



I, Cassandra Nuñez, do hereby declare as follows:

1. I am a behavioral ecologist. I currently work as an Adjunct Assistant Professor in the Department of Natural Resources, Ecology, and Management at Iowa State University. I received my Ph.D. in Behavioral Ecology from Princeton University. I received my Bachelor's degree in Laboratory Psychology from Rutgers University. My curriculum vitae is attached hereto.

2. As part of my academic work and scholarship at Princeton University, I researched the behavior and physiology of wild horses and the effects of contraceptive management, including the administration of Porcine Zona Pellucida ("PZP").

3. Researchers, including myself, have raised concerns about the repeated use of PZP.

4. I have reviewed the 2010 Final Environmental Assessment for the Clan Alpine, Pilot Mountain and Pine Nut Herd Management Areas Gather Plan (hereinafter, "2010 EA") and its discussion of PZP. What follows is my review of that plan and of more recent research on the potential effects of PZP on treated individuals and the subsequent effects to their untreated counterparts.

5. Although the Bureau of Land Management (BLM) report from 2010 is accurate regarding the information that was available at the time of its writing, it is outdated now. Recent research has demonstrated changes in mare stress and reproductive physiology, in addition to changes in male behavior. For example, we now know that mares which change groups more often (such as those treated with PZP) can exhibit increased stress levels and that this increased stress is maintained for at least two weeks after the group changes occur (Nunez, Adelman et al. 2014). Short-lived stressful situations are commonplace for several species (Sapolsky 2005); however, repeated increases in stress hormones can adversely affect cardiovascular and immune function and, in the most extreme cases, can result in adverse neurobiological effects (Sapolsky 2005).

6. In addition, research on Shackleford mares that receive PZP over extended periods are more likely to cycle, become pregnant, and subsequently give birth in the fall (Nuñez, Adelman et al. 2010) and winter (unpublished data) months. Normally, mares do not ovulate at this time of year which ensures that they do not become pregnant and subsequently give birth during the fall and winter when resources are low (gestation is approximately one year in this species). Offspring born at this time face nutritional and thermoregulatory challenges not experienced by their counterparts born during the normal foaling season (during the spring and summer), potentially making developmental benchmarks difficult to achieve (Sadleir 1969). In addition, my colleagues and I have shown that after contraception management, PZP recipients both attract and initiate more instances of reproductive behavior (Nuñez, Adelman et al. 2009) and are more often the harem male's nearest neighbor during the fall and winter (Nuñez 2011), indicating that group spreads are reduced. These changes can be important as horses typically spread out in the

fall and winter months to find scarce forage (Duncan 1985; Morel, McBride et al. 2006). Such changes represent an increase in energy expenditure and a potential decrease in nutrient intake during a time of year when sufficient energy reserves are at a premium (Sadleir 1969).

7. Also, PZP is not completely reversible. The ability of mares to become pregnant after treatment is dependent upon the number of consecutive treatments received (Kirkpatrick and Turner 2002). Mares treated for more consecutive years are more likely to exhibit the behavioral and physiological changes outlined above (Nuñez, Adelman et al. 2010), decreases in ovarian function (Kirkpatrick, Liu et al. 1992), and perhaps, permanent infertility. Shackelford mares for which treatment was halted in 2009 have yet to return to pre-contraception levels of fertility (unpublished data, see Figure 1). This effect is exacerbated in mares that received more consecutive treatments (unpublished data).

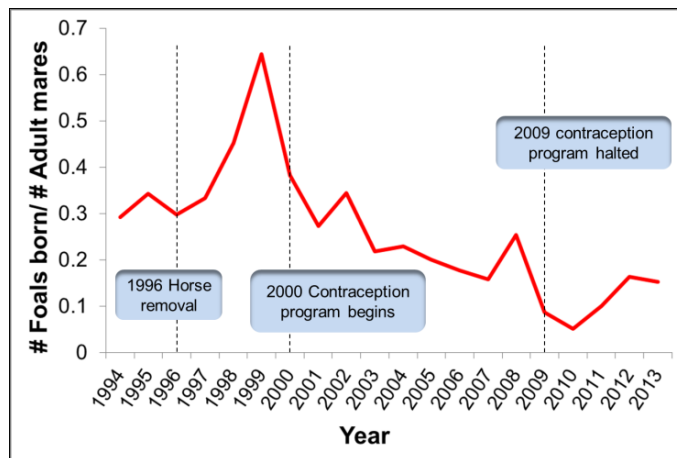


Figure 1. Pregnancy in Shackelford mares before, during and after contraception management.

8. Finally, according to the report of 2010, it would seem that the plan is to vaccinate non-reproductive females (those between 1 and 3 years old). This precludes young mares from forming the important social attachments between males and females typically made when foals are conceived. Such changes could further affect herd dynamics.

9. While I do agree that measures which decrease the number of gathers performed are generally beneficial to horse populations, I have reservations about the potential behavioral and physiological effects to horses treated with PZP and the subsequent changes in their associates' behavior. **However, it is asserted that careful scheduling of treatment can ameliorate all of the effects outlined above.** The example set by Kirkpatrick (Kirkpatrick 1995) is a good one; careful plans were made as to which animals were to be treated and how often prior to any contraceptive treatment. The National Park Service and the research team (which consisted of horse, reproductive, and population biologists, immunologists, and endocrinologists) worked together to construct the plan. It is my professional opinion that this careful planning is what has

led to the minimal changes to behavior and physiology documented in the Assateague population.

10. While the BLM's assessment of horse impacts to public lands has been deemed to lack scientific rigor (National Research Council 2013), as a biologist, I do understand that without predators to control them, prey species will very likely have adverse impacts on the lands in which they live. And I do not pretend to know or understand the challenges the BLM faces working with open populations of horses; however, if at all possible, careful planning and consultation ought to be conducted before any contraception plan is undertaken. Again, I do not know what costs such consultation would involve; they may prove to make these proposed measures infeasible. However as stewards of these populations, I believe that we have a responsibility to manage them with the most effective and humane means possible.

11. Pursuant to 28 U.S.C. § 1746, I declare, under penalty of perjury, that the foregoing is true and correct. Executed in Ames, Iowa on January 29, 2015.



Cassandra Nuñez, Ph.D.

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### **Academic and Research Appointments:**

**Adjunct Assistant Professor**, Department of Natural Resources Ecology and Management, Iowa State University, present.

**Visiting Research Scholar**, Department of Biological Sciences, Virginia Polytechnic Institute and State University, 2012–2014.

**American Association for the Advancement of Science, Science and Technology Policy Fellow**, NASA, Applied Sciences Program, Earth Science Division, 2011–2012.

**Associate Research Scholar and Lecturer**, Department of Ecology and Evolutionary Biology, Princeton University, 2004–2006; 2008–2011.

**Co-Director**, Primate Behavior, Wildlife Ecology, and Conservation Field School, Rutgers University, 2008.

**Education and Outreach Coordinator**, Phillip L. Boyd Deep Canyon Desert Research Center, University of California, Riverside, 2007–2008.

**Lecturer and Mentor**, Organization for Tropical Studies, Duke University, 2006.

**Lecturer and Mentor**, Princeton University Preparatory Program, Princeton University, 2005.

**Lecturer**, Rutgers University, 2004.

**Lecturer and Mentor**, Math and Science for Minority Students Program, Phillips Academy, 2004.

**Project Manager**, Serengeti Carnivore Disease Project, Princeton University, 2002–2003.

**Project Manager**, Zebras of Kenya Project, Earthwatch Institute, 2000–2001.

**Consultant**, National Park Service, Cape Lookout National Seashore, 1996–2000.

### **Education:**

Ph.D. 2000 Behavioral Ecology, Princeton University, Princeton, New Jersey.

B.A. 1993 Laboratory Psychology, Douglass College, Rutgers University, New Brunswick, New Jersey.

## Honors and Awards:

Writing Productivity Workshop (NSF-IOS sponsored), August 2013— selected participant  
American Association for the Advancement of Science Fellowship, 2011–2012— \$84,000.00  
Sigma Xi Grant-in-Aid of Research (GIAR), 1996— \$1,000.00  
National Science Foundation Graduate Research Fellowship, 1994–1997— \$120,000.00  
Presidential Fellowship, 1993–1999— \$40,000.00  
American Psychology Association, 1992  
Minority Undergraduate Students of Excellence Program, 1991–1993— \$6,000.00

## Teaching:

**Courses (full responsibility for all material):** Comparative Physiology (with laboratory), Animal Behavior, Conservation Biology (with field work), Junior Tutorial in Statistics, Natural History of African Mammals (with field work), Desert Ecology (day course with field work), Natural History of Desert Species (day course with field work), Invasive Species Management (day course with field work).

**Co-led courses (responsibility shared with other faculty):** Laboratory Biology, Laboratory and Field Biology.

**Teaching Assistantships (introducing material designed by others):** Evolution and Behavior of the Sexes, Ecology of Fields and Forests, Conservation Biology, Evolutionary Ecology.

**Mentorships (individuals and small groups):** I have served as a mentor to nearly 40 Princeton seniors working on their senior theses. I have taught them field and data collection techniques, and have advised them on the analysis and interpretation of behavioral and demographic data for three ungulate species: feral horses, white-tailed deer, and cattle.

**Outreach:** I created and lead interpretive hikes at the Phillip L. Boyd Deep Canyon Desert Research Center, focusing on the area's natural history and archaeological significance, and instituted the Phillip L. Boyd Deep Canyon Public Lecture Series, which continues to offer presentations by local biologists and historians.

**Instructional Development:** I have participated in the following workshops to further improve my teaching: SCALE UP Pedagogy to Create Inclusive Learning Environments (April 3, 2014), Flipping the Large Classroom (April 17, 2014), Peer Review Pedagogy to Support Authentic Engagements in Learning (April 23, 2014).

## Research Interests:

My research integrates animal behavior and physiology in the wild to answer both applied and basic questions, using feral horses as a model system.

**Contraceptive Management:** My research has shown that contracepted mares are less loyal to the band stallion; they change social groups more often, particularly during the non-breeding season. In addition, contracepted mares extend reproductive cycling into the non-breeding season. How do

these changes in behavior and reproductive physiology affect other members of the population? For example, has there been an increase in the escalation of male-male conflicts in an attempt to retain contracepted mares? Are levels of stress, measured through fecal cortisol, higher in groups that include more contracepted mares? Furthermore, how might the use of immunocontraception affect population fitness? Does stimulation of the immune system to achieve infertility select for individuals with low immunocompetence? Answers to these questions can help managers make more ethical and responsible decisions regarding the population control of species.

**Mother-Young Relationships:** How do differences in mothering strategy contribute to offspring survival? What can differences in communicative behaviors of mothers and infants tell us about the function of communication for different individuals? What do we really know about adoption in the wild? Answers to these questions can help researchers better understand the function of the juvenile stage in mammals and the importance of maternal effort and style to recruitment.

## **Publications:**

**Núñez, C.M.V., J.S. Adelman, D.I. Rubenstein.** 2014. Sociality increases juvenile survival after a catastrophic event in the feral horse (*Equus caballus*). **Behavioral Ecology**, doi: <http://beheco.oxfordjournals.org/content/early/2014/09/28/beheco.aru163>.

**Núñez, C.M.V., A. Scorolli, L. Lagos, D. Berman, A. Kane.** 2014. Management of free-roaming horses in J.I. Ransom and P. Kaczensky, eds. **Wild Equids**. The Johns Hopkins University Press. *Invited, in review*.

**Núñez, C.M.V., J.S. Adelman, J. Smith, L.R. Gesquiere, and D.I. Rubenstein.** 2014. Linking social environment and stress physiology in feral mares (*Equus caballus*): Group transfers elevate fecal cortisol levels. **General and Comparative Endocrinology**, 196: 26-33. doi: <http://dx.doi.org/10.1016/j.ygcen.2013.11.012>.

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**Núñez, C.M.V.** 2011. Management of wild horses with porcine zona pellucida: History, consequences, and future strategies, Pages 85-98 in J.E. Leffhalm, ed. **Horses: Biology, Domestication, and Human Interactions**. Nova Science Publishers, Inc. *Invited*.

**Núñez, C. M. V., C. S. Asa, and D. I. Rubenstein.** 2011. Zebra reproduction, Pages 2851-2865 in A. O. McKinnon, E. L. Squires, W. E. Vaala, and D. D. Varner, eds. **Equine Reproduction, Second Edition**. Ames, IA, Wiley-Blackwell. *Invited*.

**Núñez, C.M.V., J.S. Adelman, and D.I. Rubenstein.** 2010. Immunocontraception in wild horses (*Equus caballus*) extends reproductive cycling beyond the normal breeding season. **PLOS ONE**, 5(10): e13635. doi: <http://dx.doi.org/10.1371/journal.pone.0013635>.



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## **Scientific Meetings and Presentations:**

### **Invited talks and posters:**

“Linking social behavior and stress physiology in feral mares (*Equus caballus*): Group transfers elevate fecal cortisol levels”, Animal Behavior Society. August 2014.

“Mares gone wild: Immunocontraception alters female behavior and physiology in feral horses”, University of North Carolina, Asheville, Department of Biology, Undergraduate Seminar Series. April, 2014.

“Mares gone wild: Immunocontraception alters female behavior and physiology in feral horses”, Virginia Polytechnic Institute and State University, Department of Biological Sciences Ecology, Evolution, and Behavior Seminar Series. September, 2013.

“Immunocontraception in feral horses (*Equus caballus*) extends reproductive cycling beyond the normal breeding season”, International Wild Equid Conference. September 2012.

“Immunocontraception, social behavior, and stress in a feral horse population”, International Wild Equid Conference. September 2012.

“Behavioral effects of immunocontraception on wild horses (*Equus caballus*)”, International Society for the Preservation of Mustangs and Burros. October 2008.

“Behavioral effects of immunocontraception on wild horses (*Equus caballus*)”, Wikelski Laboratory Summit, Max Planck Institute of Ornithology. October 2008.

“The importance of safety and friends to the conservation of Grevy’s zebra”, The Living Desert, Grapes for Grevy’s Fund Raiser. March 2008.

“Desert research topics”, California Regional Environmental Educational Community Conference. December 2007.

### **Contributed talks and posters:**

“Linking social environment and stress physiology in feral mares (*Equus caballus*): Group transfers elevate fecal cortisol levels”, Society for Conservation Biology, International Congress for Conservation Biology. July 2013.

“Horses gone wild! Contraception, Promiscuity, and Pregnancy... oh my!” Nerd Nite. December 2012.

“Biodiversity research and conservation biology from space: NASA’s Biological Diversity and Ecological Forecasting Programs”, Society for Conservation Biology, North America Congress for Conservation Biology. July 2012.

“NASA Applied Sciences Program: Providing remotely sensed data for conservation and management”, Biodiversity Without Boundaries. April 2012.

“Why contracepted mares are more ‘frisky’ ”, American Association for the Advancement of Science Research Blitz. March 2012.

“Engaging NASA in the definition and development of conservation applications”, Society for Conservation Biology, International Congress for Conservation Biology. December 2011.

“Variation in the signaling between mares and foals (*Equus caballus*): Implications for the function of communication for mother and offspring”, Acoustic Communication by Animals, Third International Symposium. August 2011.

“Immunocontraception in wild horses (*Equus caballus*) extends reproductive cycling beyond the normal breeding season”, Princeton Research Symposium. Third place winner. November 2010.

“Immunocontraception in mares (*Equus caballus*) extends ovulatory cycling into the non-breeding season”, Princeton Chapter of Sigma Xi, the Scientific Research Society, Graduate and Post-Doctoral Poster Competition. First place winner. April 2010.

“Behavioral effects of immunocontraception on wild horses (*Equus caballus*)”, Princeton Chapter of Sigma Xi, the Scientific Research Society, Graduate and Post-Doctoral Poster Competition. First place winner. April 2009.

“Behavioral effects of immunocontraception on wild horses (*Equus caballus*)”, Society for Integrative and Comparative Biology. January 2009.

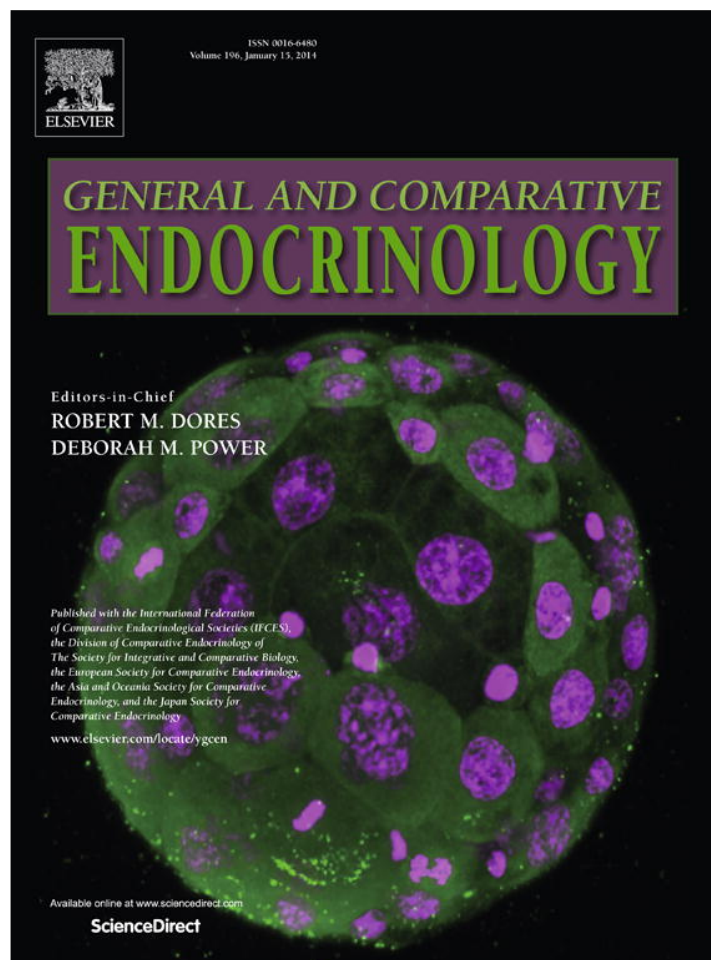
“Behavioral effects of immunocontraception on wild horses (*Equus caballus*)”, Princeton Research Symposium. Received Honorable Mention. November 2008.

“Mortality and recruitment of desert perennials as related to extreme drought: The loss of drought deciduous shrubs from low elevations”, with Edward G. Bobich, Ecological Society of America. August 2008.

“Behavioral effects of immunocontraception on wild horses (*Equus caballus*)”, Society for Conservation Biology. July 2008.

“The importance of safety in watering site choice of Grevy’s zebra (*Equus grevyi*) mothers”, Society for Conservation Biology. July 2002.

“Variation in the mother-infant relationship in wild horses; Implications for the function of the juvenile stage”, Euro-American Mammal Congress: Challenges in Holarctic Mammalogy. July 1998.



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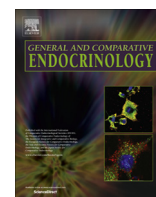
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## Linking social environment and stress physiology in feral mares (*Equus caballus*): Group transfers elevate fecal cortisol levels



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### ABSTRACT

Feral horses (*Equus caballus*) have a complex social structure, the stability of which is important to their overall health. Behavioral and demographic research has shown that decreases in group (or band) stability reduce female fitness, but the potential effects on the physiological stress response have not been demonstrated. To fully understand how band stability affects group-member fitness, we need to understand not only behavioral and demographic, but also physiological consequences of decreases to that stability. We studied group changes in feral mares (an activity that induces instability, including both male and female aggression) on Shackleford Banks, NC. We found that mares in the midst of changing groups exhibit increased fecal cortisol levels. In addition, mares making more group transfers show higher levels of cortisol two weeks post-behavior. These results offer insights into how social instability is integrated into an animal's physiological phenotype. In addition, our results have important implications for feral horse management. On Shackleford Banks, mares contracepted with porcine zona pellucida (PZP) make approximately 10 times as many group changes as do untreated mares. Such animals may therefore be at higher risk of chronic stress. These results support the growing consensus that links between behavior and physiological stress must be taken into account when managing for healthy, functional populations.

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### 1. Introduction

Social mammals typically organize themselves into coherent groups that feed, rest, and travel together (Alexander, 1974). Social living affords several benefits, but can also accrue costs, including increased competition for resources (Sterck et al., 1997), exposure to parasites and disease (Côté and Poulin, 1995), and stress (Sapolsky, 1983). Social interactions, dominance rank, and variations in population density due to territorial intrusion, predation risk, and food availability are all associated with increases in glucocorticoid levels in several vertebrate species (Creel et al., 2013).

The mammalian stress response occurs when adverse situations trigger the adrenal glands, resulting in increased secretion of glucocorticoids and/or catecholamines (Mostl and Palme, 2002). Short-lived stressful situations are commonplace for a wide range of species (Sapolsky, 2005), and the stress response is adaptive in that it helps organisms escape these dangerous or otherwise taxing

situations (Mostl and Palme, 2002; Sapolsky, 2005). For example, increases in glucocorticoids in response to adverse conditions can facilitate facultative migration (Ramenofsky et al., 2012), stimulate immune responses (Martin, 2009), and regulate food intake (Wingfield et al., 1998). When experienced chronically, however, the stress response can become pathogenic (Sapolsky, 2005). Animals that are more persistently stressed exhibit higher basal glucocorticoid concentrations (but see Dickens et al. (2009)), enlarged adrenal glands, and impaired sensitivity of the adrenal system to regulation by negative feedbacks (Sapolsky, 2005). Continued stress can also adversely affect cardiovascular function, inhibit reproduction, compromise immune function, and result in a number of adverse neurobiological effects including decreased neurogenesis, dendritic atrophy, and diminishing synaptic plasticity in the hippocampus (Sapolsky, 2005).

Feral horses (*Equus caballus*) have a complex social structure: social groups, or bands, typically consist of one (though sometimes more than one) male(s), one to several female(s), and their offspring. Excepting dispersal of both male and female offspring, stable bands will remain together for several years (Klingel, 1975). Mares will often remain in the same band for most of their adult lives and will form close associations with one another (Cameron et al., 2009). Reductions in band stability have been correlated with decreases in mare fitness, including increases in parasite load, and

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declines in body condition, fecundity, foal survival, and time spent engaged in preferred behaviors (Klingel, 1975; Linklater et al., 1999; Rubenstein, 1986). Such decreases in stability, whether due to increased male and/or female harassment (including increases in aggressive and/or reproductive behaviors) or the addition/subtraction of various group members, could induce a physiological stress response in mares, especially if stability is not restored. However, the potential effects of such behavior on mare stress physiology have not been documented. Such studies are important as they give managers and researchers a method to more quantitatively measure the physiological effects of behavioral stressors on individual health (Wikelski and Cooke, 2006). In addition, a greater understanding of the physiological responses to social instability may shed light on how the social milieu affects overall female fitness.

Here we seek to elucidate the links between social behavior and physiology in a highly social ungulate, the feral horse. Specifically, we investigate the effects of band transfers on mare stress physiology via fecal cortisol concentrations, a demonstrated indicator of individual stress level (Mostl and Palme, 2002; Wasser et al., 2000). In addition, we investigate the more general effect that increased frequency of band transfer has on mare stress physiology.

## 2. Materials and methods

### 2.1. Study area

We conducted this study on Shackleford Banks, a barrier island located approximately 3 km off the coast of North Carolina, USA. The island is 15 km in length, and varies between 0.5 and 3 km in width. The specific study area extended approximately 7.7 km from the center to the eastern end of the island.

The horse population on Shackleford Banks has been co-managed by the National Park Service and the Foundation for Shackleford Horses since 1996. The Shackleford Banks Wild Horses Protection Act stipulated that the Shackleford population consist of no less than 100 and no more than 110 horses (United States Congress, 1997), similar to the population occurring in 1981 (104 horses) (Rubenstein, 1981; Rubenstein and Dobson, 2000). At the time of this study, the population consisted of 124 and 118 horses during the breeding and non-breeding seasons, respectively. The National Park Service and the Foundation for Shackleford Horses maintain the population at this level through contraceptive management with porcine zona pellucida (PZP).

### 2.2. Study subjects

The social groups of Shackleford horses are typical of feral equids. They consist of coherent bands of one or sometimes two or three stallion(s) with one to several mare(s) and their offspring. Although the bands are predominantly non-territorial and the animals move freely within overlapping home ranges (Rubenstein, 1981), individual bands are spatially distinct from one another and individuals of particular bands rarely interact (Feist and McCullough, 1976; Rubenstein, 1981, 1986). When interactions do occur, they typically involve younger individuals engaging in play and/or exploration, the dispersal of sub-adult individuals (both male and female), and the transfer of adult females from one band to another (personal observation).

Historically, the bands on Shackleford Banks were long-lasting with most changes involving the dispersal of immature individuals (Nuñez, 2000; Rubenstein, 1981). Males sometimes fought to acquire mares from other groups, but stallions almost always retained their mares (Nuñez, 2000; Rubenstein, 1981). During a five-year study (Rubenstein, 1981), only 10.8% of the mares studied

transferred groups, as is typical in other feral horse populations (Berger, 1977; Feist and McCullough, 1976). More recently, mares treated with PZP contraception have been shown to change groups more often, making approximately 10 times more group changes than untreated mares and visiting up to 5 times as many groups (Madosky et al., 2010; Nuñez et al., 2009). During a 2.5-month study in 2008, 44% of the mares studied (in a population of 121 horses) transferred groups. Group changes typically involve aggressive herds, chases, and increased reproductive activity by both the band and new stallions, and/or aggression from the new females (personal observation). Population densities at the time of the study were equivalent to those of the 1981 population (see Section 2.1), indicating that these increases in group changing behavior are not due to significant increases in encounter rates between/among bands. All of the mares in this study received PZP at some point during their lifetime (Stuska, 2000–2010) also, see Table 1.

### 2.3. Animal welfare

All sampling was conducted in accordance with National Research Council standards (National Research Council, 2011). Given the non-invasive nature of this study, neither the Princeton University Institutional Animal Care and Use Committee nor the National Park Service deemed permitting necessary.

### 2.4. Behavioral and demographic sampling

This study was conducted by three observers during the breeding season (June–August, 2009, J. Smith, J.J. Schurle, and C.M.V. Nuñez) and two observers during the non-breeding season (December, 2009, C.M.V. Nuñez and J.S. Adelman) totaling over 181 hours of behavioral observation (84.67 h, breeding season; 96.75 h, non-breeding season). Horses were identified individually by color, sex, age, physical condition, and other distinguishing markings, including freeze brands. Ages are known from long-term records for the identified horses of Shackleford Banks (Rubenstein and Nuñez, 2009) and from National Park Service data (S. Stuska, unpublished data).

**Table 1**  
Contraception history for mares from 2000–2009.

Mare ID	Total number of years treated	Year last treated	Treatment status for 2009
Bo	2	2005	Untreated
Carrot	1	2009	Treated
Daisy	8	2009	Treated
Darcy	4	2005	Untreated
Donoma	2	2009	Treated
Dusty	6	2007	Untreated
Hardee	5	2009	Treated
Helena	6	2008	Untreated
Hercules	1	2009	Treated
Hezakiah	7	2007	Untreated
Jaquincoke	5	2006	Untreated
Kelty	1	2009	Treated
Larissa	4	2006	Untreated
Liani	4	2009	Treated
Noir	2	2009	Treated
Penelope	4	2005	Untreated
Sadie	8	2009	Treated
Sawathu	2	2009	Treated
Serenac	7	2009	Treated
Shag	5	2009	Treated
Slug	7	2008	Untreated
Texas	6	2006	Untreated
Wallace	1	2009	Treated
Zelda	2	2004	Untreated



**Table 2**  
Breakdown of sample sizes for entire study.

	Breeding (Br)	Non-breeding (NBr)	Total
Fecal samples meeting inclusion criteria	90	36	126
No. unique mares with fecal and behavioral samples	8 in Br only 12 in both seasons	4 in NBr only	24
Fecal samples/mare mean $\pm$ 1SE (range)	4.50 $\pm$ 0.84 (1–13)	2.25 $\pm$ 0.42 (1–6)	5.28 $\pm$ 0.81 (1–14)
No. unique mares changing groups	10 in Br only 3 in both seasons	3 in NBr only	16
Group changes/mare mean $\pm$ 1SE (range)	2.30 $\pm$ 0.32 (1–4)	1.50 $\pm$ 0.34 (1–3)	2.43 $\pm$ 0.29 (1–4)

We located each study group an average of 1.76 and 1.54 times per week in the breeding and non-breeding seasons, respectively. We recorded each group's GPS location and composition, noting the presence or absence of individual mares. We observed a total of 24 mares (see Table 2). Twelve mares were observed in both seasons; the remaining mares were observed in either the breeding ( $n = 8$ ) or non-breeding ( $n = 4$ ) seasons, representing 53% and 40% of the study population, respectively. Of these, 16 made group transfers (breeding season,  $n = 13$  mares; non-breeding season,  $n = 6$  mares).

Mare transfer activity was rarely witnessed directly (breeding season,  $n = 1$ ; non-breeding season,  $n = 1$ ); therefore, mare absence from a band was an important metric with which to measure the number of transfers between groups. We remained with each group for a minimum of 30 min to ensure that individuals recorded as absent were not actually nearby, but out of our sight. Transfer behavior was confirmed by the mares' presence in new bands, usually within 1–7 days of mare absence (breeding season,  $n = 28/30$ ; non-breeding season,  $n = 8/9$ ). The remaining instances were confirmed within 16 and 8 days in the breeding non-breeding seasons, respectively.

## 2.5. Non-invasive hormonal sampling

Fecal samples were collected *ad libitum* (Altmann, 1974). We only collected samples when we were certain of the mares' identity and the location on the ground. Fecal samples were collected within minutes of defecation and stored in 20 ml vials at a 2.5:1 ratio of 95% ethanol to feces (Khan et al., 2002). Samples were stored in a cooler (from 1–5 days) until they could be frozen at  $-20^{\circ}\text{C}$  (June–August) or frozen at  $-20^{\circ}\text{C}$  on the day of collection (December).

Three of the aforementioned observers (J. Smith, C.M.V. Nuñez, and J.S. Adelman) collected fecal samples. We collected samples from a total of 24 mares (see Section 2.4 and Table 2). Limitations in staff, travel logistics, and the rates of mare defecation precluded us from sampling all mares in the study area. A total of 126 samples met our timing criteria and were included in our analyses (breeding season,  $n = 90$ ; non-breeding season  $n = 36$ ). Mares contributed a total of  $4.50 \pm 0.84$  (range = 1–13) and  $2.25 \pm 0.42$  (range = 1–6) samples in the breeding and non-breeding seasons, respectively (see Table 2). We collected an average of 1.52 samples (breeding season) and 2.25 samples (non-breeding season) per day.

## 2.6. Hormone analysis

In the laboratory, ethanol was evaporated from the samples which were then freeze-dried and sifted to remove vegetative matter. 0.2 g fecal powder was extracted into 2 ml 90% methanol and then run through a prepped Oasis cartridge for solid phase extraction (Beehner et al., 2006; Khan et al., 2002). All samples were stored at  $-20^{\circ}\text{C}$  prior to assay.

Fecal glucocorticoids were quantified using modified protocols of the Immuchem Double Antibody Corticosterone RIA Kit for Rats

and Mice, MP Biomedicals, LLC, Orangeburg, NY (Beehner et al., 2006; Wasser et al., 2000), in eight separate assays. This specific method has been validated in a closely related, wild equid, the Grevy's zebra (*Equus grevyi*): the anti-corticosterone antibody detects a rise in cortisol metabolites after an individual is presented with an ACTH challenge (Franceschini et al., 2008). Moreover, fecal metabolites have been shown to increase with circulating glucocorticoids in the domestic horse (*Equus caballus*) (Merl et al., 2000; Mostl et al., 1999). We validated the assay as in Khan et al. (2002). A serial dilution of a horse fecal pool showed parallelism to the corticosterone standard curve (Linear Model; estimate = 6.30,  $t = 0.70$ ,  $P = 0.50$ ). Intra- and inter-assay coefficients of variation (%CV = [mean/SD]  $\times$  100) were  $5.7 \pm 0.6\%$  (mean  $\pm$  SE) and  $9.6\%$  for the fecal extract pool, and  $7.4 \pm 2.9\%$  and  $13.9\%$  for the high concentration controls ( $n = 8$ ). All samples were run in duplicate and any duplicate with a CV  $> 15\%$  was rerun. Mean assay accuracy (observed/expected  $\times$  100) was  $105 \pm 2.2$ .

Recently, concerns have been raised about the use of fecal metabolites in determining the physiological state of free-living species (Goymann, 2012). Individual, sex, seasonal, and dietary variation can significantly affect how hormones are metabolized. Here, we examine changes in fecal cortisol within individual, female, feral horses. The mares lived in similar ecological conditions (see Section 2.1), and we analyzed changes within season, thereby controlling for such potential differences.

## 2.7. Statistical analysis

### 2.7.1. Test 1

We analyzed the effects of group transfers on mare cortisol levels with Linear Mixed Effects Models in R, version 2.13.0 (R Development Core Team, 2011). For each female, cortisol level was analyzed with regards to its timing around group transfers, i.e., before, during, and after group transfers. These categories were defined as follows (also see Fig. 1):

- Before–cortisol levels exhibited before any group transfer(s) occurred (weeks 1–2).
- During–cortisol levels exhibited while the group transfer(s) were occurring (weeks 3–4).
- After–cortisol levels exhibited after the final group transfer occurred (weeks 6–10); these samples were collected a minimum of 7 days after the mares' last group transfer.

For this analysis, mares contributed  $3.92 \pm 0.82$  samples (range = 1–10) and  $3.83 \pm 0.75$  samples (range = 3–6) in the breeding and non-breeding seasons, respectively (also see Table 3 and Supplementary material, Fig. S1). The model included mare ID as a random effect and the following fixed effects: season (breeding/non-breeding), the timing of fecal deposition relative to the group transfer (before, during, or after), and the interaction between season and the timing of fecal deposition. We conducted this analysis in two ways.

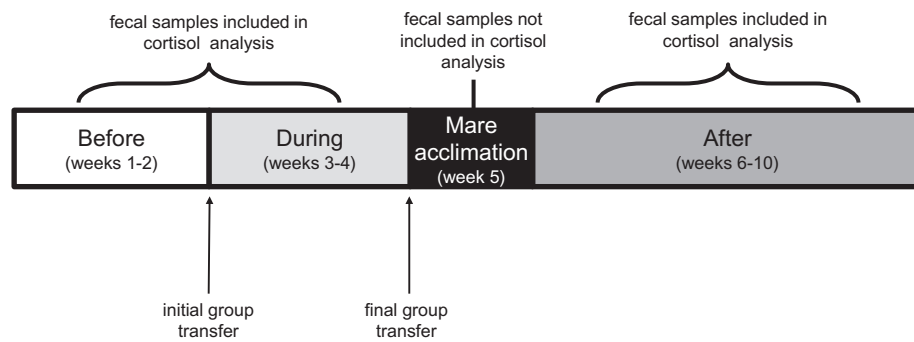


Fig. 1. Timeline for fecal sampling (Test 1).

**Table 3**  
Breakdown of sample sizes for timing relative to group transfer analysis (Test 1, Fig. 4).

	Breeding (Br)	Non-breeding (NBr)	Total
Fecal samples meeting inclusion criteria	51	23	74
No unique mares with fecal and behavioral samples	10 in Br only 3 in both seasons	3 in NBr only	16
Fecal samples/mare mean $\pm$ 1SE (range)	3.92 $\pm$ 0.82 (1–10)	3.83 $\pm$ 0.75 (3–6)	4.63 $\pm$ 0.81 (1–11)
No unique mares changing groups	10 in Br only 3 in both seasons	3 in NBr only	16
Group changes/mare mean $\pm$ 1SE (range)	2.69 $\pm$ 0.26 (1–4)	1.16 $\pm$ 0.16 (1–2)	1.81 $\pm$ 0.26 (1–4)

- Model 1: included all mares for which appropriate samples were available; not every mare contributed to all time points ( $n = 16$  mares; 74 samples; see [Supplementary material, Fig. S1](#)).
- Model 2: included only mares for which samples from all time points were available ( $n = 6$  mares; 37 samples; see [Supplementary material, Fig. S1](#)). Samples from mares for all time points were obtainable during the breeding season only.

### 2.7.2. Test 2

Cortisol levels were also analyzed with respect to the number of group transfers females made. Samples for this analysis were collected two weeks after the final group transfer. Mares contributed  $4.39 \pm 0.89$  samples (range = 1–13) and  $1.36 \pm 0.17$  samples (range = 1–3) in the breeding and non-breeding seasons, respectively (also see [Table 4](#)). The model included mare ID as a random effect and the following fixed effects: season (breeding/non-breeding), the number of group transfers made prior to fecal collection, and the interaction between season and the number of group transfers.

All of the mares in this study received PZP at some point during their lifetime ([Stuska, 2000–2010](#)); therefore, tests regarding the possible effects of infertility were not possible.

## 3. Results

As the breeding season progressed, we detected an increase in mare transfers, with a peak in the middle of the study period, after which group transfers decreased to levels exhibited earlier in the season (see [Fig. 2](#)). 65% of the mares studied transferred groups at least one time, with 45% transferring groups 2–4 times (see [Fig. 3](#)). During the non-breeding season, we detected more group transfers earlier in the study period (see [Fig. 2](#)). 37.5% of the mares studied transferred groups, with 12.5% transferring groups 2–3 times (see [Fig. 3](#)). Subsequently, 86% and 60% of the bands observed were affected (i.e. mares left/joined the bands) during the breeding and non-breeding seasons, respectively.

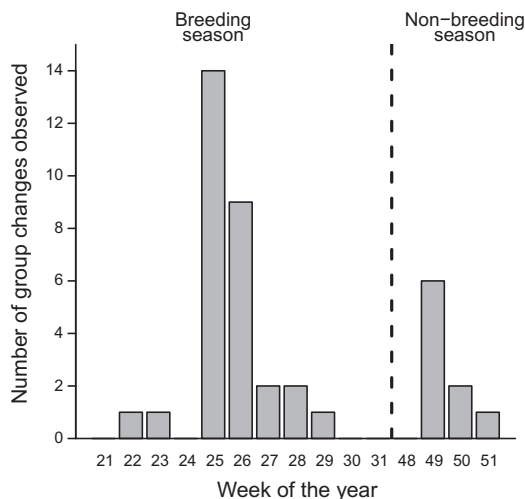
### 3.1. Timing of group transfers and cortisol levels (Test 1)

Timing of fecal deposition relative to group transfer(s) affected cortisol levels (Linear Mixed Effects Model (LME); overall model: Likelihood ratio (compared to null model) =  $-290.78$ ,  $P = 0.0008$ ;  $F_{2,53} = 3.23$ ,  $P = 0.05$ ). Season did not have a significant effect on this pattern (Season:  $F_{1,53} = 2.75$ ,  $P = 0.10$ ; Season\*Timing of fecal deposition relative to group transfer(s):  $F_{2,53} = 1.23$ ,  $P = 0.30$ ). On

**Table 4**  
Breakdown of sample sizes for number of group changes analysis (Test 2, Fig. 5).

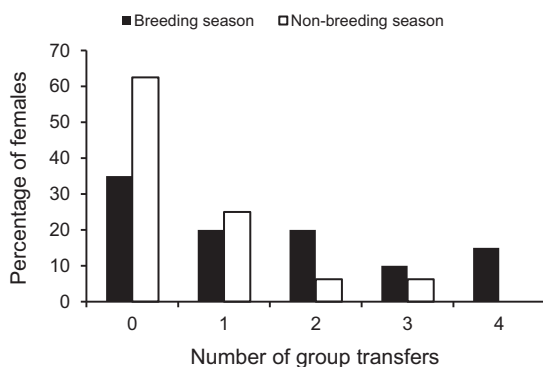
	Breeding (Br)	Non-breeding (NBr)	Total
Fecal samples meeting inclusion criteria	79	19	98
No unique mares with fecal and behavioral samples	7 in Br only 11 in both seasons	3 in NBr only	21
Fecal samples/mare mean $\pm$ 1SE (range)	4.39 $\pm$ 0.89 (1–13)	1.36 $\pm$ 0.17 (1–3)	4.67 $\pm$ 0.84 (1–14)
No unique mares changing groups	8 in Br only 2 in both seasons	2 in NBr only	12
Group changes/mare mean $\pm$ 1SE (range)	1.90 $\pm$ 0.31 (1–4)	1.50 $\pm$ 0.50 (1–3)	2.08 $\pm$ 0.31 (1–4)





**Fig. 2.** Distribution of group transfers in the breeding and non-breeding seasons.

average, mares exhibited higher cortisol levels during group transfer(s) than before group transfer(s) (LME: estimate = 7.22,  $t = 2.33$ ,  $P = 0.02$ ; Fig. 4). Cortisol levels exhibited after group transfer(s) did



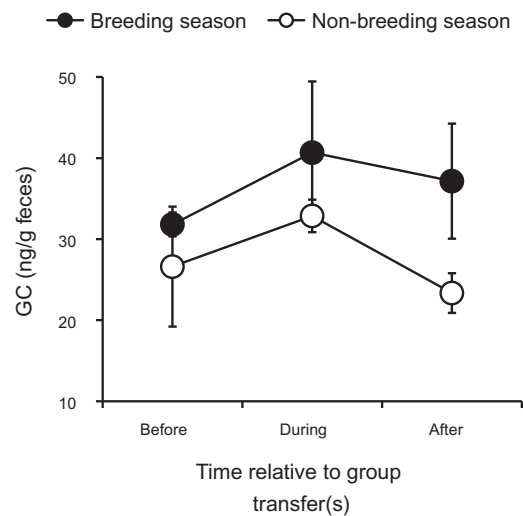
**Fig. 3.** Percentage of females making 0, 1, 2, 3, and 4 group transfers. During the breeding season (■), more mares transfer groups than do not; the majority of transferring mares make more than 1 transfer. During the non-breeding season (□), there is less transfer activity with fewer mares transferring groups; the majority of mares transferring groups make 1 transfer.

not differ from those exhibited before or during transfer(s) (before: LME: estimate = 1.90,  $t = 0.54$ ,  $P = 0.59$ ; during:  $F$ -test for linear combinations,  $F_{1,56} = 2.83$ ,  $P = 0.10$ ; Fig. 4).

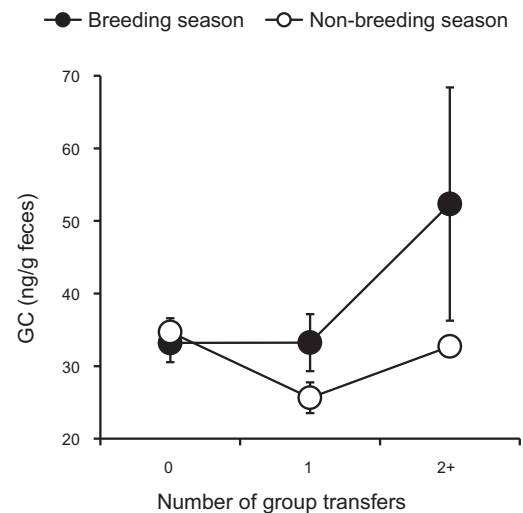
Analysis of our second model (including only mares for which we had all samples) yielded equivalent results (Linear Mixed Effects Model (LME); overall model: Likelihood ratio (compared to null model) =  $-150.90$ ,  $P = 0.0003$ ;  $F_{2,29} = 4.14$ ,  $P = 0.05$ ).

### 3.2. Number of group transfers and cortisol levels (Test 2)

Mares engaging in this behavior more frequently showed higher cortisol levels two weeks post-transfer(s) (LME; overall model: Likelihood ratio (compared to null model) =  $-382.24$ ,  $P < 0.0004$ ;  $F_{2,72} = 3.51$ ,  $P = 0.03$ ). Season did not have a significant effect on this pattern (Season:  $F_{1,72} = 0.92$ ,  $P = 0.34$ ; Season\*Timing of fecal deposition relative to group transfer(s):  $F_{2,72} = 0.67$ ,  $P = 0.51$ ). Two weeks after their final group transfer, mares making 2+ transfers exhibited higher cortisol levels than mares making 0 transfers (LME: estimate = 9.61,  $t = 2.58$ ,  $P = 0.01$ ; Fig. 5) and marginally higher cortisol levels than mares making only 1 group transfer



**Fig. 4.** Mare cortisol level and the timing relative to group transfer(s) in the breeding (●) and non-breeding (○) seasons. Mares showed increases in cortisol during group transfer behavior.



**Fig. 5.** Mare cortisol level 2 weeks post-group transfer(s) during the breeding (●) and non-breeding (○) seasons. Mares making more group transfers exhibited higher cortisol levels 2 weeks post-behavior than did mares making fewer group transfers.

( $F_{1,73} = 3.32$ ,  $P = 0.07$ ; Fig. 5). Mares making only 1 group transfer exhibited cortisol levels similar to mares making 0 changes (LME: estimate = 0.24,  $t = 0.07$ ,  $P = 0.94$ ; Fig. 5).

## 4. Discussion

Here we show for the first time that social instability has significant impacts on stress physiology in feral mares. Specifically, we show that mare cortisol levels increase during group transfers (Test 1) and that mares making more band transfers exhibit higher cortisol levels two weeks following transfer behavior (Test 2). Although we did not detect an effect of season on this pattern, the data show trends towards higher cortisol levels during the breeding season (see Figs. 4 and 5). This is not surprising as certain elements of stallion/mare aggression are elevated at this time (Stevens, 1990), but see Romero (2002). Regardless, the patterns of cortisol increase are consistent across season, indicating that the changes in mare cortisol levels are due to their group transfer behavior and not to season alone. Our results show that social

instability and the resultant behaviors, including increased reproductive interest and aggression, are integrated into a mare's physiological phenotype.

Many social changes are associated with group transfers: mares changing groups will often experience behavioral stressors in the form of harassment from both the male(s) (including herds, chases, and increased reproductive interest) and resident females (including kicks, chases, and bites) (Kearns, 2009; Madosky, 2011; Monard and Duncan, 1996; Rutberg, 1990). We cannot precisely determine which of these behaviors drive the patterns recorded in this study. However, the discrete increases in fecal cortisol occurring during group transfers indicate a direct link between the mares' social environment and their physiological response (Test 1). Moreover, the fact that higher cortisol levels are maintained for at least two weeks post-transfer behavior suggests that decreases in social stability have at least some lasting effect on mare physiology (Test 2). We know much about the effects of social environment on mare behavior and fitness (Cameron et al., 2009; Kaseda et al., 1995; Linklater et al., 1999). Our data give insights into how that social environment may be translated into fitness costs for mares via their stress physiology.

The mares in this study exhibited increases in cortisol (7.22 ng/g) comparable to the highest levels exhibited by Grevy's zebra (~10 ng/g) during captivity after capture and relocation (Franceschini et al., 2008). These data indicate that group transfer behavior incurs a significant cost to mares. Although such stress in itself is not inherently detrimental to animal fitness or well-being (Moberg, 2000), the stress response can become pathogenic when experienced chronically (Sapolsky, 2005). On Shackleford Banks, N.C., mares transferring bands more frequently are often subject to a negative feedback loop: their behavior leads to harassment which induces additional group changes, resulting in further harassment and so on (Madosky, 2011), also see Linklater et al. (1999). Results from the present study suggest that such mares are likely experiencing increased stress levels at more regular intervals, and may therefore be at higher risk of chronic stress.

Moreover, mares on Shackleford that are contracepted with porcine zona pellucida (PZP) have been shown to make up to 10 times more group transfers than untreated mares (Madosky, 2011; Nuñez et al., 2009). Direct tests of treated versus untreated mares were not possible in this study (see Section 2.2). However, mares contracepted for the first time in January 2009 and those receiving repeated applications (range = 2–8) over several years (Stuska, 2000–2010) exhibit the same increases in cortisol levels with group transfer behavior (see Supplementary material for analysis and Fig. S2). These results show that PZP treatment itself does not increase cortisol levels in recipient animals. However, changes in the frequency of group transfer behavior by consistently infertile mares (Madosky et al., 2010; Nuñez et al., 2009) may put them at higher risk of chronic stress. PZP contraception has also been conducted on Assateague Island National Seashore (Kirkpatrick, 1995), a site ecologically similar to Shackleford Banks. Currently, no systematic studies of group changing behavior with PZP treatment have been conducted there, thus limiting our ability to compare the behavior of Shackleford and Assateague mares. A study addressing potential changes to treated mares' activity budgets, aggression, and spatial relationships with the stallion on Assateague found no differences (Powell, 1999), indicating little to no effect of PZP treatment on mare behavior. However, it is worth noting that control mares in the Assateague study had been treated with PZP for three consecutive years before testing, and that different behavioral effects have been found in populations other than Shackleford Banks (Ransom et al., 2010, 2013).

Our results may seem to contrast those showing that contracepted mares (on both Assateague Island and Shackleford Banks) live longer and are in better body condition than uncontracepted

mares (Kirkpatrick and Turner, 2007; Nuñez et al., 2010). However, longevity and body condition are not the only measures of animal health. High condition scores and long life can often be maintained despite cumulative changes to other physiological systems in response to recurring stressful events (McEwen and Wingfield, 2003). For example, repeated stressors can result in more chronically dysregulated glucocorticoid secretion, chronically elevated food consumption, insulin resistance, and increased deposition of fat which, in some species, can contribute to high condition scores (Leibowitz and Hoebel, 1997; McEwen and Wingfield, 2003; Sapolsky, 2005). Moreover, research with crimson finches (*Neochmia phaeton*) has shown that accepted condition measures do not reliably predict reproductive success or survival (Milenkaya, 2013), suggesting that the value of condition scores for assessing integrative animal health may be insufficient.

How then are we to understand the relevance of increased cortisol in mares that change groups more often? The benefits of lower stress environments (to animals in general) and band stability (to feral horses in particular) have been well documented. Persistently stressed animals show a range of deleterious effects (Sapolsky, 2005), and in feral horses, band instability has been associated with decreased time spent in preferred behaviors, increased offspring mortality, and increased parasite load (Kaseda et al., 1995; Linklater et al., 1999). All of these factors can be explored further; however, future studies investigating parasite burden more closely may yield the most relevant information (Rubenstein and Hohmann, 1989). Because parasites can have important effects on host fitness, such measures could reveal much about overall animal health and ability to fight off infection (Booth et al., 1993). In addition, this metric would offer managers an important, non-invasive tool with which to further quantify animal well-being.

Our results present new evidence that should be considered when evaluating management tools. We show that social stressors are integrated into an animal's physiology via increased cortisol which, when experienced at increased frequency, can increase the risk of chronic stress (Sapolsky, 2005). In addition, such behavioral changes can decrease the mares' ability to form the stable social relationships with one another that are important to decreasing overall male harassment (Cameron et al., 2009). Furthermore, given the high degree of sociality in feral horses, the behaviors of these individuals have the potential to affect the population as a whole. For example, on Shackleford, increases in reproductive behavior (by mares) in the post-breeding season has resulted in increased male attentiveness (Nuñez et al., 2009), which in turn, limits mare movement and foraging efficiency (Rubenstein, 1986; Sundaresan et al., 2007) during a time of year when the conservation of resources is of utmost priority. Similar shifts in the reproductive behavior (Ransom et al., 2010) and subsequent parturition in feral mares (Ransom et al., 2013) have been shown in the Little Book Cliffs, McCullough Peaks and Pryor Mountains populations in the western United States. In the former study, treated mares received 54.5% more reproductive behaviors than did their untreated counterparts (Ransom et al., 2010). In addition, factors that usually determine rates of reproductive behavior directed toward untreated mares (by the stallion) did not exist for treated mares. Typically, stallions engage in higher rates of reproductive behavior with mares aged 9–14 years that are more likely to produce viable offspring. This preference did not hold with treated mares; harem males showed higher rates of reproductive behavior with these females, regardless of mare age and survival probability of the subsequent offspring. In the latter study, Ransom et al. (2013) investigated the effects of prior PZP treatment(s) on the timing of parturition in feral mares after fertility was regained. Time to regain fertility ranged between 1.5–8 years and was highly dependent upon the number of treatments received. On average,

these post-treated mares exhibited peaks in parturition 31.5 days later than did untreated mares (but see Kirkpatrick and Turner (2003)). Subsequently, post-treated mares were more likely to give birth as forage was declining, resulting in fewer resources available to mares and offspring during late term pregnancy and lactation. Such cascading behavioral effects are not uncommon in social species. For example, alterations to female song preference in brown-headed cowbirds (*Molothrus ater*) induce increased solicitation of males by other females, changes in male dominance structure, and a less stable and connected social network (Maguire et al., 2013).

While the changes to feral horse behavior and physiology discussed here could help reduce population numbers, the mechanisms by which such reductions are achieved (decreased social stability and social connectedness amongst mares, increased male/female aggression) also affect the welfare of these animals. Such effects may be of limited concern when population reduction is an urgent priority. However, their consideration is vital if managers are to maintain healthy, functional populations, particularly in social species like the feral horse, in which the manipulation of individuals can have implications for the population as a whole.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ygcen.2013.11.012>.

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# Immunocontraception in Wild Horses (*Equus caballus*) Extends Reproductive Cycling Beyond the Normal Breeding Season

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## Abstract

**Background:** Although the physiological effects of immunocontraceptive treatment with porcine zona pellucida (PZP) have been well studied, little is known about PZP's effects on the scheduling of reproductive cycling. Recent behavioral research has suggested that recipients of PZP extend the receptive breeding period into what is normally the non-breeding season.

**Methodology/Principal Findings:** To determine if this is the case, we compiled foaling data from wild horses (*Equus caballus*) living on Shackleford Banks, North Carolina for 4 years pre- and 8 years post-contraception management with PZP (pre-contraception, n=65 births from 45 mares; post-contraception, n=97 births from 46 mares). Gestation lasts approximately 11–12 months in wild horses, placing conception at approximately 11.5 months prior to birth. Since the contraception program began in January 2000, foaling has occurred over a significantly broader range than it had before the contraception program. Foaling in PZP recipients (n=45 births from 27 mares) has consistently occurred over a broader range than has foaling in non-recipients (n=52 births from 19 mares). In addition, current recipients of PZP foaled later in the year than did prior recipient and non-recipient mares. Females receiving more consecutive PZP applications gave birth later in the season than did females receiving fewer applications. Finally, the efficacy of PZP declined with increasing consecutive applications before reaching 100% after five consecutive applications.

**Conclusions/Significance:** For a gregarious species such as the horse, the extension of reproductive cycling into the fall months has important social consequences, including decreased group stability and the extension of male reproductive behavior. In addition, reproductive cycling into the fall months could have long-term effects on foal survivorship. Managers should consider these factors before enacting immunocontraceptive programs in new populations. We suggest minor alterations to management strategies to help alleviate such unintended effects in new populations.

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## Introduction

The extirpation of predator species has resulted in the expansion of free-ranging ungulate populations in North America [1], necessitating their regulation. Immunocontraceptive management has become increasingly popular as culling programs are seldom well-received by the general public. In females, the most common form of immunocontraception, porcine zona pellucida (PZP), stimulates the production of antibodies that bind sperm receptors on the egg's surface, thereby preventing sperm attachment and fertilization [2].

In recent decades, the wild horse (*Equus caballus*) has become a model for evaluating the effects of PZP, thanks chiefly to the seminal nine-year study of contracepted mares on Assateague Island National Seashore [3]. Subsequent research on this population has shown that PZP has little to no effect on recipient physiology or behavior [4,5,6]. For example, in Assateague horses, PZP has no effect on the duration of individual estrous cycles [6],

and researchers have reported only minor ovulation failure and depressed urinary oestrogen concentrations with repeated applications [4]. However, behavioral research on the horses of Shackleford Banks, North Carolina, and other wild ungulate species suggests that PZP affects the reproductive physiology of recipient animals [7,8,9]. In each of these studies, females treated with PZP extended reproductive behaviors into the non-breeding season. While these results are consistent with an extension of ovulatory cycling into the post-breeding season when most females are normally anovulatory [10,11], this possibility has not yet been tested.

Mares are seasonally polyestrous and extended estrous periods have been documented [11]. Tropical species, for example, are less strictly seasonal, and in some cases, reproduce throughout the year [12,13]. In addition, variability in the cycling schedules and receptivity of individual mares [14], and the performance of estrous behavior and copulatory activities during the non-breeding season [15] have been documented in temperate species. Such



variation in ovulatory scheduling and receptivity suggest that the seasonality of reproductive behavior in *Equus caballus* females is characterized by a substantial degree of plasticity. As the physiological state of contracepted animals has been significantly altered, the possibility of prolonged extended cycling is even more feasible.

In wild horse societies, the harem is the core social group, consisting of usually one, but sometimes two or three harem male(s), one to several female(s), and their offspring [16,17,18,19,20,21]. Harem males will sometimes fight to acquire mares from other groups, but stallions almost always retain their mares [18,19,20,22]. In temperate environments, food availability is lower during the fall and winter months and free-ranging horses will alter their activity to maximize food intake and reduce energetic costs [23,24]. Mares are typically anovulatory at this time and sexual behavior in males is largely absent [11,14].

On Shackleford Banks, increased reproductive behavior in the post-breeding season by mares has resulted in increased male attentiveness [9]. Such behavior (by males) has been shown to restrict the movement of females, thus reducing their grazing efficiency [21,25]. The occurrence of this behavior during a time of year when animals typically increase group spread to acquire adequate forage [19,24], represents a change in behavior fundamental to the animals' survival [26,27]. Offspring conceived during the post-breeding season are likely subject to decreased resource availability as lower quality forage can affect mares' ability to produce sufficient milk [27]. Finally, regardless of the timing of titer decline post-treatment [9,28,58], when anti-PZP antibody titers decrease during the fall months [28,29], extended reproductive cycling among recipient mares will increase their chances of conception, thereby lowering the vaccine's overall efficacy. Determining whether PZP recipients are likely to extend reproductive cycling is therefore of great importance if managers are to limit animal numbers while still maintaining functional, healthy populations.

Here we use the birth dates of foals to estimate dates of conception for PZP recipient and non-recipient mares on Shackleford Banks, North Carolina. Gestation length in wild horses is 11–12 months [30]. Therefore, dates of conception can be reliably estimated as approximately 11.5 months prior to birth. Breeding normally occurs from March through August, with most births occurring in April and May [11]. Given the extension of reproductive behavior in PZP-treated mares [9], we hypothesize that PZP recipients will extend cycling into the non-breeding season more often than will non-recipients. Therefore, when PZP recipients conceive and give birth, they will do so later on average and over a wider range of months than will non-recipients.

## Methods

### Study area

This study was conducted on Shackleford Banks, a barrier island located approximately 3 km off the coast of North Carolina, USA. The island was 15 km in length, and varied between 0.5 and 3 km in width. The horse population on Shackleford Banks has been co-managed by the National Park Service and the Foundation for Shackleford Horses since 1996.

### Study subjects

The reproductive units of Shackleford horses are typical of feral equids. They are coherent harem groups of one, or sometimes two or three stallion(s), one to several mare(s) and their offspring [18]. Predominantly, the harem groups are not territorial and animals

move within overlapping home ranges, although this has not always been the case [18].

## Management

**PZP Contraception.** In January 2000, the National Park Service began the application of PZP for the purposes of immunocontraception. The National Park Service administers PZP in the spring (late February through April) each year. Mares receive their initial treatment at 1.5–2 years of age. Each injection includes 100 micrograms of PZP with an adjuvant (combined at the darting site). Initial doses include Freund's Complete Adjuvant, Modified, Mycobacterium butyricum (Calbiochem #344289). All succeeding doses include Freund's Incomplete Adjuvant (Sigma #F5506). In a given year, an average of 63% of all reproductive mares are inoculated with the vaccine (range = 37–88%). The authors of the present study are not and have never been in charge of making management decisions regarding this herd.

**Gathers and Removals.** The National Park Service began their management of the Shackleford Banks population prior to the use of PZP, performing five gathers between November 1996 and January 2000. During these gathers the majority of the population was rounded up and individuals testing positive for equine infectious anemia were removed and either euthanized or quarantined. As such forms of management could conceivably influence reproductive cycling, we analyzed foaling dates before and after gathers (but before contraception management) using a linear mixed effects model (see Statistical Analyses).

From January 2000 – January 2008, 38 foals (conceived due to contraception failure or administration scheduling) were removed from the island for the purpose of population control. The majority of removals (92%) were conducted in the January following the foals' birth.

## Foaling Data

We recorded foaling data before contraception management (1995–1997) during a study of mother-infant behavior [31]. We obtained foaling data for 2000 and post-contraception years (2001–2008) from the National Park Service at Cape Lookout National Seashore, North Carolina. Although PZP was first administered in 2000, foals born in this year were conceived before contraception management. As such, we considered 2000 a pre-contraception year.

We identified individual horses by color, sex, age, physical condition, and other distinguishing markings including freeze brands. We monitored pregnant mares 2–3 times per week to ensure accurate estimation of foaling date. Births are not commonly witnessed, as mares will generally give birth away from their harem group [31]. This does not significantly affect the determination of birth dates because typically, mares return to their groups within hours (depending on the length of the foaling process and the mares' and foals' condition afterward). In the event that we did not locate mares for more than one week, the birth dates of new foals were estimated by comparing the condition of the foals' coat, mane and tail hair, and locomotor ability to that of foals for which the exact age was known. Using these methods, we were able to estimate birthdays within a range of 1–2 days to 1 week. Focal animals in this study were observed from a minimum of 15 m away at all times. The observers did not obstruct or manipulate the animals' natural behavior in any way. As such, approval for this study by a review board or ethics committee was unnecessary.

We designated births as coming from current recipient, prior recipient, or non-recipient mares. Current recipients received PZP

treatment the year they conceived (the year previous to foaling). Prior recipients received PZP treatment at some point earlier in their lifetime, but not the year of conception. Non-recipients had never received PZP at the time of conception.

### Physical Condition

Physical condition is an important factor to consider when monitoring reproductive behaviors. Animals in better condition will have more resources to allocate to mating behavior and physiology than will animals in poorer condition. We assessed mare condition via rump scoring. We determined rump scores examining the curvature of the line between the tailbone and the point of the hip. Scores were based on a scale from 1 to 5; a score of 1 being the poorest [32].

### Weather Data

We collected all climatology data from the Morehead City WNW Station at 34° 44'N; 76° 44'W, approximately 8 km from the study site. We obtained all data from the National Climate Data Center (2009) [33].

### Statistical Analyses

We analyzed data in R version 2.11.1 (R Core Group, Vienna Austria) and JMP, version 7 (SAS, Cary NC, USA). We used Fligner-Killeen tests to determine if variation in foaling dates differed before and after contraception management and between PZP recipients and non-recipients. This test is more conservative than Fisher's or Bartlett's tests and is less sensitive to outliers and non-normal data [34]. Results from these tests show Bonferroni corrected *P*-values.

We used linear mixed effects models to analyze monthly weather patterns. These statistical models included year as a random effect and the following fixed effects: linear through fourth order polynomial functions of month (to account for nonlinear fluctuations in temperature and rainfall), management regime (before or after contraception management), and two-way interactions with management regime. We selected best fit models using Akaike's Information Criterion adjusted for small sample size (AICc). Because temperature data were temporally autocorrelated, we included an autoregressive moving average correlation structure [35]. For each year of the study, we calculated the mean of the monthly residual values from both the rainfall and temperature models from July through November. We included these terms in the mixed effects models described below to determine if weather affected foaling date.

To test for differences in foaling date with PZP treatment, while controlling for pseudo-replication and unequal variances, we used a linear mixed effects model. The model included mare ID as a random effect and a function allowing different variances across groups (pre-contraception management, post-contraception management non-recipient, post-contraception management current recipient, and post-contraception management prior recipient) [35]. We included mare age as a covariate, as this has been suggested to influence female reproductive behaviors and physiology [36,37]. Additionally, a one-way ANOVA revealed no differences in mare age among treatment groups ( $F_{2,84} = 2.03$ ,  $P = 0.14$ ), suggesting that age did not confound differences among groups. From the above mixed effects model, differences in foaling dates among current, prior, and non-recipients were calculated using an F-test for linear combinations [35].

As gathers could have affected foaling date, we used a linear mixed effects model to test for differences between foaling dates before and after gathers (but before contraception management). After controlling for weather and mare age, mean foaling date did

not differ before and after gathers ( $P = 0.63$ ). Additionally, the range of birth dates did not differ significantly between these groups (SD before gathers = 1.04, SD after gathers = 0.77,  $P = 0.36$ ). Finally, AICc from our analysis of foaling dates (see Results) suggested that combining data before and after gathers yielded a more parsimonious model than did separating those categories (AICc separating pre- and post-gathers = 551.02, AICc combining pre- and post-gathers = 550.60). Consequently these groups were combined as pre-contraception management animals for all analyses.

To determine whether the number of PZP applications (total or consecutive) or the number of years between pregnancies (total or consecutive) influenced foaling date, we ran separate linear mixed effects models using these as predictor variables. Each model used only data from PZP recipients and included mare ID as a random effect. To determine if results from this analysis could be influenced by year, we ran a linear mixed effects model on post-management, non-recipients predicting foaling date by year.

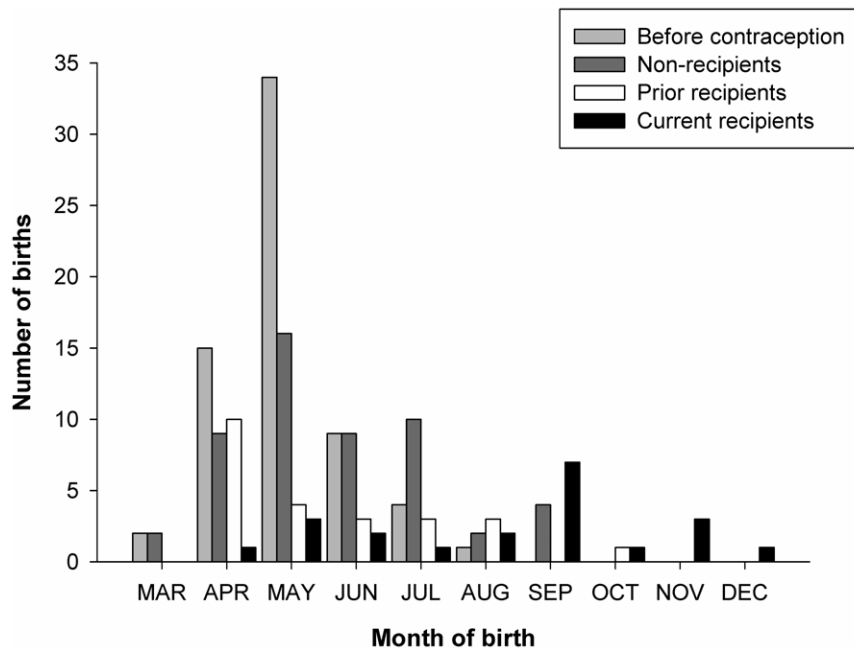
We analyzed PZP efficacy using a generalized linear mixed effects model with a binomial error distribution and mare ID as a random effect [38]. This analyzed the probability of conceiving using age at first PZP application and the number of consecutive PZP applications as fixed effects. All mixed effects models initially included two-way interactions, which were removed if their *P*-values were  $>0.10$ .

## Results

### Foaling

After contraception management, foaling occurred over a broader range of months than before contraception management (see Fig. 1, SD pre-contraception = 0.91 months, SD post-contraception = 2.04 months; Fligner-Killeen Test for Homogeneity of Variances: Median Chi Square = 28.10,  $P < 0.0001$ ; pre-contraception:  $n = 65$  births from 45 mares over 4 years; post-contraception:  $n = 97$  births from 46 mares, over 8 years). In the eight years following contraception management, mares receiving PZP foaled over a broader range of months than did non-recipient mares (see Fig. 1, SD recipients = 2.40 months, SD non-recipients = 1.54 months; Fligner-Killeen Test for Homogeneity of Variances: Median Chi Square = 11.76,  $P = 0.001$ ; recipients:  $n = 45$  births from 27 mares; non-recipients:  $n = 52$  births from 19 mares).

On average, current PZP recipients gave birth 3.36 months later than did pre-contraception mares, according to our linear mixed effects model, which controlled for heterogeneity of variances, weather influences, and mare age (estimate = 3.36, SE = 0.51,  $t = 6.64$ ,  $P < 0.001$ ; overall model: Likelihood ratio (compared to null model): 40.79, generalized  $r^2 = 0.48$ ,  $P < 0.001$ ). Mares that had received PZP earlier in their lifetime, but not during the year of conception (prior recipients), gave birth 0.90 months later than pre-contraception mares on average (estimate = 0.90, SE = 0.40,  $t = 2.27$ ,  $P = 0.03$ ). Non-recipient mares that never received PZP themselves, but gave birth after the general population was managed with PZP, gave birth 1.01 months later than pre-contraception mares on average (estimate = 1.01, SE = 0.25,  $t = 4.04$ ,  $P = 0.001$ ). This translates into current PZP recipients giving birth 2.34 months later than non-recipient animals and 2.46 months later than prior recipients (F-tests for linear combinations:  $F > 16$ ,  $P < 0.001$  for each comparison). Furthermore, birth dates were about 0.38 months later for each degree centigrade above average in the latter half of the breeding season and 0.05 months later for each centimeter of rain above average (temperature residuals July-Nov: estimate = 0.38, SE = 0.13,  $t = 2.84$ ,  $P = 0.001$ ; rainfall residuals July-Nov: esti-



**Figure 1. The distribution of births for mares on Shackleford Banks, NC, pre-contraception and post-contraception management.** Mares gave birth over a wider range of months after the onset of contraception; this effect was more pronounced in PZP recipients than non-recipients.

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mate = 0.05, SE = 0.03,  $t = 1.83$ ,  $P = 0.07$ ). In general, mares gave birth 0.05 months earlier for every year of age (estimate = -0.05, SE = 0.02,  $t = -2.03$ ,  $P = 0.05$ ).

Among mares treated with PZP, those receiving a higher total number of applications foaled later in the season than did those receiving fewer applications, with each additional application associated with a 0.5 month delay in foaling date (Linear Mixed Effects Model: estimate = 0.55, SE = 0.21,  $t = 2.61$ ,  $r^2 = 0.65$ ,  $P = 0.01$ , see Fig. 2A). In addition, each consecutive PZP application was associated with a 0.8 month delay in foaling date, on average (Linear Mixed Effects Model: estimate = 0.83, SE = 0.23,  $t = 3.64$ ,  $r^2 = 0.65$ ,  $P < 0.0008$ , see Fig. 2B). While the consecutive and total number of PZP applications were highly correlated, comparing the AICc between the prior two models suggested that the number of consecutive PZP treatments more accurately predicted month of birth than did the total number of PZP treatments (AICc total = 202.96, AICc consecutive = 196.83). The number of years that mares did not conceive (total or consecutive), however, did not correlate with foaling date (Linear Mixed Effects Model: total years: estimate = 0.31, SE = 0.26,  $t = 1.19$ ,  $r^2 = 0.57$ ,  $P = 0.24$ ; consecutive years: estimate = 0.26, SE = 0.28,  $t = 0.92$ ,  $r^2 = 0.44$ ,  $P = 0.36$ ). It is unlikely that these patterns resulted from a general trend across years, as birth dates among non-recipient animals did not change with calendar year (Linear Mixed Effects Model:  $r^2 = 0.17$ , estimate = 0.05, SE = 0.09,  $t = 0.57$ ,  $P = 0.57$ ).

### Mare Condition

A generalized linear model, which controlled for mare age, showed that among post-contraception animals, recipient mares (both current and prior) were in better physical condition, with rump scores approximately 0.4 points higher than those of non-recipients (overall model (compared to null model): Chi Square = 7.74,  $P = 0.05$ ; PZP treatment: estimate = 0.38, SE = 0.15, Chi Square = 6.13,  $P = 0.01$ ; age: estimate = -0.005,

SE = 0.06, Chi Square = 0.007,  $P = 0.93$ ; PZP treatment  $\times$  age: estimate = -0.02, SE = 0.06, Chi Square = 0.13,  $P = 0.72$ ).

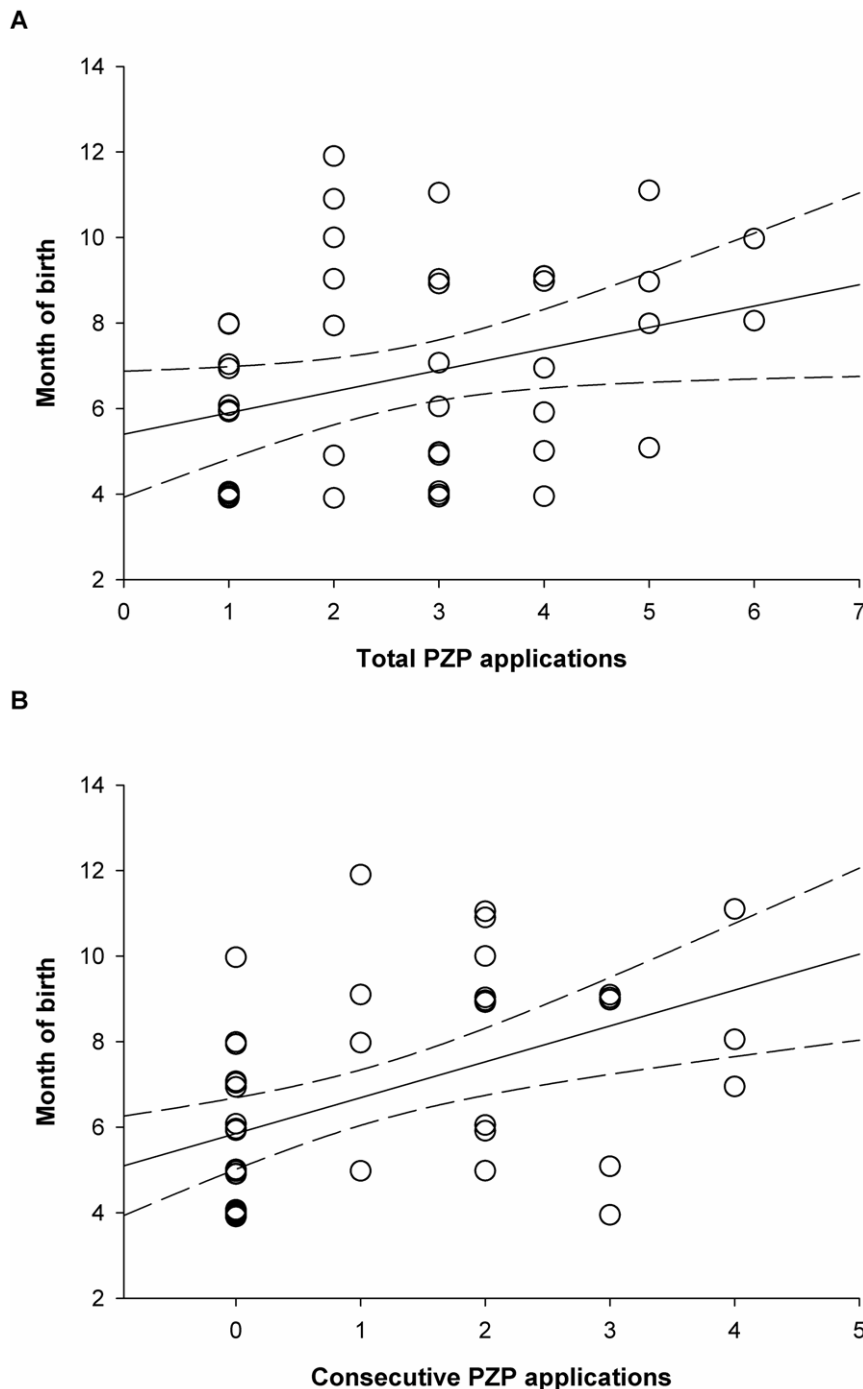
### Weather

The best fit model of monthly mean temperature showed that overall temperatures were approximately  $0.6^\circ\text{C}$  warmer after the onset of contraception management (see Fig. 3A; overall model: Likelihood ratio (compared to null model) = 358.40,  $P < 0.0001$ , generalized  $r^2 = 0.88$ ; month: estimate = -8.49, SE = -0.97,  $t = -8.78$ ,  $P < 0.0001$ ; month<sup>2</sup>: estimate = 3.88, SE = 0.29,  $t = 13.43$ ,  $P < 0.0001$ ; month<sup>3</sup>: estimate = -0.45, SE = 0.03,  $t = -13.70$ ,  $P < 0.0001$ ; month<sup>4</sup>: estimate = 0.015, SE = 0.001,  $t = 12.20$ ,  $P < 0.0001$ ; contraception management (before): estimate = 0.58, SE = 0.27,  $t = -2.13$ ,  $P = 0.06$ ). The best fit model of monthly precipitation showed no overall differences between the periods before and after contraception management, but there were significant interactions between month of the year and the periods before and after contraception (see Fig. 3B; overall model: Likelihood ratio (compared to null model) = 38.43,  $P < 0.0001$ , generalized  $r^2 = 0.20$ ,  $P < 0.0001$ ; month: estimate = -11.19, SE = 4.96,  $t = -2.25$ ,  $P = 0.03$ ; month<sup>2</sup>: estimate = 3.66, SE = 1.46,  $t = 2.51$ ,  $P = 0.01$ ; month<sup>3</sup>: estimate = -0.38, SE = 0.17,  $t = -2.27$ ,  $P = 0.02$ ; month<sup>4</sup>: estimate = -0.01, SE = 0.006,  $t = 1.88$ ,  $P = 0.06$ ; contraception management (before): estimate = 6.99, SE = 4.22,  $t = 1.66$ ,  $P = 0.12$ ; month  $\times$  contraception management (before): estimate = -2.97, SE = 1.43,  $t = -2.07$ ,  $P = 0.04$ ; month<sup>2</sup>  $\times$  contraception management (before): estimate = 0.22, SE = 0.11,  $t = -2.03$ ,  $P = 0.04$ ).

### PZP Efficacy

We defined PZP efficacy during the year of administration as the number of vaccinated mares that did not become pregnant divided by the total number receiving the vaccine. Across the first four consecutive PZP applications, this efficacy declined from 97% to 87%, returning to 100% after five or more consecutive applications (see Fig. 4). A generalized mixed effects model shows that this pattern





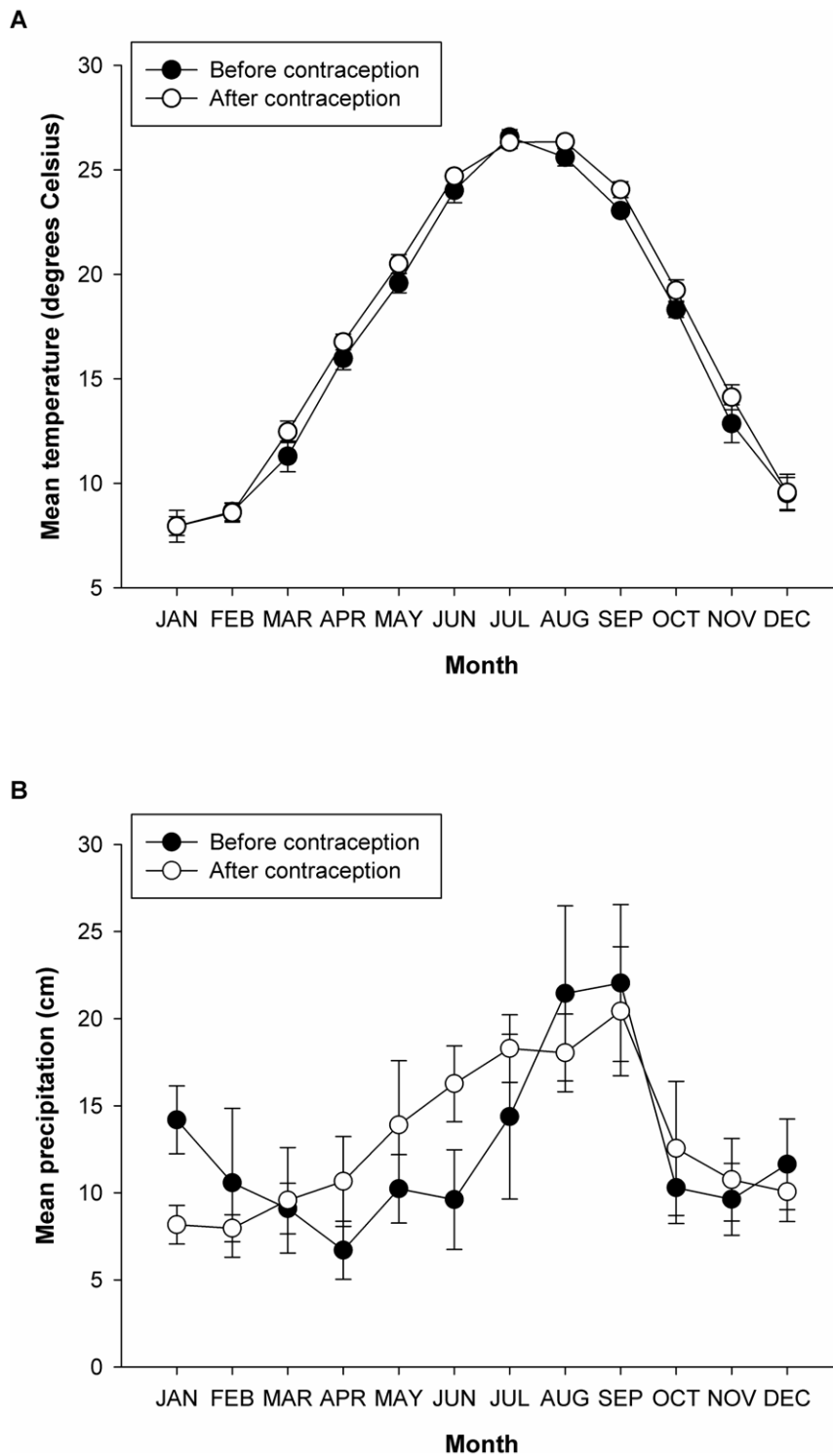
**Figure 2. Birth month and number of A) total PZP applications, and B) consecutive PZP applications.** In the events of ties, month of birth has been jittered by 0.2 years to allow clear visualization of every individual. Mares receiving more applications of PZP foaled later in the year on average than did mares receiving fewer applications. Although the number of total and consecutive applications is highly correlated, AICc suggests that the number of consecutive applications explains more of the variation in the data.  
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is significant, even when controlling for mare age (overall model with binomial error distribution: Log Likelihood =  $-61.79$ ,  $P = 0.01$ , generalized  $r^2 = 0.17$ ; consecutive PZP applications: estimate =  $2.98$ , SE =  $1.20$ ,  $z = 2.49$ ,  $P = 0.01$ ; (consecutive PZP applications) $^2$ : estimate =  $-0.51$ , SE =  $0.22$ ,  $z = -2.33$ ,  $P = 0.02$ ; age at first PZP application: estimate =  $0.10$ , SE =  $0.05$ ,  $z = 1.84$ ,  $P = 0.07$ ). Prior research has shown that five to seven years of consecutive PZP treatment can be associated with ovulation failure [4]. The present

dataset is consistent with this result, as no mare receiving the vaccine for five or more consecutive years became pregnant.

## Discussion

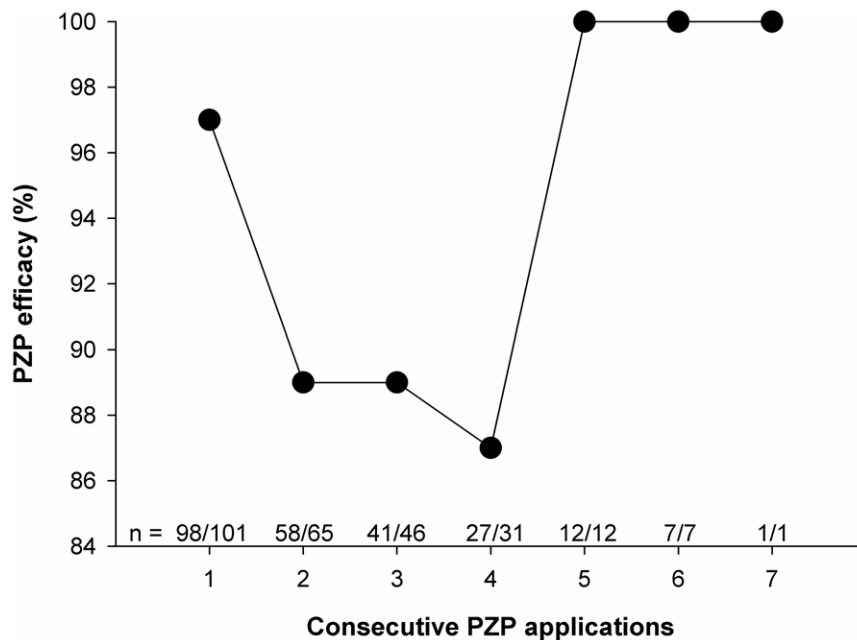
Here we show that PZP recipients exhibited a change in their reproductive schedule: recipient mares gave birth over a broader time period than did non-recipients, with current recipients giving



**Figure 3. Weather data for six years pre-contraception (1995–2000) and eight years post-contraception (2001–2008) management.** Data were collected from Morehead City, North Carolina, approximately 8 km from the study site (Shackleford Banks, North Carolina). Temperatures (A) were marginally warmer post-contraception than they had been pre-contraception. Overall rainfall (B) did not differ before and after contraception, though the seasonal patterns were marginally different pre- and post-contraception. doi:10.1371/journal.pone.0013635.g003

birth later in the year than prior recipient and non-recipient mares. Given that gestation in wild horses lasts approximately 11 to 12 months [30], this change indicates a corresponding change in the schedule of ovulatory cycling. Contraception with porcine

zona pellucida is popular amongst managers specifically because it effectively reduces the odds of conception without the application of exogenous steroids [2]. Long-term studies on Assateague Island have reported that PZP has little to no effect on reproductive



**Figure 4. PZP efficacy and number of consecutive PZP applications.** PZP efficacy was defined as the number of recipient mares that did not become pregnant divided by the total number of mares receiving the vaccine. Across the first four consecutive applications, PZP efficacy declined, returning to 100% after five or more consecutive applications (5–7 applications have been shown to result in ovulation failure and decreased oestrogen levels [5,40]).

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hormone levels, the schedule of reproductive cycling, or the social behaviors of recipient animals [4]. However, studies in other wild horse populations have shown that recipient mares both initiate and receive more instances of reproductive behavior during both the breeding [39] and non-breeding seasons [9]. This study provides the first evidence that mares treated with PZP can extend ovulatory cycling beyond the normal breeding season. This suggests that populations of wild ungulates can vary in their response to similar contraceptive treatment. Careful consideration of baseline population dynamics should be made prior to treatment in order to fully assess possible PZP effects.

### Foaling Date

Mares receiving PZP at any point during their lifetime gave birth over a broader time period than did non-recipient animals. This larger variance among PZP mares is likely driven by the fact that current recipients gave birth later than did mares receiving fewer applications. Increases in the average interbirth interval for recipient mares did not seem to be driving this result, as foaling date was not affected by the number of years (cumulative or consecutive) that mares failed to conceive. This discrepancy may be due to high variability in the conception and foaling dates of treated mares. First, it is less likely that an animal vaccinated with PZP will conceive at all, thus reducing sample size. Second, due to contraceptive failure, some treated mares will conceive during the normal breeding season, further increasing variability. Interestingly, prior to the application of PZP, the average month of birth did tend to increase with interbirth interval (Linear Mixed Effects Model: estimate = 0.30, SE = 0.17,  $t = 1.82$ ,  $r^2 = 0.06$ ,  $P = 0.07$ ) [22], demonstrating at least some plasticity in the scheduling of reproductive cycling in Shackleford mares. On Assateague, PZP recipients experience normal reproductive cycling and mate at

rates similar to non-recipients [40]. However, when such behavior fails to result in conception over several years, it follows that individuals extending reproductive cycling will be able to achieve conception later in the year if the contraceptive effects of PZP have decreased sufficiently [28,29].

Because feral horses are highly social, such changes can have cascading effects on other group members and throughout the population. Our research has shown that after contraception management, PZP recipients both attract and initiate more instances of reproductive behavior [9] and are more often the harem male's nearest neighbor during the fall/winter (Nuñez, unpublished data), indicating that group spreads are reduced. Such changes represent an increase in energy expenditure and a potential decrease in nutrient intake during a time of year when sufficient energy reserves are at a premium [27]. Moreover, early foal development in unmanaged populations typically occurs during the spring and summer when resources are plentiful [11,27]. Offspring born in the fall/winter months face nutritional and thermoregulatory challenges not experienced by their counterparts born during the normal foaling season, potentially making developmental benchmarks difficult to achieve [27].

Such predictions are not consistent with data from Assateague Island where mares show increased survival, only minimal physiological side effects, and no behavioral or demographic changes [4,5,6]. In addition, foal survival does not differ between foals born in or out of the normal foaling season [41]. However, on Shackleford Banks, recipient mares change groups more often, elicit and receive more instances of reproductive behavior, and receive more harassment from harem males [9,42]. Given these differences in mare response to PZP management in the two populations, it follows that predictions based on the data from one site are not necessarily applicable to the other.

These population differences may be due to the scheduling of PZP administration at the two sites. When the contraception

program on Assateague began in 1994, the priorities for treatment followed a hierarchical approach based on the previous breeding success of the population, ensuring that all mares were given an opportunity to reproduce [3]. Females for which there was a high priority for treatment included those that had produced at least one surviving offspring. Low priority females included those that were less than four years of age. Females greater than four years old that had not produced surviving offspring did not receive treatment. In addition, the plan stipulated that only mares that had produced at least three surviving offspring or two generations of offspring would receive more than three consecutive years of treatment. Foals were not to be removed as removal increases a mare's reproductive success in the subsequent year [43,44,45]. Finally, it was recognized that this plan was subject to change as the population numbers decreased [46]. In the present study, Shackleford mares were contracepted between 1.5 and 2 years of age and received an average of  $3.4 \pm 0.2$  (mean  $\pm$  standard error) consecutive years of contraception, regardless of their productivity. To further control population numbers, foals born to these mares (due to contraception failure or changes in the application schedule), were likely to be removed. This difference in PZP administration and subsequent discrepancy in early life experience may contribute to the behavioral differences between the populations, as the ability to conceive with a harem male is likely critical to establishing lasting harem fidelity [16] and the retention of foals (until at least two years of age) is important to maintaining normal reproductive function [43,44,45].

### Possible Mechanisms

Although the effect was more pronounced in recipients of PZP, both recipients and non-recipients showed a wider range of foaling dates after contraception management (after 2001). While relatively rare, such extended periods of estrous have been documented in several equine species. Tropical species, for example, have been observed to reproduce throughout the year [12,13,47]. Similarly, studies of temperate species have shown that individuals can vary significantly in reproductive timing [14] and estrous behaviors during the non-breeding season [15]. Our data show that Shackleford mares exhibit at least some plasticity in their reproductive cycling. This plasticity enables mares to time their reproductive cycling according to ecological, sociological, and physiological cues.

For example, our results show that the reproductive changes exhibited by Shackleford mares correlate with warmer temperatures occurring later in the calendar year, after contraception management. Increases in rainfall late in the breeding season also correlate (albeit weakly) with later births. Both warmer temperatures and increased rainfall could result in higher resource availability [27] and afford females the additional reserves necessary to extend reproductive cycling into what is typically the non-breeding season.

The physical condition of mares may also play an important role in the extension of reproductive cycling. On Shackleford Banks, recipient mares are currently in better physical condition than are non-recipients. This is likely due to the fact that successfully contracepted mares are unconstrained by the costs of pregnancy and lactation [48]. Recipient mares will therefore have more resources to allocate to additional reproductive cycles. This effect of PZP, coupled with warmer temperatures occurring later in the year, may act to increase a mare's chances of conceiving later in the calendar year, if PZP antibody titers are sufficiently low [29].

Additionally, extended cycling in non-recipient mares could be influenced by the physiology and behavior of recipients.

Shackleford males exhibit higher rates of sexual behavior towards recipient females during both breeding and non-breeding seasons [9,42]. These overt social stimuli may entrain some non-recipients to continue reproductive behaviors and cycling into the early fall. Such stimuli are commonly used to induce receptivity in several domestic species including horses [49], pigs [50], and cows [51]. In the wild, courtship signals from conspecifics advance gonadal cycles or maturation in several taxa, including mammals [52,53,54], birds [55], amphibians [56], and reptiles [57]. Given the importance of social cues in the timing of reproduction among such diverse species, this possibility warrants further investigation in Shackleford mares.

Finally, the declining efficacy of PZP with increased consecutive applications is likely a contributing factor to the later foaling dates of recipient mares. Lyda and colleagues' research with captive, wild mares has shown that antibody titers against PZP remain high for up to ten months after initial treatment [28]. In addition, research with both Shackleford and Assateague horses has shown that initial applications of PZP are often effective over multiple years [9,58], suggesting that antibody titers can remain high for longer. However, laboratory research has shown considerable variability in anti-PZP titers [29], as did Lyda and colleagues' work in which half the mares treated with PZP and Freund's Complete Adjuvant fell below contraceptive levels within the ten months of study [28]. Our data show that increasing the number of consecutive applications can reduce the single year efficacy of PZP by roughly 10%, indicating that either antibody titer or reactivity can decrease more rapidly with consecutive applications. Such patterns could result from the induction of immunological tolerance [59], which reduces responsiveness to self-tissues or repeatedly encountered, non-pathogenic antigens [60]. PZP is designed to mimic host tissue and induce an immune response against self tissue: the recipient's own zona pellucida [2]. As such, it seems reasonable that at least some animals would mount tolerance mechanisms to combat this autoimmunity. In addition, the repeated application of a specific antigen generates an antibody response that is increasingly more specific to that particular antigen [29]. The antibodies produced by mares against porcine zona pellucida should, therefore, become less cross reactive with horse zona pellucida over time. Of course, PZP efficacy will vary depending on mare age and timing of inoculation [61]. Regardless, if PZP recipients extend reproductive cycling and behavior into the non-breeding season, any decrease in efficacy that leaves them fertile in the fall/winter will help drive increases in late season conception.

Although the removal of offspring can induce estrous cycling in ungulate species [62], it is unlikely that the removal of foals has influenced foaling date among PZP-treated mares on Shackleford Banks. Thirty-nine foals (conceived due to contraception failure or administration scheduling) have been removed from the island. Approximately 55% of these foals were born to non-recipient animals. The majority of foal removals were conducted in the January following foal births. Given that non-recipient animals did not give birth later than September and most recipient animals gave birth before December, it is unlikely that foal removals in January induced late-season estrus in Shackleford mares. It is equally unlikely that increases in mare condition due to the alleviation of lactation costs resulted in early resumption of estrus the following spring [27]. If that were the case, during the early spring months we would expect to see an increase in the number of foals born to mares subjected to offspring removal. This is not borne out by the data. Still, the removal of foals is ill-advised as it increases mare fecundity the following year [43,44,45].

## Management Implications

When the alternative (gather and removal) is considered, PZP is currently managers' most humane and effective option for population control. However, careful study of the animals' demography, physiology, and behavior is necessary prior to and during treatment to ensure that a) the potential effects of PZP can be assessed accurately, and b) within managerial constraints, unintended effects of PZP are ameliorated. Differences in habitat, resource availability, and demography among conspecific populations will undoubtedly affect their physiological and behavioral responses to PZP contraception, and need to be considered. For instance, while Assateague horses show no behavioral and only minor physiological responses to PZP, horses on Shackleford Banks [9,42] and in the western United States [39] alter social and reproductive behaviors in response to PZP. Our data suggest that mare condition and warming trends may present additional complications. Increases in physical condition and changes in average temperature may interact with management regimes, enabling mares to alter their reproductive physiology even further. Moreover, these data emphasize the importance of study during both the breeding and non-breeding seasons. Much of the research showing little to no effect of PZP on feral horse behavior or physiology has been performed exclusively during the breeding season [4,5,10], potentially missing important differences in recipient response.

If population numbers are managers' primary concern, our data show that giving five or more consecutive applications of PZP will result in 100% contraception efficacy. This is consistent with data from Assateague where mares receiving 5–7 consecutive PZP applications exhibited ovulation failure and decreased urinary oestrogen concentrations [5,40]. However, if managers are tasked with the maintenance of natural behaviors and foaling schedules, consecutive PZP applications should be avoided. Research has shown that one application of PZP is often effective over multiple years, exhibiting yearly efficacy declines similar to that of 2–4 consecutive treatments (on Shackleford) [9,58]. Our data show that current recipients gave birth later than both prior recipient and non-recipient animals. However, prior recipients of PZP gave birth on schedules similar to non-recipients, suggesting that breaks between treatments can ameliorate unintended behavioral and

physiological changes in recipient animals. Contraception on such schedules will still maintain lower pregnancy rates, but will allow for the birth of a manageable number of offspring which are also important to the maintenance of normal behaviors [9]. These foals should be allowed to remain in the population for at least two years as earlier removal has been shown to increase a mare's reproductive success in the subsequent year [43,44,45]. Additionally, subadult, dispersing females should be allowed to settle into harems and have at least one foal before receiving contraception [16]. Management regimes such as this would of course necessitate a higher minimum population level. Additional research is needed to determine whether these larger, but still limited population sizes could achieve management goals. If so, this could prove a cost-effective means of controlling animal numbers while maintaining their natural physiology and behavior.

The broader implications of this research are considerable. As this study suggests, the physiological and behavioral effects of PZP are not fully understood. Still, the vaccine is currently administered to many different species including white-tailed deer, *Odocoileus virginianus* [7], elk, *Cervus elaphus* [8], black bears *Ursus americanus* [63], and African elephants, *Loxodonta Africana* [64]. As with conspecific equid populations, habitat, resource, and demographic differences among species will affect their responses to PZP contraception and need to be considered. For social species like the horse, a proper balance between managing population size and maintaining a more natural physiological and behavioral regime is particularly important.

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## Author Contributions

Conceived and designed the experiments: CMVN. Performed the experiments: CMVN. Analyzed the data: CMVN JSA. Wrote the paper: CMVN JSA DIR.

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# Immunocontraception decreases group fidelity in a feral horse population during the non-breeding season

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## ABSTRACT

The behavioral effects of the immunocontraceptive agent porcine zona pellucida (PZP) have not been adequately studied. Important managerial decisions for several species, including the wild horse (*Equus caballus*), have been based on this limited research. We studied 30 horses on Shackleford Banks, North Carolina, USA to determine the effects of PZP contraception on female fidelity to the harem male. We examined two classes of females: contracepts, recipients of the PZP vaccine ( $n = 22$ ); and controls, females that have never received PZP ( $n = 8$ ). We conducted the study during the non-breeding season from December 2005 to February 2006, totaling 102.2 h of observation. Contracepted mares changed groups more often than control mares ( $P = 0.04$ ). Contracepts also visited more harem groups than did control mares ( $P = 0.02$ ) and exhibited more reproductive interest ( $P = 0.05$ ). For both contracepted and control females, the number of group changes ( $P = 0.01$ ) and number of groups visited ( $P = 0.003$ ) decreased with the proportion of years mares were pregnant. Our study shows that the application of PZP has significant consequences for the social behavior of Shackleford Banks horses. In gregarious species such as the horse, PZP application may disrupt social ties among individuals and inhibit normal social functioning at the population level.

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## 1. Introduction

Due to the extirpation of their natural predators, ungulate populations in North America have expanded, necessitating their regulation through culling or contraception management (Eberhardt et al., 1982). Immunocontraceptives are widely used to control reproduction in free-ranging ungulates (Kirkpatrick et al., 1990; Turner et al., 1992). In females, the most common form of immunocontraception, porcine zona pellucida (PZP), stimulates the production of antibodies that bind sperm receptors on the egg's surface, thereby preventing sperm

attachment and fertilization (Sacco, 1977). While PZP effectively inhibits conception in several different mammalian species (Kirkpatrick et al., 1996), little is known about its potential effects on recipient behavior. Studies in free-ranging elk (*Cervus elaphus*) and white-tailed deer (*Odocoileus virginianus*) indicate that females receiving PZP extend reproductive behaviors into the post-breeding season (McShea et al., 1997; Heilmann et al., 1998). Authors suggest that in response to repeated unsuccessful mating attempts, females continue cycling in an attempt to gain additional reproductive opportunities. Such changes in behavior can have serious consequences for social species, particularly for those that are polygynous with males defending and retaining several females.

Several studies have examined the effects of PZP application to wild horses (*Equus caballus*). These studies have focused primarily on the physiological effects (both

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reproductive and otherwise) of the vaccine (Kirkpatrick et al., 1992, 1997; Turner and Kirkpatrick, 2002). Researchers have reported no debilitating side effects to PZP recipients and only minor ovulation failure and depressed urinary oestrogen concentrations with repeated applications (Kirkpatrick et al., 1996). In addition, the contraceptive effects of PZP have been shown to be reversible, safe for pregnant females, and do not adversely affect the survivorship or subsequent fertility of offspring born to treated individuals (Kirkpatrick and Turner, 2002).

Researchers claim that the contraceptive has no effect on recipient behavior (Kirkpatrick et al., 1996, 1997; Powell and Monfort, 2001), but present no quantitative data to support their conclusions. In fact, no systematic study has specifically addressed this issue with true controls, animals that have never received PZP during their lifetime (Powell, 1999). Additionally, no study on wild horses has focused on the post-breeding period, when the effects of PZP appear most pronounced in other ungulates (McShea et al., 1997; Heilmann et al., 1998).

In wild horse societies, the harem is the core social group, consisting of usually one, but sometimes two or three harem male(s), one to several female(s), and their offspring (Feist and McCullough, 1976; Rubenstein, 1981, 1986; Linklater et al., 2000). Harem groups are typically stable units, showing very few changes in composition over months or years (Klingel, 1975). Female loyalty to the harem male and the male's ability to retain females is paramount to maintaining this stability (Feist and McCullough, 1976; Rubenstein, 1981; Goodloe et al., 2000). Decreases in harem stability have been shown to affect several aspects of mare well-being, resulting in lower overall reproductive success (Kaseda et al., 1995), less time for preferred behaviors, decreased body condition and fecundity, elevated parasite levels, and increased offspring mortality (Linklater et al., 1999).

For the most part, wild horses are non-territorial, with several harems sharing both feeding and water resources (Feist and McCullough, 1976; Rubenstein, 1981; Cameron et al., 2003). Given this ecology, decreases in the stability of individual harems have the potential to affect the interactions and social relationships of neighboring harems and thereby, may affect significant change at the population level. As such, understanding the potential effects of PZP on individual behavior is of broad importance to maintaining a functional population of feral horses.

In this study we investigate the behavioral effects of PZP on the horses of Shackleford Banks, North Carolina, USA during the non-breeding season. Specifically, we examine female propensity to switch harems, the number of harem groups visited, and the occurrence of reproductive behavior. Since the first application of the contraceptive in January 2000, a reduction in the fidelity of resident mares to their harem males has been noted, albeit anecdotally (C. Mason, personal observation). Based on this information, we hypothesized that contracepted females would change groups more often, would visit more groups, and would exhibit reproductive behaviors more often than would control mares (those never having received the vaccine).

## 2. Materials and methods

### 2.1. Study area

Shackleford Banks is a barrier island approximately 3 km off the coast of North Carolina, USA located at 34°40'04.94"N and 76°35'39.39"W. The island stretches 15 km in length, and varies between 0.5 and 3 km in width. The specific study area extended approximately 7 km and was located in the center of the island. This site contained all study animals.

Daylight hours, measured from sunrise to sunset times, ranged from 9 h and 53 min at the beginning of the study on 10 December 2005 to 10 h and 35 min at the conclusion of the study on 3 February 2006 (U.S. Naval Observatory Data Services, 2008). In Beaufort, NC, 7.8 km from the study site, average daily temperatures  $\pm$  1 S.E. for the past 20 years for December, January, and February were  $7.93 \pm 0.40$  °C,  $7.08 \pm 0.28$  °C, and  $7.95 \pm 0.26$  °C, respectively. During the present study average daily temperatures  $\pm$  1 S.E. in Beaufort, NC for December, January, and February were  $6.86 \pm 1.65$  °C,  $7.19 \pm 0.48$  °C, and  $7.26 \pm 0.30$  °C, respectively (National Climate Data Center, 2008).

### 2.2. Study subjects

The reproductive units of Shackleford horses are typical of feral equids, consisting of coherent harem groups of one or, sometimes two or three stallion(s) with one to several mare(s) and their offspring (Rubenstein, 1981). While multi-male harems are more common in some populations (Linklater and Cameron, 2000), they occur less frequently on Shackleford Banks, accounting for only 19% of all harems on the island at the time of this study. For the most part, these social units are not territorial, and the animals move freely within their overlapping home ranges.

Normally, harem groups are long lasting with most changes involving the dispersal of immature individuals (both male and female). Harem males will sometimes fight to acquire mares from other groups, but stallions almost always retain their mares (Feist and McCullough, 1976; Rubenstein, 1981).

The application of PZP for the purposes of immunocontraception was begun by the National Park Service in January 2000. At that time, eight control mares were identified; one from each of the distinct genetic lineages on the island. These mares would not receive the vaccine at any point during their lifetime. Females younger than 2 years of age were not considered for control status. These procedures determined the current age distribution of control and contracepted animals on Shackleford Banks. The authors of this study were not involved in establishing the number of control and/or contracepted animals.

We observed 30 females that organized themselves into 13 harem groups. Twenty-two mares were treated with PZP at least once between January 2000 and January 2005; the remaining control animals had never been treated. Six of the harem groups investigated contained contracepted females only; two groups contained control females only; the remaining five groups contained both contracepted and control females (see Table 1). All harems considered in this study contained only one harem male. At the time of the study, five of the control mares were pregnant; three of which were nursing foals. An additional control mare nursed a foal, but was not pregnant. Three contracepted mares were pregnant. Two of these females had not received PZP treatment the previous spring; the remaining mare's pregnancy suggests a failure of the treatment. Two other contracepted mares nursed foals; these mares had not received treatment the previous spring. The inoculation, pregnancy, and foaling records for all study animals are shown in Tables 2 and 3.

### 2.3. PZP contraception

The National Park Service administers PZP from late February through April each year. Mares are first treated at 2 years of age. Each injection contains 100  $\mu$ g of PZP plus an adjuvant (mixed at the darting site). Initial doses contain Freund's Complete Adjuvant, Modified, *Mycobacterium butyricum* (Calbiochem, Gibbstown, NJ, USA, #344289). All subsequent doses contain Freund's Incomplete Adjuvant (Sigma, St. Louis, MO, USA, #F5506).

For the animals in this study, PZP deterred pregnancy in 97% of cases when administered during the same year. This efficacy dropped to 76% in



**Table 1**  
Initial harem configurations of focal animals.

Harem male	Group type	Control females	Contracepted females	Focal females
Adam	Control	1	0	1
Edge	Control	1	0	1
Axl*	Contracept	0	2	1
Duchovny	Contracept	0	1	1
Pacino	Contracept	0	6	6
Phinius*	Contracept	0	4	1
Teddy*	Contracept	0	5	1
Toro*	Contracept	0	4	4
Clint	Mixed	1	1	2
Dionysis	Mixed	1	1	2
Satellite	Mixed	1	2	3
Stobbs	Mixed	1	2	3
Winston	Mixed	2	2	4

Four of the harems listed reside outside of the study area and were not observed as focal groups (\*). However, at least one female from each of these harems moved into focal groups during our study. The focal females column reflects only the number of individuals from each harem that were observed systematically during the study. Other columns show the initial group composition of each harem at the study's outset.

the second year after PZP inoculation. These values are similar to those published for Assateague horses, 94% and 86%, in the first and second years, respectively (Turner et al., 2007).

While we were unable to obtain blood samples for mares during this study, anti-PZP antibody titers in domestic mares remain above control levels for up to 40 weeks post-injection when using similar doses and adjuvant mixtures (Willis, 1994). The National Park Service routinely inoculates mares from February through April. Therefore, in animals inoculated in 2005, anti-PZP antibody levels would have been high during the breeding season, but were likely approaching control levels at the time of this study.

#### 2.4. Pregnancy testing

Fecal samples are collected by the National Park Service in January of each year. All pregnancy testing is completed by enzymeimmunoassay of fecal material at the Science and Conservation Center at ZooMontana in Billings, MT, USA. Using the methods of Kirkpatrick et al. (1991), water extracts of fecal samples are assayed for estrone conjugates and nonspecific progesterone metabolites. Foaling records from the summers following testing were used to supplement assay results.

#### 2.5. Behavioral and demographic sampling

The study was conducted by one observer (C.M.V. Nuñez) during the non-breeding season from December 2005 to February 2006, totaling

102.2 h of observation. Horses were identified individually by color, sex, age, physical condition, and other distinguishing markings including freeze brands. Ages are known from long-term records for the identified horses of Shackleford Banks (Nuñez, 2000).

We located each harem and noted its composition an average of four times each week. We recorded its GPS location and composition, paying particular attention to the presence or absence of females. These data allowed us to assess female willingness (or ability) to remain with their harems. The following measures were analyzed:

- Number of changes that females made, i.e. how many times females switched groups during the study.
- Number of different groups that females visited, i.e. the total number of groups in whom a female was seen during the study.
- The age of the harem male with whom a female was most often associated.
- The size of the group in which the female was most often found.

All incidences of reproductive interest (including copulation, mounting, genital sniffing, and rump rubbing) directed to and initiated by mares were recorded *ad libitum* during scan sampling (Altmann, 1974). Behaviors of reproductive interest were defined as follows:

- Mounting—male places forelimbs around a female's flank; does not include insertion of the penis.
- Copulation—male mounts female; insertion of penis achieved.
- Genital sniffing—animal (male or female) actively places the snout to the genitals of another animal of the opposite sex.
- Rump rubbing—the initiator (male or female) places the chin and/or neck on the rump of a recipient of the opposite sex; initiator rubs its neck back and forth horizontally over recipient's rump.

#### 2.6. Statistical analyses

We analyzed the effect of contraception on the number of group changes, the number of different groups females visited, and the occurrence of reproductive interest (either received or initiated by mares) using generalized linear models in R (version 2.7.1). All variables were poisson distributed and were analyzed using models with a quasipoisson error distribution and a log link function. All models were weighted by the number of times a mare was observed.

Many factors in addition to PZP treatment may affect the number of group changes, the number of groups visited, and the occurrence of reproductive interest. Such factors include mare and harem male age, group size, pregnancy status, the presence of a foal, and the percentage of females contracepted in each group (Feist and McCullough, 1976; Rutberg and Greenberg, 1990; Linklater et al., 2000). We included mare age, PZP treatment, and their interaction in the initial, maximal generalized linear models discussed above. As PZP treatment was correlated with harem male age, group size, pregnancy status, the presence of a foal, and the percentage of contracepted mares in a group, these latter terms were not included in our models to avoid multicollinearity. Non-significant terms were removed from the

**Table 2**  
Pregnancy and foaling histories for control mares.

Mare	2000	2001		2002		2003		2004		2005	
	Pr	F	Pr	F	Pr	F	Pr	F	Pr	F	Pr
Biff	+	**	+	+	+	**	+	+	+	+	+
Carrot	+	+	—	—	+	+	+	+	+	+	+
Damigo	+	+	+	+	—	—	+	**	+	+	+
Hercules	+	**	+	+	—	—	+	+	—	—	—
Julie	+	**	+	**	+	+	+	**	—	—	+
Kelty	+	+	—	—	+	+	—	—	+	+	—
Laurie	+	+	—	—	+	**	+	**	+	**	—
Wallace	—	—	+	**	+	+	—	—	+	**	+

Column headings: Pr, pregnant during post-breeding season (fall) of the listed year; F, foal present (was conceived in prior year). "+" indicates the presence of a foal or that the animal was pregnant; "—" indicates the absence of a foal or that the animal was not pregnant; "\*\*\*\*" indicates that an animal foaled, but the offspring died before reaching 1 year of age.

**Table 3**  
Inoculation, pregnancy, and foaling histories for contracepted mares.

Mare	2000		2001			2002			2003			2004			2005-6		
	PZP	Pr	F	PZP	Pr	F	PZP	Pr	F	PZP	Pr	F	PZP	Pr	F	PZP	Pr
Alexa	+	–	–	–	–	–	–	–	–	+	–	–	+	–	–	+	–
Bo	–	+	+	–	+	+	–	–	–	+	–	–	–	–	–	+	–
C'susha	n/a	n/a	n/a	n/a	n/a	n/a	n/a	–	–	+	–	–	+	–	–	+	–
Darcy	–	+	+	–	+	+	+	–	–	+	–	–	+	–	–	+	–
Doobie	+	–	–	–	+	+	+	–	–	+	–	–	+	–	–	+	–
Dotu	–	–	–	+	–	–	+	–	–	+	–	–	–	–	–	+	–
Dumé	–	–	–	–	+	+	–	+	+	+	–	–	+	–	–	–	–
Dusty	–	–	–	+	–	–	+	–	–	+	–	–	–	+	+	+	–
Hardee	+	–	–	–	–	–	+	–	–	+	+	**	–	+	+	–	–
Helena	+	–	–	–	–	–	–	–	–	+	–	–	+	–	–	+	–
Jaqui	–	–	–	+	–	–	–	–	–	+	–	–	+	–	–	+	–
Juniper	+	–	–	–	–	–	+	–	–	+	–	–	+	–	–	–	–
Larissa	n/a	n/a	n/a	n/a	n/a	n/a	n/a	–	–	+	–	–	+	–	–	+	–
Noir	–	–	–	–	–	–	–	+	+	–	–	–	–	+	**	+	–
Paula	–	+	+	–	+	+	–	+	+	+	–	–	+	–	–	+	–
Serenac	–	–	–	+	–	–	–	+	+	+	–	–	+	–	–	+	–
Shag	+	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	+
Slug	–	+	+	–	–	–	+	–	–	+	–	–	+	–	–	+	+
Sydney	–	+	+	–	–	–	+	–	–	+	–	–	–	–	–	–	+
Tatya	n/a	n/a	n/a	n/a	n/a	n/a	n/a	–	–	+	–	–	+	–	–	+	–
Wire	–	+	**	–	+	+	–	+	+	+	–	–	+	–	–	+	–
Zelda	–	+	**	–	+	+	–	+	+	+	–	–	+	–	–	–	–

Column headings: F, foal present (was conceived in prior year); PZP, contraception during the pre-breeding season (spring) of the listed year; Pr, pregnant during post-breeding season (fall) of the listed year. "+" indicates the presence of a foal, that the animal was pregnant, and/or that the animal was inoculated with PZP; "–" indicates the absence of a foal, that the animal was not pregnant, and/or that an animal was not inoculated with PZP; "\*\*\*\*" indicates that an animal foaled, but that the offspring died before reaching 1 year of age; "n/a" indicates that an animal was 0–2 years old and not eligible for contraception. PZP administration began in January 2000; foals present that year are not indicative of PZP efficacy and are not included. The status for the animals during the study period is highlighted.

models by backwards elimination. As sample sizes were limited, terms were retained if their *P*-value was less than 0.10.

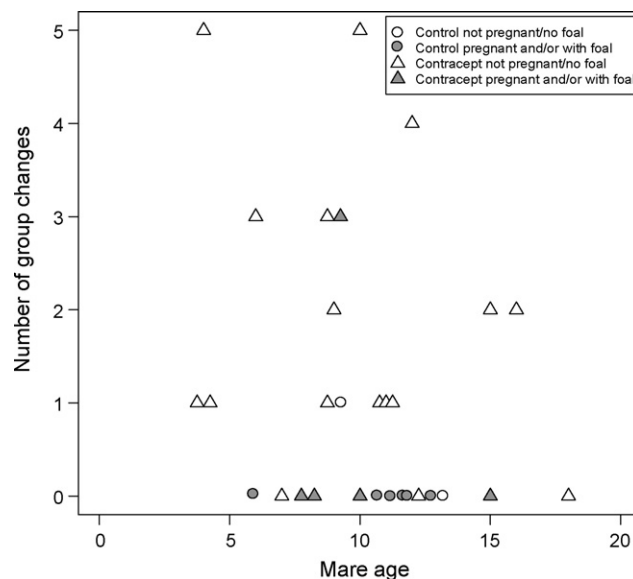
To address whether harem male age, group size, pregnancy status, the presence of a foal, and the percentage of contracepted mares in a group

had a significant influence on mare behavior, we analyzed them separately for control and PZP groups using Spearman rank correlations against the following variables: number of group changes, groups visited, and occurrences of reproductive interest (see Table 4).

**Table 4**  
Spearman's rank correlations between response variables and predictor variables that correlated with contraceptive treatment.

Predictor variable	Response variable		
	Total changes among groups	Number of groups visited	Instances of reproductive behavior
Male age	Controls: $\rho = -0.58$ , $P = 0.13$ Contracepts: $\rho = -0.16$ , $P = 0.47$	Controls: $\rho = -0.58$ , $P = 0.13$ Contracepts: $\rho = -0.18$ , $P = 0.43$	Controls: $\rho = 0.08$ , $P = 0.85$ Contracepts: $\rho = -0.03$ , $P = 0.88$
Group size	Controls: $\rho = -0.44$ , $P = 0.28$ Contracepts: $\rho = -0.28$ , $P = 0.22$	Controls: $\rho = -0.44$ , $P = 0.28$ Contracepts: $\rho = -0.22$ , $P = 0.35$	Controls: $\rho = 0.01$ , $P = 0.99$ Contracepts: $\rho = 0.02$ , $P = 0.95$
Percentage of group members contracepted	Controls: $\rho = 0.18$ , $P = 0.68$ Contracepts: $\rho = 0.11$ , $P = 0.67$	Controls: $\rho = 0.18$ , $P = 0.68$ Contracepts: $\rho = 0.09$ , $P = 0.71$	Controls: $\rho = 0.62$ , $P = 0.10$ Contracepts: $\rho = 0.13$ , $P = 0.59$
Pregnant or with foal during study	Controls: $\rho = -0.66$ , $P = 0.08$ Contracepts: $\rho = -0.41$ , $P = 0.06$	Controls: $\rho = -0.66$ , $P = 0.08$ Contracepts: $\rho = -0.41$ , $P = 0.06$	Controls: $\rho = -0.66$ , $P = 0.08$ Contracepts: $\rho = -0.12$ , $P = 0.58$

Each correlation was performed separately for control ( $n = 8$ ) and contracepted ( $n = 22$ ) groups.



**Fig. 1.** Number of group changes during the study period by mare age for control ( $n = 8$ ) and contracepted mares ( $n = 22$ ). Even when controlling for the effect of age, contracepted mares change groups more often than do controls. Filled symbols represent mares that were either pregnant or nursing a foal at the time of the study. In the events of ties, mare age has been jittered by 0.2 years to allow clear visualization of every individual.

### 3. Results

#### 3.1. Number of group changes

A generalized linear model shows that PZP treated mares changed groups significantly more often than did controls, even when accounting for mare age (analysis of deviance, overall GLM:  $F_{2,27} = 6.73$ ,  $P = 0.004$ ; PZP treatment: estimate = 1.99,  $t = 2.11$ ,  $P = 0.04$ ; mare age: estimate =  $-0.13$ ,  $t = -1.92$ ,  $P = 0.07$ , see Fig. 1). Pregnancy and/or the presence of a foal seemed to have a marginal effect (see Section 3.3). Spearman rank correlations within treatment groups show that harem male age, group size, and the percentage of contracepted mares in the group had no effect on the number of group changes (see Table 4), suggesting that their influence was not substantial.

#### 3.2. Number of groups visited

A separate generalized linear model shows that contracepted females visited significantly more groups than did control mares, again controlling for mare age (analysis of deviance, overall model:  $F_{2,27} = 6.83$ ,  $P = 0.004$ ; PZP treatment: estimate = 0.49,  $t = 2.42$ ,  $P = 0.02$ ; mare age: estimate =  $-0.06$ ,  $t = -2.39$ ,  $P = 0.02$ , see Fig. 2). As above, pregnancy and/or the presence of a foal seemed to have a marginal effect (see below). Spearman rank correlations within treatment groups show that harem male age, group size, and the percentage of contracepted mares in the group had no effect on the number of males consorted with (see Table 4).

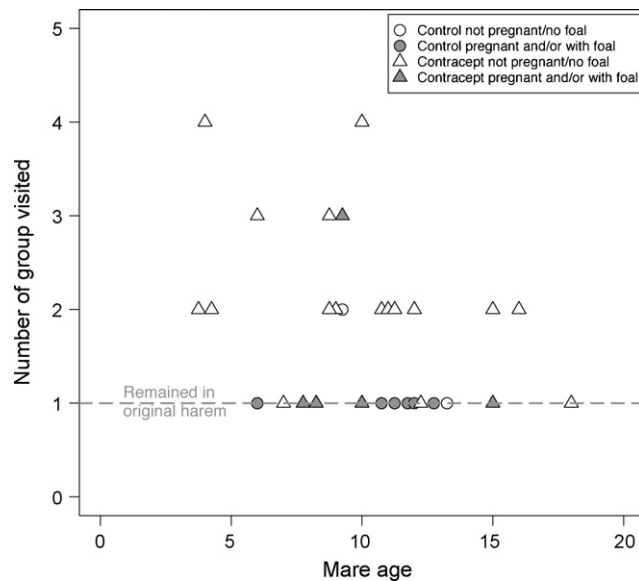
#### 3.3. Pregnancy and foal presence

Both control and contracepted mares that were pregnant and/or had foals tended to change groups less

often and visit fewer groups than did other mares (Spearman rank correlation: controls,  $\rho = -0.66$ ,  $P = 0.08$ ; contracepts,  $\rho = -0.41$ ,  $P = 0.06$ ; also see Table 4). Given this trend, we investigated whether a mare's history of pregnancy or foaling (over multiple years) affected behavior. For each female, we calculated the proportion of years pregnant and the proportion of years with a foal from January 2000 to January 2005, considering only those years in which the mare was sexually mature. A generalized linear model shows that mares pregnant for a greater proportion of years changed groups less often (overall model:  $F_{1,28} = 10.75$ ,  $P = 0.003$ ; % years pregnant: estimate =  $-3.11$ ,  $t = -2.79$ ,  $P = 0.01$ , see Fig. 3A) and visited fewer groups (overall model:  $F_{1,28} = 11.77$ ,  $P = 0.002$ ; % years pregnant: estimate =  $-1.03$ ,  $t = -3.31$ ,  $P = 0.003$ , see Fig. 3B). Mare age did not contribute significant explanatory power to these models and was thus removed. The proportion of years that mares had foals from 2000 to 2005 did not affect mare behavior (Group changes, overall model:  $F_{2,27} = 2.64$ ,  $P = 0.09$ ; % years with foal: estimate =  $-1.96$ ,  $t = -1.16$ ,  $P = 0.25$ . Groups visited, overall model:  $F_{2,27} = 4.63$ ,  $P = 0.04$ ; % years with foal: estimate =  $-1.39$ ,  $t = -1.44$ ,  $P = 0.16$ ).

#### 3.4. Reproductive interest

Contracepted mares received and exhibited more reproductive interest (see Section 2.5) than did control mares (analysis of deviance, overall GLM:  $F_{2,27} = 6.46$ ,  $P = 0.005$ ; PZP treatment: estimate = 2.04,  $t = 2.03$ ,  $P = 0.05$ ; mare age: estimate =  $-0.13$ ,  $t = -1.91$ ,  $P = 0.07$ , see Fig. 4). Spearman rank correlations within treatment groups show that harem male age, group size, the presence of a foal, and the percentage of contracepted mares in the group had no effect on the occurrence of reproductive

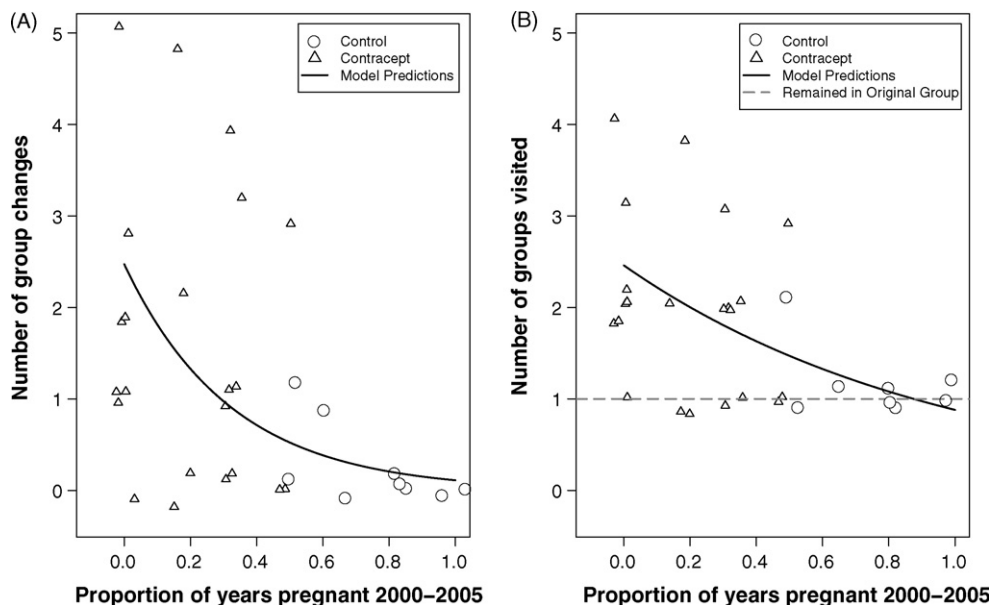


**Fig. 2.** Number of different groups visited during the study period by mare age for control ( $n = 8$ ) and contracepted mares ( $n = 22$ ). Even when controlling for the effect of age, contracepted mares visit more groups than do controls. Individuals on the dotted line did not change groups during the study. Filled symbols represent mares that were either pregnant or nursing a foal at the time of the study. In the events of ties, mare age has been jittered by 0.2 years to allow clear visualization of every individual.

interest (see Table 4). Pregnancy may have had a marginal effect on the reproductive interest received by control mares (Spearman rank correlation:  $\rho = -0.66$ ,  $P = 0.08$ ). This result is not conclusive however, since only one non-pregnant control mare received any reproductive interest. Pregnancy had no effect on the reproductive interest received or initiated by contracepted mares (Spearman rank correlation:  $\rho = -0.12$ ,  $P = 0.58$ ).

#### 4. Discussion

According to past research, contraception with PZP has little to no effect on the behavior of wild horses (Kirkpatrick et al., 1996, 1997; Powell and Monfort, 2001). The results of this study refute that assertion. Much of the aforementioned research has been based on a single island population, all studies have been conducted



**Fig. 3.** Number of group changes (A) and groups visited (B) by the proportion of years pregnant from January 2000 to January 2005. Lines show that according to the generalized linear model of the data (see Section 3.3), the number of group changes and groups visited decrease with the proportion of years mares are pregnant. Points have been jittered to allow clear visualization of every individual.

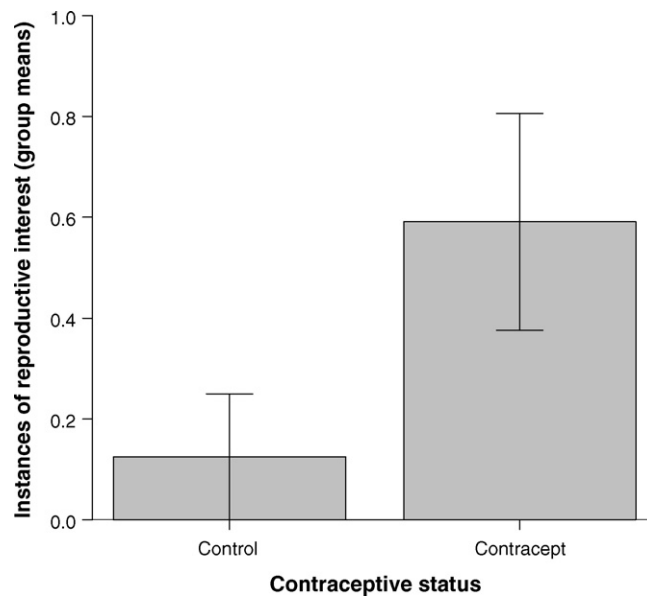


Fig. 4. Instances of reproductive interest during the study period by PZP treatment (means  $\pm$  S.E.). Contracepted mares exhibit and receive more reproductive interest than do control mares.

solely during the breeding season, and no study has had adequate controls against which to compare PZP-treated females (Kirkpatrick et al., 1997; Powell, 1999). Here, we studied horses during the non-breeding season on Shackleford Banks, North Carolina, making use of animals that had never received contraception as controls. In our study, PZP treatment increased the number of group changes, the number of different groups visited, and the occurrence of reproductive interest, both received and initiated by females. In addition, our results show that even 10 months after PZP inoculation, when anti-PZP antibody titers are likely low, the indirect behavioral effects on recipient animals remain strong. The potential implications of these results for feral horse management are of substantial importance and need to be investigated further.

#### 4.1. Fidelity to the harem male and reproductive interest

Contracepted mares are more likely to switch harem groups and visit more groups than are control mares. Decreases in mare fidelity to the harem male have debilitating consequences for harem stability (see Section 1). Resident females are often disturbed by the addition of new mares, especially if they are strangers (Monard and Duncan, 1996; Parker, 2001), and will become increasingly aggressive in their presence (Rutberg, 1990; Monard and Duncan, 1996). In addition, frequent changes to a harem's composition are likely to prohibit the establishment of a stable female dominance hierarchy, which is paramount to maintaining social cohesion among mares and overall group stability (Berger, 1977; Houpt and Wolski, 1980; Heitor et al., 2006). Moreover, the instability caused by these switching females may adversely affect the resident females' relationship with the harem male, reducing group cohesion even further. Because contracepted females do

not simply switch repeatedly between two well-known groups, but rather interact with several different groups, these detrimental effects of harem instability may be felt throughout the entire population.

Contracepted mares both receive and initiate more instances of reproductive interest than do control mares. Reproductive behavior is energetically costly (Galimberti et al., 2000). Repeated bouts of male harassment have been shown to reduce total time foraging in equid species (Rubenstein, 1986; Sundaresan et al., 2007). The relative cost of such behaviors may be especially high during the post-breeding season when resources are scarce (Stevens, 1990). In addition, the costs of this behavior may outweigh the potential benefits, i.e. increased reproductive success. Gestation in wild horses lasts approximately 11–12 months (Asa, 2002). Offspring conceived during the winter months are therefore subject to higher mortality due to the cold temperatures and poor quality forage available at birth.

The differences we observed in harem fidelity and reproductive behavior may result from prolonged estrous cycling into the post-breeding season in response to repeated failures to conceive. This hypothesis has been proposed to explain reproductive behavior during the post-breeding season in both PZP-treated elk (Heilmann et al., 1998) and white-tailed deer (McShea et al., 1997). In equids, reproductive behaviors including copulation, mounting, clitoral winking, and tail raising occur most frequently during estrous when the mare is nearing ovulation (Asa et al., 1979). Additionally, Asa et al. (1979) have shown that mare approaches to, and follows of the harem male are excellent predictors of the transition between estrous and diestrous. We propose that group changes may reflect a similar pattern, with PZP-treated mares approaching non-harem males more frequently during prolonged estrous cycling.

Extended periods of estrous, while relatively rare, have been documented in equids. Tropical species, for example, are less strictly seasonal, with some reproducing throughout the year (Grubb, 1981; Churcher, 1993). In addition, substantial variability in the cycling schedules and receptivity of individual mares (Asa et al., 1979), and the performance of estrous behavior and copulatory activities during the non-breeding season (Asa et al., 1980) have been documented in temperate species. Since the implementation of contraception, at least one winter birth, and therefore winter copulation, has occurred on Shackleford Banks (Susan Stuska, National Park Service, Cape Lookout National Seashore, personal communication). These variations in receptivity, ovulatory schedules, and foaling suggest that the seasonality of reproductive behaviors in *E. caballus* females has the potential to be quite plastic. As contracepted animals have experienced a significant alteration to their physiological state, extended cycling is even more feasible. Future work on Shackleford will test for additional estrous periods in PZP-treated mares by assaying total estrogens and progestins in fecal samples (Asa et al., 2001) during the fall and winter months.

Alternatively, mares may perceive the failure to conceive as a problem with the harem male. This perception alone may be sufficient to cause the observed differences in behavior, regardless of differential estrous cycling. Mares that did not conceive during the prior summer may then switch groups more often during the winter in an effort to prepare for the upcoming breeding season. Such group changes are likely to be less costly in the post-breeding season, as the spacing between band members increases and male herding and aggression decline during this period (Stevens, 1990). This seasonal decrease in harem male attentiveness may have contributed to the observed numbers of group changes and groups visited during this study. Given the strong relationship between contraceptive status and mare fidelity, however, it is unlikely that season is the sole cause of mare behavior. Still, additional study during the breeding season (April–August) is recommended to assess whether the changes in mare behavior result from an interaction between season and contraceptive status.

#### 4.2. The effects of pregnancy

Mare movement between groups is normally rare (see Sections 1 and 2.2). The results of this study strongly suggest that pregnancy and, possibly lactation, are important components to that stability. Regardless of contraception status, pregnant and/or nursing females tend to change groups less often, and over time, mares with a greater proportion of years pregnant are less likely to change groups. These decreases in pregnancy (and possibly lactation) may be the mechanism by which PZP treatment increases the propensity to change groups.

Additionally, decreased pregnancy and increased group switching have the potential to feedback on each other, resulting in even lower overall stability. Increased group switching has the potential to decrease mare fertility via male harassment. The more moves females make, the more male harassment they tend to receive (Rubenstein and Nuñez, 2008). Such harassment can lower female repro-

ductive success, as measured by the number of offspring surviving to independence (Rubenstein, 1986; Rubenstein and Nuñez, 2008). As evidenced by our results, such decreases in pregnancy increase the likelihood that females will change groups. These cascading effects have the potential to adversely affect entire populations (see Section 1) and are worth serious consideration when making management decisions.

#### 4.3. Management implications

If feral horse populations are to be maintained in the most natural state possible, we suggest that a small population of mares never be inoculated with PZP. Although the control mares' effects on group structure on Shackleford Banks have yet to be fully determined, the results clearly demonstrate that they are more faithful to their harem males than are contracepted mares. Mare fidelity to the harem male is important to overall harem stability. As such, it is likely, especially when one considers the sociality of these animals, that control females afford a stabilizing influence not only to individual harems, but also to the entire herd.

We also suggest that the subset of animals designated for control status be more fully representative of female demography. For example, at the time of this study, all control mares on Shackleford were between 8 and 15 years of age (see Figs. 1 and 2). This distribution does not currently afford for the behavior of very young or very old animals. Time can always provide for older individuals, but younger controls are needed to approximate the female population's age structure and natural behavior. For instance, contracepted, dispersing, subadult females likely move between more harems than they would naturally. Therefore, this demographic may adversely affect the entire herd's stability level. Allowing some portion of these animals to disperse, join harems, and reproduce normally could help to stabilize population behavior and structure.

Reevaluating the scheduling of PZP administration may also prove beneficial. An inoculation schedule that allows mares to conceive and give birth may help to ameliorate the most deleterious behavioral effects of PZP. Inoculating females every second and even every third year significantly reduces pregnancy in Shackleford Banks horses (see Section 2.3) and other wild populations (Turner et al., 2007). Contraception on such schedules will keep pregnancy rates low, but will allow for the birth of a manageable number of individuals which, according to this study, have a stabilizing influence on female behavior. Additional research is needed to determine if such contraception schedules will limit population size effectively. If so, this could provide a cost-effective means of controlling animal numbers while maintaining their natural behavior.

The broader management implications of this research are substantial. PZP has been reported to have little to no effect on the behavior of wild horses, specifically, but also wild ungulates in general (Kirkpatrick et al., 1996, 1997; Powell, 1999). The results of this study refute those claims, and in fact, highlight the pitfalls of generalizing recipient and group responses to PZP from one population to another. Moreover, these data emphasize the necessity of



study during all stages of the animals' reproductive cycle to determine the effects of contraception on social behavior. Managers of feral horse and other ungulate populations must use caution in basing contraceptive decisions upon data collected only during the breeding season and from a few, separate populations. Regardless of the ecological and sociological similarities between sites, subtle differences in factors such as demography, ready access to resources, and, as this paper suggests, seasonality, may prove important. Among different populations, such factors may shape the physiological and behavioral effects of PZP in unique and potentially unpredictable ways. Finally the trade-offs between managing population size and maintaining animal health and well-being are worth serious consideration. For social species such as the horse, such consideration is crucial if managers are to maintain behaviorally functional populations.

## 5. Conclusion

In this study, mares contracepted with PZP behaved differently from control mares. They changed groups more often, visited more groups, and both exhibited and initiated more reproductive interest. These differences in behavior have the potential to adversely affect the stability not only of individual harems, but the entire population on Shackleford Banks, North Carolina. Additional study into the mechanism behind these behavioral differences and into the scheduling of PZP administration will help ameliorate these effects.

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