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Social environment and anogenital distance length phenotype interact to explain testosterone levels in a communally rearing rodent: Part 1: The male side

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ABSTRACTBSTRACT

In vertebrates, male testosterone levels vary across the year being generally higher during the mating season relative to the offspring rearing season. However, male testosterone levels may also be associated with male anogenital distance (AGD) length (a proxy of prenatal androgen exposition), and influenced by the social group environment. In social species, it has been proposed that high levels of testosterone could be incompatible with the development of an amicable social environment. Thus, in these species, it is predicted that males have relatively low levels of testosterone. Our goal was to examine the potential association between male serum testosterone levels, season, male AGD length, and the social environment in the rodent *Octodon degus* under natural conditions. We quantified male serum testosterone levels during the mating and offspring rearing seasons, and we determined the number of females and males in each social group, as well as the composition of groups, in terms of the AGD length of the female and male group mates, from 2009 to 2019. Our results revealed that male testosterone levels covary with season, being highest during the offspring rearing season. Additionally, male testosterone levels vary with male AGD length, and female and male social group environments. More importantly, male degus exhibit low levels of testosterone that are indistinguishable from female levels during offspring rearing season. Similar to other highly social mammals, where males and females live together year-round, male amicable behavior could be the best male mating strategy, thus leading to a reduction in circulating testosterone levels.

1. Introduction

Testosterone, the main male sexual hormone, is synthetized by testes, adrenal glands, and the brain of male vertebrates (Staub and De Beer, 1997). As testes activity varies throughout the year, adult male serum testosterone levels also vary predictably within the year, being highest during mating season and lowest during the non-reproductive season (Wingfield et al., 1990; Trainor and Marler, 2001; Hau et al., 2010). This temporal variation is associated with male reproductive state and the social environment (Wingfield et al., 1990; Ketterson and Nolan, 1992; Wingfield et al., 2001; Gleason et al., 2009; Koren et al., 2019). During the mating season, male testosterone levels are high to facilitate territory acquisition, female attraction, sperm production, and development of secondary sexual traits (Wingfield et al., 2001; Hau, 2007; Hau et al., 2010), as during the mating season males typically interact agonistically with other males, but sexually with females (Wingfield et al., 1990; Ketterson and Nolan, 1992; Trainor and Marler, 2001; Gleason et al., 2009). On the other hand, male testosterone levels are generally low during the offspring rearing season because males may be exposed to parental care demands (Trainor and Marler, 2001;

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Wingfield et al., 2001; Hau, 2007), and elevated testosterone may interfere with necessary paternal care (Wingfield et al., 1990).

In species where males cohabit with females and their offspring, but do not provide parental care, low testosterone levels seem necessary to reduce male aggression towards offspring (Reburn and Wynne-Edwards, 1999). In support of this hypothesis, Schradin et al. (2009) suggest that relatively high male testosterone levels in the facultatively social African striped mouse (*Rhabdomys pumulio*) are not compatible with the expression of amicable behavior needed to allow cohabitation with the females and their offspring. Similar arguments have been raised by Holekamp and Smale (1998), Surbeck et al. (2012) and Koren et al. (2019), to explain the unexpected low male testosterone levels recorded in males of three highly social species (spotted hyenas (*Crocuta crocuta*), bonobos (*Pan paniscus*) and rock hyraxes (*Procavia capensis*)). It is well known that male testosterone levels are sensitive to social interactions (Wingfield et al., 2001; Gleason et al., 2009; Koren et al., 2019). In species where males are usually solitary and territorial, male-male interactions typically are agonistic and contribute to the increase in testosterone levels during social challenge responses (*Challenge Hypothesis*, Wingfield et al., 1990). On the contrary, in species where males usually live in multimale-multifemale social groups, male-male interactions are less aggressive and male testosterone levels remain generally low (Bales et al., 2006). Male-female social interactions also modulate male testosterone levels, where levels of this hormone rise when males interact with sexually receptive females and decrease when males interact with pregnant or lactating females (Reburn and Wynne-Edwards, 1999; Gleason et al., 2009; Aspillaga-Cid et al., 2021).

Male testosterone levels could also be modulated by male phenotype, and alternative phenotypes have been linked to different levels of male masculinization in mammals (Clark and Galef, 1998; Kaiser and Sachser, 2001; Kaiser et al., 2003). Variation in male masculinization levels can be the consequence of either maternal stress or intrauterine position phenomenon (IUP). During maternal stress, male fetuses may be exposed (i) to lower concentrations of androgens, ii) to less powerful androgens and/or (iii) to androgens but outside of the most sensitive developmental period (Ward, 1972; Ward and Weisz, 1980; Kaiser et al., 2003), thus giving rise to males with inconspicuous masculine traits. In the context of IUP, male fetuses within a litter are exposed to a gradient of androgens released from their siblings in utero. Thus, a male fetus that develops between two male siblings would be exposed to higher concentrations of androgens and develop into an adult with exacerbated male traits. In contrast, a male fetus that develops without contiguous male siblings (or between two females) would be exposed to a relatively low concentration of androgens and would develop into a male with inconspicuous masculine traits (vom Saal, 1989; vom Saal et al., 1999; Ryan and Vandenbergh, 2002; Correa et al., 2018). Taken together, prenatal exposure to high or low concentrations of androgens may result in litter and population gradients of male offspring masculinization that irreversibly modify the phenotype of individual males and persist through adulthood (Clark and Galef, 1998; vom Saal et al., 1999; Ryan and Vandenbergh, 2002). Of importance, one specific morphometric trait that varies with male masculinization gradient is the length of the anogenital tissue, or anogenital distance (AGD). Given that prenatal exposure to androgens affects the development of perineal tissue, the distance between the penis and anus is longer in males that were exposed to higher concentrations of androgens, and shorter in males that were exposed to lower concentrations of androgens (vom Saal, 1989; vom Saal et al., 1999; Ryan and Vandenbergh, 2002). Thus, AGD allows the noninvasive assessment of adult male masculinization levels (vom Saal et al., 1999; Vandenbergh, 2003).

Males with short and long AGDs differ in several physiological, reproductive, and ecological traits (Clark and Galef, 1998; vom Saal et al., 1999; Ryan and Vandenbergh, 2002; Ophir and delBarco-Trillo, 2007; Godsall et al., 2014; Correa et al., 2018). However, behavioral differences only have been studied in male domestic mice (*Mus musculus*), where compared to short AGD males, long AGD males are more aggressive, have higher probabilities of being dominant, and win more inter-male contests (Drickamer et al., 1995; Drickamer, 1996). Even though the phenomenon of male masculinization gradient is a consequence of differential exposure to testosterone during prenatal development, testosterone levels in adult males and its potential association with male AGD phenotypes remains poorly understood. The only three studies examining this link revealed that long AGD adult male Mongolian gerbils *(Meriones unguiculatus*) exhibit higher testosterone levels than short AGD males, However, in domestic mice and captive degus (*Octodon degus*), males of different AGD phenotypes do not differ in their testosterone levels (Crump and Chevins, 1989; Aspillaga-Cid et al., 2021).

The common degu represents 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). Males can be found living in multimale-multifemale groups, unimale-multifemale groups, one male-one female pairs, and less frequently, as solitary individuals or in multimale groups (Correa et al., 2018). 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) or sexual size dimorphism. Degus mate during June (austral winter) and after a gestation period of 87 ± 3 days (Rojas et al., 1982), females give birth to an average of 3.42 ± 2.71 (SD) offspring, with a range of 1–10 offspring. Females can mate immediately after parturition as they exhibit postpartum estrus (Ebensperger et al., 2013, 2019). During the offspring rearing season (austral spring), males share their burrows with lactating females, and laboratory studies suggest that males provide some, but non-essential, parental care which is not influenced by male AGD phenotype. Male parental care includes offspring huddling and retrieving, and food provisioning, and seems to be complementary to female parental care (Fulk, 1976; Ebensperger et al., 2010; Aspillaga-Cid et al., 2021). Previous studies (Soto-Gamboa, 2005; Soto-Gamboa et al., 2005) indicate that social interactions modulate male testosterone levels. Specifically, male-male agonistic interactions increase males' testosterone levels, while male-female interactions can increase or decrease male testosterone levels, depending on female reproductive status. Male-offspring interactions do not affect male testosterone levels, and solitary life is associated with the lowest levels of testosterone recorded in male degus (Aspillaga-Cid et al., 2021). Additionally, in male (and in female) 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 and cooperative behavior (Correa et al., 2013, 2021), and negatively associated with submissive and affiliative behavior (Correa et al., 2013), while in males, the behavioral consequences of male AGD gradient have not yet been studied. However, in male degus, the AGD phenotype is positively associated with male reproductive success, thus long AGD males sired more offspring, than short AGD males (Correa et al., 2018). In degus, a previous study (Correa, 2012) suggests that AGD length represents the IUP in which each male (and female) was located, and that testosterone concentrations in amniotic fluid vary depending on the IUP (Correa, 2012; Bauer et al., 2019). Thus, male degus that developed between two male fetuses have longer AGDs than males that developed between one male and one female fetus, with even shorter AGDs being observed in males 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 testosterone exposure during prenatal development (Correa, 2012). In degus, previous studies suggest that male testosterone levels vary throughout the year (Kenagy et al., 1999; Soto-Gamboa et al., 2005) however, these studies did not include a systematic testosterone sampling during the offspring rearing season. Additionally, male testosterone levels under wild conditions have not been analyzed in relation to

male AGD phenotype. Furthermore, studies that analyze the effect of social environment on male testosterone levels have been conducted in captivity, with social group environment manipulated experimentally (Bustos-Obregón and Ramírez, 1997; Ebensperger et al., 2010; Aspillaga-Cid et al., 2021), or have been conducted in wild conditions without analyzing social group effects (Kenagy et al., 1999; Soto-Gamboa, 2005; Soto-Gamboa et al., 2005).

Our aim was to examine the potential associations between focal male serum testosterone levels and (i) reproductive season (mating/offspring rearing), (ii) focal male AGD phenotype, and (iii) female and male social group environments experienced by adult male degus under natural conditions. Considering the theoretical framework of the challenge hypothesis, we hypothesized that (1) focal male serum testosterone levels vary across reproductive seasons, and we predicted that focal male testosterone levels (i) would be higher during the mating season relative to the offspring rearing season. Additionally, under the assumption that long AGD females are more aggressive, and short AGD females are affiliative and docile, and given that male-female sexual and agonistic interactions are frequent during the mating season, we hypothesized that (2) focal male testosterone levels during the mating season are associated to the interaction between focal male AGD and female social environment. Specifically, we predicted that focal male testosterone levels (ii) would be higher in long AGD males that share the social group with several long AGD females, and lower in long AGD males that share the social group with several short AGD females. We also predicted that focal male testosterone levels (iii) would be higher in short AGD males that share the social group with several, short AGD females, and lower in short AGD males that share the social group with several, long AGD females. Under the assumption that long AGD males are more aggressive, and short AGD males are less aggressive, and given that inter-male conflicts are frequent during the mating season, we hypothesized that (3) focal male testosterone levels during the mating season are associated to the interaction between focal male AGD and male social environment. Specifically, we predicted that focal male testosterone levels (iv) would be higher in long AGD males that share the social group with several long AGD males, and lower in long AGD males that share the social group, with few long AGD males, or with few or several short AGD males. Additionally, we predict that focal male testosterone (v) would be higher in short AGD males that share the social group with few or several short AGD males, and lower in short AGD males that share the social group with few or several long AGD males.

Under the assumption that long AGD females and males are more aggressive, and short AGD females and males are less aggressive, and given that during the offspring rearing season males cohabit and interact with nursing females and offspring, we hypothesized that (4) focal male serum testosterone levels during the offspring rearing season are associated to the interaction between focal male AGD phenotype, and female social group environment. Specifically, we predicted that focal male serum testosterone levels (vi) would be higher in long AGD males that share the social group with few short or long AGD females, and lower in long AGD males that share the social group with several short or long AGD females. Additionally, we predict that focal male serum testosterone levels (vii) would be lower in short AGD males, relative to long AGD males, independent of the number and AGD phenotype of their female group members. Under the assumption that long AGD males are more aggressive, and short AGD males are less aggressive, and given that inter-male conflicts also are frequent during the offspring rearing season (second reproductive event during postpartum estrus), we hypothesized that (5) focal male testosterone levels during the offspring rearing season are associated to the interaction between focal male AGD and male social environment. Specifically, we predicted that focal male testosterone levels (viii) would be higher in long AGD males that share the social group with several long AGD males, and lower in long AGD males that share the social group, with few long AGD males, or with few or several short AGD males. Additionally, we predict that focal male testosterone (ix) would be higher in short AGD males that share the social group with few or several short AGD males, and lower in short AGD males that share the social group with few or several long AGD males.

2. Material and methods

2.1. Study population

Data came from a long-term study conducted between 2009 and 2019 (11 generations) in 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. The 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*, *Acacia caven*, and *Baccharis* spp.) that on average covered 14.5 % of the field surface (Ebensperger and Hurtado, 2005). The total area examined was 2 ha and did not vary between years of study.

2.2. Live trapping and telemetry

Every year we used live trapping and radio-tracking to determine degu identity, phenotype based on AGD length, and social group membership, during both winter and spring. 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. 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 autumn 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, as we wanted extra time in case the population gave birth earlier or later than normal. Independent of the duration of the field sampling, males were captured and sampled, during the beginning of each sampling period, and thus generally coincided when most females were in estrus. 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 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 Rinconada 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 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 Supplementary Material 1-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 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 coefficient, 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 had a total sample of 227 different adult males. Of these males, 158 were captured only during one season of the study and sampled once for testosterone; 56 males were captured during two consecutive seasons (i.e., mating and offspring rearing) of the same year and were sampled twice; 10 males were captured during three different seasons and sampled three times; 2 males were captured during four different seasons and sampled four times; and 1 male was captured during five different seasons and sampled five times. For the 69 males 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., 2018). Thus, our sample size was 313 samples of testosterone from 227 males. These 313 samples, 152 were from the mating season and 161 were from the offspring rearing season. From 152 samples collected during the mating season, 15 of them were obtained from males that were alone, and 137 were obtained from males that were members of a social group. Of the 137 males that were in a social group, 13 of them were in social groups without female members, and 47 of them were in social groups without another male member. The remaining males were in social groups with at least one male and one female group member. Of the 161 samples from the offspring rearing season, 6 of them were obtained from males that were alone and 155 were obtained from males that were members of a social group. Of the 155 males that were in a social group, 3 of them were in social groups without female members and 72 of them were in social groups without another male member. The remaining males were in social groups with at least one male and one female group member.

2.5. Individual variables: male and female AGD

Individual phenotype in terms of masculinization was assessed through anogenital distance (AGD) length; the distance between the ventral anus commissure to the base of the penis (or genital papilla in females) (Vandenbergh and Huggett, 1994). We measured the AGD (mm) of all adult individuals with a digital caliper (precision 0.1 mm) at every capture event. We only measured AGD in females exhibiting non-perforated vaginas (perforated vagina is an indicator of either estrus or recent parturition, as degu females have closed vaginas for all other stages). All AGD measurements were taken by the same observer (LAC) across all eleven years. We calculated mean degu AGD length from 21.55 $\pm$ 10.05 measurements per individual (range: 4–71, n = 4129 measurements, for 227 males), resulting in a single AGD estimate per individual (Correa et al., 2018). Intra-season repeatability of male AGD length was 0.92 (measurement error 0.08, n = 2664 measurements) from 158 males examined during only one season. Inter-season repeatability of male AGD length was 0.96 (measurement error 0.04, n = 1001 measurements) in 56 males sampled during both seasons within the same year. Inter-year repeatability of male AGD length was 0.95 (measurement error 0.05, n = 464 measurements) in the 13 males sampled across years. These data suggest that male AGD length is a stable measurement within and between individuals. In wild male degus, AGD length follows a normal distribution (mean = 9.64 mm; SD = 1.1), with short and long AGD males being a rare phenotype, and intermediate AGD males being a frequent phenotype (Correa, 2012). Similar findings were reported in male 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, or without contiguous males or between two females), are less frequent and are represented in the same proportion (~15–20 % of individuals per IUP type, Vandenbergh, 2003; Hotchkiss and Vandenbergh, 2005). This representation of each AGD length phenotype approximately matches a normal distribution with limits of $\pm$1 SD. Thus, we identify short AGD males as those with AGDs $\leq$8.54 (–1SD) mm, long AGD males as those with AGDs $\geq$10.75 (+1SD) mm, and intermediate AGD male as those with AGDs between 8.55 and 10.74 mm (Correa, 2012; Correa et al., 2018). This classification only attains to identify different AGD phenotypes, because for all statistical analyses, AGD was used as a continuous variable. The mean AGD length of each male was included in our analyses as the *focal male AGD*. Before statistical modeling, we verified that body weight and AGD length were correlated during the mating season (r_p = 0.32; p value = 0.0002, n = 167), but not so during the offspring rearing season (r_p = 0.003; p value = 0.45, n = 175). The statistically significant correlation between body weight and AGD length during mating (winter) was expected because about 40 % of male population was under 180 g in body weight and had not reached their adult weight. AGD length and body weight are correlated in developing degu juveniles, but this correlation disappears when animals attain their adult body weight (Correa, unpublished data). In female degus, AGD length also varies significantly within a population, and female AGD length also represents the IUP in which females were located (Correa, 2012; Correa et al., 2016; Roff et al., 2017; Correa et al., 2021).

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 mates, and (4) *the mean group male AGD*, the mean AGD of male group mates after excluding the focal male AGD.

2.7. Measures of serum testosterone

Blood samples were obtained from all adult degus one time per season (mating and offspring rearing) during their first capture. In the offspring rearing season, blood samples of females and males were collected when females were in early lactation. No blood samples were obtained for the mating season of 2009. Samples ((~500 μL for radioimmunoassay and ~200 μL for enzyme-immunoassay, see below) were obtained by venipuncture of the saphenous vein, which was punctured with a sterile 14 G needle, allowing 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 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 in 2015, the RIA equipment was replaced by ELISA equipment due to biosafety reasons. Samples based on both techniques 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; any sample measured below the limit of detection was excluded from analysis. 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 the RIA technique, the average intra- and inter-assay variation were 7.7 % and 9.9 %, respectively. For ELISA technique, the average intra- and inter-assay variation were 10.6 % and 6.39 %, respectively.

To test whether the assay technique (RIA or ELISA) resulted in significantly different measurements of testosterone levels, we compared testosterone mean estimates between the two techniques. First, we log-transformed testosterone values and used a Student's *t*-test for independent samples with the technique as the main factor. We ran the first analysis based on all adult male and female degus in our study population, and the second analysis based on the subset of samples included in this manuscript (i.e. adult males and females with known social groups). We found no significant difference in mean testosterone levels measured through RIA and EIA in the whole population (t value = −0.823, df = 1473, p value = 0.410; n = 1475 degus from 2009 to 2019; n = 798 RIA determinations, n = 677 ELISA determinations) and within our restricted dataset (t value = 0.661, df = 834, p value = 0.508; n = 836 degus from 2009 to 2019; n = 457 RIA determinations, n = 379 ELISA determinations).

2.8. Statistical analyses

We used three different sets of models to test each of our predictions (Full models in Supplementary Material 2). To test the prediction that (i) season affects focal male serum testosterone levels, we examined model 1 (Table S1, Suppl. Mat. 2) including n = 313 male samples, all of whom were alone or in social groups of different size and composition. For our predictions (ii and iii) that male AGD phenotype and female social environment affect focal male serum testosterone levels during the mating season, we examined model 2.1 which included all elements of the female social environment and focal male AGD (Table S2, Suppl. Mat. 2) for n = 124 male samples, all of whom had at least one female group mate. For our predictions (iv and v) that male AGD phenotype and male social environment affect focal male serum testosterone levels during the mating season, we examined model 2.2 which included all elements of male social environment and focal male AGD (Table S2, Suppl. Mat. 2) for n = 90 male samples, all of whom had at least one male group mate. For our predictions (vi and vii) that male AGD phenotype and female social environment affect focal male serum testosterone levels during the offspring rearing season, we examined model 3.1 which included all elements of female social environment and focal male AGD (Table S3, Suppl. Mat. 2) for n = 152 male samples, all of whom had at least one female group mate. For our prediction that (viii and ix) male AGD phenotype and male social environment affect focal male serum testosterone levels during the offspring rearing season, we examined model 3.2 which included all elements of male social environment and focal male AGD (Table S3, Suppl. Mat. 2) for n = 83 male samples, 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 (Bartoń, 2009) packages were used to perform residual diagnostics and model selection routines, respectively.

2.9. Ethical note

The animal handling techniques and all the protocols used in this study followed the Guide of the American Society of Mammalogy, 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 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).

3. Results

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

The best model for model 1 exclusively included the season (Table S1, Supplementary Material 3-Model selection), supporting that this factor has a major impact on focal male serum testosterone levels (estimate −0.23, t value −3.62, p value: 0.0003, detailed results in Table S1, Supplementary Material 4). In focal males, higher testosterone levels were recorded during the offspring rearing relative to the mating season (mean mating season: 1.44 nmol/L, ± 0.93; range 0.27–5.98, mean offspring rearing season: 1.87 nmol/L ± 1.42; range 0.57–10.1, Fig. 1). An analysis performed with a subset of sixty-three males, which were sampled in two consecutive seasons of same year, indicated the same pattern (t value −2.021 p value: 0.045; (mean mating season: 1.37 nmol/L, mean offspring rearing season: 1.92 nmol/L).

3.2. Focal male testosterone levels, focal male AGD, and social environment during the mating season

Results from model 2.1, which analyzed the effect of female social environment and male AGD phenotype on male serum testosterone levels during the mating season, indicated that focal male testosterone levels are lower in social groups where females are more abundant (estimate −0.10, t value −2.96, p value: 0.0037, detailed results in Table S2, Supplementary Material 4, Fig. 2). Neither the mean group female AGD, nor the focal male AGD were significantly associated with testosterone levels of focal males (Table S2, Supplementary Material 3-Model selection). Results from model 2.2, which analyzed the effect of male social environment and male AGD phenotype on male serum testosterone levels during the mating season, revealed that focal male testosterone is associated with focal male AGD in a negative way (estimate −0.12, t value −2.04, p value 0.0044, detailed results in Table S3,

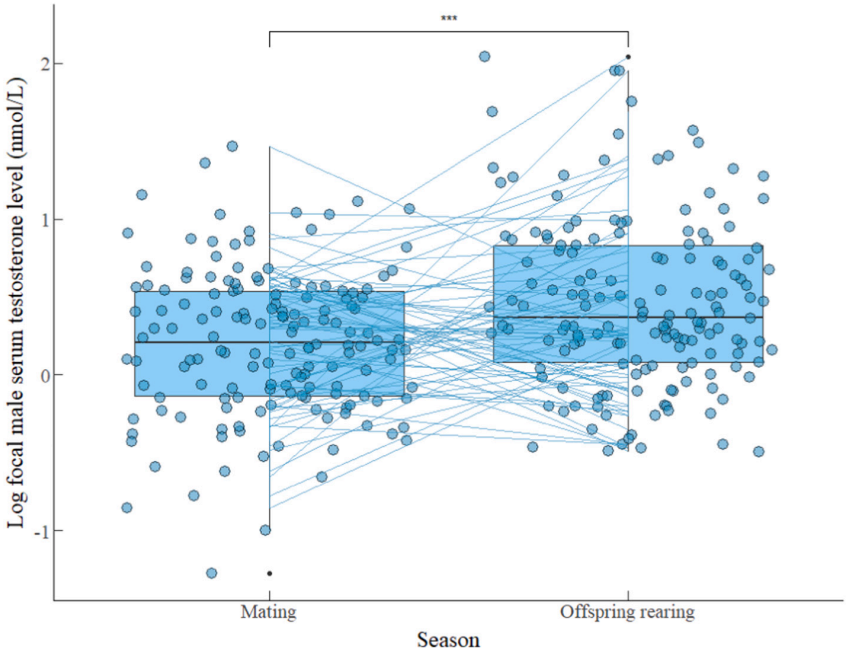

Fig. 1. Effects of season on focal male serum testosterone levels. *** indicate significant differences. The middle line in the boxplots represents median values, with their respective quartiles. Dots outside the error bars represent outlier values. Points represent the total 313 samples from the 227 males that served as replicates for this analysis. Lines between boxplots represent males whose samples were available for mating and offspring-rearing season of the same year.

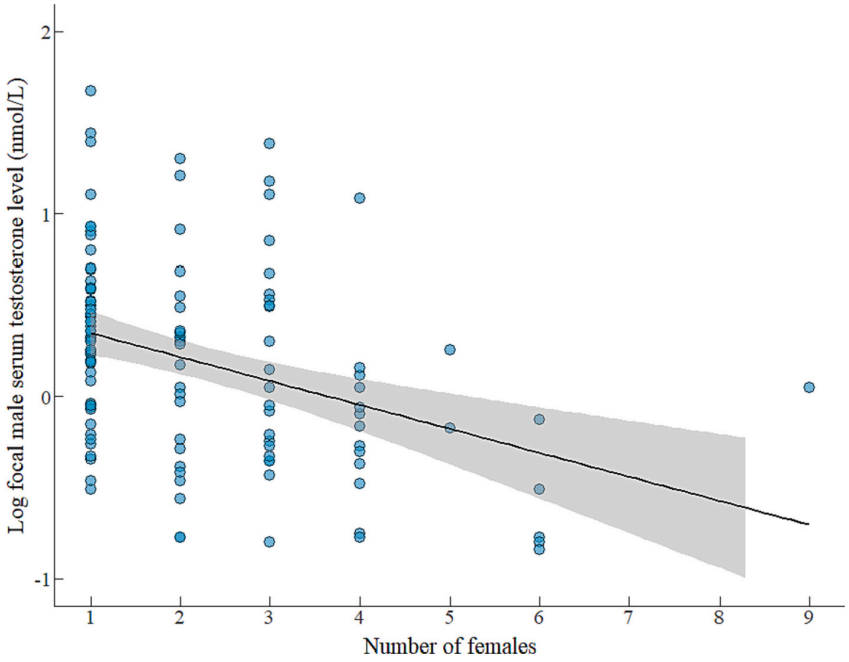

Fig. 2. Relationship between focal male serum testosterone level and the number of females in the social group during the mating season (winter). Circles represent 124 samples from 113 males that served as replicates for this analysis. The grey region indicates 95 % confidence intervals.

Supplementary Material 4). Thus, short AGD focal males exhibited higher testosterone levels compared with long AGD focal males (Fig. 3). Neither the number of males, nor mean group male AGD explained focal male testosterone variation (Table S3, Model selection, in Supplementary Material 3).

3.3. Focal male testosterone levels, focal male AGD, and social environment during the offspring rearing season

Results from model 3.1 (Table S4, Model selection, in Supplementary Material 3), which analyzed the effect of female social environment and male AGD phenotype on male serum testosterone levels during the

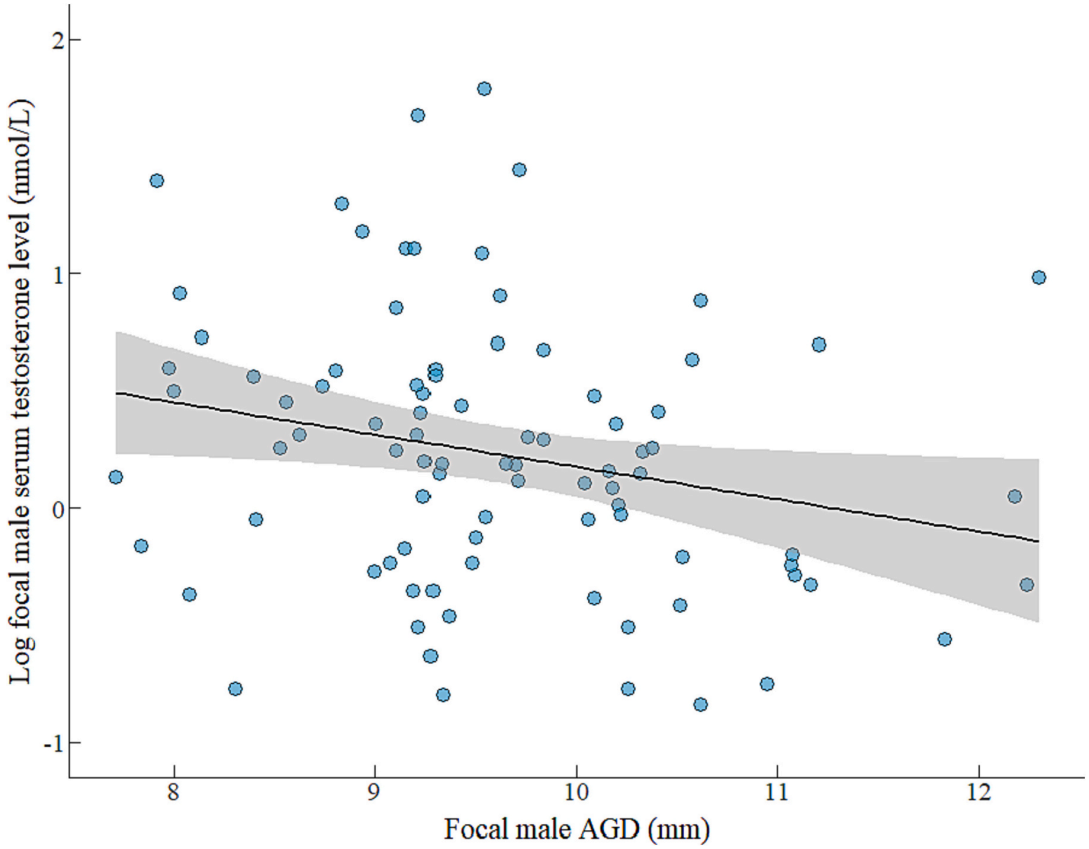

Fig. 3. Relationship between focal male serum testosterone level and anogenital distance (AGD) of focal degu males during mating season (winter). Circles represent 90 samples from 87 males that served as replicates for this analysis. The grey region indicates 95 % confidence intervals.

offspring rearing season, indicated that the focal male AGD and its interaction with the mean group female AGD was significantly associated with focal male testosterone levels (estimate 0.23, t value 2.28, *p* value: 0.0243, detailed results in Table S4, Supplementary Material 4). Thus, the highest male testosterone levels were recorded in long AGD focal males that grouped with long AGD females, while the lowest male testosterone levels were recorded in short AGD focal males that grouped with long AGD females (Fig. 4). Relatively intermediate testosterone levels were recorded in focal males of varying AGDs length that grouped with short AGD females (Fig. 4). Results from model 3.2 (Table S5, Model selection, in Supplementary Material 3), which analyzed the effect of male social environment and male AGD phenotype on male serum testosterone levels during the offspring rearing season, indicated that the focal male AGD and its interaction with the number of males in the social group is associated with focal male testosterone levels (estimate −0.26, t value −3.85, *p* value: 0.0002, detailed results in Table S5, Supplementary Material 4). Specifically, the highest male testosterone levels were recorded in long AGD focal males that were members of social groups with fewer males (Fig. 5). The lowest male testosterone levels were recorded in long AGD focal males that were in groups with a high number of other males. Intermediate male testosterone levels were recorded in short AGD focal males when in groups with few or several other males (Fig. 5).

4. Discussion

Our study revealed several novel and intriguing relationships between social conditions, male AGD phenotype, and male serum testosterone levels in degus. First, our finding that male testosterone levels were higher during the offspring rearing season than during the mating season contradicted most previous evidence from birds and mammals (Wingfield et al., 1990; Ketterson and Nolan, 1992, 1999; Hau, 2007; Gleason et al., 2009), including degus (Kenagy et al., 1999; Soto-Gamboa et al., 2005). While Kenagy et al. (1999) did not find variation in male testosterone levels between the mating and offspring rearing seasons, Soto-Gamboa et al. (2005) reported higher testosterone levels in males during June–July (mating season) compared with April (pre-breeding season). Theoretical considerations predict higher male testosterone levels during mating season, but lower levels during the offspring rearing season (Wingfield et al., 1990; Ketterson and Nolan, 1992, 1999; Hau, 2007; Gleason et al., 2009). The higher male testosterone levels during the offspring rearing season might be explained by the post-partum estrus experienced by the females during nursing (Kenagy et al., 1999; Ebensperger et al., 2013). Although this second mating event contributes significantly less to female and male reproductive success relative to the first mating event (only 8.3 % of females wean some offspring in January (Ebensperger et al., 2013, Ebensperger, unpublished data), mating during post-partum estrus likely represents an additional opportunity for males to mate and experience reproductive and behavioral challenges (Ketterson and Nolan, 1992, 1999). This sexual context could explain the "*relatively higher*" testosterone levels recorded in males during mating and offspring rearing seasons relative to the pre-breeding season (autumn) when male testosterone levels are lower (Kenagy et al., 1999; Soto-Gamboa et al., 2005). Alternatively, it is also possible that the daily timing of blood sampling was ill-matched for detecting predicted seasonal differences in male testosterone levels, as these hormone levels tend to be significantly higher with greater individual variation during the "resting" period in male birds (Needham

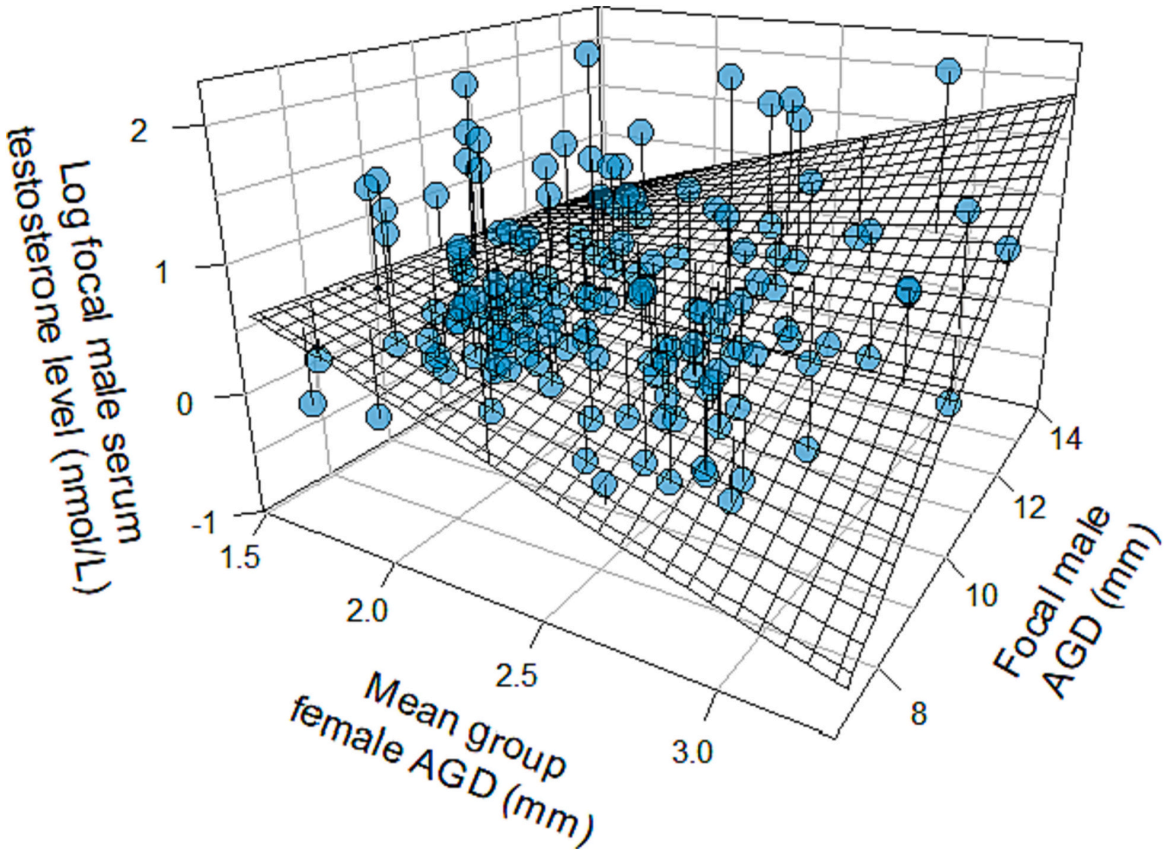

Fig. 4. Relationship between focal male serum testosterone levels, focal male anogenital distance (AGD), and mean group female AGD, during offspring rearing season (spring). Circles represent 152 samples from 145 males that served as replicates for this analysis. Black grid surface represents the model-predicted values, and vertical lines represent data deviations from the model prediction.

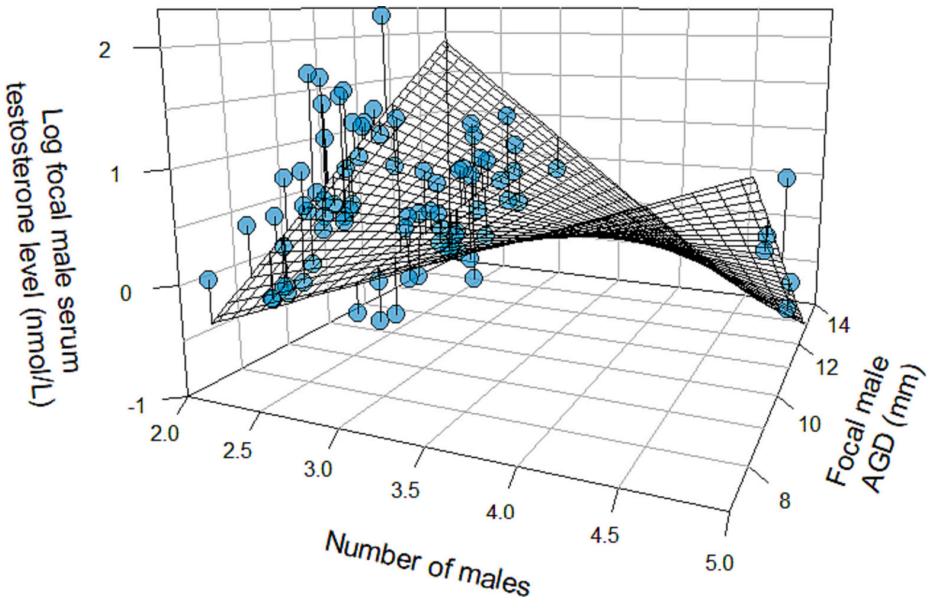

Fig. 5. Relationship between focal male testosterone levels, anogenital distance (AGD) of focal degu males, and the number of males in the social group during the offspring rearing season (spring). Circles represent 83 samples from 80 males that served as replicates for this analysis. Black grid surface represents the model-predicted values, and vertical lines represent data deviations from the model prediction.

et al., 2017) and mammals (Perret, 1985; Drea, 2007). As degus are diurnal and we collected blood samples during the morning, future studies may consider collecting night-time or GnRH-induced testosterone levels to better represent endogenous peak levels in males (Greives et al., 2021).

We found contradictory evidence, however, regarding the relationship between male AGD length phenotype and male serum testosterone levels. During the mating season, short AGD males tended to have higher testosterone levels, but during the offspring rearing season, long AGD males tended to have higher testosterone levels, but this association was modulated by social factors. Results from previous studies under laboratory conditions indicate that long AGD males exhibit higher testosterone levels compared to short AGD males in Mongolian gerbils (Clark et al., 2002), but in domestic mice and captive degus, male testosterone levels and male AGD phenotype are not associated (Crump and Chevins, 1989; Aspillaga-Cid et al., 2021). Theoretical underpinnings of the intrauterine position phenomenon suggest that prenatal exposure to testosterone can shape male phenotype through organizational effects (Clark and Galef, 1998; vom Saal et al., 1999; Ryan and Vandenbergh, 2002). Evidence from domestic mice and Mongolian gerbils indicates that long AGD males are more sensitive to testosterone than short AGD males when administrated exogenous testosterone, suggesting that factors linked to androgen receptors (quantity, affinity, distribution) could lead to greater testosterone binding and receptor activation in long AGD males (vom Saal, 1989; vom Saal et al., 1999; Clark and Galef, 1998; Ryan and Vandenbergh, 2002). We hypothesize that during the main mating season (winter), biological activity of testosterone is critical to develop and maintain male reproductive traits (e.g. sperm production, increased metabolic rate for mate searching, increased aggressive behavior for inter-male conflicts), and therefore short AGD male degus can increase their testosterone levels to activate these traits, while long AGD males do not need to do because their neuroendocrine system is already sensitive to testosterone (vom Saal, 1989; Clark and Galef, 1998; vom Saal et al., 1999; Ryan and Vandenbergh, 2002).

In relation to male testosterone levels and female social environment during the mating season, the presence of receptive females is typically associated with high testosterone levels in male birds and mammals, including rodents (Wingfield et al., 1990; Ketterson and Nolan, 1992; Wingfield et al., 2001). However, in our study, the serum testosterone levels of males were lower as the number of females in the social group increased. Our findings revealed complex effects of the female social environment on male testosterone levels. Our results partially resemble findings recorded in social and resident males of rock hyraxes (Koren et al., 2019), natal males of spotted hyenas (Holekamp and Smale, 1998), territorial males of African striped mice (Schradin et al., 2009), and dominant males in bonobos (*Pan paniscus*) (Surbeck et al., 2012), in which males that live permanently with females have lower testosterone levels, than males that are not in permanent contact with females. For instance, socially dominant male bonobos exhibit lower levels of testosterone compared with lower-ranked males, but socially interact more frequently with the females, and these interactions are affiliative (Surbeck et al., 2012). In degus 50–60 % of male degu interactions during the pre-reproductive and reproductive seasons occur with females, and these are amicable (Soto-Gamboa et al., 2005). Thus, this finding in degus supports the idea that male testosterone levels could be socially down regulated to decrease male aggressiveness within groups and allow the development of an amicable social environment (Schradin et al., 2009).

Our results similarly revealed a relatively complex influence of female social environment on male testosterone during the offspring rearing season. Specifically, short and long AGD males exhibit similarly low testosterone levels when in social groups predominated by short AGD females. However, this similarity disappears when males are in social groups predominated by long AGD females. Specifically, in social groups predominated by long AGD females (in wild conditions degu social groups are homophilic by female AGD, this means that long AGD females are grouped together, and short AGD females are grouped together Correa et al., 2021) long AGD males had significantly higher testosterone levels than short AGD males. Long AGD females have been reported to exhibit aggressive behavior in domestic mice (vom Saal, 1989), Mongolian gerbils (Clark and Galef, 1998), and degus (Correa et al., 2013), and long AGD females of domestic mice, Alpine marmots (*Marmota marmota*), and degus are socially dominant (vom Saal, 1989; Hackländer and Arnold, 2012; Correa et al., 2013). Moreover, experimental evidence from captive degus supports that long AGD female degus can be socially dominant over males (depending on social group composition), while short AGD females are mostly affiliative during intersexual interactions (Correa et al., 2013; Correa, unpublished data). We lack studies examining how AGD phenotype influences male-male interactions in degus but results from domestic mice indicate higher aggressiveness and social dominance in long AGD males (Drickamer et al., 1995). Thus, the observation of relatively high levels of testosterone in long AGD males that live with long AGD females might be expected due to potentially high frequency of intersexual agonistic interactions (Wingfield et al., 1990; Grebe et al., 2022). On the contrary, lower testosterone levels recorded in short AGD males that live with long AGD females can be explained by female aggressive behavior, which can trigger a stress response in docile males, thus inhibiting testosterone release (Hau et al., 2010). This hypothesis is plausible from a mechanistic perspective, because in degus a negative relationship between cortisol and testosterone has been recorded in wild males (Kenagy et al., 1999, but see Soto-Gamboa et al., 2005). Similar results have been described in the blue-eyed black lemurs (*Eulemur flavifrons*), an atypical mammal species (with sex-role reversal) where females are more aggressive and dominant than males. In this species, males that interact with aggressive females, experiment an environment characterized by social instability, and responds with an increase of androgens and glucocorticoids (Grebe et al., 2022).

Surprisingly, the male social environment (based on the number of males in a social group) influenced male testosterone during offspring rearing, but not so during the main mating season as predicted by the *challenge hypothesis* (Wingfield et al., 1990; Ketterson and Nolan, 1992, 1999; Hau, 2007; Gleason et al., 2009). However, we cannot discard a potential association between male testosterone and the male social environment during the mating season, as we did not measure the frequency nor the quality of degu interactions. Previous support of the *challenge hypothesis* in wild male degus comes from observations that male testosterone peaks during the mating season, a life-history stage matching the peak of inter-male agonistic interactions (Soto-Gamboa et al., 2005; Soto-Gamboa, 2005). However, most agonistic male-male interactions recorded in degus are chases rather than direct fighting (Soto-Gamboa et al., 2005), implying relatively low levels of aggression. Moreover, degus are not spatially territorial (Ebensperger et al., 2016), and both females and males' mate with multiple individuals of the opposite sex (Ebensperger et al., 2019), two conditions that prevent any female monopolization (Clutton-Brock, 1989; Kohda et al., 2002). Likely, male reproductive success depends more on male searching ability than male fighting capacity (Clutton-Brock, 1989; Kohda et al., 2002), and therefore high levels of testosterone may not be necessary. Low levels of male androgens (including testosterone) and fewer aggressive inter-male interactions have been reported in males within groups of cooperatively breeding species (Bales et al., 2006). Testosterone was relatively high in long AGD males when in groups with fewer other males, but low when in groups with several other males during the offspring rearing season, implying that inter-male agonistic interactions might negatively affect the development of an amicable social environment, which is necessary to female and offspring during the nursing season (Nguyen et al., 2009). We hypothesize that more aggressive male phenotypes (i.e., long AGD males) could have low testosterone levels to reduce the inter-male conflict within groups, thus allowing them to remain together with females. This hypothesis is plausible from a functional perspective, as previous findings in degus indicate that during

parental care tasks, male presence is stressful (Ebensperger et al., 2010) and costly in terms of female group members' fitness (Hayes et al., 2019). Overall, findings from our study and those of previous studies on captive and wild degus suggest male testosterone is sensitive to male social environment, but that effects remain more complex than was previously reported or assumed (Kenagy et al., 1999; Soto-Gamboa et al., 2005; Soto-Gamboa, 2005; Aspillaga-Cid et al., 2021).

Similar to all previous studies (Bustos-Obregón and Ramírez, 1997; Kenagy et al., 1999; Soto-Gamboa et al., 2005; Ebensperger et al., 2010; Aspillaga-Cid et al., 2021), we recorded low testosterone levels in male degus. Male testosterone levels in this study were statistically higher than those of females during the mating season (females 1.06 ± 0.79 nmol/L, mean ± SD, $n = 217$) but not statistically different from those of females during offspring rearing season (females 1.63 ± 1.03 nmol/L, mean ± SD, $n = 308$, Correa, unpublished data), despite this, some males can attain higher testosterone levels during both seasons, but these males with high levels of testosterone are very rare. We hypothesize that these low levels in males are the consequence of a combination of life-history and social traits, including (i) no sexual dimorphism in size, (ii) no territorial behavior (Ebensperger et al., 2016), (iii) a generally promiscuous mating system (Ebensperger et al., 2019), (iv) a social organization characterized by groups with multiple males and females (Hayes et al., 2019), (v) no male dominance over females (Correa, unpublished data), (vi) males who provide some (but not essential) paternal care (Ebensperger et al., 2010; Aspillaga-Cid et al., 2021), and (vii) affiliative intersexual interactions (Soto-Gamboa et al., 2005). A comparative study in male and female testosterone levels in bonobos and chimpanzees (*Pan troglodytes*) suggests that social systems may explain differences in urinary metabolites of testosterone between sexes (Sannen et al., 2003), as male chimpanzees are dominant over female and exhibit high testosterone levels, whereas bonobo females and males exhibit similar ranks (French et al., 2013; Sannen et al., 2004) and their testosterone metabolite levels overlap extensively (Sannen et al., 2003), a pattern resembling degus. Results from a bonobo study suggest that relatively small sex differences in testosterone levels might be explained by a downregulation of testosterone levels in males rather than an upregulation in females (Sannen et al., 2003). We suggest that the high levels of sociability that characterize female and male degus represents a main driver of inter-sexual similarity in testosterone levels, a pattern resembling that previously reported in hyenas (Holekamp and Smale, 1998; East et al., 2003), rock hyraxes (Koren et al., 2019), bonobos (Sannen et al., 2003; Surbeck et al., 2012), and African striped mice (Schradin et al., 2009), and where frequent interactions with the females imply low testosterone levels in males (Sannen et al., 2003), and where amicable male behavior could be a mating strategy that results in higher fitness (Holekamp and Smale, 1998; East et al., 2003; Surbeck et al., 2012).

5. Concluding remarks

Evidence from our long-term study on a wild population of degus suggests that male testosterone levels are influenced by temporal, social, and individual factors, and by the interaction between these factors, highlighting the complex regulation of male testosterone levels. Contrary to our prediction, and to the predictions of the challenge hypothesis, male testosterone levels were higher during the offspring rearing season relative to the mating season. As we expected, male testosterone levels were modulated by male AGD phenotype, and by three of the four social factors analyzed in this study, highlighting the role of social behavior on testosterone regulation. Specifically, male testosterone levels were associated with the number of females and with the AGD phenotype of female group mates. Additionally, male testosterone levels were modulated by the number of male members in the social group. Finally, we confirmed that male degus have low testosterone levels, which could be explained by their highly social nature. Thus, male degus could have low testosterone levels to facilitate permanent cohabitation and frequent interactions with females, offspring, and other males. The analyses of this extensive data set are clearly exploratory and given the multiple models investigated subjected to type 1 error (False positive). Any conclusion 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: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing. **Antonia Aspillaga-Cid:** Data curation, Formal analysis, Methodology, Software, Writing – original draft. **Carolyn M. Bauer:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Danna Silva-Álvarez:** Conceptualization, Data curation. **Cecilia León:** Data curation, Investigation, Methodology. **Juan Ramírez-Estrada:** Data curation, Investigation, Methodology. **Mauricio Soto-Gamboa:** Conceptualization, Investigation, Methodology, Supervision. **Loren D. Hayes:** Conceptualization, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. **Luis A. Ebensperger:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing.

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 male testosterone levels in a social rodent, Dryad. https://doi.org/10.5061/dryad.6djh9w16h.

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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.2024.105479.

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