Efficacy and safety of recombinant zona pellucida vaccines in domestic horse mares and current application of native porcine zona pellucida vaccines in African elephant cows

by

Margaret Bethaline Nolan

Submitted in fulfilment of the requirements for the degree
Philosophiae Doctor (PhD)

in the

Department of Production Animal Studies
Faculty of Veterinary Science
University of Pretoria

Janaury 2019

Declaration of originality

I, Margaret Bethaline Nolan, declare that the thesis, which I hereby submit for the degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

The publications included in the thesis have not been submitted previously to this or any other tertiary institution for such a doctoral degree; is my own work, my personal contribution to this work is clearly stated and I have given due recognition to the institutional policy on copyright.

.....
Margaret Bethaline Nolan

.....
Date

Summary of contribution of this work

Efficacy and safety of recombinant zona pellucida vaccines in domestic horse mares and current application of native porcine zona pellucida vaccines in African elephant cows

Margaret Bethaline Nolan

Supervisor: **Prof. M L Schulman**
Co-supervisor: **Prof. H J Bertschinger**
Degree: **Philosophiae Doctor (PhD)**
Department: **Production Animal Studies**

The use of immunocontraceptives in veterinary population management and wildlife fertility control is well-established. The porcine zona pellucida (pZP) vaccine has been successfully used in approximately 85 species. The successful application of pZP vaccine over several decades in domestic and free-roaming horses (*Equus ferus caballus*) has served to inform the development of population control management programmes for other species including the African elephant (*Loxodonta africana*). Elephant populations in South Africa, predominantly within fenced game reserves, have been humanely managed with the pZP vaccine since 2000.

Whilst the pZP vaccine meets many of the gold standard requirements for population management, it has not been without its caveats. These are partially due to the potential complications associated with both native derived ZP proteins from pig ovaries and their formulation with Freund's adjuvants. With increased demand for effective and humane population management, there is a need for improvements to current vaccine formulations for economical, regulatory and animal welfare reasons.

This research investigated the ovarian effects, immunoreactivity and safety of a novel recombinant zona pellucida (reZP) vaccine for use in horses, as both a model and target species. Additionally, alternative commercially available, effective adjuvants for inclusion

in ZP-based immunocontraceptive vaccines were tested. Finally, the current status of a pZP immunocontraception programme in managed populations of elephants in South Africa was examined.

This work investigated a potential alternative to native pZP immunocontraception in mares supported by ovarian effects, antibody titre responses and safety profiles. A reZP vaccine prepared with promiscuous T-cell epitopes of tetanus toxoid and bovine RNase formulated with Montanide™ Pet Gel A and Poly (I:C) adjuvants was shown to be a suitable alternative for immunocontraception in the horse. Additionally, the measurement of anti-Müllerian hormone (AMH) was associated with measurements of ovarian activity subsequent to immunocontraception. This may facilitate monitoring of ovarian function following immunocontraception in horses under extensive conditions which may be similarly applicable in other species.

Finally, this work showed increasing and successful application of the pZP vaccine in South African elephant populations. An effective, safe reZP vaccine holds promise for commercialisation with its associated higher yields and reduced costs in vaccine production. This will increase availability of immunocontraception for managing South African elephant populations and its potential application in other species.

Overall this work reported novel and improved alternatives to currently-employed methods for veterinary population management of horses with the potential for similar applications in elephants and other species.

Ethics statement

The author, whose name appears on the title page of this thesis, has obtained, for the research described in this work, the applicable research ethics approval. The author declares that she has observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the Policy guidelines for responsible research.

Summary of thesis

Efficacy and safety of recombinant zona pellucida vaccines in domestic horse mares and current application of native porcine zona pellucida vaccines in African elephant cows

Margaret Bethaline Nolan

Supervisor: **Prof. M L Schulman**
Co-supervisor: **Prof. H J Bertschinger**
Degree: **Philosophiae Doctor (PhD)**
Department: **Production Animal Studies**

These studies investigated the efficacy, safety and ovarian effects of immunocontraceptive vaccine formulations using horses (*Equus ferus caballus*) as a model for fertility control in African elephant (*Loxodonta* africana) populations. Additionally, the current status of the native porcine zona pellucida (pZP) vaccination programme for elephant population control in South Africa was reviewed.

In mares, the use of pZP or recombinant ZP (reZP) proteins formulated with a commercially available non-Freund's adjuvant [Montanide™ Pet Gel A and Poly (I:C)] evoked a similar anti-pZP antibody titre response to pZP formulated with Freund's adjuvants, with minimal side effects. Ovarian suppression was evident following vaccination with this novel reZP formulation, similar to anti-gonadotropin releasing hormone (GnRH) vaccination. The novel post-immunocontraceptive measurement of anti-Müllerian hormone was associated with established clinical and hormonal measurements of ovarian activity. A reZP vaccine formulated with non-Freund's adjuvants is a promising alternative for fertility control in both domestic and wildlife species.

By 2018, approximately 800 elephant cows were enrolled in the pZP-based immunocontraceptive programme across 27 South African reserves. This enrolment has grown markedly since inception in 2000. A successful effect on population growth rates

was evident utilising either individual or blanket administration strategies. Ongoing data collection is central to monitor the effects of this programme. Although collected data was insufficient to fulfil all the planned objectives, being limited primarily by the method and reporting format, this informed suggested changes. Minimum data requirements for effective review of the programme include regular population counts to establish demographics, in particular the accurate identification of young calves.

Dedication

To my Daddy, Patrick Donal Nolan (February 11th 1956-May 6th 2018)

Acknowledgements

I wish to express my enormous gratitude to my supervisors, Professors Martin Schulman and Henk Bertschinger. Thank you for the opportunity. It changed my life!

To the Technology Innovation Agency and Humane Society International for their financial support.

To Anne-Marie Human and Ofentse Mogoba at the VPML, thank you for the constant support and friendship.

To all at the Section of Reproduction, PAS, thank you for your friendship and words of encouragement.

To all at Waterford Farm Stud, without whom this research could not have been conducted.

To the many researchers and veterinarians who guided, directed and allowed me a glimpse into much of the amazing work they do.

To the many friends I have had the privilege to meet during this time.

To my family for their support and encouragement during the difficult times.

To my wonderful partner, who joined me on this undertaking and has supported and uplifted me throughout.

Finally, to the animals, the reason I do what I do.

Table of Content

List of figures

List of tables

List of abbreviations

GnRH	gonadotropin releasing hormone
pZP	porcine zona pellucida
FSH	follicle stimulating hormone
LH	luteinising hormone
CL	corpus luteum
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
E_2	estradiol 17**β**
AMH	anti-Müllerian hormone
TGF**β**	transforming growth factor **β**
AFC	antral follicle count
ZP	zona pellucida
AR	acrosome reaction
eqZP	equine zona pellucida
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FMCA	Freund's modified complete adjuvant
ASIS	Assateague Island National Seashore
reZP	recombinant zona pellucida
E. coli	*Escherichia coli*
TT-KK-ZP3	recombinant pZP3 linked to a promiscuous T-cell epitope of tetanus toxoid (TT) by a dilysine linker (KK), expressed by *Escherichia coli*
bRNase-KK-ZP4	recombinant pZP4 linked to a promiscuous T-cell epitope of bovine RNase (bRNase) by a dilysine linker (KK), expressed by *Escherichia coli*
MHC	major histocompatibility complex
TH	type helper cell
CD	cluster of differentiation
CTL	cytotoxic T-lymphocyte
PRR	pathogen-recognition receptors
TLR	toll-like receptor
NLR	nucleotide oligomerisation domain-like receptor
RLR	retinoic acid-inducible gene1-like receptor

DAMP	damage-associated molecular pattern
PAMP	pathogen-associated molecular pattern
APC	antigen presenting cell
TB	tuberculosis
IgG	immunoglobulin G
CITES	Convention on International Trade of Endangered Species
HiP	Hluhluwe-iMfolozi Park
KNP	Kruger National Park
ICI	intercalving interval
N&S	Norms and Standards for the Management of Elephants in South Africa
EMP	elephant management plan
DAFF	Department of Agriculture, Forestry and Fisheries
SAHPRA	South African Health Products Regulatory Authority
VPML	Veterinary Population Management Laboratory
elZP	elephant zona pellucida
CSIR	Council for Scientific and Industrial Research
BCS	body condition score
ELISA	enzyme-linked immunosorbent assay
LSD	least significant difference
ANOVA	analysis of variance
PBS	phosphate buffered saline
EIA	enzyme immunoassay
$NaHCO_3$	sodium bicarbonate
BSA	bovine serum albumin
H_2SO_4	sulphuric acid
ADR	adverse drug reactions

Chapter One: Literature Review

Early work in animal population management

Due to shrinking habitat and population over-abundance, the humane control of certain wildlife populations is necessary [1]. In the context of South Africa, most biologists agree that some form of intervention is necessary when managing African elephant (*Loxodonta africana*) populations due to the destruction of habitat that may arise if elephant numbers are left unchecked [2]. Traditional lethal control programmes, however, are not always legal, wise, safe, or publicly acceptable; thus, alternative approaches are necessary [1, 3]. Efforts at controlling wildlife overpopulation by means of contraception began as early as the 1950s, and most research involved natural and synthetic steroids, or a variety of non-steroidal compounds [4]. Much of the initial work in this area, whilst pharmacologically successful, ended in failure for a variety of reasons including toxicity, passage through the food chain, adverse effects on social behaviours and health risks in pregnant animals [5]. Immunological approaches were considered for use in wildlife as early as the 1980s and the earliest recorded attempts at wildlife immunocontraception included an anti-gonadotropin releasing hormone (GnRH) vaccine tested in feral horses (*Equus ferus caballus)* in 1986 [6] followed by a native anti-porcine zona pellucida (pZP) vaccine tested in domestic and captive feral horses [7]. To date contraceptive success has been achieved in more than 85 different wildlife species with pZP vaccine, at the level of both the individual animal and the population [8]. The horse has been the focus of the vast majority of pZP immunocontraception studies [7].

Equine reproductive physiology and ovarian function

The horse is a seasonally polyoestrous species in temperate locations, with onset of the breeding season occurring in spring; associated with seasonal cues which serve to synchronise an endogenous reproductive rhythm to winter and summer periods [9]. The mare's circa-annual reproductive cycle is made up of four distinct phases. The anovulatory period of the mare can be differentiated into an autumn transitional phase, a mid-

anovulatory period and a spring transitional phase, bringing the mare back into cyclic ovarian activity [10]. The physiological breeding season or ovulatory season occurs from April to September in the northern hemisphere and correspondingly from October to April in the southern hemisphere [11]. Most mares undergo an anovulatory period outside of this, the duration and occurrence of which is associated with the age and physiological status of the mare [12]. Younger mares and mares that have previously lactated undergo a long and systematic winter anoestrous but winter ovarian inactivity is observed in only about half of other mature mares [12]. In fillies, puberty occurs at 12-18 months. This is again influenced by season [10, 13]. Senescence in old mares is rarely seen and most mares continue to cycle independent of age [10]. The oestrous cycle is the period between two consecutive ovulations [9, 14, 15]. During the breeding season in spring and summer, average oestrous cycle length is about 22 days with 5-7 days of oestrus [10]. Oestrous cycle length is also affected by reproductive stage and breed [16]. During the oestrous cycle, the uterus, cervix, vagina and endometrium of the mare undergo pronounced changes related to variations in the endocrine milieu. They can easily be differentiated by clinical examination. The equine oestrous cycle is a combination of a follicular phase, or oestrus (Figure 1-1: Pro-oestrus), and a luteal phase, or dioestrus (Figure 1-2: Metoestrus).

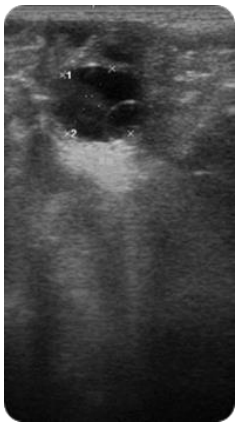


Figure 1-1 Follicular phase of the oestrous cycle: Follicle imaged *via* transrectal ultrasound of the ovary using a 5 MHz linear probe. D1: 23.7 mm; D2: 20.7 mm; Depth: 111 mm

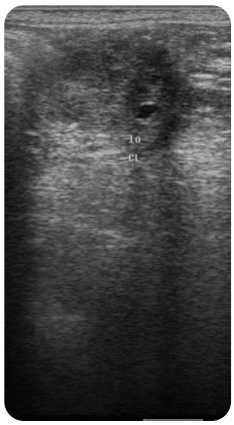
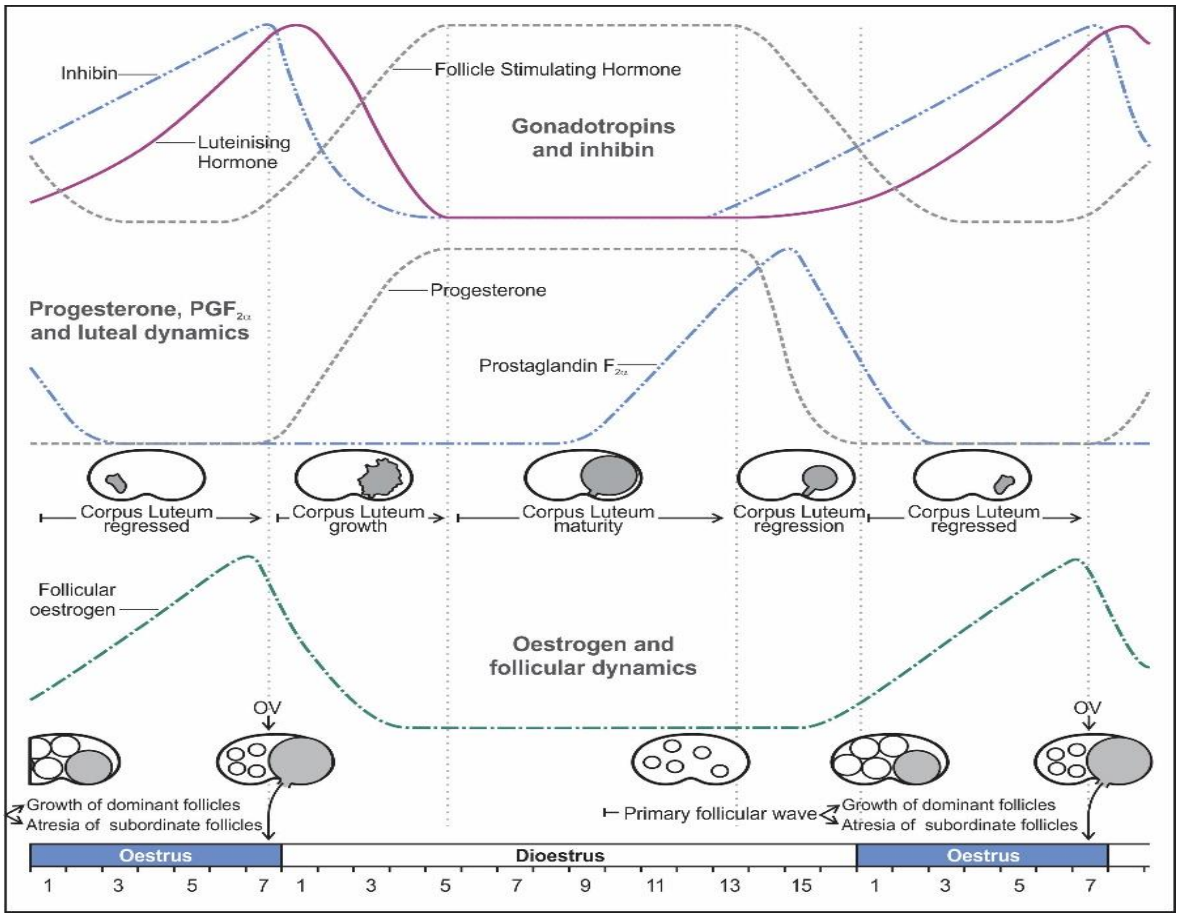


Figure 1-2 Luteal phase of the oestrous cycle: Corpus luteum imaged *via* transrectal ultrasound of the ovary using a 5 MHz linear probe. D1: 49.2 mm; D2: 38.1 mm; Depth: 111 mm

Follicular development within the ovary involves growing, ovulating, and anovulatory haemorrhagic or regressing follicles, with approximately 40,000 primordial follicles and 100 growing follicles [15]. Developing follicles are represented by early-stage, pre-antral follicles and later-stage, antral follicles. The latter, more mature stages are of clinical importance because they are the main source of reproductive steroids (oestrogens, progestogens, and androgens). These influence follicle growth and regression, uterine and cervical structural and functional changes, oestrous behaviour, oocyte development and ovulation. In the mare, growth of antral follicles occurs in wave-like patterns, categorised as major (primary and secondary) and minor waves depending on whether the largest follicle of a wave reaches ≥30 mm in association with follicle selection (major wave) or <30 mm without selection (minor wave) [17, 18]. During the anovulatory period follicular growth is minimal, marked by few follicles of a diameter >15 mm and the maximal diameter of the largest follicle does not exceed 16 mm [19]. A dominant follicle does not develop during that time. The beginning of the spring transitional period is characterised by the development of 1-3 anovulatory follicular waves before finally ovulation occurs. The spring transitional period has a variable length that ranges from 30-90 days. Its beginning is characterised by the re-initiation of follicular deviation, i.e. the development of a dominant follicle reaching a size between 20-30 mm in diameter. In addition, an increasing number of follicles with a diameter >15 mm occur [10, 19]. In general, follicular wave emergence during the oestrous cycle is associated with the growth of a group of 5-10 antral follicles (4-6 mm) within 2-3 days of one another. During the common growth phase, most follicles increase in diameter 2-4 mm/day until the largest follicle reaches 20-25 mm near the end of the common growth phase. Afterwards, either all follicles (minor wave) or all but the largest follicle (major wave) begin to gradually regress. In general, spontaneous ovulation of a single preovulatory follicle of a major wave occurs when the dominant follicle reaches about 40 mm in diameter; maximum diameter of the preovulatory follicle is related, in part, to season, breed, type of mare and number of preovulatory follicles [20].

Endocrine regulation of the oestrous cycle stems from hormones produced by the pineal gland, hypothalamus, anterior pituitary gland, ovaries, and endometrium, referred to as the hypothalamo-pituitary-gonadal axis. The hypothalamus produces GnRH, which is released in brief pulses into the hypothalamo-pituitary portal system, and stimulates the

synthesis and release of the gonadotropins; follicle stimulating hormone (FSH) and luteinising hormone (LH), from the anterior pituitary gland [21]. The ratio of circulatory LH/FSH is influenced by GnRH pulse frequency and by physiologic feedback from inhibin, oestrogens, and progesterone released from the ovaries. Low-frequency pulses of GnRH stimulate synthesis and release of FSH; and high-frequency GnRH pulses stimulate synthesis and release of LH [21]. During dioestrus (following ovulation) progesterone released from the corpus luteum (CL) suppresses the high frequency of GnRH release. Maximal progesterone concentrations are reached on day eight of the oestrous cycle and functional luteolysis occurs around day 15 and is initiated by endometrial secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the absence of a viable embryo [10]. Oestrus is characterised by the presence of follicles at different stages of development, and the simultaneous increase in the secretion of estradiol-17**β** (E2). The GnRH pulse frequency is markedly increased in response to rising concentrations of E2. Follicle stimulating hormone stimulates follicular recruitment and growth, and induction of LH receptors, and LH stimulates follicular and oocyte maturation, production of E2, ovulation, and development of the CL [15] (Figure 1-3).



OV: Ovulation

Figure 1-3 Hormone profiles and temporal relationships with follicular and luteal development during the oestrous cycle in the mare [Adapted from Ginther OJ (1992) Reproductive Biology of the Mare: Basic and Applied Aspects (2nd ed). Cross Plains, WI USA: Equiservices Publishing]

Anti-Müllerian hormone (AMH), a peptide growth factor and member of the large transforming growth factor **β** (TGF**β**) family of growth and differentiation factors, appears to play an important role in the regulation of both the number of growing follicles and their selection for ovulation [22]. It is first expressed in the granulosa cells which surround recruited primordial follicles [14] and continues to be expressed in the growing secondary follicles and tertiary follicles until they have reached the size and differentiation state at which they are to be selected for dominance by the action of pituitary FSH [22]. In the mare, circulating AMH concentrations are reportedly primarily influenced by the number of antral follicles between 6-20 mm in diameter [23].

Ovarian ageing describes the decline of the ovarian reserve with age in addition to parameters such as oocyte quality [22]. In the mare the decline of the follicular reserve with age continues until reproductive senescence is reached but this occurs at variable ages [23]. The number of primordial follicles is an important determinant for the ovarian reserve, although it is difficult to measure. However the number of growing follicles is correlated to the size of the primordial follicles stock. A marker that reflects all follicles that have made the transition from the primordial follicle pool to the growing pool may be a good indirect indication of the quantitative aspects of the ovarian reserve. Antral follicle count (AFC) using transvaginal ultrasonography in women gives an indication of the number of growing follicles and therefore, by extension, of the size of the primordial follicle pool [22]. In mares, AFC is lower in old compared to middle-aged or young mares and plasma AMH concentrations are highly predictive of AFC in middle-aged and old mares [23]. Additionally, measurements of AFC and AMH are highly repeatable within and across oestrous cycles [23]. Since AMH is produced by the growing antral follicles in the ovary up to the selection stage it supports AMH as a consistent and useful biological marker of ovarian function [22, 23].

Zona pellucida proteins

In mammals, the zona pellucida (ZP) is a glycoproteinaceous translucent matrix that surrounds the oocyte and plays a critical role in the fertilisation process [24]. It is primarily synthesised and secreted by follicles during development [24]. The ZP serves as a 'gate-keeper' by acting as a species-selective substrate during binding of the spermatozoa to the ZP of the oocyte [25]. The ZP functions in the induction of the acrosome reaction (AR), the prevention of polyspermy, and immunological protection of the early embryo [26]. The ZP is also involved in communication between the oocyte and its surrounding granulosa cells in the developing follicle [27].

In mammals, the ZP matrix is comprised of either three or four glycoproteins. In mice, ZP is composed of three glycoproteins designated as zona pellucida glycoprotein-1 (ZP1), -2 (ZP2) and -3 (ZP3) [24]. The ZP matrices of pig, cow and dog oocytes are also made up of three glycoproteins but instead of ZP1, zona pellucida glycoprotein-4 (ZP4) is present. The

ZP matrices in rat, hamster, bonnet monkey and human comprise all the four glycoproteins- ZP1, ZP2, ZP3 and ZP4 [24]. In the mare, ZP (eqZP) has been shown to consist of three glycoproteins [28]. The formation of the eqZP is a collaborative process between the oocyte and cumulus cells, and the final external layer of eqZP is a function of the cumulus cells. The collaborative secretion of ZP glycoproteins is also seen in pig, cow, dog, rabbit, marmoset and rhesus monkey. In other species, only the oocyte secretes ZP. The temporal secretion of ZP glycoproteins is variable between species relative to oogenesis [28].

The ZP glycoproteins have generally been conserved throughout the course of evolution. At the amino acid level, human ZP2 and ZP3 shows 57%, 64% and 94.2% and 67%, 74% and 93.9% homology with mouse, pig and bonnet monkey ZP2 and ZP3, respectively. Human ZP1 has 64% and 96% in common with mouse and bonnet monkey ZP1 (pseudogene in pig) and ZP4 has 68% and 92% identity with pig and bonnet monkey ZP4 (pseudogene in mouse). In addition to sequence homology of respective ZP glycoproteins among various species, there is an overall conserved backbone structure of ZP proteins [24]. Zona pellucida glycoprotein-3 acts as the putative primary sperm receptor and is responsible for acrosome induction in mice, whereas in humans (in addition to ZP3), ZP1 and ZP4 also induce the AR [24]. In pigs ZP4, together with ZP3 are involved in sperm binding [24]. It has been suggested that multiple mechanisms of sperm binding and penetration exist, in order to maximise the likelihood of success and thus aid in proliferation of the species [29].

Immunocontraception

Immunocontraception is a process that induces infertility through stimulation of the immune system and the production of antibodies in response to vaccination against endogenous components critical to the reproductive process. A number of antigens have been proposed as targets for immunocontraception. These include peptide hormones, oocyte and sperm proteins and other molecules associated with fertilisation and early embryonic development [28].

A distinct advantage of ZP-based immunocontraception is that these vaccines appear to have neither hormonal nor other deleterious side effects [29]. Reproductive cyclicity is

presumed to remain undisturbed in the short term, resulting in minimal sex-related behavioural or herd effects, as the inter-oestrous interval would be unaffected. Thus, herds of animals whose behaviour and herd integrity are based on intact reproductive cyclicity are unaffected by ZP immunocontraceptives. In both non- and seasonally breeding animals, however, it should be noted that repeated cycling throughout the year is not the norm. Furthermore, the ZP glycoproteins are structurally unique and have very little homology with somatic proteins, and so provide ideal targets for immunocontraception [29].

The ZP protein structures are highly conserved across several phyla and this facilitates potential interspecies use of native proteins [28, 29]. Zona pellucida proteins derived from pigs' ovaries are readily available in high numbers as a by-product of commercial pig slaughter houses. This conserved homology of the ZP glycoproteins has allowed the use of antigens derived from the pig to be applied in a number of species [28, 29, 30, 31]. In addition, the use of heterologous ZP obviates the necessity for a homologous source of antigens, which for most domestic and exotic species would be impossible [28].The immune response generated against pZP in combination with a suitable adjuvant cross-reacts with endogenous ZP glycoproteins in targeted females. Differences in the degree of homology between various species' ZP proteins may differ, however, which has consequences for immune recognition.

The precise molecular mechanism for the immunocontraceptive effect of pZP remains to be determined. Possible molecular explanations for the biochemical mechanism of immunocontraception could include blocking of sperm binding sites or structural changes in the ZP [28]. Anti-pZP antibodies may affect sperm-oocyte binding, either by interfering with receptors directly or through binding with nearby epitopes [32]. Similarly, anti-pZP antibodies could interrupt the AR, oocyte activation, sperm penetrations, or induce release of cortical granules resulting in premature hardening of the ZP [29]. The carbohydrate moieties have been identified as a potentially important functional component of ZP glycoproteins [28]. The glycosylation pattern of binding of distinct carbohydrate moieties to amino acids, giving rise to the glycoprotein structure of the ZP may well play a role in the subsequent immunogenicity of pZP immunocontraception. Deglycosylation of pZP3 was found to decrease its antigenic and immunogenic potential [29].

The use of ZP proteins for contraception relies on the use of suitable adjuvants as ZP proteins are weak antigens [33]. Freund's adjuvants in the form of Freund's complete adjuvant (FCA) [7], Freund's incomplete adjuvant (FIA) [34] and Freund's modified complete adjuvant (FMCA) [33, 35] have all been successfully used for inclusion in vaccine formulations for immunocontraception in free roaming African elephants (FMCA and FIA only) and in captive and feral horses.

An alternative to ZP-based immunocontraceptives are anti-GnRH vaccines. Anti-GnRH vaccines trigger production of antibodies that neutralise endogenous GnRH, which prevents receptor binding and activation of pituitary gonadotrophs. The suppression of gonadotrophin secretion causes reproductive quiescence characterised by cessation of cyclical ovarian activity in the mare [36, 37]. The commercially available anti-GnRH vaccine Improvac® is comprised of a synthetic, incomplete analogue of natural gonadotrophin-releasing factor (GnRF) which is covalently linked to a carrier protein, formulated with an aqueous non-oil based adjuvant into a ready-to use-injection [38].

Porcine zona pellucida immunocontraception in the horse

The management of feral horse populations has become an increasingly important challenge in many countries, including populations in Australasia, North America, Europe and Africa. Feral horse herds, like those of wild horses, are usually made up of small bands led by a dominant mare, containing additional mares, their foals, and immature horses of both sexes and have a complex social structure. These populations impact habitats in different ways including impacts on soil, vegetation (including overgrazing), other species and conflicts with agricultural producers [39]. Estimates for Australia indicated a feral horse population of 400,000 [40]. There are an estimated 66,976 feral horses under the management of the Bureau of Land Management in the USA, with appropriate management levels approximated at 23,771 [41]. Fertility control through immunocontraception is thus well indicted for the non-lethal management of such populations.

The horse is one of the best-studied mammals as a model and as a target species [7, 42] and an ecological model [43] for immunocontraception. The ZP vaccine was first patented as a contraceptive agent in 1976 by RBL Gwatkin for Merck and Company [34], and the first field trials in free-roaming feral horses followed in the late 1980s [7]. In the early years, studies focused predominantly on whether ZP immunisation was effective as a contraceptive and the number of inoculations required to achieve infertility. Subsequent studies investigated additional aspects of immunisation including long-term side effects on health and improving efficiency of delivery methods (Table 1-1).

Liu *et al*. [7] first demonstrated in principle the efficacy of pZP in equids by suppressing fertility in 12 of 14 captive domestic and free-roaming mares following four hand injections of pZP with aluminum hydroxide gel and/or FCA and FIA at 2-4 week intervals, with a fifth booster 6-9 months after the last injection. This study highlighted individual variability in antibody levels and also the duration of a detectable immune response. They also demonstrated that anti-pZP antibody titres >64% (expressed as an absorbance ratio of a positive reference standard) were associated with effective contraception and that a decline in contraceptive effect correlated with a decline in antibody titres.

A study in 1990 [44] used dart guns to remotely inject 26 free-roaming feral horse mares of known high fertility at Assateague Island National Seashore (ASIS), Maryland, USA with a priming dose of 65-100 µg pZP in FCA and either one or two boosters of pZP in FIA at three-week intervals. Analysis of urinary steroids indicated that only one of 26 sampled mares was pregnant five months after the final treatment. Of the 26 treated mares, 14 were boosted again a year later with a single remotely delivered dose of pZP in FIA. Only one of these 14 boosted mares produced a foal the following year, compared to 10 of 22 control mares [45]. Follow-up studies at ASIS over the next six years demonstrated foaling rates of 3.8% among pZP treated vs. 46.2% in untreated mares [46].

A 1997 report of work conducted in Nevada, USA, [47] used 127 mares in three treatment groups; pZP, control and no treatment. The pZP treatment group received an initial dose of 65 µg pZP in FCA and a booster four weeks later of 65 µg pZP and FIA. Untreated mares (n=63) produced 34 foals (53.9%), saline-injected mares (n=20) produced 11 foals (55%)

and mares receiving two inoculations about one month apart (n=44) produced two foals (4.5%).

A further 1997 study of feral horses in Nevada [48] that used three different immunocontraception protocols (pZP and FIA followed by pZP and FIA booster one month later; pZP and FCA; or pZP, FCA and additional pZP in control-release microspheres) determined that 4.5% of mares were reproductively successful following the two pronged protocol: initial treatment followed by a booster treatment.

Thereafter, in 2001, Turner *et al*. [49] treated 222 mares with either two inoculations of pZP in FCA (primary) and in FIA (booster), two inoculations using pZP in FCA and carbomer adjuvant (primary) and in FIA (booster) or one inoculation using pZP in FCA with a second pZP dose in carbomer adjuvant in controlled-release polymer microspheres, with resultant reproductive success rates of 12.8%, 10.6% and 11.3% respectively, highlighting the efficacy and potential of a simpler one dose protocol.

Investigations of the use of FMCA for adjuvanting purposes showed no significant differences in antibody titres between mares hand-injected with 65-100 μg pZP in FMCA followed by a booster of 65-100 μg in FIA and mares treated with 65-100 μg pZP in FCA followed by a booster of 65-100 μg in FIA. Seven of eight mares treated with pZP and FMCA remained above the contraceptive titre threshold after 10 months [33].

At a population level, an overview of mares on ASIS (1993-2006) treated with one or two initial shots and one or more annual boosters demonstrated a 10% foaling rate, with FMCA replacing FCA for initial injections beginning in 2002 [50]. These mares were initially inoculated as two year olds, with booster treatments at three and four years old, were then allowed to produce up to three foals and following this were re-admitted into the immunocontraception programme until death. Zero population growth was achieved within two years, with an initial population decline becoming evident within eight years, and a total decrease of 22.8% seen by year 11. Prior to initiation of the pZP inoculation programme, this ASIS herd had a foaling rate of 57.1 ±3.9% and an overall annual population growth rate of 8% [50].

Currently the most widely used form of pZP vaccine for horses is ZonaStat-H, which is similar to the original formulation [7] and is registered as a pesticide with the Environmental Protection Agency in the USA for use in free-roaming horses and burros only [51]. Other recent formulations have included pZP-22 which used controlled-release technology to replace both the initial and annual booster injection of pZP vaccine [52] and SpayVac® which makes use of multilamellar liposomes for additional adjuvanting properties [53, 54].

Reports of ovarian disruption following pZP immunocontraception were limited in the early years [Table 1-1]. Liu *et al*. [7] reported no abnormalities on histological examination of ovarian sections following 18 months of treatment (primary and five booster inoculations) in three of four ovariectomised mares. The later work of Kirkpatrick *et al*. [55] attempted to assess the long term effects of the pZP vaccine on ovarian function by monitoring urinary oestrone conjugates and non-specific progesterone metabolites from immunised feral mares in a three year vaccination protocol. This study suggested that pZP treatment for three years altered ovarian function. A later study [56] assessed faecal ovarian steroid profiles following pZP treatment in feral mares over two years and noted a high incidence of apparent ovulation failure, however, no link was demonstrated between this observation and the duration of pZP treatment. The more recently reported effects on ovarian function in the mare suggests this may be an inherent feature of ZP-based immunocontraception. Noticeable effects on ovarian activity occurred within five weeks of treatment using pZP formulated with Freund's adjuvants [57]. Effects in the short term have also been reported with differing formulations including both SpayVac [51, 54] and pZP-22 [52].

Recombinant zona pellucida vaccines

Successful vaccines induce an effective and sustained immune response, have minimal side effects and are produced cost-effectively on a large scale. Despite their reported efficacy, commercial successes for immunocontraceptive vaccines have been relatively few ascribed

primarily to poor immunogenicity of peptide self-antigens and difficulties in cost-effective production [58]. Several shortcomings associated with pZP vaccines stem from the use of the full complement of ZP proteins derived from a native source and it has been suggested that the continued successful application of ZP-based immunocontraception is contingent on the development of an effective recombinant formulation [1, 57, 59].

Recombinant ZP proteins have several compelling properties including improvements in antigen purity and structure [28]. The expression of recombinant proteins for vaccine antigen production using an *Escherichia coli* (*E. coli)* platform is well documented and benefits include high speed and yield of production, moderate production costs and scale-up capacity, no glycosylation and limited contamination risks in the form of endotoxins which can be addressed in purification processes [60].

Recently, a recombinant ZP (reZP) vaccine based on the expression of porcine ZP3 and ZP4 in *E. coli* was applied in mice [61]. To boost the antigenic potential of these proteins, ZP3 and ZP4 were expressed together with promiscuous T-cell epitopes of tetanus toxoid and bovine Rnase, respectively (TT-KK-ZP3 and bRNase-KK-ZP4). Promiscuous T-cell epitopes bind to various major histocompatibility complex (MHC) molecules, provide T-cell help that can be employed to generate humoral immune responses against self-proteins and are likely to elicit an increased immune response [61]. Following immunisation in a murine model, higher antibody titres were induced by TT-KK-ZP3 and bRNase-KK-ZP4 than their respective proteins alone. Recombinant TT-KK-ZP3 and bRNase-KK-ZP4 also elicited higher T-cell proliferation as compared to recombinant ZP3 and ZP4 thereby confirming that the promiscuous T-cell epitope of TT and bRNase also contributed to T-cell proliferation. The recombinant ZP proteins were found to be similarly capable of boosting the immune response as a native pZP treatment. Serum antibodies to the recombinant vaccine were bound to the mouse oocyte ZP complex and inhibited *in vitro* fertilisation. Furthermore, no abnormalities were detected on histological examination of mouse ovaries 120 days post-treatment following *in vivo* use of the reZP vaccine. It is worth noting, however, that any ovarian effects may have dissipated by this time.

A 2017 study [57] that compared the effects of a native pZP and a reZP vaccine both formulated with Freund's adjuvants (FMCA and FIA), on pregnancy outcome and ovarian function in a horse model demonstrated a pregnancy rate of 0% and 57%, respectively. Prolonged anoestrus was noted in 86% and 14% of pZP and reZP treated mares, respectively. No significant contraceptive effect was produced by the reZP vaccine, however, highlighting the necessity for further investigation of reZP vaccines as an alternative contraceptive in the mare. A similar study in donkey jennies [62] reported the effects of pZP and reZP (a different source to the above) on oestrous cyclicity and fertility. The vaccines were similarly formulated to that of previous pZP and reZP vaccines administered in horses, using Freund's adjuvants (FMCA and FIA). Seven of 9 (reZP), 6/8 (pZP) and 0/8 (control) jennies entered anoestrus within three months after the final vaccination treatment. No jennies in either of the reZP or pZP treated groups became pregnant compared to 6/8 control jennies. The results of these studies are encouraging, underlining the need to continue these investigations.

Adjuvant formulations for immunocontraception

Adjuvants have essentially five modes of action: immunomodulation (Th1/Th2 balance ($CD4^+$ T-cells)), presentation, cytotoxic T-lymphocyte (CTL: $CD8^+$ T-cells) induction, targeting and depot generation [63]. An immune response to a vaccine formulation relies on an interplay between innate and adaptive immune responses. The innate immune cells express various pathogen-recognition receptors (PRR) recognising infectious or foreign agents. More recently several new families of PRR have been identified including TLR (toll-like receptors), nucleotide oligomerisation domain-like receptors (NLR) and retinoic acid-inducible gene1 like-receptors (RLRs) [64]. The TLR pathways form a primary danger signal response to the presence of pathogens detected both internally and externally by tissues and cells [65]. In addition to the self/non-self discrimination against infection, danger signals from damaged cells can trigger activation of the immune system as a result of the release of molecules associated with tissue damage at the injection site. These non-infectious damage signals are referred to as damage-associated molecular patterns (DAMP) to differentiate them from pathogen-associated molecular patterns (PAMP) [64]. The non-antigen specific innate system damage signals respond to the presence of a

foreign substance or pathogen rapidly and signal the antigen-specific adaptive immune system. Efficient antigen presentation by MHC molecules on antigen presenting cells (APC) is important for the induction of the adaptive immune response [64]. The adaptive immune system consists of T- and B-lymphocytes mediated by APC, such as dendritic cells that recruit $CD4^+$ T-helper cells. Helper T-cells stimulate B-cells to mature into antibody producing cells [66].

To elicit the required immune response vaccine formulation research focuses on the additive potential of immunological adjuvants or carrier systems, such as saponins, lecithin, aluminum compounds and killed mycobacterium in combination with the antigen. This is of paramount importance for antigens with poor immunogenic capacity which includes pZP [58]. The ideal adjuvant should maximise vaccine immunogenicity without compromising tolerability or safety [67]. Freund's adjuvants have been standard components of induction protocols for many experimental animal models of disease [68]. Freund's complete adjuvant, facilitated potentially by both a TLR [69] and NLR recognition pathway [64] for an innate response, was shown to induce dendritic cell maturation, essential for induction of adaptive immune responses, and results in the enhanced ability of APC to induce T-lymphocyte activation and differentiation [64, 70]. Freund's therefore acts as an adjuvant for combined $CD4^+$ and $CD8^+$ T-cell immunity and have also been shown to act by depot effect to generate prolonged and sustained high antibody titres [70].

Freund's adjuvants have been widely used for ZP-based immunocontraception, associated with high antibody titre responses and subsequent contraceptive efficacy [7]. The use of FCA has raised concerns because of potential side effects [33], associated with toxicity, injection site reactions and false-positive Tuberculosis (TB) testing results [63]. Early studies of ZP-based immunocontraception in the horse rarely reported on injection site reactions. As investigations into alternative vaccine formulations developed injection site reactions were monitored more frequently and intensively. A 2005 immunocontraception study in mares that compared the antibody titres resulting when using FCA and FMCA for pZP

reported a single injection site reaction in a mare treated with FMCA. [33]. The methods for detection of injection site reactions were, however, not described. More recently, a study in mares investigated pZP and reZP vaccines formulated with FMCA and FIA and reported injection site swelling and/or palpable changes in muscular density affecting >95% of both treated and adjuvant control mares. Several developed overt sterile abscesses and this was observed more frequently in the reZP treated mares [57]. This study intensively monitored injection sites *via* palpation of the approximate injection sites and transcutaneous ultrasonography of the injection site area. A similar study in donkey jennies, that compared the contraceptive efficacy of pZP and reZP vaccines, also formulated with Freund's adjuvants (FMCA and FIA), produced similar injection site reactions in both treated and adjuvant control groups. Similarly, more severe reactions were observed in the reZP treated group [62]. Bechert *et al.* [54] also reported localised reactions in mares to FMCA formulations with pZP. These reactions were assessed through dissection and varied in intensity and duration and included abscessation [54]. Adjuvants, in general, have been associated with lesions resulting from an inflammatory response [36]. Whilst many of the conventional immunological adjuvants such as Freund's, bacterial toxins and non-purified crude agents (e.g. lipid A) commonly induce strong stimulant effects, they may also induce adverse side-effects upon administration. Consequently any vaccine incorporating these adjuvants is currently unlikely to be approved by regulatory authorities [58, 71]. Identification of alternative adjuvants for ZP-based immunocontraception is thus a well-recognised need. Recent developments in the formulation and delivery of acellular antigens together with investigations of newer immunological adjuvants and development of novel combination adjuvants should enhance progress in this area [58, 64].

The future of vaccine adjuvant research is heading toward developing novel combination adjuvants that consist primarily of PRR-agonists (such as TLR-3-agonists) and particulate adjuvants [64]. Montanide™ Pet Gel A, Quil-A® (a purified saponin immune stimulating complex (ISCOM) adjuvant) or Addavax™ (a squalene-oil-in-water adjuvant) may provide an alternative particulate adjuvant to Freund's adjuvants for ZP-based immunocontraception. Squalene is an oil more readily metabolised than the paraffin oil used in Freund's adjuvants and squalene oil-in-water emulsions, such as Addavax™, elicit both cellular and humoral immune responses [72]. The ISCOM vaccines, such as Quil-A®

are known to induce long-lasting antibody responses, a balanced Th1/Th2 response and a moderate induction of CTL. Quil-A® is used in a wide variety of veterinary vaccines [73]. Montanide-based adjuvants, as immunomodulators, have been used in both veterinary and human vaccines and possess a good safety profile and have been shown to work with a depot effect [74, 75]. Pet Gel A has also been used in association with both pZP and reZP vaccines [61]. Many immunological adjuvants signal *via* PRR or act as ligands for innate immune receptors [64]. With TLR-3 signalling there is a local cytokine burst that is both microenvironment- and cell-specific resulting in a local inflammatory response. This is adaptively selected to provide the greatest chance of infection control for a specified tissue [65]. This TLR-3 stimulation is not uniform, however, and may differ in specific tissue microenvironments [65]. In contrast to TLR-agonists, particulate adjuvants are not always recognised by specific PRR but they still induce adaptive immune responses [64]. The immunostimulator PRR-agonist, VacciGrade® Poly (I:C), invokes both Th1 and Th2 responses [64]. Poly (I:C) operates *via* combined TLR-3 [75] and RLR (MDA-5) [65] signalling and may assist with MHC class II expression of T-helper cell antigen presentation and recognition that can be employed for humoral immune responses against self-proteins [74]. Poly (I:C) also mobilises CD8+ T-cell involvement [64]. A safe and rapidly induced integrated immune response (antibody, $CD4^+$ and $CD8^+$ T-cell) was reported for a variation on the adjuvant combination of Pet Gel A and Poly (I:C) when formulated with a human tumour self-antigen (Montanide ISA-51 (the human equivalent of FIA) and the TLR-3-agonist Poly IC:LC) [75]. A vaccine formulation containing Poly (I:C) for use in animals was confirmed to be highly efficacious and safe in cotton rats [77].

An important limitation potentially associated with choosing a combination adjuvant approach is the increased expense and complexity of manufacturing and formulating a vaccine antigen with multiple adjuvants. Additionally the potential for more adverse effects in the treated animal should be considered. However the individual components of a combination adjuvant can often be used at a lower concentration than when administered as a stand-alone adjuvant. This may actually reduce side-effects and production costs. A third concern is that of achieving regulatory approvals for novel adjuvants and combination adjuvants in particular as safety is a primary consideration for adjuvant approval in human and animal vaccines [74]. Combining different adjuvants may result in potent formulations

that can enhance the quality and quantity of the immune response. These combinations, however, may have complex mechanisms of action [64]. An understanding of the mechanisms of action of adjuvants and any additive actions when utilising adjuvant combinations in vaccine formulations will improve insight into vaccines and their safety [64].

Ovarian suppression subsequent to zona pellucida-based immunocontraception

Effect on ovarian function, both in the short and long term, is an important determinant for the selection of a contraceptive agent [28]. The presumed immunocontraceptive mechanism of pZP in the horse involves antibody binding to the ZP sperm receptor sites and subsequent prevention of sperm-oocyte binding and fertilisation. Based on this supposition, pZP immunisation should not affect the hypothalamo-pituitary-gonadal axis, thereby permitting continuation of cyclical ovarian activity [7, 29] and associated behaviours [28]. However ZP-based immunocontraception has reportedly caused irreversible ovarian damage due to oophoritis in some species (rabbit, dog, and baboon) [78, 79, 80].

Previously, long-term vaccination effects on ovarian function in the horse were described [43], but any observed effects appeared to be reversible with time [28]. More recently, short term ovarian effects have been documented subsequent to pZP vaccination in the horse [54, 57] (Table2-1). When a mare is successfully immunocontracepted, high circulating levels of anti-pZP antibodies are measured [7]. An alternative explanation for the immunocontraceptive effects of pZP immunisation may be that during the deposition of fibrils of eqZP, anti-pZP IgG molecules might be intercalated into the resultant matrix. This combination of the IgG and eqZP matrix could cause anti-pZP antibodies to act as cross-linkers (e.g. autoimmune reactions), mimicking the zona block. These zona-blocked oocytes would not be fertilised with this effect persisting until systemic levels of IgG fall low enough to allow conception to take place. This mechanism appears most relevant in species including the horse, where cumulus cells participate in secreting ZP [28].

The hypothesised mechanisms for ZP-associated ovarian dysfunction were recently reviewed [81]. These included oophoritis associated with the high glycosylation rate of ZP proteins, oophoritis due to a CTL response, contamination with non-ZP ovarian proteins resulting from inclusion of the full complement of ZP proteins from a native porcine source, an association with Freund's adjuvants and finally as a species-dependent manifestation.

Immunisation of marmoset monkeys with deglycosylated pZP3 resulted in lower levels of ovarian dysfunction, poorer antibody responses and reduced contraceptive efficacy compared to treatment with glycosylated pZP3 [82]. Immunisation of mice with reZP3 produced in an insect expression system (without glycosylation) had neither anti-fertility nor ovarian effect despite eliciting an antibody response [83].

In mice, the detection of ZP-specific $CD4^+$ T-cells, without associated antibodies has been implicated in oophoritis [84] and these cells targeted ovarian interstitial tissues but spared developing follicles thus maintaining ovarian function [85]. When ZP-specific antibodies and $CD4^+$ T-cells were present these targeted developing follicles causing their ablation and resulted in ovarian atrophy [85]. A CTL response to ZP-based immunocontraception was only reported in 2018 by Joonè *et al.* [86]. Significant pZP-specific $CD4^+$ and $CD8^+$ T-cells were detected in mares treated with both pZP and reZP vaccines compared to pre-treatment samples. Clinical measurements of cyclic ovarian activity were, however, negatively correlated with $CD8^+$ T-cell proliferation in the pZP treated mares alone [86]. The pZP formulation in that study was also associated with high levels of contraceptive efficacy and ovarian suppression [57]. Joonè *et al.* suggested ZP-antigen uptake *via* a non-classical pathway for presentation by MHC class I and subsequent CTL stimulation [86]. The reZP treated mares showed higher $CD4^+$ and $CD8^+$ T-cell proliferation than pZP treated mares but despite this had lower antibody titres and weaker evidence of ovarian suppression and contraception [57]. This was attributed to several possible causes including differences in the particulate nature of the vaccines, affecting antigen uptake and subsequent T-cell stimulation and differences in levels and patterns of glycosylation [86]. Joonè *et al.* also hypothesised that despite higher $CD4^+$ and $CD8^+$ T-cell proliferation in reZP treated mares, that it was a CTL response to the presence of pZP2 and extra-ZP ovarian antigens in the native pZP vaccine (lacking in the reZP vaccine) that resulted in the increased levels of ovarian suppression and contraceptive efficacy. Nevertheless these results suggested a role for $CD8^+$ T-cells in ovarian suppression due to pZP immunocontraception in mares [86].

An association of Freund's adjuvants with ovarian dysfunction in ZP-based immunocontraception was proposed based on a disturbance of ovarian function in an adjuvant only control group in a primate study [86]. Later reports contradicted this by highlighting ovarian dysfunction in ZP-based immunocontraception studies that utilised non-Freund's adjuvants [79, 88].

Abnormal cyclicity subsequent to ZP-based immunocontraception has been reported in all but two of the nine most commonly studied species groups for ZP-based immunocontraception, namely in mice, rabbits, non-human primates, dogs, sheep, deer and horses [81]. African elephants and domestic cats have also been investigated as targets for ZP-based immunocontraception. In cats ZP-based immunocontraception was ineffectual [89]. In African elephants reported investigations of ovarian effects associated with ZP-based immunocontraception were limited [90, 91].

Zona pellucida-based vaccines fulfils many of the 'gold-standard' prerequisites for immunocontraception, however, while its efficacy is well documented, any longer term ovarian effects, particularly with repeated use, remain undefined [81]. Any associated ovarian suppression, at least in some species, may be an inherent feature of efficacious ZP immunocontraception.

Quantifying ovarian effects of zona pellucida-based immunocontraception

The suggestion that ovarian suppression could be an inherent feature of effective ZP-based immunocontraception across all species requires further investigation. To this end, AMH may provide a novel tool for the assessment of ovarian function during ZP-based immunocontraception [92]. Joonè *et al.* [92] reported marked suppression of AMH in mares during pZP immunocontraception. Together with associated clinical findings this suggested enhanced, reversible suppression of ovarian follicular development and, or follicular function during pZP immunocontraception.

Wildlife populations requiring contraception are typically managed under extensive conditions and, therefore, practical, minimally-invasive in-the-field methods with limited

intervention opportunities are commonly required for monitoring effects. In free-ranging species the primary goal is the creation of new knowledge of species-specific endocrine mechanisms and the subsequent application of this directed towards effective management and conservation of the species [93]. The measurement of AMH subsequent to immunocontraception in free-ranging species could potentially be informative for monitoring ovarian function [1]. In intractable species, single-non-invasive measurements are indicated, however, a reduction in the number and type of interventions may be equally advantageous. Furthermore, these measurements should be conducted to coincide with any other necessary interventions.

Table 1-1 Summary of reported ZP-immunocontraception in horses

Reference	ZP antigen	Adjuvant	Nº of treatments (primary plus boosters)	Dose of ZP *per* treatment (µg)	Lowest fertility rate achieved (%)	Effects on ovarian function
Liu *et al*., 1989 [7]	pZP	Aluminum hydroxide, FCA/FIA	4-5	27-274	14	No
Kirkpatrick *et al*., 1992 [55]	pZP	FCA/FIA	2 or 3; annual boosters	64	0	Yes
Kirkpatrick *et al*., 1995 [46]	pZP	FCA/FIA	1 to 3; annual boosters	65	8	Yes
Kirkpatrick *et al*., 1997 [47]	pZP	FCA/FIA	2	65	4.5	NI
Turner *et al*., 1997 [48]	pZP	FCA/FIA/microspheres	1 or 2	65	4.5	NI
Powell, Monfort, 2001 [56]	pZP	FCA/FIA	1-3; annual boosters	65	NI	No
Turner *et al*., 2001 [49]	pZP	FCA/FIA/microspheres /carbomer	1 or 2	65	10.6	NI
Kirkpatrick *et al*., 2002 [50]	pZP	FCA/FIA/FMCA	1 or 2; 1 or 2 annual boosters	65	10	Yes
Lyda *et al*., 2005 [33]	pZP	FCA/FIA/FMCA	2; annual boosters	65-100	NI	NI
Turner *et al*., 2007 [52]	pZP	FCA/polymer pellets	3	65	5.9	Yes
Bechert *et al*., 2013 [54]	pZP	FMCA/multilamellar liposomes	1 (long acting)	400	NI	Yes
Joonè *et al*., 2017 [57]	pZP	FMCA/FIA	2	100	0	Yes
Joonè *et al*., 2017 [57]	reZP	FMCA/FIA	2	500 (250 /peptide)	57	No

FCA: Freund's complete adjuvant; FIA: Freund's incomplete adjuvant; FMCA: Freund's modified complete adjuvant; NI: not investigated

African elephant population management-Why?

The current status of the African elephant (*Loxodonta africana)* differs depending on geographical location. The culmination of illegal killing for ivory and meat, habitat loss and conflict at the human-elephant interface has resulted in declines in elephant populations. Populations of elephant in most African countries are listed on the Convention on International Trade in Endangered Species (CITES) Appendix I and are threatened with extinction. Recently, 352,271 elephants were counted across 18 African nations demonstrating dramatic decreases in populations. The current rate of decline is 8% *per* year with an overall decline of 30% (144,000 elephants) from 2007-2014 [94].

Conversely populations of elephant in Botswana, Namibia, Zimbabwe and South Africa are stable or increasing and are listed on the CITES Appendix II (Appendix II lists species that are currently not necessarily threatened with extinction but that may become so unless trade is closely controlled [95]. These populations are a cause for concern for many small (100 km^2) to medium-sized (500 km^2) reserves [96] and create many challenges in terms of practical management [97]. Natality and immigration have a net positive influence on the size of an animal population whereas mortality and emigration have the opposite effect. Combined, this is referred to as the population flux. Populations do not attain the theoretical maximum rate of increase very often as that requires both a readily available food source and a low density of animals, resulting in negligible competition for resources [97]. However in fenced reserves with limited resources, the manipulation of the population's growth rate is of critical importance to the future survival of that population and habitat. Lack of resources can result in elephant breakouts and human-wildlife conflict issues. Additionally, an over population of elephants has negative effects on other species through habitat degradation. Such systems are isolated from emigration by fencing and some form of population control is essential to maintain an acceptable population density [97]. In 2006 it was estimated that elephant numbers in small reserves (100 km^2) in South Africa could double in as little as 10 years if left unhindered [96].

Potential veterinary contraceptive methods available for animals include surgical (gonadectomy, vasectomy and salpingectomy), hormonal (oral contraceptives, depot-injections or slow-release implants) and immunocontraception [98]. A number of methods of population control for elephants have been proposed and applied with varying success [99]. The management option of translocation is rarely available, whilst culling has become ethically unacceptable, especially to the general public. Survey data from 2001 demonstrated that relocated populations had a female bias with 0.79 males to one female and that, in these populations, almost half comprised adult and sub-adult females, indicating substantial potential for rapid population growth [97, 100]. The average annual population growth rates seen following significant translocation efforts was 8.3%, with some reserves experiencing population growth rates in excess of 15% [100]. This creates a conservation dilemma, with the impact of high elephant densities on biodiversity being in contrast with the elephant's more precarious status beyond southern Africa [99]. More recently, a review of the demographics and social dynamics of an African elephant population 35 years after reintroduction as juveniles at Hluhluwe-iMfolozi Park (HiP) noted an exponential growth rate (7.1% annually) over an 18 year period [101]. The lack of evidence for density dependent responses in elephant populations suggests the current high rates of population growth may not slow down under natural conditions. Although density feedbacks must inevitably influence population growth, it is uncertain at which stage this occurs in different elephant systems. Maximum population growth rate can be maintained in large-bodied mammals until the point when forage resources can no longer support the population [99]. However because of their long generation intervals elephant populations can lag in their response to changing forage availability, theoretically causing oscillations in the numbers of elephants and forage rather than the achievement of an equilibrium [99].

Model projections from a 2008 study that used a probabilistic age and state model (demographic parameters: maximum expected lifespan, female age at sexual maturity, average calving interval for the population, age at reproductive senescence, sex ratio of new-borns, and age-specific probabilities of survival) indicated that an annual mortality of 5.9% of the entire elephant population would be required to reduce the long-term population growth to 0% [99]. Concurrently, a mean annual mortality rate of just 0.4% was

found in small fenced reserves in South Africa [100] and 3.2% in the Kruger National Park (KNP) [102]. The low mortality rate in smaller fenced reserves may be attributable to predominantly younger animals having been introduced some 20-30 years ago [101].

Few options are currently available for policy makers in areas with high elephant densities and population growth rates. In general, either higher mortality rates can be introduced *via* culling or simulated by removal of live elephants. Alternatively, populations can be left to increase at current rates with the expectation that density feedbacks will eventually reduce the population growth rate to zero. However it has been recommended that any measures should be applied through differential management of the specific situation, with each assessed independently and suitable measures taken according to what is possible [99]. Historically, a combination of high drought mortality and predation may have kept elephant population growth in check. Today, however, artificial waterholes minimise the effects of resource limitation, elephants are relatively well protected from human predation and reserves are resource-rich, resulting in rapidly increasing populations [99]. Modelled projections of elephant population growth suggest that the current level and frequency of natural mortality in southern Africa is insufficient to prevent long-term growth of the elephant populations of the region. Therefore, interventions to increase mortality or introduce reproductive delays are indicated in attempting to limit population growth [99].

Aspects of African elephant social structure, reproductive biology and interactions

The social order of elephants is matriarchal [103]. Elephants are considered to be non-seasonal, polyoestrous breeders [104]. In the wild, cows reach sexual maturity at an age of 10-12 years [105]. The age at first ovulation is reported to be dependent on population density which is attributable to differences in density-dependent physiological, social and nutritional stressors [98, 105, 106]. The age of cows at first parturition ranges from 9-18 years [98, 105]. In translocated populations births have been observed in cows as young as nine, indicating the onset of puberty occurring as early as 7-8 years, a trend commonly observed in such populations [97, 98].

The elephant exhibits the longest spontaneous oestrous cycle of any mammal studied to date: 13-18 weeks in duration, with a 6-12 week luteal phase and 4-6 week follicular phase [105, 106]. This equates to only three to four fertile cycles *per* year. Free-ranging animals, however, are either pregnant or in lactational anoestrus, thus multiple non-conceptional cycles seem to be rare events in the wild [105]. Erratic ovarian cyclicity, especially during the dry season, has been reported in free-ranging elephant cows [90, 91]. Under favourable conditions, elephants breed throughout the year, with some evidence of distinct seasonality or oestrous synchrony. In free-ranging African elephants, conception rates and births were reported to mainly occur during the rainy season when feed resources and water are abundant [105, 106]. Under natural and wild conditions a series of non-conceiving oestrous cycles frequently observed in captive elephants, most likely do not occur.

Intercalving interval (ICI) is one of the major factors that affect population growth rate. It responds to a number of variables: most importantly resource availability and population density [98]. Gestation lasts 22 months in African elephants, which accounts for approximately 50% of the ICI. This means that for a period of two years or more after calving, cows do not show an oestrous cycle [108]. Based on behavioural studies, oestrus lasts about 4-5 days. As a polygynous mammal, mating with more than one male during a single oestrus may occur [109]. This is, however, a rare event due to the presence of a dominant breeding bull. Oestrus behaviour in the female includes walking away from the herd in an arc-shaped trail with the head tilted to the side to entice males or communicate its state (oestrus walk), producing deep roaring sounds and flicking of the tail against the vulva and then lifting and holding it in the air for short periods. When pursued, it may first run away but eventually will back up towards the bull and accept his mounting [110]. Pheromones in the urine play an important role in signalling an approaching oestrus, inducing the flehmen response in bulls [111]. When a bull approaches a herd of cows, it will inspect the anal and vestibular region and use the vomeronasal organ to detect pheromones associated with oestrus. It can be assumed that the female is in oestrus if a male engages in wrestling with intertwined trunks, reaching over and chin resting, driving, neck biting and attempting to mount [110].

Elephant management and population control using porcine zona pellucida immunocontraception

The National Norms and Standards for the Management of Elephants in South Africa (N&S) was drafted under the terms of the National Environmental Management Biodiversity Act of 2004 and came into effect on May 1st 2008. Its guiding principle states that measures to manage elephants must be informed by current scientific information and where this is insufficient, adaptive management must be central to their management [112]. The N&S stipulates that in the case of limited (smaller areas) or more extensive wildlife systems, the owner or person in control of elephants is responsible for ongoing assessment of their impact on the habitat and the ecological function of the area. The responsible person in relation to a protected area, registered game farm, private or communal land or in relation to a captive facility in which elephants are kept must prepare an elephant management plan (EMP) for submission to the relevant authority for approval [112].

The EMP, as a prerequisite for elephant ownership or management, details how owners and managers agree to the management of the elephants in their care and serves to inform decisions on any necessary interventions. The requirement for the EMP is set out at national level and is authorised and monitored by provincial nature conservation agencies and administrations. An EMP must contain general information identifying the owner and manager, contact information, farm name (including all registered farm names, numbers and portion numbers in the fenced area), the precise extent of the property and the specific enclosure where the elephants will be kept, a description of the specific land uses and activities on all neighbouring properties, the name, contact details and qualifications of an ecologist or compiler of the plan, information as to whether there is potential for enlarging the property and specifications of the perimeter fence. Included under ecological considerations are general climatic and hydrological data (e.g. rainfall, temperatures), a general description of the geology and soils, a detailed description of the vegetation, the preferred management density of elephants, other game species and numbers present on the property, information on sensitive habitats and species, information on disturbed or degraded areas and a description of all available water bodies and the distribution thereof. Additionally the following maps must be included: location, topographic and vegetation

communities of the property in its entirety. All ecological information should be collected and analysed by an ecologist using scientific methods and include a detailed report. The third part of the EMP must contain information relating to the management goals and objectives. Within this section, data on the habitat must be specified including veld condition monitoring methods and time schedules, rehabilitation programme for degraded areas, fire management plans, water provision and population management of other wildlife species and the preferred management density of such. Finally, within this section specific information pertaining to elephants must be detailed including their origin, translocation histories, projected numbers for the next five, 10 and 20 years, age profile, purpose on the property, control of population size, information on manipulation of sex and age ratios, measures to prevent poaching, provision for adequate insurance, contingency plans to deal with elephant problems, feeding scheme in case of a natural food supply shortfall and threat analysis and security plans [112].

The N&S recognised that managers needed to control the growth of elephant populations through a variety of methods. Of these methods, immunocontraception with the pZP vaccine has been identified as the most successful and practical for use in herds or groups of elephants and could be used as an alternative to culling [113]. Currently, the use of pZP vaccine in elephant populations in South Africa remains in the realm of research-only, subject to approval from the Department of Agriculture, Forestry and Fisheries (DAFF) (Section 20 of the Animal Diseases Act, 1984) and the South African Health Products Regulatory Authority (SAHPRA) (formerly the Medicines Control Council) (Section 21 Medicines and Related Substances Act, 1965). The Veterinary Population Management Laboratory (VPML) at the University of Pretoria's Faculty of Veterinary Science provides the only source of pZP vaccines in South Africa [114].

The initial study of ZP-based immunocontraception for elephant fertility control aimed to establish homology between pZP and elephant ZP (elZP) glycoproteins and the effective dose and inoculation protocol required to achieve acceptable antibody titres in zoo elephant cows [32]. This was followed by two field trials which demonstrated the efficacy and safety in wild elephants [30, 31, 115]. The latter trial carried out on free-ranging elephants in the KNP and initiated in 1996 demonstrated both the efficacy and the

reversibility of the pZP vaccine. Initial efficacy findings highlighted a 44% pregnancy rate in treated animals compared to an 89% pregnancy rate in control cows. A revised treatment protocol resulted in a 20% pregnancy rate in treated cows. One year later, following the first efficacy study, seven elephants from the treatment group that had initially received immunocontraception were selected. Four cows were boosted with pZP and adjuvant, and three were untreated. Twelve months later, the elephants were recaptured and evaluated. Transrectal ultrasound examination of the reproductive organs showed that all three untreated cows had conceived, while none of the re-vaccinated elephants were pregnant and all showed normal reproductive organs and signs of ovarian activity. This indicated that the vaccine was reversible in the short term, and that it had no obvious deleterious effect at an ovarian level [30].

The first field trial for the use of pZP vaccines for population control in elephants was initiated at the Makalali Conservancy in the Limpopo Province of South Africa in 2000. Due to its manageable size, accessibility and the availability of detailed information on individual elephants (identikits), the Makalali elephant population was ideal for this next phase [35].

Initially 18 target animals were vaccinated with 600 µg of pZP with 0.5 ml of FMCA and two booster vaccinations of pZP (600 µg) emulsified in FIA, each 2-3 weeks apart [35]. In 2004, 82% of Makalali's breeding population of cows had been contracepted. Immunocontraception required two years to noticeably affect birth rates in the reserve, as all cows pregnant at the time of the initial vaccinations calved during this interval, highlighting the putative safety of the vaccine for use during pregnancy (14 normal calves born within 21 months of the primary vaccination of their dams). Safety during lactation was also assumed, with no adverse occurrences documented in suckling calves.

The estimated projected population size for the Makalali population without immunocontraception totalled 108 animals by 2010. With reproductive control (100% efficacy) in place there was a significant reduction in population growth over the period 2003-2010. This translated to an estimated population size in 2010 of 72 versus 108 animals, or an estimated restriction of 33% in population size after 10 years of programmed contraception [97, 112, 115].

The KNP and Makalali studies conclusively demonstrated that when targeted cows can be individually identified, the pZP vaccine can be successfully and efficiently delivered remotely to free-ranging elephants in both large and small game parks.

A 2012 report [116] investigated the effects of pZP immunocontraception on the reproductive rate as well as the safety during pregnancy of elephant cows in seven private game reserves in South Africa. A total of 108 individually-identified cows were treated and monitored for 6-7 years. Primary vaccinations consisted of 600 or 400 µg pZP proteins with 0.5 ml FMCA and boosters of 400 or 200 µg pZP proteins with 0.5 ml FIA. Vaccines were delivered remotely. In the first year, cows received a primary inoculation plus two boosters 3-6 weeks apart and from the second year onwards they received annual boosters. To allow for existing pregnancies at the time of initial and booster treatments, efficacy was only assessed in year three. A 100% efficacy rate was achieved in the third year. Furthermore, 100% of cows passed the fourth, 67.6% the fifth and 47.2% the sixth year ICI respectively. The reduction in pZP dose and number of boosters from two to one in year two did not reduce efficacy. This demonstrated that it is possible to achieve a contraceptive efficacy of 100% in small to medium-sized free-ranging populations of elephants. This efficacy rate was achieved in year three after initiation of treatment and was maintained until year six.

Due to the complex social structures and family bonds present in elephant populations, the behavioural aspects are an important concern for both researchers and reserve management. The impact of an immunocontraceptive strategy for elephant population management was investigated in the Munyawana Conservancy in KwaZulu-Natal, South Africa in 2011 [117]. The aim of immunocontraception in this reserve was to decrease population growth rates without overly disrupting the social structure of the elephant herds. Therefore a programme utilising rotational lapses in vaccination administration was implemented in adult cows to increase calving ICI without preventing calving indefinitely. Young cows in this reserve were allowed to produce one calf before entering the immunocontraception programme. The short to medium term behavioural effects of this programme noted a small decrease in levels of association between individuals in a herd but no significant aberrant behavioural effects were documented. There was no marked influence on the movement of herds nor in the levels of contact between the treated cows

and bulls. This investigation therefore concluded that no significant short to medium term behavioural effects resulted with immunocontraception of this population on a herd basis. An additional outcome from this study was the lack of effect of a treatment protocol change from two to one booster treatment after the primary inoculation in the first year.

This population [117] was also mathematically modelled with variations such as age at first calving and ICI included to provide projections of growth rates and populations size over a 20 year period. The contraceptive plan served to halve the population growth rate, from a projected 7.58% to a minimum of 3.19% when first calving was delayed until a cow reached 19 years of age. The projections of population size over a 20 year period, however, would have increased by 82%. This indicated that this rate of contraception without additional methods employed to reduce animal numbers was potentially insufficient. Prior to this in 2009, similar projected decreases in population growth rates modelled for Phinda Game Reserve (within the Munyawana Conservancy), Pongola Game Reserve and Pilansberg National Park were achieved. The conclusion in these instances was that immunocontraception should be an effective tool for preventing or minimising irruption in elephants and, perhaps, other introduced ungulate species, however, achieving a population decline was not necessarily implied [118].

Reversibility is a key issue given the CITES status of elephants. While short term reversibility was demonstrated in the KNP trials (after one year of treatment), the Makalali study aimed to demonstrate reversibility in cows treated in the medium to long term. In 2005, five cows were removed from the programme to test reversibility; three cows were treated for five years, one for four years and one for three years. By 2008, four of the five cows had calved [98].

Bertschinger *et al.* [116] also investigated the reversal potential in a large scale efficacy investigation across seven reserves in South Africa. The study involved six cows that had been treated for three (n=1) and four (n=5) years. Two cows treated for four years conceived 25 and 36 months after the last treatment with pZP. The remaining four cows did not produce a calf in the remainder of the study observation period suggesting that the interval from last treatment to reversal is quite variable.

A later study [91] examining oestrous cyclicity in African elephants during pZP immunocontraception (two to three years after the start of treatment) reported anoestrus occurring in a proportion of treated cows following analysis of serial faecal progestagen metabolite levels. This was possibly attributable to seasonal effects. A treatment protocol dictating immunocontraception of all cows in this herd and the consequent lack of controls complicated any definitive conclusions. Other naturally occurring associations with anoestrus in treated animals such as immaturity, drought or lactational anoestrous similarly could not be determined. Benavides *et al.* [90] have documented a return to cyclicity after four years of pZP treatment based on resumption of ovarian cyclicity assessed through faecal progestagen metabolite concentrations highlighting luteal activity.

In small confined populations of up to 100 elephants the ideal starting point is to identify each individual animal on the property, focussing especially on the cows of breeding age. Informed by the pertinent elephant management plan, this would allow determination of the number of cows to be immunised for contraception. The population response is monitored over time and the contraception programme accordingly adapted to the needs of both the management plan and ongoing habitat response [98]. Although large long-lived species comprise a considerable portion of the biomass of many ecosystems and usually occupy key positions in their food chains, their population dynamics are often poorly understood [119]. The costs and logistics of immunocontraception are likely to remain the greatest obstacle to implementation in large elephant populations. The prospects of a reliable one-inoculation immunisation protocol would obviously simplify the operation and dramatically reduce costs. However it is possible that immunocontraception may be viable only in smaller conservation areas, where elephant numbers are low but population growth and densities are relatively high [100]. A 22 year review of the current implementation of pZP immunocontraception in elephant cows in South Africa concluded that pZP-immunocontraception delivered on an individual cow basis induced a contraception efficacy of 100%. Based on current evidence and even with application in larger populations precluding individual cow treatment, population control, however, can be achieved [114]. The effects of population density on age at ovulation, ICI and incidence of anoestrous must be taken into account when considering methods of population control. Methods that reduce population size (culling and translocation) without affecting fertility will inevitably

increase reproductive and thus population growth rates [98]. Immunocontraceptive methods that are employed for elephant population management function by preventing cows from conceiving, therefore fertility control cannot immediately reduce the population. Rather this will only happen once mortality rates exceed birth rates. Fertility control serves then to curb population expansion [97, 98, 113].

Counting elephants: Informed population management

Any large-scale contraceptive programme for elephants must be carefully designed and regularly updated to avoid collapse of herds due to dwindling numbers [120]. The contraception rate affects the population growth rate and consequently the density of elephants on the landscape [98]. To understand how contraception fits into a management system it is necessary to have an understanding of the actual population size.

Elephant census techniques fall into two classes. The first comprises those surveys where the elephants themselves are counted. These are direct counts. The second class includes surveys where signs of elephants (e.g. dung-piles, tracks, feeding signs) are counted. These are indirect counts. Direct counts of elephants can either be carried out from the air or from the ground. In savanna habitats aerial count is the most effective elephant census method. The appropriate technique to use in counting elephants is thus dependant on habitat type (vegetation type and density, and topography), size of the area to be surveyed, elephant density, and also the type of estimate required [121].

The deterministic Lotka-Volterra and Verhulst-Pearl logistic growth models which have formed the basis for most modern population theory, assume that population growth is only a function of population size and therefore that individuals are indistinguishable. This is an assumption that seems particularly inappropriate for long-lived species which are characterised by deferred and intermittent breeding, relatively high adult survivorship and correspondingly extended maximum longevities. A further problem with long-lived species concerns overlapping of biological states. Cows give birth more than once and although breeding is generally synchronised among cows in a population, their calving intervals overlap. Typically, reproductive history also differs among cows of the same cohort. For

this reason stochastic models should be employed [119].

A key issue in this process is predicting the population consequences for any particular species, given a specific level of induced infertility [120]. Therefore within the context of managed elephant populations in immunocontraception programmes, it may be appropriate to use the Hobbs *et al.* conceived method [122]. This approach encompasses two models depending on the immunocontraceptive programme. Firstly, the lifetime model, in which infertile cows remain infertile until death and secondly, the fixed duration mode, in which infertile cows experience a fixed period of infertility before returning to the fertile state.

Once the population dynamics within a managed herd are understood, it seems feasible to be able to predict the population equilibrium within the environmental maximal load for a specific location.

The key elements of modelling using the Addo Elephant National Park as an example [123], noted that as the contraception rate is increased the population growth rate declines. Thus, following a 100% contraception regime the resultant declining growth rate will result in population extinction in the medium term (50-100 years). A decline in the number of individuals in populations is expected only at contraception rates above 77% of all breeding age cows in the population. Despite implementation of such contraceptive regimes the elephant density will remain above recommended densities in the short to medium term. Increasing levels of contraceptive treatment in the population will also result in an overall aging effect on the population, with a higher number of individuals being represented in older age classes over time [96, 98]. An understanding of the demographics of the population and how they may alter when utilising fertility control therefore is imperative.

Whilst it is apparent that contraception cannot have an immediate effect on a population, assuming 100% efficacy, no more births will occur three years after implementation of a contraceptive programme. Population decline is then dependent on mortality rate, which in turn is dependent on age structure of the population and environmental factors like rainfall and disease. Contraception should be seen as a tool that can be used to prevent

rather than cure overpopulation problems with elephants. On the other hand, where an overabundance of elephants is already present, whether perceived or real, contraception can significantly curb continued population growth rate [98]. Therefore to successfully implement a contraception programme an adaptive management plan is required in tandem with an increased understanding of female reproductive physiology and improved vaccination formulations and delivery methods, with a particular focus on reversibility and safety. Any measures taken to decrease population growth must be done so with consideration for conservation of the species.

References

[1] Kirkpatrick JF, Rowan A, Lamberski N, Wallace R, Frank K, Lyda R. The practical side of immunocontraception: zona proteins and wildlife, J Reprod Immunol, 2009; 83(1-2): 151-7

[2] Kerley GIH, Landman M, Kruger L, Owen-Smith N. Chapter 3 Effects of elephants on ecosystems and biodiversity. In: Scholes RJ, Mennell KG (eds), Elephant Management: A Scientific Assessment for South Africa, Wits University Press, 2009, Johannesburg, South Africa

[3] Slotow R, Whyte I, Hofmeyr M. Chapter 8 Lethal management of elephants. In: Scholes RJ, Mennell KG (eds), Elephant Management: A Scientific Assessment for South Africa, Wits University Press, 2009, Johannesburg, South Africa

[4] Kirkpatrick JF, Turner JW. Chemical Fertility Control and Wildlife Management, Bioscience, 1985; 35(8): 485-91

[5] Kirkpatrick JF, Lyda RO, Frank KM. Contraceptive Vaccines for Wildlife: A Review, Am J Repro Immunol, 2011; 66(1): 40-50

[6] Garza F Jr, Thompson DL Jr, French DD, Wiest JJ, St George RL, Ashley KB, *et al*. Active immunization of intact mares against gonadotropin-releasing hormone: differential effects on secretion of luteinizing hormone and follicle-stimulating hormone, Biol Reprod, 1986; 35(2): 347-52

[7] Liu IKM, Bernoco M, Feldman M. Contraception in mares heteroimmunized with pig zonae pellucidae, Reproduction, 1989; 85(1); 19-29

[8] Frank KM, Lyda RO, Kirkpatrick JF. Immunocontraception of captive exotic species: IV. Species differences in response to the porcine zona pellucida vaccine, timing of booster inoculations, and procedural failures, Zoo Biol, 2005; 24(4): 349-58

[9] Nagy P, Guillaume D, Daels P. Seasonality in mares, Anim Reprod Sci, 2000; 60-61: 245-62

[10] Aurich C. Reproductive cycles of horses, Anim Reprod Sci, 2011; 124(3-4): 220-8

[11] Osborne VE. An analysis of the pattern of ovulation as it occurs in the annual reproductive cycle of the mare in Australia, Aust Vet J, 1966; 42(5): 149-54

[12] Palmer E, Driancourt M. Some interactions of season of foaling, photoperiod and ovarian activity in the equine, Livest Prod Sci, 1983; 10(2): 197-210

[13] Ginther OJV, Gastal ELD, Gastal MOD, Beg MAD. Dynamics of the Equine Preovulatory Follicle and Periovulatory Hormones: What's New? J Equine Vet Sci, 2008; 28(8): 454-60

[14] Smith ID, Bassett JM, Williams T. Progesterone concentrations in the peripheral plasma of the mare during the oestrous cycle, J Endocrinol, 1971; 47: 523

[15] Ginther OJ. Reproductive biology of the mare: Basic and applied aspects 2nd ed. Equiservices Cross Plaines, Wis. USA, 1992

[16] Heidler B, Aurich JE, Pohl W, Aurich C. Body weight of mares and foals, estrous cycles and plasma glucose concentration in lactating and non-lactating Lipizzaner mares. Theriogenology, 2004; 61: 883-93

[17] Pierson RA, Ginther OJ. Ultrasonic evaluation of the preovulatory follicle in the mare, Theriogenology, 1985; 24: 359-68

[18] Pierson RA, Ginther OJ. Follicular population dynamics during the estrous cycle of the mare, Anim Reprod Sci, 1987; 14: 219-31

[19] Donadeu FX, Watson ED. Seasonal changes in ovarian activity: Lessons learnt from the horse, Anim Reprod Sci, 2007; 100(3-4): 225-42

[20] Bergfelt DR. Chapter 11 Anatomy and Physiology of the Mare in Samper JC, Saunders WB (eds) Equine Breeding Management and Artificial Insemination (2nd ed), 2009, Saint Louis USA

[21] Irvine CH, Alexander SL. The dynamics of gonadotrophin releasing hormone, lh and fsh secretion during the spontaneous ovulatory surge of the mare as revealed by intensive sampling of pituitary venous blood, J Endocrinol, 1994; 140: 283-95

[22] Visser JA, Themmen APN. Anti-Müllerian hormone and folliculogenesis, Mol Cell Endocrinol, 2005; 234(1-2): 81-6

[23] Claes A, Ball BA, Scoggin KE, Esteller-Vico A, Kalmar JJ, Conley AJ, Squires EL, *et al*. The interrelationship between anti-Müllerian hormone, ovarian follicular populations and age in mares, Equine Vet J, 2015; 47(5): 537-541

[24] Gupta SK, Bhandari B, Shrestha A, Biswal BK, Palaniappan C, Malhotra SS, Gupta N. Mammalian zona pellucida glycoproteins: structure and function during fertilization, Cell Tissue Res, 2012*;* 349(3): 665-78

[25] Hartmann JF, Gwatkin RBL, Hutchison CF. Early Contact Interactions between Mammalian Gametes in vitro: Evidence that the Vitellus Influences Adherence between Sperm and Zona Pellucida, Proc Natl Acad Sci USA, 1972; 69(10): 2767-69

[26] Prasad SV, Skinner SM, Carino C, Wang N, Cartwright J, Dunbar BS. Structure and Function of the Proteins of the Mammalian Zona pellucida, Cells Tissues Organs, 2000; 166(2): 148-64

[27] Li R, Albertini DF. The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte, Nat Rev Mol Cell Biol, 2013; 14(3): 141-52

[28] Fayrer-Hosken R. Controlling Animal Populations Using Anti-Fertility Vaccines, Reprod Domest Anim, 2008; 43: 179-85

[29] Barber MR, Fayrer-Hosken RA. Possible mechanisms of mammalian immunocontraception, J Reprod Immunol, 2000; 46(2): 103-24

[30] Fayrer-Hosken RA, Grobler D, Van Altena JJ, Bertschinger HJ, Kirkpatrick JF. Immunocontraception of African elephants, Nature, 2000; 407(6801): 149

[31] Barber MR, Lee SM, Steffens WL, Ard M, Fayrer-Hosken RA. Immunolocalization of zona pellucida antigens in the ovarian follicle of dogs, cats, horses and elephants, Theriogenology, 2001; 55(8): 1705-17

[32] Fayrer-Hosken RA, Bertschinger HJ, Kirkpatrick JF, Grobler D, Lamberski N, Honneyman G, Ulrich T. Contraceptive potential of the porcine zona pellucida vaccine in the African elephant (*Loxodonta africana*), Theriogenology, 1999; 52(5): 835-46

[33] Lyda RO, Hall JR, Kirkpatrick JF. A comparison of Freund's Complete and Freund's Modified Adjuvants used with a contraceptive vaccine in wild horses (*Equus caballus*), J Zoo Wildl Med, 2005; 36(4): 610-16

[32] Skinner SM, Prasad SV, Ndolo TM, Dunbar BS. Zona Pellucida Antigens: Targets for Contraceptive Vaccines, Am J Reprod Immunol, 1996; 35(3): 163-74

[35] Delsink AK, Van Altena JF, Kirkpatrick JF, Grobler D, Fayrer-Hosken RA. Field applications of immunocontraception in African elephants (*Loxodonta africana*), Reproduction Suppl, 2002; 60: 117-24

[36] Stout T, Colenbrander B. Suppressing reproductive activity in horses using GnRH vaccines, antagonists or agonists, Anim Reprod Sci, 2004; 82: 633-43

[37] Schulman ML, Botha AE, Muenscher SB, Annandale CH, Guthrie AJ, Bertschinger HJ. Reversibility of the effects of GnRH-vaccination used to suppress reproductive function in mares, Equine Vet J, 2013; 45(1): 111-13

[38] Hennesy, D. Improvac® Mode of Action, Technical Bulletin, 2008, Pfizer Animal Health, Australia

[39] Nimmo DG, Miller KK. Ecological and human dimensions of management of feral horses in Australia: A review, Wildl Res, 2007; 34 (5): 408-17

[40] Pest animal risk assessment-Feral Horse, Queensland Primary Industries and Fisheries, June 2009, Australia

[41] Herd Area and Herd Management Statistics https://www.blm.gov/sites/blm.gov/files/wildhorse_programdata_2018hmastats.pdf [accessed 4 January 2019]

[42] Willis P, Heusner GL, Warren RJ, Kessler D, Fayrer-Hosken RA. Equine immunocontraception using porcine zona pellucida: a new method for remote delivery and characterization of the immune response, J Equine Vet Sci, 1994; 14(7): 364-70

[43] Turner JW, Liu IKM, Flanagan DR, Bynum KS, Rutberg AT. Porcine zona pellucida (PZP) immunocontraception of wild horses (*Equus caballus*) in Nevada: a 10 year study, Reproduction Suppl, 2002; 60: 177-86

[44] Kirkpatrick JF, Irwin LKM, Turner JW. Remotely-Delivered immunocontraception in feral horses, Wildl Soc Bull, 1990; 18(3): 326-30

[45] Kirkpatrick J, Liu I, Turner J, Bernoco M. Antigen recognition in feral mares previously immunized with porcine zonae pellucidae, J Reprod Fertil Suppl, 1991; 44: 321-25

[46] Kirkpatrick JF, Naugle R, Liu IKM, Bernoco M, Turner JJW. Effects of Seven Consecutive Years of Porcine Zona Pellucida Contraception on Ovarian Function in Feral Mares, Biol Reprod, 1995; 52: 411-18

[47] Kirkpatrick JF, Turner JW Jr, Liu IK, Fayrer-Hosken R, Rutberg AT. Case studies in wildlife immunocontraception: wild and feral equids and white-tailed deer, Reprod Fertil Dev, 1997; 9(1): 105-10

[48] Turner JW, Liu IKM, Rutberg AT, Kirkpatrick JF. Immunocontraception Limits Foal Production in Free-Roaming Feral Horses in Nevada, J Wildl Manage, 1997; 61(3): 873-80

[49] Turner JW, Liu IKM, Flanagan DR, Rutberg AT, Kirkpatrick JF. Immunocontraception in feral horses: one inoculation provides one year of infertility, J Wildl Manage, 2001; 65(2): 235-41

[50] Kirkpatrick JF, Turner A. Achieving population goals in a long-lived wildlife species (*Equus caballus*) with contraception, Wildl Res, 2008; 35(6): 513-19

[51] Roelle JE, Germaine SS, Kane AJ, Cade BS. Efficacy of SpayVac® as a contraceptive in feral horses, Wildl Soc Bull, 2017; 41(1): 107-15

[52] Turner JW, Liu IKM, Flanagan DR, Rutberg AT, Kirkpatrick JF. Immunocontraception in wild horses: one inoculation provides two years of infertility, J Wildl Manage, 2007; 71(2): 662-67

[53] Killian G, Thain D, Diehl NK, Rhyan J, Miller L. Four-year contraception rates of mares treated with single-injection porcine zona pellucida and GnRH vaccines and intrauterine devices, Wildl Res, 2008; 35(6): 531-39

[54] Bechert U, Bartell J, Kutzler M, Menino A, Bildfell R, Anderson M, Fraker M. Effects of two porcine zona pellucida immunocontraceptive vaccines on ovarian activity in horses, J Wildl Manage, 2013; 77(7): 1386-1400

[55] Kirkpatrick JF, Liu IMK, Turner JW, Naugle R, Keiper R. Long-term effects of porcine zonae pellucidae immunocontraception on ovarian function in feral horses (*Equus caballus*), J Reprod Fertil, 1992; 94(2): 437-44

[56] Powell DM, Monfort SL. Assessment: Effects of Porcine Zona Pellucida Immunocontraception on Estrous Cyclicity in Feral Horses, J App Anim Sci, 2001; 4(4): 271-84

[57] Joonè CJ, Bertschinger HJ, Schulman ML, Gupta SK, Arukha AP, Minhas V, Fosgate GT, Dieterman E. Ovarian function and pregnancy outcome in pony mares following immunocontraception with native and recombinant porcine zona pellucida vaccines, Equine Vet J, 2017; 49(2): 189-95

[58] Sharma S, Hinds LA. Formulation and delivery of vaccines: Ongoing challenges for animal management, J Pharm Bioallied Sci, 2012; 4(4): 258-66

[59] Gupta SK, Bansal P. Vaccines for immunological control of fertility, Reprod Med Biol, 2010; 9(2): 61-71

[60] Legastelois I, Buffin S, Peubez I, Mignon C, Sodoyer R, Werle B. Non-conventional expression systems for the production of vaccine proteins and immunotherapeutic molecules, Hum Vaccin Immunother, 2017;13(4): 947-61

[61] Gupta N, Chakrabarti K, Prakash K, Wadhwa N, Gupta T, Gupta SK. Immunogenicity and Contraceptive Efficacy of *Escherichia coli*-Expressed Recombinant Porcine Zona Pellucida Proteins, Am J Reprod Immunol, 2013; 70(2): 139-52

[62] Ambrosia RL, Roberts BN, Roberts TA, DeYoung BL, Peterson EW, Bertschinger HJ, Schulman ML, Crampton M, Roth R, Zyl PJ, Cameron-Blake N, Vandenplas ML, Knobel DL, French HM. Porcine and recombinant zona pellucida vaccines as immunocontraceptives for donkeys in the Caribbean, Clin Theriogenology, 2017; 9(3): 439

[63] Cox JC, Coulter AR. Adjuvants-a classification and review of their modes of action, Vaccine, 1997; 15(3): 248-56

[64] Awate S, Babiuk LA, Mutwiri G. Mechanisms of Action of Adjuvants, Front Immunol, 2013; 4: 114

[65] Nicodemus CF, Berek JS. TLR3 agonists as immunotherapeutic agents, Immunotherapy, 2010; 2(2): 137-40.

[66] Chaplin DD. Overview of the immune response, J Allergy Clin Immunol, 2010; 125(Suppl 2): S3-S23

[67] Petrovsky N. Comparative Safety of Vaccine Adjuvants: A Summary of Current Evidence and Future Needs, Drug Safety, 2015; 38(11): 1059-74

[68] Billiau A, Matthys P. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases, J Leukoc Biol, 2001; 70(6): 849-60

[69] Means TK, Wang S, Lien E, Yoshimura A, Golenbock DT, Fenton MJ. Human Toll-Like Receptors Mediate Cellular Activation by Mycobacterium tuberculosis, J Immunol, 1999; 163(7): 3920-27

[70] Herbert WJ. The mode of action of mineral-oil emulsion adjuvants on antibody production in mice, Immunology, 1968; 14(3): 301-18

[71] Claassen E, De Leeuw W, De Greeve P, Hendriksen C, Boersma W. Freund's complete adjuvant: an effective but disagreeable formula, Research Immunol, 1992; 143(5): 478-83

[72] InvivoGen, Addavax ™ information sheet, https://www.invivogen.com/addavax

[accessed: 12 September 2018]

[73] Lovgren Bengtsson K, Morein B, Osterhaus AD. Iscom technology-based matrix m adjuvant: Success in future vaccines relies on formulation, Expert Rev Vaccines, 2011; 10: 401–3

[74] Levast B, Awate S, Babiuk L, Mutwiri G , Gerdts V, van Drunen Littel-van den Hurk S. Vaccine Potentiation by Combination Adjuvants, Vaccines, 2014; 2: 297-322

[75] Poly (I:C) (HMW) VacciGrade™ Polyinosinic-polycytidylic acid – TLR-3-based adjuvant, Data sheet version # 11E06-MT, Invivogen, USA

[76] Sabbatini P, Tsuji T, Ferran L, Ritter E, Sedrak C, Tuballes K *et al*. Phase I trial of overlapping long peptides from a tumor self-antigen and poly-ICLC shows rapid induction of integrated immune response in ovarian cancer patients, Clin Cancer Res, 2012; 18(23): 6497-508

[77] Garg R, Latimer L, Simko E, Gerdts V, Potter A, van Drunen Littel-van den Hurk S. Induction of mucosal immunity and protection by intranasal immunisation with a novel respiratory syncytial virus vaccine formulation, J Gen Virol, 2013; 95: 301-6

[78] Skinner SM, Mills T, Kirchick HJ, Dunbar BS. Immunization with zona pellucida proteins results in abnormal ovarian follicular differentiation and inhibition of gonadotropin-induced steroid secretion, Endocrinology, 1984; 115(6): 2418-32

[79] Mahi-Brown CA, Yanagimachi R, Hoffman JC, Huang TT Jr. Fertility control in the bitch by active immunization with porcine zonae pellucidae: use of different adjuvants and patterns of estradiol and progesterone levels in estrous cycles, Biol Reprod, 1985; 32(4): 761-72

[80] Dunbar BS, Lo C, Powell J, Stevens VC. Use of a synthetic peptide adjuvant for the immunization of baboons with denatured and deglycosylated pig zona pellucida glycoproteins, Fertil Steril, 1989; 52(2): 311-8

[81] Joonè CJ, Schulman ML, Bertschinger HJ. Ovarian dysfunction associated with zona pellucida-based immunocontraceptive vaccines, Theriogenology, 2017; 89: 329-37

[82] Paterson M, Koothan PT, Morris KD, O'Byrne KT, Braude P, Williams A, et al. Analysis of the contraceptive potential of antibodies against native and deglycosylated porcine ZP3 in vivo and in vitro, Biol Reprod, 1992; 46: 523-34

[83] Hardy CM, Ten Have JF, Pekin J, Beaton S, Jackson RJ, Clydesdale G. Contraceptive responses of mice immunized with purified recombinant mouse zona pellucida subunit 3 (mZP3) proteins, Reproduction, 2003; 126: 49-59

[84] Rhim SH, Millar SE, Robey F, Luo AM, Lou YH, Yule T, *et al*. Autoimmune disease of the ovary induced by a ZP3 peptide from the mouse zona pellucida, J Clin Invest, 1992; 89: 28

[85] Lou Y, Park K, Agersborg S, Alard P, Tung KSK. Retargeting T cell-mediated inflammation: a new perspective on autoantibody action, J Immunol, 2000; 164: 5251-7

[86] Joonè CJ, Schulman ML, Fosgate GT, Plagis TA, Crafford JE, Gupta SK, Bertschinger HJ. Antigen-specific CD4+ and CD8+ T-cell responses in PBMC from pony mares immunized with either native or recombinant zona pellucida vaccines, Theriogenology, 2019; 126: 106-13

[87] Aitken RJ, Richardson DW, Hulme M. Immunological interference with the properties of the zona pellucida. In: Crighton DB (ed), Immunological aspects of reproduction in mammals, 1984, London, UK

[88] Gulyas BJ, Gwatkin RB, Yuan LC. Active immunization of cynomolgus monkeys (Macaca fascicularis) with porcine zonae pellucidae, Gamete Res, 1983; 7: 299–307

[89] Gorman SP, Levy JK, Hampton AL, Collante WR, Harris AL, Brown RG. Evaluation of a porcine zona pellucida vaccine for the immunocontraception of domestic kittens (*Felis catus*), Theriogenology, 2002; 58: 135–49

[90] Benavides Valades G, Ganswindt A, Annandale H, Schulman ML, Bertschinger HJ. Non-invasive assessment of the reproductive cycle in free-ranging female African elephants (*Loxodonta africana*) treated with a gonadotropin-releasing hormone (GnRH) vaccine for inducing anoestrus, Reprod Biol Endocrinol, 2012; 10: 63

[91] Ahlers MJ, Ganswindt A, Munscher S, Bertschinger HJ. Fecal 20-oxo-pregnane concentrations in free-ranging African elephants (*Loxodonta africana*) treated with porcine zona pellucida vaccine, Theriogenology, 2012; 789(1): 77-85

[92] Joonè CJ, Schulman ML, Fosgate GT, Claes ANJ, Gupta SK, Botha AE, Human A, Bertschinger HJ. Serum anti-Müllerian hormone dynamics in mares following immunocontraception with anti-zona pellucida or -GnRH vaccines, Theriogenology, 2018; 106: 214-20

[93] Kersey DC, Dehnhard M. The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation, Gen Comp Endocrinol, 2014; 203: 296-306

[94] Great Elephant Census Final Results, The Great Elephant Census http://www.greatelephantcensus.com/final-report [accessed 12 September 2016]

[95] Convention on International Trade in Endangered Species of Wild Fauna and Flora Appendices I, II and III https://cites.org/sites/default/files/eng/app/2016/E-Appendices-2016-03-10.pdf [accessed 12 September 2016]

[96] Mackey RL, Page BR, Duffy KJ. Modelling elephant population growth in small, fenced, South African reserves, S Af J Wildl Res, 2006; 36(1): 33-43

[97] Delsink AK, Altena JJ, Grobler D, Bertschinger H, Kirkpatrick J. Regulation of a small, discrete African elephant population through immunocontraception in the Makalali Conservancy, Limpopo, South Africa, S Af J Sci, 2006; 102(9/10): 403-5

[98] Bertschinger HJ, Delsink A, Van Altena JJ, Kirkpatrick J, Killian H, Ganswindt A. *et al*. Chapter 6: Reproductive control of elephants, In: Scholes RJ, Mennel KG (eds), Elephant management: A scientific assessment for South Africa, Wits University Press, 2008, Johannesburg, South Africa

[99] Woolley LA, Mackey RL, Page BR, Slotow R. Modelling the effect of age-specific mortality on elephant *Loxodonta africana* populations: can natural mortality provide regulation?, Oryx, 2008; 42(1): 49-57

[100] Slotow R, Garaï ME, Reilly B, Page B, Carr RD. Population dynamics of elephants re-introduced to small fenced reserves in South Africa, S Af J Wildl Res, 2005; 35(1): 23-32

[101] Kuiper TR, Druce DJ, Druce HC. Demography and social dynamics of an African elephant population 35 years after reintroduction as juveniles, J App Ecol, 2018; 55(6): 2898-907

[102 Whyte IJ. Conservation management of the Kruger National Park elephant population, University of Pretoria, PhD Thesis, 2001, University of Pretoria, South Africa

[103] Moss CJ, Poole JH. Relationships and social structure in African elephants in RA Hinde (ed), Primate Social Relationships: an Integrated Approach, Blackwell Scientific Publications, 1983, Oxford, UK

[104] Perry JS. The reproduction of the African elephant, *Loxodonta africana*. Proc R Soc B, 1953; 237: 93-153

[105] Allen WR. Review. Ovulation, pregnancy, placentation and husbandry in the African elephant (*Loxodonta africana*). Philos Trans R Soc Lond B Biol Sci, 2006; 361(1469): 821-834

[106] Laws RM. Aspects of reproduction in the African elephant, *Loxodonta africana*. J Reprod Fertil, 1969; S6: 193-217

[107] Plotka ED, Seal US, Zarembka FR, Simmons LG, Teare A, Phillips LG, Hinshaw KC, Wood DG. Ovarian function in the elephant: luteinizing hormone and progesterone cycles in African and Asian elephants, Biol Reprod, 1988; 38(2): 309-14

[108]Brown JL, Walker SL, Moeller T. Comparative endocrinology of cycling and non-cycling Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants, Gen Comp Endocrinol, 2004; 136(3): 360-70

[109] Short RV. Oestrous Behaviour, Ovulation and the Formation of the Corpus Luteum in the African Elephant: *Loxodonta africana*, Af J Ecol, 1966; 4(1): 56-68

[110] Poole JH. Mate guarding, reproductive success and female choice in African elephants. Anim Behav, 1989; 37: 842-49

[111] Rasmussen LE, Schulte BA. Chemical signals in the reproduction of Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants, Anim Reprod Sci, 1988; 53(1-4): 19-34

[112] Department of Environmental Affairs and Tourism, National Environmental Management: Biodiversity Act, 2004 (Act No. 10 of 2004) National Norms and Standards for the Management of Elephants in South Africa, Government Notice No. 251, 29 February 2008, South Africa

[113] Delsink AK, Van Altena JJ, Grobler D, Bertschinger HJ, Kirkpatrick JF, Slotow R. Implementing immunocontraception in free-ranging African elephants at Makalali Conservancy, J S Afr Vet Assoc, 2007; 78(1): 25-30

[114] Bertschinger HJ, Delsink A, Van Altena JJ, Kirkpatrick JF. Porcine zona pellucida vaccine immunocontraception of African elephant (*Loxodonta africana*) cows: A review of 22 years of research, Bothalia, 2018; 48(2): 1-8

[115] Fayrer-Hosken RA, Brooks P, Bertschinger HJ, Kirkpatrick JF, Turner JW, Liu IKM. Management of African elephant populations by immunocontraception, Wildl Soc Bull, 1997; 25(1): 18-21

[116] Bertschinger H, Delsink A, Kirkpatrick J, Van Altena J, Ahlers M, Dickerson T, Powrie D, Burger A. Porcine zona pellucida immunocontraception of African elephants (*Loxodonta africana*): beyond the experimental stage, In: Cain J, Marshal J (eds), IVth International Wildlife Management Congress-Cooperative Wildlife Management Across Borders: Learning in the Face of Change, The Wildlife Society, 9-12th July 2012, Durban South Africa

[117] Druce HC, Mackey RL, Slotow R. How Immunocontraception Can Contribute to Elephant Management in Small, Enclosed Reserves: Munyawana Population as a Case Study, PLoS One, 2012; 6(12): 1-10

[118] Mackey RL, Page BR, Grobler D, Slotow R. Modelling the effectiveness of contraception for controlling introduced populations of elephant in South Africa, Af J Ecol, 2009; 47(4): 747-55

[119]Wu LS, Botkin DB. Of Elephants and Men: A Discrete, Stochastic Model for Long-Lived Species with Complex Life Histories, Am Natural, 1980; 116(6): 831-49

[120] Perdok A, De Boer W, Stout T. Prospects for managing African elephant population growth by immunocontraception: a review, Pachyderm, 2007; 42: 97-107

[121] Establishing a Long Term System for Monitoring the Illegal Killing of Elephants (MIKE) Annex 3: Methods of counting elephants https://cites.org/eng/prog/mike/intro/a3.shtml [accessed 12 September 2016]

[122] Hobbs NT, Bowden DC, Baker DL. Effects of fertility control on populations of ungulates: General, stage-structured models, J Wildl Manage, 2000; 64(2): 473-91

[123] Knight M, Castley G, Moolman L, Adendorff J. Elephant management in Addo Elephant National Park. In Kerley GIH, Wilson S, Massey A (eds) Elephant Conservation and management in the Eastern Cape Terrestrial Ecology Research Unit, Nelson Mandela Metropolitan University Report vol 35, 2002, South Africa

Chapter Two: Scope of thesis

The ideal contraceptive agent for population control is non-toxic, reversible (species-dependant), has multi-year efficacy, can be remotely delivered, has minimal behavioural side-effects, is cost-effective and is efficacious in multiple species and both sexes [1].

Through decades of research, significant progress in free-ranging wildlife contraception was made and immunocontraception of free-ranging wildlife has reached the management level, with success across a large variety of species. Thus far immunocontraceptive research and management application have focused on anti-pZP and -GnRH vaccines. The evidence suggests that the pZP vaccine is an effective contraceptive that can be delivered remotely, is safe to use in pregnant animals [2], does not pass through the food chain and is reversible [3], at least after short to medium term use, and has limited documented effects on herd social structure and behaviour [4, 5]. Zona pellucida-based immunocontraception overcomes many of the early challenges of wildlife fertility control.

The variable but high degree of nucleotide sequence conservation in the genes coding for each ZP glycoprotein across species has facilitated heterologous immunisation with native pZP [6]. However the isolation and purification of this native pZP from porcine ovaries harvested at slaughter is both labour intensive, costly and associated with contamination risks from both extra-ZP proteins and infectious agents [1]. These risks limit international movement of pZP vaccine. For the continued successful application of ZP-based immunocontraception, investigations have shifted towards the development of reZP vaccines. To date this has yielded variable results [7, 8]. The development of an effective reZP vaccine is therefore warranted. In addition there is a need to move away from Freund's adjuvants for ZP-based immunocontraception for safety and regulatory reasons.

In light of more recent reports describing ovarian dysfunction or suppression subsequent to treatment [8], the clinical and laboratory monitoring of ovarian function following ZP-based immunocontraception is warranted, particularly with any novel formulation. This monitoring of target populations not amenable to clinical interventions including wildlife requires novel approaches.

This research was conducted to investigate the safety, efficacy and effect on ovarian function in the domestic horse of novel ZP vaccine formulations with non-Freund's adjuvants. In addition the current utilisation of a native pZP vaccine formulation in free-ranging African elephants in South Africa was evaluated. This research used data collected in randomised controlled studies and analysed retrospective laboratory and questionnaire derived data. The work tested the following hypotheses:

- Immunocontraception using pZP proteins formulated with non-Freund's adjuvants have similar ovarian effects as anti-GnRH vaccination in the horse
- Immunocontraception using reZP proteins formulated with non-Freund's adjuvants elicits a similar ovarian response as pZP vaccination in the horse
- Non-Freund's adjuvants used in combination with pZP proteins is an effective contraceptive in domestic horses based on humoral antibody titre response
- The use of reZP proteins with non-Freund's adjuvants is an effective contraceptive in domestic horses based on humoral antibody titre response
- Anti-Müllerian hormone concentrations will change in domestic horse mares subsequent to their immunocontraception with both native pZP or reZP proteins formulated with non-Freund's adjuvants
- The native pZP vaccine is an important and effective tool for the management of elephant populations in South African game reserves.

References

[1] Fayrer-Hosken R. Controlling Animal Populations Using Anti-Fertility Vaccines, Reprod Domest Anim, 2008; 43: 179-85

[2] Delsink AK, Van Altena JJ, Grobler D, Bertschinger H, Kirkpatrick J. Regulation of a small, discrete African elephant population through immunocontraception in the Makalali Conservancy, Limpopo, South Africa, S Af J Sci, 2006; 102(9-10): 403-5

[3] Bertschinger H, Delsink A, Kirkpatrick J, Van Altena J, Ahlers M, Dickerson T, Powrie D, Burger A. Porcine zona pellucida immunocontraception of African elephants (*Loxodonta africana*): beyond the experimental stage, In: Cain J, Marshal J (eds), IVth International Wildlife Management Congress-Cooperative Wildlife Management Across Borders: Learning in the Face of Change, The Wildlife Society, 9th-12th July 2012 Durban, South Africa

[4] Delsink AK, Van Altena JJ, Grobler D, Bertschinger HJ, Kirkpatrick JF, Slotow R. Implementing immunocontraception in free-ranging African elephants at Makalali Conservancy, J S Afr Vet Assoc, 2007; 78(1): 25-30

[5] Delsink AK, Kirkpatrick J, Altena JJ, Bertschinger HJ, Ferreira SM, Slotow R. Lack of spatial and behavioral responses to immunocontraception application in African elephants (*Loxodonta africana*), J Zoo Wildl Med, 2013; 44(4): (Suppl) 52-74

[6] Sacco, AG. Antigenic cross-reactivity between human and pig zona pellucida, Biol Reprod, 1977; 16: 164-73

[7] Gupta N, Chakrabarti K, Prakash K, Wadhwa N, Gupta T, Gupta SK. Immunogenicity and Contraceptive Efficacy of *Escherichia coli*-Expressed Recombinant Porcine Zona Pellucida Proteins, Am J Reprod Immunol, 2013; 70(2): 139-52

[8] Joonè CJ, Bertschinger HJ, Schulman ML, Gupta SK, Arukha AP, Minhas V, Fosgate GT, Dieterman E. Ovarian function and pregnancy outcome in pony mares following immunocontraception with native and recombinant porcine zona pellucida vaccines, Equine Vet J, 2017; 49(2): 189-95

Chapter Three:
Ovarian function following immunocontraceptive vaccination of mares using native porcine and recombinant zona pellucida vaccines formulated with a non-Freund's adjuvant and anti-GnRH vaccines

Margaret B Nolan[a,b], Henk J Bertschinger[a,b], Robyn Roth[c], Michael Crampton[c], Isabela S. Martins[a], Geoffrey T Fosgate[a], Tom A Stout[a,d], Martin L Schulman[a,b]

[a]Section of Reproduction, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

[b]Veterinary Population Management Laboratory, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

[c]Biomanufacturing Technologies, BioSciences, Council for Scientific and Industrial Research (CSIR), Pretoria, South Africa

[d]Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Theriogenology **120** (2018): 111-116

doi.org/10.1016/j.theriogenology.2018.07.044

An extract of this work was presented at the 12th International Symposium on Equine Reproduction, Cambridge, UK, 22-26 July 2018 and published as an abstract in *Journal of Equine Veterinary Science*

Abstract

An important determinant in the selection of any contraceptive agent is the impact on ovarian function, both in the short and longer term. In this study, ovarian activity was monitored in mares immunised with one of the following vaccine formulations; native porcine zona pellucida (pZP), recombinant zona pellucida proteins ZP3 and ZP4 (reZP), pZP & reZP combined or a commercially available anti-GnRH vaccine. The ZP antigens were prepared in an adjuvant formulation consisting of 6% polymeric adjuvant (Montanide™ Pet Gel A, Seppic, France) and 500 µg polyinosinic-polycytidylic acid – TLR-3-agonist [Poly (I:C)] HMW VacciGrade™, Invivogen, USA). A vehicle-only control group was administered the adjuvant formulation without antigen. Ovarian activity was monitored using clinical observations (transrectal palpation and ultrasonography of the reproductive tract) in addition to blood sampling for serum progesterone and anti-Müllerian hormone (AMH) concentrations while employing a low sampling frequency. Treatments and measurements were initiated in December (southern hemisphere summer) and subsequent data collection was performed in January, February, March and May. Both reZP and anti-GnRH vaccination were associated with clinically evident ovarian suppression in the short term. Ovarian activity in mares administered a reZP or anti-GnRH vaccine was significantly different to adjuvant control and pZP treated mares. Serum AMH concentrations were different between pZP and anti-GnRH treated mares 3.5 months after the final vaccination. Serum AMH concentrations were significantly correlated with mare age, serum progesterone and ovarian volume.

Introduction

A number of antigens have been proposed as targets for fertility control *via* vaccination. These include peptide hormones, oocyte and sperm proteins and other molecules associated with fertilisation and early embryonic development [1]. Two immunogens studied extensively in the horse and other species as potential contraceptive agents are gonadotropin releasing hormone (GnRH) and native porcine zona pellucida (pZP) proteins [1].

An important determinant for the selection of a contraceptive agent is the effect on ovarian function, both in the short and long term [1]. The presumed immunocontraceptive mechanism of pZP in the horse involves antibody binding to the ZP sperm receptor sites and subsequent prevention of sperm-oocyte binding and fertilisation. Based on this supposition, pZP immunisation should not affect the hypothalamic-pituitary-gonadal axis, thereby permitting continuation of cyclical ovarian activity [2, 3] and associated behaviours [1]. However pZP-based immunocontraception causes irreversible ovarian damage in some species [4, 5, 6]. By contrast, anti-GnRH vaccines trigger production of antibodies that neutralise endogenous GnRH, which prevents receptor binding and activation of pituitary gonadotrophs. The suppression of gonadotrophin secretion causes reproductive quiescence characterised by cessation of cyclical ovarian activity [7, 8]. Anti-GnRH vaccines therefore also suppress both physiological and behavioural oestrus in the immediate [7, 9, 10, 11] and longer terms [8]. Ovarian suppression onsets within three months of treatment [8-12] and is associated with decreased ovarian weight [12], length [9], volume [11, 12], and reductions in serum progesterone [9-12], oestradiol-17**β** [10], LH [12] and FSH [12] concentrations. Ovarian suppression has also been reported in mares subsequent to immunocontraception using pZP vaccine [13-15]. In this respect, apparent cessation of oestrous cyclicity and erratic cyclicity into the non-breeding season have been reported after long term treatment (>3 years) with pZP vaccines [1, 16]. More recently, 93% of mares treated with a pZP vaccine ceased cyclical activity within four months of treatment [17]. Abrogated cyclicity was associated with both consistently low serum progesterone and minimal ovarian activity as determined by clinical, macroscopic and histological examination of the ovaries. Recombinant vaccines have been developed by the expression

of porcine ZP3 and ZP4 in *E. coli* (reZP) [18]. A recent report has described the intensively monitored ovarian function and fertility of pony mares subsequent to treatment with either a pZP or a reZP vaccine [19]. Control mares retained cyclical ovarian activity throughout the trial whereas six out of seven pZP treated mares and one reZP mare entered an extended (albeit reversible) anoestrus characterised by clinically apparent ovarian suppression and basal serum ovarian steroid concentrations. Pregnancy was established in 0%, 57% and 100% of pZP treated, reZP treated and control mares, respectively [19]. A recent vaccination trial in donkey jennies studied the effects of pZP and reZP on oestrous cyclicity and fertility [20]. The vaccines were similarly formulated to that of previous pZP and reZP vaccines administered in horses [19], using Freund's adjuvants. Seven of 9, 6/8 and 0/8 jennies entered anoestrus within three months after the final vaccination for the reZP, pZP and control jennies, respectively. No jennies in the two vaccinated groups became pregnant compared to 6/8 control jennies.

The advantages of a reZP (compared to pZP) vaccine include production efficiency and the avoidance of contamination with non-ZP proteins and heat-resistant microorganisms [19, 21, 22]. However the efficacy of pZP or reZP as an immunocontraceptive agent relies on the inclusion of a strong adjuvant [23]. Freund's modified complete adjuvant (FMCA) is typically used for the primary inoculation followed by Freund's incomplete adjuvant (FIA) for booster inoculations. Freund's adjuvants can cause undesirable side effects, which can be severe and persist for months [23]. The use of alternative adjuvants that produce a similar or better immune response with less severe side effects would be advantageous.

A more complete understanding of ovarian suppression subsequent to ZP-based immunocontraception will better define the mechanism of the contraceptive effect [19]. However populations requiring contraception are typically managed under extensive conditions and, therefore, practical methods with limited intervention opportunities are commonly required for monitoring effects [21]. Anti-Müllerian hormone (AMH) has been proposed as a tool for assessing ovarian function during ZP-based immunocontraception [24] as it is reportedly a consistent [25] and useful biological marker of ovarian function [26].

The current study aimed to describe ovarian function in mares managed under extensive conditions following treatment with pZP or reZP vaccines formulated using non-Freund's adjuvants or a commercially available anti-GnRH vaccine. In addition, AMH concentrations were compared between treatment groups.

We hypothesised that immunocontraception using pZP proteins formulated with non-Freund's adjuvants would have similar ovarian effects as anti-GnRH vaccination. Furthermore, immunocontraception using reZP proteins formulated with non-Freund's adjuvants were expected to elicit similar ovarian responses as pZP vaccination. Additionally, we anticipated changes in AMH concentrations in ZP immunocontracepted mares.

Materials and methods

Mare selection, management and environment

A population of mixed breed mares (light body type: Arabian, Quarter Horse, Draught and Thoroughbred cross; age: 2-10 years) were studied from November 2016 to May 2017. Inclusion criteria were non-pregnant, normal oestrous cyclicity, good physical and reproductive health and no previous immunocontraceptive treatment [19]. Fifty barren or maiden mares were initially screened for inclusion during a 30 day monitoring period. In this group, regular oestrous cyclicity was confirmed in 26 mares on the basis of periodic changes in the serum progesterone concentration [27]. Lactating mares at the same site were recruited following re-establishment of oestrous cycle activity (assessed by transrectal palpation and ultrasonography of the reproductive tract). Thirty-nine mares were ultimately enrolled (26 maiden or barren and 13 lactating). Mares were maintained on a single extensive mountainous grassland site (3000 ha) in pre-existing groups. The study site was located at 29 ° 51' 30.8664'' S 29 ° 20' 46.9068'' E. The study occurred during the physiological breeding season [28]. The natural day length and environmental temperature range at the beginning and end of the study period were 13 h 18 m, and 7-31 °C and 10 h 20 m, and -3-24 °C, respectively.

Study design

Horses (n=39) were stratified by body condition scores (BCS: 1-9) [29], parity and age (Table 3-1) for random assignment to one of five treatment groups. Repeated measures data were gathered *via* clinical observation and venous blood collection.

Formulation of vaccines

The same adjuvant formulation was used for each of the control, pZP only, reZP only and combined pZP & reZP groups. Each vaccine dose (1 mL) was constituted by combining the antigen, 6% polymeric adjuvant (Montanide™ Pet Gel A, Seppic, France) and 500 µg polyinosinic-polycytidylic acid – TLR-3-agonist [Poly (I:C)] HMW VacciGrade™, Invivogen, USA). The amount of antigen *per* treatment was as follows: 100 µg pZP (Trumpeter Farms and Veterinary Service, Winters, California, USA) for pZP treatments; 250 µg recombinant ZP3 (containing tetanus toxoid epitope) and 250 µg ZP4 (containing bovine RNase epitope; reZP; supplied by BioSciences, CSIR, South Africa) for reZP treatments and no antigen for the control group. Multi-dose vials of each vaccine formulation were prepared, lyophilised and reconstituted with sterile water for injection.

Vaccine administration

The adjuvant control group (n=8) was treated on d=35 and again five weeks later (d=70).

The pZP only group (n=7) received an initial vaccination at d=35 followed by an identical booster vaccination after five weeks (d=70).

The reZP only group (n=8) received an initial vaccination at d=0, followed by two identical boosters at five week intervals (d=35 and d=70).

The pZP & reZP group (n=8) received an initial vaccination of pZP at d=35 followed by a booster vaccination of reZP after five weeks (d=70).

The anti-GnRH group (n=8) received an initial 2 mL vaccination containing 400 µg GnRH-protein conjugate with an diethylaminoethyl (DEAE)-dextran adjuvant (Improvac®, Zoetis, South Africa) at d=35 followed by an identical booster vaccination five weeks later (d=70).

All vaccines were administered by deep intramuscular injection into the gluteal muscle mass; boosters were administered into the contralateral musculature.

Data collection

Animals were examined and samples collected in December (d=0), January (d=35), February (d=70), March (d=105) and May (d=175). Transrectal palpation and ultrasonography of the reproductive tract was performed at d=0, d=35, d=70, d=105 and d=175. During the examination, ovarian volume, presence of follicles ≥15 mm diameter, presence of a CL (confirmed retrospectively by serum progesterone >1 ng/mL), uterine and cervical tone and the presence of uterine oedema were recorded for each mare. Oestrous cyclicity or activity was defined as the presence of follicles ≥15 mm and ovarian volume ≥25 cm^3 (prolate ellipsoid formula) and the confirmation of an ovulation (if present) was confirmed by the presence of a previously unrecorded CL or corpus haemorrhagicum in conjunction with serum progesterone levels >1 ng/mL. In the absence of a CL, oestrous cyclicity was determined on the basis of observed tubular genital tract characteristics [30]. Ovarian inactivity was defined as bilaterally small ovaries (both <25 cm^3), the absence of a CL or any follicles ≥15 mm and basal (<1 ng/mL) serum progesterone [8, 11, 19, 31].

Blood samples were collected by jugular venipuncture at d=0, d=35, d=70, d=105 and d=175. Samples were centrifuged and serum stored at -20°C until assayed

Pasture breeding

Three mature, clinically-healthy and proven fertile stallions (6-8 years of age) were selected for pasture-based breeding. One stallion was randomly selected for each of the three breeding herds and introduced in March (d=105). All stallions remained with the mares until July. Foaling outcome was assessed at the end of the subsequent physiological breeding season based on available records.

Hormone assays

Serum progesterone was measured using a chemoluminescence technique (Immulite® 1000, Siemens, Germany) [32]. Serum AMH concentrations were determined using a commercially available ELISA according to the manufacturer's instructions (AMH Gen II ELISA; Beckman Coulter, Brea, CA, USA). This assay has been validated for use in mares [33] and the detection limit of the assay was 0.08 ng/mL. Intra- and inter-assay coefficients of variation were 3.7% and 4.4% respectively, for a low AMH concentration (3.82 ng/mL), and 3.4% and 4.0%, for a high AMH concentration (16.45 ng/mL).

Statistical analyses

Data concerning the presence/absence of individual measures of normal oestrous cyclicity were compared among treatment groups using mixed effects logistic regression. Quantitative data were log transformed and analysed using mixed effect linear regression. Regression models included fixed effect terms for treatment group, sampling time (categorical with five levels), a group by time interaction (AMH only) and age to adjust for potential confounding. Mare was included as a random effect and a first-order autoregressive correlation structure was used to account for repeated sampling. *Post-hoc* tests in the mixed-effects models were adjusted using the least significant differences (LSD) or Bonferroni method. Serum AMH concentrations at each sampling time were compared among groups using one-way ANOVA with multiple *post-hoc* comparisons adjusted using Bonferroni correction of P values. Pairwise correlations were estimated using Spearman's rho or Pearson's correlation coefficient as appropriate. Statistical testing was performed using commercially available software (IBM SPSS Statistics Version 25) and significance was set at $P \leq 0.05$.

Results

Ovarian activity

Treatment groups were comparable in respect to age, parity, and BCS (Table 3-2) and all mares had evidence of cyclic ovarian activity prior to treatment (Table 3). Treatment ($P=0.001$) and time ($P<0.001$) both had a significant effect on the presence/absence of normal ovarian activity. Mares in the control and pZP treated groups expressed normal cyclical ovarian activity most commonly followed by mares within the combined pZP & reZP, reZP only, and GnRH treated groups in descending order of frequency. When summarised for all observation times, control mares were more likely to be cycling compared to reZP ($P=0.005$) and GnRH ($P<0.001$) treated mares. Similarly, pZP treated mares were also more likely to be cycling compared to reZP ($P=0.002$) and GnRH ($P<0.001$) treated mares. Five weeks after the first treatment and first booster for reZP only (d=70), 8/8 control mares, 6/7 pZP only mares, 5/8 pZP & reZP mares, 3/8 reZP only mares and 2/8 anti-GnRH mares were demonstrating normal oestrus cyclicity. Five weeks after the final booster (d=105), 5/8 control mares, 4/7 pZP only mares, 3/8 pZP & reZP mares, 1/8 reZP only mares and 0/8 anti-GnRH mares were demonstrating normal oestrus activity. By the end of the active monitoring period (d=175), 3/8 control mares, 3/7 pZP only mares, 0/8 pZP & reZP mares, 0/8 reZP only mares and 0/8 anti-GnRH mares had evidence of normal ovarian activity. At the end of the subsequent breeding season the records for seven mares were available. Three control mares foaled and one pZP only mare experienced a late-gestation abortion.

Anti-Müllerian hormone

Treatment group had a significant effect on the serum AMH concentrations collected over the entire study ($P=0.030$). Furthermore, there were significant differences among treatment groups at d=105 ($P=0.037$) and d=175 ($P=0.019$). No *post-hoc* pairwise comparisons were significant at d=105 but at d=175, mares treated with the anti-GnRH vaccine had higher concentrations compared to pZP only treated mares ($P=0.029$). The difference between pZP only treated and control mares at this time-point was not significant ($P=0.084$). Serum AMH concentrations changed over time in reZP only mares with higher concentrations at d=70 compared to d=105 ($P=0.047$) (Table 3-3). Serum AMH

concentrations were positively correlated with ovarian volume (r=0.171, P=0.035) and mare age (ordinal categories; >3 y, 3-6 y, >6 y) (r_s=0.269, P<0.001) but negatively correlated with serum progesterone (r=-0.373, P=0.014).

Discussion

The immunocontraceptive method of action of pZP in the horse, and other species, has been proposed to involve the prevention of sperm-zona binding, with oestrous cyclicity presumed to continue undisturbed [2, 3]. However the results of the present study demonstrate varying degrees of ovarian suppression across all treatment groups. Significantly more reZP only and anti-GnRH mares stopped cycling sooner after vaccination than either the control or the pZP only mares. A two-pronged treatment protocol utilising pZP as the primary inoculation and reZP for the booster had a protracted effect. Ovarian suppression following anti-GnRH vaccination has been reported and the mechanism (suppression of FSH and LH secretion) is well understood [7, 8, 11]. There is increasing evidence that, at least in some species, ovarian suppression is a contributory factor in the contraceptive efficacy of ZP vaccination [4, 5, 15, 16, 17, 19, 34]. However more research is required to define the mechanism of ZP-associated ovarian suppression.

A previous study from our research group reported higher incidences of anoestrus and a superior contraceptive effect in mares treated with pZP compared to reZP [19]. However the reZP vaccine [18] used in that study was manufactured in a different laboratory, was formulated with Freund's adjuvant and only a single booster treatment was administered. The current study is the first to report a non-Freund's adjuvant for ZP-based immunocontraception in the horse.

The reduction in ovarian activity in control mares was possibly an effect of season. A significant effect of time on ovarian activity was evident for all groups, and while this is expected in seasonally breeding animals, it was a limitation of the current study. Reproductive activity is determined by season, primarily photoperiod, and to a lesser extent nutrition and environmental temperature [35]. The physiological breeding season in the southern hemisphere is October to March but variations have also been reported

[28, 30]. Only 26 of the 50 mares initially assessed for the study were cyclic at the end of November and this delayed the initial administration of treatments and subsequent introduction of stallions for breeding. An additional limitation was the paucity of foaling records at the end of the following breeding season. Further investigations into the contraceptive efficacy, reversibility and safety of the novel formulation used in this study are warranted.

The AMH results of the present study were partially consistent with previous reports [24]. Mean AMH concentrations differed across groups at d=105, but differences between pZP only and anti-GnRH mares were only evident at d=175. The reduced sampling frequency and relatively small group sizes in this study might have contributed to the absence of other significant differences. As a result, it is not clear whether AMH is a suitable indicator of the effect of ZP vaccination on ovarian follicular activity in mares managed under extensive conditions (i.e. sampled infrequently). However AMH concentrations were correlated with ovarian volume and serum progesterone, suggesting that it can be useful under certain circumstances. Previous work by our research group noted the potential of serum AMH concentrations for monitoring ovarian function following immunocontraception in mares [24]. Samples were collected weekly from October to March in this previous work [19, 24] but only analysed for five strategic time periods. The results of the previous study served to inform the frequency of sampling and study design for the current project. Among other hypotheses, the current study investigated the premise that less intensive sampling for serum AMH would still be useful for monitoring ovarian function. This is important because most populations of horses that require immunocontraception are feral or semi-feral, greatly limiting the ease and frequency of interventions such as blood sampling. Sample collection coinciding with other interventions, such as inoculations, would enhance practicality in such circumstances. Serum AMH has less cyclical variability in the horse [25] and may therefore be less influenced by season than clinical measures of cyclicity.

Serum AMH tended to be higher in older mares and there is a need to further investigate the dynamics of AMH concentrations in younger, albeit sexually mature, mares. This positive correlation between age and AMH has also been reported in Japanese Black cows [36].

In conclusion, a non-Freund's adjuvated reZP vaccine is a promising alternative for immunocontraception in the mare when ovarian suppression is an acceptable outcome. Serum AMH concentrations following ZP-based vaccination may be used to infer reductions in both ovarian volume and serum progesterone under extensive conditions.

Table 3-1 Treatment groups sub-divided on the basis of mare distribution by: age, median (range); parity, median (range); and BCS (1-9), median (range) P>0.05

Mare information	Control (n=8)	Treatment				P value
		pZP only (n=7)	reZP only (n=8)	pZP & reZP (n=8)	GnRH (n=8)	
Age (years)	4 (2, 9)	4 (2, 8)	4 (2, 10)	3 (2, 7)	4 (2, 7)	1.000
Previous parities	1 (0, 3)	1 (0, 3)	1 (0, 5)	1 (0, 3)	1 (0, 4)	1.000
BCS (1-9)	5 (4, 7)	6 (4, 7)	5 (4, 7)	5 (3, 7)	6 (2, 8)	0.700

pZP, native porcine zona pellucida vaccine; reZP, recombinant porcine zona pellucida vaccine; GnRH, anti-GnRH vaccine (Improvac®); body condition score (BCS)

Table 3-2 Number of mares displaying ovarian activity or inactivity at each time-point during anti-ZP or -GnRH vaccination or adjuvant only (control) treatment

Time-point	Control (n=8)		Treatment							
			pZP only (n=7)		reZP only (n=8)		pZP & reZP (n=8)		GnRH (n=8)	
	Active	Inactive	Active	Inactive	Active	Inactive	Active	Inactive	Active	Inactive
d=0	8	0	7	0	8	0	8	0	8	0
d=35	8	0	7	0	8	0	8	0	8	0
d=70	8	0	6	1	3	5	5	3	2	6
d=105	5	3	4	3	1	7	3	5	0	8
d=175	3	5	3	4	0	8	0	8	0	8

pZP, native porcine zona pellucida vaccine; reZP, recombinant porcine zona pellucida vaccine; GnRH, anti-GnRH vaccine (Improvac®)

Table 3-3 Mean (95% CI) serum anti-Müllerian hormone concentrations (AMH; ng/mL) in mares over a 6 month period during anti-ZP or -GnRH vaccination or adjuvant only (control) treatment

Time-point	Control (n=8)	Treatment				P value[†]
		pZP only (n=7)	reZP only (n=8)	pZP & reZP (n=8)	GnRH (n=8)	
d=0	1.21 (0.58, 2.56)	0.80 (0.36, 1.78)	0.88$^{\Delta\ominus}$ (0.46, 1.70)	0.64 (0.29, 1.40)	0.89 (0.53, 1.50)	0.380
d=35*			0.70$^{\Delta\ominus}$ (0.41, 1.19)			
d=70	1.04 (0.59, 1.84)	0.68 (0.39, 1.20)	0.96$^{\Delta}$ (0.50, 1.83)	0.64 (0.37, 1.09)	0.93 (0.39, 2.19)	0.730
d=105	0.80^{a} (0.33, 1.96)	0.47^{a} (0.38, 0.57)	0.44$^{a,\ominus}$ (0.18, 1.07)	0.67^{a} (0.39, 1.18)	1.02^{a} (0.60, 1.72)	0.037
d=175	0.96^{ab} (0.48, 1.92)	0.45^{a} (0.21, 0.95)	0.67$^{ab,\Delta\ominus}$ (0.33, 1.35)	0.90^{ab} (0.43, 1.87)	1.16^{b} (0.69, 1.96)	0.019
P value[²]	**0.180**	**0.330**	**0.049**	**0.140**	**0.690**	

pZP, porcine zona pellucida vaccine; reZP, recombinant porcine zona pellucida vaccine; GnRH, anti-GnRH vaccine (Improvac®); *reZP only treatment group additional measurement; [†]Based on 1-way ANOVA comparing AMH among groups within each time-point. Means with different superscripts (letter) differ significantly after *post-hoc* testing incorporating Bonferroni correction; [²]Based on mixed effects linear regression comparing AMH over time within each treatment group including a random effect for mare to account for the repeated sampling and fixed effects of age and time-point. Means with different superscripts (symbol) differ significantly after *post-hoc* testing incorporating Bonferroni correction

References

[1] Fayrer-Hosken R. Controlling Animal Populations Using Anti-Fertility Vaccines, Reprod Domes Anim, 2008; 43: 179-185

[2] Liu IKM, Bernoco M, Feldman M. Contraception in mares heteroimmunized with pig zonae pellucidae, Reproduction, 1989; 85(1): 19-29

[3] Barber MR, Fayrer-Hosken, RA. Possible mechanisms of mammalian immunocontraception, J Reprod Immunol, 2000; 46: 103-24

[4] Skinner S, Timmons T, Schwoebel E, Dunbar BS. Immunization with zona pellucida proteins results in abnormal ovarian follicular differentiation and inhibition of gonadotropin-induced steroid secretion, Endocrinology, 1984; 115: 2418-32

[5] Mahi-Brown CA, Yanagamachi R, Hoffman J, Huang TTF. Fertility control in the bitch by active immunization with porcine zona pellucida: use of different adjuvants and patterns of estradiol and progesterone levels in estrous cycles, Biol Reprod, 1985; 32: 671-772

[6] Dunbar BS, Lo C, Stevens V. Effect of immunization with purified porcine zona pellucida proteins on ovarian function in baboons, Fertil Steril, 1989; 52: 311-18

[7] Stout T, Colenbrander B. Suppressing reproductive activity in horses using GnRH vaccines, antagonists or agonists, Anim Reprod Sci, 2004; 82: 633-43

[8] Schulman ML, Botha AE, Muenscher SB, Annandale CH, Guthrie AJ, Bertschinger HJ. Reversibility of the effects of GnRH-vaccination used to suppress reproductive function in mares, Equine Vet J, 2013; 45(1): 111-13

[9] Tshewang U, Dowsett K, Knott L, Trigg T. Preliminary study of ovarian activity in fillies treated with a GnRH vaccine, Aust Vet J, 1997; 75: 663-67

[10] Elhay M, Newbold A, Britton A, Turley P, Dowsett K, Walker J. Suppression of behavioural and physiological oestrus in the mare by vaccination against GnRH, Aust Vet J, 2007; 85: 39-45

[11] Botha AE, Schulman ML, Bertschinger HJ, Guthrie AJ, Annandale CH, Hughes SB. The use of a GnRH vaccine to suppress mare ovarian activity in a large group of mares under field conditions, Wildl Res, 2008; 35(6): 548-54

[12] Garza F Jr, Thompson DL Jr, French DD, Wiest JJ, St George RL, Ashley KB, Jones LS, Mitchell PS, McNeill DR. Active Immunization of Intact Mares against Gonadotropin-Releasing Hormone: Differential Effects on Secretion of Luteinizing Hormone and Follicle-Stimulating Hormone, Biol Reprod, 1986; 35(2): 347-52

[13] Kirkpatrick JF, Lyda RO, Frank KM. Contraceptive Vaccines for Wildlife: A Review, Am J Reprod Immunol, 2011; 66(1): 40-50

[14] Nunez CMV, Adelman JS, Mason C, Rubenstein DI. Immunocontraception decreases group fidelity in a feral horse population during the non-breeding season, Appl Anim Behav Sc, 2009; 117(1): 74-83

[15] Kirkpatrick J, Liu I, Turner J, Naugle R, Keiper R. Long term effects of porcine zonae pellucidae immunocontraception on ovarian function in feral horses (*Equus caballus)*, J Reprod Fertil, 1992; 94: 437-44

[16] Nunez CMV, Adelman, JS, Carr, HA, Alvarez, CM, Rubenstein, DI. Lingering effects of contraception management on feral mares (*Equus caballus*) fertility and social behavior, Conserv Physiol, 2017; 5(1): https://doi.org/10.1093/conphys/cox018

[17] Bechert U, Bartell J, Kutzler M, Menino A, Bildfell R, Anderson M, Fraker M. Effects of two porcine zona pellucida immunocontraceptive vaccines on ovarian activity in horses, The J Wildl Manag, 2013; 77(7): 1386-1400

[18] Gupta N, Chakrabarti K, Prakash K, Wadhwa N, Gupta T, Gupta SK. Immunogenicity and Contraceptive Efficacy of *Escherichia coli*-Expressed Recombinant Porcine Zona Pellucida Proteins, Am J Reprod Immunol, 2013; 70(2): 139-52

[19] Joonè CJ, Bertschinger HJ, Gupta SK, Fosgate GT, Arukha AP, Munhas V, *et al*. Ovarian function and pregnancy outcome in pony mares following immunocontraception with native and recombinant porcine zona pellucida vaccines, Equine Vet J, 2017; 49(2): 189-95

[20] Ambrosia RL, Roberts BN, Roberts TA, DeYoung BL, Peterson EW, Bertschinger HJ, Schulman ML, Crampton M, Roth R, Van Zyl PJ, Cameron-Blake N, Vandenplas ML, Knobel DL, French HM. Porcine and recombinant zona pellucida vaccines as immunocontraceptives for donkeys in the Caribbean, Clin Theriogenology, 2017; 9(3): 439

[21] Kirkpatrick JF, Rowan A, Lamberski N, Wallace R, Frank K, Lyda R. The Practical Side of Immunocontraception: Zona Proteins and Wildlife, J Reprod Immunol, 2009, 83(1-2): 151-7

[22] Gupta SK, Bansal P. Vaccines for immunological control of fertility, Reprod Med Biol, 2010; 9: 61-71

[23] Lyda RO, Hall JR, Kirkpatrick JF. A comparison of Freund's Complete and Freund's Modified Adjuvants used with a contraceptive vaccine in wild horses (*Equus caballus*), J Zoo Wildl Med, 2005; 36(4): 610-16

[24] Joonè CJ, Schulman ML, Fosgate GT, Claes ANJ, Gupta SK, Botha AE, *et al*. Serum Anti-Müllerian Hormone Dynamics in Mares Following Immunocontraception with Anti-Zona Pellucida or -Gnrh Vaccines, Theriogenology, 2018; 106(2): 214-20

[25] Claes A, Ball BA, Scoggin KE, Esteller-Vico A, Kalmar JJ, Conley AJ, Squires EL, *et al*. The interrelationship between anti-Müllerian hormone, ovarian follicular populations and age in mares, Equine Vet J, 2015; 47(5): 537-541

[26] Ireland JJ, Smith GW, Scheetz D, Jimenz-Krassel F, Folger JK, Ireland JLH, *et al*. Does size matter in females? An overview of the impact of the high variation in the ovarian function and fertility, utility of anti-Müllerian hormone as a diagnostic marker for fertility and causes of variation in the ovarian reserve in cattle, Reprod Fertil Dev, 2011; 23: 1-14

[27] Smith ID, Bassett JM, Williams T. Progesterone concentrations in the peripheral plasma of the mare during the oestrous cycle, J Endocrinol, 1971; 47: 523

[28] Osborne VE. An Analysis of the Pattern of Ovulation as It Occurs in the Annual Reproductive Cycle of the Mare in Australia, Aust Vet J, 1966; 42(5): 149-54

[29] Henneke DR, Potter GD, Kreider JL. Relationship between condition score physical measurements and body fat percentage in mares, Equine Vet J, 1983; 15(4): 371-2

[30] Dowsett KF, Knott LM, Woodward RA, Bodero DAV. Seasonal variation in the estrous cycle of mares in the subtropics, Theriogenology, 1993; 39 (3): 631-53

[31] Aurich C. Reproductive cycles of horses, Anim Reprod Sci, 2011; 124 (3-4): 220-28

[32] Berlin D, Steinman A, Raz T. Post-Partum Concentrations of Serum Progesterone, Oestradiol and Prolactin in Arabian Mares Demonstrating Normal Maternal Behaviour and Arabian Mares Demonstrating Foal Rejection Behaviour, Vet J, 2018, 232: 40-45

[33] Gharagozlou F, Akbarinejad V, Youssefi R, Rezagholizadeh A. Low Concentration of Anti-Müllerian Hormone in Mares with Delayed Uterine Clearance, J Equine Vet Sci, 2014; 34: 575-77

[34] Joonè CJ, Schulman ML, Bertschinger HJ. Ovarian dysfunction associated with zona pellucida-based immunocontraceptive vaccines, Theriogenology, 2017; 89: 329-37

[35] Nagy P, Guillaume D, Daels P. Seasonality in mares, Anim Reprod Sci, 2000; 60-61: 245-62

[36] Koizumi M, Kadokawa H. Positive correlations of age and parity with plasma anti-Müllerian hormone concentrations in Japanese Black cows, J Reprod Dev, 2017; 63(2): 205-209

Chapter Four:
Serum antibody immunoreactivity and safety of native porcine and recombinant zona pellucida vaccines formulated with a non-Freund's adjuvant in horses

Margaret B Nolan[a,b], Martin L Schulman[a,b], Alma E Botha[a], Anne-Marie Human[b], Robyn Roth[c], Michael C Crampton[c], Henk J Bertschinger[a,b]

[a]Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

[b]Veterinary Population Management Laboratory, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

[c]Biomanufacturing Technologies, BioSciences, Council for Scientific and Industrial Research, Pretoria, South Africa

Vaccine

doi.org/10.1016/j.vaccine.2019.01.053

An extract of this work was presented at the 12th International Symposium on Equine Reproduction, Cambridge, UK, 22-26 July 2018 and published as an abstract in *Journal of Equine Veterinary Science*

Abstract

Commercial and regulatory limitations associated with native porcine zona pellucida (pZP) vaccines formulated with Freund's adjuvants may be overcome by developing effective recombinant ZP vaccines (reZP) and identifying alternative adjuvant formulations. A two-part preparatory study used 15 geldings and identified potentially effective alternative adjuvant formulations based on anti-pZP antibody response following treatment with pZP formulated with Addavax (AddaVax ™, Invivogen), Quil A (Quil-A® Adjuvant, Invivogen), Quil A and Poly (I:C) (HMW VacciGrade™, Invivogen), Pet Gel A (Montanide™ Pet Gel A, Seppic) and Pet Gel A and Poly (I:C). Injection site reactions, rectal temperature and respiratory and heart rates were also monitored for three days post-treatment. Suitable anti-pZP antibody titres were seen in response to Pet Gel A and Pet Gel A and Poly (I:C). Subsequently in 31 mares, following administration of pZP, reZP and a combination of pZP and reZP proteins prepared in Pet Gel A and Poly (I:C), both serum anti-pZP and -reZP antibody responses were monitored. In addition, safety was assessed for up to seven days post-treatment by inspection and palpation of gluteal intramuscular injection sites and measurement of rectal temperature. The measured antibody titres in all treatment groups differed significantly to an adjuvant control group ($P<0.001$). Temporal changes in both anti-pZP and -reZP antibody titres in all ZP treatment groups were similar to patterns reported previously in various species vaccinated with pZP formulated with Freund's adjuvants. There were no differences in anti-pZP antibody titres between the pZP and reZP treated groups ($P>0.05$). Side effects were mild and transient in nature. This represents the first application of a reZP vaccine formulated with non-Freund's adjuvants evoking a similar antibody titre response to native pZP vaccination in mares.

Introduction

The induction of antibodies against zona pellucida (ZP) proteins for the control of fertility was first reported in 1972 [1]. In the absence of suitable adjuvant formulations ZP proteins are weak antigens [2]. Efficacy has, however, been demonstrated *via* the combination of native porcine zona pellucida (pZP) proteins formulated with Freund's modified complete adjuvant (FMCA) and Freund's incomplete adjuvant (FIA) for primary and booster immunisations, respectively. These immunocontraceptive vaccines have been used for more than 30 years in populations of both horses [3, 4] and white tailed deer [5] and for 18 years in African elephants [6, 7, 8]. In total, more than 90 zoo and wildlife species have been treated with pZP formulated with Freund's adjuvants to achieve fertility control [9]. In most, the primary treatment was followed by a booster after two or three weeks or, in African elephants, after five weeks [8]. The duration of the contraceptive effect was approximately one year in most species including African elephants and horses and single annual boosters were required to maintain this effect [10, 11, 12]. A liposomal pZP formulation containing cholesterol, a phospholipid and FMCA provided both a prolonged contraceptive effect in horses [13] and anti-pZP antibody titres in elephants [14]. Reversibility of this formulation has, however, not been demonstrated which may be problematic for the conservation of threatened species including the free-ranging African elephant.

The presumed immunocontraceptive mechanism of pZP vaccination involves antibody binding to the ZP sperm receptor sites and subsequent prevention of sperm-oocyte binding and fertilisation. Based on this supposition, pZP immunisation should not affect the hypothalamic-pituitary-gonadal axis, with continuation of cyclical ovarian activity [15]. Ovarian suppression in the equid has however, been reported in recent years [16]. It has been suggested variously that this suppression may be associated either with vaccine contamination by non-ZP proteins in the native derived pZP formulations or as a sequel to the use of Freund's adjuvant, although this has yet to be fully defined [17]. T-cell involvement has also been proposed as a cause of ovarian dysfunction subsequent to pZP vaccination [18]. A clear link between ovarian functionality and a helper T-cell-mediated (CD4+) response has been demonstrated in mice after ZP-vaccination, manifesting as either

interstitial oophoritis excluding developing follicles and ovarian function interference [19] or as an effect on the developing follicles resulting in interference with ovarian function [20] dependent on the absence or presence of a detectable anti-ZP antibody response, respectively. A later study by Lloyd *et al*. (21) further demonstrated the association between a normal adaptive immune response (including a cell-mediated antibody response) and decreased fertility following ZP-vaccination. These studies collectively demonstrated the role of CD4+ and antibodies in ZP-vaccine associated ovarian dysfunction (18). T-helper cells play a role in the production of antibodies by B-lymphocytes but it is the cytotoxic T-lymphocytes (CD8+) that produce a direct cytotoxic effect. No overt confirmation of CD8+ involvement in ovarian dysfunction subsequent to ZP- vaccination was demonstrated until Joonè *et al.* in 2019 (22). Significant proliferation of CD8+ T-cells were detected in mares immunised with a pZP and reZP vaccine. Additionally, CD8+ T-cell levels were highly correlated with clinical measurements of ovarian cyclical activity in pZP vaccinated mares (22). An apparent link exists between the immunogenicity and contraceptive efficacy of a ZP vaccine and its effect on ovarian dysfunction (18).

Appropriate delivery systems for antigen presentation [23, 24] and effective cellular and humoral immune potentiators [23, 25] are required for ZP immunocontraception formulations. A recent approach to optimise vaccine immune responses utilises different adjuvant combinations that stimulate both Th1 and Th2 mediated responses [23, 26], which may be a useful prerequisite for successful ZP-based vaccination.

Previous investigations in mice and dogs have utilised alternative (i.e. non-Freund's) adjuvants including Pet Gel A, Alum and CP20, 961 in combination with ZP-antigens [27, 28]. In a murine model study [23] that used Pet Gel A for adjuvanting purposes, purified ZP3, the putative primary sperm receptor [29], was expressed with promiscuous T-cell epitopes of tetanus toxoid (TT-KK-ZP3) and ZP4 with a promiscuous T-cell epitope of bovine RNase (bRNase-KK-ZP4). These two treatment formulations elicited respective high antibody titres as well as T-cell responses. A decrease in fertility was also reported. An additional treatment group was primed with pZP and received a booster with combined TT-KK-ZP3 and bRNase-KK-ZP4. This treatment protocol demonstrated the highest antibody titres for all antigens (pZP, ZP3 and ZP4). The application of combined TT-KK-ZP3 and bRNase-KK-ZP4 formulated with Freund's adjuvants in pony mares [16] resulted in an

ineffective contraceptive effect coupled with a poor anti-pZP antibody titre response. Interestingly, the reZP formulation used in this study resulted in higher T-lymphocyte responses (both pZP-specific CD4+ and CD8+) than was seen in the pZP treated mares [22]. However no correlations existed between T-cell response and recorded clinical measures of cyclical ovarian activity of reZP treated mares (22). This same reZP formulation when administered in donkeys was associated with 100% contraceptive efficacy [30]. The post-synthesis treatment of the recombinant proteins used in the donkeys [30] differed from that used in the mare study [16, 22].

Injection site reactions associated with vaccine formulations containing Freund's adjuvants are well established in laboratory animals [31]. In the horse, probably the most-frequently studied species, as far as pZP-based immunocontraception is concerned, until very recently, injection site reactions following the use of Freund's adjuvants were rarely reported. A recent study in pony mares investigating pZP and reZP [16] formulated with FMCA (primary) and FIA (booster), reported injection site swelling and/or palpable changes in muscular density in over 95% of both treated and adjuvant control mares. Several developed overt sterile abscesses and this was observed more frequently in the reZP treated mares. The authors speculated that the higher frequency of abscesses may have been due to the presence of promiscuous T-cell epitopes in this formulation. A similar study in donkey jennies, which also compared pZP and reZP formulated with Freund's adjuvants, produced similar injection site reactions in both treated and adjuvant control groups. Similarly, more severe reactions were observed in the reZP-treated group [30]. Bechert *et al*. also reported localised reactions varying in intensity and duration, including overt abscessation in mares treated with a pZP liposomal mixture formulated with FMCA in an aqueous solution [13]. This comparison of injection site reactions in response to differing formulations and treatment protocols was however, confounded by the differing methods of measurement used. Additionally, previous studies in feral horses [3, 4] seldom monitored injection site reactions on a daily basis and consequently, injection site reactions may have been under reported, particularly in the 2-3 weeks after inoculation.

Whilst Freunds' adjuvants with ZP-based vaccines are associated with high antibody titres and subsequent contraceptive efficacy [3], the identification of an appropriate alternative commercially-available adjuvant with a satisfactory safety profile is indicated. Whilst many

of the conventional immunological adjuvants such as Freund's, bacterial toxins and non-purified crude agents (e.g. lipid A) typically induce strong immunogenic effects their administration also frequently induces adverse side-effects. Consequently any vaccine incorporating these adjuvants is unlikely to be approved by regulatory authorities [31, 32, 33, 34]. Vaccine adjuvants based on polymer technologies, such as Montanide™ Pet Gel A, are currently available for veterinary application and have been tested for safety and efficacy in several antigenic and animal models [35], including the horse [36]. Such adjuvants should be further investigated for use with immunocontraceptive vaccines.

Coupled with these issues, reliance on the native-derived proteins for pZP vaccine formulations remains an obstacle to efficient production (economical and immunogen purity), and its distribution and movement internationally [37, 38]. Several shortcomings associated with pZP vaccines, including extra-ZP ovarian proteins or other contaminants, stem from the use of the full complement of ZP proteins derived from a native source and it has been suggested that the continued successful application of ZP-based immunocontraception and standardisation of dose is contingent on the development of an effective recombinant formulation [16, 37, 38].

Recombinant ZP proteins have several compelling properties including improvements in antigen purity and structure [39]. The expression of recombinant proteins for vaccine antigen production using an Escherichia coli (*E. coli*) platform is well documented and reported benefits include high speed and yield of production, moderate production costs and scale-up capacity, no glycosylation and limited contamination risks in the form of endotoxins which can be addressed in purification processes [40].

The aims of this study were to identify a suitable non-Freund’s adjuvant formulation for delivery of ZP proteins and to apply this formulation in a subsequent study to monitor antibody titres, injection site reactions and rectal temperature in mares following their immunisation with native pZP proteins, reZP proteins or a combination of pZP and reZP proteins.

Materials and methods

Study 1

Subject selection, environment and management

Fifteen male horses (geldings) of mixed-breed type were studied from February to May 2016. Inclusion criteria were clinical health, adult status and normal body weight (range 306-458.5 kg). Horses were maintained at a single site at the South African Police Services Mounted Academy in Potchefstroom, North West Province, South Africa. Horses were maintained outdoors in four large paddocks providing ample space for freedom of movement and exhibition of normal behaviours.

Study design

Recruited horses were assigned to one of five treatment groups in this randomised controlled study. Treatments and measurements were initiated in February (d=0), repeated in April (d=35) and final measurements were taken in May (d=70).

Vaccine formulations

The antigen used in each formulation was native pZP (Trumpeter Farms and Veterinary Service, Winters, CA, USA) [3] and the dose *per* treatment was 100 µg.

Addavax (n=3): *per* dose (primary and booster) 500 µL squalene-based oil-in-water nano emulsion adjuvant (AddaVax ™, Invivogen, USA) was mixed with 500 µL phosphate buffered saline (PBS) containing the antigen.

Quil A (n=3): *per* dose 500 µg lyophilised purified saponin (Quil-A® Adjuvant, Invivogen, USA) reconstituted in 250 µL sterile water mixed with 500 µL PBS containing antigen and 250 µL physiological saline.

Quil A & Poly (I:C) (n=3): *per* dose 500 µg purified saponin reconstituted in 250 µL sterile water was mixed with 250 µL PBS containing antigen and 500 µg Polyinosinic-polycytidylic acid – TLR-3-based adjuvant (Poly (I:C) HMW VacciGrade™, Invivogen, USA) in 500 µL sterile water.

Pet Gel A (n=3): *per* dose 100 µL high molecular weight polyacrylic polymer in water adjuvant (10%; Montanide™ Pet Gel A, Seppic, France) was mixed with 500 µL PBS

containing antigen and 400 µL physiological saline.

Pet Gel A & Poly (I:C) (n=3): *per* dose 100 µL Pet Gel A (10%) mixed with 250 µL PBS containing antigen, 500 µg Poly (I:C) in 500 µL sterile water and 150 µL physiological saline.

Vaccine administration

Formulations were prepared on site and volumes were standardised at 1 mL *per* treatment. Primary vaccinations were administered in February (d=0) and single boosters 35 days later (d=35). All vaccines were administered by deep intramuscular injection (19-gauge needle) into the gluteal muscle mass. Boosters were administered into the contralateral musculature.

Sample collection and observations

Blood samples were collected by jugular venipuncture at d=0, d=35 and d=70 for measurement of serum anti-pZP antibody titres. Samples were centrifuged and serum stored at -20° C until assayed. Prior to and for three days following treatment, safety and side effects were assessed. The injection sites were assessed subjectively by visual inspection and palpation for changes including heat and swelling and scored using a three point scale (category 0 = no reaction; 1 = palpable reaction; 2 = visible reaction with or without pain upon palpation). Rectal temperatures were measured using a digital thermometer (Kruuse, Langeskov, Denmark) and respiratory and heart rates were recorded.

Study 2

Subject selection, environment and management

Thirty-one mixed-breed horse mares (light body type: Arabian, Quarter Horse, Draught and Thoroughbred cross; age: 2-10 y) were studied from November 2016 to May 2017, during the physiological breeding season in the southern hemisphere. Inclusion criteria were oestrous cyclicity, non-pregnant status, good clinical and reproductive health and no previous immunocontraceptive exposure. Mares were maintained on a single extensive mountainous grassland site (3000 ha) in pre-existing groups. The study site was located near Underberg, KwaZulu-Natal Province, South Africa.

Study design

Recruited subjects were stratified by body condition scores (BCS 1-9) [41], parity and age and assigned to one of four treatment groups in this randomised controlled study. Treatments and measurements were initiated in December (d=0) and repeated in January (d=35) and February (d=70) and further measurements were taken in March (d=105) and May (d=175).

Antigens used

Native pZP vaccine (Trumpeter Farms and Veterinary Service, Winters, CA, USA) was prepared according to standard methods [3]. Recombinant ZP3 and ZP4 proteins (reZP; supplied by Biomanufacturing Technologies, Biosciences, CSIR, South Africa) were expressed in *E. coli* according to Gupta *et al*. [24] with several modifications. Briefly, the antigen sequences were produced according to GenBank accession numbers NP_999058 and NP_999210, respectively, encoding porcine ZP3 (amino acid (aa) residues 20–421) and porcine ZP4 (aa residues 23–463) without the signal peptide and transmembrane-like domain. Zona pellucida 3 contained the tetanus toxin (TT; aa residues 830–844) at its N-terminus. Similarly, ZP4 incorporated the promiscuous T-cell epitope of bovine RNase (bRNase; aa residues 94–104). Both expressed products were confirmed by LC-MS peptide mapping. Doses of antigen used *per* immunisation were 100 µg and 500 µg (250 µg ZP3 and 250 µg ZP4) for pZP and reZP, respectively.

Vaccine formulations

The antigens were formulated in 6% Pet Gel A and 500 µg Poly (I:C) and were lyophilised in multi-vials. The same formulation was used for the adjuvant control group without addition of antigen.

Vaccine administration

Vaccines were reconstituted with sterile injection water immediately prior to administration to provide a treatment volume of 1 mL. All vaccines were administered by deep intramuscular injection (19-gauge needle) into the gluteal muscle mass. Boosters were administered into the contralateral musculature.

Adjuvant control group mares (n=8) were treated with adjuvant on d=35 with an identical booster on d=70.

The pZP only group (n=7) were treated on d=35 with 100µg of native pZP and adjuvant with an identical booster on d=70.

The reZP only group (n=8) were treated on d=0 with 500µg reZP and adjuvant and again on d=35 and d=70 with identical boosters. The reZP only treatment was started earlier so all groups' period of assumed maximal antibody titre (d=105) would align.

The pZP & reZP group (n=8) were treated on d=35 with 100 µg pZP and adjuvant and on d=70 with 500µg reZP and adjuvant.

Sample collection and observations

Blood samples were collected by jugular venipuncture at d=0, d=35 d=70, d=105 and d=175 for measurement of serum anti-ZP antibody titres. Samples were centrifuged and serum stored at -20° C until assayed. Prior to and for up to seven days following treatment, safety and side effects (injection site reactions and rectal temperature) were assessed as described in Study 1. Additionally, a concurrent investigation [42] in Study 2 monitored ovarian function subsequent to immunocontraception. Briefly, transrectal palpation and ultrasonography of the reproductive tract was performed at d=0, d=35, d=70, d=105 and d=175. Ovarian activity or inactivity was described dependent on detection of the presence or absence of follicles >15 mm, ovarian volume > or <25 cm3 (prolate ellipsoid formula) and serum progesterone levels > or <1 ng/mL.

Anti-pZP and -reZP antibody titre assays (Study 1 and 2)

Anti-ZP antibody response was measured by enzyme immunoassay (EIA), using a modification of a method previously described [16]. All tested sera were assayed in duplicate and expressed as a proportion of a positive reference standard at the same dilution rate. For the anti-pZP antibody assay (Study 2) the positive reference standard consisted of pooled sera from the pZP only treatment group at time of assumed maximal titre (d=105). For the anti-reZP antibody assay (Study 2) and anti-pZP antibody assay (Study 1) the positive reference standard consisted of previously stored pooled sera from mares treated with a pZP vaccine containing Freund's adjuvants [16]. Ninety six well plates (Nunc

Immunoplate F76 Maxisorp, South Africa) were incubated at 2-8 °C for 16 h with 1 µg (pZP or reZP (0.5 µg ZP3 and 0.5 µg ZP4)) in 100 µL coating buffer (2.94% $NaHCO_3$, 1.59% Na_2CO_3, pH 9.6) *per* well. Plates were washed with PBS containing 0.05% Tween 20 and then blocked with 0.03% BSA in PBS for 16 h at 2-8 °C. Plates were then incubated with serial dilutions of standard and test serum samples at 37 °C for 1 h (anti-pZP antibody assay (Study 1) 1:1000 to 1:16000 for test samples and 1:1000 to 1:64,000 for positive reference serum; anti-pZP antibody assay (Study 2) 1:250 to 1:4000 for test samples and 1:250 to 1:16,000 for positive reference serum; anti-reZP antibody assay 1:8000 to 1:128000 for test samples and 1:4000 to 1:512,000 for positive reference serum). Wells containing PBS were used as blanks (negative controls). After washing, antibodies were detected by incubating plates with recombinant protein G-horseradish peroxidase (LTC Tech South Africa, Johannesburg, South Africa) at 37 °C for 1 h. After further washing, plates were developed with trimethylene blue (SureBlue™). The reaction was stopped by adding 50 µL of 2 mol/L H_2SO_4 *per* well. Absorbance at 450 nm was measured using a microplate photometer (Multiskan™ FC). Antibody response was measured as the mean sample absorbance (minus blank) expressed as a proportion of the mean absorbance (minus blank) of the positive reference standard at the same dilution for each plate. The overall proportion positive was calculated as the average value over three dilutions. Intra- and inter-assay coefficients of variation were 9.07% and 16.32% for the anti-pZP antibody (Study 1), 4.02% and 10.83% for the anti-pZP antibody (Study 2) and 5.72% and 7.79% for the anti-reZP antibody assays, respectively.

Data analyses (Study 1 and 2)

Data were assessed for normality through the plotting of histograms, calculation of descriptive statistics and the Shapiro-Wilk test for normality.

Quantitative data were analysed using mixed effect linear regression. For statistical interrogation of group differences of categorical safety data, injection site reactions were reclassified as either present or absent. Similarly, elevated rectal temperatures were reclassified as ≥39 °C or <39 °C and were compared among treatment groups using mixed effects logistic regression (Study 2). Regression models included fixed effect terms for treatment group, sampling time (categorical with three/five levels) and a group by time

interaction. Horse age and BCS were also included as fixed effects. Horse was included as a random effect and a first-order autoregressive correlation structure was used to account for repeated sampling. Post-hoc tests in the mixed-effects models were adjusted using the least significant differences (LSD) method. Additionally, binomial logistic regression analysis was performed to investigate anti-pZP and –reZP antibody titres as a predictor of ovarian inactivity in response to treatment. A first-order autoregressive correlation structure was used to account for repeated sampling. . Statistical testing was performed using commercially available software (IBM SPSS Statistics Version 25) and significance was set at $P \leq 0.05$.

Results

Study 1

Anti-pZP antibody titre

Treatment, time and the treatment by time interaction all had a significant effect on anti-pZP antibody titres collected over the entire study (All $P<0.001$) (Figure 4-1). Overall the anti-pZP antibody titres of the Addavax group were significantly lower than the Quil A ($P=0.028$), Quil A & Poly (I:C) ($P=0.011$), Pet Gel A ($P<0.001$) and Pet Gel A & Poly (I:C) ($P<0.001$) treated groups. The Quil A and Quil A & Poly (I:C) treated groups were significantly lower than the Pet Gel A ($P=0.008$ and $P=0.020$, respectively) and Pet Gel A & Poly (I:C) groups ($P=0.007$ and $P=0.017$, respectively). No differences were evident between the Quil A and Quil A & Poly (I:C) treated horses ($P=0.591$) and the Pet Gel A and Pet Gel A & Poly (I:C) treated groups ($P=0.933$).

Injection site reactions, rectal temperature, respiratory rate and heart rate

Following the primary vaccination there were no notable increases in rectal temperature. Following the booster, however, increases in temperature were observed in the Quil A & Poly (I:C) and both Pet Gel A groups. The highest rectal temperatures were measured in the Pet Gel A & Poly (I:C) treatment group. By the second day post-treatment all temperatures had returned to normal levels with the exception of the Pet Gel A & Poly (I:C) treated group which returned to normal levels three days post-treatment. An increase in localised

swelling was seen in the Quil A group and the two Pet Gel A groups following the primary treatment. The booster was associated with noticeable swellings in all groups except the Addavax group. The two Pet Gel A groups displayed more injection site reactions, these, however, were no longer detectable within a week of treatment administration.

Respiratory rate increases were not evident following administration of any formulation but rather seemed to increase in association with increased environmental temperatures (environmental temperature reached a 39 °C maximum during the primary treatment administration on February 29th 2016 and subsequently a 26 °C maximum on the first day of the booster treatment administration on April 4th 2016). Heart rates remained consistent in all groups during both observation periods.

Study 2

Anti-pZP antibody titre

Treatment, time and the treatment by time interaction all had a significant effect on anti-pZP antibody titres (all $P<0.001$). The fixed effect terms of age and BCS had no effect on anti-pZP antibody titres ($P=0.474$, $P=0.085$, respectively). Overall, anti-pZP antibody titres changed significantly at each time point from d=0 until d=75 ($P<0.001$), steadily increasing until d=105 followed by a decline at d=175 (Figure 4-2). No significant differences were measured between pZP only and reZP only treated mares but reZP only and pZP only treated mares' titres differed to pZP & reZP treated mares, with lower concentrations in pZP & reZP treated mares ($P=0.009$, $P<0.001$, respectively) (Figure 4-3).

Anti-reZP antibody titre

Treatment, time and the treatment by time interaction all had a significant effect on anti-reZP antibody titres (all $P<0.001$). The fixed effect terms of age and BCS had no effect on anti-reZP antibody titres ($P=0.156$, $P=0.478$, respectively). Overall, anti-reZP antibody titres changed significantly at each time point from d=0 until d=175 ($P<0.001$), following a similar temporal pattern to that for anti-pZP antibody titres (Figure 4-4). In this instance the reZP only treated mares showed significantly higher titres than both pZP only and pZP & reZP treated mares (both $P<0.001$). The pZP only group and pZP & reZP group also differed ($P=0.037$) (Figure 4-5).

Injection site reactions and rectal temperature

Injection site reactions were observed in 22%, 55%, 46% and 47% of examinations in adjuvant control, pZP only, reZP only and pZP & reZP treatment groups, respectively. Elevated rectal temperatures (≥38.4 °C) occurred in 25%, 25%, 33% and 29% of examinations in adjuvant control, pZP only, reZP only and pZP & reZP treatment groups, respectively.

Treatment, time and the treatment by time interaction all had a significant effect on the incidence of injection site reactions (all $P<0.001$). Considerably more injection site reactions occurred in the reZP only group compared to both the adjuvant control and pZP only groups (both $P<0.05$). No other significant treatment group differences were seen. The occurrence of injection site reactions increased with each subsequent treatment administration ($P<0.05$). All reactions were both mild (category 1=97.5%) and transient, resolving within seven days of treatment administration. A similar pattern was observed for the post-treatment occurrence of elevated rectal temperatures. Treatment, time and the treatment by time interaction all had significant effects (all $P<0.001$), with a higher incidence of elevated temperature with each subsequent treatment administration ($P<0.05$), however, all had returned to within normal limits within seven days. No significant differences were seen between individual treatment groups.

Ovarian activity

An assessment of the causal relationship indicated that anti-pZP and –reZP antibody titres were a significant predictor of ovarian inactivity ($P=0.001$ 95% CI [0.644, 2.537], $P=0.001$, 95% CI [1.321, 4.758], respectively).

Discussion

The adjuvant combinations chosen for investigation were selected for their respective immunomodulatory components according to manufacturer's specification; Pet Gel A as an antigen carrier and cell and non-cell mediated potentiator [36, 43]; Addavax [44] and Quil A [45] for Th1 and Th2 response and Poly (I:C) as a TLR-3 agonist [46].

Study 1 showed that both Pet Gel A groups performed better than the other groups in invoking anti-pZP antibody titres. Furthermore, in combination with Poly (I:C), this increased the antibody response to native pZP. There was no significant difference in titres achieved between the two Pet Gel A groups, but T-cell proliferation analysis notwithstanding, further investigations of the combined Pet Gel A and Poly (I:C) were indicated.

Subsequent discussions with the manufacturers (Seppic, France) suggested that Pet Gel A concentration could be reduced from 10% to a 6% polymeric preparation without affecting overall antibody response. The side effects observed with both Pet Gel A formulations were confined to rapidly resolving local swelling and temperature reactions. These two variables, unlike heart and respiratory rate measurements, proved most informative in monitoring post-treatment reactions.

The results of this preparatory study informed the design and formulations used subsequently in Study 2.

Study 2 is the first to describe the immune response of horses following vaccination with pZP and reZP proteins formulated with non-Freund's adjuvants. In this study, anti-pZP antibody titres following vaccination with native pZP, reZP or pZP & reZP formulated with a combination adjuvant of Pet Gel A and Poly (I:C) showed temporal changes similar to previous reports in mares vaccinated with pZP formulated with Freund's adjuvants [16]. Furthermore, there was no difference in anti-pZP titres in mares treated with pZP only or reZP only formulations. The different treatment protocols between groups used in this study may arguably have played a role in the high anti-pZP antibody titre response in the

reZP only group. Multiple boosters are reportedly required for non-glycosylated synthetic vaccines to improve immunogenicity (47).Previously, this research group reported a poor anti-pZP antibody response in pony mares following reZP treatment [16]. In the current study, higher anti-reZP antibody titres were seen in the reZP only treated mares than in those receiving the other ZP treatments. The reZP vaccine used in the earlier study was sourced from a different laboratory and manufactured differently, formulated with Freund's adjuvant and only a single booster treatment was administered. It has been previously asserted that 70-80% of the pZP antigen is likely accounted for by ZP3, and when injected, the mare may produce substantially more antibodies against ZP3 than the other ZP proteins. The pZP only mares in the current study supported this assertion by producing high anti-reZP antibody titres [48, 49]. The higher anti-reZP titres in the reZP only group may be associated with the presence of TT and BRNase epitopes. The additional booster in this instance did not appear to bolster the anti-reZP antibody titre response. After a single booster treatment, the anti-reZP antibody titres at d=70 measured in the reZP treated mares was already significantly higher than all other groups at a corresponding measurement (d=105). The reZP only group maintained higher anti-reZP titres until the end of the observation period, which may be a feature of the additional booster. Mares primed with pZP and boosted with reZP did not achieve the highest anti-reZP or anti-pZP antibody titres in contradiction to a previous report where mice were used as a model species [27]. Reports of undesirable side effects vary with the use of Freund's adjuvants for ZP-based immunocontraception. This may be at least partially due to the limitations associated with clinical monitoring in feral horse populations rather than their absence. The current study, similar to more recent investigations [16, 30], monitored injection sites closely, however, in this instance there was minimal local reactivity and all reactions and elevated rectal temperatures were mild and transient in nature.

The commercial and regulatory limitations of both native pZP immunocontraceptive vaccines and Freund's adjuvants may be overcome through the use of reZP proteins expressed with promiscuous T-cell epitopes of tetanus toxoid and bovine RNase formulated with a commercially available polyacrylic polymer in water adjuvant that provides good antigen delivery (6 %; Montanide™ Pet Gel A) [42, 43] and a TLR-3 agonist (500 µg Poly (I:C)) [46].

An interesting, though not entirely unprecedented, finding [3] was the strongly significant relationship with anti-ZP antibody titres and ovarian inactivity. However, the contraceptive efficacy of the formulations used in this study requires further investigation. Additionally, the cell-mediated immune response following the use of these novel vaccine formulations should be assessed.

In conclusion, this was the first reported administration of a reZP vaccine formulated with non-Freund's adjuvant evoking a similar antibody titre response to a native pZP vaccine in mares.

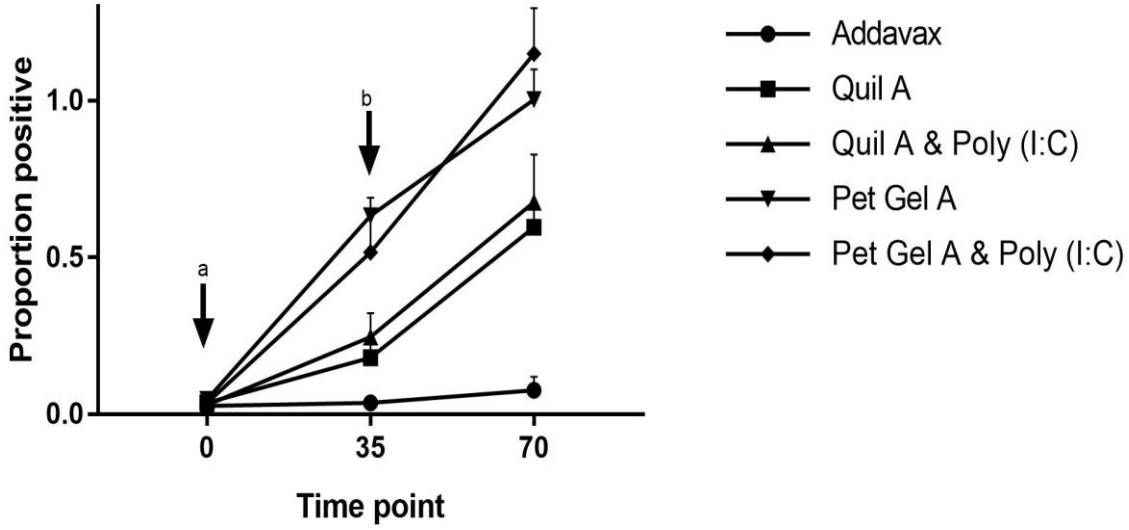


Figure 4-1 Study 1 (geldings) mean anti-pZP antibody response expressed as a proportion of the positive standard (+ s.e.) for all treatment groups [Addavax: n=3; Quil A: n=3; Quil A & Poly (I:C): n=3; Pet Gel A: n=3; Pet Gel A & Poly (I:C): n=3] at successive time-points 0 (d=0), 35 (d=35) and 70 (d=70)

a: primary vaccination; b: booster vaccination

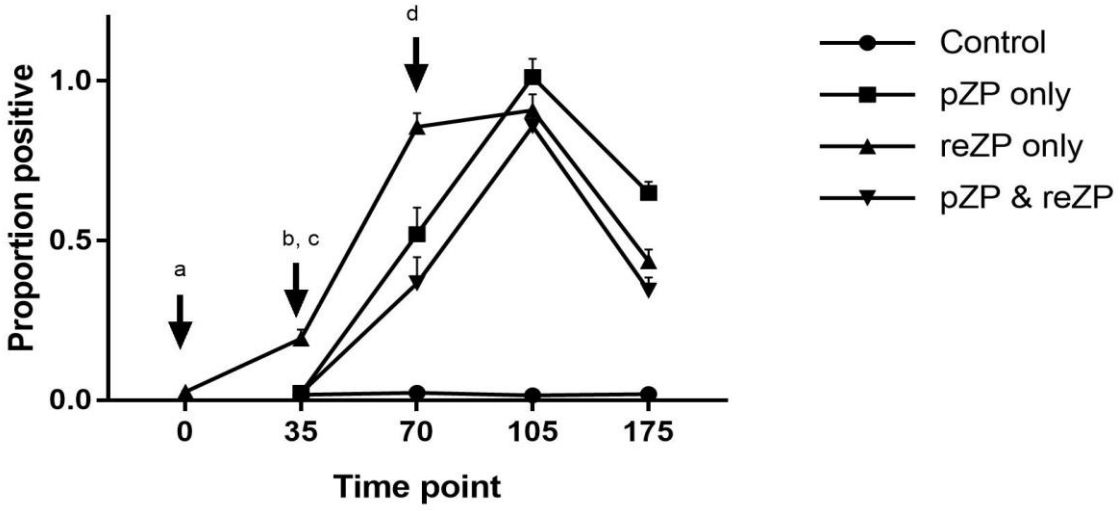


Figure 4-2 Study 2 (mares) mean anti-pZP antibody response expressed as a proportion of the positive standard (+ s.e.) for all treatment groups (Adjuvant control: n=8; pZP only: n=7; reZP only: n=8; pZP & reZP: n=8) at successive time-points 0 (d=0: reZP only), 35 (d=35: reZP; d=0 values plotted for all other groups), 70 (d=70), 105 (d=105) and 175 (d=175)

a: primary vaccination (reZP only); b: booster vaccination (reZP only); c: primary vaccination (adjuvant control, pZP only, pZP & reZP); d: booster vaccination (all groups)

Pairwise Comparisons[a]

(I) GROUP_PZP	(J) GROUP_PZP	Mean Difference (I-J)	Std. Error	df	Sig.[e]	95% Confidence Interval for Difference[e]	
						Lower Bound	Upper Bound
Group 1	Group 2	-.538[*,c,d]	.036	27.166	.000	-.613	-.463
	Group 3	-.467[*,c]	.033	22.440	.000	-.536	-.399
	Group 4	-.374[*,c,d]	.034	25.194	.000	-.443	-.304
Group 2	Group 1	.538[*,c,d]	.036	27.166	.000	.463	.613
	Group 3	.071[c]	.035	24.492	.055	-.002	.143
	Group 4	.165[*,c,d]	.036	26.930	.000	.090	.239
Group 3	Group 1	.467[*,d]	.033	22.440	.000	.399	.536
	Group 2	-.071[d]	.035	24.492	.055	-.143	.002
	Group 4	.094[*,d]	.033	22.067	.009	.026	.162
Group 4	Group 1	.374[*,c,d]	.034	25.194	.000	.304	.443
	Group 2	-.165[*,c,d]	.036	26.930	.000	-.239	-.090
	Group 3	-.094[*,c]	.033	22.067	.009	-.162	-.026

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: pZP_Titres.

c. An estimate of the modified population marginal mean (I).

d. An estimate of the modified population marginal mean (J).

e. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Figure 4-3 Study 2 (mares) anti-pZP antibody response group comparisons for all treatment groups (Group 1 Adjuvant control: n=8; Group 2 pZP only: n=7; Group 3 reZP only: n=8; Group 4 pZP & reZP: n=8) based on regression analysis including fixed effect terms for treatment group, sampling time (categorical with five levels), a group by time interaction, age and BCS. Horse was included as a random effect and a first-order autoregressive correlation structure was used to account for repeated sampling. *Post-hoc* tests in the mixed-effects models were adjusted using the least significant differences (LSD) method.

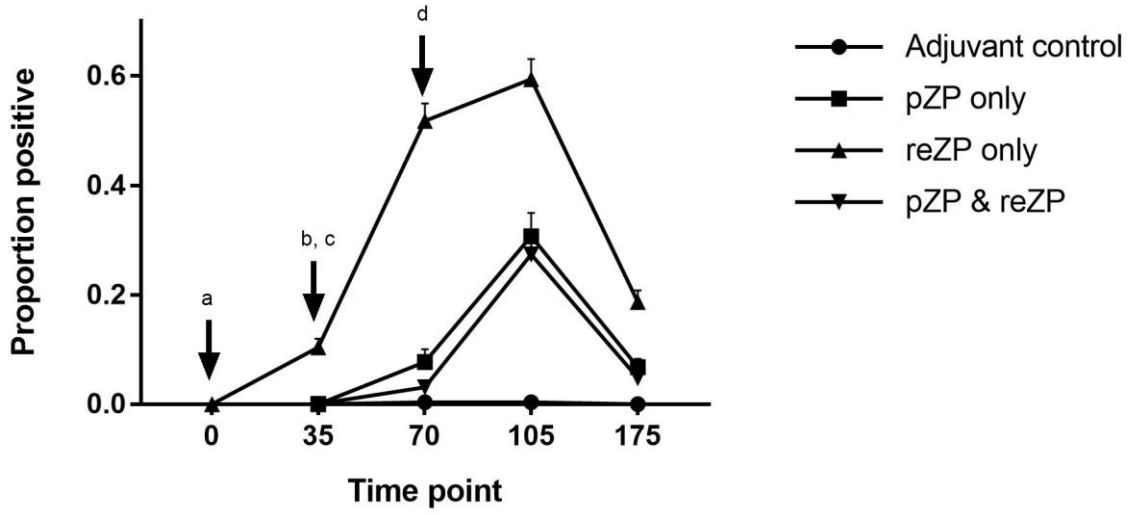


Figure 4-4 Study 2 (mares) mean anti-reZP antibody response expressed as a proportion of the positive standard (+ s.e.) for all treatment groups (Adjuvant control: n=8; pZP only: n=7; reZP only: n=8; pZP & reZP: n=8) at successive time-points 0 (d=0: reZP only), 35 (d=35: reZP; d=0 values plotted for all other groups), 70 (d=70), 105 (d=105) and 175 (d=175)

a: primary vaccination (reZP only); b: booster vaccination (reZP only); c: primary vaccination (adjuvant control, pZP only, pZP & reZP); d: booster vaccination (all groups)

Pairwise Comparisons[a]

(I) GROUP_PZP	(J) GROUP_PZP	Mean Difference (I-J)	Std. Error	df	Sig.[e]	95% Confidence Interval for Difference[e]	
						Lower Bound	Upper Bound
Group 1	Group 2	-.127[*,c,d]	.017	55.170	.000	-.161	-.093
	Group 3	-.285[*,c]	.015	44.658	.000	-.316	-.254
	Group 4	-.090[*,c,d]	.016	51.511	.000	-.122	-.058
Group 2	Group 1	.127[*,c,d]	.017	55.170	.000	.093	.161
	Group 3	-.158[*,c]	.017	48.448	.000	-.191	-.125
	Group 4	.037[*,c,d]	.017	54.515	.037	.002	.071
Group 3	Group 1	.285[*,d]	.015	44.658	.000	.254	.316
	Group 2	.158[*,d]	.017	48.448	.000	.125	.191
	Group 4	.195[*,d]	.015	43.929	.000	.164	.226
Group 4	Group 1	.090[*,c,d]	.016	51.511	.000	.058	.122
	Group 2	-.037[*,c,d]	.017	54.515	.037	-.071	-.002
	Group 3	-.195[*,c]	.015	43.929	.000	-.226	-.164

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: REZP_ZP.

c. An estimate of the modified population marginal mean (I).

d. An estimate of the modified population marginal mean (J).

e. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Figure 4-5 Study 2 (mares) anti-reZP antibody response group comparisons for all treatment groups (Group 1 Adjuvant control: n=8; Group 2 pZP only: n=7; Group 3 reZP only: n=8; Group 4 pZP & reZP: n=8) based on regression analysis including fixed effect terms for treatment group, sampling time (categorical with five levels), a group by time interaction, age and BCS. Horse was included as a random effect and a first-order autoregressive correlation structure was used to account for repeated sampling. Post-hoc tests in the mixed-effects models were adjusted using the least significant differences (LSD) method.

References

[1] Ownby CL, Shivers CA. Antigens of the hamster ovary and effects of anti-ovary serum on eggs, Biol Reprod, 1972; 6: 310-18

[2] Lyda RO, Hall JR, Kirkpatrick JF. A comparison of Freund's Complete and Freund's Modified Adjuvants used with a contraceptive vaccine in wild horses (*Equus caballus*), J Zoo Wildl Med, 2005; 36(4): 610-16

[3] Liu IKM, Bernoco M, Feldman M. Contraception in mares heteroimmunized with pig zonae pellucida, J Reprod Fertil, 1989; 85: 19-29

[4] Kirkpatrick JF, Liu IKM, Turner JW. Remotely-delivered immunocontraception in feral horses, Wildl Soc Bull, 1990; 18: 326-30

[5] McShea WJ, Monfort SL, Hakim S, Kirkpatrick JF, Liu IKM, Turner JW, *et al*. Immunocontraceptive efficacy and the impact of contraception on the reproductive behaviours of white-tailed deer, J Wildl Manage, 1997; 61: 560-69

[6] Fayrer-Hosken RA, Grobler D, Van Altena JJ, Kirkpatrick JF, Bertschinger H. Immunocontraception of free-roaming African elephants, Nature, 2000; 407: 149

[7] Bertschinger HJ, Delsink AK, Kirkpatrick JF, van Altena JJ, Ahlers M, Dickerson T, Powrie D, Burger A. Porcine zona pellucida immunocontraception of African elephants (Loxodonta africana): beyond the experimental stage, In: Cain J, Marshall J (eds), IVth International Wildlife Management Congress, 9-12 July 2012, Durban, South Africa

[8] Bertschinger HJ, Delsink A, van Altena JJ, Kirkpatrick JF. Porcine zona pellucida vaccine immunocontraception of African elephant (Loxodonta africana) cows: A review of 22 years of research, Bothalia, 2018; 48(2): 1-8

[9] Frank KM, Lyda RO, Kirkpatrick JF. Immunocontraception of captive exotic species. IV. Species differences in response to the porcine zona pellucida vaccine and the timing of booster inoculations, Zoo Biol, 2005; 24: 349-58

[10] Kirkpatrick JF, Turner A. Achieving population goals on long-lived wildlife with contraception, Wildl Res, 2008; 35: 513-19

[11] Rutberg A, Naugle RE. Population-level effects of immunocontraception in white-tailed deer, Wildl Res, 2008; 35: 494-501

[12] Delsink A, Van Altena JJ, Grobler D, Bertschinger H, Kirkpatrick JF, Slotow R. Implementing immunocontraception in free-ranging African elephants at Makalali conservancy, J S Afr Vet Assoc, 2007; 78: 25-30

[13] Bechert U, Bartell J, Kutzler M, Menino A, Bildfell R, Anderson M, Fraker M. Effects of two porcine zona pellucida immunocontraceptive vaccines on ovarian activity in horses, J Wildl Manag, 2013; 77(7): 1386-1400

[14] Bechert U. The response of African elephants to a single dose of SpayVac®, a pZP contraceptive vaccine, over a seven-year period, Pachyderm, 2016; 57: 97-108

[15] Barber MR, Fayrer-Hosken, RA. Possible mechanisms of mammalian immunocontraception, J Reprod Immunol, 2000; 46: 103-24

[16] Joonè CJ, Bertschinger HJ, Gupta SK, Fosgate GT, Arukha AP, Munhas V, *et al*. Ovarian function and pregnancy outcome in pony mares following immunocontraception with native and recombinant porcine zona pellucida vaccines, Equine Vet J, 2017; 49(2): 189-95

[17] Kirkpatrick JF, Liu IMK, Turner JW, Naugle R, Keiper R. Long-term effects of porcine zonae pellucidae immunocontraception on ovarian function in feral horses (*Equus caballus*), J Repro Fertil, 1992; 94(2): 437-44

[18] Joonè CJ, Schulman ML, Bertschinger HJ. Ovarian dysfunction associated with zona pellucida–based immunocontraceptive vaccines, Theriogenology, 2017; 89: 329-37

[19] Rhim SH, Millar SE, Robey F, Luo AM, Lou YH, Yule T, *et al*. Autoimmune disease of the ovary induced by a ZP3 peptide from the mouse zona pellucida, J Clin Invest, 1992; 89: 28

[20] Lou Y, Park K, Agersborg S, Alard P, Tung KSK. Retargeting T cell-mediated inflammation: a new perspective on autoantibody action, J Immunol, 2000; 164: 5251–7

[21] Lloyd ML, Papadimitriou JM, O'Leary S, Robertson SA, Shellam GR. Immunoglobulin to zona pellucida 3 mediates ovarian damage and infertility after contraceptive vaccination in mice, J Autoimmun, 2010; 35: 77–85

[22] Joonè CJ, Schulman ML, Fosgate GT, Plagis TA, Crafford JE, Gupta SK, Bertschinger HJ. Antigen-specific CD4+ and CD8+ T-cell responses in PBMC from pony mares immunized with either native or recombinant zona pellucida vaccines, Theriogenology, 2019; 126: 106-13

[23] Apostólico J de S, Lunardelli VAS, Coirada FC, Boscardin SB, Rosa DS. Adjuvants: Classification, modus Operandi, and Licensing, J Immunol Res, 2016; 6: 1–16

[24] Goto N, Akama K. Histopathological studies of reactions in mice injected with aluminium-adsorbed tetanus toxoid, Microbiol Immunol, 1982; 26(12): 1121-32

[25] Olive C. Pattern recognition receptors: sentinels in innate immunity and targets of new vaccine adjuvants, Expert Rev Vaccines, 2012; 11(2): 237-56

[26] Lee S, Nguyen MT. Recent advances of vaccine adjuvants for infectious disease, Immune Netw, 2015; 15(2): 51-7.

[27] Gupta N, Chakrabarti K, Prakash K, Wadhwa N, Gupta T, Gupta SK. Immunogenicity and contraceptive efficacy of Escherichia coli- expressed recombinant porcine zona pellucida proteins, Am J Reprod Immunol, 2013; 70: 139-52

[28] Mahi-Brown CA, Yanagimachi R, Hoffman JC, Huang TT Jr. Fertility control in the bitch by active immunization with porcine zonae pellucidae: use of different adjuvants and patterns of estradiol and progesterone levels in estrous cycles, Biol Reprod, 1985; 32(4): 761-72

[29] Prasad SV, Skinner SM, Carino C, Wang N, Cartwright J, Dunbar BS. Structure and Function of the Proteins of the Mammalian Zona Pellucida, Cells Tissues Organs 2000; 166(2): 148-64.

[30] Ambrosia RL, Roberts BN, Roberts TA, DeYoung BL, Peterson EW, Bertschinger HJ, Schulman ML, Crampton M, Roth R, van Zyl PJ, Cameron-Blake N, Vandenplas ML, Knobel DL, French HM. Porcine and recombinant zona pellucida vaccines as immunocontraceptives for donkeys in the Caribbean, Clin Theriogenology, 2017; 9(3): 439

[31] Billiau A, Matthys P. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases, J Leukocyte Biol, 2001; 70(60): 849-60

[32] Cox JC, Coulter AR. Adjuvants-a classification and review of their modes of action, Vaccine, 1997; 15(3): 248-56

[33] Sharma S, Hinds LA. Formulation and delivery of vaccines: Ongoing challenges for animal management, J Pharm Bioallied Sci, 2012; 4(4): 258-66

[34] Claassen E, De Leeuw W, De Greeve P, Hendriksen C, Boersma W. Freund's complete adjuvant: an effective but disagreeable formula, Research Immunol, 1992; 143(5): 478-83

[35] Parker R, Deville S, Dupuis L, Bertrand F, Aucouturier J. Adjuvant formulation for veterinary vaccines: Montanide™ Gel safety profile, 2nd Vaccine Global Congress, Boston 2008 Procedia in Vaccinology, 2009; 1: 140–7

[36] Cauchard A, Bertrand F, Barrier-Battut I, Jacquet S, Laurentie M, Barbey C, *et al*. Assessment of the safety and immunogenicity of Rhodococcus equi-secreted proteins combined with either a liquid nanoparticle (IMS 3012) or a polymeric (Pet Gel A) water-based adjuvant in adult horses and foals-Identification of promising new candidate antigens, Vet Immunol Immunopathol, 2014; 157: 164– 74

[37] Kirkpatrick JF, Rowan A, Lamberski N, Wallace R, Frank K, Lyda R. The Practical Side of Immunocontraception: Zona Proteins and Wildlife, J Reprod Immunol, 2009, 83(1-2): 151-7

[38] Gupta SK, Bansal P. 2010 Vaccines for immunological control of fertility, Reprod Med Biol, 2010; 9: 61-71

[39] Fayrer-Hosken R. Controlling Animal Populations Using Anti-Fertility Vaccines, Reprod Domest Anim, 2008; 43: 179-85

[40] Legastelois I, Buffin S, Peubez I, Mignon C, Sodoyer R, Werle B. Non-conventional expression systems for the production of vaccine proteins and immunotherapeutic molecules, Hum Vaccin Immunother, 2017;13(4): 947-61

[41] Henneke DR, Potter GD, Kreider JL. Relationship between condition score physical measurements and body fat percentage in mares, Equine Vet J, 1983; 15(4): 371-2

[42] Nolan MB, Bertschinger HJ, Roth R, Crampton M, Martins IS, Fosgate GT, Stout TA, Schulman ML. Ovarian function following immunocontraceptive vaccination of mares using native porcine and recombinant zona pellucida vaccines formulated with a non-Freund's adjuvant and anti-GnRH vaccines, Theriogenology, 2018;120: 111-16

[43] Safety and efficacy profile of Montanide™ Pet Gel A dedicated to sensitive veterinary species. 2010. Technical bulletin. S.Deville Scientific marketing, Seppic, France

[44] InvivoGen, Addavax ™ information sheet, https://www.invivogen.com/addavax

[accessed: 12 September 2018]

[45] InvivoGen Quil-A® Adjuvant information sheet https://www.invivogen.com/quila

[accessed 12 September 2018]

[46] Poly (I:C) (HMW) VacciGrade™ Polyinosinic-polycytidylic acid – TLR-3-based adjuvant, Data sheet version # 11E06-MT, Invivogen, USA

[47] Paterson M, Wilson MR, Morris KD, van Duin M, Aitken RJ. Evaluation of the contraceptive potential of recombinant human ZP3 and human ZP3 peptides in a primate model: their safety and efficacy, Am J Reprod Immunol, 1998; 40:198–209

[48] Wassarman PM. Zona pellucida glycoproteins, Annu Rev Biochem, 1988; 57: 415–42

[49] Mask TA, Schoenecker KA, Kane AJ, Ransom JI, Bruemmer JE. Serum antibody immunoreactivity to equine zona protein after SpayVac vaccination, Theriogenology, 2014; 84 (2): 261-7

Chapter Five:
A review of the current status of porcine zona pellucida immunocontraception of elephant cows in South Africa

Margaret B Nolan[a,b], Henk J Bertschinger[a,b], Martin L Schulman[a,b]

[a]Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

[b]Veterinary Population Management Laboratory, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

Manuscript in preparation

Abstract

Most of South Africa's elephant populations exist in relatively small fenced areas, many of which are privately owned. As a consequence, local elephant overabundance has led to adverse impacts on habitat. In South Africa the current Norms and Standards for Managing African Elephants recommends the use of the porcine zona pellucida (pZP) vaccine for elephant population management. Monitoring programmes subsequent to the use of pZP in elephants are required for both effective population management and regulatory reasons. This study reviewed the population data on current and previous use of pZP in managed elephant populations in South Africa and provided information on the game reserves utilising this management method. The data was sourced both from participating game reserves and administering veterinarians and *via* retrospective analysis of laboratory records. There was evidence of broad-scale adoption of this management method across a range of elephant populations, with sustained increases in both the number of reserves enrolled in the programme and the number of vaccines doses supplied. The number of reserves enrolled in pZP immunocontraception programmes since inception in 2000 has increased markedly. Thirty-four game reserves have been noted in the record for the use of pZP immunocontraception in elephants, although not all remain active participants. In 2017, 27 actively-participating game reserves used pZP vaccination, with a total treated population of 811 cows. Immunocontraception on both an individual and herd basis is effective for population management when treatment is initiated with a primary and booster inoculation in year one and continued with a subsequent annual booster thereafter. Compliance with mandatory reporting and record keeping remains poor. A number of measures can be implemented to address this problem to facilitate future successful management of the elephant pZP immunocontraception programme.

Introduction

Elephant populations in Botswana, Namibia, Zimbabwe and South Africa are stable or increasing and are listed in the Convention on International Trade in Endangered Species (CITES) Appendix II [1]. Apart from the large population of elephants in the Kruger National Park (KNP), most of South Africa's elephant populations exist in relatively small fenced areas, many of which are privately owned [2]. In some reserves, local elephant overabundance has led to adverse impacts on habitat and a number of management approaches to counteract this have been undertaken [2]. In 2008 the Norms and Standards (N&S) for Managing African Elephants were drafted by the South African Department of Environmental Affairs and Tourism (updates are currently pending) [3]. The N&S recognised that managers needed to control the growth of elephant populations and immunocontraception with the porcine zona pellucida (pZP) vaccine was identified as a suitable method to be used as a non-lethal alternative to culling [4].

The use of the pZP vaccine for elephant fertility control is well documented. Following proof of concept studies initiated in 1996 [5, 6, 7], the first field trial for the use of pZP vaccines for population control in elephants commenced in 2000 at the Makalali Conservancy in the Limpopo Province of South Africa. Collectively, these studies demonstrated that when targeted free-ranging cows are individually identified, pZP vaccine can be successfully applied using remote delivery techniques in both large and small game reserves. This will affect fertility and subsequently population growth rates [4, 8, 9]. A later report [10] further demonstrated the efficacy of pZP treatment and reported the effects on reproductive rates in seven private game reserves. This report showed a contraceptive efficacy rate of 100% in small to medium-sized free-ranging populations. Continued recruitment of new populations into the pZP immunocontraception programme ensued. In 2018 a 22 year review [11] of the current implementation concluded that pZP immunocontraception delivered on an individual cow basis was associated with an efficacy rate of 100%. In addition, population control is achievable with application in larger populations precluding individual cow identification (herd or 'blanket-treatment'). The use of the pZP vaccine is now well-established for elephant population management. The administration of pZP vaccine at a population level, however, requires ongoing monitoring according to the needs

of a specific elephant management plan (EMP) and habitat response. Recent population modelling exercises reported that with contraceptive efficacy rates of 100%, a population extinction would be reached in 50-100 years [12]. Achieving a measurable decline in the number of individuals in a population requires the contraception of 77% of all breeding cows [13]. Immunocontraception cannot have an immediate effect on a population, but assuming a 100% efficacy, no further births will occur during the third year after implementation of a programme. Population decline then becomes dependent on mortality rate, which in turn is dependent on a population's age structure, environmental factors like rainfall, disease or any other interventions. An understanding of the demographics of the population and how this may alter when utilising fertility control is obviously an important consideration. Key to this is an understanding of the natality and mortality rates and any immigration or emigration, to fully assess changes in population based on any interventions [9].

The pZP vaccine is an Unregistered Medicine in terms of Section 21 of Act 101, the Medicines and Related Substances of 1965, and is subject to approval by the South African Health Products Regulatory Authority (SAHPRA) (formerly the Medicines Control Council). Annual progress reports to the SAHPRA detailing particulars of the unregistered medication, treating veterinarian, treated animal, outcome of treatment and any adverse drug reactions (ADR) are required. The production and use of the vaccine is also subject to approval from the Department of Agriculture, Forestry and Fisheries (DAFF) (Section 20 of the Animal Diseases Act, 1984). Furthermore, the N&S states that pZP should be administered by a registered veterinarian and must be monitored to evaluate the effects of the programme in accordance with the National Environmental Management: Biodiversity Act, 2004 [3]. In 2003, the Veterinary Population Management Laboratory (VPML), of the Section of Reproduction, Department of Production Animal Studies, University of Pretoria, Onderstepoort, South Africa, started producing the pZP vaccine [11]. The current vaccine formulation consists of 400 μg (primary) and 200 μg (booster) of solubilised porcine zona pellucida proteins produced from pigs' ovaries obtained from abattoirs [11] formulated with Freund's modified complete adjuvant (FMCA) and Freund's incomplete adjuvant (FIA) for primary and booster inoculations, respectively [11]. In year one a primary inoculation is administered, followed by a booster after five weeks, and

thereafter with a single annual booster [11].

This study aimed to investigate the status and scale of pZP immunocontraception for management of elephant populations in South Africa by:

- identifying pertinent data for monitoring of pZP immunocontraception programmes,
- creating a questionnaire to obtain this data from all participating game reserves,
- collecting all available data from participating game reserves and
- cross-referencing self-reported game reserve data with previously collected laboratory data for creation of a single data repository for reporting and monitoring purposes.

At the conclusion of the study period, analysis of data obtained from the respondents and additional retrospective data sourced from laboratory records provided insight into the current status of pZP immunocontraception implementation.

Materials and Methods

Background information

New populations of elephants are enrolled in the pZP immunocontraception programme on a continual basis. In order to obtain pZP vaccine a game reserve must directly request this from the VPML which is the sole authorised producer of pZP vaccine in South Africa. These requests must all be accompanied by a veterinary prescription from the administering veterinarian. A game reserve must additionally provide an approved EMP for the target population. Based on the number of vaccine requests a database on the number of doses supplied (recorded in association with veterinarian and/or game reserve identification) is maintained by the VPML. Annual self-reporting by the game reserve or the administering veterinarian is then used to establish the approximate number of treated cows. This forms the basis for annual reporting by the VPML to the SAHPRA in accordance with Section 21 authorisations. These records were reviewed to identify game reserves that currently utilise or have previously used pZP vaccination in attempting to establish the number of treated elephants since the programme's inception.

Methods

- In this qualitative, descriptive study a questionnaire was prepared identifying pertinent data in relation to treated populations (Appendix 1). This questionnaire was divided into three sections and included environmental considerations, elephant population histories and demographics and treatment administration details. The data identified for collection was based on the minimum information required for an EMP, Section 21 of Act 101, the Medicines and Related Substances of 1965 reporting and Section 20 of the Animal Diseases Act, 1984 reporting. The collected data provided information describing the location, size and ecology of participating game reserves, the targeted elephant populations' size and histories, the treatment administration procedures and the number of treated elephants in South Africa.
- Contact information for enrolled reserves and administering veterinarians was collected.
- Requests were made *via* e-mail, telephone and third-parties for on-site visits to facilitate direct communication and data collection with each reserves' management.
- On-site visits and/or remote communications were conducted.
- Follow-up requests were made when required.
- Completed questionnaires, additional data provided in the form of population count databases and reports and informed consent forms for use of the collected data were collated into a single database and cross-referenced with existing VPML records for quality control and auditing purposes.

Results

Summary of data

In 2005 there were seven game reserves enrolled in the programme and this had increased to 11 by 2010. In the period from 2010-2017, three of the original enrolled reserves ceased treatment and 19 additional game reserves were enrolled in the programme.

In 2016 there were 23 known game reserves, across four provinces (Gauteng, Eastern Cape, KwaZulu-Natal and Limpopo Provinces) that were then or had previously used pZP as part

of their elephant management approach. The approximate number of elephants treated in that calendar year based on the number of vaccine doses supplied was 611. Annual reports provided by game reserves and administering veterinarians indicated that 536 cows had been treated.

Laboratory records indicated vaccine sales to 6, 15, 17, 22 and 22 game reserves in 2013, 2014, 2015, 2016 and 2017, respectively (Figure 5-1). Treatments are generally administered prior to the summer season for logistical reasons. However in the 2017 season several reserves administered treatment later in the summer period and so laboratory records for vaccine supply were recorded early in 2018. In addition to the 15 game reserves identified in 2014, records indicated sales to three administering veterinarians/technicians without associated game reserve identification. In 2016 (22 recorded sales), 10/22 (45.5%) treatment reports were submitted by administering veterinarians and game reserves returned 8/22 (36.4%) questionnaires and made 5/22 (22.7%) EMPs available. In 2017, 13/22 (59.1%) game reserves previously identified in the 2016 laboratory record of sale, were once again apparent. In addition there were four newly enrolled reserves (three EMPs available with four reports of treatment by an administering veterinarian) and five reserves that although not identified in the 2016 vaccine sales records, were currently or had previously used pZP vaccination during the preceding four years (two EMPs available, five reports of treatment by administering veterinarians and two returned questionnaires). One additional questionnaire was returned by a reserve that had ceased treatment in 2011 (Table 5-1). Of 27 questionnaires distributed, 11 (40.7%) were returned by the end of the data collection period.

By the data collection cut-off date of April 2018 the number of identified reserves that are currently or were previously enrolled in the immunocontraception programme totalled 34, with 27 game reserves actively-participating in the programme (administering vaccines on an annual basis). These reserves were distributed across four provinces, with one in Gauteng, five in the Eastern Cape, 13 in KwaZulu-Natal and 15 in Limpopo Provinces, representing four biomes. These reserves ranged in size from 50-960 km^2 (Table 5-2). The total population range of elephants in game reserves utilising pZP immunocontraception was between 6 and 578 individuals, with between 3 and 176 cows treated in each of these

reserves (Table 5-3).

Records indicated the treatment of 811 cows in 2017. This represents populations across 25 individually identified game reserves. The number of reserves identified as actively-enrolled in the programme was 27, however, specifics on treated populations from two reserves in 2017 were not available by the cut-off period (Table 5-3).

Data from returned questionnaires

The data provided from four of the 11 returned questionnaires provided no information of population or treatment effects. The summarised data from questionnaires and additional suitable data (population count databases and reports) sourced from a further three game reserves giving a response total of 10 game reserves is reported below.

1. Karongwe Private Game Reserve

Four cows originally treated in 2007 had all calved by 2009. Following this period, no more calves were recorded. In 2012, two additional cows were enrolled. These newly enrolled cows had both produced calves in 2011 and subsequently no calves have been recorded. Treatment was administered to individually-identified cows.

2. Phinda Game Reserve

This reserve self-reported an annual calving rate of 6.07% since year three after inception of the immunocontraception programme. The pre-treatment annual calving rate for this population was 22.32%. Treatment was administered to individually-identified cows.

3. Thaba Tholo Game Farm

This reserve initiated treatment in 2004 in eight cows (>17 years). In the period 2012-2017, two calves were reported. The pZP programme was interrupted for six years following adoption of a programme of vasectomy of adult bulls. An additional five new animals >10 years have been added to this population since inception of the original programme. Treatment was resumed in 2013 and 12 cows (>15 years) are currently receiving pZP treatment. No calves were reported in 2017.Treatment was administered to individually-identified cows.

4. Thornybush Game Reserve

This reserve initiated treatment in 2005 with no reported calvings from year three until 2011 when treatment ceased. Subsequently the fences separating this reserve from KNP have been removed. This has resulted in significant changes in population size and movements with a reported population of 354 in 2017. Treatment was administered to individually-identified cows.

5. Welgevonden Game Reserve

This reserve initiated treatment in 2006 in 35 cows. The number of cows receiving treatment had increased to 66 by 2017 with 2-6 calves recorded annually from year three after implementation (compared with 14 in the year that treatment started). A 'blanket treatment' approach has been utilised since 2010. In 2008, 2009 and 2010 one, four and eight cows did not receive treatment, respectively (Table 5-4). There were no recent population size estimates.

6. Zimanga Private Game Reserve

This reserve initiated treatment in 2010. An annual calving percentage prior to this was reported as 5-7%. Since 2013, a single calf has been recorded (from an untreated cow). Although the population count indicated an increase of two animals, it was unknown if this was due to either an additional calf or a previously unaccounted for adult. Treatment was administered to individually-identified cows.

7. Tembe Elephant Park

This is an older and established reserve with an age-equilibrated elephant population. The treatment approach in Tembe Elephant Park is on a herd or 'blanket treatment' basis, with individually identified cows (radio-collars) being used to locate family units. Although detailed information was available from Tembe Elephant Park, several discrepancies were apparent. From the reserve's submission it appeared that the population (excluding adult bulls) had grown from 54 in 1993 to 179 in 2007. From 2004-2007 the number of calves born annually varied from 10-16. Treatment was initiated in 2007 and from 2008-2017 the number of calves in the 0-2 years category had declined to one. The total population,

excluding bulls, reported in 2017 was 91 (Table 5-5). Since the data collection cut-off date, additional clarification was sought on the discrepancies in the reported data. The variations in the total population depended on the number of herds that were successfully located during a specific treatment administration operation. Furthermore, in some years specified family units were excluded from treatment and subsequently not recorded within the population count. The total population at Tembe Elephant Park has stabilised since 2007, however, with little or no increases by 2017.

8. Hluhluwe-iMfolozi Park (HiP)

This reserve initiated treatment in 2014. In 2018 HiP reported a population of 326 elephants (total number within located herds containing targeted cows for immunocontraception). Within these herds 47 calves aged 0-2 years were reported. In the first year of treatment (2016), this number was recorded as 352 elephants and 68 calves aged 0-2 years. Proportionally, this suggested a reduction in calving rates. Treatment was administered on a herd basis.

9. Ithala

This reserve initiated treatment in 2014. The population recorded in 2014 was 125 elephants with 26 calves aged <1 year. In 2018 the population was 154 with 13 calves aged <1 year. Treatment was administered on a herd basis.

10. Amakhala

This reserve initiated treatment in 2008 in three cows. Three calves were born in 2009. From 2009-2017 cows have been excluded and admitted to the programme on an individual basis, with one cow calving 34 months after her most recent vaccination. No calves have been born to cows continuously treated for at least three years. Treatment was administered to individually-identified cows.

Discussion

This study described the size, location and habitat of game reserves administering pZP vaccines to elephants, the different treatment approaches and the current status of elephant population management in South Africa.

The number of elephant cows that are currently receiving pZP vaccination had increased considerably since inception in 2000. This study's data for 2017 vaccinations showed a treatment number of 811 cows across 27 actively-participating game reserves. Speculatively, the current level of pZP vaccination may be partially driven by an increased awareness and understanding of the method. A recent workshop for stakeholders was hosted by the Elephant Specialist Advisory Group on elephant population control methods [14]. The workshop's outcomes [14] supported this study's data, as both reflected an appreciable level of acceptance and highlighted the need for increased vaccine production.

The total estimated population of elephants in 2016 South Africa was 18,841, up from 17,847 since 2006 [2]. There is great potential for the expansion of the immunocontraception programme for elephant management in South Africa. Examples of pZP immunocontraception applied to animal population management programmes that may inform and support the future of elephant management include populations of feral horses on Assateague Island National Seashore, Maryland, USA and urban white-tailed deer on Fire Island, New York, USA [15].

An initial assessment of the effects of pZP vaccination on elephant population dynamics can only be established in year three following initiation of treatment [11]. As a consequence eight of the current subscribers to the pZP programme have yet to see its effects on annual calving rates. Sixteen of the 26 reserves (73.1%) currently and historically enrolled in the programme for longer than this period had not supplied data to accurately review efficacy rates. The data required to monitor efficacy are total population counts and the identification of infant calves, ideally on an annual basis. This count was not routinely reported on most of the game reserves investigated in this study. An additional factor

complicating this reporting had been the recent need to shift attention to the imperative of anti-poaching efforts by all game reserves with rhinos in South Africa.

From the seven participating reserves reported by Bertschinger *et al.* 2012 [10], four were still participating in the programme in 2017. Detailed information was only available for three of these reserves. Phinda Game Reserve reported ongoing success with a reduced annual calving rate. Welgevonden Game Reserve reported calves of <1 year and had recently employed (August 2017) an elephant monitor in order to correctly assess the changes in population. As a result future data from this population should prove useful. Ka'Ingo Private Game Reserve had also reported a calf <1 year of age in 2017 (treatment re-initiated in 2014) based on a report from the administering veterinarian. No other data was available for this game reserve. Thornybush, also an original subscriber to the programme ceased treatment in 2011. No calves were reported in the period 2008-2011 at this game reserve.

Game reserves that enrolled subsequent to the original report of Bertschinger *et al.* 2012 [11], that have participated for more than three years and provided sufficient data to make inferences from, namely Karongwe, Thaba Tholo, Zimanga, Tembe, HiP, Ithala and Amakhala, all reported positive results in managing population growth rates. This was reflected by decreased annual calving rates or calf counts.

An important consideration for identification of suitable cows to include in the programme was the assumed age of sexual maturity, many reserves chose to exclude a cow until after she had produced her first calf. At Thaba Tholo Private Game Reserve, treatment was ended in 2007 subsequent to surgical vasectomies being carried out on their older bulls. However pZP treatment resumed in 2013, after the birth of calves that were presumed to have been sired by younger non-vasectomised bulls. Alternatively, or additionally, some of these calves may have resulted from untreated younger cows becoming pregnant and calving. By comparison on two other reserves, namely, Karongwe and Thornybush Private Game Reserves, a practice of early enrolment of cows prior to their assumed age of sexually maturity had resulted in lower reported calving rates of one and none, respectively. The age of enrolment into the immunocontraception programme was dependent on the

reserve objective, with either an aim of no calves or a reduced calving rate. The age of cows at first ovulation was reportedly dependent on population density associated with differences in density-dependent physiological, social and nutritional stressors, with the age of cows at first parturition ranging from 9-18 years [16]. Furthermore, in relocated populations (including several of the treated populations) a female bias was reported [17]. In most populations with either a skewed age (towards younger elephants) or sex profile it is critical to correctly identify cows of potential breeding age. As an older, established reserve, Tembe Elephant Park had an age-equilibrated population. There, pZP treatment was applied on a herd basis with individually identified cows used to differentiate between breeding groups. This strategy had stabilised this population. This outcome supported pZP immunocontraception additionally being successfully applied when using 'blanket treatment' for population management.

There were limited reports of side effects associated with vaccine administration, observed matings and cow-bull interactions across all treated populations.

Additionally, and perhaps an empirical observation, is that any failure of the pZP contraception programme would have been reported to the VPML. To date, no such reports have been received.

The major limitations of this investigation were the disappointing compliance and the format and accuracy of the available data. There are obligatory monitoring requirements from both regulatory and research standpoints. Through on-site communications it became apparent that participants were not all aware of the research status of the overall project and their obligation for ongoing data collection. Additionally, confusion regarding the onus of responsibility for reporting became apparent in the course of this investigation. Several elephant managers had assumed that this was the sole responsibility of the administering veterinarian, and vice-versa.

In addition to these limitations it was apparent that much of the requested data was unavailable. An additional complication was that in certain cases where comprehensive data existed, a reluctance to share data was manifest. It can be speculated that this was at

least partially ascribable to a perceived lack of awareness regarding the obligations for data reporting and associated responsibilities. This was despite records reflecting that all indemnity and agreement documents had in fact been signed both at treatment initiation and with annual booster vaccine supply applications.

The practice of bulk ordering of vaccine doses by administering veterinarians without individual reserve identification and reporting was an additional unanticipated limitation that became apparent during the course of this study. Until 2017, all treated populations were outlined under one laboratory approval from the SAHPRA. A recently implemented system (2017) of individual authorisations by the SAHPRA is likely to assist in overcoming this limitation.

Whilst the VPML maintains records of all distributed doses of vaccine, to date this vaccine information had only been recorded by vaccine dose (in mg). This variable is inadequate in accurately representing the actual number of cows successfully treated on each reserve. This accuracy is limited by being associated with complicating factors at the point of vaccine delivery including drug wastage and the vagaries of the darting process.

The data collection methods and format applied in this pilot also proved to be important limitations. This was highlighted by several of the responses. Tembe Elephant Park and HiP provided seemingly comprehensive submissions, including several data spreadsheets and reports. These did not, however, directly answer many of the specified questions and the data was conflicting and difficult to interpret. Two other submissions provided descriptive information containing limited quantitative data. Furthermore, the detailed nature of the data requested *via* the questionnaire may have been negatively perceived. It had been reported that the findings from survey data lacking prior meticulous data screening are destined to be equivocal [18]. Whilst every effort was made to coordinate on-site visits at all known enrolled game reserves, this was not always feasible and so remote communication methods were employed. This may also have contributed to the poor compliance. To facilitate compliance with future data requests a generic, less detailed template should be formulated and provided to the enrolled reserves to accompany each vaccine order. Alternatively a template tailored specifically for individual treated

populations could be provided. Such simplification strategies have been observed in association with improved response rates [18]. Additionally, an awareness of the obligation for data reporting should be encouraged perhaps *via* an ongoing educational process. This due compliance could also be incentivised.

Despite the questionnaire's low response rate, the exercise was useful in identification of several apparent limitations in both the data collection and format methodology. Furthermore, this investigation identified several strategies to potentially improve future reporting efforts. The N&S requires a monitoring programme to evaluate the effects of any contraception programme for elephant management [3]. Beyond this study's objectives, it was concerning that these statutory requirements were in many cases apparently not being complied with. With increased availability of data it will be feasible to more accurately assess the effects of this method at a population level. Furthermore, with this increased knowledge it will be possible to inform recommendations on future implementation strategies. This can be achieved using a simulation of deterministic forces and stochastic events, both natural and managed, on an annual basis, for example using the available freeware Vortex programme [19].

Despite the relative paucity of data collated, the available information was sufficient to strongly support the assertion that pZP immunocontraception was an effective method of elephant population control.

In summary, the enrolment in the pZP immunocontraception programme had increased dramatically since 2014. Immunocontraception with pZP vaccine was established as an effective method both as an individual and herd treatment strategy for elephant population management. Various measures have been implemented and additional measures were identified that will improve data reporting practices. These include individual game reserve registrations and authorisations, provision of simplified data reporting templates, encouragement of compliance and clarification of enrolment criteria for both game reserve management and administering veterinarians.

Table 5-1 Summary of data sources for all game reserves identified as previously or currently (2016/2017) using pZP immunocontraception

Game reserve	Laboratory record of sale	Returned questionnaire	Annual self-reporting by administering veterinarian	EMP declared
	Y/N	Y/N	Y/N	Y/N
Addo (Nyati; Kuzuka)	Y	Y	Y	Y
Amakhala	Y	N	Y	N
Amakhosi	Y	Y	Y	N
Blue Canyon	Y	N	Y	Y
Camp Jabulani	Y	N	N	N
Dinokeng	Y	N	N	N
HiP	Y	N	Y	Y
Ithala	Y	N	Y	Y
Ka'Ingo	Y	N	Y	N
Kapama	N	N	n/a	N
Karongwe	N	Y	n/a	N
Kwandwe	Y	Y	N	Y
KwaZulu Pvr.	Y	N	Y	N
Lalibela	Y	N	Y	N
Mabilingwe	Y	N	Y	Y
Mabula	N	N	n/a	N
Makalali	Y	N	N	N
Manyoni	Y	Y	N	N
Mkhuze Falls	Y	N	N	N
uMkhuze	Y	N	Y	N
Mokolo	Y	N	Y	N
Nambiti	Y	N	N	N
Phinda	Y	Y	N	Y
Phumba	Y	N	N	N
SanWild	Y	N	N	N
Shelanti	Y	N	Y	Y
Tembe	Y	N	Y	Y
Thaba Tholo	Y	Y	N	N
Thanda	Y	Y	N	Y
Thornybush	N	Y	n/a	Y
Venetia Lim.	Y	Y	Y	N
Welgevonden	Y	N	Y	N
Western Sh.	Y	N	Y	N
Zimanga	Y	Y	N	N

EMP: Elephant Management Plan; Y: yes; N: no; HiP: Hluhluwe iMfolozi park; n/a: not applicable; KwaZulu Pvr: KwaZulu Private Reserve; Venetia Lim.: De Beers Venetia Limpopo; Western Sh.: iSimangaliso Western Shores

Table 5-2 Game reserve size, provincial location, biome and bioregion for all reserves identified as previously or currently using pZP immunocontraception

Game reserve	Reserve size (km^2)	Provincial location	Biome (bioregion)[†]
Addo (Nyati; Kuzuka)	140;150	EC	Albany thicket; Nama-Karoo
Amakhala	75	EC	Albany thicket
Amakhosi*	50	KZN	Savanna (Zululand dry thronged, Lowveld, Lowland tall grassland
Blue Canyon	115	LP	Savanna
Camp Jabulani	nd	LP	Savanna
Dinokeng	185	GP	Savanna (Bushveld)
HiP	960	KZN	Savanna
Ithala	290	KZN	Savanna
Ka'Ingo	165**	LP	Savanna
Kapama	130	LP	Savanna
Karongwe	81	LP	Savanna (Lowveld, Bushveld)
Kwandwe	195	EC	Succulent thicket (Karroid shrubland, Afromontane, Riverine thicket)
KwaZulu Pvr.	185	KZN	Savanna (Bushveld)
Lalibela	105	EC	Savanna
Mabilingwe	83	LP	Savanna (Waterberg mountain/Sandy bushveld, Thornveld)
Mabula	80	LP	Savanna
Makalali	260	LP	Savanna (Lowveld)
Manyoni	230	KZN	Riparian, Savanna, Thicket (Lowveld)
Mkhuze Falls	nd	KZN	Savanna
uMkhuze	400	KZN	Savanna
Mokolo	165**	LP	Savanna
Nambiti	220	KZN	Savanna (Thornveld, Riverine bush)
Phinda	234.73	KZN	Savanna/Sand forest (Bushveld, Lowveld, Freshwater wetlands, Thicket)
Phumba	nd	EC	Albany thicket
SanWild	70	LP	Savanna
Shelanti	146	LP	Savanna (Roodeberg bushveld)
Tembe	300	KZN	Savannah (Sandveld)
Thaba Tholo	371	LP	Savanna (Semi-arid bushveld
Thanda	134.35	KZN	Savanna (Lowveld bushveld)
Thornybush	142	LP	Savanna (Granite lowveld)
Venetia Lim.	360	LP	Savanna (Mopane woodland)
Welgevonden	370	LP	Savanna
Western Sh.	250	KZN	Savanna
Zimanga	58	KZN	Savanna (Zululand lowveld)

[†]The Vegetation of South Africa, Lesotho and Swaziland, L Mucina, MC Rutherford (eds), SANBI, Pretoria 2006; EC: Eastern Cape; *Formerly part of Mkhuze Falls prior to 2012; KZN: KwaZulu-Natal; LP: Limpopo; nd: no data; GP: Gauteng; **Mokolo and Ka'Ingo combined 165 km^2

Table 5-3 Number of pZP treated elephant cows and elephant populations at treatment initiation, last recorded treatment and treatment administration method for all identified reserves previously or currently using pZP immunocontraception; and current game reserve enrolment status in 2017

Game reserve	1st treatment			Last treatment			Administration method	Observations 2017	Enrolment status 2017
	Year	Total population	Nº treated	Year	Total population	Nº treated			
Addo*	2013	150	48	2017	nd	65	herd/individual	injection site reactions	active
Amakhala	2008	6	3	2017	6	3	individual	nd	active
Amakhosi	2013	22	7	2017	28	nd	herd	nd	active
Blue Canyon	2017	nd	20	2017	nd	20	nd	nd	active
Camp Jabulani	nd	nd	nd	2016	15	nd	nd	nd	inactive
Dinokeng	nd	nd	nd	2017	nd	5	individual	nd	active
HiP	2014	183**	69	2017	578	176	herd	nd	active
Ithala	2014	125	60	2017	186	59	herd	nd	active
Ka'Ingo	2005	9	4	2017	14	6	individual	calves <1 y	active
Kapama	nd	nd	nd	2014	nd	5	individual	nd	inactive
Karongwe	2007	13	4	2015	18	6	individual	matings	inactive
Kwandwe	2012	57	13	2017	58	20	herd	nd	active
KwaZulu Pvr.	2014	nd	18	2016	nd	18	herd	calves <2 y	inactive
Lalibela	2017	31	9	2017	31	9	individual	nd	active
Mabilingwe	2017	24	5	2017	24	5	individual	nd	active
Mabula	2002	11	4	2015	nd	3	individual	nd	inactive
Makalali	2000	53	18	2017	nd	25	individual	nd	active
Manyoni	2014	24	13	2017	31	14	individual	nd	active
Mkhuze Falls	2013	nd	8	2017	nd	10	nd	nd	active

uMkhuze	2014	76	36	2017	95	30	herd	nd	active
Mokolo	2014	10	5	2017	10	5	individual	nd	active
Nambiti[ǂ]	2015	nd	21	2017	nd	nd	nd	nd	active
Phinda	2004	92	19	2017	100	44	herd	nd	active
Phumba	2016	nd	4	2017	nd	5	individual	nd	active
SanWild	nd	nd	nd	2016	nd	nd	nd	nd	inactive
Shelanti	2017	13	8	2017	13	8	individual	nd	active
Tembe	2007	152	71	2017	119	70	herd	nd	active
Thaba Tholo	2004	16	8	2017	23	12	individual	nd	active
Thanda	2012	17	7	2017	18	13	individual	nd	active
Thornybush	2005	35	19	2011	354***	nd	herd	nd	inactive
Venetia Lim.	2015	246	76	2017	267	110	herd	injection site reactions	active
Welgevonden	2005	117	35	2017	nd	66	herd	calves <1 y	active
Western Sh.	2015	75	28	2017	80	30	herd	nd	active
Zimanga	2010	24	4	2017	26	6	individual	matings	active

*Nyati & Kuzuka section; **population of herds containing treated cows only; ***2017 population estimate after fences removed; ~no data; [ǂ]population reportedly moved to Somkhanda Community Game Reserve

Table 5-4 Welgevonden Game Reserve; numbers of treated, newly enrolled and cows with missed treatment and number of calves (age not given) from 2005-2017

Welgevonden Game Reserve				
Year	**N° treated**	**N° newly enrolled**	**N° missed treatments**	**N° calves reported**
2005	n/a	n/a	n/a	3
2006	35	nd	nd	14
2007	37	nd	nd	4
2008	38	nd	1	2
2009	38	nd	4	5
2010	45	7	8	6
2011	56	11	nd	nd
2012	58	2	nd	3
2013	62	4	nd	nd
2014	66	4	nd	4
2015	71	5	nd	5
2016	67	nd	nd	3
2017	66	nd	nd	6

n/a: not applicable; nd: no data

Table 5-5 Tembe Elephant Park; elephant population (excluding adult bulls), number of pZP treated cows, number of calves (0-2 years) and mortalities annually from 2007-2017

Tembe Elephant Park				
Year	**Population**	**N° treated**	**N° calves (0-2 y)**	**N° mortalities**
2007	179	76	16	nd
2008	125	61	8	4
2009	144	59	10	2
2010	149	59	5	8
2011	136	63	2	4
2012	107	49	3	5
2013	114	61	4	4
2014	119	59	4	nd
2015	119	60	8	nd
2016	117	67	3	nd
2017	91	66	1	nd

nd: no data

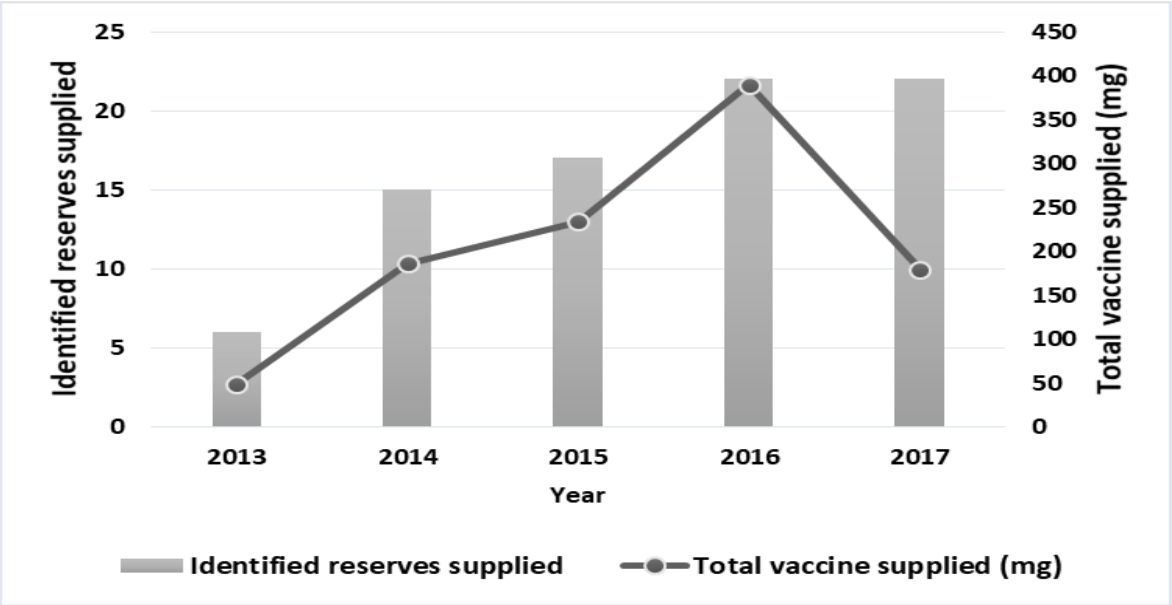


Figure 5-1 Number of identified reserves and total vaccine supplied (mg) from laboratory records between 2013-2017

References

[1] Convention on International Trade in Endangered Species of Wild Fauna and Flora Appendices I, II and III https://cites.org/sites/default/files/eng/app/2016/E-Appendices-2016-03-10.pdf [accessed 12 September 2016]

[2] IUCN African Elephant Status report 2016. An update from the African Elephant Database. Thouless CR, Dublin HT, Blanc JJ, Skinner DP, Daniel TE, Taylor RD, Maisels F, Frederick HL, Bouché P https://portals.iucn.org/library/sites/library/files/documents/SSC-OP-060_A.pdf [accessed 10 September 2018]

[3] Department of Environmental Affairs and Tourism, National Environmental Management: Biodiversity Act, 2004 (Act No. 10 of 2004) National Norms and Standards for the Management of Elephants in South Africa, Government Notice No. 251, 29 February 2008, South Africa

[4] Delsink AK, Van Altena JJ, Grobler D, Bertschinger HJ, Kirkpatrick JF, Slotow R. Implementing immunocontraception in free-ranging African elephants at Makalali Conservancy, J S Afr Vet Assoc, 2007; 78(1): 25-30

[5] Fayrer-Hosken RA, Brooks P, Bertschinger HJ, Kirkpatrick JF, Turner JW, Liu IKM. Management of African elephant populations by immunocontraception, Wildl Soc Bull, 1997; 25 (1): 18-21

[6] Fayrer-Hosken RA, Bertschinger HJ, Kirkpatrick JF, Grobler D, Lamberski N, Honneyman G, Ulrich T. Contraceptive potential of the porcine zona pellucida vaccine in the African elephant (*Loxodonta africana*), Theriogenology, 1999; 52(5): 835-46

[7] Fayrer-Hosken RA, Grobler D, Van Altena JJ, Bertschinger HJ, Kirkpatrick JF. Immunocontraception of African elephants, Nature, 2000; 407(6801): 149

[8] Delsink AK, Van Altena JF, Kirkpatrick JF, Grobler D, Fayrer-Hosken RA. Field applications of immunocontraception in African elephants (*Loxodonta africana*), Reproduction (Suppl), 2002; 60: 117-24

[9] Delsink AK, Van Altena JJ, Grobler D, Bertschinger H, Kirkpatrick J, Slotow R. Regulation of a small, discrete African elephant population through immunocontraception in the Makalali Conservancy, Limpopo, South Africa, S Af J Sci, 2006; 102: 403-5

[10] Bertschinger H, Delsink A, Kirkpatrick J, Van Altena J, Ahlers M, Dickerson T, Powrie D, Burger A. Porcine zona pellucida immunocontraception of African elephants (*Loxodonta africana*): beyond the experimental stage, In: Cain J, Marshal J (eds), IVth International Wildlife Management Congress-Cooperative Wildlife Management Across Borders: earning in the Face of Change, The Wildlife Society, 9-12th July 2012, Durban, South Africa

[11] Bertschinger HJ, Delsink A, Van Altena JJ, Kirkpatrick JF. Porcine zona pellucida vaccine immunocontraception of African elephant (*Loxodonta africana*) cows: A review of 22 years of research, Bothalia, 2018; 48(2): 1-8

[12] Knight M, Castley G, Moolman L, Adendorff J. Elephant management in Addo Elephant National Park, GIH Kerley, S Wilson, A Massey (eds) Elephant Conservation and management in the Eastern Cape Terrestrial Ecology Research Unit, Nelson Mandela Metropolitan University Report vol 35, South Africa

[13] Mackey RL, Page BR, Duffy KJ, Slotow R. Modelling elephant population growth in small, fenced, South African reserves, S Afr J Wildl Res, 2006; 36(1): 33-43

[14] Garaï M, Bates L, Bertschinger H, Delsink A, Pretorius Y, Selier J, Zitzer H. Non-lethal elephant population control methods: Summary of the first workshop of the Elephant Specialist Advisory Group of South Africa, Bothalia, 2018; 48(2): a2357

[15] Kirkpatrick JF, Rowan A, Lamberski N, Wallace R, Frank K, Lyda R. The Practical Side of Immunocontraception: Zona Proteins and Wildlife, J Reprod Immunol, 2009, 83(1-2): 151-7

[16] Bertschinger HJ, Delsink A, Van Altena JJ, Kirkpatrick J, Killian H, Ganswindt A. *et al*. Chapter 6: Reproductive control of elephants, In: Scholes RJ, Mennel KG (eds), Elephant management: A scientific assessment for South Africa, Wits University Press, 2008, Johannesburg, South Africa

[17] Slotow R, Garaï ME, Reilly B, Page B, Carr RD. Population dynamics of elephants re-introduced to small fenced reserves in South Africa, S Afr J Wildl Res, 2005; 35(1): 23-32

[18] Sage Research Empirical Findings on Quality and Comparability of Survey Data. In: Assessing the Quality of Survey Data Jörg Blasius, Victor Thiessen (eds), SAGE Publications Ltd, London, 2012, Print ISBN: 9781849203326 Online ISBN: 9781446251874 http://dx.doi.org/10.4135/9781446251874 Print pages: 15-32 Access Date: August 29, 2017

[19] Lacy RC, Pollak JP. Vortex: A stochastic simulation of the extinction process. Version 10.0. 2014, Chicago Zoological Society, Brookfield, Illinois, USA

Appendix 1:
Questionnaire

1. <u>Environmental considerations</u>

a) Reserve
b) Reserve size (km^2)
c) Reserve objective
d) Provincial location
e) Biome (Savanna or Grassland)
f) Bioregion-broad vegetation type
(Granite Lowveld/Central sandy bushveld/Zululand Lowveld/West Maputaland clay bushveld/Waterberg mountain bushveld/Central sandy bushveld/Western sandy bushveld etc. Mucina, Rutherford 2010)
g) Historical and current annual rainfall (5 y data sufficient)
h) Historical and current environmental temperature (5 y data sufficient-winter & summer average)

2. <u>Animals</u>

a) Population (n)
b) Population history
(Original location, orphans, translocations, interval at current reserve)
c) Population demographics **<u>(age and sex profile of entire herd broken down by year-beginning prior to immunocontraception and each year thereafter to present</u>)**
d) Date of count
e) Method of count
(Ground/Air/Direct/Sample/Dung etc.)
f) Method of age determination
(Direct shoulder height measurements/Field assessment relative to known individuals)
g) Method determining mother-offspring relationship
(Detailed birth records/Field assessment-likely based on behaviour)
h) Mean age of cows at first calving
i) Mean calving interval prior to immunocontraception

3. <u>Treatment</u>

a) Start of treatment (month, year)
b) Age of treated cows in Year 1
c) Cows calved before treatment (n)
d) Estimated mean calving %* before treatment
e) Mean annual calving %* during Year 1 and Year 2 of treatment
f) Mean annual calving %* during Year 3, 4, 5 + (where applicable)
**per number of cows deemed to be of breeding age*
g) Treatment protocol - **<u>itemised by each year of immunocontraception conducted [number of treated cows and age of each treated cow (specify whether a cow was given initial inoculation or booster)]</u>**
(Time and number of treatment & subsequent boosters (month, year)Treatment & booster delivery method /Identification method for treatment animals-if collared include collar number and historical movement data-particularly interested in movement data immediately prior to a treatment period and subsequent to treatment as available to note any changes in movement patterns and location following treatment)
h) Observations (prior to and subsequent to immunocontraception)
(Observed incidences of mating/Observed incidences of oestrous, incidences of attacks on vehicles/people, incidences of attacks on other animals, breakouts/break-ins to lodges/buildings)
i) Details of any bulls undergoing GnRH vaccine treatment or vasectomised bull

Chapter Six:
Summarising discussion

Chapter One introduced immunocontraception with the native porcine zona pellucida (pZP) vaccine as an established and effective method of population management through fertility control successfully applied for population management to more than 85 zoo and wildlife species [1]. This chapter broadly reviewed the relevant literature. Much of the formative work was conducted in free-roaming and domestic horses [2] and in the South African context its application focused primarily on African elephants [3]. An overview of reproductive physiology and ovarian dynamics of the mare, ZP proteins and their role and function in mammalian fertilisation, the targeting of ZP proteins for fertility control and the development and subsequent successful application of pZP immunocontraception in the horse was presented. The limitations of the currently utilised pZP vaccine formulations were highlighted. Zona pellucida proteins are poor antigens, requiring the addition of adjuvants in a vaccine formulation. Freund's adjuvants have commonly been used in vaccine formulations for pZP immunocontraception. Despite their continued use these adjuvants are associated with regulatory limitations and adverse reactions. For these reasons, the identification of alternative adjuvants for ZP-based immunocontraception was important. The application of a recombinant zona pellucida (reZP) vaccine was reviewed [4] and the importance of development of a recombinant formulation was highlighted.

Ovarian suppression subsequent to pZP immunocontraception and the requirement to monitor this effect was discussed. Ovarian suppression following pZP vaccination was previously perceived as a negative or abnormal outcome, however, more recently it has been proposed as a desirable (or inconsequential) outcome that mimics natural episodic events in reproductive cyclicity in the horse [5]. The methods used to monitor ovarian function following pZP vaccination in both free-ranging domestic and wildlife species require innovative approaches as clinical examination of these populations is rarely feasible. The measurement of anti-Müllerian hormone (AMH) was identified as a potentially useful minimally-invasive biomarker for ovarian function, which may also prove useful in the study of ovarian function in immunocontraception studies [6].

The primary focus of this research, the mare, occupies a dual space in the development and application of pZP immunocontraception serving as both a model organism and target species. The relevance of novel ZP-based vaccine formulations in South Africa was discussed and a justification for the application of a horse model [7-28] to elephants for this research was highlighted [29-36].

The successful application of pZP immunocontraception programmes for management of elephant populations is dependent on monitoring. This is associated with challenges including population counts, differing reserve management priorities and methods for monitoring ovarian function. These factors all limit data collection essential for evaluating the success of any immunocontraception programme. Similar to the horse, improvements in vaccine formulations for elephants are critical for vaccine production, regulatory compliance and animal welfare concerns.

The second chapter identified the characteristics of the ideal immunocontraceptive for population management and reiterated the major concerns with the current pZP vaccine formulations. The use of native derived pZP proteins is associated with high production costs, variable vaccine quality and purity including disease risks and restricted international movement. The current reliance on Freund's adjuvants limits the potential commercialisation of a ZP-based immunocontraceptive due to associated safety concerns. Monitoring the effects of ZP immunocontraception on ovarian function in free-ranging and wildlife species requires investigation of alternative minimally-invasive methods. These important questions shaped the presented hypotheses investigated by this thesis.

The primary objective of the study reported in Chapter Three was to compare the effects on ovarian function of treatments with both native pZP and reZP vaccines formulated with non-Freund's adjuvants and an anti-GnRH vaccine. Previously treatment with the recombinant proteins TT-KK-ZP3 and bRNase-KK-ZP4 resulted in successful fertility control in mice [37] and donkey jennies [38]. Its reported application in mares was less clearly defined [4]. The current study investigated both novel ZP vaccine formulations and treatment protocols for mares using native porcine and recombinant ZP antigens. The well-documented effect on ovarian function of anti-GnRH vaccines was additionally included in

the study design for comparative purposes. Mares that received a pZP treatment were more likely to maintain cyclical ovarian activity, followed by mares in a combined pZP & reZP, reZP only and GnRH treated groups in descending order of frequency. This study reported the first successful application of a reZP vaccine in mares. Importantly, this outcome was achieved using a novel non-Freund's adjuvant formulation consisting of Pet Gel A and Poly (I:C). Surprisingly, the pZP treatment had a limited effect on ovarian function despite reported ovarian suppression as an inherent feature of pZP immunocontraception in the horse [4, 5]. This may have been associated with pZP quality, highlighting the importance of ZP protein purity and dose control for immunocontraception. The addition of promiscuous T-cell epitopes in the reZP formulation may have enhanced its ovarian effects supported by intermediate results in mares treated with a combination of pZP & reZP. Furthermore, ovarian suppression following reZP treatment suggested that contamination with non-ZP ovarian proteins, glycosylation and carbohydrate moieties reportedly associated with native derived pZP vaccine were not a primary cause of ovarian suppression subsequent to ZP-based immunocontraception. The inclusion of Poly (I:C) is associated with high proliferation of cytotoxic T-lymphocytes, through both toll-like receptor (TLR) and retinoic acid-inducible gene1 like receptor (RLR) signalling. This in association with the promiscuous T-cell epitopes incorporated in the reZP only treatment may also account for the increased ovarian effects seen in this group. When AMH concentrations were compared between groups, a less clearly defined image emerged. The reZP only treatment group showed changes over time in AMH concentrations, with reduced concentrations after the final treatment, suggesting a cumulative effect of the multiple boosters on small follicles possibly mediated by cytotoxic T-lymphocytes involvement. Differences between treatment groups, however, were only seen between the pZP only and GnRH treated mares. Nevertheless, some interesting correlations between AMH and additional parameters were noted, namely, ovarian volume and serum progesterone. A further interesting although undefined outcome was the association between AMH and mare age. From this study it was asserted that the measurement of AMH subsequent to ZP-based immunocontraception may prove informative.

Chapter Four reported on the second objective of the mare study, investigating the efficacy of the novel reZP vaccine formulation. Although the contraceptive efficacy of this novel vaccine formulation was not fully investigated, vaccine efficacy was measured *via* the antibody titre response and the hormonal analysis described in Chapter Three. The safety of these vaccine formulations was also assessed. This chapter included both a preliminary study using geldings and an in-the-field- application using mares. In the geldings, the safety and efficacy of five different adjuvant formulations with pZP were investigated using clinical and serological data. These results informed the subsequent mare study where pZP, reZP and a combined treatment of pZP followed by reZP formulated with Pet Gel A and Poly (I:C) were applied under extensive conditions. Based on the measurement of anti-pZP and –reZP antibody titres a primary pZP and booster reZP treatment in combination with the combined adjuvant formulation is an inferior formulation to that of a three-treatment reZP and a two-treatment pZP formulation. The additional reZP booster and extra-ZP ovarian proteins, glycosylation and carbohydrate moieties of the pZP antigen may have increased the immunostimulatory aspects of these formulations. These results strongly supported this novel reZP vaccine formulated using non-Freund's adjuvants for immunocontraception in the horse. It is suggested that the action of this reZP formulation was mediated through antibody, $CD4^+$ and $CD8^+$ T-cell involvement and additionally this formulation may rely on the suppression of ovarian function.

Chapter Five reviewed the current status of the pZP immunocontraception programme of elephant cows in South Africa. This was the largest single data collection exercise conducted since the programme's inception in 2000. During this interval, the number of participating reserves had increased, particularly since 2014, to 27 actively-participating reserves by 2017. This data showed that 811 cows are currently being treated. It was evident that pZP immunocontraception could be successfully applied both to cows on an individual-identification basis and also a herd basis using a 'blanket treatment' strategy.
The study identified information assumed pertinent for the monitoring of immunocontracepted elephants. A questionnaire was formulated and supplied to all known participants in the immunocontraception programme. Data was submitted by both participating reserves and administering veterinarians and cross-referenced with laboratory records of supply. Despite a low response, several important limitations of the

currently-applied monitoring programmes were identified, namely, the data collection methods, data format and accuracy and poor reporting compliance. This study showed that continuous treatment monitoring is essential to assess emerging effects. Aerial population counts including the identification of new calves, are practically feasible during treatment administration, particularly in savanna biomes. The Veterinary Population Management Laboratory is only responsible for the collection of data pertaining to the production and supply of vaccine as regulated by the South African Health Products Regulatory Authority. This study was conducted assuming the availability to the researchers of the obligatory data collected by participating game reserves in terms of the Norms and Standards for Managing African elephants in South Africa. Much of this data was, however, unavailable or incomplete. This highlighted a significant concern that is the responsibility of both national and provincial regulatory agencies.

Future directions

Current local demand and global inquiries for humane elephant population management supports further development of ZP immunocontraception methodologies. The outcomes from the mare studies showed that a novel reZP vaccine formulated with non-Freund's adjuvants provided a successful alternative, overcoming many of the limitations associated with currently administered native pZP vaccines. Further studies of the contraceptive efficacy of this vaccine formulation and administration protocol in the mare should ensue prior to its testing in elephants.

Additional studies to investigate the reversibility of ovarian suppression and timing of resumption of fertility subsequent to prolonged treatment periods and practical, in-the-field methods to monitor ovarian suppression are warranted. The measurement of AMH concentrations, successfully applied in mares under extensive conditions, may similarly prove useful to infer cyclical ovarian activity in elephant cows [39]. Additionally, AMH secretory patterns reportedly do not necessitate intensive longitudinal sampling [39, 40, 41]. This would, however, require the development of a novel remote sampling system.

Improving and optimising data collection methods, data format and data reporting in combination with the clearly observed trend of increased implementation of pZP immunocontraception for elephant population management is indicated. This will facilitate meaningful future population modelling exercises to inform successful implementation of this programme.

Concluding statements

- The mare was an informative model for future investigation of novel ZP-based vaccine formulations and minimally-invasive monitoring of ovarian function in large herbivores such as the elephant cow.
- Immunocontraception using pZP proteins formulated with non-Freund's adjuvants may be only partially effective for immunocontraception reliant on ovarian suppression alone.
- A reZP vaccine including promiscuous T-cell epitopes of tetanus toxoid and bovine RNase formulated with Pet Gel A and Poly (I:C) was a promising alternative to native pZP immunocontraception in the horse.
- Ovarian suppression subsequent to reZP vaccination may be an integral component of successful fertility control in the horse.
- Treatment of elephants with pZP on a 'blanket-treatment' or population basis was an effective method of population management in South Africa.
- Future studies were indicated to evaluate the suitability and efficacy of a reZP vaccine for application in elephants.
- Future studies were indicated to assess the utility of measuring serum AMH as a minimally-invasive, in-the-field method to monitor ovarian function in the elephant subsequent to ZP-based immunocontraception.

References

[1] Frank KM, Lyda RO, Kirkpatrick JF. Immunocontraception of captive exotic species: IV. Species differences in response to the porcine zona pellucida vaccine, timing of booster inoculations, and procedural failures, Zoo Biol, 2005; 24(4): 349-58

[2] Liu IKM, Bernoco M, Feldman M. Contraception in mares heteroimmunized with pig zonae pellucidae, Reproduction, 1989; 85(1); 19-29

[3] Fayrer-Hosken RA, Grobler D, Van Altena JJ, Bertschinger HJ, Kirkpatrick JF. Immunocontraception of African elephants, Nature, 2000; 407(6801): 149

[4] Joonè CJ, Bertschinger HJ, Schulman ML, Gupta SK, Arukha AP, Minhas V, Fosgate GT, Dieterman E. Ovarian function and pregnancy outcome in pony mares following immunocontraception with native and recombinant porcine zona pellucida vaccines, Equine Vet J, 2017; 49(2): 189-95

[5] Joonè CJ. Porcine zona pellucida immunocontraception in the mare: Clinical, endocrinological and immunological effects, PhD Thesis, 2016, University of Pretoria, South Africa

[6] Kersey DC, Dehnhard M. The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation, Gen Comp Endocrinol, 2014; 203: 296-306

[7] Ginther OJ, Gastal EL, Gastal MO, Bergfelt DR, Baerwald AR, Pierson RA. Comparative study of the dynamics of follicular waves in mares and women, Biol Reprod, 2004; 71: 1195-201

[8] Ginther OJ, Beg MA, Gastal EL, Gastal MO, Baerwald AR, Pierson RA. Systemic concentrations of hormones during the development of follicular waves in mares and women: a comparative study, Reproduction, 2005; 130: 379-88

[9] Carnevale EM. The mare model for follicular maturation and reproductive aging in the woman, Theriogenology, 2008; 69: 23-30

[10] Mihm M, Evans ACO. Mechanisms for dominant follicle selection in monovulatory species: a comparison of morphological, endocrine and intraovarian events in cows, mares and women, Reprod Dom Anim, 2008; 43: 48-56

[11] Baerwald AR. Human antral folliculogenesis: what we have learned from the bovine and equine models, Anim Reprod, 2009; 6: 20-9

[12] Baerwald AR, Walker RA, Pierson RA. Growth rates of ovarian follicles during natural menstrual cycles, oral contraception cycles, and ovarian stimulation cycles, Fertil Steril, 2009; 91: 440-9

[13] Gastal EL. Recent advances and new concepts on follicle and endocrine dynamics during the equine periovulatory period, Anim Reprod, 2009; 6: 144-58

[14] Gastal EL, Gastal MO, Wischral A, Davis J. The equine model to study the influence of obesity and insulin resistance in human ovarian function, Acta Sci Vet, 2011; 39: 57-70

[15] Ginther OJ. The mare: a 1000-pound guinea pig for study of the ovulatory follicular wave in women, Theriogenology, 2012; 77: 818-28

[16] Alves KA, Alves BG, Gastal GD, De Tarso SG, Gastal MO, Figueiredo JR, Gambarini ML, Gastal EL The Mare Model to Study the Effects of Ovarian Dynamics on Preantral Follicle Features, PloS One, 2016; 11(2): 0149693

[17] Liu IKM, Bernoco M, Feldman M. Contraception in mares heteroimmunized with pig zonae pellucidae, Reproduction, 1989; 85(1): 19-29

[18] Kirkpatrick JF, Irwin LKM, Turner JW. Remotely-Delivered immunocontraception in feral horses, Wildl Soc Bull, 1990; 18(3): 326-30

[19] Kirkpatrick J, Liu I, Turner J, Bernoco M. Antigen recognition in feral mares previously immunized with porcine zonae pellucidae, J Reprod Fertil Suppl, 1991; 44: 321-25

[20] Willis P, Heusner GL, Warren RJ, Kessler D, Fayrer-Hosken RA. Equine immunocontraception using porcine zona pellucida: a new method for remote delivery and characterization of the immune response, J Equine Vet Sci, 1994; 14(7): 364-70

[21] Kirkpatrick JF, Naugle R, Liu IKM, Bernoco M, Turner JJW. Effects of Seven Consecutive Years of Porcine Zona Pellucida Contraception on Ovarian Function in Feral Mares, Biol Reprod, 1995; 52: 411-18

[22] Kirkpatrick JF, Turner JW Jr, Liu IK, Fayrer-Hosken R, Rutberg AT. Case studies in wildlife immunocontraception: wild and feral equids and white-tailed deer, Reprod Fertil Dev, 1997; 9(1): 105-10

[23] Turner JW, Liu IKM, Rutberg AT, Kirkpatrick JF. Immunocontraception Limits Foal Production in Free-Roaming Feral Horses in Nevada, J Wildl Manage, 1997; 61(3): 873-80

[24] Turner JW, Liu IKM, Flanagan DR, Rutberg AT, Kirkpatrick JF. Immunocontraception in feral horses: one inoculation provides one year of infertility, J Wildl Manage, 2001; 65(2): 235-41

[25] Turner JW, Liu IKM, Flanagan DR, Bynum KS, Rutberg AT. Porcine zona pellucida (PZP) immunocontraception of wild horses (*Equus caballus*) in Nevada: a 10 year study, Reproduction Suppl, 2002; 60: 177-86

[27] Turner JW, Liu IKM, Flanagan DR, Rutberg AT, Kirkpatrick JF. Immunocontraception in wild horses: one inoculation provides two years of infertility, J Wildl Manage, 2007; 71(2): 662-67

[28] Kirkpatrick JF, Turner A. Achieving population goals in a long-lived wildlife species (*Equus caballus*) with contraception, Wildl Res, 2008; 35(6): 513-19

[29] Fayrer-Hosken RA, Brooks P, Bertschinger HJ, Kirkpatrick JF, Turner JW, Liu IKM. Management of African elephant populations by immunocontraception, Wildl Soc Bull, 1997; 25 (1): 18-21

[30] Fayrer-Hosken RA, Bertschinger HJ, Kirkpatrick JF, Grobler D, Lamberski N, Honneyman G, Ulrich T. Contraceptive potential of the porcine zona pellucida vaccine in the African elephant (*Loxodonta africana*), Theriogenology, 1999; 52(5): 835-46

[31] Fayrer-Hosken RA, Grobler D, Van Altena JJ, Bertschinger HJ, Kirkpatrick JF. Immunocontraception of African elephants, Nature, 2000; 407(6801): 149

[32] Delsink AK, Van Altena JF, Kirkpatrick JF, Grobler D, Fayrer-Hosken RA. Field applications of immunocontraception in African elephants (*Loxodonta africana*), Reprod Suppl, 2002; 60: 117-24

[33] Delsink AK, Van Altena JJ, Grobler D, Bertschinger H, Kirkpatrick J. Slotow R. Regulation of a small, discrete African elephant population through immunocontraception in the Makalali Conservancy, Limpopo, South Africa, S Af J Sci, 2006; 102: 403-5

[34] Delsink AK, Van Altena JJ, Grobler D, Bertschinger HJ, Kirkpatrick JF, Slotow R. Implementing immunocontraception in free-ranging African elephants at Makalali Conservancy, J S Afr Vet Assoc, 2007; 78(1): 25-30

[35] Bertschinger H, Delsink A, Kirkpatrick J, Van Altena J, Ahlers M, Dickerson T, Powrie D, Burger A. Porcine zona pellucida immunocontraception of African elephants (*Loxodonta africana*): beyond the experimental stage. In: Cain J, Marshal J (eds), IVth International Wildlife Management Congress-Cooperative Wildlife Management Across Borders: Learning in the Face of Change, The Wildlife Society, 9-12th July 2012, Durban, South Africa

[36] Bertschinger HJ, Delsink A, Van Altena JJ, Kirkpatrick JF. Porcine zona pellucida vaccine immunocontraception of African elephant (*Loxodonta africana*) cows: A review of 22 years of research, Bothalia, 2018; 48(2): 1-8

[37] Gupta N, Chakrabarti K, Prakash K, Wadhwa N, Gupta T, Gupta SK. Immunogenicity and contraceptive efficacy of *Escherichia coli*-expressed recombinant porcine zona pellucida proteins, Am J Reprod Immunol, 2013; 70: 139-52

[38] Ambrosia RL, Roberts BN, Roberts TA, DeYoung BL, Peterson EW, Bertschinger HJ, Schulman ML, Crampton M, Roth R, Van Zyl PJ, Cameron-Blake N, Vandenplas ML, Knobel DL, French HM. Porcine and recombinant zona pellucida vaccines as immunocontraceptives for donkeys in the Caribbean, Clin Theriogenology, 2017; 9(3): 439

[39] Dow TL, Roudebush W, Parker FN, Brown JL. Influence of age and gender on secretion of anti-Müllerian hormone in Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants, Theriogenology, 2011; 75(4): 620-27

[40] Weenen C, Laven J, Von Bergh A, Cranfield M, Groome N, Visser J, *et al*. Anti-Müllerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment, Mol Hum Reprod, 2004; 10: 77-83

[41] Ireland JLH, Scheetz D, Jimenez-Krassel F, Themmen APN, Ward F, Lonergan P, *et al*. Antral follicle count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of young adult cattle, Biol Reprod, 2008; 79: 1219-25

Ethical approval certificates

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Contraceptive efficacy of different immunocontraceptive vaccines applied to mares in a pasture breeding system: a population study**
PROJECT NUMBER	**V124-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Margaret B Nolan**

STUDENT NUMBER (where applicable)	**UP_16402597**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	***Equus caballus* (domesticated)**
NUMBER OF SAMPLES	**50**
Approval period to use animals for research/testing purposes	**October 2016 – October 2017**
SUPERVISOR	**Prof ML Schulman**

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 4 November 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**A census of elephant cows undergoing porcine zona pellucida (pZP)-immunocontraception in South Africa: Considerations for a Population Model/Prospective analysis of potential population trends**
PROJECT NUMBER	**V143-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**MB. Nolan**

STUDENT NUMBER (where applicable)	**UP_16402597**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL ANIMALS	**Elephants**	
NUMBER OF ANIMALS	**700**	
Approval period to use animals for research/testing purposes		**November 2016-November 2017**
SUPERVISOR	**Prof. ML Schulman**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 28 November 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**A census of elephant cows undergoing porcine zona pellucida (pZP)-immunocontraception in South Africa: Considerations for a Population Model/Prospective analysis of potential population trends**
PROJECT NUMBER	**V143-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**MB. Nolan**

STUDENT NUMBER (where applicable)	**UP_16402597**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL ANIMALS	**Elephants**	
NUMBER OF ANIMALS	**700**	
Approval period to use animals for research/testing purposes		**January 2017-January 2018**
SUPERVISOR	**Prof. ML Schulman**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 24 January 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15