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FERTILITY CONTROL FOR WILD HORSES: IMMUNOCONTRACEPTION

JOHN W. TURNER, JR., Ph.D.
MEDICAL COLLEGE OF OHIO

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I am pleased to see this Wild Horse Forum occurring. The recent history of the feral horse, especially in the western United States, has been a time of great difficulty. The greatest difficulty has of course been for the horses, which have been caught in the dusty storms of powerful politics and management decisions with little or no data base. The hard times for the horses have been intensified by the availability of only one management tool - roundups, followed by sale and/or slaughter.

However, I have optimism that we can leave much of that behind as we turn to what is happening now and how it may brighten the feral horse future. The subject which I will address is fertility control, with a focus on immunological or vaccine-based contraception. Since our research team has been studying feral horse reproduction for many years, I would like to explain how we have come to immunocontraception.

It was 1973 when my colleague Jay Kirkpatrick, at Eastern Montana College, and I, at the Medical College of Ohio, first became aware that feral horse populations were rising. We thought of contraception early on, but had to first explore the literature and further develop a database on feral horse reproduction and behavior. By 1978 we were ready to test several contraceptive agents in captive ponies at the University of Pennsylvania Veterinary School. Our initial focus was on stallion contraception, since our own field studies and most reports in the literature at the time indicated that harem studs generally maintained good harem integrity and did not permit breeding by subordinate or outside males. We also focused on sex steroids, which seemed most promising at the time. At the University of Pennsylvania we performed a study with domestic ponies which demonstrated that a timed-release version of the male sex hormone, testosterone, effectively inhibited sperm production while maintaining harem-related behavior. Between 1980 and 1984 we did field testing and follow-up on this agent, called microencapsulated testosterone propionate (MTP) in the Challis horse range in

unacceptable. While potent synthetic estrogens might be effective in smaller volume, they exhibited poor biodegradability and could be passed through the food chain, a consideration of special concern regarding wildlife which might eat the carcass (including protected species such as the golden eagle). We also considered that the USDA, which has banned the use of estrogenic steroids in cattle, would disallow the use of these steroids in any species which may be consumed by humans, including horses and deer. Both these species are candidates for management by fertility control and are also established food sources in various countries. Many of the horses already removed from western U.S. ranges are purported to have become human food. At this time in our research, an alternative, non-steroidal contraceptive seemed highly desirable.

Interestingly, as a result of information exchange at a 1986 conference in Bishop, California on feral horses, we became aware of a non-steroidal, immunological contraceptive. This vaccine, prepared by Irwin Liu, at the University of California, Davis, was highly potent and effective in small volumes, making it a good candidate for remote delivery. Because the vaccine seemed very promising for our intended applications, we embarked on a collaborative study to field test it on feral horses inhabiting Assateague Island National Seashore. For those who wish to read the scientific details of our feral horse contraception studies, I have attached a copy of 2 of our recent *Wildlife Society Bulletin* publications (References # 1 and 2). I will however, outline the important aspects of this work below.

The most widely studied contraceptive vaccine, and the one we used, is called PZP. This is the abbreviation for porcine zona pellucida, which is the coating around the eggs from pig ovaries. This coating plays an important role in fertilization of the egg by the sperm. If some of this PZP is injected into females of another species, that species will make antibodies against its own ZP which will bind to the ZP and thereby prevent sperm from fertilizing the eggs. This type of vaccine has been used successfully in a number of species, including baboon, monkey, rabbit, rat, dog and horse. In a study using domestic mares, our colleague, Dr. Liu, demonstrated that the PZP vaccine effectively inhibited fertility in 13 of 14 treated mares and that the effect lasted approximately one year. When the mares were bred a year later, pregnancy rates were again normal, and healthy foals were born. With this encouraging database we began the Assateague field study.

The Assateague feral horse population has been on the island for several hundred years, and since the late 1970's population and lineage records have been

recently developed adjuvant that will not cause abscesses. The second disadvantage is that complete immunization has required 2 injections, about 3 weeks apart. This is clearly unacceptable for management purposes, and we have been developing a timed-release vaccine which will provide complete immunization in a single injection. The single injection will contain the initial inoculation plus a second dose sequestered in a bioedegradeable polymer matrix (much like timed-release cold capsules) which will breakdown in the body over a 3-week period, releasing the second dose of vaccine.

We have tested this timed-release pattern of vaccination in a preliminary study in 3 mares and have produced levels of PZP antibody identical to antibody levels in successfully contracepted mares given the usual 2-injection protocol. The final bioengineering of this timed-release vaccine is underway. We are also pursuing the engineering of a 2-year vaccine, similar to the above timed-release vaccine, but also containing a timed-release booster dose which will release after 1 year. The biotechnology for this capability already exists, but the formulation for our specific vaccine still must be developed.

Since many participants in this wild horses form are involved in management, I would like to share a breakthrough in the monitoring of population reproductive function which has come from our contraceptive work. As I have stated, we strongly believe that future management of feral horses must be done with a minimum of handling. We have therefore developed methods for monitoring reproductive status, including pregnancy testing and estrus cycles, via measurement of sex steroid metabolites in urine and feces collected from the ground. This technology has proved of great value in answering questions such as contraceptive effectiveness and reversibility without having to wait for foaling each year. The levels of sex hormone metabolites in urine and feces increase sufficiently to be detectable by 1-3 months of pregnancy. For those who are interested in the details of these studies I have included information from 3 representative journal papers (References # 3,4,5) which we have published on this subject.

As a final comment I would like to direct your attention to a more philosophical aspect of the wild horse contraception issue. Dr. Jay Kirkpatrick and I have been pushing for fertility control for feral horses since 1975. Now that it is finally on the horizon we must begin to address some important concerns regarding its use. We strongly believe that any fertility control method requiring capture with immobilization or restraint is unacceptable. We have seen it and have participated in it. As a consequence we have dedicated

ourselves to finding alternatives.

Part of the intellectual evolution of humans has involved the development of beliefs about how people should act. These beliefs are often expressed by the words "civilized" and "humane." We have talked of supporting the rights of animals for a long time, and we now stand at the threshold of putting words into action regarding the wild horse. I believe that there is only one thing that may stand in the way of achieving our positive human potential in this issue - our narrow perspective. Remember, the whole issue of non-lethal control centers around humane treatment. If the method of control is not humane, then what has been accomplished?

NOTE: The complete journal articles for the topics addressed in this paper are on the pages which follow.

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REMOTELY-DELIVERED IMMUNOCONTRACEPTION IN FERAL HORSES

JAY F. KIRKPATRICK, *Department of Biological Sciences, Eastern Montana College, Billings, MT 59101*

IRWIN K. M. LIU, *Department of Reproduction, School of Veterinary Medicine, University of California, Davis, CA 59616*

JOHN W. TURNER, JR., *Department of Physiology, Medical College of Ohio, Toledo, OH 43699*

Reducing fertility among free-roaming feral horses (*Equus caballus*) has been the goal of numerous studies over the past 16 years (Kirkpatrick et al. 1982, Goodloe et al. 1988, Plotka et al. 1989). Initial experiments by Kirkpatrick et al. (1982) and Turner and Kirkpatrick (1982) resulted in an 83% decrease in foaling among feral mares bred by stallions which were first immobilized and then treated with injectable microencapsulated testosterone propionate (mTP). Although the treatment decreased sperm count and motility, the high costs and the stress caused by immobilization or capture made it clear that the contraceptive agent needed to be delivered remotely. In a second study Kirkpatrick and Turner (unpublished data) demonstrated the pharmacological effectiveness of mTP in stallions, but difficulty was encountered in remotely delivering a sufficient mass of the steroid.

Recently, attention has turned to contraception in the feral mare. Experiments with ethinylestradiol-progesterone Silastic® implants (Vevea et al. 1987, Plotka et al. 1988, Plotka et al. 1989) showed pharmacological promise, but the technique required capture, restraint, and field surgery to place the implants intraperitoneally. An alternative to steroid-induced fertility control is immunocontraception. A conjugated form of luteinizing hormone releasing hormone (LHRH) has been used successfully to raise antibodies in captive feral mares (Goodloe et al. 1988), and solubilized porcine zonae pellucidae (PZP) injections inhibited fertility in 13 of 14 domestic and captive feral mares (Liu et al. 1989).

The success of the PZP vaccine in suppressing fertility is based on its ability to inhibit fertilization or possibly implantation (Sacco et al. 1984). The porcine zona pellucida consists of 3 glycoproteins. One of those, ZP3, is the receptor molecule for sperm surface molecules (Florman and Wassarman 1985). Equine antibodies raised against PZP are thought to block the sperm receptor sites on the equine ovum, thereby preventing fertilization (Liu et al. 1989). To date, PZP has been used to successfully inhibit fertility in a number of mammals, including 5 species of nonhuman primates and in vitro fertilization in humans (Sacco 1987).

The objectives of this study were to (a) determine the effectiveness of remote delivery, (b) test the contraceptive effectiveness of a PZP vaccine in free-roaming feral mares, (c) determine the contraceptive effectiveness of the vaccine in pregnant and nonpregnant mares, and (d) evaluate the safety of the vaccine for use in pregnant mares.

MATERIALS AND METHODS

Forty-six sexually mature mares were selected for the study from among the approximately 100 feral mares inhabiting Assateague Island National Seashore, Maryland. The ages and fertility records, some dating back as far as 1974, were known for almost all animals on the island (Keiper and Houpt 1984). Ages ranged from 3 to 18 (mean = 9.12, SD = 4.45 years). The mares chosen for treatment were not randomly selected. Instead, they were selected because of their high fertility rates, which averaged about 10% higher (51.7%) than the overall herd rate (approximately 40%) annually for the preceding 3 years. The PZP vaccine was prepared from porcine ovaries (Liu et al. 1989) and stored frozen at −5 C until used in the field.

The inoculation was prepared as an emulsion, of 0.5 cc of vaccine (equivalent to approximately 5,000 zonae or 64.3 μg of protein) in phosphate buffer and 0.5 cc of Freund's Complete Adjuvant. The second and third inoculations were the same as the first except for the addition of 0.5 cc of phosphate buffer solution and substitution of 0.5 cc of Freund's Incomplete Adjuvant for the complete adjuvant. The 2 vaccine components were mixed in the field, using 2 10-cc glass syringes joined with a plastic connector. After 100 strokes the emulsion was loaded into a 3.0-cc self-injecting plastic dart which was tipped with a 3.81-cm barbless needle. The needles were rinsed with 70% EtOH prior to being loaded into the rifle.

National Park Service regulations prohibited the capture or handling of any horses during the course of the study. Twenty-one mares were darted from the ground, at distances from 25 to 30 meters, in the hip region, using a Pax-Arms® 0.527-caliber capture gun. Between 29 February and 10 March 1988, 26 mares received an initial inoculation of vaccine. Eight of the mares were acclimated to humans, and the initial vaccine delivery was accomplished with a 3-cc syringe and a jab-stick and thereafter by dart. Between 12 and 21 March, 26 of the 29 mares received a second inoculation by dart, as described above. Three of the mares became extremely wary, could not be approached for the second inoculation, and were dropped from the experiment. Between 16 and 25 April, 18 of the 26 mares which received the second inoculation received a third inoculation, which was identical to the second. The 6 control mares, which were selected from the original 46 mares, received only phosphate buffer and adjuvant in 2 inoculations, between 3 March and 29 March. Identifying markings were recorded for each horse, and the animals were observed throughout April for abscesses at the sites of injection.

A minimum of 2 inoculations is required in horses in order to raise sufficiently high antibody titers for a minimum of 6 months (Liu et al. 1989). The schedule of inoculations used in this study was based on the spacing of inoculations in the 1 previous study with horses (Liu et al. 1989) and the breeding and foaling activity patterns of the Assateague horses, which peak in May and June (Keiper and Houpt 1984). The first inoculation causes antigen recognition and temporary increases in antibody titers. The second inoculation causes increased titers which last several months, and each subsequent inoculation increases the duration of high titers.

During October 1988, 5 months after the last inoculation and 2 months after the breeding season, the mares were located and identified, and the number of foals was recorded. Urine samples were collected from each of the 26 treated and 6 control mares, without capture, by extracting the urine from the soil or aspirating it directly from the ground immediately after urination. The urine samples were assayed for estrone conjugates (E_1C) and indexed to creatinine (Cr) concentrations (Kirkpatrick et al. 1988) and for nonspecific progesterone (Po) metabolites (iPdG) (Kirkpatrick et al. 1990). Pregnancy determinations were made on the basis of the urinary E_1C and iPdG concentrations.

In August 1989, the mares were again located, identified, and observed for the presence of foals. The 1989 foal production for the treated mares was compared to foal production (1) for the same group of mares for 1987 and 1988, (2) for the 6 control mares for 1989 and (3) with 11 untreated mares for 1989. The validity of these comparisons is based on long-term records of reproductive success among the Assateague horses (Keiper and Houpt 1984) which demonstrate that foaling patterns are consistent from year to year and that the probability of a mare having a foal is independent of her foaling success the previous year. Finally, in August 1989, a random sample of 7 uncaptured treated mares was tested for pregnancy by means of urinary steroid metabolites (Kirkpatrick et al. 1988, Kirkpatrick et al. 1990) in order to test reversibility of the vaccine's antifertility effect. Differences in foaling rates among treated, control, and untreated groups were tested for significance by means of binomial probability distribution (Freedman et al. 1978:231, 236).

RESULTS

Three abscesses were observed among the 26 horses treated. The abscesses appeared at the site of injection approximately 48 hours following the third treatment, were about 10–25 mm in diameter, and drained from 6 to 9 days after treatment. Complete healing had occurred within 14 days following treatment.

Of the 26 treated mares, 14 were pregnant at the time of inoculation (57.6%) and all 14 produced foals in the spring of 1988, approximately 1–3 months after the last inoculation of PZP vaccine. The 6 control mares produced 2 foals in 1988. By October 1988, a foal belonging to 1 of the treated mares had disappeared and was presumed dead. Another foal belonging to a treated mare died during the fall of 1988 as a result of a leg injury. All other foals born to treated or control mares were in good health in August 1989 as yearlings. During the 18 months following inoculation, only 3 mares moved to different bands.

Urinary E_1C and nonspecific Po metabolite concentrations in mid-October 1988 indicated there was 1 pregnancy among the 26 treated mares. None of the 18 mares receiving 3 inoculations were pregnant, and 1 of 8 receiving

Table 1. Foaling rates for treated and untreated mares for pretreatment and post-treatment years, Assateague National Seashore, 1987 through 1989.

Group	Inoculations/ horse	No. horses	% of mares producing foals (no. foals)		
			Pretreatment		Post-treatment
			1987	1988	1989
Treated	3	18	50.0 (9)	51.1 (11)	0.0 (0)
Treated	2	8	62.4 (5)	37.4 (3)	12.4 (1)
Control	0	6	33.3 (2)	33.3 (2)	50.0 (3)
Untreated	0	11			45.4 (5)

2 inoculations was pregnant. Three of 6 control mares were pregnant. Mean urinary E_1C and iPdG concentrations of nonpregnant treated and control mares (0.12 ± 0.35 SE μg/mg creatinine [Cr] and 3.42 ± 0.486 ng/mg Cr, respectively; $n = 28$) were lower than those of pregnant mares (3.41 ± 0.723 μg/mg Cr and 227.82 ± 89.7 ng/mg Cr, respectively; $n = 4$) ($t = -12.59$, 30 df E_1C; $t = -9.47$, 30 df, iPdG, $P < 0.001$).

By August 1989, 1 and 3 live foals were present among the 26 treated and 6 control mares, respectively, as precisely predicted by the urinary hormone metabolite measurements (Table 1). Post-treatment foaling rate for the treated mares (3.8%, $n = 26$) was less ($P < 0.002$) than that for the 2 pretreatment years (53.8%), for control mares in 1989 (50.0%), and for untreated sexually mature mares in the study area in 1989 (45.4%). Three of 7 randomly selected treated mares were determined to be pregnant in August 1989, based on urinary estrone conjugates and iPdG.

DISCUSSION

The choice of Freund's Complete Adjuvant for the first inoculation and Freund's Incomplete Adjuvant for the second and third was based on the work of Liu et al. (1989). While only 3 abscesses were noted in this study, the evaluation of other adjuvants which are less likely to cause abscesses is an important direction for future research.

No previous studies have been conducted in which pregnant animals of any species were vaccinated with PZP. In this study the immunosuppression which accompanies pregnancy did not interfere with the effectiveness of the antifertility effects of PZP vaccine, the pregnancies were successful, and the foals healthy. These are important considerations because the use of this vaccine for management will likely include pregnant mares among the treated animals.

A major advantage of the PZP vaccine is the small volume required and the aqueous base, both of which facilitate administration by dart. Remote delivery eliminates the need to capture horses, the attendant costs, and the likelihood of injury to horses, although our experience did not include long-distance darting of extremely wary feral mares. An advantage of PZP is the reversibility of the vaccine's contraceptive effects. Liu et al. (1989) demonstrated that captive treated horses that failed to conceive after PZP treatment could breed successfully the following year, as did at least 3 of 7 free-ranging mares in this study. The issue of reversibility is politically as well as biologically important because it is unlikely that public opinion will favor irreversible sterilization among feral horses.

A final advantage of the PZP vaccine is the protein nature of the contraceptive antigen. This characteristic precludes the possibility of passage of the antifertility agent through the food chain. In most circumstances some treated animals will die from natural causes and a variety of predators and scavengers will feed

upon the carcass. Protein, unlike steroids, and particularly synthetic steroids, cannot be accumulated intact in the predators' and scavengers' tissues. In addition, protein vaccination avoids urinary and fecal contamination by poorly metabolized steroids, and especially those synthetic estrogenic steroids which have high potency and high resistance to biodegradation.

Despite the return of normal fertility among PZP-treated horses reported by others and in this study, the long-term effects of continuous PZP immunocontraception have not been described. In the domestic rabbit (*Oryctolagus cuniculus*) (Wood et al. 1981), the domestic dog (*Canis familiaris*) (Mahi-Brown et al. 1985), and the baboon (species not given; Dunbar et al. 1989) there are data that suggest the antibody response of the treated animal attacks not only the mature ovum, but oocytes and other ovarian tissues, with resulting changes in estradiol and progesterone secretion. These effects have not been demonstrated in any of the 4 other species of nonhuman primates studied or in horses. Histological studies of ovaries among captive PZP-treated horses revealed no changes 3 years after treatment, and plasma progesterone values during treatment were consistent with normal cyclicity (Liu et al. 1989).

Behavioral integrity of treated animals is important, particularly in the case of social animals such as the horse. Bands with treated horses remained intact during the 18-month duration of this study, and the exchange of 3 mares between bands was within accepted limits for the Assateague herd during the previous 3 pretreatment years.

These results suggest that PZP immunocontraception is a possible alternative for controlling fertility in feral horse populations. However, the requirement for at least 2 inoculations for successful fertility inhibition is a weakness, and the current limitations of remote delivery are impediments for the use of PZP in management. The 3 mares which received only 1 inoculation were extremely wary and not approachable for a successful second inoculation. If this form of immunocontraception is to become an effective management tool for controlling feral horse populations, it must first be developed as a single-dose vaccine. Technology to convert the PZP antigen into a single-dose vaccine currently exists in the form of microencapsulation. This process, which provides a sustained release of drug, has been used successfully with contraceptive steroids (Kirkpatrick et al. 1982) and antigenic protein (Eldridge et al. 1989). Recently, the specific porcine zona antigenic proteins have been produced with monoclonal tissue cultures, eliminating the need for time-consuming preparation from fresh ovarian tissue and providing a potentially inexpensive source of the vaccine (Takagi et al. 1988). The effectiveness and safety of this form of immunocontraception can also be improved through the use of monoclonal proteins, because the pure receptor protein, ZP3, can be produced instead of the entire spectrum of zonae proteins which were used in this study. Experiments are under way to assess the effectiveness of a single annual booster inoculation, once antigen recognition has occurred. If a booster is effective, as it appears to be on the basis of urinary steroid metabolites, it is probably possible to incorporate the booster in an initial inoculation which delivers an initial bolus of antigen, a second pulse of microencapsulated antigen a month later, and the microencapsulated booster a year later.

CONCLUSIONS

This study provides the first description of successful fertility inhibition among uncaptured free-roaming mammals by means of remotely delivered immunocontraception. Remote inoculation of feral mares with PZP was an effective means of fertility inhibition and did not affect intact pregnancies. The process was reversible, it did not affect social integrity

of horse bands, and the vaccine cannot be passed through the food chain. The impact of PZP contraception is on fertilization, and no hormones are involved which might impinge upon the brain and change behavior directly. Coupled with remote pregnancy testing by means of urinary and fecal steroid metabolites, the remote delivery of PZP offers a potential noncapture technology for feral horse contraception. This in turn makes public acceptance of contraceptive control of mammalian wildlife more likely than with approaches that require capture and handling.

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"IN MY EXPERIENCE . . ."

NEW DEVELOPMENTS IN FERAL HORSE CONTRACEPTION AND THEIR POTENTIAL APPLICATION TO WILDLIFE

JOHN W. TURNER, JR., *Department of Physiology, Medical College of Ohio, Toledo, OH 43699-0008*

JAY F. KIRKPATRICK, *Department of Biological Sciences, Eastern Montana College, Billings, MT 59101*

The concept of sex steroids as contraceptives is not new and was originally directed toward fertility control in humans (Pincus et al. 1958). In the 1960's the development of extended-action steroids as contraceptives was explored (reviewed, Beck et al. 1980), and application of this technology to captive exotic animals was pioneered in the 1970's (Seal et al. 1976). In the light of rapidly increasing problems of wildlife overpopulation, and continued advances in contraceptive technologies, new approaches to fertility control in wild, free-roaming animal populations are now being examined (Kirkpatrick and Turner 1985, 1991). Because of local overpopulations of free-roaming feral horses (*Equus caballus*) in some areas of North America and the highly publicized nature of management efforts to control these populations, the feral horse has been the focus of a number of contraception studies in free-roaming populations (Kirkpatrick et al. 1982, Turner and Kirkpatrick 1982, Plotka and Vevea 1990).

This article examines approaches to fertility control in feral horses, including currently available and experimental antifertility agents and delivery systems, and their potential for adaptation to various free-roaming species, particularly ungulates. As a first step, it is useful to list the characteristics of the ideal wildlife fertility control agent. First, it has to provide a high degree of effectiveness across a given breeding season. Second, it has to be free of harmful side effects to the animals receiving it, including pregnant animals. Third, the ideal contraceptive should be reversible. The genetic pool in each population exists in the dynamic state, with each reproductively active animal having the potential to influence the pool. Impact on the process of natural selection will be minimized when a fertility control program is reversible. There are also important social and political reasons demanding reversibility of wildlife contraception (Kirkpatrick and Turner 1985). Fourth, the ideal agent will be relatively inexpensive. However, no fertility control program can compete on a cost-effectiveness basis with a management method such as hunting, where the public not only provides the manpower but also provides revenue. Fifth, the ideal agent should have a flexible duration of action, so that a single treatment can act for a predetermined period or number of breeding seasons. Sixth, the agent should have minimal to no effect on social organization or behavior. Finally, the ideal agent should be capable of being delivered remotely. The capture or immobilization of large numbers of animals, regardless of the skill of the management team, may lead to injuries, mortality, and monetary expense that will ultimately be unacceptable (Turner and Kirkpatrick 1986).

FERAL HORSE CONTRACEPTION

Contraception in feral horses has focused on steroids and vaccines, with delivery methods including surgical implants or intramuscular (i.m.) injection in immobilized animals and i.m. injection by remote-delivery projectile. However, the subject is not a simple one. A number of variations on these basic approaches have been explored, and an awareness of attendant advantages and disadvantages has emerged during the course of several feral horse studies.

One of the first issues faced in the feral horse studies was which sex should be targeted for fertility inhibition. Although ovulation inhibition in domestic mares by pharmacological doses of progestins had been demonstrated by Loy and Swan (1966), and confirmed by subsequent studies (Ginther 1979), the social structure of feral horse herds made males seem a preferable target (Turner and Kirkpatrick 1986). In data from 14 of 16 herds surveyed, dominant stallions controlled and bred harems of several (range 2–24, average 5) females, preventing males from outside the harem and subordinate males from breeding (Kirkpatrick and Turner 1986). Because sexual behavior and harem maintenance behavior were regulated by testosterone (Turner and Kirkpatrick 1982), we reasoned that any agent that could satisfy the basic characteristics of the ideal contraceptive while permitting maintenance of normal testosterone levels would be a promising candidate.

In a screening study 4 potential antifertility agents were evaluated in 24 pony stallions (Turner and Kirkpatrick 1982). The agents were α-chlorohydrin (nonsteroidal), 2 long-acting formulations of testosterone (testosterone cypionate [TC] and microencapsulated testosterone propionate [MTP]) and the potent, long-acting synthetic estrogen Quinestrol (17α-ethinylestradiol 3-cyclopentyl ether). The α-chlorohydrin was unacceptable because of neurotoxic side effects. The Quinestrol and both androgens were effective. We chose to use androgens, which had less potential for contamination of the environment.

Within 6–8 weeks after treatment initiation with i.m. injection of MTP (2.6 g/100 kg) or TC (1.7 g/100 kg, monthly 6×), significant decreases from control values occurred in sperm number and sperm motility, while libido scores (based on vulval sniffing, flehmen, erection, and mounting) did not change. These effects persisted for approximately 6 months (treatment phase). In the recovery phase, the affected parameters had returned to control values. No side effects were observed. In this preliminary study, the treatment decreased sperm production but did not compromise the normal sexual behavior of the male. Presumably a harem stallion given this treatment in the field would maintain his harem while being infertile.

MTP was chosen for a field trial on the basis of its more extended action in a single injection. The MTP, prepared by Southern Research Institute (Birmingham, Ala.), consisted of microdroplets of testosterone propionate coated with a nontoxic biodegradable polymer of varying thickness. The basic principle is that a thick coating biodegrades more slowly than a thin coating. By varying the thickness of the coating, it is possible to achieve delay times for MTP release ranging from several days to more than 6 months. By including a range of coating thicknesses in a single injection, hormone presence in the blood can be continuous throughout the release period. Both release rate and duration depend on the chemical characteristics of the agent which is microencapsulated. The current technology has been refined to potentially provide steroid preparations with up to an 18-month release period capability (T. Tice, Southern Research Institute, Birmingham, Ala., pers. commun.).

In an initial field study, 10 harem stallions in the Challis Horse Range in Central Idaho were immobilized from a helicopter and injected directly with MTP several months prior to the 1980 breeding season (Kirkpatrick et al. 1982). Pretreatment foaling, in 1980, was sim-

ilar in control and treated bands. In the summer of 1981, 83% fewer foals were produced among mares in the harems of treated stallions. In 1982 the foal counts in the treated bands had returned to pretreatment (1980) levels. Sexual behavior was evaluated from 1980 to 1982 using standard male parameters of mounting, intromission, and ejaculation. A sociosexual scent marking behavior, exhibited by males (Turner et al. 1981), was used as an index of harem maintenance behavior. There were no differences in stallion behavioral parameters between treated and control animals in the years monitored (1980–1982), with the exception that mating behavior continued further into the summer in treated bands. This probably reflected continued estrus cycling in the mares due to infertile matings (C. Asa, St. Louis Zoo, St. Louis, Mo., pers. commun.).

On the basis of these data, we concluded that a single injection of MTP given several months prior to the breeding season significantly decreased the fertility relative to untreated controls for a single breeding season, did not interfere with stallion behavior, and permitted a return to normal fertility in the breeding season of the following year.

Despite the encouraging outcome of this study, we found the method for delivery of the drug to be unacceptable. Factors such as the cost (approximately $50.00 per dose of etorphine and reversal agent for an equid), the immobilization-treatment-recovery time, and the danger to the animals made immobilization undesirable. We therefore focused on a method for remote delivery of the drug without the intermediate immobilization step (Harder and Peterle 1974), by loading the antifertility agent into a dart to permit administering the MTP directly.

In a trial to establish the feasibility of this approach, 15 feral horse bands in a 64-km^2 area of the Challis Horse Range were located from helicopter. After harem stallions were identified by observing characteristic movement patterns in the band response to the helicopter, remote delivery capability was demonstrated by firing a paint ball from a paint gun (Nelson Paint Company, Iron Mountain, Mich.). Thirteen of the 15 stallions were hit on target in the first pass, and the remaining 2 stallions were marked on a second pass with an average elapsed time of 5.25 minutes from locating a band to hitting the target. Most of this time was used in approach, descent, and maneuvering the horses into a safe path of movement. Usually less than 15 seconds elapsed from the beginning of close pursuit to firing.

A second issue which emerged from the field test was whether to treat males or females. The vast majority of feral horse herds have a single dominant male breeding the harem (Keiper and Houpt 1984), and treating males would be more cost and time efficient. However, it appeared that treatment could be delivered to several horses with relative ease and speed after a band of horses was within firing range. This potentially lessened the time advantage of treating males. Because helicopter time would be the major cost in treatment, the cost advantage may also be minimal. Pursuing remote delivery for female fertility control also offered the potential to increase population management flexibility and permit the possible application of this technology to nonharem species.

Between February 1986 and August 1987 on Assateague Island National Seashore (Maryland), we attempted to determine the antifertility effectiveness of MTP delivered remotely to stallions and of microencapsulated norethisterone (MNET) delivered remotely to mares. MNET is a potent synthetic progestin which has been shown to be a safe and effective extended-action antifertility agent in primates (Beck et al. 1980), acting primarily by blocking ovulation. The microencapsulated form of MNET, prepared by Southern Research Institute (Birmingham, Ala.), was designed to release over a 6-month period from the time of administration (March 1986) through the entire breeding season.

In the male study 4 harem stallions, each with a harem of proven fertility, were treated by remote delivery in February–March 1986. The 14 mares of proven fertility which were associated with the 4 treated stallions exhibited a fertility rate of 28.9% during the foaling season of 1987 (Kirkpatrick and Turner 1987). The foaling rate for a control population of 15 fertile mares for the 1987 season was 45.4%, and the foaling rate for the experimental mares for the previous 5 years ranged from 42% to 50%.

In the MNET study the drug was administered in February–March 1986 by remote delivery to 6 mares of proven fertility. No inhibition of fertility was observed (Kirkpatrick and Turner 1987). The study data did not permit determination of whether the failure was due to the agent, the dose, or the mode of delivery. It appears that the method of delivery was not the cause of failure, because the remote delivery method did work for males. Although progestin-mediated contraception has proven effective in some other species (reviewed, Kirkpatrick and Turner 1985), it may be that protestins are simply ineffective as contraceptive agents in feral mares. Plotka et al. (1988) were unsuccessful in suppressing estrus for longer than 5 weeks in captive feral males with Silastic® implants containing large amounts (24 g) of progesterone.

Valuable information in these Assateague Island studies was derived from technical problems associated with the remote administration of microencapsulated steroid. First, treatment administration occurred during several very cold days (−10 C), such that the increased viscosity of the carboxymethyl cellulose used to suspend the microcapsules sometimes interfered with rapid injection. It was thus necessary to keep the carrier warm prior to delivery. Second, the suspension of microcapsules tended to settle out and clump in the dart if not delivered within 10 minutes of initial mixing. Third, delivery of nonimmobilizing drugs (i.e., no handling of animal) necessitated barbless or micro-barbed darts which would ultimately fall out. Thus, velocity and trajectory had to be regulated carefully to ensure injection without rebound. Fourth, while it was possible to remotely deliver the effective amount of microencapsulated steroid with multiple injections in these studies, this would be unacceptable for routine use. The volume : dose ratio must be reduced sufficiently to permit administration of the complete dosage in a single dart.

If the problem of drug volume can be overcome, there is another remote delivery method which may be promising. R. Goodloe, R. J. Warren, and D. C. Sharp ("Sterilization of feral horses by immunization against LHRH," presentation, Wildl. Dis. Assoc. Conf., Univ. Ga., 7–11 Aug 1988) have successfully delivered antifertility agents to feral horses in a biodegradable bullet fired from a CO_2-powered rifle (Ballistivet, Inc., Minneapolis, Minn.). The hollow 0.25 caliber bullet is made of a compressed food-grade material. Once the bullet is lodged, biodegradation occurs over 24 hours, and the agent is freed for action. Maximum deliverable volume is 0.3 cc.

While the remote delivery dart or bullet methods cannot presently be easily used to administer steroids due to excessive volumes required for available steroids, they may be useful with water-soluble agents which can be delivered at high concentration, lyophilized, or in low volume. Most of the water-soluble reversible contraceptive agents currently being studied are vaccines.

Immunoantifertility

Immunoantifertility currently appears to be 1 of the most promising areas of contraceptive technology. The general principle is that antibodies are raised in the individual against some structural or functional protein or peptide involved in the reproductive process. The presence of the antibodies hinders or prevents some aspect of the reproductive process. Suc-

cessful immunocontraception has been achieved by raising antibodies against (1) gonadotropin releasing hormone (GnRH) in both sexes, (2) spermatozoa, and (3) ovarian zona pellucida. The latter has received the most extensive investigation.

GnRH is a hypothalamic peptide which regulates pituitary gonadotropin release. The gonadotropins, follicle stimulating hormone, and luteinizing hormone (LH), in turn regulate aspects of gonadal function, including gamete production. Thus, reproduction may be inhibited by immunizing an individual against self GnRH, which makes the GnRH unavailable for biological actions. Anti-GnRH has been used successfully to reduce fertility in several species, including pigs (*Sus scrofa*) (Esbenshade and Britt 1985), rats (*Rattus norvegicus*) (Ladd et al. 1988, Ladd et al. 1989), and rabbits (*Oryctolagus cuniculus*) (Ladd et al. 1988). In a study of domestic ewes (*Ovis aries*), Roberts and Reeves (1988) reported that immunization against either LH or a combination of estradiol-ovalbumin and testosterone-ovalbumin resulted in marked reduction of lambing relative to albumin-immunized controls.

Two contraceptive studies using anti-GnRH in the horse have been reported. In 1 study a conjugated form of GnRH was used successfully to raise antibodies in captive feral mares, but contraceptive results were poor (R. Goodloe, R. J. Warren, and D. C. Sharp, 1988, unpubl. presentation). Using a similar approach, Dowsett et al. (1990) suppressed GnRH in colts and reduced testosterone concentrations for up to 20 weeks post-immunization, suggesting contraceptive potential in males.

One major drawback of using antibodies against GnRH, LH, or sex steroids as a means of inducing infertility is that gonadal steroid production or steroid bioavailability will be decreased by these manipulations. Thus, steroid replacement will be required to ensure integrity of both reproductive and social behavior of the population involved.

Immunization of individuals against gametes or gamete proteins has the distinct advantage of avoiding steroid/behavioral effects, and this approach currently appears to be promising for immunocontraception. Antibodies to spermatozoa have been causatively implicated in human infertility (Menge 1980, Bronson et al. 1984). Spermatozoal or testicular extracts used to immunize individuals of several species have been shown to decrease fertility via both pre- and post-fertilization effects (Carron et al. 1988, Edwards 1964, Menge and Naz 1988). Antibodies raised against a recently isolated sperm-specific glycoprotein antigen found in the sperm cell plasma membrane (Naz et al. 1986) have been shown *in vivo* and *in vitro* to inhibit aspects of fertilization in several species (Naz 1988, Menge and Naz 1988, Herr et al. 1990).

In the female, active immunization of several species with porcine zona pellucida (PZP) has been associated with reduced fertility (reviewed, Henderson et al. 1987, Shivers and Liu 1982), and antizona antibodies have blocked *in vitro* fertilization in humans (Sacco et al. 1981). To date, reported side effects of PZP immunization have included some alteration in ovarian follicular growth and function in rabbits (Skinner et al. 1984), monkeys (*Saimiri* sp.) (Sacco et al. 1983), dogs (*Canis familiaris*) (Mahi-Brown et al. 1985), and baboons (*Papio* sp.) (Dunbar et al. 1989), with potential irreversibility reported for dogs. It may be possible to avoid the potential side effect problems of PZP antibodies by using cumulus oophorus matrix antibodies, which are unlikely to react with younger follicles. Rabbit oophorus matrix has been shown to effectively inhibit human fertilization *in vitro* (Tesarik 1989).

It should be noted that many of the initial PZP studies utilized high antigen concentrations for immunization. At lower concentrations side effects may be minimal to nonexistent. This appears to have been the case for PZP immunocontraception in the mare. In a recent study with captive feral and domestic mares, Liu et al. (1989) successfully produced

reversible immunoinfertility by immunization of mares with PZP. Pregnancy was prevented for approximately 8 months in 14 of 15 mares. When antibody titers had decreased to lower levels in 4 monitored mares, they conceived normally.

In a subsequent study of free-roaming feral mares on Assateague Island, Kirkpatrick et al. (1990) determined the effectiveness of remote delivery PZP immunocontraception. Between February and April of 1988, 26 Assateague mares of proven fertility received 2 or 3 inoculations (1 ml each) with PZP vaccine in adjuvant. The treatments were administered remotely via dart rifle as described for the other Assateague studies. Only 1 foal was born to the 26 treated mares, and among untreated control mares there was a 50% pregnancy/foaling rate. Regarding reversibility, 14 of the nonpregnant, PZP-treated mares were given a remotely delivered PZP booster inoculation in February or March 1989. Pregnancy determinations based on urinary steroids (Kirkpatrick et al. 1988) were made in samples collected in the fall of 1989. Results revealed only a 7.2% pregnancy rate in these mares, as compared to a 41.6% pregnancy rate among the 12 PZP-immunized mares which did not receive a booster inoculation (Kirkpatrick et al. 1991). Of 16 mares of similar age never treated with PZP, 43.7% were pregnant in the fall of 1989. These findings demonstrate the reversibility of treatment and the effectiveness of an immunization booster.

POTENTIAL FOR WILDLIFE CONTRACEPTION

From the standpoint of both antifertility agents and delivery techniques, a fair armamentarium for feral horse contraception already exists. The potential for broadened application of contraceptive technology to other wildlife populations has not yet been explored, although contraceptive efficacy has been reported for a number of domestic and captive exotic species (Kirkpatrick and Turner 1985). By carefully assessing the reproductive patterns, behavior, habits, and environment of a given free-roaming species, it may be possible to adapt existing contraceptive technology to assist in the management of some species. In this regard a brief discussion of advantages and disadvantages of currently available technology (Table 1) may be useful.

Major contraceptive agents and procedures have already been presented. However, to summarize, the agents are primarily natural and synthetic sex steroids and immunotropic protein and peptide antigens. The steroids are able to act over extended time periods via structural modifications to the molecule, microencapsulation, or gradual release from Silastic® polymer rods. Although steroids have the advantages of being well researched, biologically active in most vertebrates, and often active orally, they also have several serious disadvantages. Among captive feral mares, placement of Silastic® rod implants containing estradiol and progesterone (Vevea et al. 1987, Plotka et al. 1988) met with limited success in controlling fertility. Although Plotka and Vevea (1990) reported successful inhibition of fertility in captive feral mares given Silastic® rod implants containing ethinylestradiol, the use of such synthetic steroids, which often exhibit poor biodegradability, raises the issue of possible consumption by nontarget species, including humans. This circumstance makes acceptance for registration with regulatory agencies such as the FDA, USDA, and the EPA unlikely.

The use of natural steroids, which are rapidly metabolized, may minimize the biodegradability issue. However, the dosages of these steroids must be relatively large in order to inhibit fertility. This may limit the administration of the agents to surgical implants, which necessitates the undesirable circumstances of capturing and handling the target animals. Thus the potential seems low for the use of steroids for contraception use in free-roaming

Table 1. Current wildlife contraceptive delivery systems, route of administration, agents, and characteristics of potential target species of their use.

Delivery system	Route (I.M. vs. oral)	Agent		Target animal		
		Type[a]	Format[b]	Size (large or small)	Style (secretive or exposed)	Habitat (cover or open)
Capture and chute	IM	S, N, I	SI, E, ILA, V	L	E	C, O
Live trap and restraint	IM	S, N, I	SI, E, ILA, V	L, S	SE	C, O
Immobilizer	IM	S, N, I	SI, E, ILA, V	L	E	O
Remote delivery	IM	S, N, I	SI, E, ILA, V	L	E	O
Bait or food	O	S, N	E, ILA[c]	L, S	SE, E	C, O

[a] Agent type: Steroid (S), Non-steroid chemical (N), Immunological (I).
[b] Agent format: Subdermal implants (SI), Encapsulation (E), Intrinsic long-action (ILA), Vaccination (V).
[c] Steroids only.

wildlife. One exception to this may lie in the use of long-term, nonsurgical subcutaneous (sc) implants of steroids in certain smaller mammals which can easily be live trapped. In a recent study Bickle et al. (1991) successfully inhibited fertility (no litters in 23 treated females) in free-roaming ($n = 4$) and captive ($n = 19$) female skunks (*Mephitis mephitis*) given subcutaneous implants of levonorgestrel (Norplant®), a progestational steroid. The implant was a 2.5 × 30 mm flexible rod inserted sc into the neck via trocar. While the possibility of consumption by nontarget species remains, this issue may be minimized, for example, when treatment is applied to an urban population of skunks, in which predation and scavenging are minimal.

The immunological approach to wildlife contraception appears promising on the basis of the feral equid data demonstrating a high degree of effectiveness and reversibility. Immunocontraceptives have the advantage of high potency for low volume delivery. In addition, they do not have potential for contaminating the environment and do not have behavioral effects. Potential disadvantages also must be considered. For example, immunocontraceptives are not active orally without modification, may require more than 1 inoculation for the initial immunization, and may be variably effective across species. However, with the use of biodegradable polymer coatings it may be possible to provide oral delivery of active vaccine (Saffran et al. 1990). Furthermore, microencapsulation, which permits timed-release of the agent, can potentially eliminate the need for multiple inoculation. The potential side effects of long-term use are unknown, with the extreme possibilities including permanent infertility or escape from the antifertility effect over a period of years.

Assuming the availability of a viable wildlife contraceptive agent, a method of delivery must be chosen. The 2 pathways routinely used for getting chemicals into an animal are oral and intramuscular. In some respects the delivery aspect of wildlife contraception is the most variable and difficult to accomplish because of the wide variety of species and habitats. Put simply, however, the possibilities are "hands-on" or "hands-off." The "hands-on" methods include round-up and capture, live trapping, or chemical immobilizer. Once captured or trapped the animal can be darted, hand-injected, or implanted with the antifertility agent. The viability of the "hands-on" approach will depend on the size, accessibility, and numbers of the targeted species. For example, skunks can readily and safely be live-trapped, and it may be feasible to treat sufficient numbers to eventually limit their population in a given urban area. Access to species such as large ungulates can often be accomplished with chemical immobilizer, and treatment can be made in the field at the immobilization location. However, the already discussed disadvantages

of immobilizer in terms of cost and danger to the animal may well outweigh the on-site "hands-on" advantage, particularly when dealing with large numbers of animals.

In contrast, the "hands-off" methods provide an on-site remote delivery methodology without capture, live trapping, or immobilization. Remote delivery methods include the use of baits and the use of a gun which fires a dart or plastic bullet containing the antifertility agent. The earliest wildlife contraception efforts used baits (Marsh and Howard 1969), and this approach remains potentially viable for many species, especially for small mammals and birds (Kirkpatrick and Turner 1985). Two common disadvantages of baits are the lack of target specificity and poor bait acceptance (Harder and Peterle 1974).

These disadvantages are not shared by remote delivery methods using a projectile fired from a gun. Despite its limited usage to date for contraception, this form of remote delivery has several distinct advantages over "hands-on" methods. Perhaps most importantly it reduces the incidence of harassment, injury, and death in the target animals. In our experience with feral horses remote delivery darting has proven to be a far more cost-efficient and time-efficient method than capture and handling, requiring fewer personnel and equipment by eliminating the capture-immobilization step.

However, there will undoubtedly be circumstances where the delivery method used is dictated by situation, animal characteristics, and habitat. For example, darting of animals from a blind at waterholes may be useful in arid areas, and firing darts from a helicopter may be effective for some large ungulates in open or semi-open terrain. Live trapping and injection or baits may be preferable for some smaller species inhabiting burrows or dense underbrush.

SUMMARY

During the past decade the problem of overpopulation of many wildlife species on preserves of limited area and in urban parks has reached crisis proportions despite existing management efforts. The development of contraceptive technology for free-roaming wildlife may become essential. Using research studies of contraception in free-roaming feral horses as a contextual framework for critical analysis, an evaluation of the potential for wildlife contraception is presented. Topics include species-specific requirements regarding choice of sex, type of agent, and method of delivery. Agent types include steroidal, nonsteroidal, and immunocontraceptives. Considerations of delivery include release characteristics of the agents and capture versus remote delivery. In the continuing development of contraceptive technology for wildlife it is important to address, in addition to efficacy, issues of environmental and animal safety, reversibility, and cost effectiveness.

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Pregnancy determination in uncaptured feral horses based on steroid metabolites in urine-soaked snow and free steroids in feces

JAY F. KIRKPATRICK
Department of Biological Sciences, Eastern Montana College, Billings, MT 59101, U.S.A.
S. E. SHIDELER
Department of Reproduction, School of Veterinary Medicine, University of California, Davis, CA 95626, U.S.A.
AND
J. W. TURNER, JR.
Department of Physiology, Medical College of Ohio, Toledo, OH 43699, U.S.A.

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Urine-soaked snow from 13 uncaptured feral mares was collected and measured without extraction for estrone conjugates (E_1C) and nonspecific immunoreactive pregnanediol-3-glucuronide (iPdG) by enzyme immunoassays. The hormone values were indexed to creatinine (Cr). Mares that produced foals had urinary E_1C values of 7.30 ± 1.39 (SE) μg/mg Cr versus 0.096 ± 0.084 μg/mg Cr for mares that did not produce foals. The difference was significant ($P < 0.001$). Nonspecific iPdG concentrations for mares producing foals was 167 ± 80.33 ng/mg Cr versus 7.04 ± 1.69 ng/mg Cr for mares that did not produce foals. The difference was significant ($P < 0.0025$). Urine samples collected directly from the ground from 34 uncaptured feral mares were measured for E_1C and nonspecific progesterone metabolites and compared with fecal total estrogen concentrations in matched fecal samples, measured by means of radioimmunoassay. Both E_1C and iPdG concentrations differed significantly ($P < 0.001$) between mares producing foals and those that did not. Mean fecal total estrogen concentrations for mares producing foals was 3.18 ± 0.70 ng/g feces versus 0.552 ± 0.08 ng/g feces for those that did not produce foals. The difference was significant ($P < 0.001$). The correlation coefficient between urinary E_1C and fecal total estrogens was 0.928. The results indicate that both urine-soaked snow and fecal samples can be used to reliably assess pregnancy in uncaptured free-roaming feral horses.

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De la neige imbibée d'urine de 13 juments sauvages en liberté a été recueillie et les concentrations de composés d'estrone (E_1C) et de prégnanediol-3-glucuronide immunoréactif non spécifique (iPdG) y ont été mesurées, sans extraction, par des tests immunologiques enzymatiques. Les concentrations hormonales ont été déterminées relativement à la créatinine (Cr). Les juments qui avaient un poulain avaient des concentrations urinaires d'E_1C de 7,30 ± 1,39 (SE) μg/mg Cr, comparativement à 0,096 ± 0,084 μg/mg chez les juments sans poulain. La différence était significative ($P < 0,001$). La concentration d'iPdG non spécifique était de 167 ± 80,33 ng/mg Cr chez les juments avec petit et de 7,04 ± 1,69 ng/mg Cr chez les juments sans petit. La différence était significative ($P < 0,0025$). Des échantillons d'urine de 34 juments sauvages en liberté ont été recueillis directement du sol et soumis à des analyses afin de mesurer les concentrations d'E_1C et des métabolites non spécifiques de la progestérone (Po); ces concentrations ont été comparées aux concentrations totales d'oestrogènes dans des échantillons correspondants de matières fécales soumis à des tests radioimmunologiques. Les concentrations d'E_1C et d'iPdG différaient toutes deux significativement ($P < 0,001$) chez les juments avec petit et chez les juments sans petit. Les concentrations totales moyennes d'oestrogène dans les matières fécales des juments avec petit, 3,18 ± 0,70 ng/g fèces, différaient significativement ($P < 0,001$) des concentrations observées chez les juments sans petit, 0,552 ± 0,08 ng/g fèces. Le coefficient de corrélation entre la concentration urinaire d'E_1C et la concentration totale d'oestrogène fécal a été évalué à 0,928. Les résultats indiquent que la neige imbibée d'urine et les échantillons de matières fécales peuvent servir à déceler efficacement la grossesse chez des juments sauvages en liberté.

Introduction

The ability to determine pregnancy in uncaptured wild animals is a useful tool for the wildlife biologist. Kirkpatrick et al. (1988) demonstrated that pregnancy could be determined in uncaptured feral mares by recovering urine from soil and measuring estrone sulfate (E_1S), a conjugated metabolite of plasma estrone in horses. The method is accurate, but procedures for extracting urine from the soil are often time-consuming and certain types of soils have the potential to interfere with hormone or creatinine assays. Two strategies for simplifying noncapture pregnancy testing in free-roaming animals are to measure reproductive hormones in either urine-soaked snow or fecal samples. DelGiudice et al. (1988, 1989) and Mech et al. (1987) were able to measure winter condition in white-tailed deer and wolves, respectively, by measuring certain electrolytes, urine nitrogen, and creatinine in urine-soaked snow; however, the sensitivity of these tests is significantly less than that required for steroid hormone analysis. This study was carried out to determine if pregnancy could be diagnosed in feral horses by measuring steroids or their metabolites in feces and urine-soaked snow. Two experiments were carried out. Estrone conjugate (E_1C) and nonspecific immunoreactive pregnanediol-3-glucuronide (iPdG) concentrations were measured in urine-soaked snow, indexed to creatinine (Cr), and compared between mares that produced foals and those that did not. In a second experiment, fecal total estrogens were measured and compared with urinary E_1C and iPdG concentrations in pregnant and nonpregnant mares.

Methods

Urine-soaked snow samples were collected from 13 sexually mature feral mares on the Pryor Mountain National Wild Horse Range,

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Montana, in December. Each horse was identified by means of specific markings and observed at 100–200 m until urination occurred. Urine-soaked snow was scraped from the site with a plastic spoon, taking care to recover only stained snow, and placed in a 20-mL glass vial, allowed to thaw, and stored frozen at −5°C until assay. Urine samples, collected directly from the ground, and matched fecal samples were collected from 34 sexually mature mares on Assateague Island National Seashore, Maryland, in October. The urine samples were collected and stored as described by Kirkpatrick et al. (1988); the fecal samples were placed in plastic bags, sealed, and stored at −5°C until assayed.

The urine samples collected from snow were assayed by enzyme immunoassay (EIA) for the estrone conjugates (E_1C) estrone-3-glucuronate and estrone-3-sulfate as described by Munro et al. (1989). Each sample was diluted 1:100 in distilled H_2O and 20 μL was taken to assay. The antibody (R522) has equal cross-reactivity for both the glucuronate and sulfate conjugates of estrone. The inter- and intra-assay coefficients of variation were 13% ($n = 10$) and 10% ($n = 15$), respectively. The assay for urinary iPdG was described by Shideler et al. (1990) and Kirkpatrick et al. (1990). Samples were diluted 1:1 and 20 μL was taken to assay. The inter- and intra-assay coefficients of variation were 11:45% ($n = 10$) and 10.04% ($n = 15$), respectively. To account for differences in urine concentration and dilution caused by mixing with snow, each sample was analyzed for creatinine by the microcolorimetric method of Taussky (1954). E_1C values are given as micrograms per milligram of creatinine and iPdG values as nanograms per milligram of creatinine. The 13 mares were located and identified during the following summer and observed for the presence of foals. The specificity of the assays was previously validated by high-performance liquid chromatography (Kirkpatrick et al. 1990). In addition, dilutions of 1:2, 1:4, 1:8, and 1:16 were assayed and compared for parallelism with the standard curve.

The urine samples from the 34 Assateague Island mares, collected from the ground, were assayed for E_1C and nonspecific iPdG immunoreactivity as described earlier. Matched fecal samples were assayed for total free estrogens. Each 0.5-g sample was placed in a glass scintillation vial and approximately 1000 cpm (16.7 Bg) of ^{3}H-17β estradiol (17β-E_2) (New England Nuclear) in 20 μL of water was added to assess procedural losses. Each sample was extracted with 11 mL of chromatography-grade ethyl acetate – hexane (3:2, v/v) on a reciprocal shaker for 5–12 h. Eight millilitres of the organic phase was transferred to 13 × 100 mm glass tubes and dried at 37°C under N_2. The residue was suspended in 1.0 mL of assay buffer, vortexed briefly, and incubated for 12 h to place into solution as much of the extracted estrogens as possible. To assess procedural losses, 0.5 mL of assay buffer was transferred to scintillation vials and counted in 10 mL of cocktail (Aquasol, New England Nuclear). The remaining 0.5 mL was assayed for total estrogens by I^{125} RIA, using an anti total estrogen antibody (ICN Biomedical, Carson, California) and ^{3}H-estradiol (^{3}H-E_2) as the standard. Cross-reactivity of the antibody with estrogens was 100% for 17β-estradiol (17∂-E_2) and estrone, 9.0% for estriol, and 7.0% for 17∂-E_2. All other steroids, including androgens, progestins, and corticosteroids, cross-reacted at <0.01%. The coefficient of variation for intra-assay precision was 5.7% and recovery was 62.18 ± 6.45 (SE) %. Results are presented in nanograms of total estrogens per gram of feces. Confirmation of pregnancy was accomplished by foal counts. Mean values for hormone concentrations were compared for statistical significance with Student's *t*-test.

Results

Five of the 13 Pryor Mountain mares (38%) produced foals in 1989. These five mares had E_1C concentrations ranging from 2.71 to 10.57 μg/mg Cr, with a mean of 7.3 ± 1.39 (SE). The eight mares that did not foal had E_1C concentrations ranging from nondetectable to 0.68 μg/mg Cr, with a mean of 0.096 ± 0.084. The difference between mean E_1C concentrations for mares that produced foals and those without was significant at the $P < 0.001$ level of confidence. Mares with foals had iPdG concentrations ranging from 47.27 to 469.23 ng/mg Cr, with a mean of 167 ± 80.33. Those without foals had concentrations ranging from 1.23 to 16.81 ng/mg Cr, with a mean of 7.04 ± 1.69. The difference between mean nonspecific concentrations for mares that produced foals and those that did not was significant at the $P < 0.025$ level of confidence. Creatinine values for the 13 horses ranged from 0.11 to 0.814 mg/mL, with a mean of 0.321 ± 0.063. All urine-soaked snow samples were collected 180–200 days postconception.

Twenty-eight of the 34 Assateague mares did not deliver foals and had a mean urinary E_1C concentration of 0.11 ± 0.034 μg/mg Cr, compared with 3.47 ± 0.735 μg/mg Cr for the 6 mares that did produce foals. The difference was significant at the $P < 0.001$ level of confidence. The 28 mares that did not produce foals had a mean iPdG concentration of 3.6 ± 0.499 ng/mg Cr, which differed significantly ($P < 0.001$) from mean concentrations for the 6 mares that did produce foals (215.8 ± 83.4 ng/mg Cr). The mean fecal total estrogens for the 28 nonpregnant mares was 0.552 ± 0.08 ng/g feces and differed significantly ($P < 0.001$) from a mean value of 3.18 ± 0.70 ng/g feces for the 6 pregnant mares. The coefficient correlation (r) between urinary E_1C and fecal total estrogens was 0.928. All urine and fecal samples from Assateague Island were collected approximately 120–180 days postconception.

Discussion

These data indicate that pregnant mares can be distinguished from nonpregnant animals by measuring either E_1C or iPdG in urine-soaked snow, or by measuring fecal total estrogens. The Cr levels in samples collected from urine-soaked snow were similar to those reported for urine collected directly from domestic horses with catheters (Evans et al. 1984) or in samples of soil soaked with urine from feral horses (Kirkpatrick et al. 1988), and indicate that dilution by snow is not great, nor does it interfere with the hormone assays. The water content of snow may vary from sample to sample, but indexing hormone values to Cr concentrations will account for these differences, as well as for differences in urine concentration. E_1C values, measured by enzyme immunoassay (EIA), were also similar to E_1S values for pregnant and nonpregnant horses reported previously but measured by RIA (Kirkpatrick et al. 1988; Evans et al. 1984), and dilutions demonstrated parallelism with the standard curve.

Among the Assateague horses, urinary E_1C, iPdG, and fecal total estrogens were all reliable indicators of pregnancy in this study, and the strong correlation between urinary E_1C and fecal total estrogens supports the use of fecal samples for pregnancy diagnosis. In mammals, estrogens and other steroids are metabolized in the liver, conjugated with sulfates and glucuronates, and secreted into the gastrointestinal tract via bile. Some steroid hormones reach the gastrointestinal tract without change in structure or solubility. In a species-dependent manner some steroids are excreted directly with the feces, while a portion of their conjugates is resorbed into the blood and excreted in the urine or returned to the bile. It is the urinary pathway of steroid excretion that formed the rationale for the urinary estrogen and progestin conjugate analyses that have been used successfully in zoo biology (Loskutoff et al. 1983). The fecal steroids, however, add an important new dimension to the study of reproduction and problems of wildlife biology, where urine collection can be difficult.

It is important to note that all fecal samples in this study were collected 120–180 days postconception. Mostl et al. (1984) and Bamberg et al. (1984) have demonstrated a time-dependent

increase in fecal estrogens in pregnant domestic cows and mares, with discriminating values occurring at about 90 days. Thus, one limitation of the fecal approach is inability to determine pregnancy early in gestation. Urinary E_1C, however, can be used with a high degree of accuracy after day 40 of pregnancy in mares (Evans et al. 1984).

The difference between fecal estrogen values reported in this paper (ranging from 0.30 to 5.82 ng/g) and those in the study by Bamberg et al. (1984), which were in the 100–300 ng/g range, cannot be easily explained, but two factors probably contributed. First, significantly different antibodies were utilized in the two studies. Mostl et al. (1983) demonstrated that in pregnant cows, the concentration of 17∂-E_2 is 10× that of other estrogens, but this is not true for horses. The antibody used by Mostl et al. had a 30% cross-reactivity with this biologically weak estrogen, and therefore obviously binds with a number of other steroids. This lack of specificity is not important, however, because the cow excretes primarily 17∂-E_2. In contrast, the horse, which secretes very little 17∂-E_2, produces estrogens that are either more immunoreactive or in significantly larger quantities that those found in the cow. The precise nature of the immunoreactive fecal estrogens remains to be demonstrated. Secondly, the differences in values between the two studies might be attributed to the extraction methods used. Initial attempts to extract estrogens using the methods of Mostl et al. (1984) met with little success, and seldom recovered more than 15–20% of ^{3}H-E_2. Consequently, the ethyl acetate – hexane extraction method, which is widely used for extracting estrogens and which recovered in excess of 60%, was used. Why the extraction methods and recovery success of Mostl et al. (1984) could not be reproduced and why the difference in extraction methods reported in this study should lead to such significant differences in free steroid values remain unexplained. Thus, care must be taken to avoid generalizations regarding quantitative evaluations with nonquantitative, nonspecific assays, despite success in differentiating pregnant from nonpregnant animals.

The differences in iPdG values between the pregnant and nonpregnant horses were significant; however, using this metabolite alone appears to be less reliable than using E_1C. The highest value in a nonpregnant animal was 16.81 ng/mg Cr, compared with the lowest value in pregnancy of 47.27 ng/mg Cr. This relatively small difference probably reflects either the relativity low plasma progesterone concentrations in the horse, particularly in the second half of pregnancy, or the ability of this assay to detect the metabolites of the 5-α reduced progestins which are found during the second half of gestation in the horse. Progesterone concentrations reach a peak of about 15 ng/mL plasma between days 60 and 120 postconception (Holtan et al. 1975), then decline to low concentrations. In contrast, extremely high plasma estrone concentrations occur during the same period of pregnancy in the horse, persist, and reach levels as high as 160 ng/mL (Cox 1975).

The use of iPdG alone could be confounded by the presence of a persistent corpus luteum, which can produce plasma progesterone values as high as those found during pregnancy (Stabenfeldt et al. 1974). Thus, iPdG should be measured only along with E_1C for pregnancy determination. Nevertheless, iPdG can be measured in urine-soaked snow or in soil and values will reflect plasma progesterone concentrations and ovarian activity.

There are advantages and disadvantages to the use of either feces or urine for pregnancy diagnosis in feral mares, and the method selected must be matched to the field conditions and the resources of the investigator. Urinary estrogen conjugate analysis provides a method that requires no extraction and can be applied as early as 40 days postconception. The enzyme immunoassays are inexpensive (about $0.05/assay) and accurate but the antibodies and conjugates are not commercially available. Also, collection of urine samples requires a significant investment of time. Collection of fecal samples is easy, requires about one-fourth of the time taken to collect urine samples, and the assays are commercially available. Extraction is necessary, however, and assays cost in excess of $1.00/sample.

Although this study was confined to feral horses, applications to the study of many free-roaming species can be pursued with this methodology. These applications include the determination of fetal loss rates, and of foaling, calving, and fawning rates where neonatal mortality might obscure true rates. An important consideration in designing experiments of this nature is that each species has its own particular metabolic end-products and pathways of excretion for reproductive steroids. Assays must be selected with care and validated for each species. Nevertheless, extension of this strategy to field studies is a logical and potentially valuable next step.

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Reference # 4

Pregnancy Determination in Uncaptured Feral Horses

J.F. Kirkpatrick, L.H. Kasman, B.L. Lasley, and J.W. Turner, Jr.

Journal of Wildlife Management 52(2):305-308, 1988

Abstract

The urinary excretion of estrone sulfate (E_1S) by 25 free-roaming feral horses (*Equus caballus*) was measured by radioimmunoassay applied to extracts of urine-soaked soil. Twelve of 15 mares have E_1S concentrations > 1.0 μg/mg creatinine ($\bar{x}$ = 2.64 ± 1.02 [SD]) produced foals. All 10 mares with E_1S concentrations < 1.0 μg/mg creatinine ($\bar{x}$ = 0.44 ± 0.26) did not foal. Extracting urine from soil and measuring E_1S and creatinine can be used to determine pregnancy in free-roaming feral horses without the stress of capture or immobilization.

Reference # 5

Pregnancy Determination in Uncaptured Feral Horses by Means of Fecal Steroid Conjugates

J.F. Kirkpatrick, S.E. Shideler, B.L. Lasley, and J.W. Turner, Jr.

Theriogenology 35(4):753-759, 1991

Abstract

This study was carried out to develop an accurate, rapid and inexpensive method for diagnosing pregnancy in uncaptured feral horses by analysis of fecal steroid metabolites and to compare the accuracy of this method with diagnosis by urinary estrone conjugates (E_1C). Paired urine and fecal samples were collected from 40 sexually mature feral mares during August and October. Urine samples were extracted directly from the soil and analyzed by enzymeimmunoassay (EIA) for E_1C. Water extracts of fecal samples were assayed by EIA for E_1C. Water extracts of fecal samples were assayed by EIA for E_1C and nonspecific progesterone metabolites (iPdG). Urinary E_1C, fecal E_1C and fecal iPdG concentrations for seven mares which produced foals were 3.9 ± 1.3 (SEM) 5 μg/mg creatinine, 4.2 ± 0.8 ng/g feces and 1.411 ± 569.6 ng/g feces, respectively. Urinary E_1C and fecal E_1C and iPdG concentrations for the 33 mares which did not produce foals were 0.1 ± 0.0 μg/mg creatinine and 0.5 ± 0.1 and 32.8 ± 4.5 ng/g feces, respectively. These differed ($P < 0.01$) from values in mares which produced foals.