Multi-year fertility reduction in free-roaming feral horses with single injection immunocontraceptive formulations

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Running Title: multi-year fertility reduction

Abstract

Context. Contraception is increasingly used as a management technique to reduce fertility in wildlife populations, but the feasibility of contraceptive formulations has been limited until recently as they have required multiple treatments to achieve prolonged infertility.

Aims. We tested the efficacy and evaluated potential side effects of two contraceptive formulations, a porcine zona pellucida (PZP) formulation, SpayVac®, and a gonadotrophin releasing hormone (GnRH) formulation GonaCon-B™, in a population of free-roaming feral horses (*Equus caballus*). Both formulations were developed to provide several years of infertility with one injection.

Methods. Females were treated in June 2005 with either GonaCon-B™ (n=24), SpayVac® (n=20), adjuvant only (n=22), or received no injection (n=18). Females were monitored for fertility status year round for three years post treatment.

Key results. Both contraceptive treatments significantly reduced fertility for three years. Fertility rates for GonaCon-B™ mares were 39%, 42% and 31% respectively and 37%, 50%, and 44% for SpayVac® mares. During the same seasons, 61%, 67% and 76% of control females were fertile. We found no significant effects from contraceptive treatment on the sex ratio of foals, birthing season, or foal survival.

Conclusions. These results demonstrate that both vaccines are capable of significantly reducing fertility for several years without boosters.

Implications. Contraceptive vaccines examined in this study represent a useful tool for the management of feral horses, due to their being efficacious for three years in the absence of booster immunisations.

Additional keywords: field study, GnRH vaccine, immunocontraception, population control, PZP vaccine, side effects, wild horses

Introduction

The overabundance of wildlife is an ongoing problem, with many pest species increasing to numbers that become detrimental to both the environment and other wildlife species (e.g., elephants (*Loxodonta africana*), Whyte *et al*. 1998; white tailed deer (*Odocoileus virginianus*), Warren 1997; feral horses (*Equus caballus*), Wagner 1983; Canadian geese (*Branta canadensis*), Conover and Chasko 1985). Traditionally, populations of overabundant wildlife were controlled by lethal control or permanent removals. However, mortality manipulation can cause increased population growth due to compensatory growth (Garrott and Taylor 1990; Kirkpatrick and Turner 1991), changes to animal movements (Henderson *et al*. 2000) and public objections (Stout *et al*. 1997; Lauber *et al*. 2007). Consequently, non-lethal methods of control, usually involving fertility manipulations, are becoming increasingly advocated when traditional culling methods are impractical or undesirable (Kirkpatrick 2007).

Contraception has become a relatively widely used non-lethal technique to manage overabundant wildlife populations and is usually considered a more humane and politically acceptable long-term approach to population control because animals remain in the wild and most treatments are reversible (Oogjes 1997; Kirkpatrick 2007). In particular, immunocontraception, which uses an animal's own immune system to inhibit fertility, has been tested extensively in many wildlife species to prevent pregnancy, particularly formulations that block oestrous cycling or fertilisation through GnRH immunisation (Miller *et al*. 2000a; Baker *et al*. 2002; Killian *et al*. 2006; Conner *et al*. 2007) or treatment with porcine zona pellucida (PZP) (Kirkpatrick *et al*. 1992; 1996; Turner *et al*. 1996; 1997; Fayrer-Hosken *et al*. 2000; Miller *et al*. 2000b; Turner *et al*. 2001; Delsink *et al*. 2002; Naugle *et al*. 2002; Walter *et al*. 2002; Rutberg *et al*. 2004; Lane *et al*. 2007; Turner *et al.* 2007). While biologically effective, contraceptive formulations also need to be practical and cost-effective for management, preferably using only a single dose.

In the USA, feral horses are federally managed and protected under the Free-Roaming Wild Horse and Burro Protection Act of 1971 and continue to overpopulate rangelands where they can negatively impact grasslands, riparian areas, soils and other wildlife species (Rogers 1990; Beever and Brussard 2000; Levin *et al*. 2002; Beever and Brussard 2004; Zalba and Cozzani 2004; Beever and Herrick 2006). Despite this trend, lethal control is largely unacceptable to the public. Past management techniques have focused on the 'round-up and adopt-out' method, but there have been concerns about the efficacy of these programs and their impact on animal welfare (Kirkpatrick 2007). Contraceptive formulations have been extensively researched in feral horses (PZP; Kirkpatrick *et al*. 1990; Kirkpatrick *et al*. 1996; Turner *et al*. 1997; 2001; 2002; 2007) and show promise for population control that may be more cost effective than removals over the long term (Bartholow 2007). However there is a need for a formulation that lasts for more than one year without the need for boosters or multiple injections in order to decrease the costs and stress associated with multiple gathers.

Concerns have been raised over the potential side-effects of contraceptive treatments (see Nettles 1997), yet there has been little research conducted that addresses side effects in comparison to efficacy studies (Gray and Cameron 2010). Most research on side effects has focused on breeding behaviour and body condition of treated females. For example, several studies have shown the contraceptives alter breeding behaviour of females which results in a lengthened breeding season and later offspring birth dates (McShea *et al.* 1997; Heilmann *et al.* 1998; Miller *et al.* 2000b). However, a study on PZP showed no difference in the month that foals were born to females where contraception was waning (Kirkpatrick and Turner 2003). Several studies have also reported increased body condition of treated females in wild species (McShea *et al.* 1997, Turner and Kirkpatrick 2002). While increases to body condition can be desirable, it could impact offspring sex ratios as females that experience increased condition tend to have more sons (Cameron 2004).

Regardless of treatment, side-effects should be studied on managed species in order to minimise impacts to behaviour, physiology and population health.

We tested efficacy of contraceptive formulations in free-roaming feral horses in the Virginia Range in Nevada. We used two different formulations which were developed to reduce fertility over multiple years following one vaccination. The first formulation, SpayVac®, is a liposome encapsulated porcine zona pellucida (PZP) that acts to prevent pregnancy by blocking fertilisation by targeting the zona pellucida of the ovum. A single dose can last for several years (Brown *et al*. 1997). This contrasts with other PZP formulations that require multiple doses to achieve multiple years of infertility (Turner *et al*. 2001; 2002), although a vaccination that lasts between two and three years has been developed recently (Turner *et al.* 2007). SpayVac® has been successfully used as a single dose, multi-year contraceptive in fallow deer (*Dama dama*) (Fraker *et al*. 2002), white-tailed deer (Hernandez *et al*. 2006), grey seals (*Halichoerus grypus*) (Brown *et al*. 1997), and captive feral horses (Killian *et al*. 2008). The second formulation, GonaCon-B™, is a gonadotrophin-releasing hormone (GnRH) vaccine that consists of synthetic GnRH conjugated with a blue mollusk protein. Once injected, antibodies are produced and bind to endogenous GnRH. The antibody-GnRH complex is too large to diffuse from the blood into the anterior pituitary, resulting in a significant reduction in the production of follicle-stimulating hormone (FSH) and luteinising hormone (LH), thereby preventing ovulation (Miller *et al.* 2008). This formulation has been effective in white-tailed deer (Miller *et al*. 2000a), bison (*Bison bison*) (Miller *et al*. 2004), feral swine (*Sus scrofa*) (Killian *et al*. 2006; Massei *et al*. 2008), and captive feral horses (Killian *et al*. 2008). Both formulations alone would not elicit a large or long-term response, thus AdjuVac™, an oil based adjuvant (a non-specific immune stimulant), was used to increase the immune response. Our main objectives were to test two contraceptive formulations in free-roaming horses to determine 1) the effectiveness of these contraceptives to induce infertility up to three years after administration of a single dose and 2) potential side effects on foal survival, birth sex ratio, and birthing season.

Methods

Study site and animals

The study was conducted in the Virginia Range, southwest of Reno, Nevada. The range is approximately 145,000 hectares of private land, with a small percentage developed for houses and industry. The mountain range consists of sagebrush communities (*Artemesia tridentata*) with groups of pine (*Pinus jeffreyi, Pinus monophylla*) and juniper (*Juniperus osteosperma*) in higher elevations. Other common plant species include bluegrass (*Poa secunda*), rabbitbrush (*Chrysothamnus nauseosus*), bitterbrush (*Purshia tridentata*), and cheatgrass (*Bromus tectorum*). Other than feral horses, other ungulates in the range included mule deer (*Odocoileus hemionus*) and pronghorn (*Antilocapra americana*). The predators in the range were mountain lions (*Puma concolor*), bobcats (*Lynx rufus*) and coyotes (*Canis latrans*).

The population of feral horses is classified as estray by the Nevada Department of Agriculture since they range on private property and are therefore not protected under the Wild Horse and Burro Protection Act. Consequently, they are legally defined as feral livestock, and the Nevada Department of Agriculture is responsible for the management of the population. The population size has fluctuated from over 1200 in 2004 to around 1400 in 2009, and is currently being managed with the use of both removals and contraception. The long term management goal is to decrease population size and stabilise population growth.

Vaccine types and treatment protocol

We selected a treatment site for both ease of capture, the large number of horses present in the valley, and permission from private land owners. Horses were rounded up with a helicopter on June 15th and 16th 2005. Horses were held in purpose-built yards and restrained in a hydraulic squeeze chute for treatment. We treated every female over one year of age with 1 cc intramuscular dose in the left side of the neck in the serratus ventralis muscle with one of four treatments: a) GonaCon-B™ (n = 20), b) SpayVac® (n = 20), c) control consisting of adjuvant only (AdjuVac™) (n = 19), or d) control consisting of no injection (n = 18), but every other capture aspect was equivalent. Both GonaCon-B™ (1000ug of conjugate; NWRC, Fort Collins, CO) and SpayVac® (400ug of conjugate; ImmunoVaccine Technologies (IVT) Halifax, Nova Scotia) were prepared as a water-in-oil emulsion in AdjuVac™ (NWRC).

All females were aged by tooth eruption and wear patterns (after Tutt 1968), examined for overall health status, and were freeze branded for permanent identification on the left side of the rump. A total of 77 females ranging in age from one to 20 years were treated systematically over the two day period allowing for random distribution of treatment across all age categories. All horses were immediately released after treatment. In addition to the 77 mares, a small group of females (n = 7) were treated with GonaCon-B™ (n = 4) or adjuvant only (n = 3) in May of 2005 in a different part of the range. These horses were captured by a feed trap and processed using the same protocol. They were grouped with the rest of the mares in all of the analyses. All handling and treatment procedures were carried out by and under the supervision of veterinarians from the Nevada Department of Agriculture and were approved by the University of Nevada, Reno Animal Care and Use Committee (Protocol # A06/07-47).

Field observations

We monitored mares at least weekly from the ground to determine fertility rates until December 2008. The treatment group to which each mare belonged was not known to the field observer. Mares were judged fertile if a foal was born at any time during the year or if a female was noticeably pregnant, judged conservatively by an enlarged belly and swollen teats. If females were not seen until after the foaling season and no foal was present, they were recorded as unknown and excluded from the analysis. In order to examine impacts of contraception on foals, we compared fertile treated mares (contraceptive failures) to control mares. During field observations, we would record the birth of any foal, the sex of the foal, and foal survival to weaning.

Faecal sampling and steroid hormone assay

To supplement visual observations, we collected faecal samples opportunistically from August to May yearly to determine pregnancy of mares that may have lost a foal before being visibly pregnant. The behaviour of the horses was monitored throughout the year and when a horse defecated during the observation period, the time and horse identity was recorded. The faecal sample was collected once collection would not disturb the horses.

Only samples with 100% certainty of which horse they came from were collected in order to be as conservative as possible. The number of faecal samples collected from an individual ranged from 1-32.

Faecal samples were stored in -20°C freezers until extraction. We used the extraction protocol of Asa *et al*. (2001). Samples were thawed and approximately 0.5 g of wet faecal material was placed in a vial with 5 ml of modified phosphate-saline buffer (Shideler *et al*. 1993) and shaken overnight at room temperature. Samples were centrifuged for one hour and the supernatant was stored at -80°C until the assay was conducted. The remaining faecal material was dried in a 100°C oven overnight and weighed the next day. Hormone values are based on the dry weight of the faecal samples and are presented as ng/g of dried faeces.

Faecal progesterone assays were run at the Saint Louis Zoo and have been used to verify reproductive status of females in many species (Asa *et al*. 1996; Asa *et al*. 2001; Munson *et al*. 2001). We analysed extracted faecal samples for progesterone metabolites with a commercially available radioimmunoassay kit (Progesterone Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The progesterone antibody in this kit cross-reacted with multiple progesterone metabolites: progesterone (100%), 17α-hydroxyprogesterone (3.4%), 5α-pregnan-3,20-dione (9.0%), and 5β-pregnan-3,20-dione (3.2%). We used stripped calf serum to prepare all standards and to dilute all faecal sample extracts. Faecal samples were run against a calibration curve of progesterone from stripped calf serum. Extraction buffer was added to tubes containing standards and calf serum was added to faecal extracts to equalise the matrices of the samples and standards. All samples, standards and quality controls were run in duplicate.

The interassay coefficients of variation were 19%, 23%, 28.5% for low, medium, and high quality control standards for 7 different assays. The assays were validated for faecal extracts by demonstrating parallelism between serial dilutions of faecal samples (from 1:2 to 1:16) to the standard curve. The displacement curves were parallel to the standard curve with correlation coefficients $r^2>0.99$. Recovery of known amounts of hormone added to faecal supernatants (diluted in calf serum) at several different concentrations were 92.3 ± 3%. We biologically verified faecal progesterone samples from 19 mares by comparing their progesterone levels to our observations of their pregnancy and foaling status.

Statistical analysis

We determined the efficacy of contraceptive treatment by comparing the proportion of fertile females in each treatment group to control females across the combined foaling seasons. Females were classified as being either fertile or infertile based on visual monitoring of reproductive status or faecal progesterone levels. If any female was not seen on a regular basis or had no faecal samples collected, they were excluded from the analysis. We used a linear mixed model analysis with restricted maximum likelihood estimation to determine treatment effects on fertility rates. We used individual identification as a random variable repeated across three foaling seasons. A chi-squared test was used to test for differences between foal sex ratios, seasonality of births and foal survival. We defined the foaling season to include April, May and June (Kirkpatrick and Turner 2003). Results are shown as means ± standard errors where appropriate.

Results

Efficacy

At the time of treatment, there was no difference in the ages among all treatment groups ($F_{3,79}$=0.48, P=0.694). There was no difference in fertility rates between control mares and adjuvant only mares across the three years ($F_{1,30}$ = 0.737, P = 0.397; Table 1), and so they were combined for all further analyses. Treatment with contraception significantly reduced fertility for all combined foaling seasons ($F_{2,72}$ = 5.811, P = 0.005). Post hoc comparisons revealed that both contraceptive treatments significantly differed from control mares for all foaling seasons combined (GonaCon-B™: t_{75} = -3.138, P = 0.002, SpayVac®: t_{68} = -2.314, P = 0.024).

We supplemented fertility data with four additional mares that were not seen on a regular basis, but had faecal samples tested for progesterone metabolite levels from October through March. We found two females that were probably not pregnant (both <50 ng/g progesterone) and two females that were probably pregnant (both >1800 ng/g progesterone) (Asa *et al*. 2001). We confirmed our visual observations of fertility status with faecal progesterone levels in 17/19 mares (control: n = 10, treated: n = 7) from faecal samples that ranged from 8-24 samples per female. Pregnant mares had mean faecal progesterone of 2426 ± 228 ng/g and non-pregnant females averaged 105 ± 10 ng/g. The other two mares, neither looked visibly pregnant but had faecal progesterone levels that indicated pregnancy, were one SpayVac® treated mare which lost her foal between March and April 2006 (likely post partum), and one GonaCon-B™ treated mare who lost her foetus in October 2005. The cause of these losses is unknown.

Side effects of treatment

During our monitoring efforts, we found no abscesses or inflammation at the injection sites in the mares observed after treatment. There was no difference in the seasonality of births across the three years between these treated mares and controls ($\chi^2_1 = 0.630$, $P = 0.427$), with the majority of all foals being born in April (46%) and May (46%) (Fig. 1). There was no treatment effect on foal survival ($\chi^2_3 = 0.002$, $P = 0.969$). There was also no difference in the foal sex ratio between treatments across three foaling seasons ($\chi^2_3 = 0.939$, $P = 0.816$), as 48% and 50% of the foals were male in the treated and control mares respectively.

Discussion

As with many wildlife species, free-living horse populations continue to present a management problem, and past management methods are increasingly unfeasible or ineffective. Consequently, contraceptive treatments have become more widely tested to reduce herd numbers and population growth, especially with the development of a contraceptive agent which provides multi-year contraception after a single dose (Turner *et al.* 2007; Killian *et al*. 2008). We show that free-living horse fertility rates were decreased by contraceptive formulations with the use of single injection vaccinations. Both GonaCon-B™ and SpayVac® reduced fertility rates in every foaling season, suggesting that both formulations were successful at preventing pregnancy for multiple years. In addition, both treatments consistently reduced fertility within individual females throughout the three years. Our results indicate that long term contraception of free-roaming horses is possible with both GonaCon-B™ and SpayVac® and provide viable alternatives for long term population control in feral horses. Furthermore, both GonaCon-B™ and SpayVac® have been shown to be effective in several other wildlife species (Brown et al. 1997; Miller et al. 2000a; Fraker et al. 2002; Miller et al. 2004; Hernandez et al. 2006, Killian et al. 2006; Massei et al. 2008). While we only report fertility rates three years post treatment, we expect, based on our captive trials (Killian *et al.* 2008), that treated mares will continue to be effectively contracepted for the next two years and we are continuing to monitor these females for the duration of that time.

While we found a reduction in fertility, our efficacy was lower than most previous studies of contraception in free-living horses (Kirkpatrick *et al*. 1990; Turner *et al*. 2002; 2007). Although this may have been due to the treatment method, this seems unlikely since all mares received the full dose while in the squeeze chute. It is likely in the first foaling season (2006) that some mares were already pregnant when we treated them in 2005, thus reducing the appearance of efficacy for the first season because they were already pregnant and these formulations are not supposed to interfere with pregnancy. This would not explain our lower efficacy rates in the second and third season. It is possible that our lower efficacy rates are due to our sampling techniques. We monitored our horses more intensively than other studies by locating horses at least weekly and used highly conservative estimates of pregnancy and foal production since horses may abort foetuses throughout the year or may lose foals shortly after birth (Keiper and Houpt 1984; Lucas *et al*. 1991). If individuals are not closely monitored, these events would be missed and would increase the appearance of contraceptive efficacy and also fertility in control females. In our study, mares that were only seen late in the foaling season without a foal were excluded from the analysis since it was not possible to determine if they had lost their foal or had not foaled at all. The inclusion of these mares would significantly boost our efficacy rates. This suggests that constant monitoring of individuals may give a more accurate representation of fertility rates.

Our lower efficacy rates may result from inter-individual differences in responsiveness to the treatment. We noticed that certain mares consistently responded to the treatment, while others did not (e.g., they were pregnant or foaled). Variation in response to different contraceptive formulations and adjuvants has been demonstrated (Garrott *et al.* 1998, Fayrer-Hosken *et al.* 2002, Frank *et al.* 2005), but the cause of that variation has not been investigated. Killian *et al.* 2008 showed higher efficacy rates in captive feral horses using the same treatments on the same species from the same population we studied. The captive mares had a much better response to the treatment and showed higher levels of fertility reduction for the first two years post treatment [e.g.,100% and 83% for SpayVac® treated mares and 94% and 60% for GonaCon treated mares (Killian *et al.* 2008)]. We hypothesise that this may be due to differences in condition between captive and wild mares since captive mares are all in good body condition and have access to better-quality nutrition, which may increase immune functioning compared to animals in poor condition (Houston et al. 2007).

Impacts on foals

Another limitation of contraceptive treatment is the potential for side effects (Nettles 1997). We investigated how contraceptive treatment could impact foals born to females in which the contraception failed. Feral horse populations differ in the timing of their birthing season, but the majority of foals are born in the spring (Feist and McCullough

1975; Keiper and Houpt 1984; Berger 1986). Foal survival is related to season of birth, as foals are more likely to survive when they are born in the spring than in the winter. Previous studies have shown that PZP treatments can lead to increased oestrous cycling during the nonbreeding seasons (McShea *et al*. 1997; Miller *et al*. 2000b; Curtis *et al*. 2002). These side effects can result in offspring born out of season leading to reduced survival (e.g., white-tailed deer, McShea *et al*. 1997). While we found two foals born in July, a majority of the foals were born in the spring months of April and May to both treated and control females and this is consistent with other feral horse populations (Kirkpatrick and Turner 2003). These data suggest that contraceptive treatment does not significantly impact the timing of foal births or foal survival in feral horses. Contraceptive treatment has been shown to increase body condition of mares (Turner and Kirkpatrick 2002). These changes to body condition can impact the sex of the foal as females that experience an increase in body condition at time of conception have more males (Cameron and Linklater 2007). In our study, we found no evidence that the sex ratio of foals changed due to contraceptive treatment failure. It would be interesting to continue to monitor sex ratios in mares after the contraceptive has ceased to be effective, since mares that have not foaled for several years should be in better body condition (e.g., Turner and Kirkpatrick 2002), which could lead to an increase in male births (e.g. Monard *et al*. 1997; Cameron and Linklater 2000; 2007).

Summary

A current drawback of most contraceptive vaccines is the need for annual inoculations and therefore, the need for multiple gathers. For many wildlife managers, this issue alone makes contraception an impractical option. Fertility control of pest or invasive species has become increasingly important for management, especially when lethal control is unacceptable or impractical. Our findings demonstrate that new contraceptive formulations are able to reduce fertility in females for several years without the need for boosters. We conclude that these formulations would be useful for managing feral horse populations, but also other species that require long term population control because they reduce the costs and stress associated with multiple treatments. We also emphasise the importance of sampling methods when studying efficacy rates in wild populations in order to get an accurate representation of fertility rates in treated and control individuals.

Acknowledgements

This research was funded by grants from the Nevada Arid Rangeland Initiative NOA15353CG, 110615253BS, 140115253BS, 140215253BS, Hatch Grant 13215353BS and a research grant from the Animal Behavior Society. We thank the National Wildlife Research Center and ImmunoVaccine Technologies for providing all of the contraceptive formulations. We are grateful to anonymous land owners for access to their land for both treatment and observations. We thank Mike Holmes for managing the population and assisting with the research. We thank Joan Bauman from the St. Louis Zoo Endocrinology Lab for her valuable guidance with the faecal hormone analysis.

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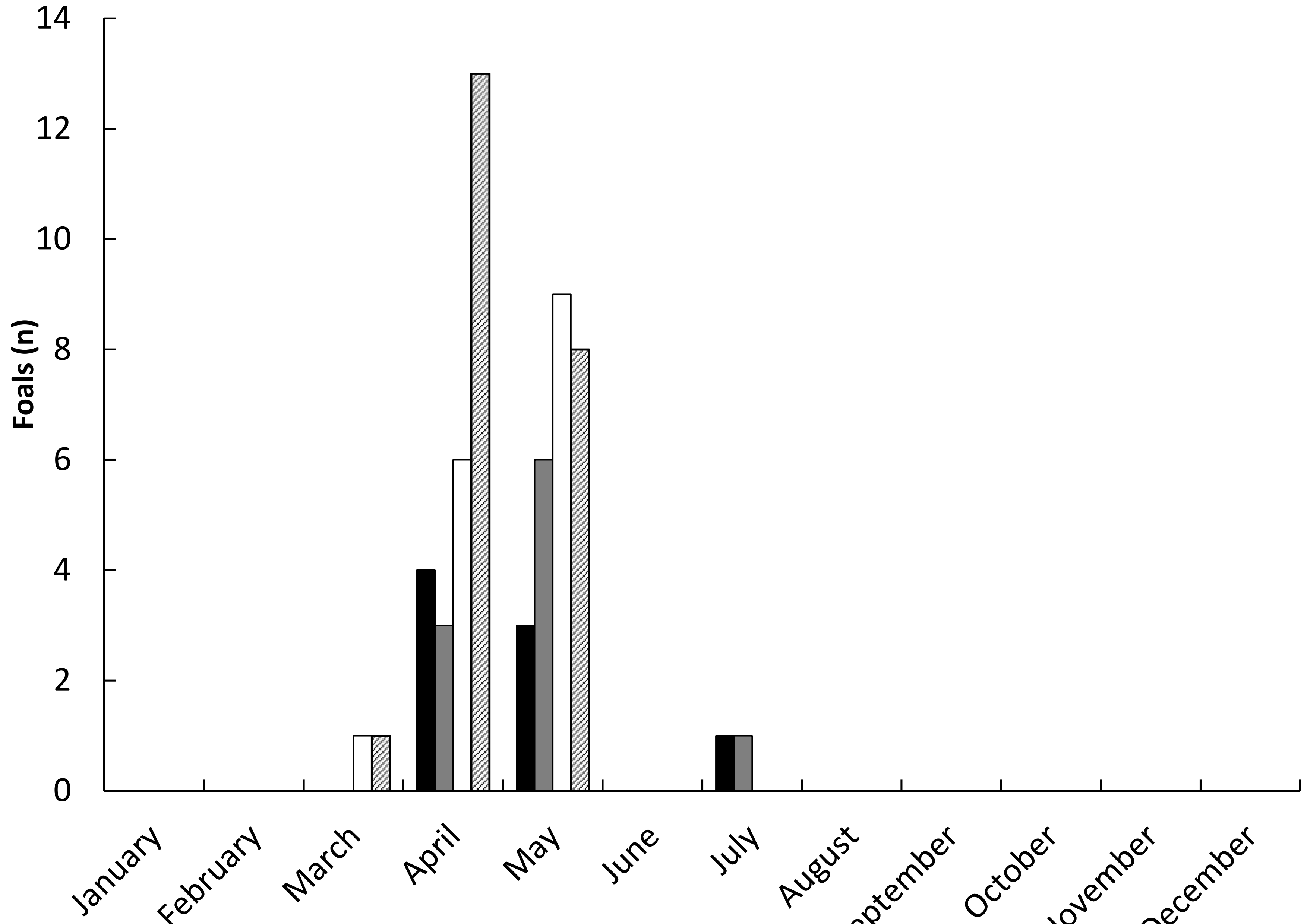
Table 1. Fertility rates of treated and control females for three foaling seasons. Both treatments significantly reduced fertility for the combined three foaling seasons (*P*<0.01). N = the number of mares observed in each season and the % represents the percentage of those mares that were fertile.

Treatment	2006 (*n*)	2007 (*n*)	2008 (*n*)
Control (no injection)	60% (15)	69% (13)	86% (14)
Adjuvant only	63% (16)	65% (20)	67% (15)
SpayVac®	37% (14)	50% (16)	44% (16)
GonaCon-B™	39% (18)	42% (12)	31% (16)

Fig. 1. The number of foals born in each month of the year from control mares (unfilled), adjuvant only mares (striped), SpayVac® mares (gray) and GonaCon-B™ mares (black). Data pooled over three foaling seasons and only from foals with known birth dates.



Foals (n)
14
12
10
8
6
4
2
0
January
February
March
April
May
June
July
August
September
October
November
December