

DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE GRANT APPLICATION Follow instructions carefully. Type in the unshaded areas only.		LEAVE BLANK FOR PHS USE ONLY.	
		Type	Activity
		Review Group	Number
		Council/Board (Month, Year)	Formerly
		Date Received	
1. TITLE OF PROJECT (Do not exceed 56 typewriter spaces.) Ovarian Function in Mares Chronically Treated with PZP			
2. RESPONSE TO SPECIFIC PROGRAM ANNOUNCEMENT ☐ NO ☐ YES (If "YES," state RFA number and/or announcement title) Number: Title: Academic Research Enhancement Award			
3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ☐ NEW INVESTIGATOR			
3a. NAME (Last, first, middle) Kirkpatrick, Jay Franklin		3b. DEGREE(S) BS, MS, Ph.D.	3c. SOCIAL SECURITY NO. 162-32-1363
3d. POSITION TITLE Associate Professor of Physiology		3e. MAILING ADDRESS (Street, city, state, zip code) Department of Biological Sciences Eastern Montana College 1500 North 30th Street Billings, Montana 59101	
3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT Biological Sciences			
3g. MAJOR SUBDIVISION Arts and Sciences			
3h. TELEPHONE (Area code, number, and extension) (406) 657-2342			
4. HUMAN SUBJECTS ☒ 4a. NO ☐ YES If "YES," exemption no. or IRB approval date 4b. Assurance of compliance no.		5. VERTEBRATE ANIMA ☐ 5a. NO ☒ YES 5b. Animal welfare assurance no. None	
6. DATES OF ENTIRE PROPOSED PROJECT PERIOD From (YYMMDD) Through (YYMMDD) 92/03/01 94/12/31		7. COSTS REQUESTED FOR FIRST 12-MONTH BUDGET PERIOD 7a. Direct Costs 7b. Total Costs \$ 26,337 \$ 35,294	8. COSTS REQUESTED FOR ENTIRE PROPOSED PROJECT PERIOD 8a. Direct Costs 8b. Total Costs \$ 73,686 \$ 100,597
9. PERFORMANCE SITES (Organizations and addresses) Eastern Montana College Billings, MT 59101 and Assateague Island National Seashore Route 2, Box 294 Berlin, MD 21811		10. INVENTIONS (Competing continuation application only) ☐ NO ☐ YES If "YES," ☐ Previously reported ☐ Not previously reported	
12. TYPE OF ORGANIZATON ☒ Public: Specify ☐ Federal ☒ State ☐ Local ☐ Private Nonprofit ☐ For Profit (General) ☐ For Profit (Small Business)		11. NAME OF APPLICANT ORGANIZATION Eastern Montana College ADDRESS (Street, city, state, and zip code) 1500 North 30th Street Billings, MT 59101	
15. NAME OF OFFICIAL IN BUSINESS OFFICE Kenneth Heikes TELEPHONE (Area code, number, and extension) (406) 657-2155 TITLE Administrative Vice President ADDRESS Eastern Montana College Billings, MT 59101		13. ENTITY IDENTIFICATION NUMBER 81-600-1642W Congressional District Eastern Montana	
		14. ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT TOWARDS A BIOMEDICAL RESEARCH SUPPORT GRANT Code: 20 Identification: Other Academic	
16. NAME OF OFFICIAL SIGNING FOR APPLICANT ORGANIZATION Bruce Carpenter TELEPHONE (Area code, number, and extension) (406) 657-2300 TITLE President ADDRESS Eastern Montana College Billings, MT 59101			
17. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application. Willful provision of false information is a criminal offense (U.S. Code, Title 18, Section 1001).		SIGNATURE OF PERSON NAMED IN 3a (In ink. "I" table.)	DATE
18. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense (U.S. Code, Title 18, Section 1001).		SIGNATURE OF PERSON NAMED IN 16 (In ink. "Per" signature not acceptable)	DATE

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DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the experimental design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. DO NOT EXCEED THE SPACE PROVIDED.

This proposal describes a long-term study of immunocontraception of feral mares with porcine zonae pellucidae (PZP) and the effects on fertility and ovarian endocrine function. Twenty-six mares have already been immunized with PZP and fertility inhibition was successful. Ten of those mares have been given an annual PZP booster inoculation for three additional years, with >90% fertility inhibition. Five of these 10 mares will be boosted annually, through 1994 (7 consecutive years of PZP treatment), 5 will be treated through 1992 (5 consecutive years of PZP treatment), and 10 additional mares will be immunized and boosted through 1994. This will permit the study of (1) animals immunized over 1,2,3,4,5,6,7 years, (2) animals immunized and permitted to return to fertility, and (3) a group of females which were in utero when their mothers were initially immunized. In addition to fertility assessment, normalcy of ovarian function will be determined by indirect measurements of plasma estradiol (E₂) and progesterone (Po) during seasonal estrus. Urinary estrone conjugates (E₁C) will be measured by enzyme immunoassay (E1A) in order to reflect plasma E₂ concentrations. Urinary Po metabolites (iPdG) will be measured by E1A to reflect circulating Po. Specific ovarian dynamics to be monitored will include follicular phase E₂ secretion, ovulation, luteal phase progesterone secretion, and luteolysis. The use of urinary steroid metabolites to monitor ovarian function will preclude the need to capture or handle horses.

KEY PERSONNEL ENGAGED ON PROJECT

NAME, DEGREE(S), SSN	POSITION TITLE AND ROLE IN PROJECT	DEPARTMENT AND ORGANIZATION
Kirkpatrick, Jay F. B.S., M.S., Ph.D. 162-32-1363	Associate Professor of Physiology	Biological Sciences Eastern Montana College

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*Type density and pitch must conform to limits provided in Specific Instructions on page 12.	
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Number of publications and manuscripts accepted for publication <i>(Not to exceed ten)</i> : _____	
Other items <i>(list)</i> :	

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PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR: **Kirkpatrick, Jay F.**

DETAILED BUDGET FOR FIRST 12-MONTH BUDGET PERIOD						FROM	THROUGH	
DIRECT COSTS ONLY						DOLLAR AMOUNT REQUESTED (Omit cents)		
PERSONNEL (Applicant organization only)		1	2	3				
NAME	ROLE IN PROJECT	TYPE APPT.	% OF APPT.	EFFORT ON PROJ.	SALARY	FRINGE BENEFITS	TOTALS	
Kirkpatrick, Jay F.	Principal Investigator	0.75	21.8	16.3	7000	1705	8705	
To be named	Field Asst.	PT	100	100	3000	18	3018	
To be named	Field Asst.	PT	100	100	3000	18	3018	
Denise Clayton	Lab Tech.	PT	100	100	1260	586	1846	
SUBTOTALS →							16,587	
CONSULTANT COSTS								
EQUIPMENT (Itemize)								
Refrigerator							700	
Pipets							600	
SUPPLIES (Itemize by category)								
Chemicals							700	
Disposable glassware/pipet tips, etc.							1000	
EIA supplies (titer plates, covers, antibodies, etc.)							1000	
Telinject tranquilizer gun, darts, accessories							2500	
TRAVEL								
DOMESTIC							2000	
FOREIGN								
PATIENT CARE COSTS								
INPATIENT								
OUTPATIENT								
ALTERATIONS AND RENOVATIONS (Itemize by category)								
CONSORTIUM/CONTRACTUAL COSTS								
Preparation of PZP antigens by I.K.M. Liu							1500	
OTHER EXPENSES (Itemize by category)								
Sample shipping costs							200	
Lyme disease tests for field assistants							150	
TOTAL DIRECT COSTS FOR FIRST 12-MONTH BUDGET PERIOD (Item 7a) →					\$ 26,337			

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BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		1st BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL (Salary and fringe benefits) (Applicant organization only)		16,587	16,612	16,637		
CONSULTANT COSTS						
EQUIPMENT		700				
SUPPLIES		5,200	2,700	2,700		
TRAVEL	DOMESTIC	2,000	2,000	2,000		
	FOREIGN					
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
CONSORTIUM/ CONTRACTUAL COSTS		1,500	1,500	1,500		
OTHER EXPENSES		350	350	1,350		
TOTAL DIRECT COSTS		26,337	23,162	24,187		

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD (Item 8a)—————→

\$ 73,686

JUSTIFICATION (Use continuation pages if necessary): Describe the specific functions of the personnel, consultants, and collaborators. For all years, explain and justify any unusual items such as major equipment, foreign travel, alterations and renovations, patient care costs, and tuition remission. For additional years of support requested, itemize and justify any significant increases or decreases in any category over the first 12-month budget period. Identify such significant changes with asterisks against the appropriate amounts. If a recurring annual increase or decrease in personnel or other costs is anticipated, give the percentage. In addition, for COMPETING CONTINUATION applications, justify any significant increases or decreases in any category over the current level of support.

Personnel: PI and 2 grad. assts. will spend 3 months/annually on AINS, collecting daily urine samples. Grad. assts. will be working 6-day weeks for 3 months, without stop. PI will also spend March on AINS administering PZP to mares, and October, collecting urine samples for pregnancy testing; PI and technician will spend approx. 3 months/annually analyzing urine samples and data. **EQUIPMENT:** Refrigerator/freezer space is currently saturated; we currently use a Pax-Arms® tranquilizer gun to administer PZP, but recent work with the Telinject® system at the Bronx Zoo proves this to be superior. **CONTRACTUAL COSTS:** Dr. I.M.K. Liu, of UC-Davis will prepare the PZP antigen (see letter in appendix). **TRAVEL:** The PI will be required to make 3 trips/year between Billings and AINS; grad. assts. will have travel costs covered, but will be from the PA/MD area.

BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants listed on page 2. Begin with the Principal Investigator/Program Director. Photocopy this page for each person.

NAME	POSITION TITLE	BIRTHDATE (Mo., Day, Yr.)
Kirkpatrick, Jay F.	Assoc. Professor of Physiology	02/24/40

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
East Stroudsburg State College (PA)	BS, MS	1962, 64	Biology
Cornell University	Ph.D.	1971	Reproductive Physiology
University of Pennsylvania (New Bolten Cnt)	Post-doc	1973 Summer	Steroid analysis
University of California, Davis	Post-doc	1989 Spring	E1A/HPLC of steroids

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

1970-76; 78-81; 85-91 - Associate Professor of Physiology, Eastern Montana College (EMC)
1976-78; 81-85 - Acting Dean, School of Arts and Sciences, EMC
Chairman, Billings Interhospital Institutional Review Board, 1978-88
National Advisory Committee on Animal Damage Control, U.S. Department of Agriculture, 1988 -
Vice President, Board of Directors, Deaconess Research Institute, 1988 -
Eastern Montana College Foundation Distinguished Professorship in Research, 1979
Burlington Northern Foundation Award for Research, 1986, 1990

Kirkpatrick, J.F., L. Kasman, B.L. Lasley and J.W. Turner, Jr. (1988) Pregnancy determination in uncaptured feral horses. J. Wildl. Manage. 52:305-308.

_____ and M.S. Gilluly (1988) Transferrin and hemoglobin polymorphism in feral horses. Northwest Sci. 62:21-27.

_____ and J.W. Turner, Jr. (1989) Contraception as an alternative to traditional deer management techniques. In: Deer Management in Urbanizing Regions, S. Lieberman (ed). Humane Society of the U.S. (in press).

_____, B.L. Lasley and S.E. Shideler (1990) Urinary steroid evaluations to monitor ovarian function in exotic ungulates. VII. Urinary progesterone metabolites in the equidae assessed by immunoassay. Zoo Biol. 9:341-348.

_____, I.M.K. Liu and J.W. Turner, Jr. (1990) Remotely-delivered immunocontraception in feral horses. Wildl. Soc. Bull. 18:326-330.

_____, S.E. Shideler and J.W. Turner, Jr. (1990) Pregnancy determination in uncaptured feral horses based on free steroids in feces and steroid metabolites in urine-soaked snow. Can. J. Zool. 68: ____ - ____.

_____, I.M.K. Liu, J.W. Turner, Jr. and M. Bernoco (1991) Antigen recognition in mares previously immunized with porcine zona pellucida. J. Reprod. Fert. (Suppl.) (in press).

_____, S.E. Shideler, B.L. Lasley and J.W. Turner, Jr. (1991) Pregnancy determination in uncaptured feral horses by means of fecal steroid conjugates. Theriogenology (in press).

_____ and J.W. Turner, Jr. (1991) Contraception in horses. In: Equine Reproduction, J.L. Voss and A. McKinnon (eds). Philadelphia, Lea and Febiger (in press).

Lasley, B.L. and J.F. Kirkpatrick (1991) Monitoring ovarian function in captive exotic species and free-roaming wildlife by means of urinary and fecal steroids. J. Zoo Wildl. Med. (in press).

Bickle, C.A., J.F. Kirkpatrick and J.W. Turner, Jr. (1991) Contraception in striped skunks (Mephitis mephitis) with Norplant® implants. Wildl. Soc. Bull. (in press).

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OTHER SUPPORT

(Use continuation pages if necessary)

FOLLOW INSTRUCTIONS CAREFULLY. Incomplete, inaccurate, or ambiguous information about OTHER SUPPORT could lead to delays in the review of the application. OTHER SUPPORT to be listed here refers to all current or requested support whether related to this application or not. If there are changes subsequent to submission, notify the executive secretary of the initial review group.

For each of the key personnel named on page 2, list, in three separate groups: (1) all currently active support; (2) all applications and proposals pending review or funding; and (3) applications and proposals planned or being prepared for submission. Include all Federal, non-Federal (e.g., for-profit, pharmaceutical, foundations), and institutional research, training, and other grant, contract, and fellowship support at the applicant organization and elsewhere. If part of a larger project, identify the principal investigator/program director and provide the data for both the parent project and the subproject. If none, state "none."

For each item give: (a) the source of support, identifying number and title; (b) percentage of appointment on the project; (c) dates of entire project period; (d) annual direct costs; (e) a brief description of the project; (f) whether the item overlaps, duplicates, or is being replaced or supplemented by the present application; delineate and justify the nature and extent of any scientific and/or budgetary overlaps or boundaries; and (g) any modifications that will be made should the present application be funded.

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR:

(1) CURRENTLY ACTIVE SUPPORT: (a)

- Immunocontraception of the Assateague feral horses; NPS grant CA-1600-3-0005
b) 20% c) 3/91-12/92 d) \$12,466 e) an evaluation of PZP immunocontraception of feral horses
f) the present application will enable a long-term study of immunological contraception with respect to fertility and ovarian function. These parameters are not addressed in the original NPS project. g) NA
- Chemical fertility control in the skunk; Eppley Foundation for Research b) 5% c) 7/88-6/91 d) \$25,000
e) an attempt to contracept skunks with Norplant implants, to control urban populations in quarantined rabies areas. f) NA g) NA
- Mechanisms of reproductive self-regulation in free-roaming ungulates assessed by non-capture methods. NSF grant DCB-8922800 b) 20% c) 6/90-12/93 d) \$91,294 e) an attempt to define the physiological mechanisms which regulate density-dependent reproductive self-regulation, utilizing urinary and fecal steroid metabolites. The study focuses on bison in Yellowstone National Park. f) NA g) NA
- Planning grant for contraceptive studies in wildlife; Humane Society of the U.S. b) 5% c) 7/90-6/91
d) \$12,000 e) development of strategies for contraception of wildlife f) NA g) NA

(2) Pending
None

RESOURCES AND ENVIRONMENT

FACILITIES: Mark the facilities to be used at the applicant organization and briefly indicate their capacities, pertinent capabilities, relative proximity and extent of availability to the project. Use "other" to describe the facilities at any other performance sites listed in Item 9, page 1, and at sites for field studies. Using continuation pages if necessary, include an explanation of any consortium/contractual arrangements with other organizations.

- ☒ Laboratory: The present endocrine laboratory consists of 600 sq. ft., is under the supervision of the PI, and is available for this project. It is located in a relatively new facility (1979). Two additional labs of 667 and 252 sq. ft. house specialized equipment and are dedicated to research. The endocrine laboratory was remodeled in 1990, under an NSF grant.
- ☐ Clinical:
- ☐ Animal:
- ☒ Computer: The Department has access to six Macintosh (SEs) computers and a DEC 20 main frame. None of these are dedicated for research and must be shared.
- ☒ Office: The PI has office space within a few feet of all laboratories described above.
- ☒ Other (Assateague Island): The NPS makes available living quarters and vehicles for all research work on the island. A letter of support can be found in the appendix.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Beckman LS-100 c liquid scintillation counter (a backup for RIA analysis in case of problems with E1A); Dynatech MR700 microtiter reader; IEC-B20A refrigerated centrifuge; rotating shaker for E1A incubation; NML 5000 gamma counter (for RIA backup); pH meters, mixers, limited refrigerator/freezer space.

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as secretaries, machine shop, and electronics shop, and the extent to which they will be available to the project.

A 1.0 FTE secretary is available on a first come-first serve basis. A 1.0 FTE science technical advisor is available for support. Dr. Michael Dennis, a Ph.D. immunologist is available to trouble-shoot E1A problems should they arise.

Since 1980, EMC has graduated 4,467 graduates with the BA/BS. Of these, approximately 113 have received the BA/BS in Biological Sciences or Chemistry. Of these, nine have received the MD, one the OD, four the Ph.D. in health related sciences, two the DDS, and three the DVM, for a total of 19. Additionally, two are currently in medical school, one in dental school and one in veterinary school. Psychology has approximately 10 graduates who have earned the Ph.D. but I cannot be specific about the exact number or the precise nature of their advanced degrees.

INTRODUCTION: This application is a revision of a prior Academic Research Enhancement Award (AREA) application (1 R15 HD26898-01), submitted in 1989. The proposed research includes the active immunization of feral mares with a porcine zona pellucida vaccine (PZP) from one to seven years (prior work will permit this within the 36 month restriction for AREA grants), and an evaluation of the long-term effects of PZP immunization upon ovarian endocrine function. The latter will be accomplished through the use of non-capture evaluation of urinary estrone conjugates and non-specific progesterone metabolites during seasonal estrus.

The original application received a 197 priority score. At the time of the original application, the urinary assay systems for monitoring ovarian endocrine sequelae were established but I had not yet demonstrated that I could in fact measure the major and subtle ovarian endocrine events in the free-roaming, uncaptured, cycling feral mare on a day-to-day basis. Data from a pilot study in 1990 is included in this revised application, and clearly demonstrates the sensitivity, accuracy, and feasibility of the methodology (see pages 15-16). Also, a submitted Ms describing the pilot study has been enclosed.

There were only two criticisms in the summary statement for the original application. The first was that I hadn't included a letter from Dr. I.M.K. Liu, of the University of California-Davis, which indicated that he would be supplying the PZP antigen. A letter from Dr. Liu is included in this application. The second criticism was that the study was mainly descriptive. I assumed that meant that while I might be able to describe the ovarian endocrine changes which might accompany long-term immunization with PZP, I was not able to demonstrate the precise mechanism(s) which cause those changes. The sensitivity of the non-capture monitoring system described herein makes it clear that we can localize the dysfunctional ovarian event, i.e., folliculogenesis, ovulation, luteinization. More important is the assessment of *what* long-term effects we can expect after five, six or seven years of continuous PZP immunization, and whether there is recovery from these effects. ***These represent data which currently are not available for any mammalian species.*** That is the focus of this study. I have made arrangements with the National Park Service to recover ovaries for histological examination from any treated mares which are killed by cars or have to be destroyed for humane reasons. This will enhance our ability to seek the precise causes of long-term dysfunction. New or revised sections of this application are found in brackets [--].

Kirkpatrick, Jay F.

132-62-1363

A. SPECIFIC AIMS:

The specific aims of this project are:

- (1) To evaluate the contraceptive effectiveness and long-term antifertility effects of up to seven years of annual PZP immunization in a population of feral mares.
- (2) To assess the effects of PZP immunization on reproductive success in offspring exposed *in utero* to PZP via maternal immunization.
- (3) To examine the dynamics of cyclical ovarian steroid secretion in chronically immunized infertile mares, in previously immunized but fertile mares, and in recruiting mares previously exposed to immunization *in utero*.
- (4) To compare the antifertility effects of PZP immunization and ovarian dynamics in mares with those found in other mammalian species.

B. BACKGROUND AND SIGNIFICANCE: In 1986, Assateague Island National Seashore (AINS) and the National Park Service embarked upon a research program designed to halt growth of the island's feral horse population by contraception. Following the immunization of 26 mares of proven fertility with porcine zonae pellucidae (PZP) in 1988, inhibition of fertility has been almost 100%. [In 1989, in a continuation of these immunocontraceptive studies, 14 of the immunized non-pregnant mares were given booster injections of PZP and in 1990 and 1991, ten of these mares were given booster PZP inoculations for the third and fourth consecutive years. Seven of these mares were monitored for ovarian endocrine function by means of urinary steroid metabolites. Details of this research and the results to date are given below.] In view of the encouraging data, we feel that this herd of horses represents a unique model for investigating the long-term effects of PZP immunocontraception upon ovarian endocrine function.

Currently a promising immunocontraceptive approach involves active immunization with PZP and its associated glycoproteins. The zona is a non-cellular layer of acidic glycoprotein which envelopes the mammalian oocyte and preimplantation embryo up until the time of implantation (Sacco 1987). The glycoprotein membrane is produced by the oocyte (Wassarman et al. 1984; Leveille et al. 1987) and is composed of several protein fractions. In the pig a 55,000 molecular weight fraction has been shown to be the primary candidate contraceptive antigen. This fraction, referred to as ZP3, has been shown to be the specific zona receptor for sperm recognition and attachment (Sacco et al. 1984, 1989) and the acrosome reaction (Saling 1991).

As early as 1977 it was demonstrated that isolated zonae were effective in preventing fertilization in hamsters (Gwatkin et al. 1977), rats (Tsunoda and Chang 1976a), and mice (Tsunoda and Chang 1976b). Studies directed at defining the antigenic properties of zonae protein from various species of mammals indicate a wide variety of immunogenicity among zonae of the cat, dog, rabbit, pig and rat, since not all zonae antigens are equally effective in raising antibodies (Maresh and Dunbar 1987). Sacco (1977), Hasegawa et al. (1985), and Trounson et al. (1980) all demonstrated that human and porcine zonae share common antigens and thereby suggested that the porcine antigen may be useful in human contraception.

As expected, PZP immunization causes significant antibody titers in non-human primates and has been evaluated in the marmoset (Aitken et al. 1984), cynomolgus monkeys (Gulyas et al. 1983), squirrel monkeys (Sacco et al. 1983, 1987), bonnet monkeys (Bamezai et al. 1986), [and baboons (Dunbar et al. 1989)]. These studies indicate that the administration of PZP or ZP3 or its epitopes and subsequent fertility interruption were often accompanied by disruptions of ovarian function and cycles. In some cases the disruptions were temporary [but in others the disruptions were long-term].

Some abnormalities in the menstrual cycle of cynomolgus monkeys appeared after PZP administration. Menses was interrupted and the expected mid-cycle E₂ elevations were absent for several cycles (Gulyas et al. 1983). Three to 5 months after the last PZP booster immunization, menses and E₂ peaks returned to normal. In another species of monkey, Sacco et al. (1987) found endocrine and histological abnormalities in response to ZP3 injection. Immunized monkeys demonstrated cyclical E₂ and P, but these cycles were different, quantitatively, than those of untreated monkeys and interpreted as non-ovulatory cycles. By 300-400 days, E₂ and P values returned to normal levels and patterns.

One of the greatest concerns regarding the potential use of PZP, ZP3, or any immunogenic fragment, is the possible permanent loss of fertility and the disruption of the reproductive endocrine sequelae associated with normal ovaries. Mahi-Brown et al. (1985) found that immunization of bitches led to long-term alterations of estrous cycles and abnormal steroid profiles. Immunization of rabbits with PZP or highly purified ZP3 also leads to disruption of hormone profiles (Wood et al. 1981; Skinner et al. 1983). Treatment of these animals with hCG failed to induce ovulation and ovaries recovered from these rabbits were only 50% the weight of control ovaries. By six weeks after the last PZP administration Skinner et al. (1983) found a significant decrease in primary, secondary and tertiary follicles. By the 20th week there was almost a complete absence of growing follicles and hCG administration was incapable of inducing

progesterone secretion. These side effects have led investigators to suggest that the mechanism of action of antibodies to PZP in the bitch and the rabbit is focused upon the ovary rather than upon fertilization block. [Among 10 baboons immunized with ZP3, all showed decreased plasma estrogen levels and reduced numbers of antral follicles. Two of these animals failed to ovulate, and five became amenorrheic by 8 months post-immunization (Dunbar et al. 1989). A more recent study with rabbits (Keenan et al. 1991) implicates zona carbohydrate moieties as the cause of ovarian dysfunction.]

Only four studies using PZP have been conducted in horses. In addition to my own study, described below in Section C, Liu et al. (1989) demonstrated that PZP could render mares infertile, that normal fertility returned during the following breeding season and that serum P concentrations and ovarian histology revealed no abnormalities after a single year's treatment.

Several factors may play significant roles in long-term alterations of ovarian function. Firstly, PZP-induced antibodies have been shown to bind with the developing follicular zona pellucida as well as the zona of mature ova (Henderson et al. 1988). This suggests that the antibodies disrupt some developmentally critical cellular communication between differentiating follicular cells and the growing oocyte (Skinner et al. 1990) which ultimately leads to the destruction of a portion or all of the oocyte pool (Skinner et al. 1984). Secondly, the duration of contraceptive efficacy is directly related to the amount of PZP antigen administered over time (Henderson et al. 1988) and to the resulting antibody titers (Millar et al. 1989), and ovarian dysfunction may likewise be related to PZP dose. Patterson and Aitken (1990) however, provide evidence that suggests antibody titers alone cannot explain ovarian dysfunction. PZP antigen doses of 200 µg in squirrel monkeys (Sacco et al. 1987), 360 µg in cynomolgus monkeys (Gulyas et al. 1983) and 5000 µg in baboons (Dunbar et al. 1989) have resulted in varying degrees and durations of ovarian endocrine function, all of which suggest interference with developing follicular ova. A total of seven 60 µg doses given to marmosets did not result in similar dysfunction (Henderson et al. 1988). It is noteworthy that in horses of 300 kg or more, an initial dose of 130 or 195 µg and 65 µg annual doses are sufficient for contraceptive efficacy of greater than 95 %. Despite this relatively small dose, there is evidence for long-term effects upon folliculogenesis after three years of treatment. Thirdly, the purity of the PZP antigen may be a factor in the possible alteration of ovarian function. A majority of the early studies with rabbits and dogs, non-human primates, and now horses, which demonstrated ovarian dysfunction, utilized relatively crude preparations of PZP, which included the entire complement of zonae protein. More recent studies have used preparations of higher purity and even genetically engineered epitopes of

the ZP3 protein. Dunbar et al. (1989) used both ZP1 and ZP3, which share a common epitope, but serious ovarian dysfunction still occurred in baboons after 49 weeks and nine cycles. Alloimmunization with PZP proteins has not yielded acceptable results, clearly because the antigen is not recognized as foreign. However, conjugation of synthetic murine ZP with keyhole limpet hemocyanin (KLH) has produced a vaccine which produces an acceptable contraceptive effect in mice without any evidence of long term ovarian dysfunction after initial immunization and booster inoculations with 100 µg antigen doses (Millar et al. 1989). Finally, the duration of treatment may contribute to long-term effects. Very small doses of high purity may produce long-term effects through cumulative effects upon follicular pools. [No other mammals aside from the horses in my own studies have been treated regularly over four years]. Thus, our current understanding of long-term effects is poor. Taken collectively, the work of Liu et al. (1989), which demonstrated normal ovarian function in mares after one-year treatment with unpurified PZP, and my studies with feral mares, using very small doses of the same antigen, suggest that the lengthy duration of treatment may be an important factor for this observed ovarian dysfunction in mares.

I have chosen to pursue this project with the mare as a model, for several reasons. Firstly, and perhaps most important, 18 months is the longest study of a discrete population of animals involving PZP immunization. [At the onset of this proposed project, 10 mares will have been immunized annually for five years, permitting a longer-term study of immunocontraception and establishing baseline data for a true long-term study of 8 + years. Secondly, the development and validation of non-capture methods of monitoring ovarian steroids through urinary steroid conjugate measurements, provides an efficient, accurate and inexpensive approach to the study of ovarian dynamics]. Thirdly, this study permits the first opportunity to examine the effects of PZP on reproduction in mares exposed to the immunogen *in utero*. Fourthly, while there are some aspects of horse reproductive physiology which are distant from those of the human, the fundamental relationship and patterns of folliculogenesis, luteal formation and function, and E₂ and P are remarkably similar to those of humans and permit the examination of this immunocontraceptive approach and any effects it may have on normal ovarian dynamics in mares with legitimacy. Finally, compared with other studies, the 65 µg doses are very small, but successful, and may approximate minimal dosages designed for humans. Collectively, the data generated by this investigation should prove valuable in the formulation of future studies involving the active immunization of non-human primates.

C. PROGRESS REPORT/PRELIMINARY STUDIES: Between February and May, 1988, I immunized 26 free-roaming feral mares with 2x or 3x 65

µg of heat solubilized PZP. Initial administration of the antigen, with Freund's Complete adjuvant, followed by subsequent inoculations with Freund's Incomplete adjuvant, was accomplished remotely, using barbless darts and a capture gun. In October, 1988, urine samples were collected from all 26 treated mares and six control mares, and pregnancy was diagnosed on the basis of urinary estrone conjugates. Only one pregnancy occurred among 26 mares receiving PZP inoculations. Difference in foaling rate between treated mares and untreated mares was highly significant (Kirkpatrick et al. 1990a). In February of 1989, a single 65 µg dose of PZP was administered to 14 of the previously immunized and non-pregnant mares in order to assess the effectiveness of a single booster administration one year following initial antigen application. Only a single mare produced a foal among these 14 and the foaling difference between treated and untreated mares was highly significant (Kirkpatrick et al. 1991). [During March, 1990 and 1991, 10 of these 14 mares were given another 65 µg booster of PZP, and during May and June, 1990, urine samples were collected from seven of these mares, along with four control mares, and urinary estrone conjugates and non-specific progesterone metabolites (Kirkpatrick et al. 1990b) were measured in order to evaluate ovarian function. None of the 10 mares conceived during summer, 1990, and four of the seven monitored for ovarian function had significantly lower E₁C concentrations and failed to ovulate (Kirkpatrick et al. submitted)].

[Only a single study has been reported which evaluated the effects of PZP immunization upon pregnant animals, the subsequent progress of the pregnancy, or the health of the offspring (Sacco et al. 1981). This study indicated that PZP antibodies do cross the mouse placenta and attach to follicles in the fetal ovary.] The mares in our study were initially immunized between February and April of 1988, and the foaling rate among these mares later that spring, was 60%. This foaling rate was normal for this population of mares, the foals were born healthy, and survival is within normal ranges for this population. Retrospectively we can see that immunization during the 10th or 11th month of pregnancy does not interfere with pregnancy, the health of the foals or the antifertility effects of immunization.

My ability to monitor ovarian endocrine function by means of urinary steroid conjugates developed over a five year period working with B. L. Lasley, (University of California-Davis). In initial studies we successfully diagnosed pregnancy in free-roaming horses by measuring urinary estrone sulfate (E₁S) (Kirkpatrick et al., 1988). That same test was used to diagnose pregnancy among the treated Assateague mares. In March of 1989, under the sponsorship of an NSF Research Opportunities Award, in B. L. Lasley's laboratory at UC-Davis, I was successful in measuring equine urinary non-specific progesterone metabolites (iPdG) by

means of an enzyme immunoassay (EIA) (Kirkpatrick et al. 1990b). [Thus, at the present time, I am capable of measuring urinary correlates of both serum E₂ and P, non-invasively, and thereby monitor the major events of ovarian dynamics. Examples of a ovulatory conceptive cycle (Fig. 1A), an ovulatory non-conceptive cycle (Fig. 1B), and parturition (Fig. 1C) among control mares and a non-ovulatory PZP-treated mare (Fig. 1D), based on urinary steroid metabolites in Assateague mares are illustrated below. Note the sensitivity of this model by the rise in E₁C following parturition, which represents an attempt at post-partum folliculogenesis in the mare in Fig. 1C].

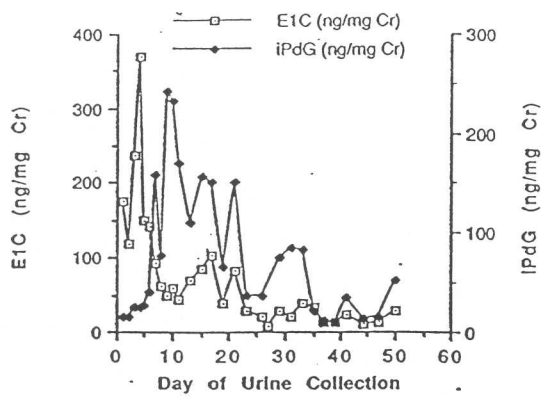


Fig. 1A. Ovulatory non-conceptive Cycle, control mare.

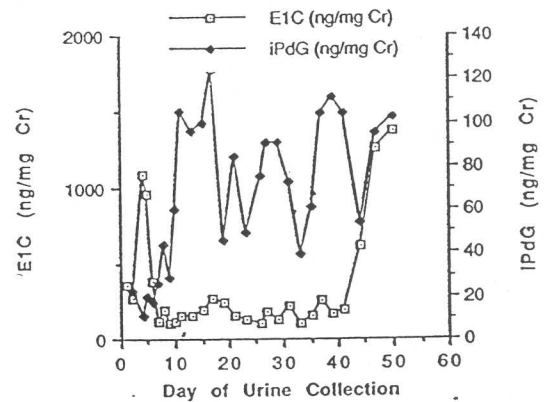


Fig. 1B. Ovulatory conceptive cycle, control mare.

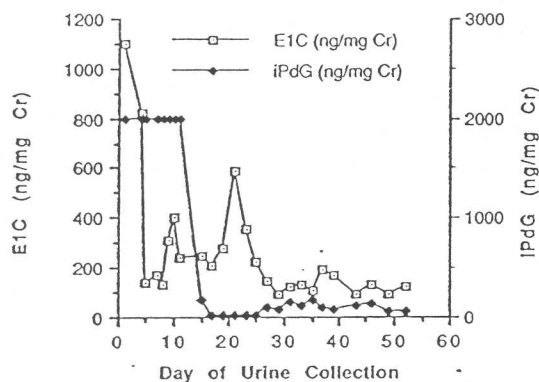


Figure 1C. Parturition in a control mare.

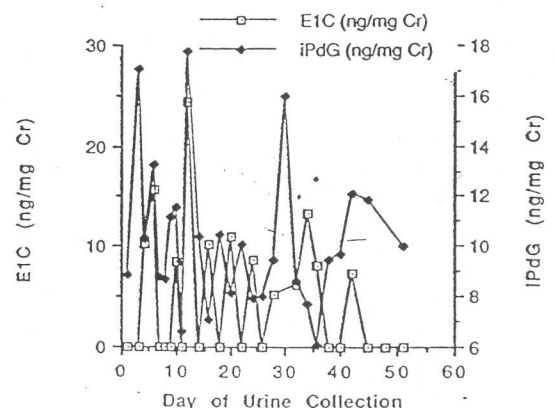


Figure 1D. Depressed E₂ and absence of ovulation in a chronically treated mare

D. EXPERIMENTAL DESIGN:

1. **PREPARATION OF PZP:** PZP antigen will be prepared by Dr. Irwin Liu, School of Veterinary Medicine, University of California, Davis, as

described by Liu et al. (1989). A letter confirming his cooperation is appended.

2. IMMUNOCONTRACEPTION SCHEMA: [At the start of this proposed project, I will have access to approximately (A) 8 mares given a single initial PZP immunization in 1988, (B) 10 mares given the initial immunization plus a 65 µg booster in each of the years 1989, 1990, and 1991, (C) four-year old females (N=4) which were exposed to the vaccine *in utero*, and (D) an unimmunized population (N=25-30), which can be used for a new series of inoculations and for controls. Half of the 10 mares initially treated in 1988 and with the boosters thereafter would receive a fifth dose, and half will be removed from PZP treatment. The initial population was chosen non-randomly, with a bias for high fertility rates. All new experimental populations will be selected randomly from among sexually mature mares. The complete experimental schema is summarized in Table 1. In this way, I can examine animals with no treatment (N=10), one treatment (N=18), two treatments (N=10), three treatments (N=10), four treatments, (N=10), five treatments (N=5), six treatments (N=5), and seven treatments (n=5) and one, two, and three years post-treatment. Additionally, during 1992 through 1995 I can examine reproductive success and ovarian endocrine function in the mares exposed *in utero*]. Treatment will be as previously described, by means of barbless darts fired from a capture gun. The PZP is administered directly into the hip or gluteal muscles at a range of 25-40 meters and no tranquilizers, anesthetics or analgesics are used. The use of Freund's Complete adjuvant raises an occasional abscess at the injection site. Five such abscesses have been observed in 144 dartings since 1988. Each abscess is about 20-30 mm in diameter, appears within two days of darting, drains on the seventh to tenth day without untoward effects. Thus far I have darted horses approximately 150 X in this population without incident or injury to the horses.

All animals selected, with the exception of the newly recruited mares, will be of proven fertility. Initial administration of the antigen/adjuvant combination (0.5 cc PZP, consisting of approximately 5000 zonae or 65 µg of protein, 0.5 cc of adjuvant, and 0.5 cc of phosphate buffer) will occur in March. Freund's Complete adjuvant will be used for initial immunizations and Freund's Incomplete adjuvant for subsequent inoculations. I realize that Freund's adjuvants will not be practical for use in humans, but since we began our project on Assateague with these adjuvants I do not think it wise to introduce a new variable by changing adjuvants.

3. CONTRACEPTIVE ASSESSMENT: Contraceptive assessment will be made on the basis of pregnancy diagnosis, as determined by urinary and fecal E₁C and iPdG, and live foal counts. Foaling differences between

TABLE 1
IMMUNOCONTRACEPTION SCHEMA

Exptl. Group	N	1988	1989	1990	1991	1992	1993	1994
1	18	X	-	-E	-E	-	-	-
2	5	X	X	X-E	X-E	X-E	X-E	X-E
3	5	X	X	X-E	X-E	X-E	-E	-E
4	10	-	-	-	-	X-E	X-E	X-E
5	4 *	-	-	-	-	-E	-E	-E
6	10 **	-	-	-E	-E	-E	-E	-E

(X = PZP immunization; E = ovarian endocrine assessment; * = *in utero* mares; **= controls)

treated and experimental groups will be tested for significance by means of binomial probability distribution.

4. ASSESSMENT OF OVARIAN DYNAMICS: The mare is a seasonal breeder, with a winter anovulatory season characterized by low E₂ and ovarian P, a spring and fall transitional season, characterized by anovulatory E₂ cycles and low P, and a light-dependent breeding season characterized by classic E₂ and P cycles and accompanied by ovulation (Ginther 1979). On Assateague Island, the ovulatory season begins in late April and terminates rather abruptly in August (Keiper and Houpt 1984). This ovulatory season consists of cyclical estrous periods of 6.8 ± 2.3 days separated by a diestrus of 15 ± 2.1 days (Back et al. 1974).

The estrous cycle is characterized endocrinologically by increasing plasma E₂ from day -8 to days -2 or -1, when a peak concentration of total E (50 pg/ml) and E₂ (12 pg/ml) is reached. Following ovulation, E₂ concentrations decline to ≈ 2 pg/ml. This same pattern of E₂ secretion is seen during the spring and fall transitional anovulatory seasons and is often accompanied by behavioral estrus.

The main source of P in the non-pregnant mare is the corpus luteum, with ranges between ≈ 50 pg/ml and 1.0 ng/ml plasma. Seven to eight days prior to the onset of estrus, P levels decline, reaching 0.5 ng/ml or lower, which is required for successful ovulation (Ginther 1979). Both E₂ and P reflect the dynamics of the functioning ovary. E₂ is the primary steroid of follicular origin and the best indicator of follicular health and viability. The formation of the corpus luteum is a requisite consequence of ovulation and its primary secretory product is progesterone. Thus, these two steroids are excellent markers of ovarian dynamics. The equine follicle, in some ways, is a sound model for studying human follicular dynamics because of similarity in the timing of events. These follicles

have a relatively long interval from the onset of estrus to ovulation and are extremely predictable with regard to E₂ secretion (Ginther 1979). The temporal pattern of circulating E₂ and P in the cycling mare is almost identical to that of the bitch (Mahi-Brown et al. 1985) and differs from that of the non-human primate (Gulyas et al. 1983) only in that the E₂ peak occurs one or two days prior to ovulation, and the post-ovulatory rise in P is a bit more pronounced. This similarity will permit the comparison of time-related endocrine events between the mare, primates and the bitch, as well as control mares.

Until recently most of what is known regarding cyclic E₂ and P secretions in the mare have been derived from direct RIA of plasma or urinary free steroids. The collection of blood is not possible in this study and reason alone dictates that only minor concentrations of non-polar free steroids will be excreted in the urine. Recently it has been demonstrated that urinary estrone conjugates are the primary metabolic products in equids, can be measured by enzyme immunoassay (EIA), and reflect plasma E₂ concentrations (Evans et al. 1984; Kasman et al. 1984; Lasley et al. 1989). Both pregnancy and follicular dynamics can be evaluated. Kirkpatrick et al. (1988;1990a) demonstrated that urine can be extracted from various soils and urinary E₁C measured to accurately reflect pregnancy and other reproductive events. It is noteworthy that, while plasma E₂ circulates in the low pg range, E₁C is measured in the ng and µg range.

[Recently, Kirkpatrick et al. (1990b) applied an EIA for non-specific progesterone metabolites to mare urine and were successful in monitoring cyclic progesterone changes. While the specific identities of the progesterone metabolites are as yet unknown, they have a high correlation with plasma Po (Kirkpatrick et al. 1990b].

Urine will be collected from uncaptured immunized and control mares on Assateague Island as described by Kirkpatrick et al. (1988) at a minimum of every third day, and more frequently when possible, between April 1 and June 30 of each of the three years in this project. This collection regimine has been used successfully to characterize the the estrous cycle of the mare by means of plasma E₂, P, and LH (Kirkpatrick and Turner 1983), and by urinary E₁C and iPdG (Kirkpatrick et al. submitted- see appendix). In order to normalize E₁C and iPdG concentrations to urine concentrations, which vary significantly among the island horses, creatinine concentrations will be measured by the method of Taussky (1954) and hormone metabolite concentrations reported as ng or µg/mg creatinine.

Using these urinary steroid assessments, ovarian dynamics will be monitored for mares as indicated in the schema above. Pregnancy will be assessed in October of each year by urinary E₁C and iPdG. Annual foal

counts will be conducted each August. [With the assistance of Park Service personnel, any treated mares which must be destroyed due to vehicle collisions or diseases such as equine encephalitis will be necropsied and the ovaries recovered, preserved and prepared for histological examination. A letter of cooperation is provided by the NPS in the appendix].

5. ANALYSIS OF DATA: Quantitatively, there are often significant differences in circulating E₂ and P concentrations in normal mares, thus means for urinary E₁C and iPdG concentrations within experimental and control groups will be compared for significant differences at the 0.05 level of confidence. The endocrine data from immunized mares will be evaluated for evidence of normal estrous cycles, folliculogenesis, ovulation and luteolysis, and compared with values for control mares with conceptual cycles.

Pregnancy rates and foals produced will be compared between all experimental groups and from among a group of 10-15 control mares. Control groups will be matched to experimental groups on the basis of age, past reproductive history, and social rank in order to minimize bias for these three parameters (some mares produce foals more frequently than others of the same age and social rank; foaling rates are age-specific, and there may be differential foaling rates for mares of different social rank).

E. HUMAN SUBJECTS: None

F. VERTEBRATE ANIMALS: Approximately 35-40 free-roaming feral mares will be involved in this study. The breed is unknown, and the ages of the mares involved in the study will range from three to 20. The age and reproductive history of each mare is known. Ten of these animals were selected because they represent a discrete population with a four-year history of PZP immunization. These animals receive no veterinary care from the National Park Service, nor are members of the public permitted to have contact with the animals. The only contact with humans in the course of this project will involve the administration of a 3 cc plastic, self-pressurized dart, fired from a capture gun. The darts are barbless and fall out on their own after a short time. Animal welfare committee assurance is attached.

G. CONSULTANTS/COLLABORATORS: None

H. CONSORTIUM/CONTRACTURAL ARRANGEMENTS: The only contractural arrangement involves payment for the preparation of the PZP, by Dr. Irwin K. Liu, Department of Reproduction, School of Veterinary Medicine, University of California-Davis.

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