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Synthesis

Twenty years of SpayVac® research: potential implications for regulating feral horse and burro populations in the United States

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Abstract: There are currently >75,000 feral horses (*Equus ferus caballus*) and burros (*E. asinus*) on U.S. public lands, yet the Appropriate Management Level (AML) is set at just under 27,000. Wildlife managers, conservation biologists, and livestock ranchers are concerned about the impacts that these free-ranging horses have on shared rangelands. Immunocontraceptive vaccines may have the greatest potential to regulate horse population numbers once AML is reached; however, the vaccine must have multi-year efficacy to be both technically feasible and cost-effective. Immunocontraception based on porcine zona pellucida (PZP)-specific antigens is highly tissue-specific, targeting the ova, and blocking sperm binding through antibody occupation of ZP receptors on the ova. ZonaStat-H and PZP-22 are PZP-based vaccines; however, their contraceptive efficacy does not last long enough to meet management needs. SpayVac® achieves multi-year efficacy with a single dose, without boosters, because the PZP antigens are encapsulated within liposomes (multi-layered, submicroscopic vesicles), which gradually release PZP glycoproteins to antigen-presenting cells over an extended period of time. We review results from SpayVac trials in horses and other species to deepen our understanding of how the vaccine works and how it may best be applied to regulate free-ranging horse populations at AML. We examine 3 studies in horses, which used different SpayVac formulations and delivery methods, to learn more about the relationship between antibody titers and contraceptive efficacy, as well as potential mechanisms of action (e.g., preferential stimulation of IgG4/7 antibody isotypes). Additional research to explore possible effects of injection site location, antigen purity, role of PZP antibodies, and different formulations (e.g., dose, adjuvant) is needed for efficacious application to free-ranging herds.

Key words: *Equus asinus*, *Equus ferus caballus*, feral horses, immunocontraception, population regulation, porcine zona pellucida (PZP) vaccine, SpayVac

Managing populations of free-ranging horses *(Equus ferus caballus)* and burros *(E. asinus)* is a significant challenge, involving animal-welfare, environmental, sociological, public policy, and economic dimensions. These non-native equids have few native predators, which contributes to high survivorship rates and annual population growth rates of 15–20% (Garrott 2018). Wildlife managers, conservation biologists, and cattle ranchers have concerns about the impacts that free-ranging horses have on rangelands shared by native ungulates and domestic stock (Danvir 2018). Salter and Hudson (1980), in a study of the spatial and foraging relationships among horses, elk (*Cervus canadensis*), mule deer (*Odocoileus hemionus*), moose (*Alces alces*), and cattle (*Bos taurus*), found the greatest potential for resource competition was between horses and cattle, with a 66% overlap in their summer diets, although the contemporaneous spatial overlap was less. Ostermann-Kelm et al. (2009) found that free-ranging horses in a desert environment in California compacted soils, increased erosion, and decreased plant cover.

Free-ranging horse populations that inhabit federal lands in the western United States are protected by the Wild Free-Roaming Horses and Burros Act (WFRHBA) of 1971 (Public Law 92-195), which requires the Bureau of Land Management (BLM) to manage the populations "…to achieve and maintain a thriving natural ecological balance…" By the summer of 2017, the numbers on public lands had increased

to >75,000 (D. Bolstad, BLM, personal communication), far exceeding the Appropriate Management Level (AML), which is currently set at 26,715 (BLM 2018).

Under the original WFRHBA, the BLM was permitted to humanely kill excess horses, and from 1981–1982, 47 horses were euthanized. However, the resulting public outcry effectively ended the further killing of healthy horses (Garrott 2018). Younger horses may be adopted by members of the public, but about half of the 84,741 horses removed between 2000 and 2009 ended up in long-term holding (Vincent 2010). At present, the off-range population is >45,000 horses and costs approximately $50 million per year to maintain (Garrott 2018). Considering the costs of capture and transportation, and a captive lifespan of 15 years (Garrott and Oli 2013), each horse maintained in an off-range facility costs taxpayers close to $25,000. Accommodating the present captive population will have a final cost of approximately $1.25 billion. However, this does not include the additional estimated 50,000 horses currently on the range that exceed the AML.

Contraception has been recognized as a potentially useful approach for controlling the fertility of free-ranging horse populations, thus reducing or possibly even eliminating the need to remove horses from their natal ranges (Kirkpatrick and Rutberg 2001, National Research Council 2013). Contraception efforts for free-ranging populations have focused primarily on females because a single fertile male can inseminate numerous females (Garrott and Siniff 1992). Several contraceptive techniques have been tried in horses, including hormone implants (Plotka et al. 1988), intrauterine devices (Killian et al. 2008), and more recently, permanent non-surgical methods of sterilization that target specific reproductive organs or functions (e.g., equine maternal recognition of pregnancy; Swegen and Aitken 2016, Hall et al. 2017). Surgical sterilization, such as ovariectomy, is highly invasive and less cost-effective as a population management tool. Animal welfare issues are also raised by conducting surgeries under field conditions.

Immunocontraceptive vaccines may have the greatest potential to help the BLM regulate horse populations at the AML; however, a vaccine must have multi-year efficacy to be both technically feasible and cost-effective (National Research Council 2013). Two main classes of contraceptive vaccines have been developed based on gonadotropin-releasing hormone (GnRH) and porcine zona pellucida (PZP) antigens. In this paper, we briefly review these classes of contraceptive vaccines prior to focusing on SpayVac's performance in several species over the past 20 years, and we end with a discussion about future research needed to maximize SpayVac's performance in the field.

GnRH vaccines

The GnRH vaccines have met varying degrees of success in a variety of species. The GnRH is a hormone produced in the hypothalamus, and its effect on reproduction is primarily via the pituitary gland, which secretes additional hormones in response to binding GnRH (e.g., follicle-stimulating hormone, luteinizing hormone). It is these hormones that subsequently affect the gonads, secondary sexual characteristics, and behavior. Serum concentrations of GnRH normally start increasing at the onset of puberty (Wiemann et al. 1989), so age of the animal at the time of GnRH vaccine administration is an important consideration. For example, a juvenile (7-year-old) Asian bull elephant (*Elephas maximus*) that received multiple GnRH boosters over the course of 6 years demonstrated stunted growth in addition to diminished size of reproductive glands and penile atrophy (Lueders et al. 2014).

In 2009, the U.S. Department of Agriculture, National Wildlife Research Center obtained U.S. Environmental Protection Agency (EPA) regulatory approval for the use of GonaCon™ in deer, and a second version was approved for use in horses in 2013. GnRH vaccines typically require boosters and result in suppressed reproduction and reproductive behavior in a variety of species, including horses (Elhay et al. 2007) as well as zebras (*E. zebra*) and African elephants (*Loxodonta africana*; Botha et al. 2008). Baker et al. (2017) found that a single injection of GonaCon in mares (n = 29) resulted in a 36% ($P < 0.047$) and 31% ($P < 0.053$) decrease in foaling compared to controls (n = 28) during years 2 and 3 post-injection, respectively.

Effects of GnRH vaccination on reproduction have been variable by species as well as gender. For example, Valades et al. (2012) found that

Improvac® (GnRH-based vaccine produced overseas) was not able to affect the estrous cycle of female African elephants; yet the same vaccine significantly decreased testicular and accessory sex gland sizes in bull African elephants (Lueders et al. 2017). Importantly, GnRH vaccines may have serious side effects (Kirkpatrick et al. 2011) because GnRH receptors are located in a variety of tissues in addition to reproductive organs, including the nervous system (Lopez et al. 2002), bladder (Bahk et al. 2008), and heart (Skinner et al. 2009). Studies have not focused on these potential side effects.

PZP vaccines

In contrast, immunocontraception based on PZP-specific antigens is highly tissue-specific, targeting primarily the ova, and is thought to block sperm binding through antibody occupation of ZP receptors on the ova (Dunbar and Schwoebel 1988, Skinner et al. 1990, Benoff 1997). When a sperm attaches to the ZP, the acrosome reaction is initiated and fertilization ensues (Bedford 2008). While this is likely the primary mechanism of action for PZP vaccines, other mechanisms have been suggested, including autoimmune oophoritis in dogs (*Canis lupus familiaris*; Brandon et al. 1998) and direct effects on the ovary resulting in an absence of growing follicles in sheep (*Ovis* spp.; Stoops et al. 2006) and horses (Bechert et al. 2013). Zona antibodies may disrupt cellular communication between differentiating follicular cells and the developing oocyte, and this may lead to the destruction of a portion or all of the oocyte pool (Skinner et al. 1984). Most PZP vaccines are made with partially purified porcine ZP, and other follicular proteins could potentially be involved in eliciting ovarian responses in vaccinates (Stoops et al. 2006).

Because PZP is a weak immunogen, a powerful adjuvant is required to stimulate an immune response. The AdjuVac™ adjuvant uses *Mycobacterium avium* in mineral oil (Miller et al. 2004). However, the most commonly used adjuvant at present for many contraceptive vaccines, including SpayVac, is Modified Freund's Adjuvant (MFA), which is made with fractionated cell walls from *Mycobacterium butyricum*, a common soil bacterium that does not produce false-positive tuberculosis test results. Inflammatory responses to vaccines containing Freund's Complete Adjuvant (FCA) are more severe compared to those that accompany AdjuVac (Powers et al. 2007). Lyda et al. (2005) compared PZP antibody titers in horses treated with PZP vaccines formulated with either MFA or FCA and found consistently greater antibody titers in the MFA vaccinated group, although the differences were not significant.

The potential effect of PZP-based immunocontraception on social structure and behavior in herd animals has been explored by Kirkpatrick et al. (1995) and Powell (2000), who reported no differences among vaccinated mares with respect to activity budgets, hierarchy within the herd, or interactions with stallions. However, Nuñez et al. (2010) found that some PZP-treated mares foaled later in the year and suggested that differences in habitat, resource availability, and mare fitness affects their physiological and behavioral responses to PZP contraception, which could ultimately also affect foal survivorship.

ZonaStat is a PZP-based vaccine that requires a booster 3–4 weeks after the initial inoculation and subsequent annual boosters to maintain contraceptive efficacy (Liu et al. 1989). ZonaStat-H was approved by the EPA for use in feral horses and burros in 2012, and ZonaStat-B is a sub-label of the same vaccine approved for use in white-tail deer (*O. virginianus*) in 2017. To extend the duration of efficacy without using boosters, PZP-22 was developed. This product uses timed-release pellets that contain PZP and an adjuvant and are administered by hand injection simultaneously with the primary vaccine dose. It has a reported efficacy of 22 months (Turner et al. 2007). More recently, PZP-22 was delivered by dart injection to a group of bait-trapped mares, and the foaling rate was reduced by 79% compared to controls during the first year but by only 38% in the second year (Carey et al. 2017, Rutberg et al. 2017). This level of performance still does not meet the management needs of the BLM (Roelle et al. 2017).

SpayVac

SpayVac was developed in the early 1990s as a contraceptive vaccine to control the population of grey seals (*Halichoerus grypus*) along the Atlantic coast of Canada. As with other PZP vaccines, SpayVac uses PZP glycoproteins as

Table 1. Percent of grey seals (*Halichoerus grypus*) that were pregnant with and without SpayVac VacciMax treatment on Sable Island, Nova Scotia, January 1992–1997 and 2002.

	Fertility rates during years post-treatment[a]						
Years post-treatment	0	1	2	3	4	5	10
Control (n = 104)	100%	72%	70%	63%	71%	75%	70%
SpayVac (n = 101)	100%	47%	11%	9%	15%	12%	14%
Decreased fertility (%)	N/A	37%	85%	86%	79%	84%	80%

[a] Years 0–5 data from Brown et al. 1997*a*; year 10 data from R. Brown, Immunovaccine, Inc., personal communication.

the antigen along with an adjuvant such as MFA or AdjuVac. Unlike ZonaStat, SpayVac achieves multi-year efficacy with a single dose, without boosters, because the PZP antigens are encapsulated within liposomes, which gradually release PZP glycoproteins to antigen-presenting cells over an extended period of time. Liposomes are multi-lamellar, concentric spheres made up of phospholipid bilayers separated by aqueous compartments and may themselves be considered immunological adjuvants (Allison and Gregoriadis 1974, Shek and Sabiston 1982, Gupta et al. 1993). The result is a longer-lasting, more robust immune response that obviates the need for boosters for several years.

Immunovaccine, Inc. (Halifax, Canada) has developed patented liposome-based formulations that improve the response of the immune system to vaccines. VacciMax™ is a water-in-oil emulsion that has been demonstrated to generate a significantly greater immune response compared to that produced by an alum-adjuvant vaccine (MacDonald et al. 2010). DepoVax® is a novel antigen delivery formulation that undergoes a lyophilization step, with the components resuspended in oil prior to injection, creating a unique formulation that targets the immune system in an active process (Brewer 2018). The DepoVax formulation would have greater utility in the field as a portable vaccine because it can be lyophilized and subsequently reconstituted when needed.

To explore ways to improve SpayVac, formulations have been varied based on different amounts of PZP (ranging from 100–400 μg PZP), type of adjuvant used (FCA, MFA, or AdjuVac), and whether the vaccine incorporated either the VacciMax or DepoVax platform.

Immunovaccine, Inc. produced all SpayVac formulations described in this paper. The PZP was isolated as previously described by Brown et al. (1997*b*) and incorporated in phosphate buffered saline (pH 7.4). Lipids containing lecithin and cholesterol at a ratio of 10:1 (0.2 g lecithin and 0.02 g cholesterol/dose) were added to the PZP solution to form multilamellar liposomes. For the VacciMax platform, the prepared PZP liposomes mixture was combined with MFA to form a water-in-oil emulsion. For the DepoVax platform, the prepared PZP-liposomes mixture was first lyophilized, and then the lyophilized preparation was reconstituted with MFA prior to administration. Bicinchoninic acid-containing protein assays and gel electrophoresis were used to control quality. Standard bioburden testing according to U.S. Pharmacopeia Convention (USP) methods further ensured purity and safety of vaccines.

Results from SpayVac trials in other species can contribute to our understanding of how the vaccine works, and how it might be successfully applied to manage feral horse populations at the AML. Below, we summarize results from trials using different formulations of SpayVac in different species, discuss the application of this information, and consider management implications and future research directions.

SpayVac in grey seals

Every January, grey seals gather in large numbers to give birth, briefly nurse their pups, and mate on Sable Island, which lies off the east coast of Canada (Brown et al. 1997*a*). Following a lactation period of just 2–3 weeks, the females mate and depart the rookery.

It was during this brief period in January 1992 that 101 females were treated with SpayVac VacciMax (100 μg PZP and FCA) and another

104 females served as controls, receiving just FCA and liposomes. All of the treated seals had just given birth and were therefore proven breeders, and because they had been branded during an earlier population study, they were of known age.

Because the seals were inoculated just 1–2 weeks before mating, there would not have been enough time for PZP antibody titers to fully develop, and no significant reduction in fertility was expected during the first year post-treatment (Table 1). During years 2–5 and 10 post-treatment, fertility rates in the control animals ranged between 70–75%, while fertility in the SpayVac-treated animals ranged from 9–15%, which was an 80–85% decrease. The results during the first year post-treatment were intriguing because there was a 37% reduction in the number of pups produced by SpayVac-treated seals.

In seals, development of the fertilized ovum is arrested at the blastocyst stage, and only later is it implanted in the uterus (Davies 1953). Perhaps PZP antibodies attached to the ZP, which surrounds the blastocyst, and subsequently interfered with implantation. Normally, 25–30% of the seals that breed in any given year will not breed in the next (Table 1).

SpayVac in deer

Overabundant populations of white-tailed deer in urban and suburban areas contribute to animal–vehicle collisions, disease transmission, adverse impacts on other wildlife, and damage to vegetation (McShea et al. 1997). In many communities, hunting is not possible or favored, making contraception an appealing option for controlling population size. Because of the lack of an effective contraceptive vaccine, Boulanger et al. (2012) took the extreme step of surgical sterilization. However, because of density-dependent changes in mortality and survivorship, a large proportion (80% or more) of a closed population needs to be captured and treated to affect a decline in numbers (Boulanger et al. 2012). At roughly $1,000 per animal, this process is very expensive and highly invasive (Boulanger et al. 2012). Long-lasting, single-dose immunocontraception offers a more economical and less invasive alternative.

Following the successful trial with seals (Brown et al. 1997*a*), Fraker et al. (2002) conducted a field experiment with a free-ranging population of fallow deer (*Dama dama*) on James Island, British Columbia. Adult females treated with SpayVac VacciMax ($n = 41$) were followed for up to 3 years post-treatment (Table 2). None of the treated females became pregnant compared to 96% of the untreated culled deer that served as controls; however, due to accessibility challenges, only 19, 10, and 5 treated does were examined during years 1, 2, and 3, respectively. The treated does apparently continued to exhibit estrus because they were observed being mounted by antlered males as late as early April (the peak of the rut was late October).

White-tailed deer have been the subject of additional trials. The first of these took place at the Johnson Space Center in Houston, Texas, USA (Hernandez et al. 2006; Locke et al. 2007). Thirty-eight adult does were inoculated with SpayVac VacciMax (Table 2), none of the treated deer gave birth during the first 2 years post-treatment (n = 32 and 20 for years 1 and 2, respectively, due to mortality losses), and only 1 of 8 treated deer observed in year 3 gave birth compared to 78–100% of control does that fawned each year. Vaccination 1 month prior to the breeding season provided enough time for development of antibody titers and control of fertility.

Another study, using captive white-tailed deer in Pennsylvania, USA (Miller et al. 2009), demonstrated contraceptive efficacy of 80–100% for 5 years with a single injection of SpayVac VacciMax (Table 2; 1 doe fawned during years 4 and 5). In the same study, 4 does were treated with an injection of SpayVac DepoVax, and half of these does produced a fawn during year 3 (Table 2).

SpayVac in cats and elephants

SpayVac DepoVax and VacciMax formulations have also been tested in domestic cats (*Felis catus*), where high PZP-antibody titers were reported; however, there was no reduction in litter size (Gorman et al. 2002). Levy et al. (2005) used zona pellucida isolated from cows (*Bos taurus*), cats, ferrets (*Mustela* spp.), dogs, and mink (*M. vison*) in SpayVac VacciMax with no effect on fertility.

Bechert and Fraker (2016) vaccinated 6 captive African elephants with SpayVac (n = 3 with the VacciMax and n = 3 with the DepoVax

Table 2. Percent of fallow (*Dama dama*) and white-tailed (*Odocoileus virginianus*) deer that were pregnant with and without SpayVac treatment.

Deer species	Formulation	Treatment / control groups	% Fertile years post-treatment		
			1	2	3
Fallow[a] in British Columbia	100 μg PZP + FCA + VacciMax	SpayVac (*n* = 41) Control (*n* = 111 average/year)	0 (*n* = 19) 96	0 (*n* = 10) 97	0 (*n* = 5) 97
White-tailed[b,c] in Texas	200 μg PZP + AdjuVac[d] + VacciMax	SpayVac (*n* = 38) Control (*n* = 11 average/year)	0 (*n* = 32) 78	0 (*n* = 20) 78	13 (*n* = 8) 100
White-tailed[e] in Pennsylvania	200 μg PZP + AdjuVac + VacciMax	SpayVac (*n* = 5) Control (*n* = 12 average/year)	0 100	0 100	0 100
	200 μg PZP + AdjuVac + DepoVax	SpayVac (*n* = 4) Control (*n* = 12 average/year)	0 100	0 100	50 100

[a] Fraker et al. 2002
[b] Hernandez et al. 2006
[c] Locke et al. 2007
[d] AdjuVac produced by the U.S. Department of Agriculture, National Wildlife Research Center, Ft. Collins, Colorado, USA
[e] Miller et al. 2009

formulation). The PZP antibody titers were first detected 4 weeks post-vaccination but did not peak until the end of year 1, after which they remained consistently elevated through the end of year 7 in most of the elephants (Bechert and Fraker 2016). Elephants vaccinated with the VacciMax formulation took longer to develop antibody titers compared to the DepoVax vaccination group. Additional research is needed to determine actual contraceptive efficacy and potential long-term effects on fertility in these animals.

SpayVac in horses

The 3 trials conducted in horses used a variety of SpayVac formulations and adjuvants (Table 3). (Note that Gray et al. [2010] reported they had used SpayVac on free-ranging horses, but this was not accurate [Fraker and Brown 2011], and Gray et al. [2011] subsequently corrected their error).

In the first horse study, Killian et al. (2008) observed pregnancy rates of 0, 17, 17, and 17%, respectively, 1–4 years after treatment of mares with a single SpayVac VacciMax dose (*n* = 12), compared to 75, 75, 88, and 100% for untreated mares (*n* = 8). Bechert et al. (2013) found that 3–4 months post-vaccination with SpayVac VacciMax (*n* = 7) or DepoVax (*n* = 7), 93% of all treated mares ceased cycling as evidenced by lower serum concentrations of progesterone ($P < 0.025$) and smaller ovaries with fewer follicles ($P < 0.001$) compared to controls (*n* = 7), while other organ systems were unaffected. This was the first time such effects on the ovaries of horses had been studied.

A subsequent study in ponies, using a different PZP vaccine formulation (100 μg PZP administered in the rump followed 5 weeks later by a 100 μg PZP booster injection; *n* = 7), documented similar ovarian findings based on transrectal ultrasound and basal serum concentrations of progesterone (Joonè et al. 2017*a*). Additionally, after breeding, all PZP-treated mares were infertile compared to 100% fertility in the control group. The PZP vaccination effects on the ovaries were reversed 10 months after initiation of treatment (Joonè et al. 2017*a*).

The third trial using SpayVac in horses was conducted by the U.S. Geological Survey using DepoVax (*n* = 30) and VacciMax (*n* = 30) formulations administered in the rump (Roelle et al. 2017; Table 3). These investigators found that pregnancy rates for the VacciMax treatment group were lower ($P < 0.001$) compared to controls for 3 years following the single injection. The fertility rate for the treated group was 13, 47, and 43% compared to 100, 98, and 100% in controls for 1–3 years post-vaccination, respectively. Although fertility in the mares treated with the DepoVax formulation was only

Table 3. SpayVac formulations and administration sites in horse (*Equus caballus*) trials.

Author	PZP dose	Formulation	Adjuvant	Injection site
Killian et al. 2008	400 μg	VacciMax	AdjuVac	Neck
Bechert et al. 2013	200 μg	VacciMax	MFA	Neck
		DepoVax	MFA	Neck
Roelle et al. 2017	200 μg	VacciMax	MFA	Rump
		DepoVax	MFA	Rump

17% in the first year, this increased to 76% in year 2, and this group was removed from the study.

The results observed by Roelle et al. (2017) were not as robust as expected, given earlier results from both Killian et al. (2008) and Bechert et al. (2013). Differences in vaccine formulations and injection sites (i.e., neck versus rump) among these studies may account for these variable results and are explored in more detail below.

Neither Killian et al. (2008) nor Roelle et al. (2017) found an absolute correlation between antibody titer, measured by ELISA, and infertility. In both cases, individual mares that were consistently infertile did not necessarily have the highest antibody titers, and the ranges of titers observed in fertile and infertile treated mares showed considerable overlap. Previous studies have demonstrated a positive correlation between levels of PZP antibodies produced and contraceptive effect (Liu et al. 1989, Turner et al. 1997), and similarly in the Roelle et al. (2017) study, antibody titers in the range of 50–60% of the positive reference serum effectively contracepted 90–95% of the mares.

The predominant antibody class in equine serum and the major component of humoral immunity is IgG. Horses have 7 different IgG isotypes (Wagner et al. 2004), and in a recent study (Bechert and Wagner 2017) using serum samples from VacciMax SpayVac-treated mares ($n = 30$) in the Roelle et al. (2017) study, a fluorescent bead-based assay was used to distinguish IgG isotype responses against PZP. The IgG1 antibodies were generally higher in treated mares that were infertile compared to those that were infertile, but only IgG4/7 antibodies were significantly higher in infertile mares during years 1 and 2 post-vaccination ($P < 0.05$).

The IgG4/7 antibodies are produced in response to many antigens, and responses are typically long-lasting; thus, SpayVac's potential ability to preferentially stimulate IgG4/7 antibodies may contribute to its long-term immunocontraceptive efficacy (Bechert and Wagner 2017). The fluorescent bead-based assay increased the analytical sensitivity to detect anti-PZP antibodies compared to the ELISA, resulting in more pronounced differences in antibody detection between the fertile and infertile vaccinates.

Both Bechert et al. (2013) and Roelle et al. (2017) reported vacillating reactions at SpayVac injection sites in mares over a period of months. These results suggested initiation of a secondary immune response. The SpayVac liposome platform likely resulted in prolonged exposure to antigen. However, a key difference between the Roelle et al. (2017) and earlier Killian et al. (2008) and Bechert et al. (2013) studies is the location of the injection site. SpayVac vaccinations administered in the neck were injected virtually on top of 1 of 2 primary lymph nodes (the superficial cervical lymph nodes), whereas vaccinations administered in the rump (Roelle et al. 2017) may have been injected into gluteal or hamstring muscles, which were further from the nearest primary lymph nodes (the subiliac).

There appears to be a direct correlation between dendritic cell "input" into the lymph and the "output" by the lymph node (Randolph et al. 2005). Miller et al. (2009) first mentioned the role that follicular dendritic cells in lymph nodes may play in SpayVac's efficacy in white-tailed deer. Antigens that induce a strong T-cell response initiate the mobilization and maturation of dendritic cells, which migrate into areas being assaulted. Follicular dendritic cells are found primarily in lymph nodes and the spleen, their receptors trap antigens opsonized by complement or antibodies, and they interact closely with B cells, which must

bind the antigen presented by dendritic cells to become future memory cells (Banchereau and Steinman 1998).

SpayVac formulations seem to support maturation and longevity of dendritic and plasma cells in bone marrow (MacDonald et al. 2010). However, additional research is needed to explore the potential impact that injection site location may have on SpayVac's efficacy.

Most PZP vaccines are made with partially purified porcine ZP, which is isolated from pig (*Sus domesticus*) ovaries obtained from slaughterhouses, and consequently, other follicular proteins could potentially be involved in eliciting ovarian responses in vaccinates (Stoops et al. 2006). Dunbar et al. (1980) first developed the mechanical PZP isolation technique used today, whereby oocytes are freed from cellular debris by sequential passages of ground tissue in a buffer solution through a series of nylon screens of decreasing pore size, and a tissue homogenizer then releases the ZP. They determined on a cellular basis that extractions were 95% pure and were 93–97% pure on an enzymatic basis, with cumulus cells being the major cellular contaminant. The antigens used to produce SpayVac derive from a similar isolation procedure (Brown et al. 1997*b*), and the specificity of PZP antibodies in the Bechert et al. (2013) study was demonstrated by immunohistochemistry, as has been done in other studies (Barber et al. 2001).

Western blot experiments have shown that immune responses to PZP vaccination in mares are against all of the PZP glycoproteins present in partially purified vaccine formulations (Liu et al. 2005); however, none of these tests eliminates the possibility that other ovarian proteins are also present. The presence of non-ZP ovarian proteins is plausible (Mask et al. 2015), and this was further suggested by the finding that recombinant ZP vaccines, which include only specific glycoproteins comprising an animal's ZP (e.g., ZP3 is the glycoprotein in mice responsible for sperm binding; Litscher et al. 2009) do not have the same contraceptive effect elicited by native PZP (Miller et al. 2000, Joonè et al. 2017*b*).

The ZP is composed of 3 or 4 major glycoproteins that differ among species in protein structure and glycosylation, and this may have contributed to species-specific differences seen in response to PZP vaccination (Prasad et al. 2000). Antibodies produced in response to PZP vaccination occupy sperm-binding sites on the ova, thereby preventing fertilization and/or interfering with egg maturation within the follicle (Dunbar and Schwoebel 1988). However, there may be other roles that PZP antibodies play. For example, in grey seals, the ZP surrounds the blastocyst and may be involved in implantation, which may explain the unexpected reduction in fertility during the first year following vaccination, as mentioned above.

Finally, dose (200 versus 400 μg PZP) and adjuvant (AdjuVac versus MFA) may also affect SpayVac's efficacy. Although the Bechert et al. (2013) study did not examine contraceptive efficacy, because 93% of the treated mares stopped cycling, we believe that vaccination probably rendered these mares infertile. Killian et al. (2008) used 400 μg PZP and AdjuVac instead of 200 μg PZP and MFA used by Bechert et al. (2013), so these variables likely play a minor role in SpayVac's efficacy.

Future research needs

We believe that SpayVac has great potential as a long-term contraceptive agent for feral horse population management in the United States because it provides multi-year, single-dose efficacy. The manufacturers of SpayVac intend to seek EPA regulatory approval to make it widely available for use in horses and deer (http://www.spayvac.com). However, research to explore differences in immune response based on injection site location and booster effects is still needed. Strengthening response to vaccination by incorporating immune stimulants in the formulation is also worth exploring (e.g., CpG; Neeland et al. 2014). Roelle et al. (2017) stated, "if even the modest results that we observed [with SpayVac] could be duplicated on a large scale…the BLM and other agencies charged with managing feral horses would have a more effective fertility control tool than has heretofore been available, especially given the fact that no booster inoculation is required with SpayVac."

A greater understanding of SpayVac's mechanism of action and long-term reproductive effects is needed so wildlife managers can efficaciously apply the vaccine to free-ranging herds, possibly in conjunction with removals.

To perfect the application of SpayVac, we plan to investigate how the vaccine elicits its immune response, considering differences in PZP isolation methods, injection sites, and formulations as well as the potential effect of boosters.

The time of year when vaccines are administered likely makes a difference in immune response, especially as related to health and nutrition. Mares that have just come through a rough winter might be expected to mount a less robust immune response compared to mares that are well fed, having spent the summer on good range. Horses are seasonally polyestrous long-day breeders, and during the winter, anestrus is characterized by a flaccid uterus and inactive ovaries. It generally takes 2 months for peak antibody titers to develop, so vaccinating mares a few months prior to the breeding season (e.g., in the spring) should result in reliable immunocontraception. However, because of its multi-year efficacy, SpayVac can be administered to horses anytime during the year. Other factors, such as environmental conditions that affect access to horses, may also have to be taken into consideration by managers. If mares need to be vaccinated while they are in anestrus, antibodies to PZP will start binding to the ZP in emerging follicles when the horses start cycling again.

Previous studies have demonstrated that antibody titers in groups of horses and several deer species can vary significantly in response to vaccination with the same immunogen, dose, and adjuvant (Kirkpatrick et al. 2011). Abolins et al. (2011) demonstrated that wild mice (*Mus musculus*) have more variable immune responses than do laboratory mice of the same species. Although a variety of internal physiologic factors can influence an animal's ability to mount an immune response, nutrition is key (Gross and Newberne 1980, Ponton et al. 2011). For example, Nalder et al. (1972) demonstrated that in rats (*Rattus norvegicus*) vaccinated with tetanus toxoid, a decrease in dietary quality (especially iron) of only 10% results in a 50% decrease in antibody titer. Therefore, also understanding how the nutritional status, potential health challenges, and environmental variables (e.g., inter-species competition, effect of climate change on vegetation) may affect horse populations is important when constructing a contraceptive vaccination strategy.

Additional research tools will be helpful in implementing contraceptive population management schemes. For example, the fluorescent bead-based assay for anti-PZP IgG4/7 detection could be used to establish reliable cut-off values for IgG4/7 that are effective, and assessing ovarian function by quantifying serum concentrations of anti-Mullerian hormone can further characterize reproductive effects of PZP vaccination (Joonè et al. 2018).

We argue that the long-term population effects of immunocontraception should also be considered when developing a vaccination strategy. For example, studies with the horse population on Assateague Island have demonstrated a significant increase in longevity of PZP-treated mares as compared to controls because the continuing energy demands of pregnancy and especially lactation leaves untreated mares with lower body condition scores and higher mortality rates (Kirkpatrick and Turner 2007). Removals done in conjunction with vaccinations may be needed to most effectively manage free-ranging horse populations (Gross 2000, Garrott 2018).

Garrott (1991) optimistically estimated that 90% of the horses are detected when wildlife managers determine population sizes. Given the many variables described above, deciding what proportion of a herd should be vaccinated will not be easy. The BLM currently uses the WinEquus population model for Herd Management Area (HMA) planning (National Research Council 2013). Model projections for immunocontracepted horses on Assateague Island predicted an average rate of population decline of 13% per year, which suggested that the management target of 80–100 horses would be reached in 5–8 years (Ballou et al. 2008). Again, slow rates of population decline have been attributed to reduced mortality and increased longevity among treated mares (Kirkpatrick and Turner 2008).

Good population models must consider an animal's life history, its social structure and mating system, but also other factors that could impact population dynamics like density dependent variables and climate change (Garrott 2018). Climate change will also undoubtedly affect feral horse populations because many

currently live in arid habitats (Saltz et al. 2006). Therefore, even once variables like vaccine formulation and administration method, herd health and nutritional status, long-term population effects, and population modeling scenarios have been considered, contraceptive strategies will need to be continually adjusted through time.

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