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Beyond aggression: androgen-receptor blockade modulates social interaction in wild meerkats

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ABSTRACT

In male vertebrates, androgens are inextricably linked to reproduction, social dominance, and aggression, often at the cost of paternal investment or prosociality. Testosterone is invoked to explain rank-related reproductive differences, but its role within a status class, particularly among subordinates, is underappreciated. Recent evidence, especially for monogamous and cooperatively breeding species, suggests broader androgenic mediation of adult social interaction. We explored the actions of androgens in subordinate, male members of a cooperatively breeding species, the meerkat (*Suricata suricatta*). Although male meerkats show no rank-related testosterone differences, subordinate helpers rarely reproduce. We blocked androgen receptors, in the field, by treating subordinate males with the antiandrogen, flutamide. We monitored androgen concentrations (via baseline serum and time-sequential fecal sampling) and recorded behavior (via focal observation). Relative to controls, flutamide-treated animals initiated less and received more high-intensity aggression (biting, threatening, feeding competition), engaged in more prosocial behavior (social sniffing, grooming, huddling), and less frequently initiated play or assumed a dominant role during play, revealing significant androgenic effects across a broad range of social behavior. By contrast, guarding or vigilance and measures of olfactory and vocal communication were unaffected by flutamide treatment. Thus, rather than regulating cooperative or communicative behavior, androgens in adult meerkats are aligned with the traditional trade-off between promoting reproductive and aggressive behavior at a cost to affiliation. Our findings, based on rare endocrine manipulation in wild mammals, show a more pervasive role for androgens in adult social behavior than is traditionally recognized, with possible relevance for understanding tradeoffs in cooperative systems.

Keywords: antiandrogen, flutamide, testosterone, aggression, communication, prosocial behavior, behavioral neuroendocrinology, subordinate male, field experiment, cooperative breeder

INTRODUCTION

Cooperative breeding, by which dominant individuals monopolize a group’s breeding efforts, is rare among vertebrates, although several theories can be invoked to explain why subordinate helpers might delay their own reproduction to care for the offspring of others (Arnold and Owens, 1998; Lukas and Clutton-Brock, 2012). The mechanisms involved in ensuring differential reproduction can differ rather dramatically across species: In some, helpers are hormonally suppressed, such that they are physiologically unable to reproduce (Arnold and Dittami, 1997; Bales et al., 2006; Schoech et al., 1991), whereas in others, helpers are behavioraly suppressed, but retain the physiological capacity to reproduce (Bennett et al., 1993; Creel et al., 1992; Khan et al., 2001; Oliveira et al., 2003). Among the latter, the role of reproductive hormones, such as testosterone (T), which might not vary substantially between breeders and helpers, remains poorly understood. Within social species, reproductive hormones often regulate (or are regulated by) the within-group interactions that are necessary to maintain stable relationships (Albers et al., 2002; Monaghan and Glickman, 1992). In males, androgen function is best understood in the context of mediating reproductive and aggressive behavior – activities that often come at the cost of paternal investment (Hegner and Wingfield, 1987; Ketterson and Nolan, 1994). Androgen function is also invoked to explain rank-related differences in courtship and competition (Wingfield et al., 1987). Nevertheless, there is recent evidence to suggest an even broader role for T in mediating adult social interaction, particularly in monogamous or cooperatively breeding species (Eisenegger et al., 2011; Gleason and Marler, 2010; Storey et al., 2006; van der Meij et al., 2012; Wang and De Vries, 1993). Here, using a wild population of the cooperatively breeding meerkat (*Suricata suricatta*), we investigated these issues by blocking the androgen-receptor system of adult, subordinate males.

Meerkats are social mongooses that live in relatively stable clans or structured groups, typically comprising a dominant breeding pair and various subordinate relatives or offspring of both sexes that contribute to pup rearing (Clutton-Brock et al., 2001). Among males, breeders and helpers express similar concentrations of T and luteinizing hormone (LH), and show comparable LH spikes in response to a GnRH challenge (Carlson et al., 2004; O'Riain et al., 2000). Thus, although the dominant male monopolizes most of a group's breeding (Griffin et al., 2003), subordinate males are not reproductively suppressed (Carlson et al., 2004) and may gain some breeding success, as well as experience raised T concentrations, during extraterritorial prospecting forays (Spong et al., 2008; Young et al., 2005, 2007). T does not correlate with aggression or dominance between male social ranks (Carlson et al., 2004) and there is no evidence that T relates to rates of pup provisioning (Carlson et al., 2006a). Yet, because behavioral endocrinologists tend to focus on understanding dominance or the differences between social ranks, little is known about the role of T in regulating subordinate male interaction in this or other species (although see: Virgin and Sapolsky, 1997). Given that dominant and subordinate animals may respond differently to the same T treatment (Fuxjager et al., 2015) or that T-associated variation in behavioral 'style' may exist within the same class (Virgin and Sapolsky, 1997), it is increasingly relevant to understand how the different social classes respond to endocrine challenges.

Meerkats are an appropriate model in which to test the proposition that androgens may regulate social behavior beyond aggression: Firstly, subordinates are far more numerous than are dominant animals and necessarily account for a large proportion of social interaction; secondly, these 'helper' males rarely reproduce, but curiously maintain androgen concentrations commensurate with those of dominant males; thirdly, access to an exceptional wild population allows us to consider social and ecological relevance, while overcoming logistical challenges that typically preclude field neuroendocrine studies (see Fusani et al., 2005).

With relatively few exceptions, typically involving avian species (e.g., Hegner and Wingfield, 1987; Schwabl and Kriner, 1991), hormones or their actions are rarely experimentally manipulated in the field (see Fusani et al., 2005), particularly to explore their relationship to the broad social repertoire. Instead, androgen-manipulation studies in laboratory animals, particularly rodents and birds, aim to improve our mechanistic understanding of isolated traits (either e.g., reproduction: Södersten et al., 1975; aggression: Searcy and Wingfield, 1980; play: Meaney et al., 1983; scent marking: Fuxjager et al., 2015; or song: Grisham et al., 2007). This historical focus can occur at the expense of gaining comparative, ecological, and evolutionary understanding of hormone action: detecting tradeoffs and constraints, for instance, requires an integrated approach (Wingfield et al., 2009).

To test the role of androgens in subordinate, male meerkats, we administered the nonsteroidal antiandrogen, flutamide, that competitively blocks the binding of androgenic hormones (primarily T) to androgen receptors (Hellman et al., 1977; Peets et al., 1974). Androgens often relate to the initiation of aggression (e.g. Virgin and Sapolsky, 1997) or the outcome of aggressive encounters (e.g. Rose et al., 1972), and androgen-mediated cues can also influence susceptiblity to aggressive attacks (Monaghan and Glickman, 1992). Consistent with studies in various species showing that flutamide administration leads to reduced adult aggression (Sperry et al., 2010; Taylor et al., 1984; Vleck and Dobrott, 1993), we expected flutamide-treated meerkats to initiate less, but receive more, aggression than their control counterparts.

Beyond the relationship to overt aggression, androgens also may be linked to other more subtly competitive or even prosocial interaction in animals. Rough-and-tumble play, for instance, which can facilitate the establishment of dominance relations among the males of certain species (Panksepp, 1981; Pellegrini, 1995), is often sexually differentiated, with males playing more vigorously than females (Boulton, 1996; Goy and Phoenix, 1971; Meaney et al., 1985). The expression of mammalian social play is masculinized through early androgen exposure (Goy and Phoenix, 1971; Olioff and Stewart, 1978; Wallen, 2005) and can be feminized through reduced prenatal exposure to androgens (Meaney and Stewart, 1981; Meaney et al., 1983). Typically, postnatal androgens do not mediate social play (Meaney et al., 1985), as neither the frequency nor vigor of play are influenced by administration of T to juvenile females (Joslyn, 1973) or by castration of juvenile males (Beatty et al., 1981; Goy, 1970; Pedersen et al., 1990). Nevertheless, few researchers have addressed the potential link between activational androgens and adult social play, largely because playful behavior tends to decrease dramatically in adulthood. Meerkats, however, continue to play as adults (Sharpe, 2005), so we might expect flutamide-treated meerkats to play less vigorously than those experiencing normal androgen action.

With regard to the role of androgens in more purely prosocial, affiliative, or even cooperative behavior, the nature of the correlations can vary considerably. Paternal care (including huddling and grooming), for instance, is generally thought to be inhibited by T (Hegner and Wingfield, 1987; Ketterson et al., 1992), but can increase with androgens in the males of various species (Desjardins et al., 2008; Gleason and Marler, 2010; Neff and Knapp, 2009; Rodgers et al., 2006; Storey et al., 2000; Trainor and Marler, 2001; Wang and De Vries, 1993). Moreover, depending on prenatal androgen exposure (Millet and Dewitte, 2006; van Honk et al., 2012), T in men can increase affiliative behavior (van der Meij et al., 2012), reduce deceit (Wibral et al., 2012), promote reciprocity (Boksem et al., 2013) and increase cooperation (Huoviala and Rantala, 2013). Meerkats show a range of prosocial behavior (including grooming, social sniffing, and huddling) and cooperative behavior (including babysitting and provisioning pups, as well as vigilance and guarding against predators: Clutton-Brock et al., 1999, 2000, 2001). If androgens in meerkats implicate the traditional tradeoff between aggression and affiliation, we might expect rates of prosocial interaction to increase with flutamide treatment. If androgens in meerkats function to increase cooperation, to the benefit of the entire group, we might expect flutamide treatment to reduce pup care or antipredator activities.

Lastly, androgens also may be involved in aspects of olfactory and vocal communication (Dryden and Conaway, 1967; Ulibarri and Yahr, 1988; Wingfield et al., 1987). In this regard, scent marking is often linked to territorial defense (Hediger, 1949; Johnson, 1973) and reproductive advertisement (Brown and Macdonald, 1985; Drea, 2015; Eisenberg and Kleiman, 1972) with dominant individuals generally marking more than subordinates (Johnson, 1973; Ralls, 1971) . Scent marking increases following early exposure to androgens and decreases if such exposure is inhibited (Epple, 1981; Turner, 1975; Ulibarri and Yahr, 1988). Postnatal T similarly mediates the frequency of scent marking (Johnston, 1981) and can also influence the chemical composition of odorants (Novotny et al., 1984). Castration causes retardation or atrophy of scent glands, with accompanying effects on odorant production (Dryden and Conaway, 1967; Epple, 1981), whereas hormone replacement restores these attributes (Dryden and Conaway, 1967). Within adult male meerkats, there is no strong evidence of rank-related differences in scent marking at latrines (Jordan, 2007), although we suspect that they might emerge in other contexts. Despite equivalence in circulating T between male ranks, anal gland secretions appear to be more pronounced in dominant males than in subordinate males (see Figure 1 in Leclaire et al., 2014) and preliminary analyses of these secretions reveal rank-related differences in chemical composition (Drea, unpublished data). Moreover, the bacterial communities associated with anal pouch secretions vary with social status (Leclaire et al., 2014). Overall, therefore, we expect that androgens might regulate certain aspects of olfactory communication in adult meerkats, such that flutamide treatment would reduce rates of scent marking.

Vocalizations likewise function in territorial defense (Bates, 1970; Peek, 1972; Hall, 2009; Shonfield et al., 2012) and reproductive advertisement (Robertson, 1986; Waas, 1988). Vocal cues are often studied in relation to T, providing evidence that the frequency or structure of vocal signals correlate with androgens (Barelli et al., 2013; Charlton et al., 2011; Evans et al., 2008; Solís and Penna, 1997; Wingfield et al., 1987). Manipulation of T prenatally, neonatally or in adulthood shows that vocalizations are regulated by androgens. Early androgen exposure masculinizes calls (Holman et al., 1995; Tomaszycki et al., 2001, 2005), whereas prenatal exposure to antiandrogens feminizes calls (Tomaszycki et al., 2001). In adulthood, increased T concentrations have been linked to increased call rate, duration or quality (Ball et al., 2003; Charlton et al., 2011; Cynx et al., 2005; Gyger et al., 1998; Ketterson et al., 1992). Conversely, castration has been shown to negatively influence call rate or signal structure (Pasch et al., 2011). As shown with androgen-receptor blockade in other species (Behrends et al., 2010), we expect flutamide treatment in meerkats to influence vocalization, potentially reducing calling rate, decreasing call duration or raising call pitch.

METHODS

Field site, study population, and research cohorts

Our subjects were members of a well-studied and habituated population of meerkats, comprising 15-20 groups that inhabit the Kuruman River Reserve and surrounding farms in the Kalahari region of South Africa (26°58’S, 21°49’E). Information about the climate, landscape, and vegetation for this region have been provided elsewhere (Clutton-Brock et al., 1998; Russell et al., 2002). All habituated members of the population are microchipped and easily identifiable from unique dye marks applied to their fur and routinely renewed without the need for capture (Clutton-Brock et al., 2008). Minimally one animal per group (typically, the dominant female) is fitted with a radio collar (Sirtrack, Havelock North, New Zealand) to facilitate locating the group when necessary.

Our main subjects, deriving from five different groups, were 24 subordinate males, 12 of which received flutamide treatment and 12 of which served as controls (see research design, below). These animals were aged 11-18 months at the start of treatment. Because meerkats of both sexes typically reach adulthood at 1 year of age (Clutton-Brock et al., 2008), but can reproduce successfully at younger ages (Young et al., 2006), we considered our subjects to be sexually mature.

Starting in 2011, we studied these animals in two cohorts. Cohort 1 included nine animals (5 flutamide, 4 controls) followed from February to March 2011, at the end of the breeding season. Cohort 1 served in a pilot study to establish our endocrine, behavioral, and surgical procedures, including treatment dosage (see Electronic Supplementary Material, ESM, §a) and to supply preliminary data (Fig. S1). Cohort 2 included 15 animals (7 flutamide, 8 controls; ESM, §b and Table S1) followed from December 2011 to January 2012, at the beginning of the following breeding season, and served in the experimental study described in detail herein. These latter subjects were closely age-matched (mean age ± standard error: 1.04 ± 0.04 years) and derived from 3 large groups totalling 96 animals (KungFu: *n* = 36; Lazuli: *n* = 30; Whiskers: *n* = 30).

Research design

We tested each focal subject of cohort 2 over a four-week period (with a one-week maximum offset between subjects). Each subject's first week served to provide baseline endocrine values and was followed by a capture day, to administer treatment, and another day of post-capture monitoring. We randomly assigned these animals to one of three treatment conditions, including flutamide (*n* = 7), placebo (*n* = 4), and no treatment or 'no-pellet' (*n* = 4), with the constraint that littermates be assigned to different treatments and that flutamide-treated animals be evenly distributed between the three groups (see ESM, §b and Table S1). Treatment was followed by another three weeks of data collection to evaluate endocrine and behavioral effects (see below). One of the flutamide-treated individuals was struck by a vehicle (along with two other non-intervention animals) and died early in the study. This animal contributed to baseline fecal and serum values only, reducing our sample for examining the behavioral effects of flutamide to *n* = 6 (2 per group).

All protocols were approved by Duke University’s Institutional Animal Care and Use Committee (Protocol Registry Numbers A171-09-06 and A143-12-05) and the University of Pretoria’s Animal Use and Care Committee (Ethical Approval Number #C074-11, to CMD). The Northern Cape Conservation Authority in South Africa provided permission for the project.

Sampling, capture, and treatment administration

We visited our focal groups 3-5 days per week, during both a morning (0600-1100 h) and evening (1600-2000 h) session. We obtained ad lib fecal samples prior to treatment (to establish baseline) and across the 3-week treatment period. Whenever a subject was observed defecating, we collected the fresh sample into a plastic bag and placed it immediately on ice (in a cooler box or thermos). We stored all of the fecal samples at -20 °C within 4 hours of collection.

We performed all of the captures over the course of five consecutive days in mid December, with 1-2 capture mornings (0600-0800 h) per group. We processed maximally four subjects, in succession, per day. Shortly after emergence from their den or ‘sleeping burrow,’ we captured our subjects by gently picking them up by the base of the tail, placing them into a cloth bag, and anesthetizing them with isoflurane (Isofor; Safe Line Pharmaceuticals, Johannesburg, South Africa), administered in oxygen via face mask. We first obtained a blood sample (~ 2 mL) from the jugular vein of each individual, using a 25 G needle and 2-mL syringe. We immediately transferred blood samples to serum separator tubes (BD Vacutainer; BD Franklin Lakes, NJ, USA) and allowed them to clot at ambient temperature. Following a morning’s captures, we centrifuged the blood samples at 3000 rpm for 10 min and pipetted the serum layer into a clean Eppendorf tube. We stored serum samples on site at -20 °C until transport, on ice, along with all fecal samples (see above), to Duke University in Durham, North Carolina, where we stored samples at -80 °C until further processing or analysis.

The animals that received flutamide, at roughly 15 mg/kg/day (Table S1), or placebo underwent a minor surgical procedure performed by JD, a veterinarian licensed in South Africa. Using sterile procedures, we implanted one 21-day release pellet (either

150 mg flutamide (treatment) or carrier only (placebo), Innovative Research of America, Sarasota, FL) subcutaneously between the subject's shoulder blades. Briefly, a dorsal skin incision of 1-2 cm was made using a scalpel, a small subcutaneous pocket was created using blunt dissection, and the pellet was inserted using forceps. Incisions were sutured using dissolvable material (Vicryl). These subjects also received a subcutaneous injection of a non-steroidal, anti-inflammatory painkiller (0.2-0.3mg/kg meloxicam: Metacam, Boehringer) at the time of capture. The animals that served as no-pellet controls underwent captures and blood sampling only. After recovery from anesthetic, all of the subjects were immediately returned to their groups (20-30 min postcapture) and closely monitored throughout that and the following day. One male developed a minor infection at the implant site, for which he received a 3-day course of antibiotics (5-10 mg/kg enrofloxacin: Baytril, Bayer), injected subcutaneously, by gently lifting the skin, once per day. Animals in this population are sufficiently well-habituated that injections can be administered to conscious animals, typically while they are foraging. We suspended data collection from this animal during his period of medication.

Behavioral data collection

We began data collection two days following surgery. We conducted focal observations (Altmann, 1974) of our subjects roughly 3 days per week (average = 3.1 ± 0.35 days) across the 3-week treatment period. Morning sessions began as soon as about half of the group had emerged from the sleeping burrow. Because most prosocial interaction occurs while meerkats are clustered and sedentary, including during the brief periods spent at the burrow, we conducted a series of short (~ 5 min) 'burrow focals' (in random order) to ensure that we obtained some data from all focal subjects in a given group before the meerkats began to forage and disperse. Thereafter, we conducted longer, 30-min ‘foraging focals’ (rotating through our subjects in random order) until the group settled into its mid-day siesta. After a break of several hours, we used radiotelemetry to relocate the group, which had typically recommenced foraging. Evening sessions thus began with foraging focals and ended with burrow focals that were terminated once about half of the group had entered its sleeping burrow. Using this regimen, we collected 524 focals, representing over 130 hours of behavioral data.

We collected behavioral data in real time using the CyberTracker software package (version 3.263, CyberTracker Conservation) on handheld palm pilots (Palm T|X, Palm, Inc.). We established our data recording protocol (see ESM, §a) and ethogram (Table 1) for use both during burrow and foraging focals. For all social interaction, we included the partners and the directionality of behavior. We paused observation whenever the focal subject was out of view (e.g. if it entered a ‘bolt hole’ following a predator alarm call) and resumed observation once the focal subject was back in sight. We recorded the frequency and, in some cases, duration of behavior, which fell into the following seven categories: (1) aggression, (2) submission, (3) play (Fig. 1), (4) other prosociality, (5) vigilance, (6) olfactory communication and (7) vocal communication (see Table 1). Because occurrences of submission were so rare, we dropped this category from our analyses. Also, owing to a drought-induced shortage of pups at the time of our study, there were no opportunities to observe babysitting or pup provisioning; therefore, the only cooperative behavior included in our study were various forms of vigilance. For details about the vocal analyses, see below. In assessing intra- and inter-observer reliability for the remaining five behavioral categories, we obtained indices of concordance that were minimally 87.0% (see ESM, §c).

- Insert Table 1 and Fig 1 -

Vocal recordings and sound analysis

We assessed any potential treatment effects on vocalizations by examining the rate and acoustic structure of meerkat close calls, which are thought to be important in the maintenance of group cohesion (Manser, 1998). We conducted 5-15 min sound focals on each individual every third day during the treatment period, resulting in 5-7 recording sessions per male (12 hours of sound recordings in total). We recorded close calls during the mornings, after groups had left the sleeping burrow and the focal males had started foraging. We recorded individuals from a distance of 0.5-1.0 m with a directional Sennheiser microphone (ME66 with a K6 power module and a MZW66 pro windscreen, Old Lyme, CO, U.S.A) connected to a Marantz Professional PMD661 solid-state recorder (16bit, 44.1kHz, Marantz Japan Inc.).

We assessed the calls for quality using Cool Edit 2000 (Syntlillium Software Corporation, Phoenix, AZ, USA), selected for analyses 16-68 calls per individual, and carried out quantitative acoustic analyses in Praat v.5.3.84 (www.Praat.org). From each call, we selected four acoustic parameters, including the number of pulses, call duration (s), average pulse duration (s), and mean fundamental frequency (F0, Hz), as these have been shown to be affected by androgen concentrations in other species (Bass and Remage-Healey, 2008; Fusani et al., 1994; Pasch et al., 2011; Rek et al., 2011). We based final analyses on 554 calls for all acoustic parameters, except average pulse duration, which was based on 324 calls because the duration of all pulses in the calls could not always be realiably calculated.

Enzyme immunoassays

To prepare fecal samples for analysis, we lyophilized, pulverized, and sifted fecal samples into a fine powder within six months of collection, and stored the powder in vials at -80 °C until extraction. We extracted steroid metabolites from fecal samples following a protocol described elsewhere (Starling et al., 2010; Wasser et al., 2000). Briefly, we weighed 0.2 g of dry fecal powder and mixed it with 2 mL of 90% methanol. We placed the mixture on a rotating shaker for 30 min and centrifuged it twice, discarding the sediment each time. We stored the methanol-extracts at -80 °C until analysis.

We analysed serum and fecal extracts for circulating T and androgen metabolites (hereafter fecal T or fT), respectively, via enzyme immunoassay (EIA). We used an anti-T antibody raised in mice (Fitzgerald Industries International) that cross reacts 100% with T, 9% with dihydrotestosterone, < 1% with androstenediol, and < 0.1% with androstenedione, estriol, estradiol, and progesterone. We paired this antibody with a matched T 3-CMO-HRP conjugate (Fitzgerald Industries International). Plate sensitivity was 0.2-12.5 ng/mL. Our EIA protocol is detailed in the ESM (§d).

To assess intra-assay reliability we assayed low, medium, and high controls in 10 wells on each of two plates. The average coefficient of variation (CV) between the two plates was 9.8% (low control), 7.7% (medium control), and 5.7% (high control). Inter-plate reliability was assessed by assaying low, medium, and high controls in duplicate on each of 10 plates. The average interplate CV was 5.5% (low control), 6.4% (medium control), and 6.8% (high control). Serial dilutions of serum and fecal extracts pooled from multiple individuals produced linear displacement curves that were parallel to the T standard curve. We also combined serum and fecal pools with low, medium, and high concentrations of T prior to analysis. Recovery percentages for serum spikes were 90.2% (low control), 108.1% (medium control), 95.4% (high control), and for fecal spikes were 93.6% (low control), 110.5% (medium control), and 104.3% (high control). To assess our extraction efficiency for fecal samples, dried feces from multiple subjects were pooled and spiked with T prior to extraction. Extraction efficiency was 85.4%.

Physiological validation

One means of biological validation of fecal hormone metabolites is to show that the metabolites reveal a physiologically relevant difference across groups, detectable from varying circulating hormone concentrations (Brown et al., 2005). We performed a biological validation of fT in wild meerkats and obtained the expected age-related change in fT characteristic of male puberty (Beehner and Whitten, 2004) (see ESM, §e and Fig. S2). Another means of validation, particularly for showing a cause-and-effect relationship, is to administer a drug known to stimulate hormonal production. In this case, our administration of flutamide might also serve as a biological validation of our assay, because in sufficient doses, flutamide is known to impair the negative feedback loop, somewhat paradoxically raising T concentrations (Hellman et al., 1977). Accordingly, flutamide-treated animals might reveal an initial increase in fT relative to control animals.

Statistical analyses

We conducted our statistical analyses using R, version 2.15.2 (R Core Team, 2012), and SPSS 22.0. We set significance at $P < 0.05$. After log transformation, our endocrine data, which derived both from fecal and serum samples, were normally distributed. To determine if serum T concentrations between our experimental conditions (flutamide, placebo, no-pellet) differed prior to treatment, we ran a single ANOVA using the aov function in R. Once we determined that the placebo and no-pellet conditions did not differ (see results), we combined these two conditions and ran a single student's *t*-test to compare serum concentrations of all control subjects against those of flutamide-treated individuals.

We tested the influence of flutamide treatment on fecal T metabolites by implementing a series of generalized linear mixed models (GLMMs) using the glmmADMB package, version 0.7.4 (Skaug et al., 2013) in R, using Gaussian distributions. The log of fT (ng/g) was entered as the response variable in each model. The fixed effects in the full model were treatment (three levels: flutamide, placebo or no-pellet), treatment period (two levels: pre-treatment or treatment), and time of deposition (two levels: AM or PM). We included the individual nested within its social group as a random effect. Following Crawley (2002), we included all probable independent terms and interactions in the full model and excluded terms sequentially until the model contained only statistically significant terms.

If fT concentrations did not differ significantly between the placebo and no-pellet treatments, we pooled these two conditions in a single 'control' treatment and reran models with only two levels for the treatment factor (flutamide and control). Because treatment period (i.e. pre-treatment or treatment) influenced fT concentration, we subsequently re-ran the model within the two treatment periods. Moreover, as the response to flutamide treatment may have been different across weeks, we ran a model within each week of treatment. For all of the models, we included all probable independent terms and interactions in the full model and excluded terms sequentially until the model contained only statistically significant terms (Crawley, 2002).

For the behavioral data, we also used the glmmADMB package, version 0.7.4 (Skaug et al., 2013) to implement GLMMs with zero-inflation. Each behavioral category was entered as the response variable. The fixed effects in the full model were treatment (three levels: flutamide, placebo or no-pellet), days on treatment (continuous variable), location (two levels: burrow or forage), time of day (two levels: AM or PM), and group size (continuous variable). Individual and group identities were entered as a nested random effect in the models. The duration of each observation was accounted for as an offset in the model. If a behavior did not significantly differ between the placebo and no-pellet conditions, we pooled these two treatments in a single control treatment and reran the model with only two levels for the treatment factor (flutamide and control). As in our endocrine analyses, we included all probable independent terms and interactions in the full model and excluded terms sequentially until the model contained only statistically significant terms. For each model we used the Poisson and negative binomial distributions and selected the model with the lowest AIC value.

We analysed call rates and vocal parameters using linear mixed effects models (procedure lmer from package lme4 in R, version 1.1-7), except for number of pulses, which we analysed using general linear mixed effects models with specified poisson distribution (glmer procedure, nlme package version 3.1-118). We calculated call rates for each recording session. We used treatment (three levels: flutamide, placebo or no-pellet) as a between-subjects factor and individual identity in all models to account for multiple observations per invidiual. Call rates and average pulse duration were natural-log transformed to conform with linearity assumptions. Call rate analyses are based on 100 sound recordings.

RESULTS

Baseline androgen patterns

During the baseline week of fecal endocrine monitoring, prior to treatment administration, subordinate male meerkats that were to receive placebo, no pellet, or flutamide did not differ in their fT concentrations (ANOVA: $F_{2,11} = 0.65$, $P = 0.53$). These pre-treatment placebo and no-pellet conditions did not differ from each other (*t*-test: $t_{10.903} = 0.29$, $P = 0.78$), nor did males in the single collapsed, control group differ in their baseline fT values from males that were assigned to the flutamide condition (*t*-test: $t_{11.161} = 1.10$, $P = 0.29$). Likewise, serum T concentrations from blood samples collected at the time of capture (representing a more immediate pre-treatment baseline) did not vary by the three eventual experimental conditions (ANOVA: $\mathrm{F}_{2,11} = 0.62$, $P = 0.55$). There were also no differences in circulating T when the males assigned to the two comparable control conditions (*t*-test: $t_{4.25} = 0.54$, $P = 0.62$) were collapsed and compared against the males assigned to the flutamide condition (*t*-test: $t_{11.87} = -1.01$, $P = 0.33$). Thus, there were no baseline differences in the androgen profiles of our subjects.

Effect of flutamide on fecal androgens

During treatment, no-pellet and placebo males also did not differ in their fT concentrations, either across all weeks of treatment (z value = -0.52, $P = 0.60$) or when considering the first (z value = -1.14, $P = 0.25$) and second (z value = -0.4, $P = 0.69$) weeks separately. We had too few fecal samples from these males in week three to compare these two conditions in the last week. Given the lack of differences, we collapsed the two control categories in subsequent analyses.

Despite the absence of an overall difference in fT concentrations between flutamide and control males across the entire 3-week treatment period (z value = 1.12, *P* = 0.26), there was a clear time course in the effect of antiandrogen treatment on fT (Fig. 2). Notably, in the first week of treatment, flutamide-treated males showed the expected effect of this form of antiandrogen treatment and had significantly greater fT concentrations than did control males (z value = 3.71, *P* < 0.001; Fig. 2). Thereafter, this difference disappeared: Flutamide and control males no longer differed in fT in either the second (z value = -0.8, *P* = 0.42) or third (z value = 0.39, *P* = 0.70) weeks of treatment.

- Insert Fig 2 -

Behavioral equivalence between placebo and no-pellet conditions

Consistent with their equivalent androgen values (and intact androgen function), males in the no-pellet and placebo conditions did not differ in any of their behavioral patterns. This equivalence was true for week 1 only (see ESM, §f and Table S2), confirming that, after a 48-hour recovery period, the minor surgery for pellet implants had no effects on behavior. Moreover, the same pattern of behavioral equivalence maintained across all weeks of the study, as evidenced, for instance, by initiating (z value = -0.02, *P* = 0.98) and receiving (z value = -1.62, *P* = 0.11) high-intensity aggression (HIA; see Table 1) or initiating (z value = -1.64, *P* = 0.10) and receiving (z value = -0.09, *P* = 0.93) prosocial interaction (see ESM, §f and Fig. S3). Therefore, we collapsed the two control categories in subsequent behavioral comparisons against flutamide-treated males.

Effects of flutamide on behavior and vocal parameters

As expected, compared to all control males, flutamide-treated males initiated significantly less (z value = -2.93, *P* = 0.003; Fig. 3a) and received significantly more (z value = 2.10, *P* = 0.036; Fig. 3a) HIA (Table 2). The most frequent aggressive behavior within the HIA category was food competition, which we examined independently. Compared to all control males, flutamide-treated males initiated significantly fewer foraging competitions (z value = -2.91, *P* = 0.004). The rates of receiving foraging competition, however, were not affected by treatment (z value = 1.07, *P* = 0.29).

Flutamide treatment also altered certain aspects of social play. Compared to all control males, flutamide-treated subjects were significantly less likely to initiate play using the play-face invitation (z value = -4.32, *P* < 0.0001; Fig. 1a and Table 2). As anticipated, flutamide treatment also decreased the expression of ‘dominant’ types of play, such as pinning during wrestling (Fig. 1b). Whereas control and flutamide males were equally likely to play in a ‘subordinate’ (e.g. pinned) position (z value = -0.97, *P* = 0.33), flutamide-treated males played significantly less in the dominant position than did control males (z value = -2.09, *P* = 0.036; Fig. 3b).

Compared to all control males, flutamide-treated males also initiated significantly more prosocial behavior at the burrow after foraging (z value = 1.99, *P* = 0.046; Table 2 and Fig. 3c). We could detect no effect of receiving other prosocial interaction relative to an individual’s treatment (z value = 1.4, *P* = 0.16). Interestingly, when considering the identity of the focal subjects’ partners in all of these aggressive, playful, and prosocial interactions, the vast majority (82.3%) occurred with non-focal group members (see ESM, §f and Table S3). The minimal involvement of the dominant male and other flutamide-treated subjects suggests, respectively, that the effects of treatment were unlikely to have been biased by the dominant, male breeder in each group or confounded by having flutamide-treated animals as both the actor and recipient in given interactions.

Unlike the patterns we observed for direct social interaction, flutamide males did not differ from control males in their more solitary expression of vigilance (z value = 0.23, *P* = 0.82) or scent-marking (z value = -0.27, *P* = 0.79) behavior (Table 2, although see Fig. S1b). Also contrary to expectations, call rate was not affected by treatment (LMM: all t < 0.19, all *P* ≥ 0.8). Instead, individual identity explained a large proportion of the variation in all models, revealing high individual variability in all of the measured acoustic parameters of close calls (see ESM, §g and Table S4).

- Insert Fig 3 and Table 2 -

Time course of behavioral treatment effects

The number of days that subjects spent on treatment explained little to none of the overall variance in our GLMM models. Owing to limited sample sizes, non-normal distribution, and zero-inflation, we lacked the statistical power to further test for time-course effects in our data. Nevertheless, for comparison with the endocrine effects (Fig. 2), similar graphical representation of various types of behavior across weeks of treatment shows consistency in the relationship between flutamide-treated and control animals and, if anything, that treatment effects became stronger (rather than weaker) with time (Fig. 4).

- Insert Fig 4 -

DISCUSSION

In this first-ever, experimental manipulation of androgen action in wild meerkats, we found that androgens were involved in regulating a range of social behavior among subordinate, male helpers. Specifically, based on the effects of the antiandrogen, flutamide, we deduce that androgens facilitate various forms of aggressive and dominance interaction, influence aspects of social play, and dampen prosociality or affiliative behavior. By contrast, androgens have potentially no effect on cooperative antipredator behavior, scent marking or various parameters of close-call vocalizations. Given that T concentrations do not differ between the social classes of adult male meerkats (Carlson et al., 2004), androgens may not fully explain the social stratification and behavioral roles of breeders and helpers; nevertheless, based on present results, circulating androgens clearly play an important part in the daily, social lives of subordinate males, perhaps maintaining their reproductive potential and roaming proclivities to overcome the limited, unpredictable, and fleeting nature of their breeding opportunities.

We found no inherent bias in circulating or fecal androgen concentrations between our control and treated subjects, but we observed a significant, short-term (i.e., week-long) rise in fT concentrations as a result of blocking a subordinate male's androgen receptors with flutamide. This seemingly paradoxical result is consistent with effects of flutamide treatment observed in other studies (e.g. Stone and Clejan, 1991) and likely owes to a decrease in androgen negative feedback causing a compensatory increase in androgen production (Södersten et al., 1975). Beyond indicating that our early flutamide treatment was successful and that we had achieved an effective dosage, this result represents a second physiological validation of our assay of fecal androgen metabolites (the first being detection of pubertal endocrine changes). Nevertheless, there is great variation across studies in the impact of flutamide on circulating androgen concentrations: In some cases, significant behavioral impacts of flutamide treatment occur without any increase in T (Searcy and Wingfield, 1980) or occur even with a decrease in T (Hegner and Wingfield, 1987); in other cases, increases in T remain in effect long-term and until cessation of treatment (Stone and Clejan, 1991) or, as in our study, over only a short time span (e.g. from day 5 to 7 of a week-long treatment, despite daily injections: Södersten et al., 1975; see also Fusani, 2008). This range of physiological responses to flutamide treatment across studies could owe to the varying dosages achieved, the mode of administration used, the social context or the species tested.

The possibility exists that the decrease in fT we observed after week 1 might have indicated that, rather than lasting the full 21-day period, the pellets were exhasted after only one week. This interpretation is contradicted by the persistent behavioral effects of treatment across weeks, suggesting instead that androgen-receptor blockade remained in effect, but that feedback mechanisms may have stabilized, effectively ‘resetting homeostasis’, or that initial receptor blockade had lasting behavioral consequences, perhaps via altered receptor sensitivity (Fusani, 2008). Although we cannot distinguish between these alternative mechanistic explanations, it is clear that behavioral effects of antiandrogen treatment persisted minimally throughout the three-week study period.

These behavioral effects of flutamide administration, as expected, were manifest in meerkat aggressive behavior, with treated males initiating less, but receiving more, aggression than controls. The reduced initiation of aggression by treated males provides strong evidence for a direct effect of androgens on agonism. That treated males also received more aggression from conspecifics implicates additional indirect effects of androgens on behavior. Perhaps group members perceived a difference or ‘weakness’ in flutamide-treated males, which may have prompted an increase in the frequency with which treated subordinates were targeted. Alternately, the stability of social relations among subordinate males may be partially maintained by balanced interactions, such that a mismatch in the aggressive performance between flutamide-treated males and controls may have led to an escalation in the aggression against treated animals.

We also found that androgen-receptor blockade mediated certain aspects of social play in adult meerkats. Notably, flutamide-treated males initiated less play and were less dominant in their expression of social play than were control males. Thus, in the absence of androgenic influence, male meerkats were less bold, assertive, or competitive in their play. Although the directionality in these patterns is not unexpected, these findings provide rare evidence of activational effects of androgens on adult social play. Across mammalian taxa, prenatal, neonatal or prepubertal androgens have been shown to influence rough-and-tumble play, specifically, during infancy or juvenility (Meaney et al., 1985; Panksepp, 1981; Pedersen et al., 1990; Pellegrini, 1995). Those studies established that organizational, rather than activiational, T is important for modulating social play (Meaney et al., 1985) – a generalization that is called into question by our present findings.

Flutamide administration also affected other prosocial interaction, although in the opposite, enhancing direction. Flutamide-treated males were more likely to initiate affiliative behavior, such as grooming, huddling, and social sniffing. Combined with the depressive effects of flutamide on the initiation of aggressive or dominance behavior, these results are consistent with the hypothesis that there is an androgen-mediated trade-off between aggression and affiliation (Albers et al., 2002; Hegner and Wingfield, 1987; Ketterson and Nolan, 1994). Nonetheless, it must be noted that in some monogamous or cooperatively breeding mammals, T (either directly or following conversion to estrogen) can promote, rather than inhibit, paternal or affiliative care (Storey et al., 2006; Trainor and Marler, 2001, 2002). We might therefore have expected androgen-receptor blockade to influence various facets of meerkat cooperation, but based on vigilance behavior only, we found no such evidence. These results are in accord with a previous study that found no relation between another form of cooperation – pup provisioning – and T in subordinate males (Carlson et al., 2006a). As indicated by the relation between prolactin and babysitting (Carlson et al., 2006b), other neuroendocrine circuits may be involved in promoting pup care.

Conservatively, we might interpret that androgen function does not play a pivotal role in regulating cooperative behavior in adult meerkats; however, it is important to note that we lack information about any role androgens may play in prenatally priming meerkats for their adult behavioral repertoire. In humans, for instance, there is evidence to suggest that T's action in promoting prosociality or cooperation may stem from prenatal androgen exposure. Specifically, experimentally increasing circulating T in humans leads to an increase in cooperative behavior, but only in those individuals who had low prenatal exposure to androgens (van Honk et al., 2012).

Antiandrogen treatment also did not appear to influence scent-marking behavior, including anal marking, body rubbing, chewing, and chinning vegetation. Nonetheless, although expressed evenly among the treatment groups, scent marking occurred in only 22 focal observations (4.2%). It may be that these null results reflect a floor effect of low scent-marking frequencies by subordinate males, rather than any lack of androgenic involvement in olfactory behavior or odorant quality. Although Jordan (2007) reports no rank-related difference in male marking patterns at latrines, we suspect that the marking behavior of male meerkats may be strongly rank related in other contexts (see Leclaire et al., 2014).

Although androgens have been shown to affect vocalizations in various species, including humans (e.g. Gyger et al., 1988, Charlton et al., 2011; Baker, 1999, Damrose, 2009), we did not detect any significant effects of flutamide treatment on meerkat close calls. These null results, albeit consistent with the findings of some antiandrogen studies in avian species (Grisham et al., 2007; Schwabl and Kriner, 1991), may owe, in part, to the significant individual variability we observed: This variability confirms previous findings of individual-specific close calls in meerkats (Townsend et al., 2010), but it may have overridden any potential treatment effects. Alternately, it may be that close calls produced during foraging are particularly insensitive to the actions of androgens. Indeed, previous findings of significant androgenic or antiandrogenic effects on vocalizations have involved calls produced in the contexts of reproductive advertisement and antipredator behavior (Ball et al., 2003, Behrends et al., 2010, Charlton et al., 2011, Gyger et al., 1988). In the future, it may be worth exploring if meerkat vocalizations produced in more directed social interaction relate to circulating androgen concentrations.

In summary, we found that androgen receptor blockade had important effects in wild, subordinate male meerkats beyond modulating aggression: antiandrogens affected a broad range of social interaction, from competitive to affiliative behavior. Continued studies of equally ranked individuals are thus likely to reveal new insights into the hormonal regulation of behavioral interaction. Whereas androgens are increasingly recognized for their role in mediating social behavior, estrogens have received considerably less attention, particularly in males. Because androgens can be readily converted to estrogens, depending on local enzyme activity, addressing the role of estrogens in monogamous and cooperatively breeding species will be an important next step. In future studies, researchers should also examine the role of prenatal androgens in establishing receptor distribution that might help explain how differential activational responses may arise from animals showing roughly equivalent endocrine profiles. That influencing the action of activational androgens could have such wide-ranging effects within members of the same social class leads us to expect even more dramatic influences of organizational androgens. It is noteworthy that all of the effects we observed became evident in a relatively short time span. With longer-term endocrine manipulation, even greater effects may be revealed. In sum, experimental endocrine manipulation in the field, albeit challenging, is key to revealing the mechanisms supporting social relationships, within and between classes.

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Table 1

Ethogram for codifying meerkat (*Suricata suricatta*) behavior. The definitions for behavior, grouped by category (i.e., aggression, submission, play, prosociality, vigilance, olfactory, and vocal) and subcategory (e.g. high- vs. low-intensity aggression), are adopted primarily from meerkat studies, but also from studies of other carnivores.

Behavior by category	**Definition**	**References**
Aggression		
Bite[a]	Grabbing, with one's teeth, any part of another individual's body, ranging from quick forceful nips to prolonged or intense contact.	Clutton-Brock et al., 2006
Chin rub[a]	Touching or wiping another with one's chin, often accompanied by head shaking.	Kutsakake and Clutton-Brock, 2008
Food competition[a]	Approaching another's food item or hole, prompting a defensive response via growling, blocking approach, pushing, threatening, and biting.	Ewer, 1963; Madden et al., 2009
Hip slam[a]	Using one's hindquarters to push intensely against the side of another individual.	Clutton-Brock et al., 2006
Push[a]	Slamming one's hindquarters against another's in an interaction that can be resolved immediately or can persist for a measurable duration.	Madden et al., 2009
Threat[a]	Lunging at another individual, often accompanied by growling.	Drea et al., 1996
Block approach	Shifting one's body to prevent another's access to a resource.	Ewer, 1963; Madden et al., 2009
Chatter[b]	Breathy, repetitive clucking vocalization.	Ewer, 1963
Growl[b]	Emitting a low, rumbling vocalization.	Clutton-Brock et al., 2006
Submission		
Grovel	Adopting a crouched body posture, often while peeping.	Clutton-Brock et al., 2006
Peep[b]	High-pitched vocalization, often occurring in rapid repetition.	Clutton-Brock et al., 2006
Play		
Play bite[c]	Short nips that are not forceful; commonly expressed during wrestling and grappling, but only scored when independent of	Ewer, 1963; Wemmer and Fleming, 1974

	wrestling or grappling.	
Play bite shake[c]	Non-harmful, open-mouth contact of another individual’s body using a slow, side-to-side motion of one’s head.	Drea et al., 1996
Play chase[c]	Pursuit with a bouncy gate.	Ewer, 1963; Wemmer and Fleming, 1974
Play mount[c]	Clasping another individual’s ribcage or groin while participants are a ventro-dorsal position.	Wemmer and Fleming, 1974
Stand on[c]	Simultaneously placing both forelimbs on the torso of an individual that is either sitting or prone.	Wemmer and Fleming, 1974
Wrestle-top or wrestle-bottom[c]	Vigorous, mutual rolling around or pushing, often coupled with play biting, shaking, pawing, and clasping.	Wemmer and Fleming, 1974
Play face	Type of play initiation involving an exaggerated open mouth, often shown while in a prone body position with the tail pointing upward.	Drea et al., 1996
Other prosociality		
Groom	Moving the mouth/teeth through another’s fur; recorded as a dyadic interaction for each pair of individuals; considered as a new bout after switching to a new partner or after 1 min of inactivity.	Ewer, 1963; Madden et al., 2009
Social sniff	Olfactory investigation of another individual.	Drea et al., 2002
Sniff genitals	Olfactory investigation of individual's genital region.	Drea et al., 2002
Huddle	Body contact with another individual; recorded as one event regardless of how many individuals are involved.	Madden et al., 2009
Vigilance		
Guard[d]	Standing on the ground, on hind legs, while scanning the environment.	Clutton-Brock et al., 1999
Raised guard[d]	Standing on a raised (>10 cm) perch, on hind legs, while scanning the environment.	Clutton-Brock et al., 1999
Other vigilance	Quick scans of the environment from a quadrupedal position.	English, 2009
Olfactory		
Anal mark environment	Everting the anal pouch and rubbing it across a vertical or horizontal substrate.	Ewer, 1963; Moran and Sorensen, 1986
Chin rub environment	Wiping of the face or cheek region across a substrate.	Moran and Sorensen, 1986
Chew marking	Biting vegetation, usually accompanied by rapid head shaking.	Jordan, 2007

Vocal		
Contact or close call	Short pulsated vocalization made during foraging, but not during a direct social encounter.	Townsend et al., 2010

[a] Included in the collapsed subcategory of high-intensity aggression.

[b] Vocalization that is clearly associated with aggressive/dominance or submissive interaction, but that we did not record acoustically.

[c] Included in determining ‘dominant’ vs. ‘subordinate’ role assumed during play.

[d] Indicates behavior recorded as a state (all other behavior recorded as an event).

Table 2

Effect of flutamide treatment on the behavior of subordinate male meerkats. A 95% confidence interval (CI) excluding 0 indicates a statistically significant relationship. The *P* values and CI that indicate statistical significance are shown in bold.

Dependent variable	**Treatment coefficient**[1]	***P* value**	**95% CI**
Initiate aggression (all types)	-0.15	0.37	(-0.47) − 0.17
Receive aggression (all types)	0.21	0.17	(-0.09) − 0.51
Initiate high-intensity aggression	-0.75	**0.003**	**(-1.26) − (-0.25)**
Receive high-intensity aggression	0.43	**0.036**	**0.03 − 0.83**
Initiate food competition	-1.05	**0.004**	**(-1.75) − (-0.34)**
Receive food competition	0.40	0.29	(-0.34) − 1.14
Play face	-4.32	**<0.0001**	**(-2.65) − (-1.00)**
Dominant play	-1.18	**0.036**	**(-2.28) − (-0.08)**
Subordinate play	-0.50	0.33	(-1.51) − 0.51
Initiate prosocial behavior[2]	0.47	**0.046**	**0.01 − 0.92**
Receive prosocial behavior[2]	0.28	0.16	(-0.16) − 0.95
Vigilance	0.03	0.82	(-0.20) − 0.25
Scent marking	-0.20	0.79	(-1.67) − 1.27

[1] Positive or negative values indicate that the behavior values were higher or lower in response to the flutamide treatment than to the control treatment (including both the no-pellet and placebo conditions), respectively.

[2] Indicates prosocial behavior that occurred around the burrow system after foraging.

Figure 1: Adult, subordinate male meerkats playing. (A) The individual in the top-left corner is inviting play by showing a ‘play face’. (B) Two individuals involved in play wrestling can either occupy a dominant position (shown by the standing animal) or a subordinate position (shown by the pinned animal).

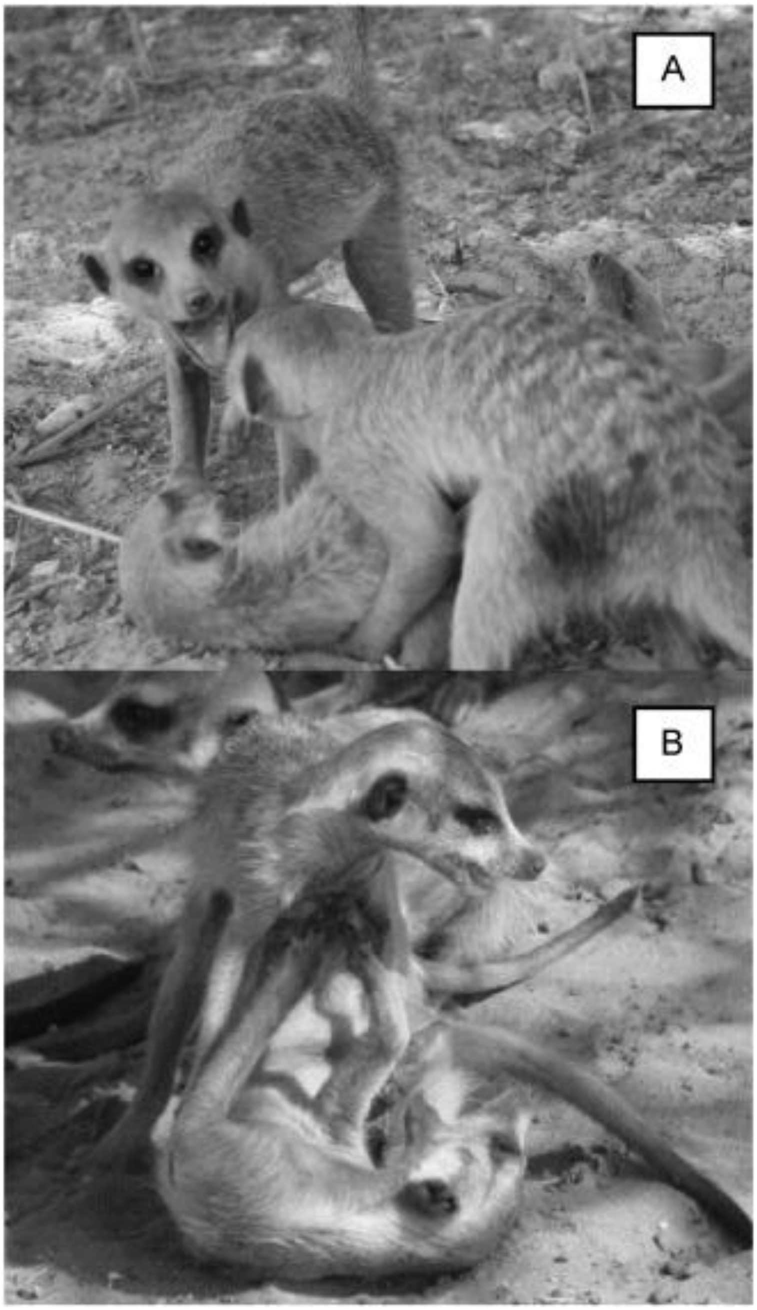

Figure 2: Fecal testosterone in adult, subordinate male meerkats across the three-week treatment period. ***, P < 0.001.

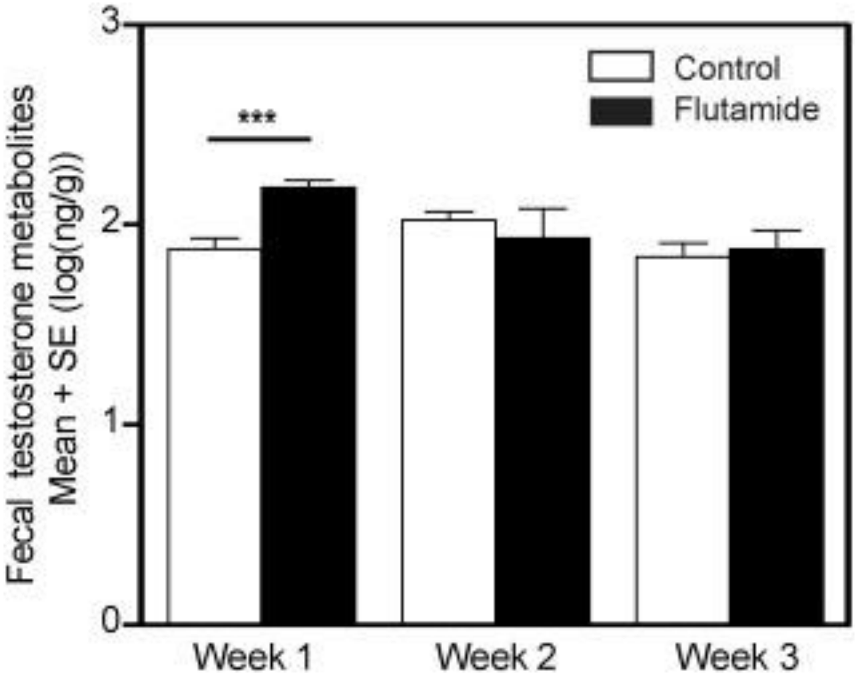

Figure 3: Effect of flutamide treatment on the frequency (per focal) of behavior in subordinate male meerkats: (A) Initiating and receiving high-intensity aggression, (B) playing in the dominant position, and (C) initiating prosocial behavior after foraging around the burrow. *, P < 0.05; **, P < 0.01.

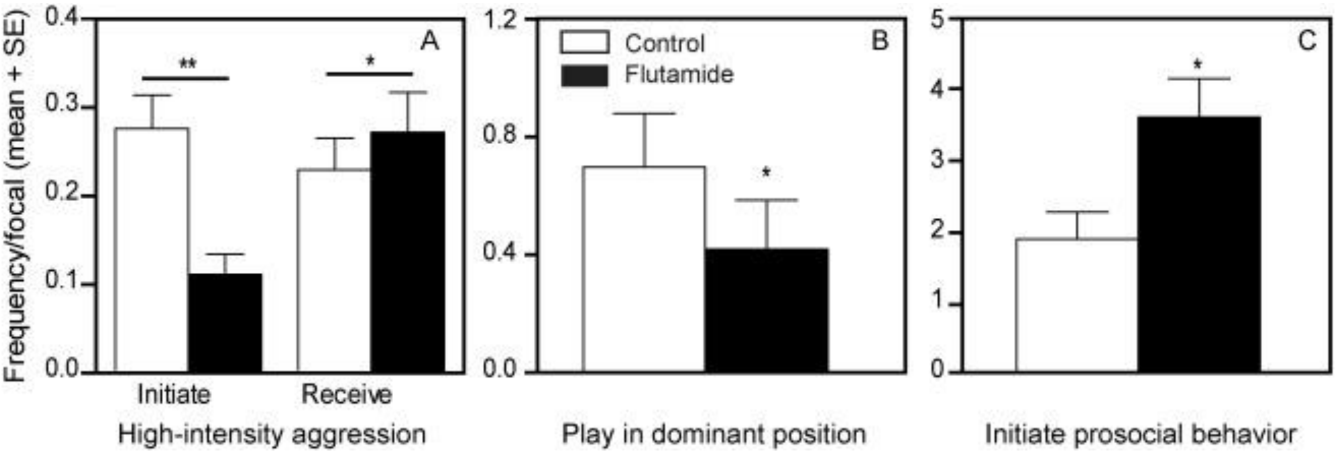

Figure 4: Behavior of adult, subordinate male meerkats across the three-week treatment period: (A) Initiating high-intensity aggression, (B) initiating foraging competition (during foraging focals), (C) rough-and-tumble play, and (D) initiating prosocial behavior.