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Effect of immunocastration with a gonadotropin-releasing hormone vaccine in a male zebra *Equus quagga boehmi*

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Abstract

Grant's zebra *Equus quagga boehmi* is an equine species commonly bred in zoos under human care. Male zebras are often castrated to prevent a surplus captive zebra population. Gonadotropin-releasing hormone (GnRH) vaccination may pose an alternative to surgical castration. The aim of this study was to evaluate the effects of an anti-GnRH vaccine on testosterone concentrations in a male zebra. A harem of one male and three adult female Grant's zebras were included in the study. The male was immunised with 4 ml (600 µg GnRH protein conjugate) of a commercially available anti-GnRH vaccine (Improvac®) administered on five occasions at an interval of 4 weeks for the first and second dose and 12-week and 6-week intervals for the remaining doses. Faecal metabolites of testosterone were determined weekly for 1 year. In addition, faecal progesterone and estradiol metabolites were measured in one female after parturition. The GnRH vaccine failed to reduce testosterone metabolite concentrations, and in one female, pregnancy was confirmed through faecal progesterone and estradiol metabolite concentrations. Furthermore, fertility of the male was verified by detection of pregnancy in all three females. Therefore, the GnRH vaccine protocol used here was not effective as immunocastration in the treated male zebra. Further investigations should consider a larger number of animals, a higher dose, and four to five injections with an administration interval of 4 weeks.

Background

In general, zebras are seasonally polyoestrous and their gestation period is about 12 months. The breeding season includes the mid-spring and summer months, although in captivity zebra births have been observed throughout the year (Nuñez et al. 2011).

Breeding programmes in zoos are focused on managing sustainable populations of both endangered and non-endangered species, preserving a large and healthy gene pool, avoiding hybridisation of taxa and reducing overpopulation (Hosey et al. 2013; Rodríguez-Guerra and Guillén-Salazar 2012). Castration is the most common surgery performed in the equine species as a contraceptive method (Blodgett 2011). It is also an accepted method in the list of measures to limit undesired reproduction proposed by the European Association of Zoos and Aquaria in its Standards for the Accommodation and Care of Animals in Zoos and Aquaria (EAZA 2022). However, castration is an invasive and irreversible procedure that removes individuals from breeding pools. Immunocastration is a theoretically reversible alternative to castration which, in males, consists of stimulating an immune response against reproductive hormones.

Gonadotropin-releasing hormone (GnRH) is produced in the hypothalamus and regulates the production of luteinising hormone (LH) and follicle-stimulating hormone (FSH) in the

pituitary gland (Ginther 1993). In the male, the production of testosterone in Leydig cells is stimulated by the action of LH and FSH. Testosterone is required for normal spermatogenesis (Ginther 1993). The goal of vaccination of males with GnRH is to stimulate production of antibodies that block the release of the endogenous hormone to cause a decrease in serum testosterone (Janett et al. 2009; Janett et al. 2012; Lueders et al. 2014). Several doses are necessary to maintain an effective concentration of circulating antibodies (Baker et al. 2018). Several species-specific commercial vaccines are available for domestic animals, but these vaccines have not been validated for use in wild animals. The most suitable vaccine and administration regimen needs to be determined for those species.

Immunocastration has been widely used in horses *Equus caballus* (Baker et al. 2018; Janett et al. 2009), domestic cattle *Bos taurus* (Janett et al. 2012), pigs *Sus scrofa* (Kubale et al. 2013), Asian elephant *Elephas maximus* (Lueders et al. 2014), deer *Cervus elaphus* (Miller et al. 2000), giraffes *Giraffa camelopardalis* (Moresco et al. 2022; Schwarzenberger et al. 2022) and dogs *Canis lupus familiaris* (Ajadi and Gazal 2016). The authors could find no reports of immunocastration of zebras.

One effective protocol used in horses includes doses of 200 µg GnRH protein conjugate (Equity®) with an administration interval of 4 weeks between the first and second doses and a booster two months after the second dose (Janett et al. 2009). The protocol used in another study included four doses of 1 mg of GnRH equivalent of the tandem peptide ovalbumin conjugate with an interval of 2 months between the first and second dose and two booster doses at 3 months (Clement et al. 2005). Improvac® is a commercial anti-GnRH vaccine available in Spain for use in pigs. The manufacturer recommends, in males, two doses of 300 µg of GnRH protein conjugate at least 4 weeks apart. In females, booster doses can be added 3 months after the second dose. In a study in horses, two doses of Improvac® were used 4 weeks apart and anti-GnRH antibodies were detected up to 200 days after the first injection (Bailly-Chouriberry et al. 2017).

The purpose of the current study was to control surplus in a captive group of Grant's zebras *Equus quagga boehmi* by following a protocol of immunocastration with a commercial anti-GnRH vaccine (Improvac®, Zoetis Belgium SA, Belgium).

Actions

Study animals

The harem consisted of one stallion (17 years), three females (14–16 years) and three foals (<1 year) housed at Bioparc Valencia, Spain. The zebra exhibit consisted of a naturalised outdoor enclosure of 3798 m2 with vegetation composed mainly of herbaceous plants, which offered a permanent source of grass. Animals had ad libitum access to water and alfalfa hay. The stallion had not previously received any contraceptive treatment. At the beginning of the study all females were pregnant. Faecal samples were taken from the first female (Female 1) after foaling (31 July) to determine progesterone and estradiol concentrations to determine the reproductive cycle phase (oestrus/dioestrus/pregnancy), in order to test the stallion's fertility and the success of immunocastration.

Immunocastration protocol

The stallion was immunised with 4 ml (600 µg GnRH protein conjugate) of Improvac®. Based on the manufacturer's recommendations and studies performed in horses (Bailly-Chouriberry et al. 2017), four doses were administered with a difference of 1 month between the first and second dose followed by boosters after 3 months, 1.5 months and 1.5 months (7 December 2017, and 4 January, 10 April, 31 May, and 25 July 2018). The initial protocol included booster doses at 3 months, but due to an increase in the stallion's sexual behaviour, the interval between the last booster doses was shortened. All injections were successfully administered intramuscularly by dart rifle.

Faecal sample collection

Faecal samples (50 g) were collected every 2–3 days from 13 December 2017 to 13 December 2018 from the stallion and from 31 July 2018 to 15 December 2018 from one of the females after foaling (11 July 2018). Samples were frozen at -20ºC within 2 hr of collection and remained stored until steroid hormone measurement (faecal testosterone metabolite (FTM) in the male and faecal progesterone and estradiol metabolites in the female).

Hormonal analysis

Hormonal analysis (validated for zebras) was carried out at the Faculty of Sciences (Universidad Autónoma de Madrid). Faecal samples were dried at 90ºC for 24 hr, and then dry samples were placed in assay tubes with 2500 µl of phosphate buffer and 2500 µl of methanol (80%) to extract hormone metabolites. Tubes were vortexed for 16 hr. Subsequently, samples were centrifuged at 4000 r.p.m. for 30 min, and the supernatant was removed and stored at -20ºC. Commercial testosterone (T), progesterone (P) and estradiol (E) enzyme immunoassay kits (Demeditec Diagnostics GmbH, Kiel, Germany) were used for quantification according to the manufacturer's instructions. Each sample was tested in duplicate and hormonal concentrations were expressed in ng/g of dry faeces.

Linearity was satisfactory. Thus, parallel displacement curves were obtained for each hormone by comparing serial dilutions of pooled faecal extracts with standard curves. The results showed parallel curves in all cases. Intra-assay coefficients of variation were 10.2% (T), 13.7% (P) and 10.5% (E). Inter-assay coefficients of variation were 9.8%, 6.6% and 4.9%, respectively. The mean recovery of steroids from faecal extracts was T 104.4%, P 101.8% and E 107.8%. The assay sensitivity was T 0.08 ng/ml, P 0.045 ng/ml and E 6.2 pg/ml. Co-measurement of the three assays was excluded based on <0.1% cross-reactivity between hormone assays.

Statistical analysis

FTM concentration was collected serially over time to assess effectiveness of treatment. The treatment was administered to the male zebra (n=1) in a single-subject design. The data obtained were repeated measurements of the same subject over a period of time. For this reason, an interrupted time series analysis was performed. The time series data were divided into five periods corresponding to intervals between the administered doses of the GnRH vaccine. The mean square successive difference test (Zar 2014) was used to see whether there was a trend in the data series. In each period, the null hypothesis was that the sequential variability among FTM concentrations was random and the alternative hypothesis was that the consecutive measures of FTM did not have random variability and were serially correlated. In cases where a trend was found (Periods 2 and 5), the nature of that trend (sign and value of the slope) was analysed by linear regression using SPSS 24.0 (IBM Corporation, New York, USA). A P value of less than 0.05 was considered significant.

Consequences

FTM consecutive measurements were only serially correlated in Periods 2 (C=0.49; gl=40; P=0.0025) and 5 (C=0.387; gl=45; P=0.005). In the other periods, consecutive measurements of FTM presented random variability. A moderate positive correlation was observed in Period 5 (R=0.403; R^2=0.163; P=0.007), but the

correlation was not significant in Period 2 (R=0.239; R^2=0.057; P=0.133) (Figure 1).

Faecal progesterone and estradiol metabolite concentrations from Female 1 show an oestrus period during 12–17 August 2018 (date of estimated conception). Concentrations of these hormones between August and December were compatible with the first months of a pregnancy (Figure 2). On 6 August 2019, this zebra gave birth. The remaining females also became pregnant during the year of the study.

This study presents the attempted immunocastration of a male zebra. Based on the female pregnancy rate (100%) and FTM concentration results, the protocol of GnRH vaccination used for this male zebra was ineffective.

Plasma concentration of testosterone varies with season, increasing to its highest concentrations in the spring and summer months (Dhakal et al. 2011). Faecal testosterone reflects testosterone secretion over a duration of several hours, minimising the circadian fluctuation that occurs in plasma due to pulsatile release of the hormone (Schwarzenberger et al. 1996). Zebras are difficult to handle and blood collection is invasive. Faecal testosterone concentrations in domestic and feral horses closely correlate with plasma testosterone concentration (Khalil et al. 2009; Opałka et al. 2010).

Several studies on immunocastration have shown suppression of testosterone secretion after vaccine administration in horses (Janett et al. 2009), cattle (Janett et al. 2012) and Asian elephants

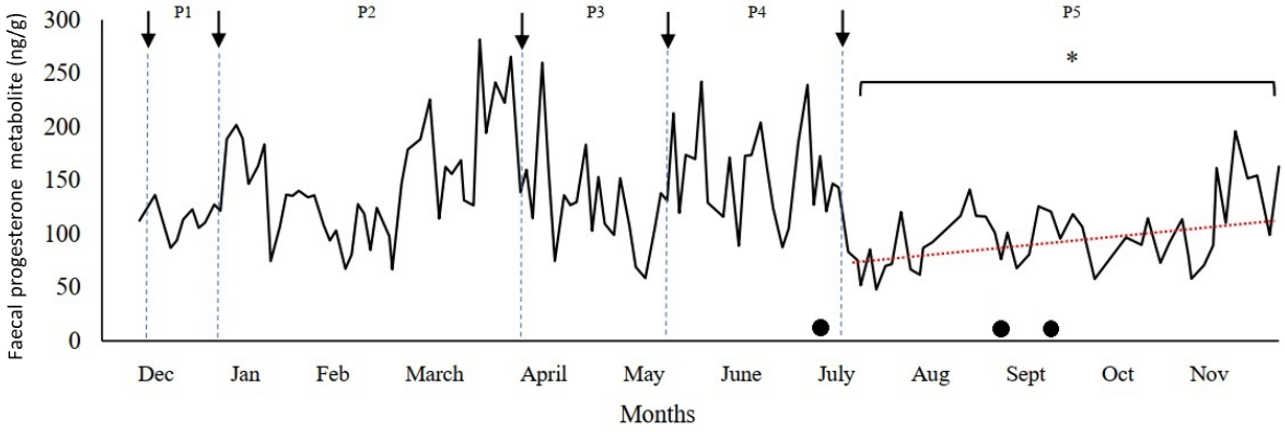

Figure 1. Faecal testosterone metabolite concentration of zebra stallion after vaccination with Improvac® (arrows) from December 2017 to November 2018. The periods between administration of each dose are shown (P1 to P5). The asterisk indicates a significant increase between the fifth dose and the end of the study (Period 5) and the red line indicates the trend line in that period. The females' parturitions are indicated with black circles.

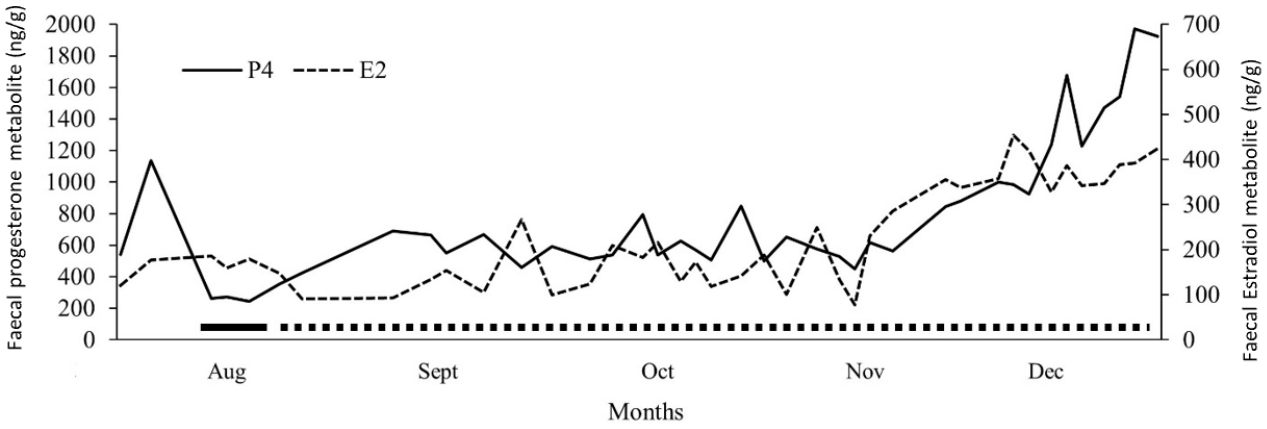

Figure 2. Faecal progesterone (P4) and estradiol (E2) metabolite concentrations of one zebra female (Female 1). The thick line indicates an oestrus period from 12 to 17 August 2018 and the dashed line shows the first months of a pregnancy.

(Lueders et al. 2014). In this study, no decrease in FTM was detected in the periods analysed. The increase in FTM observed in Period 5 could be explained by sexual activity after female parturition. However, in this last period, lower concentrations of FTM were observed, probably reflecting the end of the breeding season.

In Female 1, hormonal concentrations indicated oestrus one month after parturition. In domesticated horses, the first postpartum oestrus (foal heat) begins 7 to 9 days after birth (Ginther 1993). One month after birth, the second postpartum oestrus occurs with higher probability of pregnancy, which is the result of more complete uterine involution (Ginther 1993). Similar findings concerning postpartum oestrus pregnancy rates have been described in zebras (King 1965; Nuñez et al. 2011). The concentrations of progesterone and estradiol observed in Female 1 from August until December were similar to those reported in zebras in their first months of pregnancy (Asa et al. 2001). Furthermore, the zebra gave birth on 6 August 2019, 12 months after the postpartum oestrus, as reflected in her endocrine profile. The other two females were also pregnant. Therefore, the stallion's fertility and ineffectiveness of treatment were confirmed.

Studies on GnRH vaccines in male horses show that its effectiveness is variable, especially in mature stallions with previous sexual experience; for mature stallions the GnRH vaccine did not reduce testosterone concentration nor fertility (Clement et al. 2005; Stout and Colenbrander 2004). The extensive reproductive experience of the male zebra may have influenced the effect of treatment. Another explanation is that the male's immune response was not sufficient (Janett et al. 2009). For a comprehensive evaluation of immunisation success in this male, it would have been beneficial to evaluate anti-GnRH antibody titres. This measurement could not be performed because blood sample collection from this animal required general anaesthesia, which was high risk for the animal. Although Improvac® is a porcine vaccine, it has been used successfully in domestic (Bailly-Chouriberry et al. 2017) and wild (Ponthier et al. 2020) horses, elephants (Lueders et al. 2014), giraffes (Moresco et al. 2022; Schwarzenberger et al. 2022) and dogs (Ajadi and Gazal 2016) with different protocols. A recent study in giraffes has shown that the effective protocol for males of this species consists of four or five injections administered at 4-week intervals. In addition, adult males received more doses of GnRH protein conjugate (750 µg) than young males (300 µg) (Schwarzenberger et al. 2022). Although the protocol used in the current study was based on effective protocols in horses, it could be that the zebras needed more injections and/or a shorter administration interval as shown in giraffes (Moresco et al. 2022; Schwarzenberger et al. 2022). In addition, the male in this study was an adult with reproductive experience and the administered dose may have been insufficient.

In conclusion, the Improvac® protocol used in this study did not have any detrimental effect on testosterone concentration or fertility in this individual male zebra. Further investigation should consider a larger number of animals, a higher dose, and four to five injections with an administration interval of 4 weeks. Furthermore, it would be interesting to include females in future studies.

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