Title: Effects of Lupron and surgical castration on fecal androgen metabolite concentrations and intermale aggression in capybaras (*Hydrochoerus hydrochaeris*)

Running Title: Castration and aggression in capybaras

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Research Highlights: Chemical sterilization with leuprolide acetate resulted in down-regulation of sperm and testosterone production, but did not reduce intermale aggression among three capybara males. Similarly, subsequent surgical castration of two males was unsuccessful in managing agonistic interactions.

Abstract:

To curb agonistic interactions in a bachelor group of three male capybaras (*Hydrochoerus hydrochaeris*), a single dose of leuprolide acetate (Lupron®) was used in an attempt to chemically sterilize the males. Concurrently, fecal androgen metabolite (FAM) concentrations were quantified via enzyme immunoassay to monitor changes in testosterone production after injection of the gonadotropin-releasing hormone agonist. When Lupron proved ineffective in suppressing intraspecific aggression, surgical castration was performed on two males, with continued noninvasive endocrine monitoring. In all three capybaras, FAM concentrations increased initially as a result of the luteinizing hormone surge, but then decreased significantly following chemical sterilization. Surgical castration resulted in further, persistent declines in FAM concentrations in two males, while the third, intact male demonstrated a rise in FAM to pre-Lupron concentrations at 8.5 and 9.5 months post-administration. Despite successful suppression of sperm and testosterone production, intermale aggression continued, ultimately necessitating separation of the animals and transfer to other holding institutions. Under this set of conditions, a single Lupron dose was inadequate for suppressing intraspecific aggression in a group of three males with a pre-established history of aggression.

1. Introduction:

Capybaras (*Hydrochoerus hydrochaeris*) are a gregarious, semi-aquatic rodent species native to South America and commonly maintained in zoological collections. Given their value to commercial production in some regions, and their pest status in others, capybara social behavior has been extensively studied to inform population control and management (as reviewed in Herrera, Salas, Congdon, Corriale, and Tang-Martínez, 2011). In the wild, capybaras tend to form relatively stable, territorial groups with a sex ratio strongly biased toward females. Male social structure exhibits a rigid and strongly linear dominance hierarchy, with a single dominant male maintaining priority access to mating opportunities, food, wallowing sites, and other resources (Herrera and Macdonald, 1993; Herrera et al., 2011; Rosenfield and Pizzutto, 2019). Dominance is maintained through the expression of androgen-driven secondary sexual characteristics (e.g. enlarged nasal scent glands), display of marking behavior, defense of central positions among the herd, and aggressive interactions directed at subordinate or satellite males. Intermale agonistic interactions may range from brief, relatively harmless chases to severe bite wounds sustained by subordinates (Costa and Paula, 2006; Herrera, 1992; Herrera and Macdonald, 1993; Morreira, Macdonald, and Clarke, 1997; Rosenfield and Pizzutto, 2019; Rosenfield et al., 2019).

Given the likelihood of aggressive interactions and potential for serious injury when housed in groups, it is generally not advised to house male capybaras together for welfare reasons (AZA Rodent, Insectivore and Lagomorph TAG, 2018). However, the maintenance of heavily female-biased capybara groups is unsustainable indefinitely, resulting in a surplus of males in the global zoological population that often must be housed together. Surgical castration has been a strategy to curb undesirable agonistic behaviors, but it precludes the male from future breeding, an unfavorable outcome for conservation management. Thus, chemical sterilization has been proposed as an alternative, temporary, and less invasive means to reduce intraspecific aggression in wildlife maintained under human care (Penfold, Patton, and Jimchle, 2004).

Among chemical sterilization agents, gonadotropin-releasing hormone (GnRH) agonists like deslorelin and leuprolide acetate are commonly used in veterinary medicine. GnRH agonists are a class of drugs that function by binding to GnRH receptors, leading to an initial "flare" or acute surge of luteinizing hormone (LH) and a consequent short-term rise in circulating testosterone (Padula, 2005). Subsequently, they desensitize the pituitary gland through GnRH receptor downregulation, which reduces the secretion of gonadotropins and ultimately androgen production. GnRH agonists may theoretically modulate behavior by reducing circulating levels of testosterone (Goericke-Pesch, 2017; Padula, 2005). However, the impact of chemical or surgical castration on undesirable behaviors can be highly variable. This study evaluated the effects of a single-dose injection of leuprolide acetate (a GnRH agonist) and subsequent castration on intermale aggression and excretory fecal androgen metabolite (FAM) concentration in adult male capybaras.

2. Materials and Methods:

2.1 Animal History, Husbandry, Housing, and Social Behavior:

Three male capybaras were acquired in November 2003 and housed in the collection of the Smithsonian's National Zoological Park in Washington, DC. At the time of introduction, two siblings were from the same litter and aged 1.5 years, while the third male was 1 year of age (Table 1). Following a routine and uneventful 30-day quarantine, the males were introduced and housed together as a trio in one indoor-outdoor enclosure for 2 years. The habitat was composed of an outdoor 6 m x 6 m area on exhibit and a 4 m x 5.8 m indoor area with crushed granite flooring split into two accessible stalls. All three capybaras had access at all times to one shared indoor pool, and weather-dependent access to a shared outdoor pool. The capybaras were fed twice daily with pelleted rodent feed, hay, and mixed greens and produce, and water was provided ad libitum.

The three males were housed together uneventfully for 2 years until the time of sexual maturity. Agonistic interactions among the three males began abruptly and were first observed in early February 2005. A serious altercation ensued among the three males, resulting in severe skin lacerations in one

individual and a fractured incisor and minor wounds in the first aggressor. At this time, they were separated in adjacent enclosures during the healing process; however, visual and auditory contact remained intact.

2.2. Chemical and Surgical Interventions:

Due to its temporary effects, chemical sterilization was attempted in the initial effort to manage intermale aggression and reduce further risk of injury. Capybaras were anesthetized for physical examination and injection of leuprolide acetate (Lupron®, TAP Pharmaceuticals, North Chicago, IL) on 14 or 15 February 2005. Animals were induced with a combination of ketamine (3.0–3.6 mg/kg) and medetomidine (0.03–0.035 mg/kg), then maintained under anesthesia with supplemental oxygen and isoflurane (0.5–1%) as needed delivered via facemask. A single dose of Lupron (3.0–3.5 mg, or approximately 0.08–0.09 mg/kg) was injected intramuscularly in the left thigh of each male. Additionally, testicular measurements were taken from each male (Table 1). Following Lupron injection and reversal with atipamezole (5.5–7.0 mg), the capybaras were initially housed individually to avoid agonistic interactions during the initial testosterone surge. Each individual was prescribed a short course of haloperidol at 1 mg/kg daily to assist with the reintroduction process beginning 3 March 2005 and discontinued 21 March 2005. Slow reintroduction efforts were initiated 7 March 2005 but were ultimately unsuccessful, with capybara 1 sustaining severe wounds and injuries inflicted by enclosure-mates that required immediate veterinary attention.

Given the severity of the injuries, the decision was promptly made to surgically castrate capybaras 1 and 2 and to house all three males individually. Capybaras 1 and 2 were surgically castrated on 24 March and 4 April 2005, respectively. Both animals were induced using a combination of ketamine (3.4–3.5 mg/kg) and medetomidine (0.033–0.035 mg/kg) and maintained at a surgical plane under anesthesia using isoflurane at 1–2% delivered via facemask. Both animals were sterilized using a closed castration technique without complication. As the presumptive dominant male, capybara 3 was not surgically castrated and remained intact. Testicular tissue from capybaras 1 and 2 were submitted for histological analysis.

2.3. Fecal Processing and FAM Analysis:

Voided fecal samples were collected opportunistically from each male for fecal androgen metabolite (FAM) analyses between February and mid-December 2005. Fecal samples were attributed to specific individuals with the use of food coloring and stored frozen until time of processing and analysis as described by Putnam et al. (2015). Briefly, samples were dried in a lyophilizer (VirTis Ultra 35XL, SP Scientific, Warminster, PA), powdered, sifted, and 0.20 ± 0.02 g was weighed into 16x125 mm glass tubes (Fisherbrand, Thermo Fisher, Pittsburgh, PA). Five ml of 90% ethanol:10% de-ionized water was added to each sample along with ~20,000 dpm ^{3}H–testosterone tracer (NEN Radiochemicals, Perkin Elmer, Boston, MA) to determine procedural loss. For 20 minutes, samples were boiled in a 95°C water bath and maintained at a 5-ml volume with the addition of 100% ethanol as needed. Samples were then centrifuged at 500 x *g* for 20 minutes (Sorval RC 3C Plus, Kendro Laboratory Products, Newtown, CT), the supernatant recovered and 5 ml of 90% ethanol:10% de-ionized water added to the pellet, which was vortexed (pulse rate 1/second, speed 65; Glas-Col, Terre Haute, IN) for 30 seconds. Samples were re-centrifuged (15 minutes, 500 x *g*) and the supernatants combined and dried down under forced air. One ml of 100% methanol was then added to dried sample extracts, evaporated to dryness, and reconstituted in 1 ml of preservative-free buffer (0.2 M NaH_2PO_4, #S8282; 0.2 M Na_2HPO_4, #S7907, Sigma Aldrich, St. Louis, MO; 0.15 M NaCl, #S271, Fisherbrand; pH 7.0). After vortexing for 15 seconds, samples were placed in an ultrasonic cleaner water bath (Cole Parmer Instrument Company, Vernon Hills, IL) for 15 minutes. Sample extracts were further diluted 1:50 – 1:250 in buffer as needed. All sample extracts and dilutions were stored in polypropylene tubes at -20 °C until analysis. Average fecal extraction efficiency was 76.0 ± 0.9% based on recovery of ^{3}H-testosterone added samples prior to extraction.

A single-antibody enzyme immunoassay (EIA) utilizing a polyclonal antibody to testosterone (R156/157, C.J. Munro, University of California, Davis, CA) and testosterone-horseradish peroxidase ligand (lot: 2005/2006, SCBI, Front Royal, VA) (Putman et al., 2015) was used to measure FAM concentrations. Briefly, testosterone antibody in coating buffer (0.015M Na2CO3, #S2127; 0.035 M

NaHCO3, #S8875, Sigma Aldrich, St. Louis, MO; pH 9.6) was adsorbed onto a flat-bottomed, high-binding 96-well microtiter plates (Nunc-Immuno, Thermo Fisher) and incubated for 8 hours at 4˚C. The plates were washed five times (0.05% Tween 20, #P1379, Sigma Aldrich; in 0.15 M NaCl solution) followed by the addition of 0.05 ml standard (0.47–12.00 ng/ml, Catalog #46923, Sigma Aldrich), internal control or diluted sample in duplicate, and then 0.05 ml horseradish peroxidase solution. Plates were washed four times after incubation for 2 hours at RT, and 0.1 ml ABTS solution (0.04 M ABTS diammonium salt, #0400, Amresco, Solon, OH; 0.5 M H2O2 #BP2633, Fisherbrand, in 0.05 M citric acid buffer, #C0759, Sigma Aldrich; pH 4.0) was added to each well. All EIA plates were read on a microplate reader (MRX, Dynex Technologies, Chantilly, VA) at 405 nm (ref. 490 nm) when the optical density (OD) of the 0.00 ng/ml standard was ~1.00 (range 0.90–1.10). The testosterone EIA was validated for capybara fecal extracts by demonstrating parallelism between standard curves and serially diluted fecal extracts, and recovery of testosterone standard added to fecal extracts.

3. Results:

3.1 Testicular Histology:

Gross and histologic findings of the testes at the time of castration in two capybaras showed signs of testicular atrophy and fertility suppression following Lupron injection. Reduced sperm production in the seminiferous tubules was evident in both testes from both individuals, with minimal to no sperm storage noted in the epididymides. Mild to moderate hyperplasia of the interstitial cells was also observed in both males. These findings suggest that the Lupron did significantly reduce the production of sperm in capybaras 1 and 2.

3.2 FAM Concentrations:

Data are reported as ng/g dry feces. The intra-assay coefficients of variation (CV) between duplicates for all samples were <10%. Inter-assay CV for two internal controls (high and low concentration) were 3.52% and 6.78%, respectively (n = 13).

FAM concentrations for each male following Lupron injection and surgical castration are depicted in Figure 1, with descriptive statistics summarized in Table 2. In all three males, chemical sterilization with Lupron resulted in an initial surge of FAM concentrations as expected. For capybaras 1 and 2, FAM concentrations peaked at 5375.6 ng/g and 2131.2 ng/g at 4 and 6 days post-injection, respectively. After the initial FAM surge, concentrations then averaged 1292.3 ng/g and 782.1 ng/g, respectively. For the intact capybara 3, FAM concentrations peaked at 3824.8 ng/g 1 day following Lupron injection, followed by a decline to a mean of 702.5 ng/g over the remainder of the study period.

Following surgical castration, capybaras 1 and 2 demonstrated a sustained reduction in FAM for the remainder of the study (Fig. 1). Post-castration FAM concentrations dropped to means of 231.6 ng/g and 244.3 ng/g, respectively (Table 2). Conversely, the intact capybara 3 demonstrated FAM peaks of 1595.8 ng/g and 2131.2 ng/g at approximately 8.5 and 9.5 months post-Lupron, respectively.

3.3 Final Outcomes:

Intermale aggression in capybaras was insufficiently reduced by chemical and surgical castration in this study. All three capybaras were maintained as individuals, in exhibits separated by steel pipe barriers which restricted physical contact, but allowed for visual, auditory, and olfactory stimuli. Despite this individual housing arrangement, physical barriers, and chemical followed by surgical castration, aggressive behavior among the males continued, characterized by posturing and aggressive encounters at the gates. Due to this persistent and dangerous aggressive behavior, capybara 3 was transferred to a different institution in April 2006, while the remaining males were housed individually until transfer to another holding institution in September 2009.

4. Discussion:

Noninvasive monitoring of reproductive status and condition through fecal steroid analysis is an effective and valuable tool for the reproductive management of exotic species. Testosterone metabolites

excreted in feces reflect pooled activity over several hours, and thus better reflect average concentrations than measures of circulating testosterone (Brown, Terio, and Graham, 1996; Schwarzenberger, 2007). In this study, assessing FAMs proved to be an effective means of monitoring reproductive condition in male capybaras.

In all three males, the administration of Lupron caused an initial increase in FAM concentrations, followed by a gradual reduction. For capybaras 1 and 2, surgical castration largely resulted in persistently low but detectible concentrations for the remainder of the study. The residual excretion of FAM suggests an extra-gonadal source of androgens, possibly through production by the adrenal cortex or through peripheral conversion of adrostenedione (Middlebrook and Schoener, 2020). That could also account for the single surge in FAM in capybara one month after castration (1731.8 ng/g on 1 December 2005), or it may be due to sample contamination with urine. For capybara 3, which retained testes throughout the study, Lupron induced an initial surge and subsequent decrease similar to the other two individuals; however, this pattern appeared to be subject to greater fluctuation. At approximately 8.5 and 9.5 months post-injection, FAM concentrations of capybara 3 increased to 1595.8 ng/g and 2131.2 ng/g, respectively, comparable to levels prior to Lupron administration. These spikes may indicate the waning efficacy of Lupron consistent with a recrudescence period, and potentially a ramping up of reproductive activity in preparation for the breeding season.

The male capybaras in this study demonstrated lower FAM concentrations following chemical sterilization with Lupron and subsequent surgical castration, but did not have sufficiently lowered levels of aggression, ultimately necessitating their separation. These results are comparable to earlier studies that demonstrated the immunocontraceptive GonaCon – an anti-gonadotropin-releasing hormone vaccine – effectively suppressed steroidogenesis and fertility when used in male capybaras, but secondary sexual characteristics and intermale aggression were preserved (Rosenfield and Pizzutto, 2019; Rosenfield et al., 2019). Similarly, Lupron did not extinguish agonistic behaviors in male capybaras under the specific circumstances and conditions in our study.

The efficacy of GnRH agonists in mitigating unwanted aggression in conspecifics has been explored in a variety of other wildlife species. Deslorelin appears to be efficacious in curtailing interspecific aggression, especially in carnivores, but this pattern does not hold true for all taxa (Bertschinger et al., 2001; DeCaluwe, Wielebnowski, Howard, Pelican, and Ottinger, 2016; Harley, Power, and Stack, 2018; Molter, Fontenot, and Terrell, 2015; Norton et al., 2000; Penfold et al., 2002; Raines and Fried, 2016; Rowland, 2011; Vinke, van Deijk, Houx, and Schoemaker, 2008). Lupron in particular had promising results in suppressing musth-associated behaviors in an Asian elephant bull (de Oliveira, West, Houck, and Leblanc, 2004), but had minimal to no effect on aggression in collared lemurs and rut-associated behaviors in red deer (Barrell, Schaafsma, Ridgway, Wellby, and Miller, 2009; Ferrie, Becker, Wheaton, Fontenot, and Bettinger, 2011). Likewise, the success of surgical castration in eliminating intraspecific aggression can be variable and unpredictable in both wildlife and domestic species (Demas, Moffatt, Drazen, and Nelson, 1999; Farhoody et al., 2018; Ferrie et al., 2011; Garde, Pérez, Vanderstichel, Dalla Villa, and Serpell, 2016; Takeshita, Huffman, Kinoshita, and Bercovitch, 2017).

As studied extensively in domestic dogs, the expression of aggressive behaviors in males is not solely testosterone-driven and can be influenced by additional factors; thus, the effects of chemical or surgical sterilization may be challenging to predict (Farhoody et al., 2018; Garde et al., 2016; Neilson, Eckstein, and Hart, 1997). The apparent age-dependent expression of androgen-driven behaviors (including aggression directed towards conspecifics) suggests that testosterone may play a role in agonistic behaviors that become learned with age (as reviewed in Goericke-Pesch, 2017). These learned behaviors may then persist despite the removal of hormonal drivers. Capybara males have a low gonadosomatic index and high proportion of non-spermatogenic testicular tissue, suggesting heavy investment in androgen production (Moreira et al., 1997; Costa and Paula 2006; Paula and Walker, 2013). It has been speculated that this reproductive strategy emphasizes chemical signaling and behavioral maintenance of social status over sperm output (Herrera and Macdonald, 1993; Moreira et al., 1997). Interestingly, the presumed dominant male (Capybara 3) did not achieve the highest FAM concentrations pre- or post-intervention, although there appear to be a lot of intrinsic variability. This could suggest that he was not actually dominant, or that other

factors not captured here determine dominance, but monitoring over a longer period pre-intervention might elucidate more consistent patterns.

Unfortunately, behaviors were not formally quantified via an ethogram or other methods in this study, so it is possible that changes in aggression levels occurred but were not perceived. However, the priority objective was to minimize harm, and address ethical concerns given the severity of aggression and serious risk of injury to all capybaras. For subsequent or similar studies in these or other species, rigorous objective measurement of behaviors would be essential to determine the effects of these interventions on conspecific aggression.

Under the specific set of circumstances in this study, a single Lupron injection did not sufficiently suppress aggression in a bachelor group of male capybaras with established social antagonism. However, this study alone does not allow for conclusive determination of the efficacy of Lupron. For future attempts to maintain bachelor groups of capybaras, strong consideration should be given to age and timing of chemical interventions. In this study, the three males had already reached sexual maturity and developed agonistic behaviors prior to veterinary intervention. It is possible that chemical or surgical castration of capybaras at a younger, pre-pubertal age to prevent rather than treat aggression may have more favorable effects on group dynamics. However, it should also be noted that age is often not a reliable predictor of the effects of castration on problem behaviors (Neilson et al., 1997). Additionally, with no published guidelines for Lupron dosing at the time that these interventions were undertaken, it is uncertain whether higher or repeated doses might have produced greater suppression of intermale aggression. The use of deslorelin implants or other GnRH agonists for the control of problem behaviors also should be explored under more ideal conditions before definitive conclusions are drawn on efficacy for the control of aggressive behaviors.

5. Conclusion:

While contraception via single-dose Lupron and surgical castration had observable effects on excretory FAM concentrations in a group of three male capybaras, they did not abolish the expression of secondary sexual characteristics or agonistic interactions in this group. Subsequent studies and attempts at housing males together should consider earlier pre-pubertal interventions prior to the onset of conspecific aggression, and the addition of in study design for objective measurement of behavioral effects. Due to the possibility of persistent intermale aggression and risk of serious injury, caution is advised when housing capybaras in bachelor groups.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author, JHY, upon reasonable request.

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Table Legends:

Table 1. Summary of adult male capybaras (*Hydrochoerus hydrochaeris*) treated with Lupron (n=3) and subsequently castrated (n=2).

Table 2. Summary of fecal androgen metabolite (FAM) concentrations (ng/mg dried feces) for approximately 1 week before and 5-7 weeks after injection of Lupron in three male capybaras, followed by subsequent surgical castration in two of the males.

Figure Legend:

Figure 1. FAM concentrations as determined by enzyme immunoassay for capybara bachelor group, February through December 2005. Dates of Lupron injection are denoted with (*), while surgical castration dates for Capybaras 1 and 2 are indicated by (†).