because of this, SGID was removed from both sets of models as a random factor before model selection routines to prevent model over-fitting due to the presence of singularity (Barr et al., 2013). We subjected each sub-model to model selection routines, where the best models were chosen by their AICc values and average model weight (Burnham and Anderson, 2002).

All statistical analyses were performed in R 4.1.3 (R Core Team, 2023). Linear mixed models (LMM) were fitted with the package LME4 1.1–31 (Bates et al., 2015). DHARMa 0.4.6 (Hartig, 2022) and MuMIn 1.46.0 (Bartón, 2009) packages were used to perform residual diagnostics and model selection routines, respectively.

2.9. Ethical note

Animal handling techniques and protocols used during this study adhered to the Guide of the American Society of Mammalogists for the use of wild animals in research (Sikes et al., 2016). All protocols implemented during this study were approved by the Scientific Ethical Committee for the Care of Animals and the Environment of the Pontificia Universidad Católica de Chile (CBB-155, 2012 resolution, supervised and approved 03/03/2015, CBB-170509009 resolution, supervised and approved 08/2020), and by the Bioethics Committee for the Use of Animals in Research of the Universidad Austral de Chile (DID-03/09 resolution, supervised and approved 10/06/2009), and followed the Chilean Ethical Legislation (Permits 1–31/2009, 3881/2012, 2826/2013, 6975/2017 and 2890/2019, by the Servicio Agricola y Ganadero). Blood sampling was performed by well-trained veterinarians (CL and JR-E).

3. Results

3.1. Effect of season (mating/offspring rearing) on focal female testosterone levels

Results from selected model 1 (Table S1, Supplementary Material 4-Model selection) indicates that season explains variation in female testosterone levels. Specifically, females exhibited higher testosterone levels during the offspring-rearing season (or spring) relative to the mating (or winter) season (mean mating season: 1.06 nmol/L ± 0.79 SD; range 0.27–5.31, $n = 217$, mean offspring rearing season: 1.63 nmol/L ± 1.03 SD; range 0.32–7.23, $n = 308$; Table 1; Fig. 1). An analysis performed with a subset of 133 females, which were sampled in two consecutive seasons of same year, indicated the same pattern (t value: −6.059; p value: < 0.0001; (mean mating season: 1.04 nmol/L ± 0.80; mean offspring rearing season: 1.60 nmol/L ± 1.12)).

3.2. Female testosterone levels, female AGD phenotype, and social environment during the mating season

For model 2.1, which analyzed the effect of female AGD phenotype and the female social environment on female serum testosterone levels during the mating season, the model selection routine selected the null model (Table S2, Supplementary Material 4-Model selection). This means that female AGD phenotype (Fig. 2, panel A), the number of females in the social group, and the mean group female AGD, are not significantly associated with female serum testosterone during the mating season. Results from model 2.2, which analyzed the effect of female AGD phenotype and the male social environment on female serum testosterone levels during the mating season, revealed that focal female testosterone levels are associated with the male social environment (Table S3, Supplementary Material 4-Model selection). There was a statistically significant factor interaction between the number of males in the social group and mean group male AGD on female serum testosterone during the mating season (Table 2). Specifically, higher testosterone levels were recorded in females of social groups with an increasing number of long AGD males, but lower testosterone levels

were recorded in females when in social groups with multiple short AGD males. Intermediate testosterone levels were recorded in females that shared group membership with relatively few short and/or long AGD males (Fig. 3).

3.3. Female testosterone levels, female AGD phenotype, and social environment during the offspring rearing season

For model 3.1, which analyzed the effect of female AGD phenotype and the female social environment on female serum testosterone levels during the offspring rearing season, the model selection routine selected the null model (Table S4, Supplementary Material 4-Model selection). This means that female AGD phenotype (Fig. 2, panel B), the number of females in the social group, and the mean group female AGD are not significantly associated with female serum testosterone during the offspring rearing season. Similarly, for model 3.2, which analyzed the effect of female AGD phenotype and the male social environment on female serum testosterone levels during the offspring rearing season, the model selection routine selected the null model (Table S5, Supplementary Material 4-Model selection). This means that female AGD phenotype (Fig. 2, panel B), the number of males in the social group, and the mean group male AGD are not significantly associated with female serum testosterone during the offspring rearing season.

4. Discussion

Our study revealed that female serum testosterone levels vary in relationship to season and the male social environment, but not in relationship to female AGD phenotype and the female social environment. As we predicted, female testosterone levels were higher during the offspring-rearing season (spring) relative to the mating season (winter). This finding may reflect greater adrenal activity during the offspring-rearing season relative to the mating season. Greater activation of the adrenal glands could result in the secretion of androgen precursors such as DHEA (dehydroepiandrostenone), DHEA-S (dehydroepiandrostenone sulfate), and androstenedione by the adrenal cortex, which could be metabolized in peripheral tissues to produce testosterone (Staub and De Beer, 1997; Ketterson et al., 2005; Rosvall et al., 2020). Greater adrenal activation during the offspring-rearing season vs. the mating season may be due to the high energetic expense associated with breeding effort. In previous studies in the same wild population, both Kenagy et al. (1999) and Bauer et al. (2014) recorded how blood cortisol levels of adult female degus peak during the offspring-rearing season, and Ebensperger et al. (2013) recorded a positive association between female reproductive effort and cortisol levels. Although we are hypothesizing that the higher testosterone levels recorded during the offspring-rearing season could be explained by an increase in the production of androgen precursors by the adrenal gland, we do not rule out that the ovaries could be the main source of female testosterone, like in female humans (*Homo sapiens*, Staub and De Beer, 1997). In female degus, ovaries are active during the offspring rearing season, as females exhibit a postpartum estrus (Ebensperger et al., 2013, 2019). Additionally, we have evidence that during the mating season, female testosterone attain their higher concentrations around the period of estrus, which is when the majority of testosterone samples were obtained in this study (see Table S1 and Fig. S1, Suppl. Mat 1-estimated estrus date). Therefore, high testosterone levels in female degus during the offspring-rearing period could also be explained by heightened ovarian activity. Future studies that determine the origin of female testosterone (ovary or adrenal) are necessary to determine the role of each organ in female testosterone production. Additionally, studies that examine the interaction between the hypothalamus-pituitary-adrenal axis and the hypothalamus-pituitary-gonadal axis in degus also are necessary to understand the feedback between glucocorticoids and androgens (Toufexis et al., 2014).

During both mating and the offspring rearing season, we found no significant relationship between female testosterone levels and female AGD length phenotype, a finding that met our predictions. This contradicts findings in male rodent species, as Mongolian male gerbils with long AGDs have higher testosterone levels (Clark et al., 2002) and domestic male mice of different AGD phenotypes have similar testosterone levels (Crump and Chevins, 1989). In male degus, the association between male testosterone levels and male AGD length phenotype can be negative or positive depending on the season and male social environment (Correa et al., 2024). In females, only two previous studies have examined the potential association between female serum testosterone levels and female AGD phenotype (vom Saal, 1989; Correa et al., 2013). Results from these captive studies suggest that in domestic mice as in degus, female testosterone levels are not associated with female AGD phenotype. Results from this study, carried out under wild conditions and with a large sample size, confirm previous findings and suggest that the female masculinization gradient may be consequence of testosterone organizational effects (Correa et al., 2013; Bauer et al., 2019).

Contrary to our predictions, we found that female testosterone levels were predicted by the male social environment, but not by the female social environment. Under the challenge hypothesis (Wingfield et al., 1990; Rosvall, 2013), that also can be extended to females (Grebe et al., 2022; Rosvall et al., 2020), female testosterone levels are expected to increase in situations/conditions/times when inter-female conflict is high, and/or when female competition is intense, as seen during the mating season in naked, Natal, and Damaraland mole-rats, meerkats, ring-tailed lemurs, blue-eyed black lemurs (*Eulemur flavifrons)* and

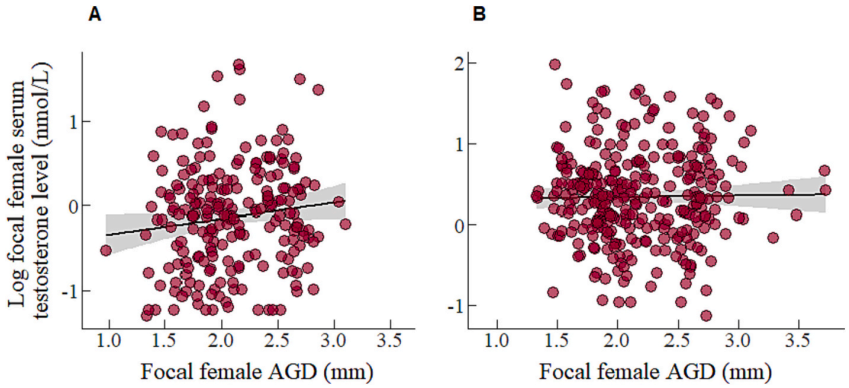

Fig. 2. Relationship between log focal female serum testosterone levels and focal female AGD during the mating season (winter) and offspring rearing season (spring). Circles represent 217 samples from 195 females during mating season (panel A) and the 307 samples from 268 females during offspring season (panel B) that served as replicates. The grey region indicates 95 % confidence intervals.

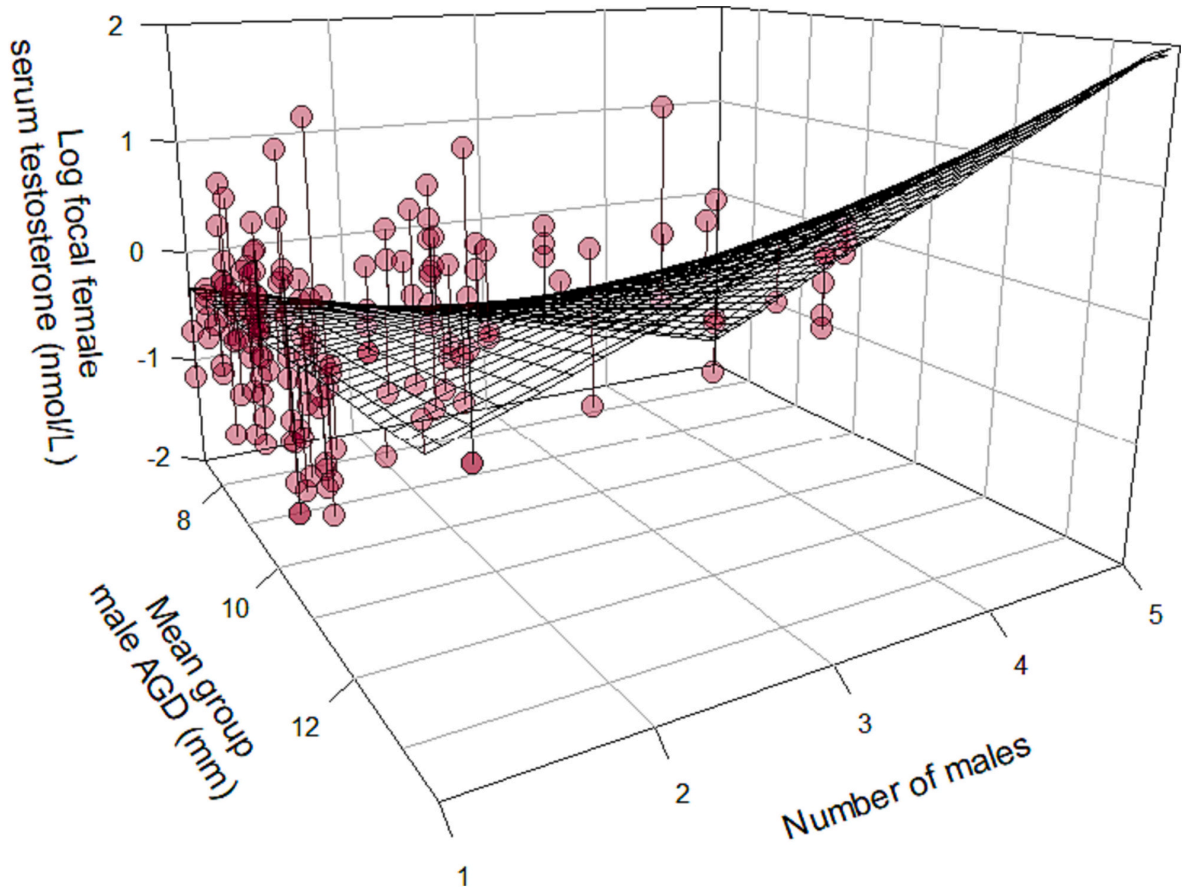

Fig. 3. Relationship between log focal female serum testosterone levels, the number of adult males in the social group, and the mean group male AGD during the mating season (winter). Black surface represents the model-predicted values, and the circles represent 159 samples from 141 females that served as replicates for this analysis; vertical lines represent data deviations from the model prediction.

Table 1
Full results from model 1, seasonal differences in female serum testosterone levels. A total of $n = 525$ female samples from 334 females were used in the analysis.
Model proposed: female testosterone levels = season + year + Degu ID.
Model selected: female testosterone levels = season + year + Degu ID.

Random effects	Variance	Standard deviation
Degu ID	0.07	0.26
Year	0.02	0.15
Residual	0.25	0.50

Fixed effects	Estimate	Standard error	t value	*p* value
Intercept	0.35	0.06	6.07	<0.0001
Season	−0.43	0.05	−8.93	<0.0001

several bird species (Clarke and Faulkes, 1997; von Engelhardt et al., 2000; Clutton-Brock et al., 2006; Ketterson et al., 2005; Young et al., 2006; Drea, 2007; Lutermann et al., 2013; Rosvall et al., 2020; Drea et al., 2021). However, we found no significant relationships between female degu testosterone levels and the female social environment. Nevertheless, we cannot rule out that testosterone levels do not underlie inter-female conflict, as we were not able to measure social interactions. However, and in opposition to the predictions of the challenge hypothesis expanded to females (Grebe et al., 2022; Rosvall et al., 2020), we found that higher female testosterone levels were recorded during

Table 2
Full results for model 2.2 female serum testosterone levels, during the mating season (winter). A total of n = 159 female samples from 141 females were used in the analysis.
Model 2.2 proposed: Female testosterone levels during the mating season = n° of males + mean group male AGD + focal female AGD + (n° of males x mean group male AGD x focal female AGD) + year + Degu ID + SGID.
Model 2.2 selected: Female testosterone levels during the mating season = n° of males + mean group male AGD + (n° of males x mean group male AGD) + year + Degu ID.

Random effects	Variance	Standard deviation
Degu ID	0.08	0.29
Year	0.03	0.16
Residual	0.22	0.47

Fixed effects	Estimate	Standard Error	t value	*p* value
(Intercept)	0.79	0.95	0.83	0.4079
Mean group male AGD	−0.09	0.10	−0.99	0.3225
n° of males	−1.40	0.66	−2.12	0.0357
Mean group male AGD x n° of males	0.14	0.07	2.10	0.0377

the offspring rearing season rather than during the mating season. This pattern is similar in male degus, where males also attain higher testosterone levels during the offspring rearing season (Correa et al., 2024). Thus, general findings from recent studies in degus (this study, Correa et al., 2024), suggest that seasonal variation in testosterone levels, in female and male degus, are not in agreement with the predictions from the original challenge hypothesis (Wingfield et al., 1990) nor with the predictions from the challenge hypothesis expanded to females (Grebe et al., 2022; Rosvall et al., 2020).

In agreement with our predictions, we found that during the mating season (winter), females with higher testosterone levels grouped with multiple long AGD males, whereas females with lower testosterone levels grouped with multiple short AGD males. We lack studies examining how AGD phenotype influences male behavior in degus, but results from domestic mice indicate that long AGD males display higher aggressiveness and social dominance (Drickamer et al., 1995; Drickamer, 1996). We hypothesize that a social group environment comprised of several long AGD males could be socially stressful for females, potentially because of the high frequency of agonistic interactions between the males (Correa et al., 2024). Our findings, together with evidence from previous studies that indicate that females may experience some costs when cohabiting with males (Ebensperger et al., 2010; Hayes et al., 2019), suggest that females are sensitive to the male social environment and that they respond with endocrine changes to male stimuli (Ebensperger et al., 2010; this study). In a parallel with females, male degus also exhibit variation in their testosterone levels when they cohabit with long AGD females (Correa et al., 2024). Together results from males and females suggest that long AGD females and males constitute a social stimulus that can modulate the testosterone profiles of their group mates of the opposite sex, suggesting that in degus, intersexual social interactions are more relevant that intra-sexual interactions towards influencing testosterone dynamics. This last finding opens the door to the exploration of the challenge hypothesis (Wingfield et al., 1990), but extended to intersexual interactions (Grebe et al., 2022), as in degus, male and female testosterone seems to be most sensitive to intersexual rather than to intrasexual interactions.

Male degus had significantly higher testosterone levels than females during the mating season (males: 1.44 ± 0.93, females: 1.06 ± 0.79 nmol/L), but not during the offspring-rearing season (males: 1.87 ± 1.42, females: 1.63 ± 1.03 nmol/L). Similar results have been reported bird species where males, rather than females, provide all parental care. In these species with parental *sex role reversals*, males have higher testosterone levels than females during the mating season, but these differences disappear during the parental care season, as males reduce and females increase their testosterone levels, respectively (Lipshutz and Rosvall, 2020). However, data from degus (this study, Correa et al., 2024), indicate that both sexes increase their testosterone levels from mating to the offspring rearing season, although females show a more marked increase. Furthermore, while male testosterone levels are significantly higher than female levels during the mating season, this sex difference disappears by the offspring rearing season. This was a surprising result, as rock hyraxes (*Procavia capensis*) have been the only mammal species thus far with no reported sex differences in testosterone levels (Koren et al., 2006; Drea, 2009; Koren and Geffen, 2009; French et al., 2013). In mammals, males are generally the aggressive and dominant sex, although there are at least nine species exhibiting reversed sexual dimorphism or that do not fit this expected sex difference in behavior (French et al., 2013). French et al. (2013) named these species "*atypical mammals*", where females are equally or more aggressive than males and/or are similarly dominant. French et al. (2013) examined if higher aggressiveness and dominance of females of atypical mammal species could be explained by higher testosterone levels, and only rock hyraxes have been recorded to exhibit females with higher or similar testosterone levels than males. Thus, higher female aggressiveness/dominance of atypical mammals could result from organizational effects of testosterone, or from activational effects of other androgens like androstenedione (French et al., 2013; Goymann and Wingfield, 2014; Lipshutz and Rosvall, 2020; Drea et al., 2021; Grebe et al., 2022). Androstenedione is a relatively weak (or even inactive) androgen released by female adrenal glands and ovaries and is converted into testosterone in peripheral tissues (Staub and De Beer, 1997). This androgen has been proposed as a candidate hormone involved in the phenotypical and behavioral masculinization of female spotted hyenas, ring tailed lemurs, blue-eyed black lemurs, and meerkats (Goymann et al., 2001; Drea, 2007; Drea et al., 2021; Grebe et al., 2022). Degus could be another atypical species as males are not dominant over females (Correa, unpublished data) and do not always exhibit higher testosterone levels than females (this study, Correa et al., 2024). Future studies are needed, however, to examine potential differences in aggressiveness between male and female degus. Degus similarly share other attributes with atypical species, including a complex social organization based on multimale-multifemale social groups (Hayes et al., 2019), a social structure characterized by the communal rearing of offspring (Ebensperger et al., 2004), males that are non-infanticidal (Ebensperger, 2001) and provide non-essential paternal care (Ebensperger et al., 2010; Aspillaga-Cid et al., 2021), intersexual interactions that are mostly affiliative (Soto-Gamboa et al., 2005), and males that are as affiliative as the females (Correa, unpublished data). Degus are also monomorphic and not territorial (Ebensperger et al., 2016), and both sexes mate with multiple partners from the same and neighboring social groups (Ebensperger et al., 2019). Thus, we hypothesize that similar to some sex-role reversal, monogamous, and colonial birds (Ketterson et al., 2005; MØller et al., 2005; Goymann and Wingfield, 2014; Lipshutz and Rosvall, 2020), and similar to atypical mammals, including the rock-hyraxes (Saanen et al., 2003; Koren et al., 2006; Koren and Geffen, 2009; French et al., 2013; Drea et al., 2021), the unique social organization, social structure, and mating system of degus could explain the absence of strong sex differences in testosterone levels.

5. Conclusions

Evidence from our long-term study on a wild population of degus demonstrates that female testosterone levels are influenced by season and the male social environment, but not by female AGD phenotype nor female social environment. As predicted, female testosterone levels were higher during the offspring-rearing season. Additionally, females of different AGD length have similar levels of testosterone, suggesting that the female AGD masculinization gradient is consequence of testosterone organizational effects. Contrary to our predictions, female testosterone levels were not significantly associated with the female social environment but were sensitive to the male social environment. These findings agree with previous results in wild and captive degus. Finally, we found that male testosterone levels were only slightly higher than female testosterone levels during the mating season and were no different from those of females during the offspring-rearing season. Thus, degus are the second mammalian species, after rock hyraxes, in which males and females have similar testosterone levels. These observations, in association with the amicable behavior of males and lack of male dominance over females, suggests that degus could be considered an atypical species. The analyses of this extensive data set are clearly exploratory and given the multiple models investigated, results may be influenced by type 1 error (False positive). Any conclusions derived from these analyses should thus be considered cautiously and would require confirmatory analyses by predetermined, preregistered protocols.

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CRediT authorship contribution statement

Loreto A. Correa: Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Antonia Aspillaga-Cid:** Writing – review & editing, Writing – original draft, Software, Methodology, Data curation. **Cecilia León:** Supervision, Methodology, Data curation. **Carolyn M. Bauer:** Writing – review & editing, Writing – original draft, Supervision, Investigation. **Juan Ramírez-Estrada:** Supervision, Methodology, Data curation. **Loren D. Hayes:** Writing – review & editing, Supervision, Software, Resources, Methodology. **Mauricio Soto-Gamboa:** Supervision, Methodology, Investigation, Conceptualization. **Luis A. Ebensperger:** Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors have no competing interests.

Data availability

Analyses reported in this article can be reproduced using the data provided by Correa, LA. Data for: social environment and masculinization level interact to explain testosterone levels in a communally rearing rodent: part 2: the female side Dryad: doi:https://doi.org/10.5061/dryad.crjdfn3b3.

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