# Controlled-release components of PZP contraceptive vaccine extend duration of infertility

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**Abstract.** Successful immunocontraception of wildlife relying on repeated access to individuals for boosters has highlighted the need to incorporate primer and booster immunisations into one injection. We have investigated use of controlled-release polymers (lactide–glycolide) in small pellets to provide delayed *in vivo* delivery of booster porcine zona pellucida (PZP) antigen and adjuvant. This report reviews pellet-making methodology, *in vitro* testing of controlled-release pellets and *in vivo* effects of controlled-release PZP vaccine. We assessed 3 different manufacturing approaches for producing reliable, cost-effective pellets: (1) polymer melting and extrusion; (2) solvent evaporation from polymer solution; and (3) punch and die polymer moulding. *In vitro* testing of release patterns of controlled-release formulations, towards development of a 3-year duration vaccine, provided estimates for *in vivo* use of pellet preparations. These *in vitro* studies demonstrated protein release delay up to 22 months using 100% *l*-lactide or polycaprolactone polymers. For *in vivo* tests, pellets (1-, 3-, and 12-month release delay) serving as boosters were administered intramuscularly with PZP/adjuvant liquid primer to wild horses (*Equus caballus*), white-tailed deer (*Odocoileus virginanus*) and African elephants (*Loxodonta africana*). Horse field studies assessed fertility via offspring counts and/or faecal-hormone pregnancy testing. Treatment decreased fertility 5.3–9.3-fold in Year 1 and 3.6-fold in Year 2. In preliminary testing in deer, offspring counts revealed treatment-associated fertility reduction of 7.1-fold Year 1 and 3.3-fold Year 2. In elephants, treatment elevated anti-PZP titres 4.5–6.9-fold from pretreatment (no fertility data).

## Introduction

Contraception has become a substantive means of managing several species of free-roaming wildlife and captive exotics. In the light of growing interest in this area, the need for agents that are maximally both user and recipient friendly has become apparent. Two agent characteristics that partially address this need are efficient agent delivery and extended duration of effectiveness in a single treatment. The impetus for developing a controlled-release, extended-duration immunocontraceptive originated from studies with free-roaming wild horses. Early studies employed a protocol of two or three separate immunisations (primer plus one or two boosters) about 1-month apart (Kirkpatrick *et al.* 1990). This protocol was necessary to satisfy requirements for optimal and sustained immune response with maximal immunomemory for subsequent annual boosters. Although the treatments were effective, the high costs and daunting logistics of (1) multiple accessing of wild horses for darting or (2) maintaining wild horses in captivity between injections made the multiple-injection

protocol impractical for most circumstances. Since controlled-release polymer technology was already in use for delivery of cancer chemotherapeutics and some vaccines (Chang 1976; Carter *et al.* 1988; Linhardt 1989; Wang *et al.* 1990, 1991), we began investigations of this technology for possible application in a single-injection porcine zona pellucida (PZP) immunocontraceptive.

This report reviews and updates salient aspects of the testing of a one-injection, multi-year immunocontraceptive that employs controlled-release polymer components to achieve efficient vaccine delivery and extended vaccine effect. The vaccine utilises PZP antigen plus adjuvant to provide contraception (Kirkpatrick *et al.* 1990, 1991*b*). Polymer-based controlled-release vaccine delivery has been studied predominantly in horses to date (Turner *et al.* 2001, 2002, 2007), and this is reflected in the present report. However, preliminary data applying this technology to white-tailed deer and African elephants are also presented for broader perspective. The

content of this review is divided into three sections: (1) *in vitro* studies; (2) *in vivo* studies; and (3) research assessment and management implications.

## *In vitro* studies

### Methods

The endpoint for *in vitro* testing has been the temporal pattern of protein release from controlled-release formulations into incubation medium. Early *in vitro* studies (1992–1996) focussed on controlled-release polymers mixed with vaccine and formulated as microspheres suspended in liquid for delivery (Turner *et al.* 2002). However, repeated difficulties were encountered with delivery of microspheres, due to the tendency of these particles to settle out of the liquid vehicle, clump in the syringe needle and clog it. This persistent and unresolvable problem led to development of solid pellets as the delivery medium, and all subsequent studies have utilised pellet formulations.

### Polymers and pellets

The polymers used to make pellets (and information regarding their projected release attributes) were obtained from Birmingham Polymers (Birmingham, AL; now Lactel/Durect, Inc., Pelham, AL). Polymer information is presented in Table 1. Lyophilised PZP and water-soluble saponin adjuvant (QS-21, human-grade; QA-21, veterinary-grade Vrbac Corp., Brussels, Belgium) were mixed by trituration to form a homogeneous matrix with the inert, non-toxic polymers. The mixture was then pelletised by one of three methods briefly described below: heat/extrusion (H/X), cold/evaporation (C/V) or pressure/moulded (P/M).

The H/X method involved melting the polymer–vaccine mixture in a Dynisco extruder (Morgantown, PA) and pushing it through a small aperture to form a long strand of diameter 0.6 mm (to fit inside a 14-gauge hypodermic needle). The strand was then cut to ~1.6-mm length pellets. This method had the advantage of bulk pelleting capability, but ~50% of the ingredients were lost during manufacture, and the heat (100–113°C) could partially degrade the PZP. For this reason we examined two alternate methods that did not employ heat. In assessing the viability of these two methods, we used H/X pellets (of proven efficacy) as a reference base.

The C/V pellets were formed at <20°C by homogeneous suspension of vaccine ingredients in polymer that was rendered as a viscous liquid in the organic solvent dichloromethane. This liquid was then injected into 14-gauge Silastic tubing moulds 5 cm each in length. Gradual evaporation of solvent from the moulded polymer–vaccine mixture across 48 h at 4°C yielded a firm strand inside the tubing. The mould was cut away, and the cylindrical strand was then cut into 1.6-mm lengths, yielding 26–30 pellets. Loss of ingredients during pellet manufacture was 15–20%. This method required ~3-fold more personnel time per pellet than the H/X method.

The P/M pellet-making method employed a low-pressure punch-and-die system. We designed an aluminum mould with 40-pellet capacity that was based on a standard mould used for small-scale pharmaceutical tablet/suppository production. Each half of the mould contained a row of half cylinders, which became whole cylinders for pellets when the mould halves were clamped together. We had the mould manufactured locally (Bollinger Tool and Die Co., Toledo, OH). The pellet-making process consisted of (1) thorough mixing (trituration) of finely powdered vaccine ingredients, polymer and colour dye, (2) loading the powder into the mould, (3) warming the mould in an incubator for 20–30 min while periodically applying pressure to the die chambers by a spring-loaded punch array. This method employed a temperature of 80–85°C, which was well below the denaturation temperature for this vaccine (J. Turner, unpubl. data). The polymer–vaccine mixture became pliable at this temperature, allowing pressure from the punch array to form the pellets in the die cylinders. The halves of the multi-pellet mould were then separated, and the exposed pellets were removed. Relative to H/X pellets, these product pellets were of similar hardness (based on force required to cut with a single-edged razor blade and to crush in a mortar and pestle) and similar physical appearance at 10× magnification (similar translucence and lack of air bubbles or fracture lines). The loss of materials was 8–14% (compared with >50% for H/X method), and the per-pellet personnel time was ~1.5-fold greater than for H/X pellets. An advantage of the P/M method is that no minimum number of pellets must be produced to obtain reasonable production efficiency (the H/X method requires a 100-pellet minimum of each type). Thus, the P/M method can be advantageous for short-notice production and for application to small numbers of animals.

### Controlled-release pellet contents

Three polymer–vaccine formulations were prepared by the H/X method and tested *in vitro*. Contents of the various pellets are presented in Table 1. Lyophilised PZP was prepared as previously described by Liu *et al.* (1989). The adjuvant was QS-21 or QA-21. Dyes for colour coding were commercial non-toxic food dyes. Polymers were finely ground and passed through a #60 sieve (250 μm). These were projected to release respectively in windows of 1–3 months, 3–5 months and 10–12 months. For all pellets, homogeneity was assured by trituration mixing of powdered ingredients (polymer, PZP, adjuvant, dye) using a mortar and pestle and examination of produced powder by microscope (10–20×). The cylindrical pellets (~0.6 mm × 1.6 mm) weighed 7.5–10.0 mg and the ratio of vaccine ingredients to polymer (termed '% loading') was <9%. For optimal pellet performance maximal loading should be <10% (D. Flanagan, pers. comm.). Pellets were stable at room

**Table 1. Characteristics of polymer pellets employed in one-injection controlled-release porcine zona pellucida (PZP) vaccine of known effectiveness**

| Pellet type (expected release delay) | Actual release delay (days) | Polymers | Ratio (%) | PZP (µg) | Adjuvant (µg) (QA-21) |
|---|---|---|---|---|---|
| 1 month | 30–60 | Lactide : glycolide | 65 : 35 | 100 | 200 |
| 3 months | 75–100 | Lactide : glycolide | 85 : 15 | 100 | 200 |
| 12 months | 340–370 | *d*, *l* lactide | 100 | 250 | 500 |

temperature but were nonetheless refrigerated for extended storage.

Since the pellet-making process involved potential protein-denaturing conditions (heat for H/X process; organic solvent for C/E process), we assessed viability of PZP exposed to these pellet-making conditions (P/M-type pellets have not been tested to date). We determined conservation of immunoreactivity by Ochterlony immuno-diffusion and titre response of mares immunised with these PZP preparations. No significant effect on PZP of heat or solvent exposure was observed (Liu *et al*. 2005; Turner *et al*. 2007).
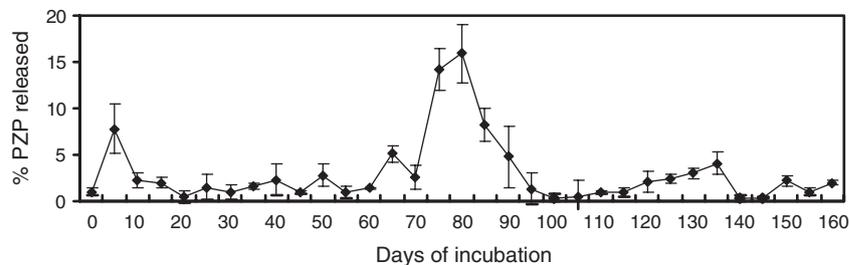
### In vitro *testing methods*

For all pellets, we determined characteristics of PZP release by measuring PZP concentrations in the incubation medium across time using a sandwich-type enzyme-linked immunosorbent assay (ELISA) for PZP (Turner *et al*. 2002). The *in vitro* environment for a given pellet consisted of 1–3 cc of 37°C bacteriostatic PBS gently and continuously rocked back and forth (one motion per second). Every 3–5 days for up to 23 months, we removed the medium for assay and added the same volume of new medium to the pellet vial. We performed the ELISA on 100 μL of each medium sample. We did not measure the release of adjuvant from pellets, but expected release characteristics to be similar to PZP based on similar water solubility (C. Kensil, Antigenics, Inc., pers. comm.). Some testing of pellets employed bovine serum albumin (molecular weight similar to PZP) in place of PZP. Protein release in those tests was measured by commercial protein assay (Pierce BCA Kit, Rockford, IL). Protein assay sensitivity was 0.5 $\mu g\,mL^{-1}$ incubation fluid.

*In vitro* group data are presented as mean values ± standard error (s.e.). However, statistical analysis was not applied, since the purpose of these studies was to examine release patterns, which were visually obvious.

### In vitro *findings*

Release patterns for PZP vaccine-containing pellets have been previously reported (Turner *et al*. 2002). However, in light of the focus of this report on pellets, relevant details are provided in Table 1, and a sample *in vitro* PZP release pattern is presented in Fig. 1.

### C/V *pellets*

We examined the C/V method towards avoiding the disadvantages of H/X method: heat denaturation of PZP and high materials loss. Although the C/V method did achieve these goals, C/V pellets performed suboptimally *in vitro*, releasing ~80% of the total PZP before the desired release window (Fig. 2). Nonetheless, these studies included release characteristics of extremely water-resistant polymers (100 *l*-lactide and polycaprolactone) in pellets that could theoretically delay vaccine release by up to 2 years. One useful outcome of the *in vitro* testing of these polymers in C/V-type pellets was the finding during a 23-month incubation of presumptive 20-month pellets that both polymers can delay some protein release for up to 20 months (Fig. 2). Although only ~20% of the protein remained in these pellets after the first 18 months of incubation, across the next 5 months more than half of the remaining protein was released during only 1 month (Month 20 for polycaprolactone; Month 21 for 100 *l*-lactide).
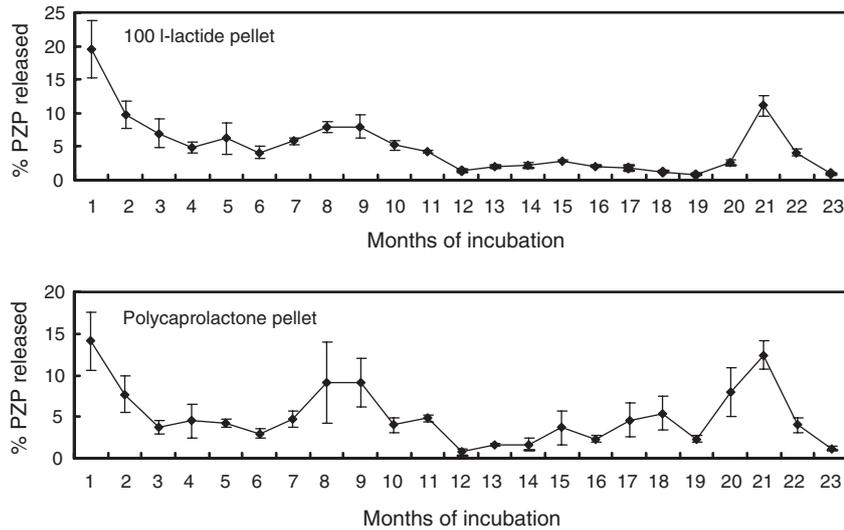
### P/M *pellets*

*In vitro* protein-release testing of 1-month P/M-type pellets containing bovine serum albumin (an inexpensive stand-in for PZP) was carried out as previously described for H/X pellets. The protein release pattern showed <12% initial release followed by <5% thereafter (except 7% on Day 20), with the major release (45.2%) occurring across Days 40–48 (Fig. 3). Release returned to pre-surge values thereafter.

### In vivo **studies**

### *Animals*

These studies report effectiveness of controlled-release PZP vaccine in domestic and free-roaming wild horses (*Equus caballus*) and in free-roaming white-tailed deer (*Odocoelius virginanus*) and African elephants (*Loxodonta africana*). The principal investigators were J. Turner (horse), A. Rutberg (deer) and H. Bertschinger (elephant). Horse study sites were USA Department of Interior Bureau of Land Management (BLM) Herd Management Areas in Nevada (USA) for free-roaming horses and University of California, Davis (I.K.M. Liu) for domestic horses. The deer study site was Fripp. Island, SC (USA). The elephant study site was in Limpopo Province, South



**Fig. 1.** *In vitro* release pattern of porcine zona pellucida (PZP) from heat/extrusion (H/X) pellets of presumptive 3-month release delay. Each point is the average of 6 pellets (8 mg per pellet). Each pellet was a matrix of lactide : glycolide polymer and PZP (100 μg) incubated at 37°C in phosphate-buffered saline (PBS; pH 7.2) with gentle rocking motion (one per second). Assay was by enzyme-linked immunosorbent assay (ELISA).

**Fig. 2.** *In vitro* porcine zona pellucida (PZP) release (as percentage of total pelleted PZP) from polymer pellets of cold/evaporation (C/V) type with presumptive release delay of up to 20 months. Each pellet contained PZP (400 μg) plus QA-21 adjuvant (500 μg). Each monthly value is the average from 10 pellets across four separate 7-day samplings of incubation medium and is presented as % of total PZP placed in each pellet. Lower limit of detection was 0.5 μg protein per mL phosphate-buffered saline (PBS) measured by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).
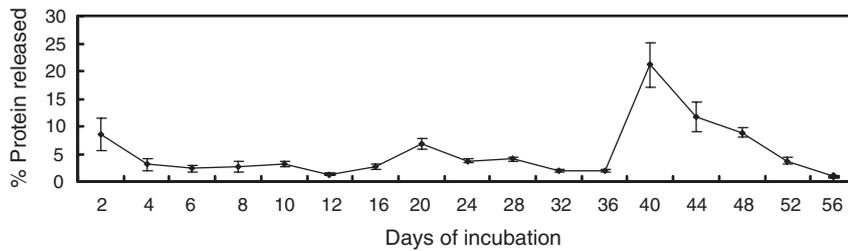


**Fig. 3.** Percentage of total pelleted bovine serum albumin (BS) released from P/M-type pellets. Each point is the average of 6 pellets (mass = 10 mg/pellet), which were derived from a mixture of lactide : glycolide (50 : 50) and bovine serum albumin (BSA; 750 μg), incubated at 37°C in phosphate-buffered saline (PBS; pH 7.2) with gentle rocking motion (one per second).

Africa. All animals were studied under auspices of respective specific Animal Care and Use protocols for each organisation. Data for horses include both new and previously published files, with the latter clearly referenced. Deer and elephant data represent preliminary studies.

## Vaccine

PZP controlled-release vaccine was delivered via hip i.m. injection composed of (1) a primer dose of 100-μg PZP in 0.5 cc phosphate-buffer solution (PBS) emulsified with 0.5 cc Freud's adjuvant (complete = FCA or modified = FMA) and (2) 1-month and 3-month pellets (for 1-year vaccine) or 1-month, 3-month and 12-month pellets (for 2-year vaccine). All pellets used *in vivo* were prepared by the H/X method (except C/V type in one deer group) and contents of each pellet type were as previously described.

## Statistics

Titre data are presented as mean value ± s.e. Domestic horse titre data for treatment *v.* control were sufficiently different to obviate statistical assessment. Wild horse data were previously published and statistically assessed in those reports (Turner *et al.* 2002, 2007). Elephant titre data were assessed by *t*-test and deer fertility data were assessed for treated *v.* control differences by binomial probability distribution (Zar 1984).

## Horse studies

Subsequent to *in vitro* testing and before testing in free-roaming horses, *in vivo* assessment of presumptive 1-year and 2-year controlled-release vaccine was performed in captive mares at U.C., Davis. Mares were titre tested via periodic jugular blood sampling across 42 weeks (for 1-year vaccine) and 24 months (for 2-year vaccine). Anti-PZP antibody titres were measured using an

ELISA developed for this purpose and previously described (Liu *et al.* 1989). The positive reference titre was produced by standard two-injection vaccine averaged across six mares from a prior study (Liu *et al.* 1989).

We previously reported *in vivo* anti-PZP titre response to 1-year vaccine containing 1- and 3-month pellets (Turner *et al.* 2002). To summarise those data: relative to a standard two-injection protocol (known to provide 1 year of infertility), the 1-year vaccine was not different in titres across a 42-week monitoring period. By Week 3, the titre response to both vaccine types was >100% of positive reference control and remained so through Week 10. By Week 42, levels were 49–55%. Previous studies comparing titres and fertility in the same mare have shown excellent contraception associated with titres near or above 60% of positive reference control (Turner *et al.* 2002). In a subsequent study, not previously reported, we assessed titre response pattern in domestic mares given 2-year vaccine (containing 1-, 3-, 12-month pellets). Treated mares exhibited a rapid anti-PZP titre rise across 3 weeks to reach $1.4 \times$ positive reference control titre (Fig. 4). These titres gradually decreased but remained above or near positive reference control (100%) for 45 weeks. Thereafter titres declined more rapidly, but remained elevated (54%) at 53 weeks. Control mares, given saline plus blank pellets, showed titres near or below reliable detection throughout the study.

In addition to studies in domestic horses, free-roaming wild mares were field-tested with PZP vaccine. Several herds in Nevada were given 1-year controlled-release vaccine between 1995 and 2001, and these studies have been previously reported (Turner *et al.* 2001, 2002). Likewise, a field test of 2-year controlled-release vaccine was performed in Nevada from 2000 to 2005 (Turner *et al.* 2007). Methods and Results of these studies are summarised below in the context of controlled-release vaccine efficacy. All studies utilised adult horses captured in routine BLM gathers, and mares scheduled for return to the range were treated, by hand-injection or by darting in field corrals. The 1-year and 2-year PZP vaccine used in these studies was the same as described elsewhere in this review. Fertility was determined by foal counts and/or faecal-sample pregnancy test (Turner *et al.* 2001). Pregnancy was assessed by the concentrations of oestrone conjugates and immunoreactive pregnanediol glucuronide in aqueous extracts of faecal material from individually identified mares. These conjugates were measured via ELISA as described by Kirkpatrick *et al.* (1991*a*).

Summary data compiled from our previously reported field studies with 1-year vaccine (Turner *et al.* 2001, 2002) are presented in Table 2. Mares given 1-injection, 1-year vaccine by hand or by dart had fertility rates 0.19 and 0.39, respectively, compared with those of untreated mares (0.61–0.63). Mares given standard two-injection, 1-year vaccine averaged 0.12 of the fertility of untreated mares. All treated groups returned to normal fertility the following year.

More recently, in our field study of hand-injected 2-year PZP vaccine effectiveness in 96 mares (Turner *et al.* 2007), we observed fertility rates of 53.6% for pretreatment and 5.2, 14.9, 31.6 and 46.2% across the ensuing 4 years, evidencing marked infertility effect of 2-year vaccine in Years 1 and 2, partial infertility remaining in Year 3 and return to normal fertility in Year 4.

### Deer study

As part of a larger study of one-injection PZP preparations performed on Fripp. Island, white-tailed deer were treated with pellet-based controlled-release PZP vaccine. In February and March (2005, 2006), adult female deer were captured via chemical immobilisation, ear-tagged, measured, weighed, blood-sampled and hand-injected with one of several PZP
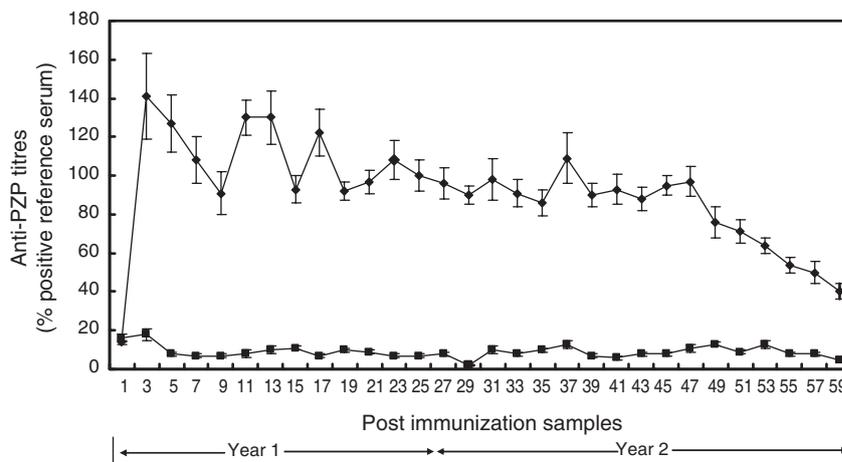


**Fig. 4.** Antibody response in domestic mares to controlled-release porcine zona pellucida (PZP) vaccine of presumptive 2-year duration. Treatment was standard PZP/adjuvant primer injection plus 1-, 3-, 12-month pellets (*n* = 10; ◆). Control was saline injection plus blank pellets (*n* = 5; ■). Positive reference serum (100%) was highest titre average of six previously studied mares given standard two-injection PZP vaccine. First sample was on the day of immunisation.

**Table 2. Survey of field tests of 1-year controlled-release vaccine in wild horses[A]**

| No. of mares[B] | Vaccine type[C] | Delivery format[D] | Fertility (% foaling) | |
| --- | --- | --- | --- | --- |
| | | | Year-1 | Year-2 |
| 295 | Untreated | None | 64 | 61 |
| 376 | Standard 2-injection (no pellets) | Hand injection | 8 | 60 |
| 48 | Primer plus 1-, 3 months pellets | Hand injection | 12 | 58 |
| 114 | Primer plus 1-, 3 months pellets | Dart | 25 | 50 |

[A]Modified from Turner *et al.* (2002).
[B]Data are composite of 1–4 separate study sites in Nevada.
[C]Adjuvant in all pellets was a saponin (QS-21).
[D]All mares treated in field corrals or stock chute during routine horse gathers associated with US government wild horse and burro program.

preparations. At capture, females received a priming injection of 100-μg PZP/FMA and a set of one each 1-month, 3-month and 12-month H/X or C/V pellets, hand-injected via trocar. Treated deer that could be located again were recaptured 1 and 2 years later (2006, 2007), and blood was drawn for pregnancy testing via an ELISA for pregnancy-specific protein B (Biotracking, Moscow, ID).

In 160 adult white-tailed does that were captured and pregnancy tested before the treatment period, 133 (83%) were pregnant (Table 3). Of 32 does given 2-year vaccine containing H/X-type pellets, nine were recaptured 1 year later and none of these was pregnant (0% fertility). Of 12 does given 2-year vaccine with C/V-type pellets, two of eight recaptured were pregnant (25.0% fertility). Four does (three H/X, one C/V) were recaptured in Year 2, and one doe (H/X) was pregnant (overall 25.0% fertility). Thus, among all does given pellet-containing 2-year vaccine, fertility rates were 11.7% in Year 1 and 25.0% in Year 2. Relative to pretreatment, Year-1 fertility rates were reduced in treated deer ($P < 0.01$ for H/X, $P < 0.05$ for C/V; binomial probability distribution). Year-2 data were insufficient for analysis. Return to fertility remains to be determined.

*Elephant study*

Two groups of elephant cows were treated in South Africa. One ($n = 6$) was given standard two-injection PZP vaccine, albeit using 400-μg PZP (instead of 100 μg) in the primer dose and 200 μg (instead of 100 μg) in the booster. The larger doses were used in consideration of large elephant bodyweight. These free-roaming animals were accessed by remote darting from a helicopter. The second group ($n = 3$) was captive and hand injected with 1-year controlled-release vaccine consisting of primer (400-μg PZP/FMA) plus two pellets each of the 1-month, 3-month and 12-month type per cow.

Titres were calculated as percentage of positive reference control value. A peak titre value of 100% was assigned to the six two-injection elephant cows in the present study, since the same vaccine dose and protocol provided up to 80% contraceptive efficacy in previous elephant studies (Fayrer-Hosken *et al.* 2000). Thus, these two-injection cows averaged anti-PZP titres of 100%, at 3 months post immunisation. In the three cows given 1-year (pellet-containing) PZP vaccine, anti-PZP titres were 162.1 after 3 months (Table 4). The titre difference was significant ($P < 0.01$, *t*-test), giving preliminary indication of more vigorous effect of pelleted vaccine than standard two-injection vaccine in this species. Titre monitoring remains in progress and fertility has not been assessed.

**Research assessment and management implications**

Considerable progress has been made in the use of controlled-release technology to improve PZP immunocontraception for wildlife. A major technical development in this area has been the

**Table 3. Effect of one-injection, 2-year controlled-release porcine zona pellucida (PZP) vaccine on fertility in white-tailed deer**

| Condition | Doe fertility[A] | | | |
| --- | --- | --- | --- | --- |
| | Year 1 | | Year 2 | |
| Untreated[B] | 133/160 | (83.1%) | – | |
| H/X treated[C] | 1/9 | (0%)[D] | 1/3 | |
| C/V treated[C] | 2/8 | (25.0%)[E] | 0/1 | (25.0%) |

[A]Determined by pregnancy-specific β-protein enzyme-linked immunosorbent assay (ELISA; Biotracking, Moscow, ID): denominator = total doe *n*; numerator = fertile doe *n*; parentheses = % of does producing fawns.
[B]Fertility tested at original capture.
[C]Recaptured 1 or 2 years later (H/X = heat-extruded pellets; C/V = cold-evaporation pellets).
[D]Different from untreated ($P < 0.01$, binomial probability distribution).
[E]Different from untreated ($P < 0.05$, binomial probability distribution).

**Table 4. Anti-porcine zona pellucida (PZP) titre response in African elephants given PZP vaccine**

| Vaccine type | *n* | Anti-PZP titres[C] | |
| --- | --- | --- | --- |
| | | (% positive reference control ± s.e.) | |
| | | PRE(zero time) | POST(3 months) |
| Two-injection vaccine[A] | 6 | – | $100.0 \pm 14.2$ |
| Controlled release[B] | 3 | $16.2 \pm 3.3$ | $162.1 \pm 16.3$[D] |

[A]Delivery by dart; primer (400 μg PZP/ Freund's modified adjuvant (FMA)), booster (200 μg PZP/Freund's incomplete adjuvant (FIA)).
[B]Delivery by hand injection; primer (400 μg PZP/Freund's modified adjuvant (FMA)), pellets (two each of 1 month and 3 months type). One- and 3 months pellet = 100 μg PZP/200 μg QA-21; 12 months pellet = 250 μg PZP/500 μg/ QA-21).
[C]Assay via enzyme-linked immunosorbent assay (ELISA) at 1 : 10 serum dilution; measurement via microtitre plate reader.
[D]Different from two-injection value and pretreatment value ($P < 0.01$, *t*-test).

transition from microspheres to pellets. A major functional development has been the use of pellet-based vaccine boosters to enable 2 years of contraception in a single injection, thus limiting the need to re-access animals for treatment. *In vitro* assessment of various controlled-release polymer formulations has been a beneficial and cost-efficient weeding-out step in pellet development. Despite the inability to project *in vivo* performance from *in vitro* data, reasonable estimates can be made. The need for *in vivo* testing remains essential and should include both antibody titre measurement in blood and actual fertility assessment via pregnancy test and/or offspring counts. *In vivo* assessment should include pretreatment, treatment (contraceptive effect) and follow-up (return to fertility) data. To date, positive outcomes of *in vitro* release-delay studies and *in vivo* titre studies associated with controlled-release pellets have correlated well with field-treatment contraceptive success in horses (Turner *et al*. 2002; Liu *et al*. 2005) and deer (Turner *et al*. 1992; Rutberg *et al*. 2004). Although controlled-release pellet technology has shown promise for eventual routine management use, several obstacles remain to be surmounted. An issue that faces use of controlled-release, multi-year PZP vaccine is possible delay in return to fertility (Kirkpatrick *et al*. 1995; Turner *et al*. 2007). Thus, controlled-release PZP vaccine must be assessed regarding population-level effects of both initial treatment and retreatment. If controlled-release capability can eventually extend infertility to 3 or 4 years, this issue will likely become even more pressing. Another, perhaps simpler, issue is the reliable delivery of vaccine by remote darting. Currently available darts cannot reliably provide sufficient or rapid enough pressure to propel both vaccine primer emulsion and two pellets into muscle tissue. Partial loss of vaccine ingredients sometimes occurs owing to backflow from the injection site or incomplete expulsion from the dart needle or both. This is evidenced by data in Table 2 showing half the contraceptive efficacy in dart-injected *v.* hand-injected mares given 1-year PZP vaccine. This circumstance for darting is both costly and time consuming. In treating wild horses gathered in routine BLM horse management, the delivery problem has been surmounted by separate hand-injections of primer emulsion by syringe and pellets by trocar. The latter has been 100% effective in pellet delivery in >200 horses (J. Turner, unpubl. data). Development of a trocar-containing dart (now in progress) may resolve this issue. In addition to delivering primer vaccine emulsion, this dart also propels the pellets in the needle barrel with a metal rod upon dart firing.

The database for pellet use in horses indicates that controlled-release PZP vaccine is a viable option in this species. Because controlled-release pellets of the 2-year vaccine provide <24 months (21–22 months) of contraceptive-level titres (Fig. 4), application of vaccine is best done in the autumn or winter of a given year to maximise infertility for two breeding seasons. The same preference may eventuate for 3-year vaccine, since the longest-term pellets for this version of vaccine release at 19–21 months rather than 24 months. Although effectiveness of PZP contraception has been previously established for deer (Turner *et al*. 1992; Rutberg *et al*. 2004) and elephants (Fayrer-Hosken *et al*. 2000; Delsink *et al*. 2002), use of controlled-release pellets as boosters has not been reported. Controlled-release vaccine data for deer and elephant in this report are preliminary but encouraging. In deer the limited

evidence shows markedly reduced fertility in Year 1 with controlled-release, 2-year vaccine. Although the finding of only one in four recaptured does with fawns in Year 2 is promising, no conclusions can be drawn from this small '*n*'. Interestingly, C/V pellets, which did not maintain anti-PZP titres in mares, yielded a 58% reduction in doe fertility. The reason for this difference is unknown but may lie in species differences in responsiveness to a given PZP exposure pattern. Elephant data to date are insufficient for conclusions regarding pelleted vaccine, but the vigorous titre response (62% greater than for 2-injection vaccine) is encouraging. It is unlikely that this difference was due to incomplete two-injection vaccine delivery by dart, since previous studies of PZP vaccine delivered to elephants by dart have shown effective (2–4-fold) fertility reduction (Fayrer-Hosken *et al*. 2000; Delsink *et al*. 2002).

Although controlled-release technology is not new, the extended release-delay intervals to which PZP vaccine use has aspired are unexplored territory. Much of the field success has stemmed from trial and error forays and *in vitro* inferences. However, emergent from this has been the efficacious transition from a two-injection contraceptive lasting 1 year to a one-injection contraceptive lasting 2 years. Continued efforts should target refinements in controlled-release capabilities, ease of use and more extended contraceptive duration through controlled-release technology.

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