MANAGEMENT

Immune response of African elephants to a single dose of SpayVac®, a pZP contraceptive vaccine, over a seven year period

Ursula Bechert[1,] and Mark A. Fraker, MA[2]*

[1]School of Arts and Sciences, LPS, 3440 Market Street, Suite 100, University of Pennsylvania, Philadelphia, PA 19104, USA
[2]Terramar Environmental Research, Ltd., 8617 Lochside Drive, Sidney, BC, V8L 1M8, CANADA
*corresponding author: bechertu@sas.upenn.edu

Abstract
As free-ranging African elephant *(Loxodonta africana)* populations become increasingly confined to smaller ranges, concerns grow about human–elephant conflicts and negative impacts on flora and fauna. Current elephant population management alternatives include translocation, culling, dispersal techniques, surgical sterilization, and contraceptive vaccines. Porcine *zona pellucida* (pZP) immunocontraception may have the greatest potential to control fertility because it has proven efficacy, and vaccines are easy to handle and safe to administer. SpayVac® (ImmunoVaccine) is a pZP vaccine that has demonstrated single-dose, multi-year contraceptive efficacy in other wildlife species, which would make it practical and economical for field application if it performs similarly in African elephants. Over a 7-year period, we assessed the immune response of African elephants to two SpayVac formulations: non-aqueous (n=3) and aqueous (n=3) emulsions. pZP antibody titers raised by the non-aqueous SpayVac formulation were first detected 4 weeks post-vaccination but did not peak until after 1 year, after which they remained consistently elevated through 7 years. This study demonstrated the ability of a single-dose of non-aqueous SpayVac to elicit an antibody response in African elephants for 7 years. Additional research is needed to determine actual contraceptive efficacy and potential long-term effects on fertility.

Résumé
Alors que les populations d'éléphants d'Afrique en liberté (Loxodonta africana) sont de plus en plus confinées à des habitats plus petits, les inquiétudes grandissent au sujet des conflits hommes-éléphants et des impacts négatifs sur la flore et la faune. Des alternatives actuelles de gestion des populations d'éléphants comprennent la translocation, l'abattage, les techniques de dispersion, la stérilisation chirurgicale et les vaccins contraceptifs. L'immunocontraception à la Pellucina Zona Porcina (pZP) peut avoir le plus grand potentiel de contrôler la fertilité, car elle a prouvé son efficacité, et les vaccins sont faciles à manipuler et sûrs à administrer. Le SpayVac® (ImmunoVaccine, Inc., Halifax, Nouvelle-Écosse, B3H 0A8, Canada) est un vaccin pZP qui a démontré l'efficacité d'une dose unique contraceptive sur plusieurs années chez d'autres espèces sauvages, ce qui le rendrait plus pratique et économique pour l'application sur le terrain s'il se révèle performant de la même façon chez les éléphants d'Afrique. Au cours d'une période de 7 ans, nous avons évalué la réponse immunitaire des éléphants d'Afrique à deux formulations SpayVac: des émulsions non aqueuses (n = 3) et aqueuses (n = 3). Des titres d'anticorps pZP soulevés par la formulation de SpayVac non aqueuse ont d'abord été détectés 4 semaines après la vaccination, mais ne culminaient qu'à 1 année, après quoi ils sont restés constamment élevés pendant 7 ans. Cette étude a démontré l'aptitude d'une dose unique de SpayVac non aqueuse de provoquer une réponse d'anticorps chez les éléphants d'Afrique pendant 7 ans. Des recherches supplémentaires sont nécessaires pour déterminer l'efficacité réelle de la contraception et les effets potentiels à long terme sur la fertilité.

Introduction

Wildlife management challenges are diverse. In the case of elephants, there is a disastrous depletion populations in some areas; while in others increased concentrations of animals due to restrictions on their movement necessitates a long-term, acceptable method of population control. Overabundant species, even native ones, can reduce biodiversity by monopolizing resources, spreading infectious diseases, and changing species composition or relative abundance (Noss 1990). In some regions of Africa (e.g., northern Botswana), elephant *(Loxodonta africana)* population densities have climbed, primarily because these animals are increasingly confined to limited spaces: hemmed in by human settlements, agricultural development, fences, civil war, and poaching (Chase and Griffin 2009). Pressures to control elephant population size exist because of their potential negative impact on flora and fauna (Kerley and Landman 2006; Guldemond and van Aarde 2007) and, in particular, because of human–elephant conflict (Pinter-Wollman 2012), which is one of the biggest issues facing elephant conservation today. Several options, such as chemical repellants and physical barriers (Davies et al. 2011), have been pursued to minimize human–elephant conflict, but in view of burgeoning human populations and the need to minimize habitat encroachment, it is ultimately the number of elephants that must be controlled.

Current population management options include: 1) translocation (Pinter-Wollman et al. 2009); 2) culling (Walker et al. 1987); 3) dispersal through establishment of water boreholes (Chamaille-Jammes et al. 2007) or corridors to connect and expand available habitat (Chase and Griffin 2011); 4) surgical sterilization (Foerner et al. 1994; Stetter et al. 2006); and 5) contraceptive vaccines (Kirkpatrick et al. 2011). All of these options have cost–benefit ratios that differ from one situation to the next. Translocating elephants is always costly; it is feasible only for small numbers of animals, and is not always effective (Pinter-Wollman 2012). Culling has become ethically problematic, and post-traumatic stress disorder (PTSD) is increasingly encountered in elephant herds that have experienced culling or poaching (Bradshaw et al. 2005). PTSD symptoms can include abnormal startle responses, depression, unpredictable asocial behavior, and hyper-aggression. Passive dispersal through the use of boreholes can eventually result in greater densities of elephants over time (Milewski 2000), and expansion of habitat space has limits. However, the development of trans-frontier conservation areas addresses many of the socioeconomic challenges inherent in conservation issues and promotes integrity and function of whole ecosystems (Jones et al. 2012). Surgical sterilization (e.g., vasectomy; Stetter et al. 2006) is costly and has no potential for managing large, free-ranging herds (Bokhout et al. 2005). Contraceptive vaccines can be based on Gonadotropin-releasing hormone (GnRH) or porcine *zona pellucida* (pZP) (Perdok et al. 2007); however, there is little currently published about GnRH vaccines, and they may not be effective in female African elephants (Valades et al. 2012). Presently, pZP vaccines are most effective when applied in small, enclosed conservation areas (Delsink 2006); the requirement for annual boosters of current vaccines precludes their use in larger, free-ranging populations (Druce et al. 2011). Combinations of these tools applied in concert with other management strategies appear necessary to successfully address particular elephant population management challenges (Owen-Smith et al. 2006).

Of the options, pZP immunocontraception has been touted as the most promising option because the vaccines are safe, tissue-specific, cost effective, and relatively easy to administer (Kirkpatrick et al. 2011; Ahlers et al. 2012). The potential effect of pZP-based immunocontraception on social structure and behavior in herd animals has been explored by researchers who reported either no differences (Kirkpatrick et al. 1995, Powell 2000) or minimal differences (Ransom et al. 2010) between vaccinated and control mares with respect to activity budgets, hierarchy within the herd, or interactions with stallions; unlike GnRH vaccines, which suppressed behavioral and physiological estrus (Elhay et al. 2007, Botha et al. 2008). Thus concerns about potential behavioral side effects of pZP vaccines in elephants have largely been dispelled (Delsink et al. 2013). The primary limitation of current pZP vaccines is the frequency with which they typically need to be administered: initially three times with two boosters given 2–4 weeks apart, and then annually (Delsink et al. 2006; Fayrer-Hosken et al. 1999). A single-dose immunocontraceptive vaccine that can elicit sustained antibody titers over several years with one treatment would be advantageous because elephants would require less frequent handling, thereby minimizing costs as well as exposure to stress and risk of injury. In horses, efforts to produce pZP vaccines that provide a sustained release of antigen have been attempted by incorporating antigen in either microcapsules or pellets (Liu et al. 2005); however, the effectiveness of these slow-release products to extend the period of infertility

has been limited and inconsistent (Turner et al. 2008). SpayVac® (ImmunoVaccine is a pZP vaccine that uses a unique liposome technology and has delivered single-dose, long-lasting immunocontraception in a variety of species including fallow deer (*Dama dama;* Fraker et al. 2002), white-tailed deer (*Odocoileus virginianus*; Rutberg et al. 2013, Locke et al. 2007), horses (*Equus caballus;* Killian et al. 2008), and grey seals (*Halichoerus grypus;* Brown et al. 1997a). In all cases, a single dose of SpayVac raised antibody titers that were highly effective for several years. For example, after treating mares with a single dose of SpayVac containing 400 µg of pZP and the adjuvant AdjuVac™ (National Wildlife Research Center, U.S. Department of Agriculture, Ft. Collins, Colorado 80526, U.S.A.), annual reproductive success over the next four years was 0%, 17%, 17%, and 17% compared to 75%, 75%, 88%, and 100% for untreated controls (Killian et al. 2008).

We used two SpayVac formulations in this study: 1) an aqueous Modified Freund's Adjuvant (MFA) emulsion, and 2) a non-aqueous formulation that also incorporated MFA. The latter formulation was of particular interest because it has an extended shelf life and need not be stored frozen, which are important considerations for a vaccine that is to be used in remote areas. The objectives of this 7-year study were: 1) to compare the ability of aqueous (emulsion) and non-aqueous SpayVac formulations to raise serum concentrations of pZP antibodies and 2) to determine the length of time that antibody titers were sustained in African elephants.

Table 1. Female African elephant treatment groups for trials of pZP immunocontraception: name, age at start of study, and location

Treatment Group	Elephant	Age (yr)	Location
Non-aqueous	Hadari	22	Nashville Zoo
	Kimba	27	Cheyenne Mountain Zoo
	Cinda	34	Sedgwick County Zoo
Aqueous	Alport	46	San Antonio Zoo
	Alice	35	Wildlife Safari
	Stephanie	34	Sedgwick County Zoo

Materials and methods

Animals

Our subjects were six captive female African elephants held in North American zoos (Table 1). They had been monitored prior to this study to determine estrous cyclicity based on serum concentrations of progesterone, and only one female was not acyclic. Procedures were approved by each facility's Institutional Animal Care and Use Committee. Blood samples were collected from ear veins using 20-gauge catheters, and initial samples were used to confirm the absence of pre-vaccination serum pZP antibody titers, and perform complete blood counts (CBCs) and serum biochemical profiles for evaluation of health status. Samples collected for serology were allowed to clot overnight at 4°C, centrifuged for 10 minutes at 1,300 g, and stored at –20°C until time of analysis. Whole blood samples for CBCs were collected in tubes containing EDTA and analyzed on-site the same day.

Elephants were vaccinated in 2005 with one of two SpayVac formulations containing 600 µg of pZP glycoprotein by intramuscular (IM) injection in the gluteal muscles using 7.6 cm long 18 or 20-gauge needles. Post-immunization, animals were visually monitored for potential side effects or inflammation at the injection site. Blood samples were collected weekly for 8 weeks, then every 2 weeks for the next 8 weeks, and finally monthly for an additional 5 months (17 samples/elephant). Antibody titers were then monitored annually for up to 7 years. Serum was kept frozen at –20°C until time of analysis to determine anti-pZP antibody titers.

SpayVac vaccine preparation

Frozen pig ovaries were obtained from a slaughterhouse (Garden Province Meats, Charlottetown, Prince Edward Island, Nova Scotia, C1E 2A1, Canada) and were the source of the pZP for vaccines prepared by Immunovaccine Inc. The vaccines were prepared as previously described by Brown et al. (1997b). Briefly, frozen pig ovaries were gradually thawed in an incubator at 5°C, trimmed of excess tissue and placed in washing buffer (2°C, 10 mM sodium phosphate, 125 nM sodium chloride, 3 mM sodium citrate, 2 mM EDTA and 3 mM sodium azide, pH 7.4). Throughout the procedure, all tissues and fluids were kept cool using ice baths. Trimmed ovaries were ground using a Moulinex Multi-Grinder with openings of 7 mm. Washing buffer was added during the grinding procedure to carry the ground materials through the grinder. The ground tissue was passed through a series of nylon screens with sequential pore sizes of 1410, 500, 209, and 153 µm. Material collected

on the 1410 µm screen was ground for a second time with the Moulinex grinder with grill openings of 4 mm. Porcine oocytes, which are approximately 100 µm in diameter, were captured on a 74 µm screen. Material on the 74 µm screen was filtered again through a 153 µm screen to remove more debris and the oocytes were collected on a 74 µm screen. Oocytes were placed in a Wheaton tissue homogenizer (30 mL) equipped with a Teflon plunger. The plunger was pushed to the bottom of the tube and up to the top until microscopic examination indicated that most oocytes were broken and free of cytoplasm. The cell ghosts were collected on a nylon screen (48 µm pore size) with the aid of reduced pressure and washed with buffer. Microscopic examination was used to assess when a pure suspension of intact ZP was achieved. The ZP were suspended in Tris buffer (20 mM, pH 8.0) and incubated at 75°C in water for 25 minutes. The suspension was vortexed and centrifuged at 16,000 g for 5 minutes. The supernatant fluid containing ZP was removed, dialyzed overnight, freeze dried and stored at –20°C.

Purified pZP was suspended in phosphate-buffered saline (PBS; pH 7.4) at a final concentration of 600 µg per dose. Lipids containing lecithin and cholesterol were added at a ratio of 10:1 (0.2 g lecithin and 0.02 g cholesterol/dose; Lipoid, to the pZP solution to form multilamellar liposomes.

Half of the prepared pZP liposomes solution was mixed with MFA (Calbiochem, La Jolla, California 92037, U.S.A.) to form a water-in-oil (aqueous SpayVac) emulsion (1:1, v/v; 1 mL/dose). The second half of the prepared pZP-liposomes mixture was lyophilized (non-aqueous SpayVac) and later reconstituted with MFA for a final deliverable dose volume of 0.5 mL (Karada et al. 2010). To control quality, a bicinchoninic acid-containing protein assay and gel electrophoresis were used. Standard bioburden testing according to United States Pharmacopeia Convention (USP) methods further ensured purity and safety of vaccines.

All SpayVac vaccines were shipped to zoos in pre-loaded, ready-to-inject syringes. Permits required for the transportation of pZP products and elephant sera across the USA–Canada border were obtained (05US105093/ CA05CWIMO126; 07US154918/ 07CA00713CWHQ; 12US64538A/ 12CA00464CWHQ).

Measurement of antibody titers

Serum samples were coded prior to analysis to avoid bias and were analyzed in batches at 6 and 12 months and then annually to 7 years. Lyophilized pZP was dissolved in Dulbecco's phosphate buffered saline to a concentration of 1.0 mg/mL; the mixture was gently shaken, heated for 30 min at 37°C and diluted to 1 µg/mL with coating buffer (0.02 M NaHCO3, 0.03 M Na2CO3, and 0.003 M NaN3). A 96-well polystyrene microtiter plate (Bio-Rad) was coated with 100 µL/well of this solution and then incubated overnight at 4°C. The next day, the plate was washed with Tris-buffered saline-Tween 20 (0.01 M Tris, 0.05% M Tween-20, and 0.15 M NaCl; TBS-T) and blocked with 3% gelatin (Bio-Rad) for 30 min at 37°C. Following two washes with TBS-T, the plate was then incubated overnight at 4°C with two-fold serial dilutions of elephant sera (1:50 to 1:6,400) or buffer only. A reference serum, from an elephant previously immunized with SpayVac (in another study) and demonstrating a pZP-specific antibody response, was included with each plate. Rabbit anti-elephant serum diluted 1/500, was next added to the plate and incubated at 37°C for 1 hour. This was followed by incubation with alkaline phosphatase conjugated Protein A at a dilution of 1/1000 for 1 hour at 37°C. The plate was developed by incubating for 1 hour at 37°C with substrate solution containing 1 mg/Ml 4-nitrophenyl phosphate disodium salt hexahydrate The optical density of each well was measured at 405 nm using a Microplate reader. Half maximal effective concentration (EC50) titers were calculated using a 5 Parameter Logistic (5PL) non-linear regression model (Masterplex ReaderFit) and as a percentage of the reference serum at the same dilution.

Results

Animals

All of the CBC and serum chemistry values at the beginning of the study were within normal reference ranges. Mild swelling was seen around the vaccine injection site in Hadari (non-aqueous formulation), which lasted for approximately 2 weeks and then spontaneously resolved. Slight stiffness in the vaccinated leg lasted for a day in Alice, who received the aqueous emulsion.

Antibody titers

Antibody titers to pZP in response to the non-aqueous SpayVac formulation were seen 4 weeks post-vaccination but didn't reach peak concentrations until 1 year later (Table 2). Log titer graphs demonstrate

consistently elevated levels through 7 years post-vaccination (Figure 1). Because antibody titers in response to the aqueous SpayVac formulation were not as robust as those raised by the non-aqueous formulations during the first year (i.e., titer values were comparably lower), no subsequent collections were made from aqueous SpayVac-injected elephants. However, serum samples inadvertently submitted 2, 4, and 5 years post-vaccination for Stephanie (an aqueous SpayVac vaccinate) and subsequently analyzed demonstrated antibody titers within the range of non-aqueous SpayVac vaccinates.

Table 2. Serum pZP antibody titers in elephants in response to single-injection SpayVac® non-aqueous and aqueous immunocontraceptive vaccine formulations. *NA: not analyzed

Titer	NON-AQUEOUS			AQUEOUS EMULSION		
Months post-vax	Hadari	Kimba	Cinda	Alport	Alice	Stephanie
0	0	0	0	0	0	0
0.5	0	0	0	0	0	0
1	400	100	200	0	0	0
1.5	400	200	200	0	0	0
2	800	200	2554	131	0	0
3	800	400	200	859	0	0
4	800	400	400	722	0	0
5	1600	800	400	574	0	25
7	3200	400	200	491	0	25
9	1600	200	NA	603	762	25
12	4160	1600	3711	1248	1813	NA
24	3200	2772	3839	NA	NA	1448
36	3242	2558	3598	NA	NA	NA
48	4326	1473	6895	NA	NA	3280
60	2514	1238	1588	NA	NA	1283
72	2660	2307	3355	NA	NA	NA
84	4289	3683	NA	NA	NA	NA

Estrous cyclicity

Elephants included in this study were either acyclic prior to vaccination, based on serum concentrations of progesterone being consistently at baseline (Kimba, Hadari, Stephanie, Alice, and Alport), or were unable to conceive (Cinda). The goal of breeding elephants to maintain sustainable populations in captivity precluded use of cycling females in this study; however, our only goal initially was to evaluate the ability of SpayVac to elicit an immune response, not to evaluate actual contraceptive efficacy. Cinda appeared to be cycling normally from 1999 through 2003 based on weekly serum progesterone concentration profiles. Median estrous cycles during this time were 12 weeks in length (range: 11–17 weeks) characterized by 9-week luteal and 5-week follicular phases; however, she was unable to conceive after multiple insemination attempts in 2002–03. Serum concentrations of progesterone were only determined on a monthly basis after 2004 and did demonstrate luteal phases (progesterone concentrations above 0.20 ng/mL). In 2008 (approximately 3 years after the SpayVac injection), weekly serum concentrations of progesterone were determined during a 4-month period, and cycling was still evident.

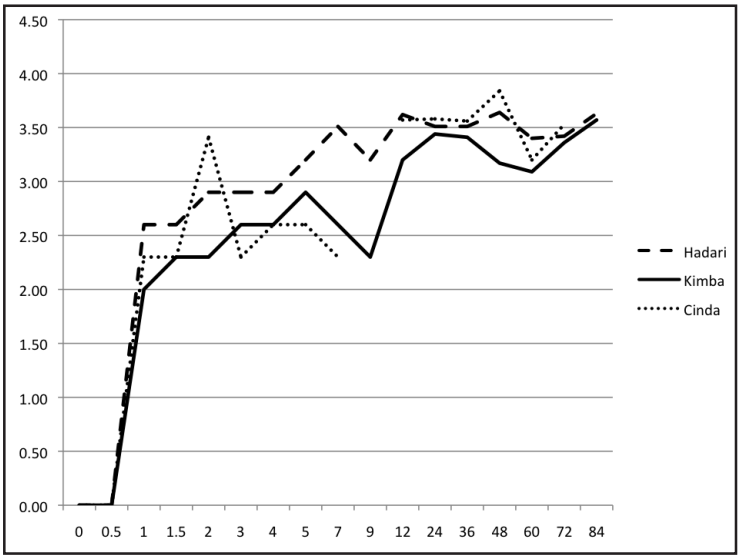

Figure 1. Porcine zona pellucida antibody titers expressed as log titers in elephants vaccinated with a single-dose, non-aqueous SpayVac® formulation

Discussion

Fayrer-Hosken et al. (1997) were among the first to explore the use of a pZP vaccine as a contraceptive agent in African elephants. In their study, anti-pZP antibodies rose significantly ($p<0.05$) in a captive female elephant after vaccination with an initial 400 µg pZP dose followed 4 weeks later by a 600 µg booster. Antibody titers in this individual remained elevated for 7 months (Fayrer-Hosken et al. 1997), and in three elephants receiving an additional 600 µg booster at 10 months, antibody levels remained elevated for an additional 12–14 months (Fayrer-Hosken et al. 1999). In our study, we demonstrated the ability of a single-dose, non-aqueous SpayVac vaccine to elicit an antibody response that remained strong for at least 7 years. It is also possible that the aqueous SpayVac formulation elicits a similar immune response; however, antibody titers appear to take longer to develop. How much longer pZP antibody titers would have remained elevated in these elephants is not known; however, grey seals vaccinated with a single-dose of SpayVac (Brown et al. 1997a) maintained high titers for at least 10 years (Robert Brown, personal communication).

Zonae pellucidae contain three major glycoproteins (ZP1, ZP2 and ZP3) encoded by three gene families (ZPA, ZPB and ZPC) that are highly conserved across species (Yurewicz et al. 1998). Generally, ZP1 is believed to provide structure to the ZP matrix, ZP2 binds to acrosome-reacted sperm and prevents polyspermy, and ZP3 is thought to serve as the primary sperm ligand (Lefievre et al. 2005). High-affinity sperm-binding sites are constructed by ZP proteins in different ways in pigs and mice (Yurewicz et al. 1998), and differences in ZP structure and glycosylation likely contribute to the species-specific differences seen in response to pZP vaccination (Prasad et al. 2000; Lyda et al. 2013).

Vaccine formulations differ depending on the adjuvant and pZP isolation methodology used. A study by Jones et al. (1992) demonstrated that rabbits immunized with solubilized isolated pZP (which contains ZP1, ZP3α, and ZP3β), ZP3 (purified ZP3α and ZP3β), and ZP3β-endo-β-galactosidase digested glycoproteins had a significant decrease in the number of primary, secondary, and tertiary follicles compared with controls ($p<0.01$). Purified ZP3α or deglycosylated ZP3β did not produce such histopathological changes. It is possible that the different pZP isolation methods used by Liu et al. (1989, 2005) and Brown et al. (1997b) result in qualitatively different antibodies, which may be more important than the absolute titers achieved. Dunbar et al. (1980) and Gwatkin et al. (1980) first developed the mechanical pZP isolation technique used today, whereby oocytes are freed from the cellular matrix by sequential passages of ground tissue through a series of nylon screens of decreasing pore size, and a tissue homogenizer then releases the ZP. On a cellular basis, extractions were 95% pure and they were 93–97% pure on an enzymatic basis, with cumulus cells being the major cellular contaminant (Dunbar et al. 1980). Differences in the pZP isolation procedures of Liu et al. (2005) compared to those of Brown et al. (1997b) include use of a ganged razor blade apparatus instead of a Moulinex Multi-Grinder, fewer washes and screen passages, and extended heat solubilization (50 min at 70oC versus 25 min at 75oC for Brown et al.). It is possible that pZP vaccine antigens include different proportions of ZP1, ZP3α, and ZP3β, ZP3β-endo-β-galactosidase digested glycoproteins, and deglycosylated ZP3β, which stimulate different immune responses in vaccinates. All 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). 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), and immunohistochemical staining of ovarian tissue from mares vaccinated with SpayVac also demonstrated a ZP-specific immune response (Bechert et al. 2013). However, none of these tests eliminates the possible presence of other ovarian proteins, which could also play a role in eliciting the ovarian histopathology seen in some animals.

Immunocontraceptive vaccines based on recombinant pZP would avoid the presence of other ovarian proteins. Miller et al. (2000) were the first to test recombinant vaccines in deer, based on observations that pZP-injected deer formed antibodies that cross-reacted with RC55, RC75α, and RC75β (Skinner et al. 1994), and that pZP3α shares a 66% protein sequence identity with 55 kDa rabbit ZP (Yurewicz et al. 1993). They compared the effectiveness of 500 µg of antigen in Freund's Complete Adjuvant using pZP, RC55, RC75α, or a combination of RC55, RC75α and RC75β. The pZP-treated does experienced an 87% reduction in fawning compared to 33% in RC55-treated does, 27% in RC75α-treated does, and 0% in the combination-treated does (Miller et al. 2000). The development of an effective recombinant ZP vaccine

would ensure consistency and would facilitate large-scale production.

The duration of antibody production in response to pZP vaccination varies within and between species even when using the same vaccine formulation (Lyda et al. 2013). In the current study, elephants developed maximal titers 12 months following vaccination with non-aqueous SpayVac and log titers remained between 3.0 and 3.5 for 7 years. A similar pattern was observed in an earlier study with horses where log titers peaked 2 months post-vaccination with non-aqueous SpayVac and then remained elevated at approximately 5.0 for the duration of the study (4 months; Bechert et al. 2013). Neither study evaluated actual contraceptive efficacy, so whether or not these titers rendered animals infertile is not known. Anti-pZP antibody titers above a certain threshold level are clearly linked to infertility in a variety of species (Liu et al. 1989; Turner et al. 1997). In the Bechert et al. (2013) study, 3 to 4 months after vaccination, 93% of SpayVac-injected mares (n=14) ceased cycling as evidenced by significantly lower serum concentrations of progesterone ($p<0.025$) and smaller ovaries with fewer follicles ($p<0.001$). Whether or not this would have resulted in permanent infertility is not known; however, a similar recent study, which extended 10 months post treatment did demonstrate a return to cyclicity in treated, temporarily anestrous mares (Joone et al., 2016).

Modeling (Druce et al. 2011) and field trials (Delsink et al. 2007) using pZP vaccines in African elephants have shown that 100% contraceptive efficacy is not required to effectively manage fertility. In fact, pZP vaccines have been used to effectively manage small populations of African elephants for over 6 years (Delsink et al. 2006; Delsink et al. 2007; Bertschinger et al. 2012). However, these vaccines required annual boosters, which make this option ineffective for managing larger, free-ranging populations of elephants like those in northern Botswana. An immunocontraceptive vaccine that can sustain antibody titers over multiple years with just a single treatment is advantageous because elephants would require fewer treatments, thereby minimizing stress and risk of injury to animals and workers, as well as containing costs. Potentially this could allow larger, free-ranging populations to be contracepted.

Efforts to maintain sustained titers over an extended period have also included the incorporation of pZP in either microcapsules or pellets (Liu et al. 2005); however, more effective oil-based adjuvants, such as MFA, cannot be incorporated into these matrices (Kirkpatrick et al. 2011), and the effectiveness of slow-release pellets, at least in horses, has been inconsistent (Turner et al. 2008). Vaccine trials in African elephants using different formulations of lactide-glycolide pellets have been attempted (VanRossum 2006), but the results have also been inconclusive (Bertschinger et al. 2008). Liposomes are multi-lamellar, concentric spheres made up of phospholipid bilayers separated by aqueous compartments and may themselves serve as immunological adjuvants (Allison and Gregoriadis 1974; Gupta et al. 1993). A VacciMax formulation containing Hepatits B antigen (similar to the aqueous version of SpayVac), when administered to rabbits, created a significantly greater humoral response compared to that produced by alum-adjuvanted vaccines (MacDonald et al. 2010), and this enhanced response was correlated with an increased number of ZP-specific plasma cells in the bone marrow. SpayVac may similarly stimulate an increased production of plasma cells in elephants; most long-lived plasma cells are found in the bone marrow (Bortnick et al. 2012).

The predominant serum antibody in African elephants is IgG, and up to five subclasses of IgG have been demonstrated (Kelly et al. 1998). Nine IgG genes are contained in the African elephant genome, which is a larger number than has been found in the genome of any other placental mammal examined so far, although their functionality is not known (Guo et al. 2011). Cross-reactivity between anti-Asian elephant IgG and African elephant IgG in serum was weak based on capture enzyme-linked immunosorbent assay (ELISA) results (28.2%; Kania et al. 1996); thus species-specific differences in the effectiveness of SpayVac are possible. How SpayVac would perform in Asian elephants *(Elephas maximus*) is not known. Species-specific differences are further suggested by the difference in performance of GnRH vaccines in African and Asian elephants. Even though GnRH vaccines do not appear to be effective in female African elephants (600 µg of GnRH [Improvac®,] administered twice 5–7 weeks apart; Valades et al. 2012), complete ovarian cycle suppression occurred in an Asian elephant given Repro-BLOC® (Amplicon Vaccine), a recombinant ovalbumin-GnRH fusion protein, subcutaneously as a series of four boosters of increasing dose ranging from 3 to 30 mg (Boedeker et al. 2012). However, differences in the dosing regimen may also account for the differences seen.

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 populations of mice have more variable immune responses than do laboratory mice. A variety of internal physiologic factors can influence an animal's ability to mount an immune response, and nutrition is key (Gross and Newberne 1980; Ponton et al. 2011). For example, Nalder et al. (1972) demonstrated that in rats vaccinated with tetanus toxoid, a decrease in dietary quality of only 10% results in a 50% decrease in antibody titer. Wild elephants forage and graze throughout the year, and seasonal dietary changes are significant. Plants can be low in key nutrients like Cu or Zn, which may lead to clinically significant immune deficiency (Cunningham-Rundles et al. 2005). Micronutrient deficiencies, including in vitamins B12, A, C, and E and minerals Zn, Fe, Se, and I, commonly affect humoral and cellular immune function (Katona and Katona-Apte 2008; Rivera et al. 2003), whereas protein calorie malnutrition more specifically affects the cellular immune system (Cunningham-Rundles et al. 2005; McMurray 1981). Additionally, several studies have shown that helminth loads can impair the efficacy of immunization in a variety of species (Rivera et al. 2003; Pederson and Babayan 2011).

Whether treatment with SpayVac could potentially have affected the estrous cycles of elephants in this study is unknown, because all but one were acyclic 'flatliners' prior to vaccination, with serum concentrations of progesterone remaining at baseline (Brown 2000). Estrous cycles in African elephants normally average 14 weeks in length and are composed of 9-week luteal and 5-week follicular phases (Bechert et al. 1999). The median estrous cycle length for Cinda from 1998 to 2004 was 13 weeks (range: 11–15 weeks) with normal luteal and follicular phases, and a 4-month glimpse at her estrous cycle in 2008 demonstrated continued cycling. Fluctuations in the length of the estrous cycle are normal and can range from 8 to 12-week luteal and 4 to 6-week follicular phases; however, cycle length within individual animals is usually consistent (Brown 2000). It seems unlikely that vaccination in 2005 with SpayVac (when this study was initiated) affected ovarian function in Cinda. The mechanism of contraception for pZP vaccines is believed to be the blocking of sperm receptors in the ZP, which, in addition to sperm binding, appears to play a role in granulosa cell differentiation and folliculogenesis (Prasad et al. 2000). It is plausible that pZP antibodies may interfere with ZP function, affecting follicular development in some species, like horses, where an erosion of the follicle pool, lack of ovulation, and low serum concentrations of progesterone were reported in response to vaccination with SpayVac (Bechert et al. 2013). However, Cinda continued to experience elevations in serum concentrations of progesterone for several years after vaccination, indicating that ovulation was occurring.

Elephants receiving the non-aqueous SpayVac formulation demonstrated a more robust immune response compared to those that received the aqueous emulsion, as evidenced by greater antibody titers. The reason for this is not known; however, initial results between the same two SpayVac formulations were similar in horses, with non-aqueous SpayVac treated mares having higher antibody titers (Bechert et al. 2013). It is also possible that the aqueous SpayVac formulation elicits a similar immune response in African elephants; however, antibody titers take longer to develop. The non-aqueous formulation would have greater utility in the field as a portable vaccine, because it can be lyophilized and subsequently reconstituted with MFA when needed.

In conclusion, of the non-lethal alternatives for managing elephant numbers in the wild, SpayVac has perhaps the greatest potential because of its demonstrated single-dose, multi-year performance in multiple species. Because ZP vaccines are tissue-specific to the reproductive tract, there are fewer side effects as compared to GnRH vaccines. SpayVac should be tested in African elephants to demonstrate actual contraceptive efficacy and separate trials should be conducted with Asian elephants. Long-term effects on the reproductive system also need to be assessed, because field application strategies will differ based on reversibility of contraception. Wildlife managers need to know how often and how many elephants should be vaccinated to effectively implement immunocontraceptive vaccines in free-ranging herds, possibly in conjunction with culls, translocations, or dispersal efforts.

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