

The use of a GnRH vaccine to suppress mare ovarian activity in a large group of mares under field conditions

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Abstract. The aim of this study was to evaluate the effect of active immunisation against GnRH on ovarian activity and serum progesterone concentrations in a large group of mares (10 control and 55 experimental) under field conditions as a model for wildlife species such as zebra and African elephants. Within the experimental group, mares were subdivided into three age categories: Category 1 (4 years and younger, $n = 26$), Category 2 (4–10 years old, $n = 18$), and Category 3 (≥ 11 years old, $n = 11$). Experimental mares were vaccinated intramuscularly with 2 mL (400 μg) of the GnRH vaccine Improvac (Pfizer Animal Health, Sandton, South Africa). Control mares received the same amount of saline solution. The vaccinations were repeated 35 days later. The ovaries and reproductive tracts of each mare were examined by means of rectal palpation and ultrasonography on Days 0, 35 and 70. Blood was collected weekly for determination of serum progesterone concentration until Day 175. On Day 35 after primary vaccination all of the control mares and 14.5% of the experimental mares showed evidence of ovarian activity on the basis of clinical examination and serum progesterone concentration. On Day 70, all control mares and none of the experimental mares showed evidence of cyclic activity. No age-related effect within treatment groups was found. The serum progesterone concentration indicated that all experimental mares remained in anoestrus until Day 175. Five of the control mares fell pregnant between Days 35 and 70. The five non-pregnant control mares continued to cycle until the end of the observation period. Having achieved such promising results in this trial we now plan to test the GnRH vaccine in Burchell's zebra mares and African elephant cows.

Introduction

Contraception as a means of population management of captive wildlife species is well established. Methods that have been used include separation of sexes, surgical techniques and steroid hormone implants. The safety, practicality and reversibility of methods have enjoyed more attention in recent years, particularly where endangered and rare species are involved. Because of developments during the last 15 or so years it has become possible to successfully apply some methods for the management of free-ranging wildlife populations. Typical examples of these are the use of the porcine zona pellucida (pZP) vaccine to control reproduction in wild horses (Kirkpatrick *et al.* 1982, 1990; Kirkpatrick and Turner 2002) and white-tailed deer (Naugle *et al.* 2002; Rutberg *et al.* 2004).

In mares, the control of reproductive activity is a vital management aspect from both a fertility and behavioural perspective. Anecdotally, oestrous behaviour may negatively influence the performance ability of mares engaged in work and competition events (Elhay *et al.* 2007).

In the past, fertility control and the suppression of aggressive and sexual behaviour in horses was successfully managed by administration of progesterone or progestagens, in oral or injectable formulation, to simulate dioestrus. The high costs and the frequent administration associated with the administration of

progesterone or progestagens and the short duration of action made their use impractical. A potential negative effect on future reproduction after repeated administration of progesterone or progestagens in prepubertal fillies has also been described (Skelton *et al.* 1991).

The major alternative to pharmacological manipulation for long-term control of reproductive cyclicity and behaviour involves the surgical removal of both ovaries (ovariectomy). Ovariectomy is invasive and also associated with various complications. After this procedure, some mares may still show signs associated with oestrous behaviour (Ginther 1979; Asa *et al.* 1980; Hooper *et al.* 1993). The factors of cost, associated trauma, potential postoperative complications, production setbacks and the irreversible loss of breeding potential make this method difficult to implement (D'Occhio 1993). Ovariectomy is therefore considered undesirable unless circumstances are exceptional.

Recent studies on the use of gonadotropin-releasing hormone (GnRH) vaccination for suppression of fertility, aggressive and sexual behaviour have shown promising results. Vaccination entails the administration of a modified form of the GnRH hormone in order to stimulate the production of anti-GnRH antibodies. These antibodies then bind to the endogenous GnRH and inhibit the natural binding of this molecule to its

receptors on the pituitary gonadotropes. This results in suppression of secretion of both follicle-stimulating hormone and luteinising hormone at the level of the pituitary.

Immunocastration by means of GnRH vaccination as an alternative to surgical castration to control unwanted male characteristics and behaviour has been studied in male animals of different species, including bulls (Jago *et al.* 1997), stallions (Malmgren *et al.* 2001; Stout 2001; Turkstra *et al.* 2005; Burger *et al.* 2006), Chinese pigs (Zeng *et al.* 2001), lambs (Janett *et al.* 2003), rats, dogs and rams (Ferro *et al.* 2004), African elephants (Bertschinger *et al.* 2004; Delsink *et al.* 2004), cats (Levy *et al.* 2004; Robbins *et al.* 2004), puppies (Jung *et al.* 2005) and feral swine (Killian *et al.* 2006).

The use of the commercially available GnRH vaccine (Improvac, Pfizer Laboratories, Sandton, South Africa) in boars eliminates boar taint and enhances growth performance in uncastrated male pigs (Dunsha *et al.* 2001; Chumkam and Ravungsook 2003).

In stallions and colts the semen quality of animals vaccinated with a GnRH vaccine deteriorated but the production of semen was never completely suppressed (Dowsett *et al.* 1996; Malmgren *et al.* 2001; Turkstra *et al.* 2005; Burger *et al.* 2006). The shedding status of stallions infected with equine viral arteritis was also converted to non-shedder status in vaccinated stallions (Burger *et al.* 2006).

The effect of GnRH vaccination in female animals of various species (including deer, wild rats and bison) has also been reported. With the use of an early form of a GnRH vaccine it appeared that GnRH vaccination is not an effective means of contraception in deer (Becker *et al.* 1999). However, a larger and more comprehensive study on immunocontraception of white-tailed deer (Miller *et al.* 2000) showed immunocontraception of deer to be effective. Female white-tailed deer treated with GnRH immunocontraceptive vaccine showed fewer oestrus cycles per female than either females treated with a porcine *zona pellucida* (PZP) vaccine or an untreated control group (Curtis *et al.* 2001). In wild rats, GnRH vaccine was found to be more effective than mouse *zona pellucida* peptide vaccine (Miller *et al.* 1997). Finally, a single dose of GnRH vaccine prevented pregnancy in female bison for ≥ 1 year (Miller *et al.* 2004).

In mares, the few studies to date report a successful but variable effect on suppression of ovarian activity and corresponding prevention of oestrous behaviour in adult mares. These studies have, in most instances, been limited by relatively small numbers of mares: in 10 mares (≥ 2.5 years of age) (Garza *et al.* 1986), in six mares (2 years of age) (Tshewang *et al.* 1997), in four mares (age range: 6–14 years) (Dalin *et al.* 2002), in seven mares (Stout *et al.* 2003), in 18 mares (age range: 3–17 years) (Imboden *et al.* 2006). In only one study was a relatively large population of 48 mares (age range: 3–12 years) studied (Elhay *et al.* 2007). In addition, an effect of mare age on treatment response has been reported in some studies (Dalin *et al.* 2002; Stout *et al.* 2003), with significant injection-site reactions observed in older mares (Stout *et al.* 2003).

The aim of the present study was to investigate the effects of active immunisation against GnRH on reproductive cyclicality in a large group of mares of different age categories and to monitor the effect of vaccination and mare age on injection-site reactions. A secondary aim of the study was to use the domestic mare study as a

model for contraception of zebra mares and African elephant cows.

Materials and methods

Experimental design

A total of 65 mares, of either barren or maiden reproductive status, of various horse breeds and between 3 and 17 years of age from the Mounted Unit of the South African Police Services were used for this trial. The trial was approved by the Animal Use and Care Committee of the Faculty of Veterinary Science of the University of Pretoria (V068/05). The mares were housed in outdoor paddocks or extensive pastures for the duration of the trial. They were assigned to either a control ($n=10$) or an experimental ($n=55$) group. For management purposes, all experimental mares were excluded from breeding attempts whereas certain control mares were later assigned for breeding. Within the experimental group, mares were subdivided into one of three age categories: Category 1 (4 years and younger, $n=26$), Category 2 (5–10 years old, $n=18$), and Category 3 (≥ 11 years old, $n=11$).

GnRH immunisation

On Day 0, mares from all three age categories of the experimental group were injected with 2 mL Improvac (400 μg RnRF-protein conjugate) (Pfizer Animal Health, Sandton, South Africa) intramuscularly into the left gluteus muscle, whereas all mares from the control group received 2 mL of a sterile saline solution using a 20-gauge, 1 $\frac{1}{2}$ " needle (Terumo Corporation, Tokyo, Japan). The subsequent booster vaccination (2 mL) was administered into the right gluteus muscle 35 days after the primary vaccination (J. F. Kirkpatrick, pers. comm.).

Transrectal monitoring of the reproductive tract

On Day 0, all mares were examined by means of transrectal palpation and ultrasound of the internal genitalia using a 5-MHz linear array probe (Aloka Echocamera SSD-500, Aloka Mitakashi, Tokyo, Japan) to establish their reproductive status. All clinical findings were recorded on a standardised data-capture form.

Ovarian activity was described using mean ovarian volume. The length, height and width of the left and right ovaries were estimated on transrectal palpation for each mare. These were used to calculate the ovarian volume using the prolate ellipsoid formula ($\text{length} \times \text{height} \times \text{width} \times 0.523$) (Pavlik *et al.* 2000). The mean ovarian volume for each mare was calculated as the average of the left and right ovarian volume. The diameters of all ultrasonographically visible follicles ≥ 2 cm and the presence and number of *corpora lutea* (CLs) were also recorded.

The dimensions of the uterine horns and body and cervix were also estimated on transrectal palpation and their palpable tone was scored. The observed uterine oedema patterns were scored and the presence of intraluminal uterine fluid and other ultrasonographically visible abnormalities were recorded. A subjective scoring system was used to grade the degree of observed endometrial oedema to reflect changes in circulating oestrogen and progesterone concentrations that define oestrus status (Hayes *et al.* 1985): 0 (no oedema with a homogeneous echo texture), 1 (the least amount of detectable uterine oedema),

2 (moderate amount of oedema throughout), and 3 (most obvious oedema throughout the whole uterus, typical 'wagon-wheel' appearance). The gynaecological data collection was repeated on Days 35 and 70.

Blood sampling

Blood samples were collected by means of jugular venipuncture commencing on Day 0 (6 December), approximately the midway point of the Southern Hemisphere physiological breeding season, and repeated at weekly intervals until Day 175 (29 May), which is approximately the onset of the non-ovulatory season. The blood samples were collected in plain Vacutainer tubes (BD Vacutainer Systems, Plymouth, UK) and left to allow clotting. Serum was separated by centrifugation (4000g, 10 min) on the same day as sample collection. The labelled samples were stored at -20°C until assayed for serum progesterone concentration.

Serum progesterone assay

Assay for serum progesterone concentration were conducted by means of Radioimmunoassay (Coat-a-Count, Diagnostic Products Corp, Los Angeles, CA, USA).

Injection site reactions

Mares were observed daily in their enclosures by the same operator for one week after the vaccinations for injection-site reactions. Reactions at the injection site were scored as follows: 0, no visible reaction; 1, visible swelling; and 2, visible swelling accompanied by lameness.

Statistical analysis

All data were analysed using Systat 12 (Systat Software Inc., San Jose, CA, USA), using an analysis of variance (ANOVA) of treatment and age on clinical variables for general linear models to assess the level of significance. Significance was set at $P < 0.05$

Results

Transrectal monitoring of the reproductive tract

On Day 0, all control and experimental mares showed clinical evidence of cyclic reproductive activity on clinical examination. On Day 35 after the primary vaccination, all control mares and only 8 of 55 (14.5%) experimental mares showed evidence of ovarian activity on clinical examination. On Day 70 after the primary vaccination, all control mares and none (0%) of the 55 experimental mares showed evidence of ovarian activity on clinical examination. There was no difference between the volumes of the left and right ovaries within individual mares and therefore the mean ovarian volume of each animal at each examination was subjected to further statistical analysis. The mean ovarian volumes of both groups of mares on the days of primary (Day 0) and booster (Days 35) vaccinations and on Day 70 of the trial are shown in Fig. 1.

There was a significant treatment effect on ovarian volume between the control and experimental mares; no age effect was seen within the experimental groups. All clinical variables of the reproductive tract observations recorded are summarised in Table 1.

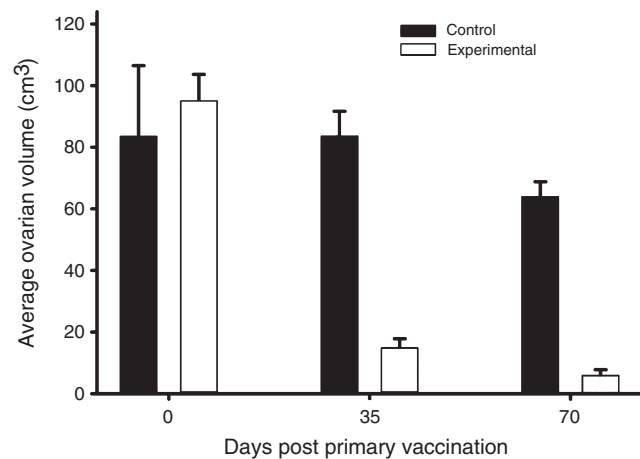


Fig. 1. Mean volume (\pm s.e.) of the ovaries in control and experimental groups on Day 0 (day of first vaccination), Day 35 (day of second vaccination) and Day 70.

Serum progesterone concentration (SPC)

The serum progesterone concentrations of the control and experimental groups over the 175-day observation period are shown in Fig. 2. Within the experimental group there was no effect of age on SPC ($P=0.452$) whereas between the two treatment groups there was a significant difference ($P < 0.001$). Five of the control mares were mated and fell pregnant between Days 35 and 70. The remaining five control mares continued to cycle until the end of the observation period (Day 175).

Injection-site reactions

The control mares showed no injection-site reactions to the intramuscular saline injections. Table 2 shows the side effects due to GnRH vaccination in experimental mares. The side effects were transient by nature and by Day 6 were no longer visible. No age-related effect was observed.

Discussion

The effects of GnRH vaccination on a large group of experimental mares on both the reproductive tract (Fig. 1, Table 1) and SPC (Fig. 2) were observed. These effects were shown to be rapid in onset and statistically significant.

Thirty-five days after the primary vaccination, only eight (14.5%) of the experimental mares showed evidence of cyclic ovarian activity. By Day 70 (35 days after the booster vaccination) all experimental mares showed suppressed ovarian activity. For this trial cyclic inactivity was characterised by the absence of any follicle >2 cm in diameter, no visible CL and SPC <1 nmol/L. This effect was significant (and bilateral, as described by Imboden *et al.* 2006), with a marked decrease in the mean ovarian volumes as well as in concurrently observed changes to the dependant variables characterising ovarian structures and the tubular genitalia (cervix and uterus). The ovaries were uniformly small and apparently inactive with follicular atresia, similar to seasonally anoestrus mares (Dalin *et al.* 2002; Elhay *et al.* 2007). The tubular genitalia also showed atrophy typical of seasonal anoestrus. Two GnRH vaccinations (Improvac)

Table 1. The number of control and vaccinated mares allocated by clinical findings at the time of examinations
 C, control mares; A1, Age Category 1 (≤ 4 years); A2, Age Category 2 (5–10 years); A3, Age Category 3 (≥ 11 years)

Clinical variable category	Day 0				Day 35				Day 70			
	C (n=10)	A1 (n=26)	A2 (n=18)	A3 (n=11)	C (n=10)	A1 (n=26)	A2 (n=18)	A3 (n=11)	C (n=10)	A1 (n=26)	A2 (n=18)	A3 (n=11)
Uterine tone												
0	4	9	9	4	3	21	13	8	1	26	16	8
1	6	11	9	5	4	4	2	2	4	0	1	3
2	0	6	0	2	3	1	3	1	4	0	1	0
3	0	0	0	0	0	0	0	0	1	0	0	0
Cervix tone												
0	2	8	2	2	1	21	12	8	1	26	16	8
1	8	12	8	7	4	4	5	2	4	0	2	3
2	0	6	8	2	4	1	1	1	4	0	0	0
3	0	0	0	0	1	0	0	0	1	0	0	0
Uterine oedema ^{A,B}												
0	4	12	11	5	6	26	17	10	1	26	18	11
1	3	8	5	4	3	0	1	1	2	0	0	0
2	2	2	2	2	1	0	1	0	1	0	0	0
3	1	2	0	0	0	0	0	0	1	0	0	0
Follicle >2 cm												
Yes	9	20	8	5	9	2	0	1	8	0	0	0
No	1	6	10	6	1	24	18	10	2	26	18	11
CL present												
Yes	8	21	13	11	5	2	1	2	9	0	0	0
No	2	5	5	0	5	24	17	9	1	26	18	11

^ATwo datasets were missing for uterine oedema with examination at Day 0, Age Category A1.

^BFive control mares were diagnosed pregnant and uterine oedema score for Day 70 of the five control mares not scored.

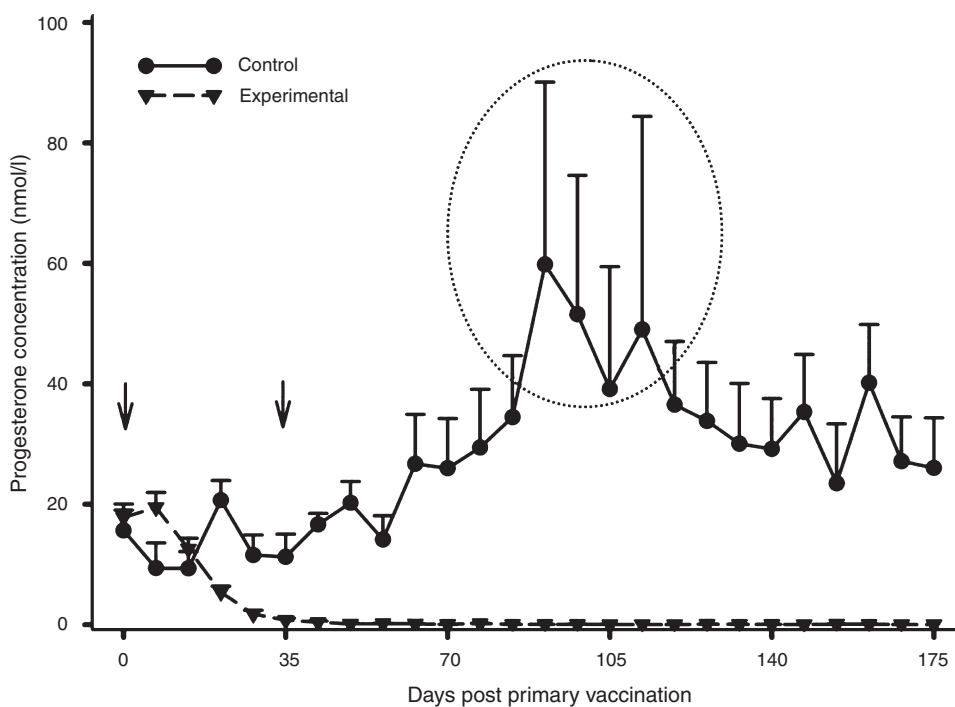


Fig. 2. Progesterone concentrations (\pm s.e.) of control and experimental mares. Arrows indicate times of vaccinations. The encircled data points reflect raised mean SPC obtained from the control mares because five mares were in the early stages of pregnancy.

Table 2. Injection-site reaction scores (number and percentage) after GnRH vaccination in experimental mares ($n = 55$)

Age category	Score=0			Score=1			Score=2		
	1	2	3	1	2	3	1	2	3
Primary vaccination (n)	26	18	11	0	0	0	0	0	0
(%)	(100)	(100)	(100)	(0)	(0)	(0)	(0)	(0)	(0)
Booster vaccination (n)	20	17	10	4	1	1	2	0	0
(%)	(76.9)	(94.4)	(90.9)	(15.4)	(5.6)	(9.1)	(7.7)	(0)	(0)

35 days apart apparently resulted in the effective downregulation of ovarian activity in all 55 experimental mares.

The small percentage of mares that still showed evidence of ovarian activity and progesterone secretion after the initial vaccination is probably due to individual variation in cyclic status at the time of initial vaccination. The eight mares (14.5%) that were still cyclic 35 days after the initial vaccination were all in the luteal phase at the time of the vaccination (on the basis of both clinical findings and SPC values of >1 nmol/L). This supports the findings that the stage of oestrous cycle and nature of ovarian structures at first administration of vaccine is of importance in terms of the observed response (Tshewang *et al.* 1997; Dalin *et al.* 2002; Imboden *et al.* 2006).

The SPC was shown to provide a reliable indication of ovarian activity for both group and individual monitoring of the response to GnRH vaccination of a relatively large number of mares. The SPC clearly characterises the effect of GnRH vaccination on the ovarian activity of the mares. Following the primary vaccination, eight mares still showed signs of ovarian activity, whereas after the second vaccination no luteal activity was evident. Baseline SPC persisted until the end of the observation period on Day 175. Once again, there was no age effect within the experimental group, contrary to the findings of Tshewang *et al.* (1997) and Dalin *et al.* (2002). On Days 42, 49 and 56 there were 3, 1 and 1 mares, respectively, with SPC >1 nmol/L. Thereafter, and until the end of the observation period, SPC of all experimental mares remained <1 nmol/L. This is well below the threshold value of 6 nmol/L quoted by Elhay *et al.* (2007). In comparison, all five non-pregnant control mares showed SPC indicative of ovarian cyclicity.

Five of the control mares became pregnant during the course of the observation period. This would explain the large increase in standard error obtained between Days 80 and 110. While the non-pregnant control mares continued to cycle until the end of the observation period (Day 175), none of the experimental mares resumed cyclicity. This probably reflects antibody titres that were still sufficiently high to neutralise endogenous GnRH. As a result, insufficient gonadotropins were available to resume normal ovarian activity. The relatively late stage of the physiological equine breeding season may also have contributed to this observation.

The efficacy achieved with the Improvac GnRH vaccine in our trial was greater than that stated in earlier reports (Garza *et al.* 1986; Tshewang *et al.* 1997; Stout *et al.* 2003). Likely explanations for this are improvements in the antigen and adjuvant used in the final vaccine formulation that evoke a better, more consistent and longer-lasting immune response.

The details of the Improvac vaccine are unknown to the authors as they constitute proprietary information with a single exception that the antigen is a RnRF-protein conjugate. Equity (Pfizer Animal Health, West Ryde, NSW, Australia) is manufactured by the same company and consists of a GnRH peptide conjugated to a protein carrier combined with an immunostimulating complex consisting of Saponin Quil A, cholesterol and dipalmitoylphosphatidyl choline as adjuvant (Elhay *et al.* 2007). The results of our trial to date, however, indicate better and more consistent results than those reported with Equity.

No adverse effects were observed in the experimental mares after the initial vaccination, which is consistent with the findings described by Dalin *et al.* (2002), but contrary to the findings of Imboden *et al.* (2006), where injection-site reactions occurred after the initial vaccination. The few animals displaying injection-site reactions during this study were observed only after the booster vaccination. All adverse reactions were found to be transient and mild, and by Day 6 were no longer visible. This is in contrast to the findings of Imboden *et al.* (2006), where the adverse effects to the vaccination with Improvac were found to be quite severe. This can possibly be due to the fact that in the present study all injections were administered intramuscularly into the gluteal muscles (J. F. Kirkpatrick, pers. comm.) and not into the neck, as described by Imboden *et al.* (2006).

This study showed a close relationship between the observed clinical response and SPC within a large group of mares. This finding supports the conclusion that SPC provides an effective method for monitoring the response to GnRH vaccination in horse mares of all ages.

On the basis of the results of our trial in mares, Improvac is a promising prospect for the contraception of zebra, African elephants and perhaps other wildlife species. The vaccine is freely available, cheap, stored at 4°C and no further preparation (e.g. mixing of antigen and adjuvant) is required before injection. Side effects at the chosen site of injection were minimal. Although of a slightly viscous nature, the vaccine has been successfully administered remotely via darting in African elephant bulls with both Dan Inject (Børkop, Denmark) and Pneu-Dart (WilliamSPORT, PA, USA) darts (H. Bertschinger, pers. comm.). Reliable reversibility of anoestrus induced by Improvac has yet to be investigated. Imboden *et al.* (2006) were unable to establish this because the mares were no longer accessible after the initial phase of the trial. Elhay *et al.* (2007) established a 100% return to folliculogenesis 8–28 weeks after the second vaccine with Equity. The mares in the present study will continue to be monitored during the 2007–08 breeding season and the data regarding reversibility of Improvac contraception will

become available in the near future. A likely advantage of a GnRH vaccine used for contraception of wildlife species such as African elephant and zebra is induced anoestrus. The females of species like African elephants and zebra, once they have reached reproduction age, spend most of their lives in a state of anoestrus. African elephants have an average intercalving interval of four years. Of this, 22 months is accounted for by pregnancy and approximately two years by lactation anoestrus (Brown *et al.* 2004). This means that cows usually conceive during the first oestrus once cycling resumes. Thus, an elephant cow probably comes into oestrus approximately only once every four years. The use of pZP immunocontraception in feral horses (Kirkpatrick *et al.* 1990) and African elephants (Delsink *et al.* 2006) causes infertility by interfering with fertilisation and the immunised females continue to exhibit oestrous cycles. The authors of the present study hypothesise that pZP is more likely to result in behavioural and social disturbance because individuals in herds are continually attracting males as a result of coming into heat. Comparative studies are needed to test this hypothesis.

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