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Field-testing a single-dose immunocontraceptive in free-ranging male capybara *(Hydrochoerus hydrochaeris)*: Evaluation of effects on reproductive physiology, secondary sexual characteristics, and agonistic behavior

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ABSTRACT

Controlling wildlife populations to mitigate human-wildlife conflicts and the spread of zoonotic diseases is an ever-growing necessity. The objective of this study was to evaluate a single-dose anti-gonadotropin-releasing hormone vaccine (GonaCon, USDA/NWRC, Fort Collins, CO, USA) as a non-lethal alternative for population control in free-ranging, synanthropic male capybara. In addition to infertility efficacy of this treatment, potential effects on the alpha male's secondary sexual characteristics and agonist behavior need to be assessed because any alterations in these factors could lead to population management failure. The treatment group ($n = 3$) received 1 mL of the anti-GnRH vaccine, intramuscularly, and the control group ($n = 2$) a 1 mL sham vaccine. Reproductive behavior and social group dynamics were monitored for 30 days prior to inoculation (June 2017) with continuous observations occurring during the study period. Antifertility effects were assessed by conducting exams of testicular morphology, semen characteristics, and histological analysis (after 270 days via hemi-gonadectomy). Compared to the control group, the testicles of the treated males had severe atrophy ($P < 0.05$), oligozoospermia and greater numbers of sperm cells in a static developmental phase. Courtship and agonistic alpha male behavior were not altered, and the group's social integrity was maintained. Results indicate there was 100% infertility in capybara males, observed throughout the study period of 18 months, and equally important, the male's alpha characteristics were not affected by the treatment, which is imperative for successful capybara population control efforts.

1. Introduction

In Brazil, where wildlife and feral animal population control is still in its early development ages, an emerging "problem" species is the capybara (*Hydrochoerus hydrochaeris*). Native to South America, these animals are the world's largest rodent, and a very

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resilient and highly proliferative species of polygynous nature, with a well-defined hierarchy in its social structure. The decrease of its natural predators (jaguar, puma, alligator, and anaconda) and the capacity to adapt quickly to rural/urban settings, attracted by abundant food supply, have made the capybara one of the most prevalent invasive synanthropic species in Brazil (Felix et al., 2014). Consequently, with capybara there are all the classical human-wildlife conflicts (HWC), such as traffic accidents, area occupation, and crop destruction, among others. The primary concern, however, is that capybara represent a serious epidemiological threat to public health, specifically, capybaras are considered to be amplifying hosts for *Rickettsia rickettsii* the etiological agent of the tick-borne disease, Brazilian spotted fever (BSF), the most lethal rickettsiosis (Meira, 2013; Labruna, 2014). The disease is transmitted by ticks of the genus *Amblyomma.* In Brazil alone, this disease has caused more than 1900 confirmed human cases since the year 2000, and numbers are steadily increasing (Sinan, 2018).

Depending on the application in free-ranging or captive wildlife, contraceptive concepts range from simple physical separation to hormone antifertility agents and immunocontraceptives, each with its merits and disadvantages. Some of the desired contraceptive characteristics include being effective in a large variety of species and in both genders, applicable remotely and as a single-dose (no booster required), long-term effects that are reversible, safe for the animal and handler with no adverse effects (physiologic or psychologic), logistically straightforward in the field, non-polluting to the environment and food-chain, and inexpensive and readily available (Rosenfield, 2016).

One contraceptive approach that combines most of these relevant characteristics is the immunocontraceptive anti-GnRH vaccine. The use of the immunocontraceptive GonaCon (GonaCon, USDA/NWRC, Fort Collins, CO, USA) induces an immune response to the inoculated antigens (synthetic GnRH peptide and other foreign proteins) because of the formation of antibodies that consequently neutralize the actions of endogenous GnRH. The resulting antibody-endogenous GnRH immunocomplex leads to an alteration in the chemical structure of endogenous GnRH and an ultimate loss in biological function because of a lack of capacity to bind to the target receptors in the anterior pituitary. Consequently, there is an inhibition of the release of FSH and LH and subsequently a relatively lesser intra-gonadal synthesis of the sex steroid hormones testosterone and estrogen than would occur in non-immunized animals.

The general objective of this study was to provide an alternative strategy to control the rapidly-growing syntrophic capybara populations and to indirectly mitigate the increasing human-capybara problems. For this purpose, there was examination of GonaCon as a potential non-lethal, non-invasive contraceptive for field-application in free-ranging capybaras, while also assessing any possible adverse effects and the effects of this treatment on the social-reproductive dynamics of capybara. There was a specific focus on the alpha male's behavior as the alpha male from a reproductive perspective, which is particularly important when considering the polygyny (harem-like) social structure of capybaras. Having an alpha male lose its courtship capacity would only provoke sub-ordinate or invading rival males to dominate the group and be the principal individual in reproductive capacity of the polygynous group, thus, negating the capacity for population management using this treatment in these groups. Having a treated alpha male maintain the dominant behavior and social status within the polygynous group would maintain group stability and minimize the overall rate of reproduction within the group.

2. Materials and methods

The project was approved by the university's ethics committee and the Brazilian Ministry of the Environments, SISBio: 54634-2. It was approved by the university's Ethics Committee on the Use of Animals in Research (CEUAVET/FMVZ/USP, protocol # 9553260816).

2.1. Area of study

The study was conducted at a manmade water pool, of approximately 247,500 m^2, surrounded by trees and extended grassy areas, and used for water-based athletics. It is part of the University City, University of Sao Paulo, south-eastern region of Brazil (23.555202, -46.722433).

2.2. Animals

In 2013, wild capybara invaded the pool area, from a nearby river, through a breach in the water canalization system. In total, two adults (one male and one female) and five pups were reported, which grew within a 4-year period to greater than 40 animals. As of May 2016, when the present study commenced, Group I represented one dominant male, 14 females, and 15 juveniles. Group II represented one dominant male, one subordinate male, five females, and three satellite males. All had free access throughout the entire area. In total, there was selection of five sexually mature capybara males that were assigned to one of two groups. The treatment group (n = 3; alpha males) was treated intramuscularly with a single-dose of 1000 μg anti-GnRH vaccine (GonaCon). The control group (n = 2; one alpha male, one sexually mature satellite male) was treated with a sham vaccine. Based on observational data, the alpha males had a proven history of fertility, and after a visual assessment, all individuals included in the study were considered to be healthy.

2.3. Identifying an alpha male

Although sexual dimorphism in capybaras is limited, especially until sexual maturity, alpha males have two distinct physical characteristics which are considered androgen-dependent (Costa and Paula, 2006; Herrera, 1992). The most prominent of these is the

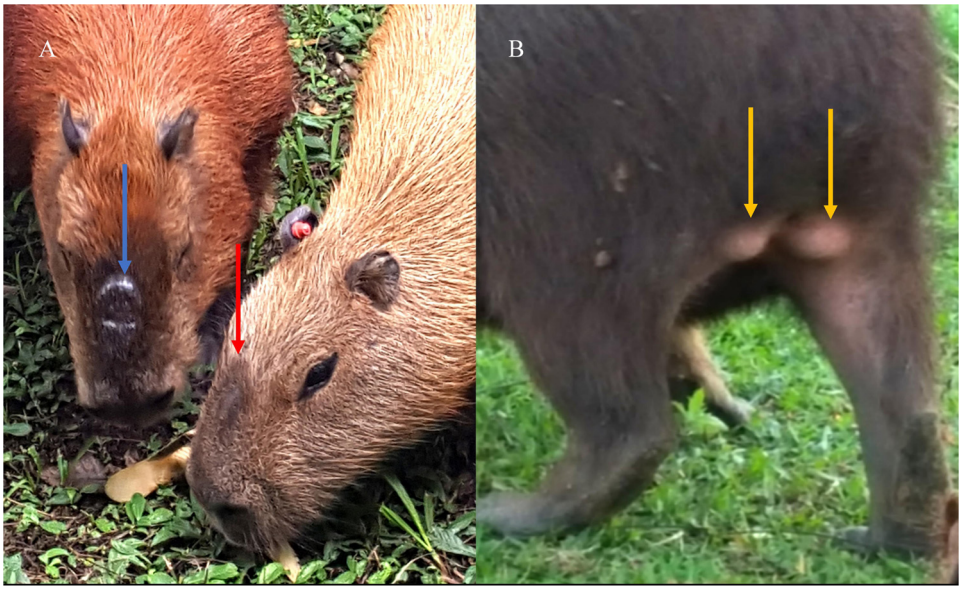

Fig. 1. Capybara Alpha Male Secondary Sexual Characteristics. A) blue arrow, showing an alpha male's nasal gland, red arrow, compared to a female's nasal gland; B) yellow arrow, two visible testes at the caudal-cranial area of the inner thigh.

morillo, a nasal gland (Fig. 1a) that, along with the perianal gland, is used for territorial marking. Results from several studies are consistent with the male with the greatest concentration of testosterone and greatest sexual activity having the largest morillo (Herrera, 1992). The second characteristic is the testicles; male capybaras do not have a scrotum and the testicles are located subcutaneously in the inguinal region (Paula and Walker, 2013). Based on observations of those conducting the present study of sexually mature/dominant males, the testes migrate bilaterally in the mid-sagittal plane to the region of the upper medial thigh (Fig. 1b), becoming distinctively visible. These two secondary sexual characteristics were considered to be indicators of the effects of the immunocontraceptive treatment.

2.4. Behavioral study

To serve as a baseline for comparing reproductive behavioral observations post-treatment, there was recording of the adult males' behaviors three times a week for a period of 1 month prior to any intervention. There was use of the continuous focal sampling method for 2 h with observational sessions being distributed evenly between morning, afternoon, and evening, totaling < 20 h. Post-treatment observations were conducted for 18 months, twice per month, using the same continuous focal sampling method for 2 h with observational periods being distributed evenly between morning, afternoon, and evening periods, totaling < 40 h of observation. To categorize behavioral traits, there was development of a simple but specific capybara alpha male agonistic and courtship behavior score that could be visually assessed.

2.5. Bio-material collection/analysis

On day 0 (first capture) there was recording of the animal's biometrics and identification of the animal with pre-prepared ear clips and an ID microchip implant, which were placed subcutaneously at the intra-scapular region. Preceding the histological analysis of the testicles, the epididymis was removed, and external dimensions were measured and there was weighing of the tissue.

2.6. Male fertility assessment

2.6.1. Semen collection

Sperm samples were collected either by electroejaculation or through pharmacologically induced urethral catheterization. Opportunistically, after hemicastration or necropsy, semen was collected by direct epididymal aspiration. The sample was transported in temperature-controlled conditions to the laboratory for immediate computer-assisted sperm analysis (CASA).

For the semen collection using urethral catheterization, there was specific selection of an anesthetic protocol consisting of ketamine (Syntec, Brazil) and dexmedetomidine (Zoetis, Brazil), a potent alpha-2 adrenergic agonist, which is believed to have relaxing effects on the smooth muscle of the ductus deferent, thereby promoting the release of semen into the urethra (Lueders et al., 2012; Pisu et al., 2017).

Semen collection by epididymal aspiration was performed in cases where a hemi-orchiectomy (in the field) was required. The removed testicles were taken to the laboratory for further processing. Spermatozoa were collected from the epididymal cauda using a

slicing technique (Nichi, 2009)

2.6.2. Sperm analysis

Immediately after seminal collection, the samples were protected from light, stored at 37 °C, and transported to the laboratory for further processing and analysis. There was analysis of samples for spermatic kinetics, concentration, sperm morphology, evaluation of mitochondrial activity, and evaluation of plasma and acrosomal membrane integrity using procedures that were previously described: Evaluation of sperm kinetics (Goovaerts et al., 2006) and sperm morphology, using the wet chamber method and a differential interference contrast microscope (DIC, Nikon® Eclipse TE300, Tokyo, Japan), (Barth and Oko, 1989); plasma membrane integrity, using eosin-nigrosine staining (Barth and Oko, 1989); acrosomal integrity, using fast-green/rose bengal staining (Pope et al., 1991); and mitochondrial activity using the DAB (Diaminobenzidine) test (Hrudka, 1987).

2.7. Collection frequencies

On days 90 and 180 of the present study, there was repeated collections of the biomaterial and biometric data. On day 270, in addition to collecting blood/semen samples and biometrics, a hemi-orchiectomy was performed for morphological and histological studies.

2.8. Morphometric analysis of testes

Immediately post-castration, the testis mass (epididymides removed) was quantified to the nearest 0.5 g, and the dimensions were measured to the nearest 1 mm using a Vernier caliper.

2.9. Histology analysis

For the histological samples, there was preparation using the following methods described by (Júnior, 2012; Paula, 1999). Morphological changes of the treatment group were evaluated using a microscope and there was comparison with values from tissues collected from the control animals.

2.10. Variables of immunocontraceptive effects on the testes

To identify alterations due to the contraceptive effects, the values for the following variables were compared to those of untreated males: testicular morphology (gross morphology weight/dimensions); and evaluation of the testicular parenchyma, assessment of the parenchymal tissues including 1) evaluations for the presence and organization of seminiferous tubules (numbers and structural arrangements within the seminiferous tubule); 2) Sertoli cells (structural integrity); 3) Leydig cells (numbers, cellular organizations); 4) basal lamina structure; 5) germinal epithelium/germ cells/8-stages of differentiation (numbers, arrangements, stage, number of necrotic cells); 6) lymphoid space (size); and 8) lumen (size).

2.11. Population survey

To support the overall evaluation of the effectiveness of the immunocontraceptive vaccine for population control, there was monthly monitoring of the two population groups, double counting the group members by direct observation, from a distance of 10 to 50 m, for a duration of 1 to 2 h, and various morning, day, evening, and night sessions. Monitoring was initiated 1 month prior to the vaccination date and continued throughout the study period.

2.12. Statistical analysis

The statistical analysis performed in the present study to compare the groups was mainly descriptive because the number of experimental units (i.e., animals) available for the study was not great enough to make concise statements due to the experimental conditions. There were, however, significant differences observed among the groups that should have great applicability in future research or field conditions. In this way, the data were analyzed using the SAS System for Windows program (SAS Institute Inc., Cary, North Carolina, USA). Differences between the treatments were evaluated using the Student *t*-test considering there was normality of the values (Gaussian distribution) and homogeneity of the variances. The significance level used to reject the H0 (null hypothesis) was 5%, that is, for a level of significance less than 0.05; it was considered that statistical differences occurred between the values for the variables evaluated in this study.

3. Results

3.1. Testicular morphology

At 9 months post-treatment, hemi-orchiectomies of the animals in the treatment ($n = 3$) and control ($n = 2$) were performed. The testis of the treated males had a 35% lesser ($P < 0.05$) - weight whereas the testicular volume was less ($P = 0.06$), compared to the

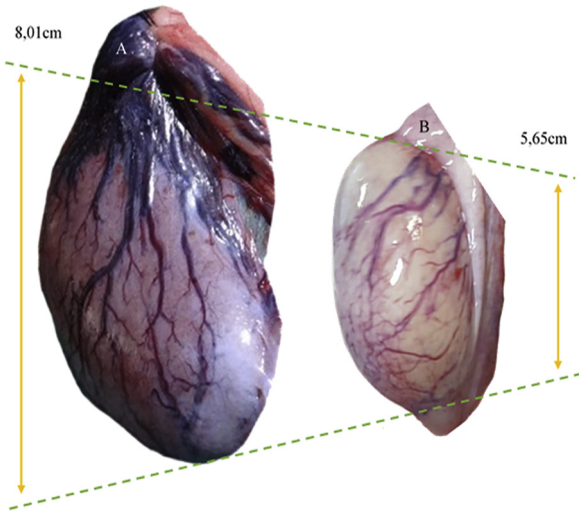

Fig. 2. Comparison Testicular Morphology. A) Testicle – control male, and B) treated male, 270 days post-treatment.

volume of the control group (Fig. 2a and b). Values are reported as the mean ± S.E.M. (Table 1).

3.2. Semen characteristics

Results from the analysis of semen (n = 5) of the animals in the control and treated group indicated there was a difference in spermatozoa concentration between groups ($P < 0.05$; Table 2). Furthermore, there was a greater percentage of total and primary spermatozoa morphology abnormalities in the animals of the treated compared with the control group ($P < 0.05$; Table 2), and there was a tendency for a greater percentage of spermatozoa with minor defects in animals of the treated compared with the control group ($P = 0.06$; Table 2).

3.3. Histological analysis

Histological examination of the testicular parenchyma of animals in the control group indicated there was an abundance in number of Leydig cells and the assessment indicated there was a well-organized spermatogenic process (Fig. 3a), while basal lamina was undamaged as a result of treatment, and the luminal space was minimal (Fig. 3c), with spermatids of each developmental stage being present (Fig. 3e).

Examination of the testicular parenchyma of treated males indicated there were disruptions in the overall cellular organization (Fig. 3b) with a decrease/absence of germinal cells (oligozoospermia) (Fig. 3d). Consistent with this finding was the substantial number of seminiferous tubules (Fig. 3f) with degenerative characteristics of the germinal epithelium for which there were cytoplasmic vacuoles and spermatogonial multinucleate giant cells.

3.4. Alpha male behavior and secondary sexual characteristics

None of the treated males had any loss of the behavioral characteristics that are typical for the alpha male and these males continued to mark the territory of the polygynous group. Agonistic behavior toward other males was maintained after the treatment was imposed on the alpha male. The only variant observed was part of the courtship behavior, whereby males continued to follow females and sniff their genitals but made very few or no attempts to mount.

3.5. Population dynamics

The actual population size of the polygynous group studied increased during the period when there were observations in this

Table 1
Testis weight and volume comparison – control and treated males 270 days post-treatment.

	Treatment group	Control group	*P*
Testicular Weight (g)	16.33 ± 10.68	45.76 ± 8.47	0.0268
Testicular Volume (mL)	19.67 ± 0.80	62.29 ± 11.71	0.0672

Values are mean ± S.E.M; Treatment group ($n = 3$) and control group ($n = 2$).

Table 2
Data for sperm collected from epididymis following unilateral orchiectomy – control and treated males 270 days post-treatment.

Variables	Control Males	Treated Males	*P*
Concentration (Spz. x 10^6/mL)	146.25 ± 3.75	18.38 ± 10.68	0.0028
Total Motility (%)	49.00 ± 19.00	11.00 ± 9.00	0.2124
Progressive Motility (%)	13.50 ± 1.50	0.00	*0.0704*
Rapid (%)	41.50 ± 0.50	9.00 ± 1.00	0.0012
Medium (%)	21.00 ± 5.00	6.00 ± 3.00	0.1439
Slow (%)	28.50 ± 0.50	50.50 ± 7.50	*0.0996*
Static (%)	9.50 ± 5.50	38.50 ± 15.50	0.2199
VAP (average path velocity, μm/s)	53.10 ± 6.70	35.50 ± 7.10	0.2132
VSL (straight-line velocity, μm/s)	27.10 ± 4.80	17.15 ± 0.35	0.1746
VCL (curvilinear velocity, μm/s)	104.20 ± 17.40	65.10 ± 9.50	0.1873
ALH (amplitude of lateral head displacement, μm)	7.85 ± 0.15	0.00	0.0122
BCF (beat cross-frequency, Hz)	26.70 ± 0.10	35.55 ± 10.25	0.5466
STR (straightness, %)	52.00 ± 3.00	48.00 ± 6.00	0.6115
LIN (linearity, %)	27.50 ± 1.50	26.00 ± 3.00	0.6985
Major defects (%)	29.00 ± 1.00	41.33 ± 0.33	0.0007
Minor defects (%)	26.50 ± 0.50	37.67 ± 2.96	*0.0622*
Total defects	55.50 ± 1.50	79.00 ± 2.64	0.0073
Plasmatic membrane integrity (%)	60.50 ± 0.50	59.67 ± 1.20	0.6376
Acrosomal membrane integrity (%)	84.50 ± 2.50	74.67 ± 10.41	0.5211
High mitochondrial activity	64.00 ± 4.00	60.67 ± 1.20	0.3944
Medium mitochondrial activity	24.00 ± 4.00	28.00 ± 1.52	0.3447
Low mitochondrial activity	7.00 ± 0.00	7.00 ± 1.00	1.0000
No mitochondrial activity	5.00 ± 0.00	4.33 ± 0.88	0.5286

Values are mean ± S.E.M; Treatment group (n = 3) and control group (n = 2); *P values indicating tendency.

study between 2014 and 2017. The results from the sensitivity analysis indicated that the most important variables were the number of births and the capacity of the area where the polygynous group resided for nutrient procurement, which were twice as important as sterilization and mortality rates in affecting size of the polygynous group. The 2018 simulation scenario (58 animals) is greater than the values for data from the first year of observation (52 animals). Detailed information on the population dynamics is provided in the supplementary material for this manuscript.

4. Discussion

To our knowledge, this present study is the first report on the use of GonaCon as a method of antifertility in *Hydrochoerus hydrochaeris*. Research findings in the present study confirm that there is a 100% effectiveness of this immunocontraceptive anti-GnRH by inhibiting the fertility of capybara alpha males over the entire study period of 18 months, without any indication that the treatment was becoming ineffective, while there was a sustained maintenance of the alpha characteristics of the dominant male, phenotypically as well as behaviorally. The research of the present study focused on three physiological fertility variables and one observational determinant to ascertain the contraceptive effects of the imposed treatment in capybara. The first and most obvious effect of the treatment was the changes in testicular weight and volume among treated males, compared with control males, indicating there was acute testicular atrophy. This testicular atrophy has also been observed in other studies where there was treatment with GonaCon in various species (D'Occhio et al., 2001; Ghoneim et al., 2012; Wicks et al., 2013).

A second important finding was that the assessment of the semen characteristics confirmed there were similar mean spermatozoa concentrations in intact and fertile (free-ranging) males to the findings in previous studies with the same species (Rodríguez et al., 2012, in supplementary material), Using the reported values as a reference, together with the data from untreated control males in the present study that were considered fertile because of reproductive history and by having a similar mean sperm concentration as those in previous studies, the findings with use of the CASA assay on the semen characteristics indicated there was a marked decrease in the sperm concentration in GonaCon-treated males (literature reference male: 127 ± 59.01 Spz. x 10^6/mL compared with control male: 146.25 ± 3.75 Spz. x 10^6/mL compared with treated male: 18.38 ± 10.68 Spz. x 10^6/mL). The lesser than typical sperm count is suggestive of an infertile individual. Values for additional sperm variables also indicate there are significant differences in sperm motility, damage, and morphology between control and treated males.

The third important aspect of the physiological evidence is based on the histological findings in the present study for which there was comparison of the testicular parenchyma between control and treated males. Extensive histological studies on testicular morphology and spermatogenesis in intact capybara males (Moreira et al., 1997; Paula et al., 1999; Paula, 2002; Costa et al., 2006) served as reference information for the control (intact and fertile) male capybaras in the present study.

Observations of the testicular parenchyma of GonaCon-treated males indicated the cells and structures of parenchyma and seminiferous tubules were abnormal as compared with those in the control animals and those described previously in capybara, with the lumen diameter being greater due to a cessation of spermatogenesis. These results indicate there is a lack of germ cell differentiation throughout all developmental stages, including preleptotene/leptotene (P/L), leptotene (L), pachytene (P), diplotene (D), and zygotene (Z), leading to a decrease in, or total absence of, rounded/elongated spermatids, resulting in oligospermia. This

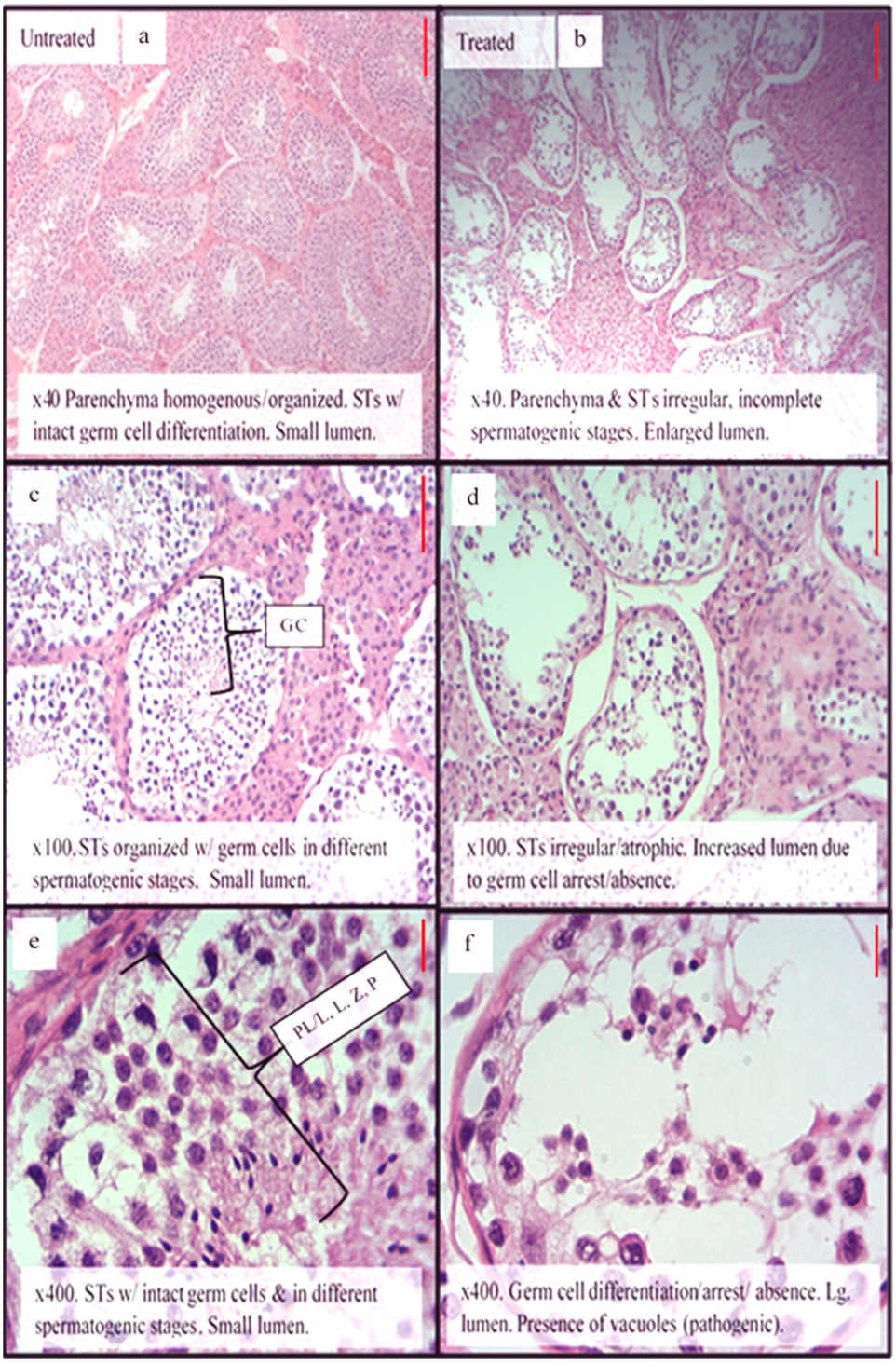

Fig. 3. Direct histological comparison of the testicular parenchyma. Control male (a, c, and e) and treated male (b, d, and f). Image a, b, c, d: Scale bar (red line) 100 μm (a, b, c, and d) and 10 μm (e and f).

cessation of spermatogenesis and testicular degeneration that subsequently progresses until there is testicular atrophy was evident in the treated capybara of the present study. The lumen of seminiferous tubules was enlarged and irregular in shape due to the depletion of spermatids. In the present study, all of the histological findings were substantiated by the lesser sperm concentrations in the semen that was collected, and data obtained using computer-assisted sperm analysis as compared to values for sperm in the males of the control group.

The basal membrane appeared intact, an important fact considering the potential reversibility of the infertility effect. The presence of large numbers of Leydig cells is a species-specific normality (Paula, 2002). Furthermore, additional evidence of the effects on the parenchyma was evident as a result of the presence of multinucleated giant cells, greater numbers and size of the lymphatic space, and the presence of vacuoles. These findings are similar to those from other studies where there was use of an anti-GnRH vaccine in males from a variety of species (Ghoneim et al., 2012; Malmgren et al., 2010; Han et al., 2013).

In regard to the observational determinants, monitoring the population dynamics and comparing the numbers of the polygynous groups using the mathematical models (available in the supplementary materials, Oswaldo and Costa, 2019) was a valuable and effective non-invasive method to measure the treatment effectiveness. The population dynamics that were present as a result of the treatment with GonaCon were more desirable than anticipated when the study was initiated. It is thought that the conception of the offspring during the initial study period occurred prior to immunization or a female was inseminated by a male other than the treatment group's alpha male. The second observational element, which is as important as the successful antifertility effect, is the preservation of the agonist behavior of the alpha male subsequent to treatment with GonaCon, including the secondary sexual characteristics (Rosenfield and Schilbach Pizzutto, 2019). Any alterations to the alpha males' phenotype or behavioral traits would eventually lead to the loss in the dominant position, consequently undoing any capybara population control effort as a result of the GonaCon treatment.

Besides a local injection site reaction and the formation of a small abscess, which is expected because of the immune response to the vaccine's adjuvant, no other adverse effects were observed. This type of response has also been documented in most of the prior studies conducted using GonaCon.

Alternative measures such as surgical procedures, for example, castration and vasectomy, are 100% effective and in certain situations, the use of the approaches is warranted, but the use of these approaches are not feasible on a larger scale or for in-the-field applications. Furthermore, and perhaps even detrimental to the polygamous group's stability and overall goal of population control, the 7 to 14 days of recovery time for a male from surgical procedures (in this case post-operation trauma) provides opportunities for untreated rivals to ascend to the alpha male status.

5. Conclusions

Considering the consolidated data, the use of GonaCon results in a satisfactory anti-fertility effect on alpha male capybaras, without any significant adverse effects on secondary sexual characteristics, agonist behavior, or pathological occurrences. The capacity to administer a single dose via remote drug delivery systems makes this immunocontraceptive a valuable non-lethal population control approach for managing synanthropic capybaras. Inhibition of the overall population growth helps to mitigate the conflicts between humans and capybaras. More importantly, regarding public health, this treatment might be an effective strategy for preventing the spread of tick-borne pathogens such as *R. rickettsia* considering the outcomes from the present study.

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Declaration of Competing Interest

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

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Appendix A. Supplementary data

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