

# PORCINE ZONA PELLUCIDA TECHNICAL DATA

## PRODUCT EFFICACY

### Description of Vaccine

ZonaStat-H is an injectable contraceptive vaccine consisting of an emulsion of two components: (a) the antigen, a naturally occurring, chemically unmodified glycoprotein, porcine zona pellucida (PZP), extracted from pig ovaries by simple physical processes and dissolved in a buffered salt solution (phosphate buffered saline); and (b) an adjuvant (modified Freund's Complete Adjuvant, mFCA, or Freund's Incomplete Adjuvant, FIA). mFCA consists of cell wall fragments from a naturally occurring, non-transmissible, non-pathogenic soil bacterium (*Mycobacterium butyricum*) suspended in a physiologically inert mineral oil and an emulsifier; FIA is identical to mFCA, but lacks the mycobacterial cell wall component.

### Mechanism of Action of ZonaStat-H

Like all vaccines, ZonaStat-H exercises its effects by stimulating a classic humoral response, *i.e.*, the B-cell-mediated production of antibodies against the glycoprotein components that comprise PZP (ZP1, ZP2, ZP3, and ZP4). The anti-PZP antibodies interfere with fertilization by binding to the ZP glycoprotein receptors that surround the egg of the treated female animal, causing steric hindrance of the zona sperm receptor, and blocking the binding and subsequent penetration of sperm.

### Published Data Supporting the Contraceptive Efficacy of ZonaStat-H

Liu et al. (1989) first demonstrated in principle the efficacy of PZP in equids by suppressing fertility in 12 of 14 (86%) captive domestic and wild horse mares (*Equus caballus*). These investigators administered 4 hand injections of PZP with aluminum hydroxide gel and/or Freund's Complete and Incomplete Adjuvants (FCA and FIA) at 2–4-week intervals, with a fifth booster injection at 6–9 months after the last injection. They also demonstrated that anti-PZP antibody titers of 64% or greater were associated with effective contraception, and that a decline in contraceptive effect correlated with a decline in antibody titers. Kirkpatrick et al. (1990) used dart guns to remotely inject 26 free-roaming wild horse mares of known high fertility at Assateague Island National Seashore (ASIS), Maryland, 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 the following autumn indicated that only one of 26 sampled mares (3.8%) was pregnant, and the following spring only one of the 26 treated mares produced foals. Of the 26 treated mares, 14 were boosted again a year later with a single remotely delivered dart containing PZP in FIA. Only 1 of the 14 boosted mares (7.1%) was diagnosed as pregnant and produced a

foal the following year, compared to 10 of 22 sham-treated and untreated mares (45.5%) (Kirkpatrick et al. 1991). Follow-up studies at ASIS over the next six years demonstrated foaling rates of 3.8% (4 foals in 105 mare-years) among PZP-treated mares vs. 46.2% in untreated mares (Kirkpatrick et al. 1995). According to the most recent data (1993–2006), mares on ASIS treated with one or two initial shots and one or more annual boosters produced 34 foals in 340 mare-years (10%), with modified Freund's Complete Adjuvant (mFCA) replacing FCA for initial injections beginning in 2002 (Kirkpatrick and Turner 2008). Zero population growth was achieved in 2 years, with an initial population decline becoming evident within 8 years, and a total decrease of 22.8% seen by year 11. Prior to initiation of the PZP inoculation program, this ASIS herd had a foaling rate of  $57.1 \pm 3.9\%$  and an overall annual population growth rate of 8% (Kirkpatrick and Turner 2008).

Hand-injection of a priming dose of 65–100  $\mu\text{g}$  PZP in FCA followed by hand-injection of 65–100  $\mu\text{g}$  in FIA has also been investigated in wild horses in Nevada. Mares were treated with either two separate injections, 4 weeks apart of PZP (n=60); one injection of PZP (n=21); 1 injection of PZP + adjuvant + controlled-release PZP in microspheres (no adjuvant) (n=22); 2 injections of placebo (saline + FIA) (n=19), or 1 injection of placebo (n=10). Additionally, pregnancy status was assessed in 63 untreated mares. Measuring reproductive success either by a positive fecal steroid metabolite diagnosis of pregnancy or by positive association with a foal, Turner et al. (1997) showed that 2 of 44 mares (4.5%) treated with 2 injections of PZP were reproductively successful, vs. 45 of 83 (54.2%) reproductively successful sham-treated or untreated mares. Of mares injected once (with or without microspheres), 7 of 29 were reproductively successful (24%). The differences in reproductive success between all treated groups and the untreated control animals were statistically significant ( $p < 0.05$ ). Using similar methods to infer reproductive success, a second study showed that 10 of 78 wild mares hand-injected with PZP in FCA followed by PZP in FIA (12.8%) were reproductively successful, vs. 45 of 72 untreated mares (62.5%) (Turner et al. 2001). Variation in efficacy between studies can be entirely accounted for by sampling error (*i.e.*, 95% confidence intervals for the proportion of PZP-treated horses reproducing overlap for all studies), although differences in injection quality, nutritional condition, and other variables might affect contraceptive effectiveness.

Modified Freund's Complete Adjuvant (mFCA) has been substituted for FCA in titer trials of captive mares. No significant difference was seen in antibody titers between mares hand-injected with 65–100  $\mu\text{g}$  PZP in mFCA followed by a booster of 65–100  $\mu\text{g}$  in FIA and mares treated with 65–100  $\mu\text{g}$  PZP in FCA followed by a booster of 65–100  $\mu\text{g}$  in FIA. Seven of 8 (87.5%) of mares treated with PZP and mFCA remained above the contraceptive titer threshold after 10 months (Lyda et al. 2005). These trials corroborate the effectiveness of mFCA- adjuvanted PZP vaccines as reported at ASIS above (Kirkpatrick and Turner 2008).

Comparable results were seen in tests of two-injection PZP protocols on free-roaming feral burros (*Equus asinus*) at Virgin Islands National Park, St. Johns, VI. In that study, 0 of 13 females darted with a priming dose of 65–100  $\mu\text{g}$  PZP in FCA and a booster of 65–

100 µg PZP in FIA produced foals in the period 12–24 months after treatment, and 1 of 3 injected only once with PZP in FCA produced foals during this period. In contrast, 6 of 11 control females either gave birth or tested positive for pregnancy in that time period, which differs significantly from the two-injection treated animals ( $p < 0.05$ ) (Turner et al. 1996). (Feral burros on the Virgin Islands are not seasonal breeders, and some were pregnant at the time of treatment.)

Contraception in horses treated with two initial doses of 65–100 µg PZP emulsified in FCA for the initial priming dose and FIA for the boosting dose followed by annual PZP-FIA boosters is fully reversible after up to five consecutive years of treatment, although mares treated for 4 or 5 years may experience a delay in return to fertility (Kirkpatrick and Turner 2002). PZP contraception also was shown to be reversible in feral burros, at least after 1–2 years of treatment; 6 of 13 (46.1%) of burros treated with PZP for one year and 18 of 39 (46.2%) untreated or control burros testing positive for pregnancy using fecal steroid analysis 2–3 years after PZP treatments stopped (Turner et al. 1996).

In addition to the evidence presented above for the efficacy of ZonaStat-H for preventing births in individually treated female wild horses and feral burros, as noted above, there is evidence that ZonaStat-H is effective at the population level. Systematic application of PZP to the wild horse population at ASIS in 1994 was associated with an immediate cessation of population growth (Turner and Kirkpatrick 2002). Continued application led to a population decline that continued from 2003 through 2007, the last year for which published data are available (Kirkpatrick and Turner 2008).

### **List of Studies Submitted for Volume II: Efficacy**

- 1 Kirkpatrick, J.F., and A. Turner. 2002. Reversibility of action and safety during pregnancy of immunization against porcine zona pellucida in wild mares (*Equus caballus*). *Reproduction Supplement* 60:197-202.
- 2 Kirkpatrick, J.F., and A. Turner. 2008. Achieving population goals in a long lived wildlife species (*Equus caballus*) with contraception. *Wildlife Research* 35:513-519.
- 3 Kirkpatrick, J.F., I.K.M. Liu, and J.W. Turner, Jr. 1990. Remotely delivered immunocontraception in feral horses. *Wildlife Society Bulletin* 18:326-330.
- 4 Kirkpatrick, J.F., I.K.M. Liu, T.W. Turner, and M. Bernoco. 1991. Antigen recognition in feral mares previously immunized with porcine zonae pellucidae. *Journal of Reproduction and Fertility Supplement* 44:321-325.
- 5 Kirkpatrick, J.F., R. Naugle, I.K.M. Liu, M. Bernoco, and J.W. Turner Jr. 1995. Effects of seven consecutive years of porcine zona pellucida contraception on ovarian function in mares. *Biology of Reproduction Monograph* 1:411-418.
- 6 Liu, I.K.M., M. Bernoco, and M. Feldman. 1989. Contraception in mares heteroimmunized with pig zonae pellucidae. *Journal of Reproduction and Fertility* 85:19-29.

7 Lyda, R.O., J.R. Hall, and J.F. Kirkpatrick. 2005. Comparison of Freund's complete and Freund's modified adjuvants used with a contraceptive vaccine in wild horses (*Equus caballus*). *Journal of Zoo and Wildlife Medicine* 36:610-616.

8 Turner, A., and J.F. Kirkpatrick. 2002. Effects of immunocontraception on population, longevity and body condition in wild mares (*Equus caballus*). *Reproduction Supplement* 60:187-195.

9 Turner, J.W., Jr., I.K.M. Liu, A.T. Rutberg, and J.F. Kirkpatrick. 1997. Immunocontraception limits foal production in free roaming feral horses in Nevada. *Journal of Wildlife Management* 61:873-880.

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11 Turner, J.W., Jr., I.K.M. Liu, and J.F. Kirkpatrick. 1996. Remotely delivered immunocontraception in free roaming feral burros (*Equus asinus*). *Journal of Reproduction and Fertility* 107:31-35.

## **TOXICOLOGY - ACUTE**

### **I. PRODUCT PROPERTIES ASSESSMENT FOR PORCINE ZONA PELLUCIDA (PZP) ANTIGEN**

#### **a) Scope for PZP**

##### **1) Applicability**

Porcine zona pellucida (PZP) antigen is the core active ingredient of the ZonaStat-H contraceptive vaccine for use in wildlife. It is intended to provide an environmentally safe, effective, and humane means of regulating wildlife populations in the islands of habitat to which they have been confined by human expansion.

##### **2) Background**

PZP is a glycoprotein isolated from pig ovaries obtained from commercial abattoir. The glycoprotein is used, like in all vaccines, as an antigen to stimulate a classic humoral response, *i.e.*, the B-cell-mediated production of antibodies against the glycoprotein components of PZP in recipient mammals. The PZP antibodies bind to these glycoprotein receptors on the non-cellular membrane (zona pellucida) that surrounds the egg of the treated animal, and causes steric hindrance of the zona sperm receptor (Henderson et al. 1988; Hasegawa et al. 1992), thereby preventing fertilization. PZP vaccine has been experimentally tested in thousands of animals during the past 20 years with no toxic effects (see below).

The chemistry of the porcine zona pellucida (ZP) family of proteins is well documented, primarily in studies from the late 1970's and 1980's (Dunbar et al., 1989; Gwatkin et al.,

1980; Hedrick and Wardrip, 1986; Liu et al., 1989; Mahi-Brown et al., 1985; Monne et al., 2008; Sacco et al., 1983; Yurewicz et al., 1987). The early studies attempted to characterize the role of ZP in the process of mammalian fertilization, *i.e.*, union of sperm with the ovulated egg. The purpose was to understand the fertilization process in order to manipulate it, *i.e.*, finding ways to enhance or inhibit fertility. It was during this time that marked interest in the possible use of this protein as an immunocontraceptive arose (Liu et al., 1989; Mahi-Brown et al., 1985). The coating (ZP) surrounding mammalian egg, and especially that from porcine ova, can readily be isolated from the ova (Gwatkin et al., 1980; Liu et al., 1989; Whitten and Russell, 1996), enabling isolation and purification of the ZP constituents. Studies to isolate ZP yielded a family of four proteins, ZP 1-4, with the protein ZP3 exhibiting the most promising qualities for use in a contraceptive vaccine (Hedrick and Wardrip, 1986; Sacco et al., 1983; Yurewicz et al., 1987).

**b) Information on Product Composition** The chemical composition of ZP is presented in Table 1 (Gwatkin et al. 1980). The two major components are protein and hexose, with the latter incorporated into the structure of each of the 4 ZP glycoprotein families.

Table 1. Chemical Components of Zonae Pellucidae\*

<u>Component</u>	<u>ng/zona</u>	
	<u>Bovine</u>	<u>Pig</u>
Protein	36.0±2.9	35.5 ±1.8
Hexose	2.2±0.2	2.5±0.1
Sialic Acid	0.06±0.01	0.02±0.00
Uronic acids	0.70±0.04	0.34±0.03
<u>Amino Sugars</u>	<u>1.57±0.12</u>	<u>3.01±0.13</u>

\* Based on three to six determinations, ± standard error of the mean (Reproduced from (Gwatkin et al., 1980).

Studies of ZP protein separation via two dimensional polyacrylamide gel electrophoresis (2D- PAGE) and 2D-PAGE immunoelectrophoresis revealed approximately 7% contamination with non-ZP materials (Sacco et al., 1983). Heat solubilization of ZP prior to PAGE yields a product that does not contain organ cross-reactive antigens (Dunbar and Raynor, 1980). This clean ZP preparation is used to prepare the contraceptive vaccine that is the subject of this registration application. Each of the 4 ZP glycoprotein families (labeled ZP1, ZP2, ZP3 and ZP4) are identifiable on 2D-PAGE and the amino acid and carbohydrate compositions have been described (Hedrick and Wardrip, 1986).

The major PZP component is ZP3, which is a 55 kD glycoprotein comprising 71% of the purified PZP. This glycoprotein serves as the primary immunogenic antigen in the PZP vaccine. An important characteristic of ZP3 is its extensive charge heterogeneity, contributed by lactose aminoglycans in the glycoprotein structure (Yurewicz et al., 1987). Lactose aminoglycans are oligosaccharides with repeating N-acetyl-lactose amine units.

Chemically, ZP3 is made up of overlapping families of charge isomers that correspond to an α- and β-glycoprotein (Yurewicz et al., 1987). The ZP3 α- and β-glycosylated proteins are both structurally and immunologically distinct, and presumably, each is the

product of a different gene. The molecular composition of the  $\alpha$  and  $\beta$  glycoproteins is not completely determined. However, the core polypeptide of the  $\alpha$  glycoprotein is 38 kD and that of the  $\beta$  glycoprotein is 35 kD. Chemical analysis indicates 12.1 mannose residues and 3.0 N-acetylgalactosamine residues per mol of  $\alpha$  glycoprotein and 10.9 mannose and 6.0 N-acetylgalactosamine residues per mol of  $\beta$  glycoprotein (Yurewicz et al., 1987).

The immunogenicity of the ZP3 is closely linked to the glycosyl residues and their positions in the molecule (Dunbar et al., 1989). Current data indicate that the species-specific antigen that generates fertilization-blocking antibodies is an 11 kD fragment of the 55 kD ZP3 protein (Monne et al., 2008).

- c) **Active ingredient Information** PZP/ZP3 details are provided below.
  - 1) PZP components are not EPA-registered.
  - 2) i) *No Chemical Abstract Service Registry:*  
chemical name = glycoprotein; common names = ZP3 and PZP
  - ii) *Molecular Formula and Molecular Weight Range:*  
PZP is a glycoprotein family (ZP1-4). Molecular formula is peptide- bonded amino acids containing oligosaccharides with repeating N-acetyl- lactoseamine units.  
Molecular weight range is 35-55 kD.
  - iii) *Nominal Concentration in Weight Percent:* 500  $\mu$ g PZP/1.03 g vaccine = 0.052%.
  - iv) *Upper/Lower Limits of Active Ingredient:*  
100-550  $\mu$ g PZP (equivalent of 5-20  $\times 10^3$  zonae) per immunization (intramuscular).
  - v) *Purpose of Ingredient:* The ingredient serves as an antigen to generate anti-PZP antibodies, which bind to ovulated eggs and block sperm attachment to the egg, preventing fertilization.
- d) **Inert Ingredients**
  - 1) *Chemical Name of Ingredients:* Chemical name = glycoprotein; common names = ZP1, ZP2, ZP4.  
The PZP glycoprotein family is composed of 4 glycoproteins ZP1, ZP2, ZP3 and ZP4.  
ZP3 is the primary active ingredient and comprises 71% of the purified PZP. ZPs 1, 2 and 4 are minimally- or un-involved in the contraceptive action and can be considered inert ingredients.
  - 2) *Nominal Concentration of Inert Ingredients:* ZP1, 2, and 4 comprise 29% of the product.
  - 3) *Upper/Lower Limits of Inert Ingredients:* Upper limit is 150  $\mu$ g and lower is 29  $\mu$ g per immunization (1.03 g.).
  - 4) *Purpose of Inert Ingredients:* Inert ingredients are unseparated, co-migrating products of PZP purification.
- e) **Impurities of Toxicological Significance Associated with Active Ingredient** No detectable toxicological impurities are present in the immunization-ready PZP preparation.
- f) **Other Impurities Associated with Active Ingredient** No other impurities are

detectable in the immunization-ready PZP preparation.

## II. REFERENCES

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## **TOXICOLOGY – SUBCHRONIC, DEVELOPMENTAL AND REPRODUCTIVE TOXICITY; GENOTOXICITY; NEUROTOXICITY; AND IMMUNOTOXICITY**

### **Description, History of Use, and Safety of Vaccine Components**

*Composition of ZonaStat-H.* ZonaStat-H is an emulsion consisting of two components: (a) a naturally occurring, chemically unmodified glycoprotein (porcine zona pellucida, PZP) extracted from pig ovaries by simple physical processes and dissolved in a buffered salt solution (phosphate buffered saline) that serves as the antigen; and (b) an adjuvant (modified Freund's Complete Adjuvant, mFCA, or Freund's Incomplete Adjuvant, FIA). mFCA consists of cell wall fragments from a naturally occurring, non-transmissible, non-pathogenic soil bacterium (*Mycobacterium butyricum*) suspended in a physiologically inert mineral oil and an emulsifier; FIA is identical to mFCA, but lacks the mycobacterial cell wall component.

*Porcine zona pellucida antigen.* Porcine Zona Pellucida (PZP) is produced following the methods of Dunbar et al. 1980. (*See, also, Product Chemistry*). Briefly, porcine ovaries are collected from freshly slaughtered female pigs at USDA-inspected slaughterhouses, and frozen immediately. Oocytes are extracted from the ovaries using a rotary-ganged razor blade device and washed with a buffered salt solution through a series of nylon screens, the last of which (74 $\mu$ m) traps the oocytes but permits dissolved proteins, erythrocytes, and other small debris particles to pass through. The isolated oocytes are then gently homogenized in buffered salt solution, and the zonae pellucidae collected on a 50 $\mu$ m screen and repeatedly washed. The isolated zonae are then heat-solubilized at 70°C for 30 minutes in phosphate buffer solution (PBS), and diluted to concentrations of approximately 5,000 zonae per 0.5 mL dose. The ZP solution is then frozen until use. Thus, the PZP antigen is extracted directly from a USDA- inspected animal food product, and is dissolved in a standard buffered salt water solution.

<sup>1</sup> Health Effects Test Guidelines, OPPTS 870.1000, Acute Toxicity Testing -- Background, EPA 712-C-98-189, August 1998, at 2.

*Adjuvant.* The purpose of a vaccine adjuvant is to boost or modulate the immune response to a given antigen. Adjuvants are especially important if the antigen alone yields a weak immune response (which is the case for PZP). In particular, the PZP antigen induces little or no immune response unless administered with an adjuvant (Bhatnager et al. 1989). Adjuvants also may allow the use of smaller quantities of antigen (if, for example, the antigen is toxic or very expensive), or improve vaccine effectiveness by boosting parts of the immune system that are not strongly stimulated by the antigen. The mechanisms of adjuvant action are still not well understood, but are generally thought to



include enhancement of presentation of the antigen to T- helper cells and cytotoxic T cells, improvement of antigen stability, and modulation of the immune response (Cox and Coulter 1997; Spickler & Roth 2003). Adjuvants also may stimulate the activity of the antigen presenting cells (such as dendritic cells and macrophages) that initiate the response of immune effector cells. Adjuvants can be associated with side effects including injection site reactions such as granulomas and sterile abscesses, systemic effects such as fever, lethargy, and loss of appetite, and sometimes autoimmune diseases (Hanly et al. 1997).

ZonaStat-H uses Modified Freund's Complete Adjuvant (mFCA) for primer injections, and Freund's Incomplete Adjuvant (FIA) for booster injections. mFCA consists of 85% Drakeol 5 NF (long-chain, hydrocarbon oil; also referred to as Light mineral oil N.F. - 776510), 15% Arlacel A (mannide monooleate ester), and 0.1% killed and dried *Mycobacterium butyricum* cell walls. FIA is the same composition as mFCA, but lacks the *M. butyricum* cell wall component. The published literature has identified no pathology associated with *M. butyricum*. Drakeol 5 NF is the trade name for a grade of light mineral oil used commonly in animal and human pharmaceuticals (including vaccines and capsules), laxatives, cosmetic additives, and other applications. Arlacel A is a trade name for mannide monooleate, an ester that acts as an emulsifier.

Freund's adjuvants were among the first developed, and the combination of Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA) remains arguably the most effective of all adjuvants (Stewart-Tull 1997; Lindblad 2000). In horses, Kirkpatrick et al. (Report 1) tested PZP vaccines with FCA/FIA adjuvants against PZP vaccines using Carbopol 934P, DEAE-dextran, and Ribi Adjuvant System (RAS); antibody titers over 92 days were significantly higher in the FCA/FIA –adjuvanted vaccines than the DEAE-dextran and RAS vaccines, but did not differ significantly from the Carbopol® vaccine. Smith et al. (1992) tested alternative adjuvants against FCA/FIA adjuvanted vaccines with low-molecular weight antigens in rabbits, and found that The FCA/FIA vaccine produced antibody titer levels 4-10X higher than vaccines adjuvanted with Ribi Adjuvant System and TiterMax®. Rutberg (2005) summarizes a series of tests of different adjuvants with PZP vaccines on white-tailed deer (*Odocoileus virginianus*), finding that the FCA/FIA adjuvant combination was more effective and consistent than Carbopol®, QA-21, RAS, and Montanide ISA 50 adjuvants.

As noted above, FCA comprises mineral oil (a distilled mixture of petroleum-based hydrocarbons), an emulsifier, and dried/killed fragments of cell walls from the bacterial genus *Mycobacterium* (*M. tuberculosis* in classical FCA, *M. butyricum* in modified FCA). FIA contains only the mineral oil and emulsifier components. During the 1950's and early 1960's, FIA was incorporated into widely-used human vaccines for influenza, tetanus, and polio vaccines; over 500,000 people received the influenza vaccine alone in the U.S. and U.K. (Stewart-Tull 1997; Lindblad 2000). Follow-up examinations at 17 years and 35 years post- injection of 18,000 American GI's receiving the FIA-adjuvanted flu vaccine showed no increase in incidence of cancer and other diseases, and showed lower incidences of side effects than the aqueous vaccine (possibly because of the higher doses needed for the aqueous vaccine) (Gupta et al. 1993; Lindblad 2000). Use of oil-

adjuvanted vaccines in humans was discontinued in the mid-1960's because of the incidence of injection-site reactions, and a few reports that components of the adjuvant promoted tumor formation in two strains of mice. However, FIA has not been found to be carcinogenic in other mouse strains (Gupta et al. 1993; Lindblad 2000). Thus, there is a documented history of safe use of the adjuvant (or similar adjuvants) contained in ZonaStat-H

Highly-purified oil adjuvants (Montanide® ISA) are in clinical trials of human vaccines, with more than 4,000 patients having been treated to date (Aucouturier et al. 2006). Because of its efficacy in raising antibody titers, FCA is used extensively in laboratory settings, but because of safety concerns, FCA has not previously been approved by FDA or USDA for use in commercial vaccines. FCA has been associated with abscess formation and inflammation at the injection site, pain, fever, autoimmune diseases, and organ damage (Gupta et al. 1993).

Our own data on the frequency and severity of side effects and the general health of treated horses (described in detail below) does not support the negative results observed with Freund's adjuvants in the laboratory. This discrepancy may be due to several reasons. First, both efficacy and the type and magnitude of side effects elicited vary with species, route of administration, and adjuvant. In that context, it should be observed that reports of side effects associated with Freund's adjuvants are derived from studies of laboratory animals, including mice, rats, hamsters, guinea pigs, and rabbits. Cats and dogs also seem to be very sensitive to FCA (e.g., Harrenstien et al. 2004; Wheir et al. 2005).

Second, dosages administered in many studies reporting side effects are extremely high relative to body weight (Stewart-Tull 1997). In one toxicity study of components of FIA in mice, for example, 0.25 ml oil were injected i.p. into juvenile male mice weighing 11 g (Hardegree and Kirschstein 1968). This dose is approximately 10<sup>4</sup> x the dose by weight administered to horses in our studies.

Finally, the composition of mineral oils has changed significantly over the 40-50 years in which this research has been done (Lindblad 2000). This is important because different fractions of the petroleum-based mineral oil have different health effects. In particular, polycyclic aromatic hydrocarbons can be carcinogenic and mutagenic, and short chain, saturated hydrocarbons are more toxic (possibly because of their solvent effects) than longer chain saturated hydrocarbons (Gupta et al. 1993; Stewart-Tull 1997; Lindblad 2000). In more recent mineral oil preparations, the unsaturated and aromatic hydrocarbons are removed, leaving behind the less reactive longer-chain saturated hydrocarbons (Stewart-Tull 1997; Lindblad 2000). These "white mineral oils" are non-carcinogenic in mice when administered dermally or by inhalation; nonmutagenic by Ames test at doses of 50-1000 µg/ml, and non-fetotoxic and non-teratogenic in rats treated by oral gavage at 5 ml /kg-day (Stewart-Tull 1997).

Another concern associated with the use of adjuvants generally, and Freund's adjuvants in particular, is the risk of aggravating autoimmune diseases associated with antigens that resemble host proteins (Billiau and Matthys 2001; Staykova et al. 2007). PZP, however,

does not cross-react with any equine somatic tissues or protein hormones, making this concern irrelevant (Kirkpatrick et al. 1996; Barber and Fayrer-Hosken 2000).

### **Mechanism of Action of ZonaStat-H**

Like all vaccines, ZonaStat-H exercises its effects by stimulating a classic humoral response, *i.e.*, the B-cell-mediated production of antibodies against the glycoprotein components of PZP (ZP1, ZP2, ZP3, and ZP4). The PZP antibodies interfere with fertilization by binding to these glycoprotein receptors on the non-cellular membrane (zona pellucida) that surrounds the egg of the treated animal, and causing steric hindrance of the zona sperm receptor (Henderson et al. 1988; Hasegawa et al. 1992).

### **Fate of Product after Injection**

Following injection, both components of the vaccine follow the common immunological and metabolic path of vaccines detected by the humoral immune system (Report 2). Briefly, both the PZP and the bacterial peptidoglycans are hydrolyzed in cells of the immune system. The breakdown products of this hydrolysis bear no resemblance to PZP or bacterial peptidoglycans, and are indistinguishable from other products of lysosomal hydrolysis. After hydrolysis, these metabolic products are excreted and eliminated from the body in forms (such as CO<sub>2</sub>, water, lactic acid, and urea) that are indistinguishable from other metabolic products. Likewise, the antibodies produced in response to ZonaStat-H injection are broken down into their component amino acids, and recycled into other body proteins or metabolized and excreted as urea, CO<sub>2</sub>, and water. PZP and the adjuvant antigens are not stored in body tissues in a stable or physiologically active form, thereby eliminating the possibility of continued exposure of the target animal to the vaccine components, or of non-target animals and humans of exposure to vaccine components from excreted products. Further, if non-target animals were to ingest vaccine components, complete digestion by stomach acids and enzymes in the stomach walls, pancreas, and small intestine would yield end products comprising amino acids and simple carbohydrates, which elicit no immune response and are bioinactive. Thus, vaccine components will not be transferred through the food chain.

Bollinger (1970a, b) used radioactively-labeled <sup>14</sup>C to trace the fate of mineral oil and mannide monooleate after injection into female rats and squirrel monkeys. He found that 85- 98% of the hexadecane component of the mineral oil remained at the injection site after 1 week, declining to 65-75% at one month, 55-65% at 3 months, and 30% at 10 months. The mineral oil tracer was “readily incorporated into lipids” (Bollinger 1970a). At 1 month, radioactivity appeared in triglycerides, sterol esters, and free sterols in the liver, at 3 months in liver phospholipids, and were absent from the liver by 10 months. Radioactive tracer also appeared as triglycerides in depot fat, a small amount of which still remained after 10 months. Radioactivity was lost slowly over time through metabolism and elimination via respiratory CO<sub>2</sub>, and there was no bioaccumulation of mineral oil or tracer. Mannide monooleate is dissipated more quickly from the injection site than mineral oil, with the oleate component largely being incorporated into lipids, with subsequent elimination through respiratory CO<sub>2</sub>, and the mannide largely eliminated from the body in urine in the form of non-esterified sugars – 25% in the first 24 hr after administration (Bollinger 1970b).

### **Method of Administration and Exposure Risk**

Zonastat-H is injected intramuscularly, either by hand-held syringe, by syringe attached to a “jab-stick” (a pole that extends the reach of the injector), or by syringe dart projected from a blow-pipe, CO<sub>2</sub>-cannister powered gun, or .22-caliber powered dart rifle. The volume of the injection is 1 mL. This method of delivery ensures that the target animal receives no aerial, oral, ocular, or general dermal exposure.

There is a nearly zero probability that non-target animals or humans will be exposed to ZonaStat-H in undischarged darts in the environment. One potential pathway of exposure of non-target animals or humans is through incidental contact with the contents of unrecovered, non-discharged syringe darts. The minimal risk of exposure via this pathway is further reduced by the following:

- Applicators administering PZP by dart should search for, and attempt to recover, all fired darts, whether or not they hit their targets. In practice, approximately 95% of all darts fired are recovered (Report 3). This procedure significantly reduces the number of unrecovered darts that remain in the environment.
- The darts (Pneu-dart® 1.0 cc darts with 1.5-inch 14-gauge needles) do not discharge spontaneously or with incidental contact. For the dart to discharge, a small weight at the rear of the body of the dart must be launched forward (through impact with a target) with sufficient velocity to fire a small gunpowder cap, which in turn sets off a larger charge, which pushes the plunger forward and empties the dart. Striking, stepping on, jiggling, biting, or otherwise casually moving or contacting the dart will not discharge or release the contents of the dart.

A second potential pathway of exposure of non-target animals to the product is by oral consumption of the injection site by a predator or scavenger. As discussed above, however, because they are broken down to amino acids and simple carbohydrates following ingestion, neither the PZP antigen nor the bacterial peptidoglycans are physiologically active if eaten (Report 2). The mineral oil component is either passed directly through the gut without absorption or broken down, reprocessed into triglycerides and other lipids, and metabolized with other lipids.

During handling of the final product and loading the product into the syringe or syringe dart, the applicator is exposed to a very small risk of dermal, oral, or ocular contact. Thus, the required training and certification process instructs and requires applicators to wear protective clothing during the preparation of ZonaStat-H for field use (*see* section on Human Exposure).

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### **Field and Laboratory Data on the Safety of ZonaStat-H**

There are extensive field and laboratory data describing the safety of ZonaStat-H in target animals using various adjuvants and injection regimens. The safety endpoints evaluated

in most of these studies included injection site abscesses; safety to, and fertility of, foals of treated mares; mare body condition; and behavior. The results concerning injection site reactions are discussed below. Effects on foals of treated mares, mare body condition, and behavior are addressed in Subchronic, Developmental and Reproductive Toxicity; Genotoxicity; Neurotoxicity; and Immunotoxicity below).

*Injection Site Reactions.* Transient adverse reactions to ZonaStat-H may occur at the injection site. Sterile granulomas (typically ~25mm in diameter) occur commonly at the injection sites of horses that have received injections of PZP emulsified in FCA or PZP emulsified in FIA delivered remotely by dart. Visible draining abscesses at the injection site are rare, although slightly more common in horses that are treated by dart than in horses that are treated by hand injection. In the initial field studies at Assateague Island National Seashore, three visible abscesses (10-25 mm diameter) were observed among 26 mares receiving 2-3 injections of PZP in FCA or FIA; all drained from 6 to 9 days after treatment (Report 4). As of July 2007, 1,841 dartings with 65-100 µg PZP/FCA or PZP/FIA of 329 individual horses at 4 locations have yielded 19 total visible abscesses (1% of all dartings), ranging from 25-50 mm in diameter; all drained within 30 days (Report 5). No visible abscesses were observed in 215 mares hand-injected with PZP/FCA and PZP/FIA in two western wild horse populations, nor were injection-site marks observed in subsequent field observations (Report 6, Report 7). A study of 15 captive mares hand-injected with an initial shot of 100 µg PZP in modified Freund's Adjuvant (mFA) followed by a booster of 100 µg PZP in FIA resulted in 1 visible abscess (following a booster injection), which drained without incident (Report 8). Inspection of injection sites of 50 captive female wild horses treated with PZP and mFCA in four different formulations yielded a rate of visible abscesses of 8% over 12 weeks after treatment; palpation at 10 months indicated that two still had palpable subcutaneous abscesses. Ultrasound examination at seven months after treatment showed muscle tissue disruption at the injection site in eight of 28 horses examined; of these, seven were slight and one was a 2-inch diameter draining abscess pocket (Report 8).

### **List of Studies Submitted for Acute toxicity**

#### Report Number

- 1 Kirkpatrick, J.F., J.W. Turner, Jr., I.K.M. Liu, P.P. Calle, and K. Goodrowe. 1993. Alternative adjuvants for use with porcine zonae pellucidae immunocontraception of captive exotic species. Final Report to the AZA.
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- 4 Kirkpatrick, J.F., I.K.M. Liu, and J.W. Turner. 1990. Remotely- delivered immunocontraception in feral horses. *Wildlife Society Bulletin* 18:326-330.



- 5 Kirkpatrick, J.F. 2007. Injection site reactions in wild horses and zoo animals in response to remotely delivered injections of porcine zona pellucida vaccine in FCA, mFCA, or FIA. Unpublished report dated July 31, 2007.
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[http://www.penreco.com/products/pdfs/whitemineraloil/Drakeol\\_LightMinOilNF-776510.pdf](http://www.penreco.com/products/pdfs/whitemineraloil/Drakeol_LightMinOilNF-776510.pdf) Information for Arlacel A, including MSDS  
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#### *Long-term Effects of ZonaStat-H on Feral and Wild Horse Condition and Longevity.*

Two published studies evaluated the long-term effects of ZonaStat-H treatment on mare body condition and mortality in wild mares on Assateague Island. In the first study (Report 4), 82 wild adult female horses were treated with PZP emulsified in FCA or FIA and were followed for up to 11 years. The animals received a single- or two-dose treatment, followed by single annual booster inoculations. Survivorship and body condition<sup>2</sup> were evaluated. Treated mares (except for lactating mares) showed better body condition as evidenced by statistically significantly higher body conditions scores after 11 years as compared to untreated wild adult mares ( $p=0.0064$ ). Treated mares also survived longer than wild adult female horses not treated with PZP following treatment. The mortality rate decreased from >10% for all adult horses and 3% for foals to <4% four years after the start of the contraceptive treatment, with mare mortality decreasing to a rate of <4% and foal mortality decreasing to 1%. New, older age classes (>21 years) of wild mares on Assateague Island began to appear 10 years after the onset of PZP treatments in the herd, indicating increased longevity.

In the second, follow-up study (Report 5), a retrospective analysis was undertaken to further evaluate the long-term effects of ZonaStat-H treatment and longevity. The four groups evaluated included: (1) 56 stallions, (2) 42 untreated mares, (3) 11 mares that had been treated for up to 2 years, and (4) 19 mares treated for 3 or more years. The mean age of death of mares treated with vaccine for > 3 years was 19.9 years, which was greater than that of untreated mares (6.4 years,  $p=0.0001$ ), mares treated < 3 years (10.2 years,  $p=0.064$ ), and stallions (10.3 years,  $p=0.005$ ).

*Effects on Fertility, Reproduction, and Development.* ZonaStat-H works to control wild horse populations by inhibiting fertility (see Volume II). However, inhibition of fertility is reversible in most circumstances. The following parameters were evaluated in 53 wild mares on Assateague Island: reversibility of contraception in mares treated over a 12-year period; survival of foals born to treated mares over this period; maintenance of pregnancy and live births in 26 mares treated while pregnant; and fertility of mares that were *in utero* when their mothers were treated with ZonaStat-H (Report 6). Contraception in wild adult female horses treated with two initial doses of 65–100µg PZP emulsified in Freund's Complete Adjuvant (FCA; initial priming dose) or Freund's Incomplete Adjuvant (FIA; boosters) followed by annual boosters was fully

<sup>2</sup> Body condition was assessed on a scale of 0 to 5 according to the shape of the rump, which is indicative of the amount of fat deposited in the rump area. A score of 0 was given to an animal in very poor condition and a score of 5 was given to an animal with extreme fat deposits. Body condition scores were assessed at the beginning of the treatment period and again 11 years later.

reversible after up to five consecutive years of treatment, although some mares treated for 4 or 5 years experienced a delay in return to fertility. None of the mares treated for 7 consecutive years returned to fertility over a 7-year interval since the last ZonaStat-H treatment. With regard to the fate of foals born to mares treated during their pregnancy, over a 12-year period, 26 such pregnancies were diagnosed during the first trimester by urinary and fecal steroid analysis; and all resulted in successful births. Of all foals born to mares treated with PZP during their pregnancy, 67 of 80 (83.8%) survived to 1 year as compared to 83.7% of foals born to untreated mares. Further, of 14 fillies born to mares treated during their pregnancy and who had lived long enough to breed by the conclusion of the study, 8 were untreated and permitted to breed, and all 8 produced live foals that survived to at least 1 year of age. These results indicate that (1) contraception with PZP is reversible for up to 5 years of consecutive treatment, and (2) PZP treatment does not adversely affect pregnancy outcomes, survival of foals, or the subsequent fertility of the female foals.

Return to normal rates of fertility two years after administration of one or two shots of PZP (FCA primer and FIA booster) also was documented among Nevada feral horses when compared to untreated controls animals. Of 25 mares sampled that received two injections, 11 became pregnant (44.0%) two years after treatment, as compared to 12 of 22 untreated mares (54.5%). Further, the mare's conditions ranged from fair to very good throughout the study (Report 7).

Pregnancy rates in free-roaming burros 12 to 24 months after the last PZP treatment (n=16) also were comparable to the pregnancy rates of untreated control animals (n=11) in St. John, U.S. Virgin Islands, as determined by fecal pregnancy testing (Report 8). The feral burros initially received either one or two injections (total dose of 130 µg PZP of FCA), followed 10–12 months later by a booster injection of 65 µg PZP in FIA. All burros appeared healthy for the entire study period, based on subjective evaluation of physical appearance, demeanor, and general behavior. Twelve to 24 months after the last treatment, 6 of the 13 (46.1%) of the treated burros became pregnant, as compared to three of six (50.0%) of the control burros and 15 of 33 (45.5%) randomly chosen burros

that were not part of the study. Further, mating behavior (*i.e.*, courtship, female estrus behavior, and male mounting and copulatory behavior) were comparable between treated and control animals. Four foals that were born to treated burros assumed to be pregnant at the time of treatment (*i.e.*, they were born within 12 months of treatment) nursed and grew normally, indicating that the treatment did not disrupt existing pregnancies.

Urinary estrone conjugates and non-specific progesterone metabolites also were used to track ovarian function in 50 free-roaming mares that were inoculated with PZP over 7 consecutive years (1–3 injections of 65 µg PZP followed by yearly booster inoculations), as compared to 33 untreated controls (Report 9). Ovulation rates among PZP-treated wild adult female horses, as evidenced by pregnancies or luteal phase progesterone metabolite patterns, were 73% after one year of treatment, 56% after 3 consecutive years of treatment, and 10% after 7 consecutive years of treatment. After a single year of treatment, 80% of PZP-treated mares showed normal levels of estrone conjugates, suggesting normal ovarian function; these numbers declined gradually after consecutive years of treatment, but some mares with decreased urinary estrone conjugate levels continued to show cyclicity. The high levels of reversibility of contraceptive effects in Report 6 also suggest that in most horses, cessation of ovulation is reversible through at least 5 consecutive years of treatment. All 11 female western wild horses known to have been pregnant in captive trials at the time of treatment with 65–100 µg PZP in mFCA/FIA successfully produced foals, all of which survived to weaning the following autumn (Report 10).

*Immunotoxicity.* A body of available evidence, as well as the longevity and body condition studies cited above, indicates that immune responses to PZP in horses (and other animals tested) occur solely and uniquely in the ovary, and consequently PZP treatment poses no risk of autoimmune reactions. In immunocytochemical studies, antibodies produced by rabbits injected with PZP did not bind or react to any of 14 horse and dog tissue types tested, including brain, heart, lung, kidney, liver, bladder, stomach, small intestine, large intestine, muscle, skin, spleen, pancreas or lymph node tissues (Report 11). Radioimmunoassays had previously shown that antibodies produced by rabbits in response to PZP injections failed to bind or react to any of 22 fluid and tissue types in pigs except for ovarian tissue (Palm et al. 1979). In rabbits fed PZP, no circulating anti-PZP IgG antibodies that crossreacted with PZP were measured (Report 11), providing evidence that no adverse immunological effects occur in nontarget animals that eat the vaccine.

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## **ECOLOGICAL EFFECTS**

### **History of ZonaStat-H Use**

Testing of ZonaStat-H and closely related vaccines began on wild horses in 1988, and has subsequently been tested on white-tailed deer, zoo animals, African elephants, and other animals (e.g., Kirkpatrick et al. 1990; Kirkpatrick and Rutberg 2001; Rutberg 2005; Rutberg and Naugle 2008; Kirkpatrick and Turner 2008; Turner et al. 2007; Delsink et al. 2006; Delsink et al. 2007; Frank et al. 2005; see also Volumes II, IV, and V of this submission for more details regarding wild horses). Adverse side effects in wild horses, white-tailed deer, elephants, and zoo animals are limited to injection site reactions, including granulomas and sterile abscesses (Delsink et al. 2007; Naugle et al. 2002). Incidence of draining abscesses is approximately 1% in wild horses, deer, and hoofstock in zoos (Kirkpatrick 2007; Naugle et al. 2002). Other reported side effects of ZonaStat-H and related vaccines are confined to changes in ovulatory patterns and breeding-related behavior that are directly linked to the vaccine's mechanism of action.

On Assateague Island, wild adult female horses treated with PZP emulsified in FCA or FIA showed better body condition and survived longer than wild adult female horses not treated with PZP. New, older age classes (>21 years) of wild mares on Assateague Island began to appear ten years after the onset of PZP treatments in the herd; mean age of death of mares treated with vaccine for > 3 yrs (19.9 yrs) was significantly greater than untreated mares (6.4 years), mares treated < 3 yrs (10.2 yrs), and stallions (10.3 yrs). Body condition also improved in the 10 years following beginning of PZP treatments for all animals except lactating mares (Turner and Kirkpatrick 2002; Kirkpatrick and Turner 2007). Data suggesting no effect or improvement in body condition also have been



reported for PZP-treated female white-tailed deer (McShea et al. 1997; Walter et al. 2003).

All available evidence indicates that PZP treatments have no effect on ongoing pregnancies in wild adult female horses. In the Assateague wild horse population, all 26 pregnancies diagnosed between month 8 and month 11 among PZP/FCA-FIA treated adult females resulted in successful births. There were no differences in probability of survival to one year between foals born to treated (N=80) and untreated mares (N=246); PZP treatment of pregnant mares did not affect the fertility of their female offspring (Kirkpatrick and Turner 2002). All 11 female western wild horses known to have been pregnant in captive trials at the time of treatment with 65–100 µg PZP in mFCA/FIA successfully produced foals, all of which survived to weaning the following autumn (Lyda et al. 2005). Similar data on safety regarding administration to pregnant females have been reported for African elephants (Delsink et al. 2006).

### **Fate of Product after Injection**

Following injection, both components of the vaccine follow the common immunological and metabolic path of vaccines detected by the humoral immune system (Report 2). Briefly, both the PZP and the bacterial peptidoglycans are hydrolyzed in cells of the immune system. The breakdown products of this hydrolysis bear no resemblance to PZP or bacterial peptidoglycans, and are indistinguishable from other products of lysosomal hydrolysis. After hydrolysis, these metabolic products are excreted and eliminated from the body in forms (such as CO<sub>2</sub>, water, lactic acid, and urea) that are indistinguishable from other metabolic products.

Likewise, the antibodies produced in response to ZonaStat-H injection are broken down into their component amino acids, and recycled into other body proteins or metabolized and excreted as urea, CO<sub>2</sub>, and water. PZP and the adjuvant antigens are not stored in body tissues in a stable or physiologically active form, thereby eliminating the possibility of continued exposure of the target animal to the vaccine components, or of non-target animals of exposure to vaccine components from excreted products.

Further, if non-target animals were to ingest vaccine components, complete digestion by stomach acids and enzymes in the stomach walls, pancreas, and small intestine yields end products comprising amino acids and simple carbohydrates, which elicit no immune response and are bioinactive. Rabbits fed adjuvanted PZP proteins had no anti-PZP antibody titers, nor did control and treatment groups differ in the number or stage of embryos produced (Report 1). Likewise, mice and rabbits fed PZP directly in phosphate buffered saline (PBS), or fed PZP in alginate microspheres with or without a cholera-toxin adjuvant, showed no significant rise in anti-PZP antibody titers, nor was there any difference in litter size in these animals (Report 3). Thus, vaccine components will not be transferred through the food chain. Bollinger (1970a, b) used radioactively-labeled <sup>14</sup>C to trace the fate of mineral oil and mannide monooleate after injection into female rats and squirrel monkeys. He found that 85–98% of the hexadecane component of the mineral oil remained at the injection site after 1 week, declining to 65–75% at one month, 55–65% at 3 months, and 30% at 10 months. The mineral oil tracer was “readily incorporated into lipids” (Bollinger 1970a). At 1 month, radioactivity appeared in triglycerides, sterol

esters, and free sterols in the liver, at 3 months in liver phospholipids, and were absent from the liver by 10 months. Radioactive tracer also appeared as triglycerides in depot fat, a small amount of which still remained after 10 months. Radioactivity was lost slowly over time through metabolism and elimination via respiratory CO<sub>2</sub>, and there was no bioaccumulation of mineral oil or tracer. Mannide monooleate is dissipated more quickly from the injection site than mineral oil, with the oleate component largely being incorporated into lipids, with subsequent elimination through respiratory CO<sub>2</sub>, and the mannide largely eliminated from the body in urine in the form of non-esterified sugars – 25% in the first 24 hr after administration (Bollinger 1970b).

### **Fate of Product in the Environment**

Because of the very small volume of product used for treatment, the high recovery rate of darts, and the near-impossibility of release of the product from unused or undischarged darts (discussed below), it is expected that only miniscule amounts of ZonaStat-H would enter the environment. Second, the product is only effective when injected; neither oral consumption nor casual dermal contact induces antibody production or contraception. Finally, whatever product might enter the environment would quickly lose its effectiveness.

### **Method of Administration and Exposure Risk in Non-Target Species**

Zonastat-H is injected intramuscularly, either by hand-held syringe, by syringe attached to a “jab-stick” (a pole that extends the reach of the injector), or by syringe dart projected from a blow-pipe, CO<sub>2</sub>-canister powered gun, or .22-caliber powered dart rifle. The volume of the injection is 1 mL, which comprises 100 µg of the PZP antigen dissolved in 0.5 mL PBS and 0.5 mL mineral-oil based adjuvant (mFCA or FIA). The small volume used and the highly targeted delivery system preclude any general environmental effect or exposure of non-target terrestrial or aquatic animals.

It should be noted that the amounts of protein antigen used in a typical application of ZonaStat-H are orders of magnitude smaller than the amounts of BtCry proteins used in agricultural applications previously approved by EPA. Sims and Reim (1997), for example, calculate that BtCryIIA proteins would be released at a rate of approximately 486 g/acre of planted transgenic cotton (or 8.1 mg/plant). EPA (2001) assumed a production of 259 g BtK protein per acre of corn, and of 1.44 g Cry1Ac delta protein per acre of cotton (Clark et al. 2005 note with puzzlement the discrepancy between the Sims and Reim and EPA estimates). Using the higher figures, a typical application of ZonaStat-H to 100 adult female horses would use approximately as much total PZP protein as the amount of BtCry protein contained in a single transgenic cotton or corn plant. Thus, the total amount of PZP antigen used in ZonaStat-H applications is environmentally insignificant.

Although one potential pathway of exposure of non-target animals is through incidental contact with the contents of unrecovered, non-discharged syringe darts, there is a nearly zero probability that non-target animals will be exposed to ZonaStat-H via this route. The risk of exposure via this pathway is reduced by the following:

- Applicators administering PZP by dart should search for, and attempt to recover, all fired darts, whether or not they hit their targets. In practice, approximately 95% of all darts fired are recovered (Report 4). This procedure significantly reduces the number of unrecovered darts that remain in the environment.
- The darts (Pneu-dart® 1.0 cc darts with 1.5-inch 14-gauge needles) do not discharge spontaneously or with incidental contact. For the dart to discharge, a small weight at the rear of the body of the dart must be launched forward (through impact with a target) with sufficient velocity to fire a small gunpowder cap, which in turn sets off a larger charge, which pushes the plunger forward and empties the dart. Striking, stepping on, jiggling, biting, or otherwise casually moving or contacting the dart will not discharge or release the contents of the dart.

A second potential pathway of exposure of non-target animals to the product is by oral consumption of the injection site by a predator or scavenger. As discussed above, however, because they are broken down to amino acids and simple carbohydrates following ingestion, neither the PZP antigen nor the bacterial peptidoglycans are physiologically active if eaten, the components are not stored in a stable or physiologically active form in the body, and they are excreted into the environment as CO<sub>2</sub>, water, lactic acid, and urea (Report 2). The mineral oil component is either passed directly through the gut without absorption or broken down, reprocessed into triglycerides and other lipids, and metabolized with other lipids.

Finally, to retain its immunological efficacy, ZonaStat-H requires that the PZP glycoprotein antigen retain its conformation and glycosylation structure. In the environment, where the PZP antigen and other components of ZonaStat-H would be exposed to microbial degradation, sunlight, chemical and pH changes, and fluctuating temperatures, degradation of the antigen and adjuvant would be expected to be rapid. In addition, the PZP antigen alone is a very weak immunogen, and induces little or no immune response unless administered with an adjuvant (Bhatnagar et al. 1989). When prepared as directed, however, the PZP antigen/FCA- FIA adjuvant emulsion breaks down within 48 hours. Consequently, one would expect rapid loss of biological activity of ZonaStat-H in the environment.

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### **List of Studies Submitted for Ecological Effects**

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## **HUMAN EXPOSURE**

## Description and Safety of Vaccine Components

*Composition of ZonaStat-H.* ZonaStat-H is an emulsion consisting of two components: (a) a naturally occurring, chemically unmodified glycoprotein (porcine zona pellucida, PZP) extracted from pig ovaries by simple physical processes and dissolved in a buffered salt solution (phosphate buffered saline) that serves as the antigen; and (b) an adjuvant (modified Freund's Complete Adjuvant, mFCA, or Freund's Incomplete Adjuvant, FIA). mFCA consists of cell wall fragments from a naturally occurring, non-transmissible, non-pathogenic soil bacterium (*Mycobacterium butyricum*) suspended in a physiologically inert mineral oil and an emulsifier; FIA is identical to mFCA, but lacks the mycobacterial cell wall component.

*Porcine zona pellucida antigen.* Porcine Zona Pellucida (PZP) is produced following the methods of Dunbar et al. 1980. (See also Product Chemistry, above). Briefly, porcine ovaries are collected from freshly slaughtered female pigs at USDA-inspected slaughterhouses, and frozen immediately. Oocytes are extracted from the ovaries using a rotary-ganged razor blade device and washed with a buffered salt solution through a series of nylon screens, the last of which (74µm) traps the oocytes but permits dissolved proteins, erythrocytes, and other small debris particles to pass through. The isolated oocytes are then gently homogenized in buffered salt solution, and the zonae pellucidae collected on a 50 µm screen and repeatedly washed. The isolated zonae are then heat-solubilized at 70°C for 30 minutes in phosphate buffer solution (PBS), and diluted to concentrations of approximately 5,000 zonae per 0.5 mL dose. The ZP solution is then frozen until use. Thus, the PZP antigen is extracted directly from a USDA- inspected animal food product, and is dissolved in a standard buffered saltwater solution.

*Adjuvant.* The purpose of a vaccine adjuvant is to boost or modulate the immune response to a given antigen. Adjuvants are especially important if the antigen alone yields a weak immune response (which is the case for PZP). In particular, the PZP antigen induces little or no immune response unless administered with an adjuvant (Bhatnager et al. 1989). Adjuvants also may allow the use of smaller quantities of antigen (if, for example, the antigen is toxic or very expensive), or improve vaccine effectiveness by boosting parts of the immune system that are not strongly stimulated by the antigen. The mechanisms of adjuvant action are still not well understood, but are generally thought to include enhancement of presentation of the antigen to T- helper cells and cytotoxic T cells, improvement of antigen stability, and modulation of the immune response (Cox and Coulter 1997; Spickler & Roth 2003). Adjuvants also may stimulate the activity of the antigen presenting cells (such as dendritic cells and macrophages) that initiate the response of immune effector cells. Adjuvants can be associated with side effects including injection site reactions such as granulomas and sterile abscesses, systemic effects such as fever, lethargy, and loss of appetite, and sometimes autoimmune diseases (Hanly et al. 1997).

ZonaStat-H uses Modified Freund's Complete Adjuvant (mFCA) for primer injections, and Freund's Incomplete Adjuvant (FIA) for booster injections. mFCA consists of 85% Drakeol 5 NF (long-chain, hydrocarbon oil; also referred to as Light mineral oil N.F. -

776510), 15% Arlacel A (mannide monooleate ester), and 0.1% killed and dried *Mycobacterium butyricum* cell walls. FIA is the same composition as mFCA, but lacks the *M. butyricum* cell wall component. The published literature has identified no pathology associated with *M. butyricum*. Drakeol 5 NF is the trade name for a grade of light mineral oil used commonly in animal and human pharmaceuticals (including vaccines and capsules), laxatives, cosmetic additives, and other applications. Arlacel A is a trade name for mannide monooleate, an ester that acts as an emulsifier.

As noted above, FCA comprises mineral oil (a distilled mixture of petroleum-based hydrocarbons), an emulsifier, and dried/killed fragments of cell walls from the bacterial genus *Mycobacterium* (*M. tuberculosis* in classical FCA, *M. butyricum* in modified FCA). FIA contains only the mineral oil and emulsifier components. During the 1950's and early 1960's, FIA was incorporated into widely-used human vaccines for influenza, tetanus, and polio vaccines; over 500,000 people received the influenza vaccine alone in the U.S. and U.K. (Stewart-Tull 1997; Lindblad 2000). Follow-up examinations at 17 years and 35 years post- injection of 18,000 American GI's receiving the FIA-adjuvanted flu vaccine showed no increase in incidence of cancer and other diseases, and showed lower incidences of side effects than the aqueous vaccine (possibly because of the higher doses needed for the aqueous vaccine) (Gupta et al. 1993; Lindblad 2000). Use of oil-adjuvanted vaccines in humans was discontinued in the mid-1960's because of the incidence of injection-site reactions, and a few reports that components of the adjuvant promoted tumor formation in two strains of mice. However, FIA has not been found to be carcinogenic in other mouse strains (Gupta et al. 1993; Lindblad 2000). Thus, there is a documented history of safe use of the adjuvant (or similar adjuvants) contained in ZonaStat-H.

Highly-purified oil adjuvants (Montanide® ISA) are in clinical trials of human vaccines, with more than 4,000 patients having been treated to date (Aucouturier et al. 2006). Because of its efficacy in raising antibody titers, FCA is used extensively in laboratory settings, but because of safety concerns, FCA has not previously been approved by FDA or USDA for use in commercial vaccines. FCA has been associated with abscess formation and inflammation at the injection site, pain, fever, autoimmune diseases, and organ damage (Gupta et al. 1993).

Our own data on the frequency and severity of side effects and the general health of treated horses (summarized below, and described in detail in Volumes IV and V) do not support the negative results observed with Freund's adjuvants in the laboratory. This discrepancy may be due to several reasons. First, both efficacy and the type and magnitude of side effects elicited vary with species, route of administration, and adjuvant. In that context, it should be observed that reports of side effects associated with Freund's adjuvants are derived from studies of laboratory animals, including mice, rats, hamsters, guinea pigs, and rabbits. Cats and dogs also seem to be very sensitive to FCA (e.g., Harrenstien et al. 2004; Wheir et al. 2005).

Second, dosages administered in many studies reporting side effects are extremely high relative to body weight (Stewart-Tull 1997). In one toxicity study of components of FIA in mice, for example, 0.25 ml oil were injected i.p. into juvenile male mice weighing 11g



(Hardigree and Kirschenstein 1968). This dose is approximately 10<sup>4</sup> x the dose by weight administered to horses in our studies.

Finally, the composition of mineral oils has changed significantly over the 40–50 years in which this research has been done (Lindblad 2000). This is important because different fractions of the petroleum-based mineral oil have different health effects. In particular, polycyclic aromatic hydrocarbons can be carcinogenic and mutagenic, and short chain, saturated hydrocarbons are more toxic (possibly because of their solvent effects) than longer chain saturated hydrocarbons (Gupta et al. 1993; Stewart-Tull 1997; Lindblad 2000). In more recent mineral oil preparations, the unsaturated and aromatic hydrocarbons are removed, leaving behind the less reactive longer-chain saturated hydrocarbons (Stewart-Tull 1997; Lindblad 2000). These “white mineral oils” are non-carcinogenic in mice when administered dermally or by inhalation; nonmutagenic by Ames test at doses of 50–1000 µg/ml, and non-fetotoxic and non-teratogenic in rats treated by oral gavage at 5 ml /kg-day (Stewart-Tull 1997).

### **Mechanism of Action of ZonaStat-H**

Like all vaccines, ZonaStat-H exercises its effects by stimulating a classic humoral response, *i.e.*, the B-cell-mediated production of antibodies against the glycoprotein components of PZP (ZP1, ZP2, ZP3, and ZP4). The PZP antibodies interfere with fertilization by binding to these glycoprotein receptors on the non-cellular membrane (zona pellucida) that surrounds the egg of the treated animal, and causing steric hindrance of the zona sperm receptor (Henderson et al. 1988; Hasegawa et al. 1992).

### **History of Safe ZonaStat-H Use**

Testing of ZonaStat-H and closely related vaccines began on wild horses in 1988, and has subsequently been tested on white-tailed deer, zoo animals, African elephants, and other animals (*e.g.*, Kirkpatrick et al. 1990; Kirkpatrick and Rutberg 2001; Rutberg 2005; Rutberg and Naugle 2008; Kirkpatrick and Turner 2008; Turner et al. 2007; Delsink et al. 2006; Delsink et al. 2007; Frank et al. 2005; *see, also*, Volumes II, IV, and V of this submission for more details regarding wild horses). Adverse side effects in wild horses, white-tailed deer, elephants, and zoo animals are limited to injection site reactions, including granulomas and sterile abscesses (Delsink et al. 2007; Naugle et al. 2002). Incidence of draining abscesses is approximately 1% in wild horses, deer, and hoofstock in zoos (Kirkpatrick 2007; Naugle et al. 2002). Other reported side effects of ZonaStat-H and related vaccines are confined to changes in ovulatory patterns and breeding-related behavior that are directly linked to the vaccine’s mechanism of action.

ZonaStat-H has been extensively handled and administered to wild horses, white-tailed deer, and zoo animals by researchers, wildlife biologists, and zoo veterinarians with no reports of harm to applicators. At Assateague Island National Seashore, Maryland, between 1994 and 2007, 901 female wild horses were darted with PZP by two applicators without report of harm to applicators (Kirkpatrick and Turner 2008). Since 2004, ZonaStat-H has been administered by hand-injection or jab-stick to an estimated 1800 western wild horses on 47 herd management areas by researchers and federal Bureau of Land Management personnel trained as described below and in Report 1, also with no

reports of harm to personnel handling the vaccine (GAO 2008). At the National Institute of Standards and Technology, 1630 PZP treatments were administered by hand-injection or dart to 311 female white-tailed deer between 1994 and 2006 by 8 applicators, with no reports of harm to applicators (Rutberg and Naugle 2008). As of 2005, more than 600 captive animals had been treated by zoo veterinarians with ZonaStat-H (by hand injection, jab-stick, or dart); according to current (12/2008) records, 136 zoos have administered ZonaStat-H to captive animals, with no reports of harm or adverse effects to applicators (Frank et al. 2005; K. Frank, Science and Conservation Center, Billings, MT, pers. comm.).

### **Preparation, Administration and Exposure Risk**

During handling of the final product and loading the product into the syringe or syringe dart, the applicator is exposed to a very small risk of dermal, oral, or ocular contact in the event of equipment failure or other mishap. Zonastat-H is injected intramuscularly, either by hand-held syringe, by syringe attached to a “jab-stick” (a pole that extends the reach of the injector), or by syringe dart projected from a blow-pipe, CO<sub>2</sub>-cannister powered gun, or .22-caliber powered dart rifle. The volume of the injection is 1 cc.

The applicator prepares the ZonaStat-H emulsion in the field (see product label). Briefly, the applicator draws 0.5 cc adjuvant (mFCA or FIA) from a glass vial with a 1.5-inch needle attached to a 5.0 cc glass syringe. The PZP antigen, which is stored frozen in 0.5 cc PBS in a plastic vial, is defrosted and drawn out of the vial with the same needle and syringe. The needle is removed, and a second 5.0 cc glass syringe is attached to the first using a Luer lock connector. The applicator then pushes the PZP-adjuvant mixture back and forth between the glass syringes 100 times, until the emulsion is thick and white. The emulsion is then forced into one syringe, and the other syringe is removed from the Luer-Loc. A 2.0 or 3.0 cc plastic syringe is then connected to the Luer-Loc and the ZonaStat-H emulsion is transferred to the plastic syringe. It is then removed from the Luer-Loc, and an 18g., 1.5-inch needle is attached. The ZonaStat H can then be hand injected, inserted into a jabstick, or transferred to a 1 cc Pneu-dart® dart with a 14 g., 1.5-inch needle for remote delivery. The tip of the dart needle is dipped in Vaseline to prevent accidental leakage.

Accidental dermal or ocular exposure to ZonaStat-H and its components may occur during preparation of the product or its loading into the plastic syringe or dart through breakage of syringes, inadvertent dislodging of the Luer-Loc, mistimed pressure on a syringe plunger, or during washing of mixing syringes. Exposure may also occur via needle stick with a syringe needle. The Pneu-dart® dart is not pressurized, and cannot discharge spontaneously or through incidental contact. For the dart to discharge, a small weight at the rear of the body of the dart must be launched forward (through impact with a target) with sufficient velocity to fire a small gunpowder cap, which in turn sets off a larger charge, which pushes the plunger forward and empties the dart.

### **Restricted Use and Training Requirements**

Application of ZonaStat-H is restricted to trained applicators. Applicators will be instructed in specific safety precautions to prevent accidental dermal or ocular exposure

or needle stick. Precautions required of applicators include:

1. “One-hand” insertion of needle into adjuvant vial and replacement of plastic safety cover over needle;
2. Proper disposal of used needles and darts in sharps containers;
3. Proper disposal of syringes in clearly marked “Biohazard” bags;
4. Use of high-quality glass syringes to prevent breakage;
5. Wearing of latex or vinyl examination gloves during all operations in which accidental dermal exposure could occur, including washing of mixing syringes;
6. Washing site of needles stick or cut with soapy water and disinfection of wound with alcohol or other disinfectant or antiseptic.

### **Post-application exposure**

Although one potential pathway of human exposure is through incidental contact with the contents of unrecovered, non-discharged syringe darts, there is a nearly zero probability that humans will be exposed to ZonaStat-H via this route. The minimal risk of exposure via this pathway is further reduced by the following:

- Applicators administering PZP by dart should search for, and attempt to recover, all fired darts, whether or not they hit their targets. In practice, approximately 95% of all darts fired are recovered (Report 1). This procedure significantly reduces the number of unrecovered darts that remain in the environment.
- The darts (Pneu-dart® 1.0 cc darts with 1.5-inch 14-gauge needles) do not discharge spontaneously or with incidental contact. For the dart to discharge, a small weight at the rear of the body of the dart must be launched forward (through impact with a target) with sufficient velocity to fire a small gunpowder cap, which in turn sets off a larger charge, which pushes the plunger forward and empties the dart. Striking, stepping on, jiggling, biting, or otherwise casually moving or contacting the dart will not discharge or release the contents of the dart.
- Because of the very small volume of product used for treatment, the high recovery rate of darts, and the near-impossibility of release of the product from unused or undischarged darts (discussed below), it is expected that only miniscule amounts of ZonaStat-H would enter the environment. Moreover, whatever product might enter the environment would quickly lose its effectiveness.

To retain its immunological efficacy, ZonaStat-H requires that the PZP glycoprotein antigen retain its conformation and glycosylation structure. In the environment, where the PZP antigen and other components of ZonaStat-H would be exposed to microbial degradation, sunlight, chemical and pH changes, and fluctuating temperatures, degradation of the antigen and adjuvant would be expected to be rapid. In addition, the PZP antigen alone is a very weak immunogen, and induces little or no immune response unless administered with an adjuvant (Bhatnagar et al. 1989). When prepared as directed, however, the PZP antigen/FCA-FIA adjuvant emulsion breaks down within 48 hours. Consequently, one would expect rapid loss of biological activity of ZonaStat-H in the environment.

Further, following injection into the target animal, both components of the vaccine follow

the common immunological and metabolic path of vaccines detected by the humoral immune system (see Report 2 in Volume IV). Briefly, both the PZP and the bacterial peptidoglycans are hydrolyzed in cells of the immune system. The breakdown products of this hydrolysis bear no resemblance to PZP or bacterial peptidoglycans, and are indistinguishable from other products of lysosomal hydrolysis. After hydrolysis, these metabolic products are excreted and eliminated from the body in forms (such as CO<sub>2</sub>, water, lactic acid, and urea) that are indistinguishable from other metabolic products. Likewise, the antibodies produced in response to ZonaStat-H injection are broken down into their component amino acids, and recycled into other body proteins or metabolized and excreted as urea, CO<sub>2</sub>, and water. PZP and the adjuvant antigens are not stored in body tissues in a stable or physiologically active form, thereby eliminating the possibility of exposure of humans to vaccine components from excreted products. Further, if non-target animals or humans were to ingest vaccine components, complete digestion by stomach acids and enzymes in the stomach walls, pancreas, and small intestine yielding end products comprising amino acids and simple carbohydrates, which elicit no immune response and are bioinactive. Thus, vaccine components will not be transferred through the food chain to humans.

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### **List of Studies Submitted for Human Exposure**

1 Kirkpatrick, J.F. 2008. Recovery of darts containing porcine zona pellucida vaccine delivered to wild horses at three field sites. Unpublished report dated February 20, 2008.

2 Training manual. The application of porcine zona pellucida contraceptive vaccine to wild horses. Science and Conservation Center, Billings, MT.

## **ENVIRONMENTAL FATE**

### **Description of Vaccine Components**

*Composition of ZonaStat-H.* ZonaStat-H is an emulsion consisting of two components: (a) a naturally occurring, chemically unmodified glycoprotein (porcine zona pellucida, PZP) extracted from pig ovaries by simple physical processes and dissolved in a buffered salt solution (phosphate buffered saline) that serves as the antigen; and (b) an adjuvant (modified Freund's Complete Adjuvant, mFCA, or Freund's Incomplete Adjuvant, FIA). mFCA consists of cell wall fragments from a naturally occurring, non-transmissible, non-pathogenic soil bacterium (*Mycobacterium butyricum*) suspended in a physiologically inert mineral oil and an emulsifier; FIA is identical to mFCA, but lacks the mycobacterial cell wall component.

*Porcine zona pellucida antigen.* Porcine Zona Pellucida (PZP) is produced following the methods of Dunbar et al. (1980). (See also Volume III, Product Chemistry). Briefly, porcine ovaries are collected from freshly slaughtered female pigs at USDA-inspected

slaughterhouses, and frozen immediately. Oocytes are extracted from the ovaries using a rotary-ganged razor blade device and washed with a buffered salt solution through a series of nylon screens, the last of which (74 µm) traps the oocytes but permits dissolved proteins, erythrocytes, and other small debris particles to pass through. The isolated oocytes are then gently homogenized in buffered salt solution, and the zonae pellucidae collected on a 50 µm screen and repeatedly washed. The isolated zonae are then heat-solubilized at 70°C for 30 minutes in phosphate buffer solution (PBS), and diluted to concentrations of approximately 5,000 zonae per 0.5 mL dose. The ZP solution is then frozen until use. Thus, the PZP antigen is extracted directly from a USDA- inspected animal food product, and is dissolved in a standard buffered salt water solution.

*Adjuvant.* The purpose of a vaccine adjuvant is to boost or modulate the immune response to a given antigen (Hanley et al. 1997; Spickler and Roth. 2003). ZonaStat-H uses Modified Freund's Complete Adjuvant (mFCA) for primer injections, and Freund's Incomplete Adjuvant (FIA) for booster injections. mFCA consists of 85% Drakeol 5 NF, 15% Arlacel A, and 0.1% killed and dried *Mycobacterium butyricum* cell walls. FIA is the same composition as mFCA, but lacks the *M. butyricum* cell wall component. The published literature has identified no pathology associated with *M. butyricum*. Drakeol 5 NF is the trade name for a grade of light mineral oil used commonly in animal and human pharmaceuticals (including vaccines and capsules), laxatives, cosmetic additives, and other applications. Arlacel A is a trade name for mannide monooleate, an ester that acts as an emulsifier.

### **Mechanism of Action of ZonaStat-H**

Like all vaccines, ZonaStat-H exercises its effects by stimulating a classic humoral response, *i.e.*, the B-cell-mediated production of antibodies against the glycoprotein components of PZP (ZP1, ZP2, ZP3, and ZP4). The PZP antibodies interfere with fertilization by binding to these glycoprotein receptors on the non-cellular membrane (zona pellucida) that surrounds the egg of the treated animal, and causing steric hindrance of the zona sperm receptor (Henderson et al. 1988; Hasegawa et al. 1992).

### **Fate of Product after Injection**

Following injection, both components of the vaccine follow the common immunological and metabolic path of vaccines detected by the humoral immune system (Report 1). Briefly, both the PZP and the bacterial peptidoglycans are hydrolyzed in cells of the immune system. The breakdown products of this hydrolysis bear no resemblance to PZP or bacterial peptidoglycans, and are indistinguishable from other products of lysosomal hydrolysis. After hydrolysis, these metabolic products are excreted and eliminated from the body in forms (such as CO<sub>2</sub>, water, lactic acid, and urea) that are indistinguishable from other metabolic products. Likewise, the antibodies produced in response to ZonaStat-H injection are broken down into their component amino acids, and recycled into other body proteins or metabolized and excreted as urea, CO<sub>2</sub>, and water. PZP and the adjuvant antigens are not stored in body tissues in a stable or physiologically active form, thereby eliminating the possibility of continued exposure of the target animal to the vaccine components, or of non-target animals of exposure to vaccine components from excreted products.

Further, if non-target animals were to ingest vaccine components, complete digestion by stomach acids and enzymes in the stomach walls, pancreas, and small intestine yields end products comprising amino acids and simple carbohydrates, which elicit no immune response and are bioinactive. Rabbits fed adjuvanted PZP proteins had no anti-PZP antibody titers, nor did control and treatment groups differ in the number or stage of embryos produced (Barber and Fayrer-Hosken 2000). Likewise, mice and rabbits fed PZP directly in phosphate buffered saline (PBS), or fed PZP in alginate microspheres with or without a cholera-toxin adjuvant, showed no significant rise in anti-PZP antibody titers, nor was there any difference in litter size in these animals (Martin et al. 2006). Thus, vaccine components will not be transferred through the food chain.

Bollinger (1970a, b) used radioactively-labeled  $^{14}\text{C}$  to trace the fate of mineral oil and mannide monooleate after injection into female rats and squirrel monkeys. He found that 85–98% of the hexadecane component of the mineral oil remained at the injection site after 1 week, declining to 65–75% at one month, 55–65% at 3 months, and 30% at 10 months. The mineral oil tracer was “readily incorporated into lipids” (Bollinger 1970a). At 1 month, radioactivity appeared in triglycerides, sterol esters, and free sterols in the liver, at 3 months in liver phospholipids, and were absent from the liver by 10 months. Radioactive tracer also appeared as triglycerides in depot fat, a small amount of which still remained after 10 months. Radioactivity was lost slowly over time through metabolism and elimination via respiratory  $\text{CO}_2$ , and there was no bioaccumulation of mineral oil or tracer. Mannide monooleate is dissipated more quickly from the injection site than mineral oil, with the oleate component largely being incorporated into lipids, with subsequent elimination through respiratory  $\text{CO}_2$ , and the mannide largely eliminated from the body in urine in the form of non-esterified sugars – 25% in the first 24 hr after administration (Bollinger 1970b).

### **Method of Administration and Exposure Risk in Non-Target Species**

Zonastat-H is injected intramuscularly, either by hand-held syringe, by syringe attached to a “jab-stick” (a pole that extends the reach of the injector), or by syringe dart projected from a blow-pipe,  $\text{CO}_2$ -cannister powered gun, or .22-caliber powered dart rifle. The volume of the injection is 1 mL, which comprises 100 $\mu\text{g}$  of the PZP antigen dissolved in 0.5 mL PBS, and 0.5 mL mineral-oil based adjuvant (mFCA or FIA). The small volume used and the highly targeted delivery system preclude any general environmental effect or exposure of non-target terrestrial or aquatic animals.

It should be noted that the amounts of protein antigen used in a typical application of ZonaStat-H are orders of magnitude smaller than the amounts of BtCry proteins used in agricultural applications previously approved by EPA. Sims and Reim (1997), for example, calculate that BtCryIIA proteins would be released at a rate of approximately 486 g/acre of planted transgenic cotton (or 8.1 mg/plant). EPA (2001) assumed a production of 259 g BtK protein per acre of corn, and of 1.44 g Cry1Ac delta protein per acre of cotton (Clark et al. 2005 note with puzzlement the discrepancy between the Sims and Reim and EPA estimates). Using the higher figures, a typical application of ZonaStat-H to 100 adult female horses would use approximately as much total PZP protein as the amount of BtCry protein contained in a single transgenic cotton or corn



plant. Thus, the total amount of PZP antigen used in ZonaStat-H applications is environmentally insignificant.

Although one potential pathway for entry into the environment is by leakage of the contents of unrecovered, non-discharged syringe darts, the extremely small quantities involved limit environmental release. The risk of such release via this pathway is further reduced by the following:

- The applicators administering PZP by dart should search for, and attempt to recover, all fired darts, whether or not they hit their targets. In practice, approximately 95% of all darts fired are recovered (Report 2). This procedure significantly reduces the number of unrecovered darts that remain in the environment.
- The darts (Pneu-dart® 1.0 cc darts with 1.5-inch 14-gauge needles) do not discharge spontaneously or with incidental contact. For the dart to discharge, a small weight at the rear of the body of the dart must be launched forward (through impact with a target) with sufficient velocity to fire a small gunpowder cap, which in turn sets off a larger charge, which pushes the plunger forward and empties the dart. Striking, stepping on, jiggling, biting, or otherwise casually moving or contacting the dart will not discharge or release the contents of the dart.

A second potential release pathway for the product is by oral consumption of the injection site by a predator or scavenger. As discussed above, however, because they are broken down to amino acids and simple carbohydrates following ingestion, neither the PZP antigen nor the bacterial peptidoglycans are physiologically active if eaten, the components are not stored in a stable or physiologically active form in the body, and they are excreted into the environment as CO<sub>2</sub>, water, lactic acid, and urea (Report 1). The mineral oil component is either passed directly through the gut without absorption or broken down, or reprocessed into triglycerides and other lipids and metabolized with other lipids.

### **Fate of Product in the Environment**

Because of the very small volume of product used for treatment, the high recovery rate of darts, and the near-impossibility of release of the product from unused or undischarged darts (discussed below), it is expected that only miniscule amounts of ZonaStat-H would enter the environment. Moreover, whatever product might enter the environment would quickly lose its effectiveness.

To retain its immunological efficacy, ZonaStat-H requires that the PZP glycoprotein antigen retain its conformation and glycosylation structure. In the environment, where the PZP antigen and other components of ZonaStat-H would be exposed to microbial degradation, sunlight, chemical and pH changes, and fluctuating temperatures, degradation of the antigen and adjuvant would be expected to be rapid. In addition, the PZP antigen alone is a very weak immunogen, and induces little or no immune response unless administered with an adjuvant (Bhatnagar et al. 1989). When prepared as directed, however, the PZP antigen/FCA-FIA adjuvant emulsion breaks down within 48 hours.

Consequently, one would expect rapid loss of biological activity of ZonaStat-H in the environment.

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### **List of Studies submitted for Environmental Fate**

#### Report Number

1 Takashima, A., and R.M. Wooten 2008. Unpublished letter dated July 30, 2008.

2 Kirkpatrick, J.F. 2008. Recovery of darts containing porcine zona pellucida vaccine delivered to wild horses at three field sites. Unpublished report, dated February 20, 2008.