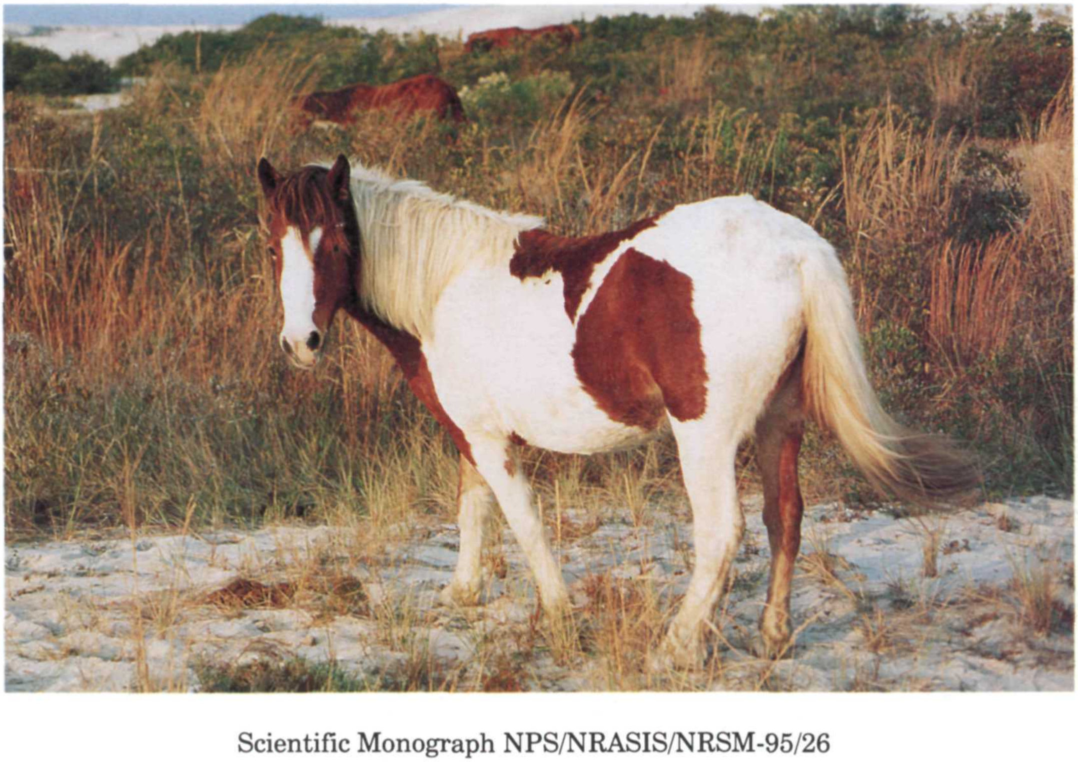
Management of Wild Horses by Fertility Control: The Assateague Experience



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Management of Wild Horses by Fertility Control: The Assateague Experience

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Abstract

Research was initiated in 1986 with the goal of managing the Assateague wild horses using fertility control. Initial experiments with steroid hormones designed to reduce sperm counts in stallions and to prevent ovulation in mares did not show promise. Immunocontraception of 26 mares in 1988 with two or three inoculations of a porcine zonae pellucidae (PZP) vaccine was 100% effective. The glycoprotein-based PZP vaccine produces antibodies that block fertilization of the ovum. The effects of the vaccine did not interfere with pregnancies in progress, could easily be delivered remotely, and did not interfere with social organization. A single annual booster inoculation was adequate to continue contraception. After 120 mare-years of PZP contraception, only four foals have been born. Reversal of contraceptive action was documented in mares after 1, 2, 3, and 4 years of consecutive treatment.

Methods were developed for the detection of pregnancy in uncaptured horses using urinary and fecal estrogen metabolites (E_1C), progesterone metabolites (iPdG), and fecal total estrogens. These same methods were applied to treated and control mares during the reproductive seasons of 1990-94 in order to assess the effects of 7 consecutive years of PZP contraception upon ovarian function. Urinary E_1C concentrations were used to assess follicular development, and urinary iPdG concentrations were used to detect ovulation. Seven consecutive years of contraception resulted in failure to ovulate and in depressed estrogen concentrations; no other health side effects were noted.

Key words: Contraception, estrogen metabolites, fecal hormones, porcine zona pellucida (PZP), progesterone metabolites, urinary hormones

Preface

The management of wildlife within the boundries of National Park Service (NPS) units varies with the classification of the unit and the particular species of wildlife involved. The enabling legislation of Assateague Island National Seashore provides for "hands-on" management of some species, such as the annual hunts for white-tailed deer (*Odocoileus virginianus*) up until 1986 and currently for the non-native sika deer (*Cervus nippon*). It also established another non-native species, the wild horse, as a "desireable exotic species." While the classification of wild horses in North America as "exotic" is specious on scientific grounds, the NPS recognized the cultural and historic value of this species on Assateague. The horse, however, presents unique problems from a management viewpoint. The horse cannot be hunted, and the potential for damage to the island's fragile ecology was recognized. The only other known control method for this species is removal and sale of animals, a practice that has met with public opposition on humane grounds. Additionally, the wild horses of Assateague are highly valued by the public, and management options were further restricted following public scrutiny. Fertility control as a management option for the horses of Assateague seemed a feasible alternative.

This study of a new and unique management option for Assateague's wild horses resulted from a desire on the part of the NPS to find methods of population control acceptable for this popular herd of animals that elicited minimal objection from the public. In 1985, when the NPS first considered the idea, there were limited data to suggest that fertility control would be successful. Nevertheless, the NPS was willing to embark upon research that held the potential for controversy and failure, as well as a unique solution not yet successful with wild ungulate populations.

The planned two-year project turned into ten years of research that ultimately brought together a myriad of scientists, representing the fields of reproductive physiology, endocrinology, immunology, behavior, vaccine development, and population biology. Although the research team had had 13 previous years of research in this field with other government agencies, the Assateague wild horse contraceptive study became a model for cooperation between the public, the NPS and the research team. The outcome was a practical, relatively inexpensive, and publicly-acceptable humane management tool.

Even more exciting was the development of a variety of non-capture, hands-off methods for studying reproduction in free-roaming wildlife, necessitated by the act of carrying out research with large popular animals within an NPS unit. These methods which were first applied to the horses of Assateague, based on the analysis of urinary and fecal steroid hormones, have since become important tools for the study of reproduction in free-roaming wildlife around the world. If the Assateague experience has one overriding theme, it is that high-risk research can be carried out in our national parks, if the potential benefits outweigh the risks, and the scientists, the NPS and the public are all willing to place the welfare of the resource ahead of personal or political considerations.

Management of Wild Horses by Means of Fertility Control: The Assateague Experience

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Background

Horses (*Equus caballus*) first inhabited Assateague Island, a barrier island off the coasts of Maryland and Virginia, about 1669 when English settlers on the mainland turned animals loose for grazing in an attempt to avoid the king's tax on fencing (Bearss 1968). An unsubstantiated addition of horses to the herd allegedly occurred in 1820, when the Spanish ship *San Lorenzo* ran aground on shoals off the island's coast. The ship was carrying 95 small horses of undetermined breed and popular legend has it that an undetermined number of these horses survived the accident and swam ashore, adding to the genetic diversity of the Assateague horses. The factual nature of this event is only speculative and National Park Service (NPS) historians discount the addition of horses from this source. During the 1970s, about 40 wild horses from the western United States were added to one of two herds inhabiting the island, in a misguided attempt to improve the genetic makeup of these horses. Only two stallions and a few mares survived the harsh conditions of the island after just a few years but there is some phenotypic evidence of this blood-line today in the Chincoteague National Wildlife Refuge (CNWR) herd and the south of the Assateague Island National Seashore. There is also some evidence for the introduction of Shetland ponies into the Assateague Island population in the early part of this century and some believe that is the origin of the piebald or pinto coloration of many of the horses (Keiper 1985).

Today, Assateague Island is made up of three distinct political units (see Fig. 1). That portion of the island in Virginia, which approximates the southern one-third of the island, is largely Chincoteague National Wildlife Refuge (CNWR) which is under the management jurisdiction of the U. S. Fish and Wildlife Service. The northern two-thirds of the island, in Maryland, constitute Assateague Island National Seashore (ASIS) which is under the management jurisdiction of the NPS. An approximate two-mile length in the middle of the Maryland portion of the island is Assateague State Park which is under the jurisdiction of the State of Maryland.

For most of the island's recorded history, the horses of Assateague were privately owned. The earliest capture and penning of the Assateague horses may have occurred in the late 1700's, for purposes of branding animals. In 1920, the round-up and selective removal of Assateague horses was formalized by citizens of Chincoteague, a small island community in Virginia, near the southern end of Assateague Island. With the creation of ASIS in 1965, those horses owned by those property holders north of the Maryland state line were permitted to remain within ASIS

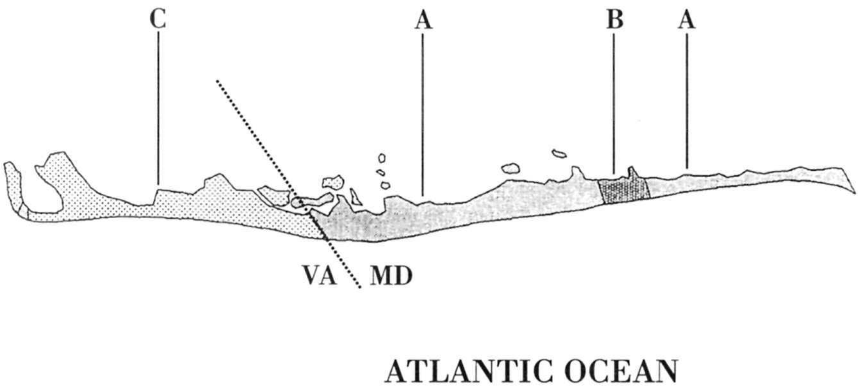


Figure 1: Map of Assateague Island. Management units include (A) Assateague Island National Seashore; (B) Assateague State Park; and (C) Chincoteague National Wildlife Refuge.

and management responsibility was given over to the NPS. The horses are considered a non-native species on the island, but the horses are considered by the public to be a cultural and historic part of Assateague; consequently, the NPS has accepted the animals' presence, labelling them a desireable feral species. The titles to the horses living on the Virginia portion of the island belong to the town of Chincoteague and the animals are permitted to graze on fenced portions of the CNWR by permit. A fence at the Maryland-Virginia border separates the two herds but some mixing of the Maryland and Virginia populations still occurs on a regular basis. Despite the fence, the genetic background, forage, climate, and general living conditions for the two herds are similar. The Virginia herd of approximately 150 animals, hereafter known as the Chincoteague horses, is managed by means of an annual public auction of foals. The herd is gathered in late July and forced to swim across the small bay separating Assateague Island from Chincoteague Island, where the foals are sold. The horses inhabiting the Maryland portion of the island will hereafter be referred to as ASIS or Assateague horses. These horses are managed as wild and free-roaming and designated as a desireable exotic species.

When ASIS was established in 1965, there were nine stallions and twelve mares on the Maryland portion of the island. By 1975 and 1984 the herd had increased to 60 and 107 animals, respectively, and in December 1994 there were 167 animals. The horses of the ASIS herd live in either harem or bachelor bands. The harem bands contain anywhere from two to twenty horses, usually including a single sexually mature stallion, mature (≥3 years old) mares, and juveniles (<3 years old) (Keiper 1976). These bands are not territorial but have distinctive home ranges which often overlap with other bands and which change seasonally as a function of insect densities. Bachelor bands are made up of stallions, perhaps two to five in number, who have not acquired their own mares. The stability of bachelor bands is less than that of harem bands, and the bachelor stallions are thought to form these loose social groups for little more than companionship with others of their kind.

The growth of the ASIS herd has led to two major management concerns. The first and most important was the impact on the island's ecology. In 1976, studies were initiated to quantify the impact of the horses upon island vegetation. Total plant utilization ranged from 0.75% to 30.5% of annual biomass production within various home ranges of horse bands. Using this data along with some broad and untenable assumptions, such as the absence of competition from other herbivores, which include white-tailed deer, sika deer, rabbits, and mice and the equal distribution of horses across the entire island, the carrying capacity was determined to be 2,440 horses. To account for questionable assumptions, 10% of the carrying capacity, or 244 animals was used as a guideline (Keiper and Zervanos 1979). This figure was further adjusted to account for various management problems and resulted in a recommended population size of 120-150 horses. This study was based on the utilization of three primary forage plants for the horses. These plants included saltmarsh cordgrass (*Spartina alterniflora*), American beachgrass (*Ammophila breviligulata*), and three-square sedge (*Scirpus americana*). In 1985, with the population at 107 horses, the first management plan for the horses indicated that, with the exception of a single band on the northern-most portion of the island, there was little evidence of measureable adverse effects on the island's vegetation (Rodgers, 1985). Another study (Stribling, 1989, 1990) suggested that grazing reduced primary productivity of all three grasses and interfered with nutrient storage and transfer. Furbish (1990, 1994) suggested that preferential grazing on saltmarsh cordgrass might be altering the species composition of the low saltmarsh community and reducing the three keystone species of the marshes, saltmarsh cordgrass, saltmarsh hay (*Spartina patens*), and spikegrass (*Distichlis spicata*).

The second management problem that emerged with the increasing numbers of horses was human-horse interactions. The popularity of the ASIS horses with the visitors was instrumental in drawing horses and people together, and as the herd size increased so did contact between humans and horses. In 1975, there were only three stallions with home ranges that overlapped with state or ASIS campgrounds, but by 1983, 40 horses lived near the campgrounds. The number of horses inhabiting land adjacent to the campgrounds cannot be accounted for by random distribution. The largest single cause for this concentration is believed to be the state park's practice of spraying for insects during the summer months. Horses have learned to migrate from as far away as 10-12 miles in order to escape the island's rather large insect load. The human-horse interactions have led to injuries to humans and injuries and death to the horses from car-horse collisions. Despite clear and well-displayed warnings forbidding the touching and feeding of the horses, the public more or less continues to violate park regulations and exacerbate the problem. Thus, while human-horse interactions constitute a management problem, they are more of a visitor problem than a horse problem.

Until 1986, management of the horses on ASIS was limited to the translocation of "problem" animals to the Chincoteague herd. In the absence of other management options, the translocation of horses to Chincoteague appeared reasonable. However, the management of the Chincoteague horses has come under intense public scrutiny and criticism. First, the wildlife refuge already has all the horses it can accomodate. Additionally, the public auction of horses on Chincoteague is perceived by segments of the public sector, and particularly by animal protection organizations as unacceptable on humane grounds. The Chincoteague horses are no longer free-roaming and live in fenced pastures. Each July the mares and foals of the year are gathered, forced to swim Chincoteague Bay, and the foals are sold in a public auction. A significant number of foals are taken from the mares too early; one animal protection group provides starter kits (nursing bottles and formula) for the young animals. There is no follow-up on the fate of the sold foals. The documented abuses of western wild horses sold through the Bureau of Land Management's (BLM) Adopt-A-Horse program lend credibility to concern over the care of the Chincoteague horses once they become private property.

The management of the Chincoteague horses is also unsound on biological grounds. First, compensatory reproduction is triggered by the removal of foals and this action has almost doubled the foal production (Keiper and Houpt, 1984; Kirkpatrick and Turner, 1991a). Thus, the translocation of publicly-owned animals into a privately-owned herd, whose management can be questioned on both humane and biological grounds, would imply approval of Chincoteague's management practices by the National Park Service. Finally, early translocations of horses from ASIS to CNWR were carried out with little concern about the genetic composition of the herd and some genetic lines have been removed from the ASIS herd over the years.

The Decision to Test Fertility Control

The decision to test fertility control as a means of controlling the ASIS horse population was based on a lack of publicly-acceptable management options. Fertility control as a means of manipulating wildlife populations was largely an untested concept prior to the 1980s. There was a 20-year history of limited experiments with various contraceptive agents and wildlife (Kirkpatrick and Turner, 1985, 1991b) but there were few data which suggested practical application to actual wildlife populations. Between 1977 and 1982, a limited set of experiments was conducted with steroid fertility control and western wild horses. The initial experiments focused on inhibiting sperm production in stallions by means of testosterone injections. Repeated injections of testosterone propionate (TP) in domestic pony stallions led to significant decreases in sperm counts and motility (Kirkpatrick 1982). In a field test in Challis, ID, in 1979, the stallions of ten bands, each of 5-17 horses, were first immobilized with approximately 300 mg succinylcholine chloride from a helicopter and then injected with 2.5 to 10 gm of a long-acting form of testosterone propionate (mTP). The hormone was microencapsulated in a biodegradable, non-toxic lactide coating. Upon intramuscular injection, this coating eroded and released the testosterone over a 4-6 month period but did not affect libido. Stallions of another ten bands were immobilized and injected with only carrier and empty microcapsules.

Due to the removal of some bands by the Bureau of Land Management (BLM), mortality, and inaccessibility of some sections of the range, accurate data were obtained from only seven of the 10 original treated bands and from eight of the control bands. Foal counts for control bands averaged 0.37 foals/mare, whereas for treated bands the average was 0.07 foals/mare. Seven of eight control bands had at least one foal, whereas only two of the seven treated bands had one or more foals. These data translated into an 83% reduction in foals among mares bred by treated stallions as compared to mares bred by untreated stallions (Kirkpatrick et al., 1982; Turner and Kirkpatrick, 1982). These data were derived from the only known contraceptive test with wild horses and led to the decision to try fertility control with the ASIS horses.

Objectives

The primary goal of the Assateague wild horse contraceptive project was to find a method for inhibiting reproduction that could be used to limit population growth of the herd on ASIS. The original objectives of this study included the following:

1. Identify a reversible contraceptive agent that would be effective, could be delivered remotely, and would not cause health problems for the treated animals.
2. Test the field delivery of a steroid hormone contraceptive agent which would lower sperm counts in stallions to the point where infertility would result.
3. Test a steroid hormone contraceptive agent, and its delivery in the field, which would prevent ovulation in mares.

As the project evolved, the additional following objectives emerged:

4. Determine if a vaccine-based contraceptive agent could be used to effectively, but reversibly, reduce fertility among mares.
5. Determine if the short-term use of this contraceptive vaccine would have any effects upon the health of pregnant mares or the social behaviors of the wild horse herds.
6. Determine if long-term use of the contraceptive vaccine would lead to irreversible infertility or serious health side-effects.
7. Determine if the use of the contraceptive vaccine could elicit a population reduction effect.

In order to achieve the contraceptive goals of this study, several ancillary objectives were developed mid-way through the project. These ancillary objectives involved non-capture methods of diagnosing pregnancy and studying ovarian function among wild horses. These objectives included:

8. Diagnose pregnancy through the use of urinary and/or fecal hormone metabolites, and
9. Detect ovulation and normal ovarian function by means of urinary hormone metabolites.

During the course of the project we tested the following hypotheses:

1. Ho^1: A slow-release form of testosterone propionate can be delivered to stallions under field condition and in sufficient quantities to induce temporary infertility.
2. Ho^2: A slow-release form of a synthetic progesterone (NET) can be delivered to mares under field conditions and in sufficient quantities to induce temporary infertility.
3. Ho^3: Two inoculations of a protein-based contraceptive vaccine can be delivered to previously untreated mares under field conditions over a four week period and in sufficient quantities to induce temporary (one year) infertility.
4. Ho^4: A single annual booster inoculation of a protein-based contraceptive vaccine will sustain infertility for an additional year.
5. Ho^5: A slow release form of the contraceptive vaccine can be delivered in a single dose to previously untreated mares which would cause effective temporary (one year) contraception.
6. Ho^6: Urine and fecal samples can be used to diagnose pregnancy in treated and untreated mares by the measurement of certain estrogen-related hormones or their metabolites.

   Ho^7: The measurement of urinary hormones can be used to assess the long-term effects of contraceptive vaccines on ovarian function in treated mares.

   Ho^8: A population reduction effect can be demonstrated among a subpopulation of the Assateague horses after application of the contraceptive vaccine.

Steroid Contraceptive Experiments

Stallion Contraceptive Experiments

At the start of the steroid contraceptive trials, in 1986, there were 14 identifiable harem bands on ASIS and four of these, M, OS, OR, and K, were included in stallion contraceptive studies. These four bands lived on the northern half of ASIS, from approximately Jim's Gut to the Ocean City Inlet. During initial discussions with ASIS personnel, it was decided that the Challis stallion contraceptive experiments should be repeated on ASIS but that delivery would be from the ground and without immobilization. The NPS also requested a protocol that could be applied to mares as well as stallions, an approach that had not yet been tested with captive animals. Two important considerations in mounting a contraceptive project with the ASIS horses were the constraints imposed on conducting research of this nature on a national seashore, and the perception of this research by the public, which places a great aesthetic and recreational value on the wild horses of the island. It would not be wise, or perhaps even possible, to immobilize animals in sufficiently large numbers to conduct the research because of the risk of injury or mortality to these popular animals. It would also be almost impossible to conduct this research out of the view of the public; thus, for the welfare of the animals as well as public perception, methods would have to be as humane as possible. Finally, there existed the possibility that the public might perceive the ASIS research effort as an attempt to eliminate the horses from the national seashore rather than just control the population.

Between 4 February and 12 March 1986, four band stallions (M, OS, OR, and K) on ASIS were treated with a d,l-lactide microencapsulated form of testosterone propionate (mTP). The four stallions collectively had 14 mares of proven fertility in their bands. Each stallion was to receive 3.47 g of mTP. The mTP was designed to release over six months and to cause oligospermia and decreased sperm motility. The dose of 3.47 g was selected because of the remote delivery and limitations on volume (1 g of lactide microcapsules = 0.579 g testosterone propionate), and the proven effectiveness of this dose in the Challis field studies (Kirkpatrick et al. 1982; Turner and Kirkpatrick, 1982).

In order to deliver 3.47 g of mTP, a total of 6 g of the microspheres had to be administered. The mTP was delivered in 6.0 cc PaxArms darts at ranges between 25-35 m. Four separate darts were required, each with 1.5 g of microcapsules suspended in methyl cellulose carrier. During June 1986, at the peak of the breeding season, the stallions were observed and the mares accompanying the treated stallions were recorded. Identity of stallions and mares was based on unique color patterns of pinto animals, unique facial and leg markings of bays and sorrels, and scars and hoof pigmentation of unmarked animals. These characteristics were recorded on left and right side and full face drawings for each horse. During August 1987, the mares were located and observed for the presence of foals.

Three technical problems with the actual administration of the drug were encountered. First, administration occurred during several very cold days (-2 to -4^{o} C) and the viscosity of the carrier, carboxymethyl cellulose, increased and sometimes interfered with fast injection. This necessitated keeping the carrier as warm as possible prior to delivery. A second problem involved the suspended microcapsules. Because the microcapsules are not dissolved, but merely suspended, they had a tendency to settle out and clump in the dart if not delivered within 10 minutes of initial mixing. Thus, when stallions wandered off before darting could occur, the dart would have to be removed from the gun and the microcapsules resuspended. Often, some of the more wary stallions presented only occasional opportunities to dart them, and the need to resuspend microcapsules often prevented timely treatment of the stallions. Thirdly, use of barbless darts required considerable skill with the gun. Almost all tranquilizer guns and darts are designed for delivering immobilizing drugs. Consequently, a dart can be fired with substantial velocity and little trajectory, and because of the barb it will remain in the animal after impact and can be removed after the animal is recovered. The delivery of non-immobilizing drugs necessitates barbless darts, which will ultimately fall out. Thus, velocity must be decreased significantly and trajectories increased in order to "lob" darts into animals and have them remain until injection is complete. Similar problems were encountered by other investigators, even with barbed darts (Seal et al. 1985).

In addition to these technical problems it proved to be very difficult to administer the requisite four darts to certain of the stallions. One stallion, K, who was acclimated to people, presented a relatively easy target provided that several days passed between dartings. Another stallion, OR, was moderately wary and caused some difficulty. Two other stallions, M and OS, were extremely wary and required many days to complete the four injections. Darts which bounced out before the completion of injection caused some loss of drug, and most doses fell short of the desired 3.47 g of active drug. Estimated actual delivered doses ranged from 1.1 to 3.4 g (Table 1).

In 1987, the 14 mares of proven fertility associated with the four treated stallions produced four foals, for a fertility rate of 28.9% (Table 2). The 28.9% foaling rate was significantly smaller ($p < 0.05$) than the 54.4% foaling rate for an untreated control population of 15 mares for the 1987 foaling season, and for the foaling rate for the treated mares for the previous five years, which ranged from 42% to 52%. One of the four foals born to mares with treated stallions was born about 15 March, which meant that it was conceived only about 14 days after the completion of treatment to stallion K. Thus, this early breeding occurred before the phramacological effects of mTP could take place.

TABLE 1. Estimated microcapsule weight and active drug delivered to stallions and aares in 1986.

Horse	Microcapsule wt. delivered (g)	Active drug delivered (g)
Stallions (mTP)		
M	4.5	2.3
OS	2.0	1.1
OR	6.0	3.4
K	5.0	2.9
Mares (mNET)		
T5	4.0	1.3
N9E	4.0	1.3
T5B	3.0	1.0
M6	3.0	1.0
N6E	2.0	0.7
T6	3.0	1.0

Active drug = microcapsule weight x 0.579 for mTP and x 0.326 for NET

TABLE 2. Fertility among mares associated with mTP-treated stallions, mNET-treated mares, and control mares in 1987.

Treatment group	Mares	Foals (%)
mTP-treated stallions	14	4 (28.9)
mNET-treated mares	6	6 (100)
Control mares	15	8 (54.4)

The behavior of treated stallions was tested for changes through the use of a behavior exclusively associated with the reproductive season of the ASIS horses, known as elimination marking behavior, or EMB (Turner et al. 1981). Socio-sexual scent marking behavior exhibited by males was used as an index of harem maintenance behavior. This was the only reproductively-associated behavior which occurred with high enough frequency to be useful and which was observable and quantifiable. Stallions mark mare eliminations (urinations and defecations) with their own urine and/or feces. This elimination marking behavior is not demonstrated by mares or immature animals, and stallions rarely mark eliminations by immature animals. This EMB is a seasonal phenomenon, occurring primarily during the reproductive season. Each EMB response by a stallion can be quanitified with a score that can reach a maximum of 5.0. Using this technique, there were no quantifiable differences in reproductive behaviors between mTP-treated and untreated stallions following treatment (Table 3).

TABLE 3. Elimination marking behavior (EMB) scores among mTP-treated and control stallions

Stallion	Observations	EMB Score ± SD
mTP-treated		
K	3	2.66 ± 1.22
OS	3	3.42 ± 0.53
M	4	2.80 ± 0.53
OR	3	3.20 ± 1.22
Control		
J	5	3.63 ± 1.74
N2E	3	2.66 ± 2.30
Pal. Pinto	2	3.16 ± 0.40
Sorrel #1	2	3.00 ± 0.00
N10D	4	3.10 ± 0.08
N6BF	3	3.70 ± 1.10
Sorrel #2	5	2.98 ± 2.11

This decrease in fertility on Assateague Island was substantially less than the 83% reduction reported by Kirkpatrick et al. (1982) for treated wild horses in Idaho. There were several probable reasons for this difference. Firstly, the Idaho stallions were first immobilized and then given the mTP by hand-injection, in doses of 3 – 10 g of active drug. There was no loss of drug during the administration. The ASIS horses were all given doses at the lowest end of the effective dose range and this may have decreased the effectiveness of treatment. A second, and more serious, problem involved the social behavior of the horses. The entire experimental design was based on two premises. The previous and extensive work by Dr. Ron Keiper of The Pennsylvania State University (Keiper 1985) indicated that band stability was high and that very little mare exchange occurred. A second premise was that there would be little migration and range overlap between bands. Both of these conditions existed and were well-documented prior to the initiation of this study.

However, immediately prior to the initiation of the contraceptive experiments, both of these premises became invalid. Stallion T, whose primary home range was Tingles Island, a small island in Sinepuxent Bay several miles south of the ASIS campgrounds, and who maintained an unusually large band of 22 horses, lost control of the band. Consequently, a large number of unattended mares roamed the portion of the island where the experiments were being conducted, disrupting the stability of other bands. At the same time, the majority of J-Band, which inhabited the marshes near the causeway, was translocated to CNWR. This also contributed to instability among bands. In turn, home ranges changed dramatically and overlap of these ranges occurred with regularity. The peaceful and structured nature of the horse bands no longer existed when the contraceptive experiments began in 1986.

Treatment of stallions with dart-delivered mTP demonstrated for a second time that it was pharmacologically possible to inhibit fertility in wild horses by reducing sperm counts and sperm motility. The method however, also proved to be impractical because of the logistics of delivering 3.47 g of the steroid in four separate doses to each stallion (Kirkpatrick and Turner, 1987; Turner and Kirkpatrick 1991). The first treatment was relatively easy among naive stallions but the animals became wary quickly and the subsequent injections became extremely difficult.

Mare Contraceptive Experiments

In a second experiment, during February and March 1986, an attempt was made to administer 1.3 g of microencapsulated northisterone (mNET), a synthetic contraceptive progestin, to six ASIS mares from a different portion of the island than where the stallions were treated. The basis for this experiment was clinical data that supported the idea that blood progesterone concentrations in excess of 0.5 to 1.0 ng/ml would inhibit ovulation in the mare (Squires et al. 1974; Noden et al. 1978; Palmer and Jousset 1975). The mNET was sequestered in the same d,l-lactide material as the mTP and was designed to release over a six month period. Each g of microcapsules contained 0.326 g of NET. The mNET, in a total mass of 4.0 g of microcapsules, was delivered as described for the stallions, in only 2 injections. The mares were observed in August 1987 for the presence of foals.

All six mares treated with mNET produced a foal in 1987, a highly improbable event among ASIS mares, whose annual foaling rates seldom exceed 55% of sexually mature mares (Keiper and Houpt 1984). These same six mares had a 50% foaling rate in 1986 and a control population of 15 untreated mares had a 54.4% foaling rate in 1987 (Table 2). This suggested that the progestin treatment actually enhanced reproduction; however, estimated doses actually delivered to mares (Table 1) fell short of the intended dose for four of six mares.

The reasons for the failure of the mNET treatments and the apparent fertility-enhancing effects were not fully understood, but contraceptive experiments conducted by the University of Minnesota, with Nevada wild horses, shed some light on the subject. In the Nevada experiments, five groups of 30 wild mares were each given Silastic implants containing 8.0 g estradiol (E), 24 g progesterone (P), 8 g E plus 8 g P, 4 g E plus 12 g P, or 12 g E plus 12 g P (Vevea et al. 1987; Plotka et al. 1988). Compared to other treatments, fewer mares receiving 8 gm E, 4 gm E plus 12 gm P, or 8 gm E plus 8 gm P displayed estrus than other treated mares, but all animals which did display estrus, whether treated or control, ovulated. These data indicated a rapid decline in plasma steroid concentrations within five weeks of receiving the steroid implants and suggested an increased metabolic clearance rate of the steroid. Thus, based on the results of the University of Minnesota study, it is probable that the injection of the ASIS mares with northisterone stimulated the clearance of all progesterone, endogenous as well as the exogenous. This in turn would lead to ovulation, based on previous studies of progesterone-induced ovulation in mares (Kenney et al. 1975). Thus, on the basis of these two experiments hypotheses Ho^1 and Ho^2 were rejected.

Lessons Learned

An element in the ultimate success of the Assateague wild horse fertility control project was the National Park Service's commitment to the goal at the conclusion of the steroid contraceptive trials. The very modest results of the stallion contraceptive trial and the outright failure of the mare contraceptive trial could have led to an abrupt end of the project. However, the research team was asked to evaluate the results and submit any additional suggestions for continued research on horse contraception. This point in time was also a critical moment for the research team who had now put 15 years of research effort into steroid contraception research for wild horses. It was a time to determine what had been learned from the Assateague trials and previous work with western horses and to realisitically assess the possibilities for future research. The first lesson was a conceptual picture of what would constitute the "ideal" wildlife contraceptive agent (Kirkpatrick and Turner, 1991b). The characteristics of this ideal agent are listed below.

1. The agent had to be effective as a contraceptive – perhaps 90-95% effective.
2. The agent had to be reversible in its contraceptive action in order to insure the genetic integrity of the herd and public acceptability of the method as a management tool for wild horses.
3. It had to be possible to deliver the agent remotely, without capturing the horse.
4. The agent had to be safe to deliver to pregnant as well as non-pregnant mares.
5. The agent had to be reasonably inexpensive.
6. The agent and methods of delivery must not cause serious health side-effects to the animals.
7. The agent must not be able to pass through the food chain to scavengers and predators.
8. The agent should have no, or minimal, effects on the social behaviors of the animal.

The steroid hormone experiments conducted on Assateague were evaluated in light of these theoretical ideal wildlife contraceptive characteristics. The steroids, given in sufficient doses, could cause effective contraception, at least in stallions, but the effectiveness fell far short of 90%. The contraceptive actions were reversible. Remote delivery of steroids was clearly not realistic in wild horses. The accurate delivery of the darts to the target animals was fairly successful and 90% of the 42 shots taken hit the intended target, but only 21 (54%) injected completely (Table 4). Steroid hormones had to be delivered in very large quantities in order to bring about a contraceptive effect. In stallions, at least 5 grams of steroid, encapsulated in slow-release compounds, had to be delivered to reduce sperm counts sufficiently (Turner and Kirkpatrick 1982). The quantity of steroid necessary to contracept mares was still unknown, but we knew it had to exceed 0.75 grams. Concurrent research on steroid contraception of mares, conducted by the University of Minnesota, suggested that contraceptive doses of estrogens and progestins had to be as high as 10 to 15 grams and even then had to be released slowly by means of Silastic implants (Plotka et al. 1988). Dart-delivery of steroids required no less than four separate treatments with very large 6.0 cc darts in order to achieve even a minimal contraceptive effect and that was clearly not practical with wild horses. It was now very clear to us that the ideal agent had to be delivered in a single dose.

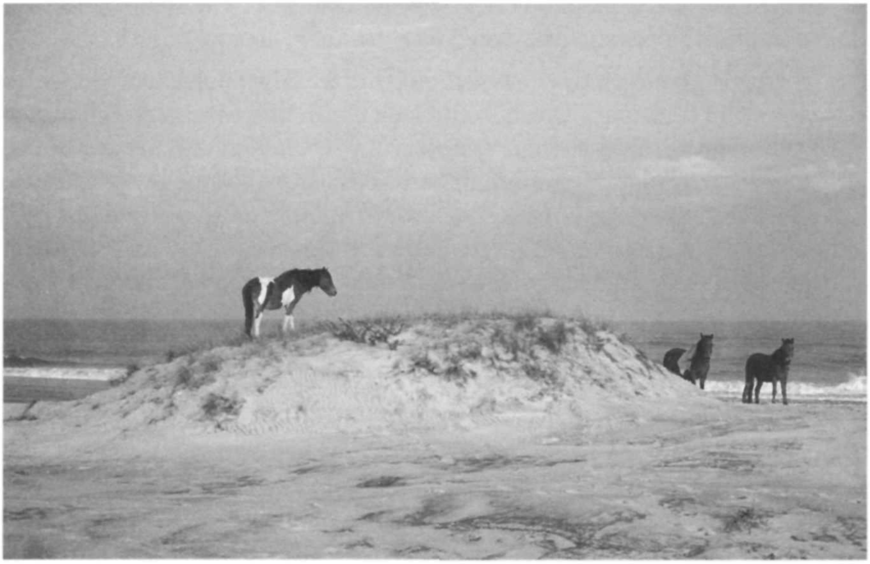
TABLE 4. Darting effectiveness during Assateague steroid hormone experiments in 1986.	
Total shots attempted	42
Hits	38 (90%)
Complete injection	21 (54%)[1]
Bounces (before complete injection)	17 (44%)[1]
Darts recovered	30 (71%)
[1]percent of total hits	

Treatment of stallions with steroids would obviously not interfere with pregnancies, but the effects of estrogenic or progestational steroids on pregnant mares were not known. This issue was particulary important because of the 340-day gestation period for horses. It is improbable that a large number of mares could be treated, at any time of the year, without including pregnant animals.The cost of steroids was relatively high, reaching more than $75/dose for pure steroids and several hundred dollars per dose for microencapsulated steroids. The long-term health effects of steroid administration on either sex were unknown. Retrospectively, examing today's knowledge of the effects of long-term steroid contraception on the health of certain captive exotic species (Kollias et al. 1984; Linnehan and Edwards, 1991), this was a very important consideration. We simply did not know enough about this aspect of steroid contraception to pursue our previous work.

A growing concern by the research team was the possibility of passage of the steroid hormones through the food chain to scavengers and predators. Natural steroids, such as testosterone, estradiol, and progesterone, are identical throughout mammals, thus passage to secondary consumers would not have serious effects. These natural hormones would simply be quickly metabolized and cleared. It is for this very reason that these hormones cannot be used effectively for contraceptives unless they can be given almost daily, an impossible task in wildlife. It is for this same reason that synthetic hormones such as testosterone propionate, northisterone, or ethinylestradiol are more commonly used for contraception. These compounds work with fewer treatments because they cannot be easly "recognized" by the liver and metabolized. Thus, they remain in circulation longer and smaller doses can exert their contraceptive influences longer. However, these same characteristics make the synthetic steroids potentially dangerous with regard to passage through the food chain. While the actual effects of synthetic steroid passage to secondary consumers was, and still is, not known, there are clear potential environmental hazards with the steroid compounds. On Assateague a combination of foxes, opposums, vultures, and a variety of gulls consume horse carcasses quickly.

At no point in our previous 15 years of research on wild horse contraception had the Bureau of Land Management (BLM) shown any concern over possible behavioral effects upon treated animals, nor had the NPS broached the subject. It was the opinion of the research team however, that wildlife contraception carried with it the moral and ethical obligation to leave behind behaviorally intact animals after treatment. This was particularly important for highly social animals such as wild horses. It was known that steroid hormones have profound effects upon the behavior of most mammals. The behavioral effects of testosterone treatment of stallions had already been tested in western wild horses (Turner et al. 1981) without any obvious adverse effects. This again proved to be the case with the mTP-treated stallions on ASIS (Kirkpatrick and Turner 1987), but the behavioral effects of estrogens and progestins in mares was not known.

These then, were the lessons learned from our 15 years of research with steroids and wild horses and from the Assateague trials. Clearly there was little to encourage the continuation of studies with steroid hormone contraception. An entirely new direction was needed.



Immunocontraception

At this point the research team asked a few fundamental questions. The first question was what, in the history of contraceptive technology, fit the characteristics of the ideal wildlife contraceptive agent ? The history of human contraceptive technology centered around steroids and we had already concluded that too many technical problems existed to continue this approach. The next and more important question was, what is the future of contraceptive technology? In the opinion of the research team, and based on the available literature and research efforts elsewhere, the answer was immunocontraception. A review of the literature in 1987 made it clear that there was increasing emphasis on immunocontraception, with vaccines directed against reproductive hormones (Al-Kafawi et al. 1974), at sperm (Primikoff et al. 1988), and at the ovum (Sacco 1987). Basically, immunocontraception is the stimulation of the body's immune system to produce antibodies that interfere with some requisite event in the reproductive process. The published literature revealed several specific approaches which might be of value in wildlife contraception but the most attractive was the possibility of blocking fertilization by means of producing antibodies against the zona pellucida (ZP) of the ovum.

The zona pellucida is a non-cellular membrane surrounding all mammalian ova and it is composed of several glycoproteins. At least two of these glycoproteins, designated ZP3 and ZP4, are thought to be the sperm receptor molecule (Florman and Wassarman 1985; Hasagawa et al. 1992). In order for a sperm to be able to attach to the ovum, and in order to cause fertilization, there must be complimentary proteins on both the surface of the sperm and zona pellucida of the ovum. The literature contained many references to porcine zonae pellucidae (PZP) which had been proven effective at blocking fertilization in various non-human primates (Sacco 1987), rabbits (Wood et al. 1981), dogs (Mahi-Brown et al. 1985), and even humans (Sacco et al. 1987). Even more intriguing was a published report on the presence of spontaneous antibodies against the zona pellucida of the domestic mare (Liu and Shivers 1982) which appeared to block fertilization.

One of those investigators, Irwin K. M. Liu, Department of Population Health and Reproduction, in the School of Veterinary Medicine at the University of California-Davis, decided to test the PZP vaccine in horses. Dr. Liu reasoned that if spontaneously-formed antibodies against the zona inhibited fertilization in mares, perhaps those antibodies could be deliberately induced with PZP. Ten captive wild mares living on a wild horse sanctuary and four domestic mares were repeatedly inoculated with the protein equivalent of 2,000 to 5,000 PZP (Liu et al. 1989).

The vaccine was prepared as described by Gwatkin et al. (1980) and Dunbar et al. (1980). Briefly, frozen/thawed porcine ovaries, acquired from slaughterhouses, were minced in cold phosphate buffered saline (PBS) using a ganged razor blade apparatus. The oocytes were separated from other tissues, including granulosa cells, by screen filtration, counted, and homogenized in a Potter-Elvehjen homogenizer. The zonae were isolated on a 48 μm screen, heat solubilized at 70^{o} C, and stored frozen in PBS. This vaccine preparation contained the whole family of zona pellucida proteins, including ZP1, ZP2, ZP3, and ZP4.

Freund's Complete adjuvant (FCA) was used for the initial inoculation and Freund's Incomplete adjuvant (FIA) was used for the three monthly booster inoculations which followed. The PZP, being a foreign protein to the mares, caused them to produce anti-PZP antibodies which in turn attached to the mares' own zonae sperm receptors and thereby blocked fertilization. Serum progesterone concentrations from the four domestic mares indicated that three of the four ovulated following treatment, but a year later only a single mare among the 14 treated animals produced a foal. The four domestic mares were retained for a second year and all four produced foals two years after their initial treatment, after antibody titers declined. Thus, reversibility of contraceptive effects was established after a single year of treatment. This seminal discovery opened the door to successful immunocontraception of wild horses.

Initial PZP Contraceptive Trial

With these encouraging results, a field test of the PZP vaccine began on ASIS in 1988. For this field test, 26 mares were remotely inoculated with approximately 5,000 PZP, or about 65 μg of protein. Mares were selected from at least eight bands, however, the stability of the original 14 bands present in 1986 was lost due to the removal of several bands for management purposes and the breakup of T-stallion's band of 22 mares, near Tingle's Island. The test mares were not selected randomly, but rather, with a bias for high fertility. The 26 mares collectively had a fertility rate for the previous three years that was 10% higher than the average fertility rate for all ASIS mares. This decision was made in order to minimize treating mares with low fertility rates, which would not rigorously test the contraceptive effectiveness of the vaccine and delivery system. The initial inoculation was given as a 1.5 ml volume containing an emulsion of PZP in 1.0 ml of PBS and 0.5 ml of FCA, between 29 February and 13 March 1988. The PZP vaccine was administered by means of barbless self-injecting 3.0 cc darts fired from a Pax-Arms 0.579 cal. capture gun. The darts were shot into the hip or gluteal muscles of the mares at ranges of 30-40 m. Pax-Arms darts are pressurized with air with a small hand-pump and inject from side ports in the needle upon impact with the animal. Between 15-30 March 1988, a second inoculation was given to all the mares in the same manner, but FIA was used as the adjuvant. Between 16-30 April 1988, 18 of the 26 mares received a third inoculation of PZP plus FIA. Six additional mares each received a sham inoculation of PBS plus FCA between 29 February and 30 April, and 11 other mares were selected as untreated controls (Kirkpatrick et al. 1990a).

Non-capture Pregnancy Testing

At this point in the project another new technology was applied to the contraceptive studies of ASIS. Results of the March – April 1988 PZP inoculations would not normally be available until August 1989, because of the 340-day gestation period in horses. Even though we had not yet obtained the contraceptive results for the first year of treatment, we were already thinking about the possibility of a single annual booster inoculation in order to sustain contraceptive effects beyond a single year. However, before a booster-inoculation study could be designed we had to know how successful the first year's PZP treatment had been. This, of course, would necessitate pregnancy testing wild horses.

Historically, there were three ways to pregnancy-test a horse. First, the horse could be restrained and palpated rectally. This is very accurate but requires highly trained personnel and capture and restraint of the mares, something that was not possible with the 26 mares on ASIS. A second common method was the Mare Immunological Pregnancy (MIP) test . This is a serological test that is based on the presence or absence of the pregnancy-specific hormone, pregnant mares serum gonadotropin (PMSG). This, however, requires blood samples which in turn again necessitates capture and restraint. The third method was to measure plasma progesterone, but this too requires blood samples. Additionally, it is not a very accurate test because its use results in significant numbers of false positives. Persistent corpora lutea are common in mares, and progesterone concentrations often remain quite high for several months after an ovulation, even when no pregnancy occurs. Also, if a mare aborts, progesterone concentrations often remain at pregnancy levels for many weeks after the abortion has occurred. Thus, the common means of pregnancy testing were useless in the ASIS setting, and the problem was defined as how to pregnancy-test a large wild animal that cannot be touched.

The solution to this probelm was a urinary pregnancy test. In the early 1980s, Dr. B. L. Lasley and his research team at the San Diego Zoo developed a urinary test for estrogen metabolites (Loskutoff et al. 1983; Lasley and Kirkpatrick 1991). These urinary tests were originally developed for diagnosing pregnancy in large intractable captive exotic species, like the rhinoceros (*Rhinoceros unicornis*) (Kassam and Lasley 1981), or in species that become highly stressed as a result of handling, like the okapi (*Okapia johnstoni*) (Loskutoff et al. 1982), in order to facilitate captive breeding efforts. By chance, the urinary estrone conjugate (E_1C) hormone assay was also useful in diagnosing pregnancy in domestic mares after day-35 of pregnancy (Evans et al. 1984). In 1986, Dr. Lasley and the author decided to evaluate the urinary E_1C pregnancy test in free-ranging wild horses inhabiting the Pryor Mountain National Wild Horse Refuge of southern Montana. Urine was collected directly off the ground, after observed eliminations, from 25 mares and analyzed for E_1C concentrations. Ten of the mares had urinary E_1C concentrations less than 1.0 μg/mg creatinine (Cr) and none produced a foal the following year; fifteen had concentrations in excess of 1.0 μg/mg Cr and 12 of these had foals at their sides a year later (Kirkpatrick et al. 1988). Creatinine is a common metabolite of protein metabolism and it is freely-filtrable in the kidney and excreted at a constant rate. The hormone values were indexed to creatinine in order to account for differences in urine concentrations. Thus, the very first attempt to pregnancy test wild horses without capture proved highly reliable.

Between 1 – 20 October 1988, approximately five to six months following the breeding season on ASIS, and six to seven months following final PZP treatment, urine samples were collected from all 26 PZP-treated mares and six control mares. When possible, the samples were collected from pooled urine immediately after the elimination. If the mare urinated in sand, the urine-soaked sand was placed in a gauze square which was then placed inside a plastic sandwich bag, and the urine was hand-centrifuged from the sand. Only 50-100 μl of urine was necessary for assay of the hormone metabolite. The samples were transported frozen to a laboratory in Billings, Montana, and assayed. The results indicated that none of the 26 PZP-treated mares was pregnant. The mean urinary E_1C concentration for the 26 PZP-treated mares and three of the control mares which did not produce foals in 1979 was 0.12 ± 0.35 μg/mg Cr, and no values for any of these 29 individual mares exceeded 1.0 μg/mg Cr. In contrast, the mean urinary E_1C concentration for the three sham-treated control mares which did produce foals in 1979 was 3.41 μg/mg Cr., and none of these three mares had E_1C concentrations which fell below 1.0 μg/mg Cr. The differences in mean values between PZP-treated and untreated mares which did not produce foals and untreated mares which produced foals in 1989, was significant ($t = 9.47$; 30 df; $P<0.001$). Thus, six to nine months prior to the foaling season we already knew the outcome of the PZP contraceptive trial was 100% successful.

While the collection of urine and analysis of E_1C was a reliable non-capture approach to pregnancy detection in the ASIS horses, the procedure was labor intensive. On the average, a mare on ASIS urinates perhaps every three hours, but they defecate with greater frequency. This casual observation was made at the same time the initial urine collections were being carried out in the fall of 1988, and it was decided to collect matching fecal samples for possible pregnancy testing. An added advantage to fecal collection was that the mares could be observed at greater distances – up to several hundred meters – and samples could be located and collected with greater ease.

Fecal samples were collected from the 26 PZP-treated mares and eight untreated mares (six of which were also used in the urinary E_1C pregnancy tests described above) at the same time that urine samples were collected. An additional 14 samples were collected from other mares, opportunistically, and various combinations of samples from these three groups were used for the different pregnancy testing procedures described below. One-half g of feces was extracted with ethyl acetate/hexane, the organic solvents were dried, and the residue was resuspended in assay buffer. Based on the dramatic rise in estrogens after day-35 of pregnancy in mares, and the concomitant rise in urinary E_1C, it was decided to analyze the fecal samples for some form of estrogen. Normally, free steroids are excreted in feces while water soluble conjugates of steroids, like E_1C, are excreted in urine. Thus, we decided to examine total fecal estrogens (TFE), which meant some combination of estradiol, estrone, and perhaps estriol. The analyses were carried out with a commercially available radioimmunoassay. The results showed significant differences between TFE in pregnant and non-pregnant mares (Table 5) (Kirkpatrick et al. 1990b). This approach proved to be reliable and offered far greater ease of collection in the field, although preparation of samples in the laboratory was more time-consuming and difficult because of the organic solvents. The analysis of samples with radioimmunoassay, instead of the enzyme immunoassays used for E_1C, was significantly more expensive.

TABLE 5. Mean concentration of urinary estrone conjugates (E_1C) and total fecal estrogens (TFE) in pregnant and non-pregnant Assateague mares in 1988.

Pregnancy Status	N	Mean Urinary E_1C (μg/mg CR)	Mean TFE (μg/g feces)
Pregnant	6	3.47 ± 0.735	3.18 ± 0.70
Non-pregnant	28	0.11 ± 0.034	0.55 ± 0.08

Pregnancy status was based on whether or not the mare produced a foal in 1989; difference in mean TFE values between pregnant and non-pregnant mares was significant ($p<0.001$). 26 of the 28 non-pregnant mares were PZP-treated.

In a final experiment to simplify remote pregnancy testing, fecal samples were collected from adult untreated mares inhabiting both ASIS and CNWR, in October 1989. Each sample was extracted with water and analyzed for E_1C with the less expensive enzyme immunoassays. Circulating estrogens are normally metabolized in the liver. In the case of estradiol in the mare, it is metabolized to estrone. In the mare, estrone is then conjugated to some molecule which makes the steroid water soluble; the two most common forms of estrone conjugates in the mare are estrone sulfate (E_1S) and estrone glucuronide (E_1G). Our assay cross-reacts with both forms of conjugated estrone and we refer to the collective group as estrone conjugates or E_1C. The E_1C is stored in the gall bladder and released into the gastrointestinal tract, where it is quickly resorbed back into the vascular space and excreted by the kidney, in urine. We reasoned that some small percentage of E_1C would not escape the gastrointestinal tract and show up in the feces. The results of our assays indicated that there were sufficient quantities of E_1C in the feces to diagnose pregnancy accurately in free-ranging horses (Table 6) (Kirkpatrick et al. 1991a). The advantage of this latter test was that it required only a water extract of the feces and utilized the inexpensive non-isotopic enzyme immunoassay. Thus, at this point we had reliable, inexpensive, and accurate pregnancy tests using both urine and feces. The urinary E_1C is the easiest to conduct in the laboratory, the least expensive, and the most accurate, but this particular hormone assay is not available commercially and access to the test is very limited. Additionally, collection of urine samples in the field is very labor intensive. The fecal E_1C and iPdG pregnancy test also requires access to the steroid metabolite assays, but requires less time in the field to collect samples. The total fecal estrogen pregnancy test can be carried out with commercially available estrogen radioimmunoassays assays, requires less time in the field for sample collection than for urine. However, preparation of the samples is time consuming, involves the cost of organic solvents, and the cost of commercial estrogen radioimmunoassays is much greater than for the enzyme immunoassays for E_1C and PdG. The choice of pregnancy test for other scientists is therefore, dependent upon access to assays and resources.

TABLE 6. Urinary and fecal E_1C mean concentrations for pregnant and nonpregnant Assateague mares in 1988.

Pregnancy Status	N	Mean Urinary E_1C (μg/mg CR)	Mean TFE (μg/g feces)
Pregnant	7	3.9 ± 1.3	4.2 ± 0.1
Non-pregnant	33	0.1 ± 0.0	0.5 ± 0.1

Difference in mean fecal E_1C concentrations between pregnant and nonpregnant mares was significant ($p<0.01$). Pregnancy status confirmed by whether or not foals were present in 1989. 26 of 33 non-pregnant mares were PZP-treated.

The only major disadvantage associated with these urinary and fecal pregnancy tests was the need to transport frozen samples to a laboratory facility, and the need for expensive instrumentation to measure hormone concentrations. We evaluated a rapid non-instrumented field test for pregnancy that might permit on-site pregnancy tests. The measurement of urinary E_1C by the instrumented enzyme immunoassays described above is based on observable color changes which are ultimately measured as differences in optical density with a spectrophotometer. Plastic test tubes were pre-coated with the anti-E_1C antibodies, and stored in the research facilities on ASIS. We prepared Whatman No. 1 filter paper disks of 7.0 mm diameter with a paper punch prior to fieldwork, and upon recovery of a urine sample, several disks were dropped into each urine sample tube. The disks were removed with forceps at the end of each day and air-dried on a non-absorbent surface. The appropriate buffers were added to the pre-coated antibody tubes and a single filter paper disk was dropped into each tube. From this point, the assay followed the same procedure and order as the instrumented tests (Shideler et al. 1990), but the intensity of the color changes were simply noted visually and subjectively. The measurement of urinary E_1C by this non-instrumented test was 100% accurate when compared with instrumented spectrophotometrically measured tests for the same hormone (Kirkpatrick et al. 1993a). On the basis of these experiments, we accepted hypothesis Ho^6.

All of these non-capture pregnancy tests were developed in the course of the Assateague fertility control research and as a result of the unique conditions of conducting this form of research with a highly valued public resource. This new technology would later have effects that reached far beyond Assateague Island, and would facilitate research in other units of the National Park Service.

In August 1989, we returned to ASIS, counted foals, and confirmed the results of the earlier urinary E_1C and fecal total estrogen pregnancy tests. No foals were born to any of the PZP-treated mares, whereas 50% of the six sham-treated control mares produced foals and 45% of 11 untreated mares produced foals (Table 7). The 11 untreated mares were selected on the basis of age-range, which fell within that of treated mares, and because they belonged to harem stallions of proven fertility. Of the 26 PZP-treated mares in 1988, 14 were pregnant at the time of inoculation and all 14 produced healthy foals in 1988, two to three months following PZP treatment. Thus, the PZP vaccine treatment had no effect upon pregnancies in progress or the health of the foals which were born. Social behaviors and herd social organization were unaffected by treatment and there was no difference in dispersion of mares between bands for treated and untreated mares (Kirkpatrick et al. 1990a).

TABLE 7. Foaling rates for PZP-treated, sham-treated, and untreated mares for pretreatment, treatment, and post-treatment years, 1987 through 1989.

			No. mares that foaled (%)		
			Pretreatment	Pretreatment	Post-treatment
Inoculations/ Group	Horse	N	1987	1988	1989
Treated	3	18	9 (50.0)	11 (51.1)	0 (0.0)
Treated	2	8	5 (62.4)	3 (37.4)	0 (0.0)
Sham-treated	0	6	2 (33.3)	2 (33.3)	3 (50.0)
Untreated	0	11	*	*	5 (45.4)

Difference in foaling rates between PZP-treated and untreated mares was significant ($p<0.002$) based on binomial proability distribution. All inoculations were given in 1988.

*Data not available.

The results of this trial indicated that the vaccine could be delivered remotely in 1.5 cc volumes, that contraceptive effectiveness was 100%, that two inoculations were as effective as three inoculations, that PZP-treatment was safe in pregnant mares, and that social behaviors were unaffected. The cost of preparing the vaccine, at that time, was $25/dose. Additionally, because of the protein nature of the vaccine, it could not be passed through the food chain. Thus, six of the eight characteristics of the ideal wildlife contraceptive were realized in this initial trial, and hypothesis Ho^3 was accepted.

PZP Booster-inoculation Experiment

The next step in validating the potential usefulness of the PZP vaccine in the ASIS wild horses was a test of the effectiveness of a single annual booster inoculation, and a test of reversibility of contraceptive effects. It was reasonable to hypothesize that a single annual booster inoculation would result in continued contraceptive antibody titers. The initial inoculation of a mammal with any protein-based vaccine results in antigen "recognition" and moderate but temporary increases in antibodies (Liu et al. 1989). Subsequent inoculations lead to higher and sustained antibody titers, and this was the rationale for the second inoculation among the Assateague mares. Thus, we predicted that a single inoculation in 1989 would extend contraceptive protection to the mares for a second year.

Between 20 February and 17 March 1989, 14 of the previously treated 26 mares were given a single booster inoculation of 65 μg PZP plus FIA, exactly as described in the first field trial (Kirkpatrick et al. 1991b). We already knew these mares were not pregnant as a result of the 1988 treatments. Six mares were sham-treated and another sixteen untreated mares having ages within the range for treated mares were chosen as controls. A year later, in 1990, only one, the mare M17G, of the 14 treated mares foaled (Table 8). The 12 mares immunized in 1988 but which were not given a booster inoculation in 1989 produced five foals. This foaling rate (41.6%) was not different from the foaling rate (44%) of 16 untreated mares, selected for similar age ranges to the treated mares and accompanying stallions of proven fertility, and demonstrated for the second time that the contraceptive effects of a single year of treatment were reversible. The reason for the birth of the single foal was not understood. Because it was not possible to measure antibody titers in this mare, we could not know if the inoculation failed to elicit a sound antibody response. Thus, on the basis of the initial experiments with the PZP vaccine, in 1988 and 1989, we accepted hypothesis Ho^4.

TABLE 8. Pregnancy and foaling rates for booster-inoculated, sham-treated, and untreated mares from 1989.

Treatment Group	No. mares diagnosed N	pregnant ($)[1] in 1989	No. mares that foaled (%) in 1990
Booster PZP	14	1 (7)	1 (7)
Sham-treated	6	3 (50)	3 (50)
Untreated	16	7 (44)	7 (44)

[1]Based on urinary E_1C measurements

The success of the single booster inoculation moved the potential of contraceptive management of the ASIS horses closer to reality. The need to inoculate each mare twice in the first year represented a labor-intensive effort, but after that first year, it was clear that only a single inoculation was necessary, something that is quite feasible on Assateague Island.

Long-Term Effects of PZP Treatment

It was still not known whether long-term treatment with PZP, defined here as three to seven consecutive years of annual treatments, would affect ovarian function, reversibility of the contraceptive effects, and the general health of the mares. Reversibility of the contraceptive effects of the PZP vaccine had been demonstrated in several species, including the horse, but only after short-term application of the vaccine (Gulyas et al. 1983; Sacco et al. 1987; Liu et al. 1989). In the dog and the rabbit, PZP immunization appeared to be very damaging to ovarian follicles and often led to cessation of ovarian function, with accompanying anovulation and depression of estrogen concentrations (Wood et al. 1981; Mahi-Brown et al. 1985). Unusually large doses of PZP (5,000 μg) caused anovulation in the baboon (Dunbar et al. 1989). These data suggested that the antibody response of the treated animal attacks not only the zona pellucida of the mature ovum, but also oocytes and possibly other ovarian tissue with resulting changes in estradiol and progesterone secretion. These effects had not been demonstrated in the horse after short-term use of the vaccine (Liu et al. 1989), but there were no available data to predict the effects of long-term use of the vaccine in this species.

The concern about the possible effects of long-term use of the vaccine upon ovarian function in the ASIS horses was based on the sensitive nature of the public's perception of these horses. One of the lessons learned during the many years of research with the western wild horses was that any contraceptive agent which was irreversible had little or no chance of being publicly acceptable. Thus, a decision was made to continue treatment of a small population of experimental horses for several more years, and to attempt to evaluate ovarian function.

This brought the focus of the Assateague contraceptive project temporarily back to remote testing of reproductive hormones in free-ranging horses. Our initial attempts to diagnose pregnancy had been very successful, but now we were faced with a more complicated question. How was the application of the PZP vaccine to the ASIS mares for three or more years affecting the function of their ovaries? Would we see the same suppression of ovarian function that others had seen with rabbits and dogs? And, more to the point, how could we study these potential effects in publicly-valued animals without intrusive methods?

On the basis of the urinary E_1C tests for pregnancy, which were measuring primarily placental estrogens, it was probable that we could also measure the ovary's abilility to produce estrogens if we could collect serial urine samples over a long period of time during the breeding season. The indirect measurement of ovarian estrogen production would provide data regarding the development of ovarian follicles during the first half of the estrous cycle. However, in order to determine if ovulation was occurring, we had to have some method for measuring progesterone or progesterone metabolites in the urine. Immediately after ovulation, the ovary forms a structure known as the corpus luteum, which is the primary source of progesterone in the non-pregnant mare. There is an abrupt rise in progesterone and then, if no pregnancy occurs, the progesterone usually declines until the end of the 21-day estrous cycle of the mare. This characteristic rise which occurs during the luteal phase, or second half of the estrous cycle, is proof of ovulation. However, in 1989 it was still not possible to measure metabolites of progesterone in the urine of equids.

Work in Dr. Lasley's laboratory at the San Diego Zoo had demonstrated that urinary progesterone metabolites could be successfully measured in a variety of captive exotic species of Artiodactyla, such as okapi (Loskutoff et al. 1982) and giraffe (Loskutoff et al. 1986). The particular progesterone metabolite was pregnanediol-3-glucuronide (PdG), but it was not present in the urine of equids. In 1989, at the University of California-Davis (Dr. Lasley moved from San Diego to Davis) a series of urine samples from pregnant and non-pregnant ASIS mares were analyzed for progesterone metabolites, using several different assay systems. This research was made possible by the National Science Foundation, which found particular interest in the non-capture pregnancy testing technology which had been developed on ASIS. One particular enzyme immunoassay utilized an antibody raised against 20α-hydroxyprogesterone and showed a significant difference in progesterone metabolite concentrations between four pregnant and four non-pregnant ASIS mares, suggesting that we were actually measuring a metabolite of progesterone in the urine of horses. In the next experiment, we acquired matched blood and urine samples from several domestic horses and measured both plasma progesterone and the unknown urinary progesterone metabolites using this new antibody. The correlation between blood progesterone concentrations and the urinary progesterone metabolites was r = 0.91 (Figure 2). Further examination of these progesterone metabolites with high performance liquid chromatography revealed that we were actually measuring three different progesterone metabolites (Kirkpatrick et al. 1990c). Although the precise nature of these metabolites, which we refer to as immunoreactive PdG-like progesterone metabolites, or iPdG, has not been characterized, the high correlation between plasma progesterone and urinary iPdG indicated that we had an assay that would permit us to detect ovulation in the ASIS mares.

We first tested the application of urinary iPdG analysis in the ASIS mares by collecting and analyzing spot samples from pregnant and non-pregnant mares in 1989. Pregnancy status was first confirmed by analyzing the urine with the highly accurate urinary E_1C assay, and then we compared urinary iPdG values to the E_1C values. The urinary iPdG results correlated well with the urinary E_1C results (Table 9) and indicated we were able to evaluate urinary progesterone metabolites in ASIS mares from samples collected under field conditions (Kirkpatrick and Turner, 1991a).

TABLE 9. Mean urinary ummunoreactive pregnanediol-glucuronide-like progesterone metabolites (iPdG) in pregnant and non-pregnant mares.

Pregnancy Status	N	Mean urinary E_1C (μg/mg CR)	Mean urinary iPdG (ng/mg Cr)
Pregnant	3	3.78 ± 5.8	227.82 ± 89.7
Non-pregnant	29	0.34 ± 0.4	3.42 ± 0.486

Difference in mean urinary iPdG concentrations between pregnant and non-pregnant mares was significant ($p<0.001$). Samples were collected in 1989.

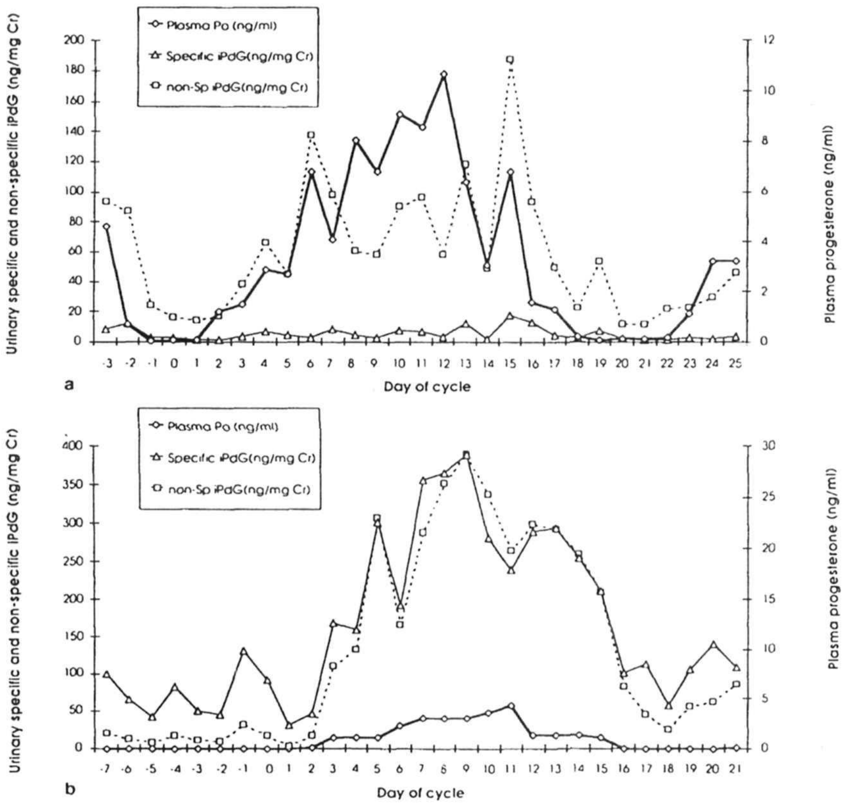


FIGURE 2. Plasma progesterone and urinary specific and non-specific iPdG concentrations in two representative cycling untreated domestic mares. Day 0 represents the day of ovulation, verified by rectal palpation (from Kirkpatrick et al. 1990c)

In May 1990, Rick Naugle, a wildlife major at The Pennsylvania State University, and I started collecting urine samples from seven mares which had been treated with the PZP vaccine for three consecutive years, and four untreated mares. The samples were collected every-other-day, for 56 days, from 4 May through 30 June, a period of time which coincides with peak breeding activity of the ASIS mares (Keiper and Houpt 1984). The samples were analyzed for E_1C and iPdG, and a 60-day pattern of ovarian estrogen and progesterone secretion became apparent when the results were plotted. Three of the untreated mares demonstrated normal ovarian activity, characterized by preovulatory estrogen peaks, concurrent progesterone nadirs at ovulation and breeding activity, and subsequent luteal phase progesterone increases after ovulation. The urinary hormone profiles are shown for these three untreated Assateague control mares in Fig. 3. The first mare (Fig. 3a) demonstrates parturition, characterized by a sudden drop in iPdG on the day of parturition. Following parturition, there is a significant rise in estrogens, signalling an attempt by the mare to have a post-partum estrus; but ovulation never occurred. The second mare (Fig. 3b) demonstrates an ovulatory non-conceptive cycle , and the third mare (Fig. 3c) demonstrates a conceptive cycle. Two of the 7 PZP-treated mares demonstrated ovulatory cycles that did not result in conception (Fig. 4a,b). Another PZP-treated mare, M17G, was pregnant as a result of conception in 1989 and demonstrated a normal, late-gestation endocrine profile. The remaining four PZP-treated mares revealed no evidence of ovulation, and urinary estrogen concentrations were significantly depressed (Kirkpatrick et al. 1992a). Thus, while there was evidence of long-term effects of the endocrine function of the ovary and the mares' ability to ovulate, we concluded that we could measure these effects with non-capture methods. On this basis we accepted hypothesis Ho^7.

The significance of this pilot study of the long-term effects of PZP contraception upon ovarian function of the ASIS mares was three-fold. First, this was the longest running experiment of PZP contraception in any species and it suggested that three consecutive years of PZP treatment may interfere with normal ovarian function, as shown by anovulation and depressed estrogen concentrations in four of seven treated mares. These data suggested that future management schemes utilizing PZP-immunocontraception would have to be carried out with extreme care and a great deal of planning in order to avoid possible irreversible infertility. Once again public perceptions and acceptability of management practices with the ASIS mares had to be considered. The possibility of inducing permanent sterilization, as opposed to reversibly inhibiting fertility, in the ASIS mares meant that the PZP vaccine would have to be used very carefully in order to both manage and preserve this herd. Secondly, and perhaps more important in the broader field of science, this was the very first demonstration that the entire reproductive cycle of a free-ranging ungulate could be monitored without capture of the animals. Thirdly, this particular experiment and the data generated by it caught the imagination of the National Institutes of Health (NIH). The PZP vaccine had been considered for a human contraceptive vaccine for several years (Sacco 1987) but the possibility of long-term effects was a serious concern and there were no data regarding effects on the ovary after many years of application. Consequently, in 1992, the NIH sponsored four years of additional study of the effects of the PZP vaccine on ovarian function and reversibility of contraceptive effects in the ASIS

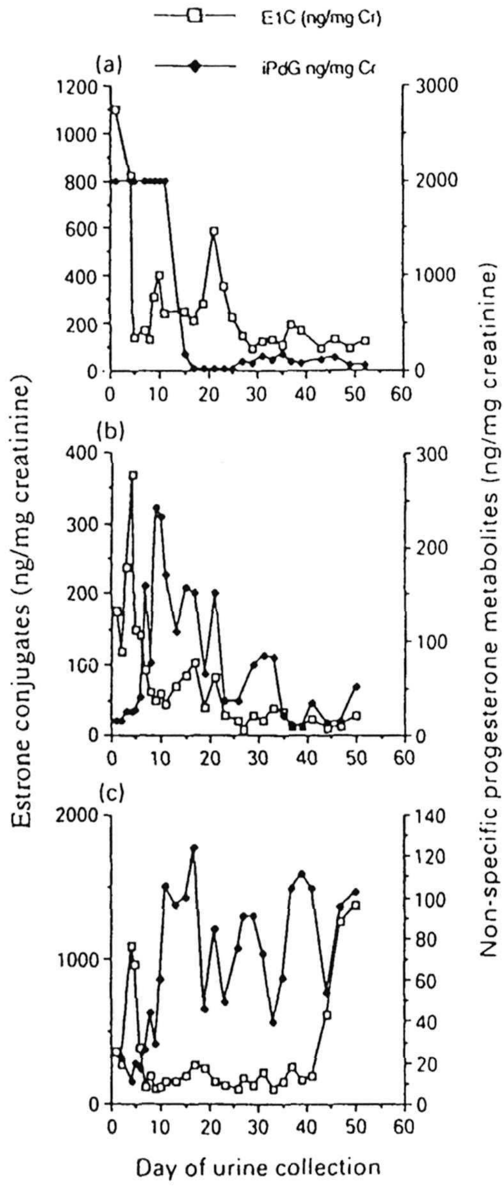


FIGURE 3. Profiles of urinary E_1C and iPdG in three untreated control mares: (a) M13 (a foal was born on Day 12), (b) an estrous cycle without conception in Speckles and (c) an estrous cycle with conception in M17GL. Day 0 was 4 May 1990 (from Kirpatrick et al. 1992a).

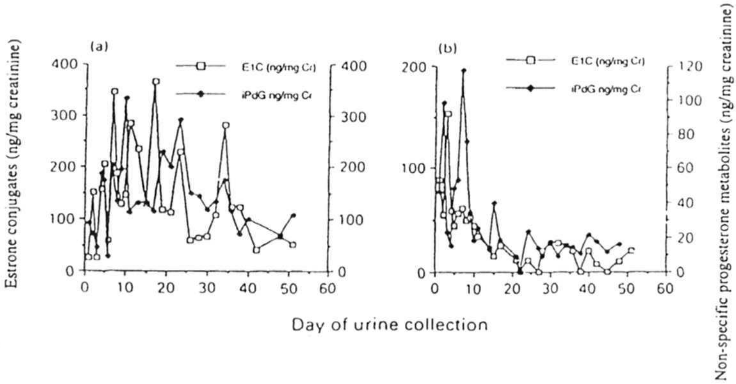


FIGURE 4. Profiles of urinary E_1C and iPdG in two mares treated with porcine zonae pellucidae: ovulatory cycles without conception in (a) M6I and (b) T2BE. Day 0 was May 1, 1990. (from Kirkpatrick et al. 1992a).

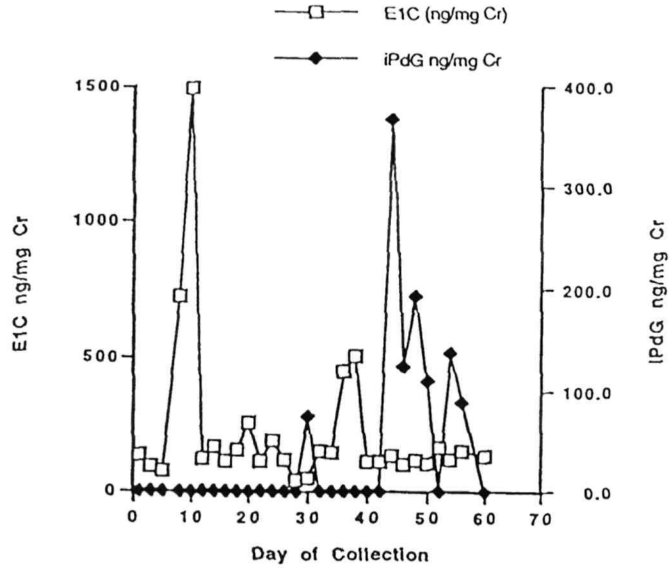


FIGURE 5. Profiles of urinary E_1C and iPdG in the mare M6I. The preovulatory estrogen rise and the luteal phase iPdG pattern indicates ovulation during the sixth year of the study and after five consecutive years of PZP-treatment. Day 0 was May 1, 1992.

mares. There was little interest by the NIH in the horses *per se*, but the opportunity to examine long-term effects in a species with ovarian dynamics similar to women appealed to the agency.

When studies of the long-term effects of PZP contraception began in 1990, the study population consisted of mares which had been treated for three consecutive years. At that time there were no mares which had been treated for only one or two years available for study. Thus, with the initiation of the NIH-sponsored research, in 1992, a new population of mares had to be treated in order to provide animals which had only one or two years of consecutive treatment.

In March 1992, 12 previously untreated mares were placed on the PZP protocol and serial urine samples were collected as described above, in 1992, and 1993 in order to study the effects of the vaccine on ovarian function during the first and second years of treatment. In still another experiment (see One-Inoculation Experiment, below), an additional 14 previously untreated mares were treated with a one-inoculation protocol in 1992. Thus, by May 1994, 52 different ASIS mares had been inoculated remotely with the PZP vaccine over a period of seven years. PZP-treatment over six years, equalling 105 mare-years (the sum of the number of years each mare was treated), resulted in four foals (3.8% foaling rate), compared to a 46.2% foaling rate for an untreated population equalling 65 mare-years. The difference in foaling rate between PZP-treated and untreated mares was highly significant ($p < 0.001$). By 1994, 48 mares received their initial PZP inoculations during the latter stages of pregnancy (280-340 days gestation) and all 48 delivered foals. Thirty-nine of these foals survived one year or more (81.2%) and this degree of survival did not differ significantly ($p < 0.05$) from the survival of 25 foals born to untreated mares (84%) during the same period of time.

Reversibility of contraceptive effects was documented in seven of 10 (70%) mares after a single year of PZP-treatment, in two of three (66%) mares after two years of treatment, in one of 10 (10%) mares after three years of treatment, and in one of two (50%) after four years of treatment. Three mares that were *in utero* at the time their mothers were first inoculated, in 1988, have survived to produce foals of their own. Whether or not mares treated for five consecutive years or more are irreversibly infertile remains to be seen.

The ovulation rate after one and two consecutive years of treatment was 73.3% (11/15) and 60% (9/15), respectively. After three consecutive years of treatment the ovulation rate declined to 55.5% (5/9) and after seven consecutive years of treatment the ovulation rate declined to 10% (1/10). Over the seven years, the ovulation rate for four control mares ranged from 75% to 100%. One mare (M6I) was withdrawn from treatment after five consecutive years of PZP inoculations and she ovulated during the sixth year of the study, but did not become pregnant (Fig. 5). She ovulated again during the seventh year.

The normal range for urinary E_1C for cycling mares is 100-385 ng/mg Cr (Daels et al. 1991). After a single year of treatment, 12 of 15 mares (80%) had urinary E_1C concentrations which fell within this normal range. The percent of mares with normal urinary estrogen concentrations declined to 46.1% (6/13) after two consecutive years of treatment. After three consecutive years of treatment 2 of six mares (33%) had normal urinary E_1C concentrations, after six consecutive years of treatment two of five mares (40%) had normal urinary E_1C concentrations, and after seven consecutive years of treatment none of the five mares had normal urinary E_1C concentrations. By the seventh year of treatment, the difference between urinary E_1C concentrations in the five treated mares (14.21 ± 0.87 ng/mg Cr) and four control mares (246.88 ± 35.53 ng/mg Cr) was highly significant ($p < 0.0001$) during the May – June collection period. All observed reproductive behaviors were correlated with elevated urinary E_1C concentrations. Seven of 16 mares (43.7%) treated for one to seven consecutive years had decreased urinary E_1C concentrations but continued to demonstrate cyclic peaks and nadirs, suggesting continued but diminished follicular activity. This typical cyclic pattern, with diminished peaks approximately 20 days apart, is shown for the mare M2E after six consecutive years of treatment (Fig. 6). The same type of urinary E_1C pattern is also seen in the mare X1 (Fig. 7) after seven years of treatment. However, M2E had urinary E_1C concentrations which were within the normal range. During the seventh year of the study, the mare M6I, which was withdrawn from treatment after the fifth year, had significantly higher ($p < 0.0001$) urinary E_1C concentrations (51.14 ng/mg Cr) than the five mares receiving seven years of treatment, but her mean urinary E_1C concentration was still significantly lower ($p < 0.005$) than four untreated control mares (Kirkpatrick et al. 1995a).

The results of this study have significance for the proposed management of the ASIS horses with PZP immunocontraception, and point to the importance of incorporating basic science studies, wherever pertinent questions arise, into applied scientific studies directed at management techniques. It was clear that the PZP vaccine was an effective inhibitor of fertility in wild horses and that it fit most of the characteristics of the ideal wildlife contraceptive outlined above. However, the decline in ovarian function, although not a debilitating health problem, suggested that permanent infertility was at least a possibility after 4 or more consecutive years of PZP treatment, and underscored the need to build in some sort of a safeguard as a management plan was formulated around the use of this vaccine. In short, any management plan would have to

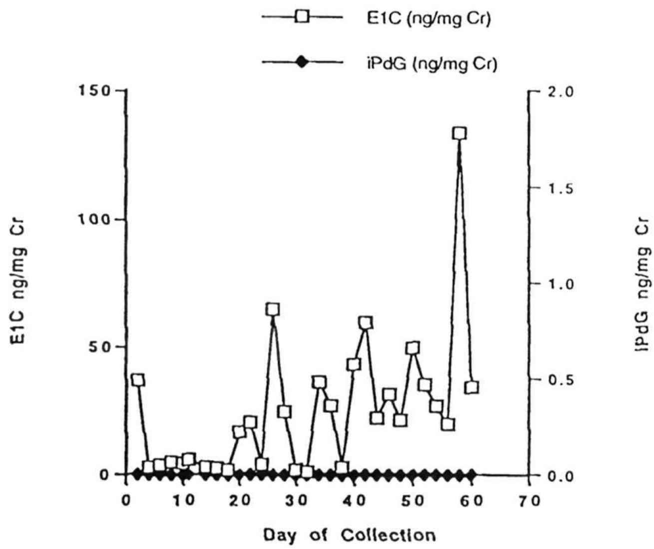


FIGURE 6. Profiles of urinary E_1C and iPdG for the mare M2E. After six consecutive years of PZP contraception there are no indications of a luteal phase iPdG pattern or ovulation, but cyclic E_1C patterns suggest the partial maturation of follicular pools. Day 0 was May 1, 1993.

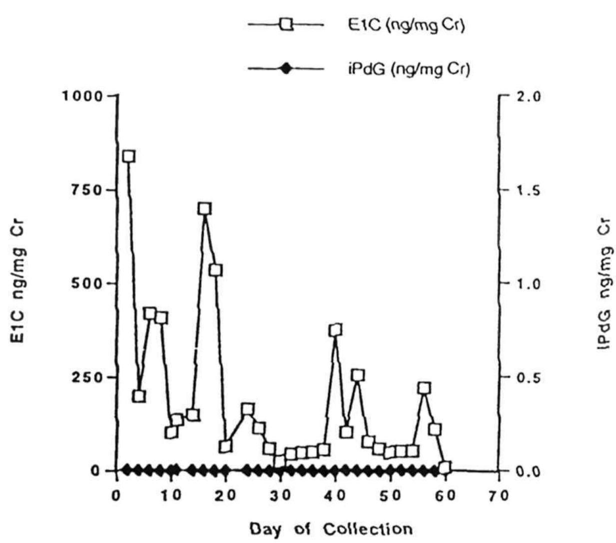


FIGURE 7. Profiles of urinary E_1C and iPdG for the mare X1, after seven consecutive years of PZP contraception. These profiles indicate cyclic estrogen waves appxoximately 20 days apart. Day 0 was May 1, 1994.

have a built-in period of treatment withdrawal in order to protect the reproductive and genetic integrity of the ASIS horses.

One-inoculation Experiment

The need to give animals at least two inoculations during the first year of treatment hindered the cost-effective use of this technique. Some mares, particularly those inhabiting the campground regions of the national seashore and the Maryland State Park, are very approachable and delivering a seond inoculation three to four weeks after the first inoculation poses little problem. However, many of the ASIS mares are not at all easily approachable and many hours, or days, can be required to deliver two inoculations to one animal. Thus, there was also a need was for a one-inoculation form of the vaccine.

Several technologies which permit slow sustained or pulsed releases of the drug exist which have been applied to both steroids and protein compounds. One of these approaches involves forming a homogenous mixture of the drug with a biodegradable non-toxic material in the form of microspheres (Eldridge et al. 1989; Wang et al. 1990). Upon intramuscular injection and contact with tissue fluids, the biodegradable material erodes and releases the drug over some predetermined period of time (Wang et al. 1991).

A study was designed to determine the effectiveness of a one-inoculation form of the PZP vaccine in Assateague mares. The PZP antigen was incorporated into lactide/glycolide microspheres, 10-50 μ in diameter, in a ratio of protein antigen to lactide/glycolide (weight:weight) by the process described by Wang et al. (1991). This lactide coating is very similar to the biodegradable coating used in the earlier experiments with steroid hormones.

In a preliminary experiment in March 1991, 10 domestic mares were used to determine the concentration and duration of anti-PZP antibody titers after inoculation with the PZP antigen or with lactide/glycolide microspheres containing PZP. On March 13, five mares received an initial inoculation of 65 μg of the PZP antigen emulsified in 0.5 ml of FCA, as previously described (Kirkpatrick et al. 1990a). Three weeks later, on 2 April, each of the five mares received a second inoculation of 65 μg of the PZP antigen emulsified in 0.5 ml of FIA. Both inoculations were given intramuscularly in the gluteal muscles, by hand injection.

The other five mares received a single inoculation of 65 μg of the PZP antigen emulsified with 0.5 ml of FCA on 13 March. The emulsification also contained an addtional 65 μg of the PZP antigen sequestered in 10-50

μ biodegradable lactide/glycolide microspheres described above. Release rates of the PZP from the microspheres were calculated to be continuous over approximately four weeks, with the greatest release rates during weeks one and four (Wang et al. 1990, 1991).

A blood sample was collected from each mare by jugular venipuncture at the time of the initial inoculation and at 20, 34, 55, 97, and 215 days following the initial inoculation. Serum was harvested and stored frozen until analysis for anti-PZP antibody titers. Antibody concentrations were determined by an ELISA assay as described by Voller et al. (1986) with modifications for horses as previously reported by Liu et al. (1989). Antibody results were expressed as a percentage of a positive reference serum, which consisted of a pool of sera with antibody titers in the medium to high range.

All 10 domestic mares had elevated anti-PZP antibody titers as a result of the PZP inoculations. Antibody titers for the 10 domestic mares are shown in Table 10. Within 20 days the antibody titers of all 10 mares were significantly ($p < 0.002$) elevated above pre-immunization levels and maximum titers occurred at 55 days post-immunization. At day-97, the antibody titers of all immunized mares ranged between 82% and 152% of positive reference values. These results suggested that there was a release of the antigen in the microspheres and that microsphere-sequestered PZP could provide similar antibody titers to a 2-inoculation protocol.

In an application of this research to Assateague, 14 previously untreated ASIS mares were darted with a Pneu-Dart 2.0 cc dart containing a single dose of the PZP vaccine which contained the PZP/lactide/glycolide microspheres described above between 1 March and 30 March 1992. These mares ranged in age from 3 to 17 years at the time of treatment and 12 were of known fertility. The emulsion containing the microspheres was administered with 1.0 cc self-injecting Pneu-Darts with a 1.5" 16 g. barbless needle, fired from a Model 171C Pneu-Dart capture rifle, at ranges of 30-45 m. All inoculations were given in the hip region. Thirty-seven untreated Assateague mares were selected for controls. The controls were not given sham injections because previous experiments have documented the lack of effects of sham treatment (Kirkpatrick et al. 1990a, 1991b, 1992a). During October 1992, seven months following their inoculations and four to six months following the breeding season, urine samples were collected from the mares as described by Kirkpatrick et al. (1992a) and analyzed for E_1C in order to determine pregnancy. During the summer of 1993, the mares were observed for the presence of foals.

TABLE 10. Anti-PZP antibody titers in domestic mares after one inoculation using PZP + PZP sequestered in lactide microspheres and after one inoculation of PZP followed by a booster three weeks later.

		Antibody titers (% of positive reference serum)					
Treatment	**Horse**	**3/13/**	**4/2**	**4/16**	**5/7**	**6/8**	**10/14**
FCA-PZP	Aldo	5	99	114	131	106	79
bolus + PZP	Mia	4	70	107	123	82	28
microspheres	Imp	6	33	70	95	98	131
	Lab	5	38	76	120	114	39
	Dee	5	56	108	106	99	26
FCA-PZP	Ant	4	90	130	131	152	115
bolus +	Con	6	66	145	135	144	58
FIA-PZP	Len	8	58	118	123	91	35
bolus 3	Rou	3	73	116	133	123	79
weeks later	Sou	4	67	147	126	136	94
Mean titer values		5	70.8	131.2	129.6	129.2	76.2

Both groups received their initial inoculations on March 13, 1991.
Booster inoculations were given on April 2.

Thirteen of the 14 Assateague mares were treated successfully with a single dart. One of the 14 darts did not discharge or inject (mare N9B) and 13 of 14 darts were recovered. One treated mare (Speckles) was killed by a car on the island causeway during the fall of 1992, but a urine sample was obtained from her a few weeks prior to her death. October 1992 urinary E_1C values indicated that two of the 14 mares (N2B and N9B) were pregnant. Foal counts during the summer of 1993 confirmed the urinary E_1C results and only N2B and N9B had foals at their sides. Of 37 untreated mares within the same age range as the treated mares, 16 produced foals (43.2%) during the same period of time, and the difference between foaling rates for the treated and untreated mares was significant ($p < 0.008$). These data indicate that a one-inoculation form of the PZP vaccine is effective in preventing pregnancy in feral horses if it can be delivered prior to the onset of breeding activity and not earlier than 200 days before the end of the breeding season. With that qualification, hypothesis Ho^5 was accepted.

Population Effects of Immunocontraception

A study was needed which would examine whether or not a population effect could be achieved through PZP immunocontraception. It would be of little importance if we were able to demonstrate that we could contracept wild mares, but we could not also demonstrate an ability to change population trends.

The ultimate objective in the use of fertility control in the management of wild horses is modification of population sizes. While it was clear that immunocontraception was effective in suppressing fertility among treated mares, it remains to be seen whether or not treatment of the entire ASIS population can be effective in changing the actual population dynamics of the herd. In 1991, Dr. Brian Underwood, National Biological Service, initiated preliminary population studies of the ASIS horse herd. A simulation model for the ASIS horse life-history was constructed using a Generalized Animal Population Projection System (GAPPS II v.3). This analysis utilized life histories which existed for the majority of horses on ASIS. These were started in 1975 by Dr. Ronald Keiper, The Pennsylvania State University, and maintained by NPS personnel and researchers since that time. The two purposes of the population modeling included a determination of whether or not contraceptive experiments through 1992 had had any effect on the population dynamics of the herd, and the creation of a model to predict the outcomes of varying treatment regimines.

Results showed a steady increase in the size of the ASIS herd, from a low of less than 40 animals in 1975 to a high of 165 animals in 1988. Between 1988 and 1992, the ASIS herd size decreased to 139 animals. While this period coincided with PZP treatment, there was a loss of approximately 40 horses due to two years of eastern equine encephalitis, which killed approximately 30 horses, and a catastrophic storm in January of 1992, which killed 10 horses on the northern portion of the island. These two events confounded the role of immunocontraception in the dynamics of the herd population. However, during that same period of time, immunocontraception experiments were responsible for the absence of approximately 30 new horses. On the basis of these data, hypothesis Ho^{8} remains untested. The continuation of this modeling will be vital to ultimately documenting the population-level effects of immunocontraception.

In 1995, an environmental assessment was prepared for management-level application of immunocontraception to the ASIS herd. The actual priorities for treatment of ASIS mares will follow a heirarchical approach that is based upon documented breeding success to ensure that all mares are given an opportunity to reproduce. For example:

High Priority for Treatment	– horses having produced two or more generations of surviving offspring,
|	– horses having produced more than one surviving offspring
|	– horses having produced one surviving offspring
Low Priority for Treament	– horses less than 4 years old
|	
No Treatment	– horses greater than 4 years old not having produced a surviving offspring

This approach was designed to maintain genetic diversity within the population during an initial period of intensive fertility control and it seeks to minimize the possibility that PZP-treatment will prevent any horse from ever reproducing. As management moves from an intense initial phase to a maintenance phase, fewer horses will require treatment and the selection process may be modified to introduce a measure of randomness and avoid the possibility that long-term immunocontraception will be biased toward horses with greater reproductive success. Additionally, no horses will be treated for more than three consecutive years, except for those having successfully produced at least three surviving offspring, or two generations of offspring. This approach is believed to offset the risk of permanent ovarian dysfunction from long-term PZP treatment.

Delivery Systems

With the initiation of the expanded studies in 1992, a new delivery system was tested. Incomplete injections of 1.5 cc of vaccine was less of a problem with the Pax-Arms self-injecting darts than injections of large volumes of microencapsulated steroids, but a significant number of darts still bounced out before injection was complete. The Pax-Arms darts, which are air-pressurized with a small hand pump, had side ports in the needle which prevented rapid injection of the viscous aqueous-oil vaccine emulsion. In 1992, Pneu-Dart darts, fired from a cartridge powered Pneu-Dart 50 cal. capture rifle were tested. The volume of vaccine was reduced to 1.0 cc and delivered in 1.0 cc self-injecting darts. These darts are small (95 mm) and light (5.4 g), inject through the front end of a 16 gauge needle, and are powered by a small powder charge. Injection is instantaneous and the darts pop out immediately after inoculation. Additionally, the rifle was capable of delivering the darts accurately up to 50 meters, even in the less than perfect conditions on ASIS. Gun discharge noise was less than that of the Pax-Arms gun and mares demonstrated less reaction. After 44 dartings in 1992, this delivery system was adopted.

Reasons for the Success of the Assateague Contraception Project

All too often, research projects carried out on public lands are viewed only in terms of success or failure and those outcomes are in turn viewed only in the context of successful or unsuccessful science. In reality, the success of important research on public lands, and in national parks in particular, results from a combination of good science, cooperation between the agency and the research team, clear understandings by both agency and team about the tasks and roles for which each are responsible, and the complex sociology of research team, agency, and visitor perceptions.

A retrospective view of the Assateague wild horse contraception study provides a picture of a research effort that is wholly unique and almost without precedent. The Assateague contraception research program was successful in achieving all its original goals, and the reasons for this success go well beyond the science and technology of the research itself. Firstly, considerable courage was demonstrated by the staff of ASIS and the regional NPS office by their willingness to explore unique but potentially controversial management practices with this valuable public resource. Both the scientific community and government agencies often seek "safe" solutions to big problems. These safe solutions do not often evoke strong public response but neither do they often advance the quality of the science or effectiveness of resulting management practices. In 1985, when the decision to pursue contraceptive research was made by ASIS, the field of wildlife contraception had little in the way of demonstrable success and, in fact, was quite controversial. Thus, the NPS was willing to embark upon high risk, but needed, research.

Secondly, the project's ultimate success rested upon the willingness of the NPS to pursue immunocontraception despite the only moderate success with the stallion steroid hormone experiment and the outright failure with the mare steroid experiment. The overwhelming history of federally-funded research in the United States is that failure immediately leads to a cessation of funding and therefore the work itself. In this case however, the park's Resource Management Specialist and Superintendent and the Regional Chief Scientist all concurred that the research should go forward as long as the scientific team believed that there were potential solutions. This faith shown by the NPS was a strong incentive to the research team to find a workable solution.

Thirdly, the research team, which had a 15 year record of research contracts with various federal agencies, was struck by the clear "can-do" attitude of the ASIS staff. Logistical support was always available as promised, emergency vehicle maintenance was carried out with dispatch, and all necessary steps were taken to clear hurdles to the research. Prior to any treatment phases, memoranda were sent to all law enforcement personnel within both ASIS and the Maryland State Park, informing officers that the researchers would be present and carrying capture guns. This avoided unpleasant interactions with park personnel and visitors. When boats were needed instead of vehicles, they were made available immediately, and in instances where additional personnel were needed, they were provided by the park in a timely fashion. Special clothing for protection from insects and safety considerations while working along roadways was provided by the ASIS staff. Radio contact between researchers and ASIS personnel was maintained, and ASIS staff even participated in time saving efforts by reporting the locations of particularly elusive horses. Perhaps the single most important dimension of this cooperative spirit was the willingness of ASIS staff to change or modify day-to-day horse management practices in order to improve the team's chances of success. For example, horses grazing along the grassy shoulders of the main road through the park, and particularly on the busy causeway, pose both a law enforcement and a safety problem. Despite numerous warnings, visitors stop in restricted zones and feed the animals, thereby causing "pony jams". To combat this problem, rangers from both ASIS and the state park chase horses from the roads. At the same time, the collection of urine samples is a much easier task for the researchers when the horses graze in these locations. A workable solution was found when ASIS provided researchers with safety vests and NPS hats, and researchers responded by keeping traffic moving and preventing visitors from feeding the animals. It was apparent throughout the nine years of research that this project was a high NPS priority and that ASIS staff were going to do everything possible to facilitate the contraceptive project. This cooperative spirit reflected itself in financial ways as well. The first six years of contraceptive research carried out on ASIS cost less than $6,000 annually in direct costs.

A fourth and very important factor in the success of this project was a strong NPS background by the research team. Invasive wildlife research is a difficult task anywhere, but particularly so in national parks. Interactions between visitors and the research team are inevitable and these experiences are not always positive. Visitors often resent the special access enjoyed by researchers, or the nature of the research itself. Researchers on the other hand often resent interference with their work, intended or unintended, by visitors. Previous employment by one of the team, as a ranger with Rocky Mountain National Park, provided a frame of reference for the research team that placed the visitor as the "owner" of a public resource and the research team as "guests". Put another way, the research team viewed itself first as representatives of ASIS and the NPS, and second as researchers. It was understood that all actions by the research team reflected upon the NPS.

The fifth major reason for success was the effort put forth by ASIS in developing an education program that fully informed the public of the reasons and the nature of the research. At the initiation of the project in 1986, it was made clear by ASIS staff that as much research as possible should be conducted out of the public' s view. It was also recognized that this was not always possible and that when work had to be conducted in view of the public, the visitors were to be told the exact nature of the work and the reasons for this work. A video tape of the actual darting of the horses was made by ASIS staff for educational purposes, and a brochure which described the research project was written for distribution at the visitor center. Finally, the research team met with the park's interpretive staff annually, taking them into the field to witness the work firsthand, explaining each year's progress, and providing props, such as expended darts, for educational purposes. Campfire programs included a description of the research.

It is often the case in national parks that researchers in the field will have more interaction with the public than the interpretive staff. As such, the research team again becomes a represenative of the NPS as well as an important educational resource. This was clearly the case on ASIS, at least during the times when actual research was being carried out. The darting of a horse or the collection of a urine sample always evoked curiosity and questions by visitors and the research team took the time to explain everything in a clear and comprehensive fashion, even to the point of slowing down their own work.

The end result of this educational effort by ASIS and the NPS background of the research team was a potentially controversial project that never once came under public criticism. Over the nine-year course of the work, the public had only two major questions when confronted with the research project. Is the NPS trying to eliminate the horses from Assateague, and are the horses being hurt in the conduct of the research? The educational efforts by ASIS made it clear that this was an effort to responsibly, and humanely, test a management option to control the horse population in order to protect the island's other resources. An ancillary but important benefit of the educational efforts was support by numerous animal welfare and animal rights organizations, demonstrated by numerous positive articles in the organizations' newsletters. In two cases, moderate financial support from a large animal protection group aided the ASIS research. The willingness of the NPS to seek a humane, as well as an effective, solution generated additional educational efforts by these organizations through their publications, and further goodwill between the public sector and the NPS.

Collectively, these were the factors that made the Assateague horse contraceptive project so successful. The implications of this success and the reasons for it go well beyond ASIS. Today, with numerous other wildlife contraceptive projects already in progress or in the planning stages, the design of the research follows "the Assateague model" with regard to the many organizational, attitudinal, and sociological dimensions of the projects.

Contributions of the Assateague Project to Science and the NPS

The influence and implications of the Assateague wild horse contraceptive project ultimately reached far beyond ASIS and its popular horses. The immediate scientific outcome of the ASIS wild horse contraceptive project was the first demonstration that an entirely new form of fertility control, immunocontraception, could be successfully applied to free-roaming wildlife. Largely because of the very limited successes or failures with hormone-based wildlife contraceptive trials in previous years, the first report of the ASIS project (Kirkpatrick et al. 1990a) generated a great deal of excitement throughout scientific communities. This in turn led to an expansion of trials with the PZP vaccine by our own team and an immediate surge in research by other groups.

Application in wild and feral equids was of immediate interest. After investigating the outcome of the ASIS experiments, the Australian government mounted a twelve million dollar effort to develop a recombinant form of the PZP vaccine for the control of equids and various other forms of feral animals on that continent. New Zealand is investigating the use of the PZP vaccine among the Kaimaniwa wild horses. Soon after the publication of the initial results on ASIS, a joint effort was mounted by the NPS and The Humane Society of the United States to test PZP contraception in non-seasonally breeding feral donkeys in Virgin Islands National Park. In the initial phase of this project, 16 donkeys were remotely immunized and only a single animal produced a foal (J. W. Turner, Jr., unpublished data).

In June, 1991, a Senate subcommittee on the control of western wild horses invited the research team to testify regarding the success of the ASIS experiments. As a direct result of this hearing a large scale contraceptive research effort was mounted by the Bureau of Land Management. This project had two phases. The first was a trial of the PZP vaccine in the management of Nevada wild horses. In December 1992, 500 wild horses were captured in the Ely district and hand-injected as they passed through chutes. The preliminary results indicated that treatment was successful (J. F. Kirkpatrick, unpublished data). The second phase involved continued development of a one-inoculation form of the vaccine. This research is seeking to exploit the development of PZP-micro*capsules*, as opposed to microspheres. The microspheres, which consisted of a homogenous mixture of PZP and lactide/glycolide matric release the PZP in a sustained fashion. The microcapsules, in contrast, release the PZP as "pulses". This work is in progress and is aimed at the large-scale control of western horses.

A second outcome of the ASIS experiments was the application of this contraceptive technology to captive exotic animals in an effort to alleviate the surplus animal problem faced by zoos. At the present time, over 60 species are being treated in more than 40 zoos on three continents (Kirkpatrick et al. 1992b; 1993b, 1995b). Positive results have been documented thus far in over 30 of those 60 species, and the results for the remaining species are still pending. In less than three years after its initial use in zoo animals, the PZP vaccine has become the second most widely used form of contraception in captive exotic species.

In the course of the zoo animal experiments, it became clear that the PZP vaccine caused significant contraceptive protection among members of Cervidae. The problem of high populations of white-tailed deer in urban areas has in recent years reached epic proportions and it was decided to test the PZP vaccine in deer. Initial trials with captive deer indicated the same high degree of contraceptive efficacy as with equids (Turner et al. 1992). Further trials with captive deer (Turner et al. in press) indicated that the contraceptive effects were reversible, a single annual booster inoculation was effective, antibody responses to the vaccine were similar to that in horses, and there were no health side effects (Turner et. al., 1995; in press). Following these experiments with captive deer, field trials were conducted with free-roaming white-tailed deer at the Smithsonian Institute's Conservation and Research Center in Front Royal, VA, and within selected communities on Fire Island National Seashore. These trials are still underway, but intitial results are promising and suggest that PZP immunocontraception may become an important management tool for urban white-tailed deer. Deer immunocontraceptive projects are now in the planning stages for numerous communities, arboretums, federal campuses in the Washington, D.C. area, military bases, city parks, and at least one Canadian national park.

The success of the ASIS wild horse experiments and those with other captive and wild species stimulated interest in PZP immunocontraception by other research groups. Dr. Robert Warren, University of Georgia, has initiated research into different delivery systems for the PZP vaccine. Trials have been conducted with lyophilized PZP packed into biodegradable biobullets (Willis et al. 1994). Porcine zonae pellucidae trials with free-roaming black-tailed deer were conducted by a research group at the University of California-Davis. Dr. Bonnie Dunbar, Baylor University School of Medicine, produced recombinant forms of the vaccine for trials with deer, and the U. S. Department of Agriculture and the Denver Wildlife Research Center, in collaboration with Dr. Dunbar, have initiated research into forms of the vaccine which might be delivered orally.

There were also human medicine implications which arose from the ASIS experiments. The PZP vaccine has long been a candidate for a human contraceptive vaccine (Sacco 1987). One of the few remaining concerns about the use of this vaccine in women is the possibility of long-term effects of the antibodies upon the ovary and the possibility of irreversible infertility, similar to that seen in dogs (Mahi-Brown et al. 1985) and rabbits (Wood et al. 1981). By 1991, the ASIS experiments were the longest running PZP vaccine experiments ever conducted with any species and the opportunity to examine long-term effects availed itself with the ASIS horses. The Reproductive Biology section of the National Institutes of Health (NIH) invited and ultimately funded a proposal from the research team to study these long-term effects using the ASIS horses as a model species.

Aside from advances directly related to immunocontraception, the ASIS project also created an entirely new approach to the study of reproduction in free-roaming wildlife through the development and application of urinary and fecal steroid metabolite analysis. In 1986, when the ASIS contraceptive project was initiated, no free-roaming species of wildlife had ever been pregnancy tested remotely, nor had the estrous cycle of any free-roaming species been characterized without capture. The first remote pregnancy testing was accomplished in wild horses, using urinary E_1C concentrations (Kirkpatrick et al. 1988), in preparation for the ASIS contraceptive research. Following this breakthrough, the technology was applied directly to the ASIS horses (Kirkpatrick et al. 1990a; 1991b). Soon after, advances in remote testing techniques were developed that included the ability to measure progesterone metabolites in equids (Kirkpatrick et al. 1990c), something that had not been accomplished prior to this time. Other advances included fecal analysis of both free estrogens (Kirkpatrick et al. 1990b) and steroid metabolites (Kirkpatrick et al. 1991a).

These advances in remote pregnancy testing were also applied to the Assateague horses to investigate questions of basic biology. One study examined the issue of induced abortion in pregnant mares caused by new band stallions (Kirkpatrick and Turner 1991c) . A previous study (Berger 1983) had suggested that when a new stallion takes over a band, he repeatedly "rapes" pregnant mares and induces them to abort. This hypothesis fits sociobiological models, but the original research made assumptions about pregnancy in mares, based on witnessed breedings, which is very unreliable in horses. When this phenomenon was reexamined on Assateague, using the urinary pregnancy testing techniques, it was shown that the phenomenon really did not exist in horses. A second study investigated the phenomenon of compensatory reproduction (Kirkpatrick and Turner 1991a) and demonstrated that the removal of young horses only stimulates reproduction among mares from which those foals were removed. The excitement by other field biologists which resulted from these reports of non-capture reproductive studies in free-roaming wildlife led to a rapid application of this technology to other species, including muskoxen, black rhinoceros, caribou, and even mountain gorillas (Lasley and Kirkpatrick 1991; Kirkpatrick and Lasley 1993).

Our own research team applied this technology to studies of reproduction in bison in Yellowstone National Park, and were soon able to detect ovulation by means of urinary progesterone metabolites (Kirkpatrick et al. 1991c), pregnancy (Kirkpatrick et al. 1992c), and even fetal loss (Kirkpatrick et al. 1993c). The ability to gather these types of data without capturing or immobilizing bison in the middle of a national park was the direct outgrowth of the development of this technology with the Assateague horses.

In March 1994, 76 mares on ASIS were immunized in the initial step of a comprehensive wild horse management program. The purpose of this treatment was to cause antigen recognition within the entire herd, establishing them as one-inoculation animals in the future. In March 1995, 68 of those mares were given their second inoculation in the first serious attempt at slowing reproduction. This marks the very first time that contraception has been applied to any population of wildlife in order to control its growth. In future years, attempts will be made to reduce the ASIS wild horse herd from the present (January 1995) 168 animals to 150. Following the attainment of that goal, the focus will shift to balancing births with mortality and the maintenance of the population at 150. Additionally, mares which have been immunized for four, five, and seven consecutive years will no longer be treated and will be monitored for the resumption of ovulation and succcessful pregnancies in order to assess the reversibility of four to seven consecutive years of treatment. A great deal of planning must yet be accomplished in order to insure that the ASIS herd will be sustained as a healthy, genetically viable population. Despite the additional work ahead, the Assateague contraceptive research has provided a useful tool for humane and publicly acceptable management of coastal barrier island wild horse populations.

Conclusions

1. Two initial inoculations of porcine zonae pellucidae can inhibit fertility among female wild horses for up to one year,
2. The PZP vaccine can be delivered remotely from ranges of 10 to 50 m by means of self-injecting darts,
3. The PZP vaccine is safe to give to pregnant mares, will not interupt pregnancies underway, and will not interfere with the health of foals exposed to either the PZP vaccine or its resultant antibodies *in utero*,
4. PZP treatment does not alter social organization of the horses,
5. Mares who were exposed to the vaccine and the resultant antibodies *in utero* are fertile upon reaching sexual maturity,
6. The contraceptive effects of treatment for up to 3 consecutive years is reversible,
7. A single annual booster inoculation of PZP will sustain contraceptive effects for a additional year,
8. A single initial inoculation of PZP microencapsulated in glycolide/lactide microspheres can inhibit fertility in mares for one breeding season,
9. Four to 7 consecutive years of PZP contraception will cause changes in ovarian function, including depressed estrogen production and anovulation,
10. Pregnancy can be diagnosed accurately in non-captured wild mares by means of steroid and steroid metabolite analysis of urine and fecal samples,
11. Ovulation, and therefore ovarian function, can be monitored remotely in non-captured wild mares by means of urinary and fecal steroid analysis.

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