EFFECT OF INTRODUCTION OF FEMALE AND MALE URINE PHEROMONES ON ESTROUS CYCLE LENGTH AND PROGESTOGEN AND TESTOSTERONE METABOLITE CONCENTRATION IN CAPTIVE RED RIVER HOGS (*POTAMOCHOERUS PORCUS*) IN NORTH AMERICAN ZOOS

A Thesis

by

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ABSTRACT

Captive red river hogs (RRH) have variable reproductive success yet potential causes are unknown. We hypothesized that non-breeding females would cycle or cycle more regularly and non-breeding males with low libido would increase concentrations of fecal testosterone metabolites in response to urine pheromone exposure. Female estrous cycles and progestogen metabolite (P4) concentrations and male testosterone metabolite (T) concentrations were compared between: 1) proven-breeder pairs (control(C); male n=3; female n=4), 2) new pairs (new male(NM); male n=4; female n=4), and 3) pairs exposed to pheromones (pheromone(P); male n=3; female n=3). Fecal samples were collected 3-5 times per week for a year. P animals had baseline sampling (6 months), followed by 2.5 month exposure to sow urine, 2-4 week wash-out, and 2.5 month exposure to boar urine. Fecals were extracted and assayed for P4 and T with ELISAs. Results were assessed for normality with PROC GLM (SAS, Cary, N.C.) and non-normal data transformed with repeated measures ANOVA (P4/T) or one-way ANOVA (cycles). There was a trend for P female's estrous cycle length to elongate (P baseline: 15.7 ± 1.5 days; P post-male: 19.3 ± 3.1 days; p=0.07) and for P male T concentrations to increase (+336.1±1.4 ng/g feces) in response to male urine pheromones. Pregnancies occurred in 2/3 C, 1/4 NM, with pseudopregnancy/pregnancy loss noted in 1/3 C and 2/3 NM females. The luteal phase P4 concentration for non-pregnant females was highest in P females and lowest in NM females (P: 3945.6 ± 158.3 ng/g feces; C: 3291.6 ± 196.3 ng/g feces; NM: 2884.5 ± 144.1 ng/g feces). Overall T concentration for males was

highest in P males and lowest in NM males (P: 909.6 ± 365.3 ng/g feces; C: 427.5 ± 353.8 ng/g feces; NM: 325.4 ± 283.2 ng/g feces). A season and treatment interaction (p<0.0001) for males and females, and acyclicity of females from August-December suggest that season confounded the results. Females housed with pregnant females were acyclic or experienced pseudopregnancy/pregnancy loss, suggestive of female reproductive suppression. In conclusion, urine pheromones may manipulate reproduction in captive RRH and consideration of the number of female RRH in housing is warranted.

DEDICATION

For my ever-curious nephew and nieces, Charles, Chloe, and Eloise Muesenfechter. Never lose your sense of wonder and know that I am always there to support and encourage you.

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NOMENCLATURE

AI	Artificial Insemination
AOB	Accessory Olfactory Bulb
ARKS	Animal Record Keeping System
ART	Artificial Reproductive Techniques
AZA	Association of Zoos and Aquariums
C	Control treatment group animals
CL	Corpus Luteum
ELISA	Enzyme-linked immunosorbent assay
ET	Embryo Transfer
FM	Female urine pheromone exposure period
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
GRB	Genome Resource Banking
hCG	Human Chorionic Gonadotropin
HRP	Horseradish Peroxidase
ISIS	International Species Inventory System
IUCN	International Union for the Conservation of Nature
IVF	In Vitro Fertilization
LH	Luteinizing Hormone
M	Male urine exposure period

MEDARKS	Medical Animal Record Keeping System
MOE	Main Olfactory Epithelium
N_e	Effective Population Size
NM	New male treatment group animals
P	Pheromone treatment group animals
P4	Progestogen metabolite(s)
PRE	Pre-urine pheromone introduction
RRH	Red River Hog (*Potamochoerus porcus*)
SPARKS	Single Population Analysis and Record Keeping System
SSP	Species Survival Plan
VNO	Vomernasal Organ
T	Testosterone metabolite(s)
W	Washout period

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1. INTRODUCTION

RRH are a widely distributed and relatively non-threatened Afrotropical suid species found in thickly wooded areas, swamps, and forests in equatorial West Africa (Berger et al., 2006). In North America, the species has become common in the zoo population due to their charisma and capability to readily adapt to mixed-species exhibits. According to the 2014 SSP Population Analysis and Breeding and Transfer Plan, the current AZA population of RRH totals 201 animals (96 males and 105 females) with a genetic diversity of approximately 84.54% (Holland et al., 2014). Inbreeding in the captive population has become a concern, with the average genetic relationship being greater than that of half-siblings due to the skewing of founder lines (Holland et al., 2014). Combating this phenomenon requires the prioritization of space to breed underrepresented animals with few or no offspring, which often involves translocation of animals to different zoos in order to match genetically valuable animals. However, many of the breeding pairs of RRH in the zoo population have low reproductive success even when they have been recently re-paired. In 2013, eight of the 33 paired RRH designated for breeding (24%) bred successfully and of the eight successful breeding pairs, three were newly paired RRH (37%) (Holland, 2013). After following the re-pairing recommendations, the pregnancy rate remained the same, regardless of whether they were a new (27%) or established (24%) pairs (Holland, 2013). The reasons for this variability in reproductive success in captive RRH are unknown.

Scant research has been conducted on reproduction in captive RRH, yet the

current practices of re-pairing animals to improve reproductive success have been unable to increase under-represented genes in the captive AZA population (Holland et al., 2014). Moreover, re-pairing is stressful for animals (Dickens et al., 2010) as it involves immobilization and translocation to a different zoo, a quarantine period, and introduction to a new environment and sounder. Further, according to the 2014 SSP Population Analysis and Breeding and Transfer Plan, if within three years the genetically valuable animals fail to reproduce, re-pairing is recommended, resulting in additional translocations of unsuccessfully reproducing animals (Holland et al., 2014).

Pheromones, olfactory chemicals which play an important role in the regulation of reproductive behavior, have been used to aid breeding initiatives in a variety of domestic and exotic species such as cows, mice, African elephants, and the giant panda (Roberts and Gosling, 2004; Rowell et al., 2003; Swaisgood et al., 2000; Wei et al., 2015; Weissenbock et al., 2009). In domestic swine, boar pheromones are known to be critical in several aspects of breeding in sows and gilts, such as the acceleration of puberty in gilts and induction of estrus, estrus behavior, and estrus synchrony in sows and gilts (Brooks and Cole, 1970; Rekwot et al., 2001). These insights have been vital in manipulation of reproduction in domestic and production animals (Delcroix et al., 1990; Dorries et al., 1991). As RRH are closely related to domestic swine, pheromones have the potential to be useful to manipulate reproduction in captive breeding pairs of RRH.

Based on this background information, I hypothesized that the introduction of female and male urine pheromones to male-female pairs of RRH will stimulate the onset of or regulate estrous cycles in females and increase T in males both of which could lead

to successful breeding in previously abstinent animals. Introduction of male urine pheromones will induce territorial behavior in males with low libido because the pheromones will stimulate T synthesis (Kempenaers et al., 2008; Wingfield, 1985). The introduced male urine pheromones, along with the production of additional pheromones from the captive male, will together create the “male effect”, causing induction of estrous cycles or improved regularity of estrous cycles in females. Female urine pheromones introduced into the environment will simulate new conspecifics in the environment, potentially aiding in estrus synchronicity in captive females paired with males. Female pheromones may also increase male libido via an increase in T production. The induction or regulation of estrous cycling and improvement in male libido could induce genetically viable pairs to breed which would eliminate the need for repeated and costly translocation and re-pairing of non-breeding animals.

2. LITERATURE REVIEW

2.1 Introduction

The scientific knowledge and application of pheromones have expanded considerably since their discovery in 1959 by Karlson and Lüscher (Karlson and Lüscher, 1959). Pheromones are chemicals or blends of substances secreted by mammals and used in chemical communication to elicit specific behavioral or physiological responses (Vandebergh, 1983). In recent decades, pheromones have been discovered to play a vital role in mammalian reproduction (Brennan and Keverne, 2004; Wyatt, 2003). Behaviorally, pheromones transmit information about the secreting animal, such as sexual identity, sexual cycles, state of arousal, age and reproductive status (Dehnhard, 2011; Heath, 2014). Pheromones also can induce physiological responses such as estrous cycling and synchronization, reproductive suppression, and increase in libido (Dehnhard, 2011; Whitten, 1958). Pheromones have aided captive breeding initiatives through their application to induce estrus synchronization or influence mate selection to create appropriate mating conditions or to optimize conditions for artificial reproductive techniques (ART) (Andrabi and Maxwell, 2007). With respect to domestic animals, Rowell et al. (2003) demonstrated the ability to synchronize estrus in cows via introduction of a novel bull. Similarly, it has been demonstrated that estrous synchrony occurs in African elephants in response to pheromone exposure (Weissenbock et al., 2009). In a study by Roberts & Gosling (2004), introduction of a competitor odor to female mice increased their attraction to an "unattractive" male. Introduction of conspecific odors in the giant panda has been

shown to elicit dramatic increases in chemoresponsiveness (Swaisgood et al., 2000) and manipulation of the complex chemical communication system of the giant panda has contributed to captive mating success in this species (Wei et al., 2015). Although the utility of pheromones to naturally induce breeding in captive species is well documented, the majority of zoological institutions utilize more invasive methods to synchronize estrus or increase libido in captive mammals (Comizzoli et al., 2000; Dehnhard, 2011). Administration of exogenous substances such as oral Altrenogest, a progestin analog, in bottlenose dolphin (Robeck et al., 2009) and killer whale (Robeck et al., 2004), human chorionic gonadotropin (hCG) in Persian leopard (Dresser et al., 1982) and ferrets (Howard et al., 1991), or intravaginal progesterone-releasing devices in scimitar-horned oryx (Morrow et al., 2000) and Mohor gazelle (Holt et al., 1996) are some of the methods to induce estrus in female animals in preparation for ART protocols. Though some of these procedures have led to successful births, the long-term effects of administering these exogenous substances has yet to be fully understood.

The majority of captive breeding initiatives are rooted in the desire to supplement a wild population or conserve the individuals left in the wild (Frankham et al., 2002). Keeping captive conditions as similar as possible to the natural environment is vital to the maintenance of animal populations that mimic their wild counterparts. The use of natural methods to breed captive animals is an important aspect of these programs. Even though our current understanding of the detailed mechanisms of pheromones remains incomplete, there have been some promising examples of the application of pheromones to naturally create optimal breeding conditions. However, to our knowledge, the

application of pheromones to the manipulation of reproductive function in captive wildlife species remains relatively limited.

The Red River Hog (RRH; *Potamochoaerus porcus*), a common species found in North American zoological collections, has variable breeding success in captivity. Inbreeding and the skewing of founder lines have become a concern in the captive population of this species, which has lead to numerous new breeding pair recommendations in recent years (Holland et al., 2014). Even after re-pairing males and females in response to breeding recommendations from the RRH SSP, the percentages of breeding and non-breeding animals remain the same (Holland, 2013). Little research has been done on the reproductive physiology of RRH and the current practices of re-pairing underrepresented individuals have been minimally effective (Holland, 2013).

Based on advances in the biological understanding and potential applications of pheromones, this study explored the usefulness of pheromones as a method to regulate estrous cyclicity in female RRH and induce breeding in RRH pairs in North American zoos.

2.2 PHEROMONES

2.2.1 Background

Olfaction is critical to animal communication (Doty, 2012). In the 1970's, concrete scientific evidence solidified the role of hormones in the olfactory modulation of behavior (Doty, 1976). Since that time, much of the research on the role of hormones

in olfactory communication has concentrated on how olfactory-based hormones influence reproductive behavior (Doty, 2012). During the early years of research on olfactory communication, substances which convey information within the body or to other conspecifics were termed "hormones" (Karlson and Lüscher, 1959; Vandebergh, 1983). The distinction between circulating hormones (such as LH and FSH) and excreted pheromones was initially made in 1959 (Karlson and Lüscher, 1959). Karlson and Lüscher delineated the broad category of hormones into hormone-like substances secreted into the blood for transmission and activation of processes within the body and those substances excreted from the body (via urine, saliva, feces, or scent glands) to elicit responses from specific recipients (Karlson and Lüscher, 1959; Vandebergh, 1983).

2.2.2 Signaling versus Primer Pheromones

Pheromones are used in intra-specific communication to cause innate reactions in conspecifics that will ultimately affect species-wide behavioral patterns (Dehnhard, 2011). Two types of pheromones have been described based on their modes of action. Signaling pheromones (also termed "releaser" pheromones) are those which lead to prompt behavioral responses, such as sniffing, licking, genital investigation, flehmen response, and various courtship behaviors (Vandebergh, 1983). These types of pheromones convey information such as age, reproductive status, gender, dominance, and nutrition (Dehnhard, 2011). Primer pheromones lead to physiological changes in the

recipient which produce long-term effects. Examples of the effects of primer pheromones include sperm production in fish, regulation of puberty and the ovarian cycle in mammals, pregnancy blocking, and termite caste determination (Vandebergh, 1983; Wyatt, 2003). Often mammals use a combination of both signaling and primer pheromones to elicit desired behavioral and physiologic effects in conspecifics.

2.2.3 Pheromones Pathways

Pheromones, whether signaling or primer are transported via body secretions (Wyatt, 2003). Pheromone producing glands vary from species to species and include salivary glands, preorbital glands, the kidneys, ureter, urethra, bladder, male accessory glands, and the rectum among others (Wyatt, 2003). A recipient conspecific detects the pheromone either through the main olfactory epithelium (MOE) in the nose which transmits the signal to the olfactory bulb in the brain or via the vomeronasal organ (VNO) in the nasal hard palate (Heath, 2014; Wyatt, 2003). Compared to signals transmitted via the MOE, the VNO has a significantly lower detection threshold, which indicates a high sensitivity to individual small molecule pheromones (Wyatt, 2003). Recent studies that compare the MOE to the VNO suggest that the VNO is specific for pheromones related to reproduction, though the complicated endocrine mechanisms involved are not understood fully (Wyatt, 2003).

The VNO is present in various forms depending on the species and transmits sensory information in a mucous stream in response to stimuli at the level of the

accessory olfactory bulb (AOB), the medial amygdala, and the centromedial hypothalamus (Boehm et al., 2005; Dehnhard, 2011; Heath, 2014; Wyatt, 2003). The VNO accessory olfactory pathway is necessary for the primer pheromone pathway, because primer pheromones stimulate limbic structures involved in sexual and neuroendocrine regulation (Keverne, 1983). As an example, in mice lesions in the VNO or AOB prevent pheromonal effects such as estrus induction or implantation blockage (Brennan and Keverne, 2004; Keverne, 1983). As pheromones have effects in the hypothalamus and can alter patterns of estrous cycling in females and reproductive behavior in males, it is likely they have direct effects on the anterior pituitary and subsequent release of Lutenizing Hormone (LH) and/or Follicle Stimulating Hormone (FSH) (Keverne, 1983; Kleiman et al., 2010).

2.2.4 Pheromones and the Endocrine System

LH and FSH, which are released from the anterior pituitary gland in response to hypothalamic gonadotropin releasing hormone (GnRH), create distinct hormone profiles during various periods of an animal's life (Senger, 1997). These reproductive hormones are responsible for activation and growth of reproductive characteristics during puberty, spermatogenesis and androgen production in males, and the ovarian cycle in females (Kleiman et al., 2010; Senger, 1997). The timing and production of these hormones vary between species, though the underlying mechanism remains very similar (Senger, 1997). In males, FSH acts on Sertoli cells in the testes to promote spermatogenesis and LH

works to stimulate androgen production in the Leydig cells (Kleiman et al., 2010). Circulating androgens remain at relatively stable levels and are required for maintenance of spermatogenesis, accessory sex glands, secondary sex characteristics, sebaceous glands, and libido (Kleiman et al., 2010).

In females, the ovarian cycle has two phases, the luteal phase and the follicular phase. During the follicular phase, ovarian antral follicles undergo growth of the antrum and development of theca and granulosa cells followed by rupture and release of an oocyte during ovulation. FSH stimulates follicular growth and, along with LH, causes maturation of the ovarian follicles, oocytes and cumulus cells, and changes in follicular steroidogenesis (Kleiman et al., 2010). After ovulation due to a surge of LH, a ruptured ovarian follicle is converted to a corpus luteum (CL), which is characterized by synthesis and secretion of high levels of progesterone from luteinized granulosa and theca cells (Kleiman et al., 2010).

Following ovulation and the formation of a CL, species such as primates and bats undergo a menstrual phase, which is characterized by relatively low circulating levels of steroid hormones (Kleiman et al., 2010). Other species undergo anovulatory or anestrus periods of the estrous cycle which also are associated with low circulating levels of ovarian steroid hormones (Kleiman et al., 2010). These hormonal-steroidal interactions are vital for reproductive processes to proceed and for successful pregnancy and parturition. The fine-tuned nature of these physiologic processes can be disrupted by factors such as social or environmental factors. Therefore, the management of animal

breeding and reproduction requires a substantial understanding of the interaction of pheromones, the central nervous system, and the reproductive organs.

2.3 ANIMALS IN CAPTIVITY

2.3.1 Captive Breeding

The maintenance of zoological collections is complicated and requires management of husbandry, diet, social environment, habitat, general health, and reproductive health (Tribe and Booth, 2003). Although often viewed as a source for entertainment, modern zoological institutions have shifted their focus from entertainment of the public to global conservation of imperiled species through education, research, and conservation initiatives (Carr and Cohen, 2011). The welfare of captive animals has improved vastly in recent years. Increasingly, it is understood that both the physical and psychological needs of captive animals must be met to maintain a good quality of life in captivity (Kleiman et al., 2010). Nutrition, behavior, habitat, and reproductive success are some of the aspects taken into consideration when dealing with maintenance of captive species. Organizations such as the Association of Zoos and Aquariums (AZA) in the United States put stringent guidelines in place to ensure optimal animal care. Creating an environment that mimics the wild requires a thorough knowledge of a species' basic life history, as well as knowledge of mammalian social organization, mating systems, and communication (Kleiman et al., 2010).

There are many reasons to maintain captive populations of species. Some captive populations act as a reservoir population for their endangered wild counterparts. The captive breeding of these species has become a vital component for the reintroduction of such species into the wild or augmentation of existing wild populations (Gordon and Gill, 1993; Swaisgood and Schulte, 2010). Long-term display and propagation of species also allows for scientific, educational, and entertainment benefits to humans (Penfold et al., 2014). Captive species also serve as ambassadors for their wild counterparts, creating a platform for education about international species conservation initiatives (Carr and Cohen, 2011; Swaisgood and Schulte, 2010). Many factors, such as high cost, lack of proper management, and sociopolitical influences, can be detrimental to the success of a captive breeding program and must be taken into consideration when designing such a program (Conde et al., 2011; Gibbons et al., 1995).

2.3.2 Creation and Maintenance of a Captive Population

The goals of bringing a species into captivity are to increase the captive population size quickly to a set carrying capacity and to retain founder genetic diversity over time (Kleiman et al., 2010). According to Frankham, et al. (2002), captive breeding and reintroduction can be defined by a six stage process which includes founding a captive population, expanding a population to the appropriate size, and maintaining the genetics of a population over many generations. Achievement of these goals is dependent on the creation of a population with appropriate age and sex ratios for

reproduction and must take into consideration the potential for loss of genetic diversity, inbreeding, and the need for adaptation of a species to captivity (Frankham et al., 2002; Kleiman et al., 2010).

When designing a captive breeding program for a species it is essential to consider the demographics of the species and the need for genetic management of the species through reproductive manipulation (Gibbons et al., 1995). Captive populations are small compared to wild populations and thus have a high risk of genetic heterozygosity which can lead to inbreeding and loss of genetic diversity (Kleiman et al., 2010). The risk for inbreeding and loss of genetic diversity in captive species can be further exacerbated depending on the specific mating system or mate choice displayed in a given species (Quader, 2005; Shuster and Wade, 2003). The mating system of a species is determined by the genetic relationships between mates and/or the number of mates per sex (Shuster and Wade, 2003; Swaisgood and Schulte, 2010). For some species, sex ratios in the wild tend to be skewed towards one sex (often female) to account for unequal investment in offspring (Wyatt, 2003). Skewed sex ratios lead to sexual competition and the evolution of mechanisms to indicate attributes such as good health, adequate resources, and enhanced survival ability (Wyatt, 2003). Some mate choice attributes may be conspicuous (i.e., bright coloration or available resources), but growing evidence demonstrates that pheromonal cues may communicate the overall health and success of a competing individual (Wyatt, 2003). Mate competition is often the driving factor behind successful breeding within species (Kleiman et al., 2010). Often, females have “selection thresholds” that require males to have an array of criteria

above an arbitrary threshold to be deemed suitable mates (Kleiman et al., 2010). If all the males in the available population fall below a given female's "selection threshold", cessation of breeding may occur (Kleiman et al., 2010). On the other hand, if a single male in the population is above the "selection threshold", genetics within the population may skew in favor of the preferred male (Kleiman et al., 2010). In the wild, large population sizes can support these evolutionary mechanisms and maintain a genetically viable population. In captive populations, which are much smaller than their wild counterparts, mating systems and mate choice have much greater impacts on genetic diversity and effective population sizes (Kleiman et al., 2010).

It is well-established that a positive correlation exists between population size and genetic diversity, with smaller populations facing a high risk of extinction due to factors such as genetic drift and inbreeding (Ogle, 2010). With respect to captive breeding for long-term display and propagation, maintenance of genetic diversity is important to the preservation of reproductive potential and survival (Frankham et al., 2002). Additionally, many captive breeding programs are designed specifically for endangered species with the intention of creating "reservoir" populations that may be eventually released to the wild (Frankham et al., 2002). Loss of genetic diversity risks the ability of these populations to adapt and evolve in response to environmental changes, thus affecting long-term sustainability (Frankham et al., 2002).

When considering the genetics of a captive population, effective population size (N_e) is critical for management of genetic diversity (Frankham et al., 2002). Effective population size refers to the individuals in a population that are able to breed and

contribute genetically to the future generations (Frankham et al., 2002; Ogle, 2010). For a wild population, short-term sustainability requires a $N_e > 50$, but for long-term maintenance of evolutionary potential a $N_e>500$ is required (Frankham et al., 2002). In captive populations, there is not enough space to maintain 500 individuals over a long period of time. However, the ability to select individuals for breeding with viable genetics enables maintenance of genetic diversity in captive populations despite such populations having fewer than the required number of individuals need for maintenance of evolutionary potential.

Creating a successful captive breeding program begins with selection of wild-caught founder numbers (Frankham et al., 2002). Ideally, the recommended minimum founding population would consist of 20-30 individuals (Frankham et al., 2002). This seemingly large number takes into account that not all wild-caught individuals will breed and that the proportion of heterozygosity retained after a single-generation bottleneck in those that do breed decreases with an increased number of contributing founders [$1 – 1/2N_e$] (Frankham et al., 2002). From a given founding population, rapid expansion to a defined target population takes precedence initially over genetic management (Frankham et al., 2002). Once the desired population size is achieved, the "maintenance phase" of a breeding program is focused on genetic management (Frankham et al., 2002). Genetic management prioritizes minimization of inbreeding, loss of genetic diversity, and protection from deleterious adaptations to captivity (Frankham et al., 2002). The majority of zoological institutions aim to minimize mean kinship to maintain genetic diversity in their captive populations (Frankham et al., 2002; Kleiman et al., 2010).

Proper genetic management of captive animals requires stringent monitoring of pedigrees and the creation of elaborate mating schemes designed to delay inbreeding and to maintain equal founder contribution throughout the population (Frankham et al., 2002).

In current zoological populations, contributing founder numbers tend to be much lower than 20-30 individuals (Witzenberger and Hochkirch, 2011), which emphasizes the need for optimal genetic management of these populations. Cooperative programs such as the Species Survival Plan (SSP), International Species Inventory System (ISIS), Animal Record Keeping System (ARKS), Single Population Analysis and Record Keeping System (SPARKS), and Medical Animal Record Keeping System (MEDARKS) have been created to keep track of information on individual animals in captive populations such as individual life histories, pedigrees, breeding history, and medical records (Ballou et al., 2010; Carr and Cohen, 2011; Tribe and Booth, 2003). These cooperative animal management programs and the various zoological institutions they support work together to manage the long term genetic viability of captive populations of animals (Lees and Wilcken, 2009; Tribe and Booth, 2003). Preferential breeding of specific individuals is required to maintain high genetic heterozygosity and effective population sizes (Ballou et al., 2010). Often translocation of individual animals from one institution to another is necessary to pair two genetically viable animals for the ultimate goal of maintenance of the genetic diversity of the population (Ballou et al., 2010; Fischer and Lindenmayer, 2000). In captivity, the introduction of a novel individual to a potential mate may result in successful breeding between the pair.

However, often captive breeding attempts are unsuccessful and a more complicated protocol to induce reproductive behaviors or result in a successful pregnancy is required (Comizzoli et al., 2000).

2.3.3 Overcoming Issues with Captive Breeding

Over the years, various ART procedures have yielded successful propagation of captive species (Andrabi and Maxwell, 2007; Comizzoli et al., 2000). However, the creation of a successful ART protocol for a given species is a slow process due to the species-specific nature of various aspects of reproduction such as seasonality, gamete physiology, and estrous cycle (Andrabi and Maxwell, 2007). In the majority of wildlife species basic reproductive biology is not understood fully, which limits the ability to develop ART protocols to assist with captive breeding (Andrabi and Maxwell, 2007).

ART have yielded successful pregnancies in numerous species via artificial insemination (AI), embryo transfer (ET), in vitro fertilization (IVF), gamete/embryo micromanipulation, semen/embryo sexing, and genome resource banking (GRB) (Andrabi and Maxwell, 2007). AI, which is the least invasive ART procedure, (Durrant, 2009), requires semen collection and analysis, selection of optimal semen samples, induction of ovulation, and ultimately deposit of the selected semen in the optimized female reproductive tract (Andrabi and Maxwell, 2007; Durrant, 2009). Due to the high cost and the fact that ART procedures often require anesthesia and intramuscular injections of exogenous hormones to induce ovulation (Andrabi and Maxwell, 2007),

other less invasive and costly means to modulate breeding in captive animals would be advantageous.

Numerous methods and substances have been utilized to induce ovulation in females (Andrabi and Maxwell, 2007; Comizzoli et al., 2000; Pukazhenthi and Wildt, 2003). Hormonal agents such as hCG or deslorelin are administered intramuscularly during specific time points in the estrous cycle to promote ovulation of ovarian follicles (Gomes et al., 2014). When utilized for short term induction of ovulation, these pharmaceuticals are effective and safe. However, the long-term effects of these agents have recently been linked to reproductive pathology in numerous species (Moresco and Agnew, 2013; Moresco et al., 2009; Munson et al., 2002; Munson et al., 2005). Extensive studies in zoo felids have indicated a positive correlation between the risk of developing reproductive diseases (i.e. endometrial hyperplasia, fibrosis, uterine neoplasia) and exposure to progestin contraceptives (Moresco and Agnew, 2013). Similar findings in zoo canids demonstrated a link between chronic exposure to progestins or GnRH agonists and reproductive pathology in females (Moresco and Agnew, 2013). Retrospective studies of the relationship between short-term and long-term use of such drugs and reproductive tract pathology are ongoing. However, as many ART protocols are currently being developed for numerous species, it is essential to determine the effect that short term exposure to such drugs may have on long-term reproductive health. Discovery of natural methods for the control of estrous cycles and reproductive behavior in captive animals would aid in the maintenance of overall and reproductive health in such animals.

2.3.4 Pheromones as a Natural Alternative

Numerous effects of pheromones have been documented in a variety of mammalian species (Delgadillo et al., 2009; Swaisgood et al., 2000; Whitten, 1958). The "male effect", or male-induced ovulation initially was reported in sheep and goats, and has been utilized in agricultural industries to control reproduction using a "clean, green, and ethical" method (Delgadillo et al., 2009; Martin and Kadokawa, 2006). Male induced ovulation involves the sudden introduction of novel males to induce anestrus females to ovulate (Pellicer-Rubio et al., 2016). This method can interrupt seasonal anestrus or shorten post-partum anestrus to increase reproduction rates in sheep, goats, and cattle (Martin and Kadokawa, 2006; Pellicer-Rubio et al., 2016).

First observed by Whitten in mice, the "Whitten effect" refers to the synchronization of estrous cycles among grouped females via male pheromone-laden urine (Whitten, 1958). This effect also occurs in rats and is linked to estrous synchronization which occurs in numerous domestic and exotic species (Vandebergh, 1983). Species such as African elephants, canids, and various ungulate species undergo estrous synchrony as well (Hradecky, 1985; Tirindelli et al., 2009; Weissenbock et al., 2009). Depending on the social system of the species, the selective advantage of estrus synchrony may cause adaptive synchrony of births and/or correspond with seasonal resource availability (Weissenbock et al., 2009). Pheromones have been linked to many reproductive phenomena such as estrous cycle suppression, regulation of puberty, and pregnancy failure (Vandebergh, 1983). Unfortunately, knowledge of female reproductive physiology and anatomy is scant in many wildlife species, which makes it

difficult to design natural breeding husbandry techniques, develop protocols for pheromone modulation of breeding, and design ART procedures, such as AI, for wildlife species (Andrabi and Maxwell, 2007).

Development of ART protocols requires a comprehensive knowledge of female reproductive physiology and anatomy and induction or synchronization of ovulation is an essential component of the development of ART protocols (Andrabi and Maxwell, 2007). Non-invasive hormone monitoring of fecal steroid metabolites is essential to determine the stage of the estrous cycle in females (Berger et al., 2006; Schwarzenberger et al., 1996). Non-invasive endocrine hormone measurement has been conducted in many captive suid species such as the red river hog, babirusa, and warthog (Berger et al., 2006). Such endocrine monitoring techniques using fecal or urine steroid metabolites have expanded the reproductive knowledge of many species, but the ability to manipulate reproductive endocrine mechanisms is necessary for the development of ART protocols. Although ART protocols have been developed for a variety of species, sometimes the drugs utilized in ART cause deleterious long-term effects on general and reproductive health (Moresco and Agnew, 2013; Pukazhenthi and Wildt, 2003). Development of non-invasive methods to modulate reproductive biology and behavior is warranted.

Pheromones have been used to modulate reproductive physiology and improve breeding success in some species (Pageat and Gaultier, 2003; Rekwot et al., 2001; Swaisgood et al., 2000). In the giant panda, conspecific odors have been shown to elicit dramatic increases in chemoresponsiveness and application of the giant panda's complex

chemical communication system has contributed to captive mating success (Swaisgood et al., 2000; Wei et al., 2015). The Order Carnivora has the greatest variety of pheromone-secreting glands in the skin and mucous membranes and, therefore, the application of pheromones to the modulation of behavior in these species is widespread (Pageat and Gaultier, 2003). In domestic cats and dogs, use of pheromone analogues has been successful in the treatment of behavioral disorders and the reduction of stress (Pageat and Gaultier, 2003). In agriculture, the movement toward animal production methods without the use of hormones and other pharmaceutical compounds has encouraged research into the application of non-invasive methods such as the "male effect" in females (Pellicer-Rubio et al., 2016). Introduction of a novel bull synchronizes estrus in groups of cows and similar techniques have been utilized in sheep (Martin and Kadokawa, 2006; Rowell et al., 2003). In domestic swine, boar pheromones are critical to successful breeding in sows and gilts (Rekwot et al., 2001). Boar pheromones are secreted primarily in saliva from the submandibular salivary gland or concentrated in the urine (Rekwot et al., 2001). Presence of boar pheromones accelerates induction of estrus and onset of puberty in gilts by about 30 days (Brooks and Cole, 1970; Rekwot et al., 2001). Furthermore, introduction of boars to groups of gilts results in induction of estrus, estrus behavior, and synchrony of estrus in sows and gilts (Brooks and Cole, 1970). These insights have been vital to manipulation of reproduction in production animals (Delcroix et al., 1990; Dorries et al., 1991). As RRH are related to domestic swine (Gongora et al., 2011), pheromones have the potential to be useful to manipulation of reproduction in captive breeding pairs of RRH.

2.4 THE RED RIVER HOG

2.4.1 Background

Red river hogs (RRH) (*Potamochoerus porcus*) are a widely distributed and non-threatened Afrotropical suid species found in thickly wooded areas, swamps, and forests in equatorial West Africa (Berger et al., 2006). The smallest of the Afrotropical suids, RRH have short, dense orange or reddish-brown pelage along their entire body, with a dorsal crest of long white hair extending along the spine and partially down a long, tapered tail which ends in a black tuft (Berger et al., 2006; Leslie and Huffman, 2015). Adults have a unique facial mask of bright white hairs encircling the eyes and jaw line with a black snout, and leaf-shaped ears with extended termini ending in long tufts of white or black hairs (Leslie and Huffman, 2015). Adult males are distinguished by long white whiskers which emphasize inflated mandibular and suborbital ridges and cone-shaped bulges on either side of the muzzle (Leslie and Huffman, 2015). These omnivorous mammals have a diet that consists of roots, bulbs, herbs, fruit, grass, eggs, small invertebrates, and dead plant and animal material (Leslie and Huffman, 2015).

In their native habitat, RRH form territorial family groups consisting of a dominant male and a group of females and their offspring, though they have also been known to aggregate to form larger sounders (Berger et al., 2006; Dayrell and Pullen, 2003). Both male and female RRH are fully grown at two years old and sexually mature at three years old (Leslie and Huffman, 2015). In captivity, females have been reported to give birth as early as 22 months of age, indicating more rapid sexual maturity in captivity (Leslie and Huffman, 2015). Both in captivity and the wild RRH are seasonal

breeders, with births in the wild occurring between the end of the dry season and the first part of the rainy season (February-July) (Leslie and Huffman, 2015). While there are records of captive females giving birth year-round, the majority of reproduction occurs during set times (North America: March-August; United Kingdom: June-October) (Leslie and Huffman, 2015). Through the use of fecal steroid hormone analysis in female RRH in European zoos, Berger et al (2006) reported an estrous cycle that typically lasts 34-37 days (Leslie and Huffman, 2015). Gestation in RRH is approximately 120-127 days, with litter sizes ranging from 1-6 piglets (Leslie and Huffman, 2015).

2.4.2 In Captivity

First exhibited at the London Zoo in 1852, the RRH had a sporadic presence in western zoos until the 1990s (Leslie and Huffman, 2015). Since that time, the species has become common in zoo collections due to its charismatic appearance, high activity level, and capability to form mixed-species exhibits (Leslie and Huffman, 2015). According to the 2014 RRH SSP, the current Association of Zoos and Aquariums (AZA) population of RRH consists of 201 animals (96 males and 105 females) with a genetic diversity of 84.54 % (Holland et al., 2014). Age structure and sex ratios remain relatively evenly distributed with a projected growth rate of 13.5 % per year (Holland et al., 2014). Considering that the median life expectancy is 12.1 year for males and 13.9 years for females and that current population birth rates exceed the suggested 12 births

per year to maintain the target population size (190 individuals), it is extremely important to prioritize space to breed RRH whose genetics are underrepresented in the captive North American population (Holland et al., 2014).

The captive AZA population of RRH is descended from eight founders and the current trajectory predicts a gene diversity of 69 % in less than 100 years (Holland et al., 2014). Much of this predicted loss of genetic diversity in the AZA population of RRH is due to the skewing of the genetic contributions from founder animals (Holland et al., 2014). Such skewing in the genetic diversity of the population has resulted in an average mean kinship of 0.1546 within the AZA population, which equates to an average relationship being greater than that of half-siblings (Holland et al., 2014). While much effort has been employed to decrease average mean kinship through the judicious re-pairing of RRH males and females of valuable and unrelated genetic backgrounds, inbreeding remains a concern (Holland et al., 2014). With such a small population produced from eight founders, inbreeding depression could lead to detrimental factors such as small litter sizes, low birth weights, and high mortality rates (Frankham et al., 2002). By equalizing founder contributions to the current population, the RRH SSP aims for 90% gene diversity retention for 100 years (Holland et al., 2014).

To maintain gene diversity, the AZA RRH population requires adequate space to breed underrepresented animals, which often involves translocating animals to different zoos to pair genetically valuable animals (Fischer and Lindenmayer, 2000; Holland et al., 2014). The process of being crated, shipped across the country, and introduced into a completely unfamiliar environment is very stressful for such animals (Dickens et al.,

2010; Fischer and Lindenmayer, 2000). The stress of relocation can affect all aspects of an animal's physiology, such as appetite, temperament, behavior, and reproductive function (Dickens et al., 2010). Relocation of animals is also expensive for all institutions involved and is associated with risks, such as illness and even the death of such animals. Unfortunately, many of the breeding pairs of RRH in the zoo population have low reproductive success (Holland, 2013). In 2014, of the recommended 40 breeder females, only seven were considered likely to reproduce (Holland, 2013). The RRH SSP recommends re-pairing of animals if genetically viable individuals do not breed within three years of being paired together (Holland et al., 2014). Another option to improve genetic diversity in the captive RRH population is importation of unrelated individuals. However, this approach is controversial with respect to wildlife conservation and is challenging to implement due to the risk of foreign animal diseases, such as African swine fever virus (Anderson et al., 1998), Zaire ebolavirus (Kobinger et al., 2011), and foot-and-mouth disease (Arzt et al., 2011). The reasons for the low reproductive success in captive RRH are unknown and there is little known about basic RRH reproductive endocrinology.

2.4.3 Issues and Solutions

Although depressed reproductive rates in captive wildlife species could be due to a number of environmental stressors, few stereotypical behaviors indicative of stress have been observed in conjunction with abnormal reproduction (Swaisgood and

Shepherdson, 2005). Therefore, the dogma within the wildlife reproduction research field states that reproductive failure in captive wildlife species is likely a physiological factor due to the "roommate effect" (Lindburg and Fitch-Snyder, 1994). The "roommate effect" occurs in individuals paired together that have become accustomed to one another (Lindburg and Fitch-Snyder, 1994). In such pairings of non-domestic suid species, chronic exposure to the same boar/sow pheromones may prevent the appropriate effects in the conspecific recipients (Lindburg and Fitch-Snyder, 1994). Although the presence of a boar has been shown to induce estrus in female domestic pigs, this effect appears to only occur under situations in which a limited amount of boar exposure occurs; continual exposure to a boar can lead to habituation and lack of estrus induction (Tilbrook and Hemsworth, 1990). In domestic pigs, gilts housed next to boars are habituated to boar stimuli which causes a reduction in detection of estrus using the "back-pressure test" (Soede, 1993; Tilbrook and Hemsworth, 1990). As domestic swine are related to wild suid species, it is presumed that the "roommate effect" can also occur in non-domestic suid species. Therefore, the cause of poor breeding success in captive RRH may be due to habituation.

To combat the declining genetic diversity in the RRH, it is necessary to mate the non-breeding, genetically viable pairs of RRH. However, once RRH are re-paired, it is possible that chronic exposure to the new mate could result in habituation, significantly lowering the chance of viable offspring. Aside from the re-pairing of animals, AI could be a useful method to maintain genetic diversity in the captive RRH population. However, an AI protocol for RRH has yet to be delineated as further understanding of

male and female RRH reproductive physiology is required prior to its development. It is possible that application of RRH pheromones could help to modulate and improve reproductive success in RRH.

2.5 THE GLOBAL SCALE

In addition to the importance of animal welfare, in recent decades zoos have emphasized the roles of conservation education and research in their mission (Carr and Cohen, 2011). Zoo animal research focuses on many scientific areas, including reproductive biology and captive breeding initiatives. Dwindling habitats due to human encroachment remain the largest threat to global biodiversity, requiring extensive *in situ* conservation actions including expansion of protected areas (Conde et al., 2011). Unfortunately, some populations of species have reached critical values in the wild and thus captive breeding has become a short-term, practical option to aid in their conservation (Conde et al., 2011). According to IUCN, in recent years captive breeding programs have played a role in the reduction of the threat level of 17 of 68 species (Conde et al., 2011; Hoffmann et al., 2010). Regardless of these successes, captive breeding remains a complicated multidisciplinary approach. Many issues such as cost, technical and equipment needs, and sociopolitical factors must be taken into consideration in the success of a captive breeding program (Conde et al., 2011; Gibbons et al., 1995).

In addition to the many logistical factors associated with captive breeding, working with endangered species is even more complicated due to the bureaucratic rules and regulations surrounding these species. The conduction of novel studies or development of ART protocols for endangered animals is not always feasible. Thus, conduction of studies in species that are taxonomically related to endangered species is often a first step to building potential methods for successful captive breeding in species more difficult to work with due to value, limited numbers, or difficulty of access (Caro and O'doherty, 1999).

The RRH (*Potamochoerus porcus*), though not an endangered species, is closely related to many other species in the Suidae family that are on the IUCN Red List of Threatened Species (Gongora et al., 2011; IUCN, 2017). Threatened or endangered species such as the Babirusa (*Babyrousa babyrussa*), Pygmy Hog (*Porcula salvania*), and Javan Warty Pig (*Sus verrucosus*) are closely related to the RRH and undergo similar reproductive difficulty in captivity. Protocols and non-invasive techniques to promote successful breeding in the RRH could then be applied to promote reproductive success amongst captive animals belonging to related, more imperiled species.

2.6 SUMMARY

The use of pheromones to stimulate successful reproduction in captive mammals is a relatively new idea (Dehnhard, 2011). However, there are promising results from studies of the reproductive effects of pheromones in giant panda (Swaisgood et al.,

2000), goats (Pellicer-Rubio et al., 2016), and sheep (Delgadillo et al., 2009). The capability of pheromones to induce or alter certain physiological aspects of mammalian reproductive behavior has been described in the past and new research in domestic ungulate species further promotes the use of pheromones for reproductive modulation in sheep and cows (Rekwot et al., 2001). The link between the VNO and the hypothalamic-pituitary axis outlines a potential pathway for the direct stimulation of steroid production and indirect increase in libido in males and of estrous cycling in females in response to pheromone cues (Tirindelli et al., 2009). Reproduction is dependent on the production of steroids, like P4, necessary for pregnancy (Senger, 1997). Monitoring the fecal steroid levels of paired animals is a non-invasive method to detect potential reproductive difficulties in males and females (Bryant et al., 2016).

As pheromones are useful in the closely related domestic swine species, introduction of pheromones to non-breeding pairs of RRH could be a promising new method to induce estrous cycling and increase testosterone levels to lead to successful breeding of genetically viable pairs of RRH. The use of pheromones would be a natural, non-invasive method to promote the optimal endocrine conditions necessary for mating behaviors to yield successful pregnancies.

3. MATERIALS AND METHODS

3.1 ANIMALS AND FECAL SAMPLE COLLECTION

All sample collection and introduction of urine was conducted under an animal care and use protocol from University of Illinois at Urbana-Champaign and through approval by each zoological institution's animal care and use committees.

Animals in this study included 10 sexually mature male and 11 sexually mature female RRH at nine AZA institutions in North America. Animals were compared among three treatment groups: proven-breeder male-female pairs (control; male n=3; female n=4), females with poor breeding history repaired to a new male (new male; male n=4; female n=4), and male-female pairs with poor breeding history that remained paired and were exposed to sow and boar pheromones (non-breeding pheromone; male n=3; female n=3) (Table A-1). To document different geographical locations, each zoo was categorized by the following: North (N; n=4), Midwest (MW; n=1), South (S; n=3), and West (W; n=1) (Table A-1).

Over the course of a year, fecal samples were collected from each animal in the study 3-5 times per week by zoo keeper staff. Keepers were instructed to collect a morning fecal sample that was freshly voided and to store it as soon as possible in a -20 °C freezer until shipment to the lab. Samples were shipped to the lab overnight on ice packs and immediately transferred upon receipt to a -20 °C freezer until extraction and analysis. To differentiate between male and female fecal samples, keepers added food dye to the food ration of a single member of a given pair of RRH to color that individual's feces.

Animals in the pheromone group had a baseline fecal sampling period of approximately six months. To ensure desensitization to enrichment tubes in which pheromones were introduced, empty tubes were placed in exhibits two weeks prior to the onset of urine introduction. After the two-week desensitization period, as a source of female pheromones urine collected from 3 harem RRH females from Birmingham Zoo was introduced into the habitat via urine-soaked hay or fabric placed into the conical enrichment tube for a two and a half month period. Fresh urine was collected and shipped overnight on ice packs to the zoos in the pheromone treatment group every two weeks. Once the fresh urine arrived, the old urine sample from the enrichment tube was switched out in favor of the new urine sample. Due to the logistics of collecting and shipping fresh urine from zoos for many consecutive months, fresh urine was only introduced to each enclosure in the pheromone treatment group every two weeks. Following the two and a half month period of exposure to female urine, there was a washout period of one month wherein the enrichment tube was in the exhibit with no sample inside of it. Thereafter, male urine from a boar from Disney's Animal Kingdom Lodge was introduced into the habitat via urine-soaked hay or fabric placed into the conical enrichment tube for a two and a half month period (Figure A-1). Collection of urine from male RRH is difficult and as most zoos only house one male with several females, our source for male pheromones was a single boar. Three zoos were contacted about the collection of boar urine. While all of the zoos contacted tried to collect urine from their male RRHs, only one, Disney's Animal Kingdom Lodge, was successful. Urine was collected easily in the soil from a group of female RRH at Birmingham Zoo.

For enrollment into the pheromone study six zoos were contacted, with only three of these zoos agreeing to participate: Safari West, Dallas Zoo, and Virginia Zoo. Reasons for not enrolling in the study included: insufficient keeper time to participate and concerns about biosecurity and disease transmission with the introduction of urine from another animal. Therefore, as only three pheromone zoos could be recruited to participate in this study, female urine was introduced first (2.5 months), followed by a washout period (1 month), and then the introduction of male urine (2.5 months) (Figure A-1). A one month washout period was selected as this is the approximate length of the estrous cycle in this species (Leslie and Huffman, 2015). Exact dates for urine introduction for each zoo are described in Table A-2.

3.2 FECAL STEROID EXTRACTION

Frozen feces were lyophilized to remove moisture, pulverized, and stored in a parafilm sealed, airtight conical at -20°C until steroid extraction. For fecal steroid extraction, five milliliters (ml) of 90 % ethanol (KOPTEC USP, King of Prussia, PA) was added to 0.2 g of dried feces in a 16 x 100 mm glass tube. To monitor extraction efficiency, 100 µl of a ^{3}H-steroid spike (~1000cpm of either ^{3}H-progesterone or ^{3}H-testosterone; Perkin Elmer, Waltham, MA) was added to a subset of animal samples (n=2 females; n=2 males). All samples, whether spiked with ^{3}H-steroid or not, were boiled at 80-90 °C for 20 minutes with the addition of ethanol as it evaporated during the boiling process. Thereafter, samples were centrifuged at 500 g for 10 minutes and the ethanol supernatants were decanted into a fresh 16 x 100 mm glass tube and the fecal

sample was reserved in its original glass tube. An additional five ml of 90 % ethanol was added to remaining fecal sample, the sample was vortexed for one minute, and then the sample was centrifuged again at 500 g for 10 minutes. The secondary supernatant was added to the first in a 16 x 100 mm glass tube and dried under compressed air in a warm water bath at 35-37 °C. Evaporated extracts were resuspended in 100 µl of pure ethanol and 900 µl of assay buffer (0.04 mol/L NaH_2PO_4 [Fisher Scientific, Waltham, MA], 0.06 mol/L Na_2HPO_4 [Fisher Scientific], 1 % bovine serum albumin [BSA; Lampire Biological, Pipersville, PA], 8.7 % NaCl [Fisher Scientific], 0.9 % Kathon [Arbor Assays, Ann Arbor, MI]). After resuspension, extracts were vortexed for one minute followed by sonication for 15 minutes, and subsequently vortexed again following sonication for 30 seconds. Extraction efficiency in the subset of samples monitored for this was assessed by counting 100 µl of each extraction in three ml of scintillation fluid (Ultima Gold, Perkin Elmer) on a liquid scintillation counter (Tri-Carb® 4910 TR Liquid Scintillation Analyzer, Perkin Elmer). Each animal's counts were conducted using a unique quench curve generated for each animal from a pool of extracted fecal samples from that animal. A quench curve had to be generated for each animal as the color of the neat extracts varied from animal to animal. The average extraction efficiency was ≥ 99 % for all samples assessed. Fecal extracts were stored in two ml cryovials (Nalgene, Rochester, NY) at -20 °C until analysis by enzyme linked immunoassay.

3.3 ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

Fecal extracts were analyzed for P4 and T metabolites by ELISA. Horseradish peroxidase (HRP) ligands and mono-clonal (progestogen, CL425, ISWE mini-kit, Arbor Assays) or poly-clonal (testosterone, R156/7, Coralie Munroe, University of California-Davis) antibodies were utilized. CL425 antibody was produced as a monoclonal antibody from cell culture derived using a single mouse cell line and R156/7 antibody produced as poly-clonal antibody made via inoculation of a rabbit. As per kit recommendations, working antibody and HRP dilutions were both 1:50 for the assessment of P4. For assessment of T, working antibody and HRP dilutions were 1:400,000 and 1:300,000, respectively. Working dilutions were selected based on a dilution matrix, which assessed dilution pairings ranging from 1:100,000 to 1:300,000 (HRP) and 1:300,000 to 1:500,000 (antibody). The dilution combination that achieved a B0 optical density of approximately 1.2 was selected for the T ELISA. Hormone assays were conducted with a competitive, double antibody ELISA with the following secondary antibodies which were coated on 96 well micro-titer plates (Corning®, Kennebunk, ME): goat anti-mouse IgG (progesterone, Arbor Assays) and goat anti-rabbit IgG (testosterone, Arbor Assays). Prior to analysis, fecal extracts were diluted 1:1000 in assay buffer for analysis of P4 from female samples and 1:500 for analysis of T from male samples. Aforementioned dilutions were chosen based on results from pooled parallelism, selecting dilutions where samples fell in the middle of the standard curve for higher accuracy of reading.

Each ELISA was validated in our lab specifically for the RRH samples specific to this study by the following protocols. Validation with parallelism for both the P4 and

T ELISAS was performed by serial dilutions of pooled fecal extracts and demonstration that the serially diluted samples produced displacement curves parallel to that of the standard curve. Cold recovery of a set amount of added P4 and T spiked to the same pooled fecal extracts was conducted such that > 90 % recovery was obtained for each steroid hormone. Inter- and intra-assay coefficients of variation were 18.2 % and 4.2 % for all P4 ELISAs and 8.0 % and 4.0 % for T ELISAs.

The procedure for running each ELISA was as follows. Fifty µl of samples, standards, and controls was added to secondary antibody coated wells followed by addition of 25 µL (P4) or 50 µL (T) antibody and HRP conjugate to each well. Plates were incubated at room temperature for two hours while shaking. Post-incubation, plates were thoroughly rinsed with wash solution (0.05 % Tween 20; Acros Organics, Geel, Belgium) followed by addition of 100 µl of TMB substrate (Moss, Inc., Pasadena, MD) and incubation for 30 minutes at room temperature. The ELISA reaction was stopped using 1N HCl Solution (Fisher Scientific) and the optical density (OD) was read at 450 nm wave length using a plate reader (BioTek Synergy HT, Gen5 Microplate Data Collection and Analysis Software, BioTek Instruments, Winooski, VT).

3.4 STATISTICAL ANALYSIS

Results were assessed for normality with PROC GLM (SAS, Cary, NC) and non-normal data was transformed prior to analysis with repeated measures ANOVA (P4/T) or one-way ANOVA (estrous cycle length). The Shapiro-Wilke statistic and skewness and kurtosis values were utilized to determine whether the data was normally distributed

or not. Results are presented herein, unless otherwise noted, as the least square mean (LSM) ± standard error of the mean (SEM). In all statistical tests, $p>0.05$ was the criterion for statistical significance.

3.4.1 Female Analysis

The onset of a luteal cycle was determined as a rise of P4 concentrations from baseline, with more than two points in a row that were greater than one standard deviation above the mean P4 concentration across all samples for a given female. The end of an estrous cycle was determined as the time point at which the P4 concentration returned to baseline. To identify significant differences between the mean estrous cycle length between groups, mean estrous cycle length was compared across groups with a one-way ANOVA, followed by Tukey's post-hoc test. For the pheromone treatment group, estrous cycle length during the baseline, female pheromone exposure period, and male pheromone exposure period was compared by one-way ANOVA. Gestation length in pregnant and pseudopregnant/pregnancy loss females was compared by unpaired T-test.

The difference between mean P4 concentrations was determined using a repeated measures ANOVA and a Tukey's post-hoc test to identify the significant differences amongst means at given time points. P4 concentrations were compared across all treatment groups over time. Additionally, using a repeated measures ANOVA and a Tukey's post-hoc test P4 concentrations were compared in the pheromone treatment group during baseline, female pheromone exposure, and male pheromone exposure

periods. Parameters in the statistical model included: treatment, month, season, and phase of the estrous cycle (i.e., luteal or follicular). Tests for interaction between treatment with month, season, and phase of the estrous cycle were conducted. Location was not included in the statistical model due to the scant number of zoos in each location category.

3.4.2 Male Analysis

Using a repeated measures ANOVA and a Tukey's post-hoc test to identify significant differences amongst given means, T concentrations were compared across treatments during the entire study period. The same analysis was repeated for the pheromone treatment group and the T concentration was compared between the baselines, female pheromone exposure period and the male pheromone exposure period. Parameters in the statistical model included: treatment, month, and season. Tests for interaction between treatment with month and season were conducted. Location was not included in the statistical model due to the scant number of zoos in each location category.

4. RESULTS

4.1 COMPARISON OF INDIVIDUAL MALE AND FEMALE FECAL STEROID PROFILES BY TREATMENT GROUP

Individual male T data over time was compared graphically to paired individual female P4 data over time (Figures A-2-4). On these figures female estrous cycle, mating, parturition, and pheromone exposure periods in the pheromone treatment group are noted. This information was received from zoo keeper staff and some zoos did a better job of collecting behavioral data than others.

4.1.1 Pheromone Treatment Group

Profiles of fecal P4 and T concentrations for the pheromone treatment group are shown in Figure A-2. Overall female response to pheromones was extremely variable, particularly with the females from Dallas Zoo (#08J638) and Virginia Zoo (#211051) (Figure A-2 B, C). Both females from these zoos had irregular cycles throughout the year, and it appears that introduction of male pheromones shut down estrous cycling. In the males from Dallas Zoo (#10K171) and Virginia Zoo (#212007) (Figure A-2 B, C), minimal change in T concentration or oscillation over time occurred in response to introduction of female or male pheromones. The female (#109064) from Safari West located in California (Figure A-2 A) had estrous cycles year around. Although minimal changes in the female's P4 profile were detected in response to pheromone introduction, the male (#108039) appeared to respond to introduction of male pheromones with a

substantial increase in T concentration approximately 30 days after male urine introduction.

4.1.2 New Male Treatment Group

Profiles of fecal P4 and T concentrations for the pheromone treatment group are shown in Figure A-3. All males have sustained, high T concentrations throughout the year. Female estrous cycles appear to be variable over the course of the year, with notable spans of acyclicity from approximately July to December, irrespective of zoo location. Removal of an old male (#2435) and introduction of a new male (#4534) at Brookfield Zoo resulted in cessation of estrous cycling in the female (#3166) (Figure A-3 B, C). Grouping two females together (Disney Animal Kingdom Lodge (DAKL); Figure A-3 D, E) resulted in a pregnancy carried to term in one female (#120138; Figure A-3 E) and pregnancy failure in the other female (#120137; Figure A-3 D). Keeper notes from DAKL indicated that the female with pregnancy failure (#120137) would repeatedly breed and get pregnant, but would consistently experience pregnancy loss. In contrast, over consecutive breeding seasons the other DAKL female (#120138) would breed and carry her pregnancy to term. Following this study, the reproductive successful female (#120138) was contracepted, at which time the other female (#120137) became pregnant and carried that pregnancy to term.

4.1.3 Control Treatment Group

Profiles of fecal P4 and T concentrations for the control treatment group are shown in Figure A-4. Overall T concentrations were lower in this group than in the P and NM groups. T concentrations appear to be elevated from July to October in two out of the three of the males. Two of the females became pregnant during the study (Figure A-4 A, C). The female from Columbus Zoo (#211051) had a long period of elevated fecal P4 of approximately 74 days, which was indicative of pregnancy. However, she never gave birth and after this 74 day period she began cycling again (Figure A-4 B). Two females housed together with a single male (Oklahoma City Zoo; Figure A-4 C, D) resulted in two pregnancies from one female (#774809) and complete lack of estrous cycling in the other female (#775816).

4.2 FEMALE RESULTS

4.2.1 Estrous Cycle Comparison

Fecal P4 concentrations (ng/g feces) for non-pregnant females during the luteal and follicular phases of the estrous cycle were significantly different within and between treatment groups (p=0.0065; Figure A-5). Between treatment groups, there was a significant difference between the P4 concentration in the luteal phase of the estrous cycle in the NM females (2884.5 ± 144.1 ng/g feces) and P females (3945.6 ± 158.3 ng/g feces; p=0.0414; Figure A-5). During the follicular phase, NM females and P females had fecal P4 concentrations of 1201.2 ± 119.3 ng/g feces and 1342.4 ± 151.8 ng/g feces, respectively. C females averaged P4 concentrations of 1080.3 ± 150.4 ng/g feces during

the follicular phase and 3291.61 ± 196.3 ng/g feces during the luteal phase of the estrous cycle (Figure A-5).

Average estrous cycle length was compared between treatment groups (Figure A-6). C females had a mean estrous cycle length of 12.0 ± 3.5 days and NM females had a mean estrous cycle length of 18.0 ± 2.2 days. Prior to pheromone exposure, P females had a mean estrous cycle length of 15.7 ± 1.5 days. Although there was no significant difference in the amount of change between estrous cycle lengths between pheromone introductions (p=0.66), the mean length of the estrous cycle became truncated (12.8 ± 2.0 days) in response to female pheromones and elongated in response to male pheromones (19.3 ± 3.1 days).

4.2.2 Urine Pheromone Treatment Group

No female in the pheromone treatment group became pregnant during the study. Overall there was no significant difference in fecal P4 concentrations amongst the different phases of the urine pheromone trial (p=0.4206). However, there was an overall significant effect between the interaction of the phase of the urine pheromone trial and the phase of the estrous cycle (p<0.0001) (Figure A-7).

During the luteal phase of the estrous cycle, mean fecal P4 concentrations were significantly different between pre-introduction (PRE) and female urine pheromone exposure (FM) (p<0.0001) and FM and male urine pheromone exposure (M) (p=0.0011). Mean P4 concentration was highest for the luteal phase of the estrous cycle during PRE (4392.6 ± 146.0 ng/g feces) and was lowest for the luteal phase of the estrous cycle

during FM (3380.9 ± 188.0 ng/g feces). For the follicular phase of the estrous cycle, mean fecal P4 concentrations were significantly different between PRE and FM (p=0.008) and FM and M (p=0.0176). Mean fecal P4 concentrations were highest during FM (1550.8 ± 173.4 ng/g feces) and lowest during male pheromone exposure (1199.6 ± 165.5 ng/g feces). Month did not have a significant effect on mean fecal P4 concentrations in the pre-introduction versus post-introduction time frames (p=0.0570). However, there was a significant interaction of season and phase (ie, pre vs post introduction) of the urine pheromone trial (Figure A-8; p<0.0001). The winter PRE P4 concentration is lower than all other time points (1796.7 ± 796.1 ng/g feces) and the spring PRE P4 concentration is higher than all other time points (4038.0 ± 832.3 ng/g feces). The fall and summer PRE P4 concentrations were 2186.5 ± 763.8 ng/g feces and 2631.1 ± 790.5 ng/g feces, respectively.

4.2.3 Non-Pregnant versus Pregnant and Pseudopregnant/Pregnancy Loss

For purposes of the analysis of fecal P4 concentrations, females within each treatment group were separated into two groups: 1) non-pregnant; 2) pregnant or pseudopregnant/pregnancy loss. This separation was done to take into account the extremely elevated fecal P4 concentrations due to pregnancy or pseudopregnancy/pregnancy loss. Individual animals that had elevated fecal P4 concentrations beyond the length of a normal cycle (i.e. > 40 days) were incorporated into the pregnant or pseudopregnant/pregnancy loss group for the duration of that particular time frame. No females from the P group became pregnant or

pseudopregnant/ pregnancy loss. Six females displayed elevated fecal P4 concentrations longer than 40 days (Figure A-3 A & D-E; Figure A-4 A-C). Of these six females, three gave birth (one female had two pregnancies to parturition over the course of the study) with an average gestation length of 112.8 ± 5.4 days. The three females that had elevated fecal P4 concentrations but did not give birth had a significantly shorter lengths of elevated P4 than the pregnant females who underwent parturition (56.7 ± 15.5 days; p=0.0009; Figure A-9). The fecal P4 concentrations for pregnant (11945.0 ± 1516.3 ng/g feces) and pseduopregnant/pregnancy loss (10236.0 ± 1746.5 ng/g feces) females were not significantly different across treatments (p=0.9151), thus the two were considered as one group for analysis.

4.2.4 Season

4.2.4.1 Non-Pregnant Females

An examination of the effect of season coupled with treatment on fecal P4 concentrations is shown for non-pregnant females only in Figure A-10. C females (2421.4 ± 191.9 ng/g feces) and females exposed to pheromones (2865.3 ± 186.8 ng/g feces) had the highest fecal P4 concentrations in the summer. On the other hand, NM females had the highest fecal P4 concentrations in the fall (2508.2 ± 151.6 ng/g feces). Within the P group, the winter P4 concentration was lower than all other seasons (2033.0 ± 201.3 ng/g feces) and the summer P4 concentration was highest of all seasons (2865.3 ± 186.8 ng/g feces), with values similar to fall (2656.4 ± 189.3 ng/g feces) and spring (2360.3 ± 192.4 ng/g feces) (p<0.05). Within the NM treatment group, P4

concentrations in winter (2327.6 ± 161.0 ng/g feces), spring (2110.4 ± 153.8 ng/g feces), and summer (2297.2 ± 150.6 ng/g feces) were dissimilar to values in fall, though similar to each other ($p<0.05$). In the C group, P4 concentrations were similar for all four seasons (Fall: 2282.8 ± 190.1 ng/g feces; Winter: 2274.2 ± 191.5 ng/g feces; Spring: 2123.5 ± 196.7 ng/g feces; Summer: 2421.4 ± 191.9 ng/g feces) ($p>0.05$). There was an overall significant interaction between treatment group and season ($p<0.0001$; Figure A-10).

4.2.4.2 Pregnant and Pseudopregnant/Pregnancy Loss Females

An examination of the effect of season coupled with treatment on fecal P4 concentrations is shown for pregnant and pseudopregnant/pregnancy loss females only in Figure A-11. C females had the highest fecal P4 concentrations in the fall (54440 ± 2775.1 ng/g feces) and newly paired females had the highest fecal P4 concentrations in the spring (4928.9 ± 2522.8 ng/g feces). Within the C group, P4 concentration in fall is higher than all other seasons and winter is lower than all other seasons (1880.7 ± 1788.1 ng/g feces) ($p<0.05$). Spring (7593.8 ± 1816.0 ng/g feces) and summer (10243.0 ± 2023.9 ng/g feces) P4 concentrations are different than winter and fall in the C group, but are similar to each other ($p>0.05$). In the NM group, P4 concentration in winter is lower than all other seasons (862.4 ± 1935.7 ng/g feces), and in spring P4 concentration is higher than the other seasons ($p<0.05$), though similar to concentrations seen in summer (3267.5 ± 2941.8 ng/g feces) ($p>0.05$).

4.2.5 Month

4.2.5.1 Non-Pregnant Females

There was an overall significant interaction between month and treatment in non-pregnant females ($p<0.0001$). Fluctuations in fecal P4 concentrations for each treatment group by month are shown in Figure A-12. For P females, fecal P4 concentrations were highest in June (2937.3 ± 310.7 ng/g feces) and lowest in January (1702.5 ± 328.9 ng/g feces). C females had the highest fecal P4 concentrations in June (2531.3 ± 273.8 ng/g feces) with the lowest in February (1892.3 ± 327.2 ng/g feces). NM females had the highest fecal P4 concentrations in November (2646.2 ± 214.7 ng/g feces) and lowest in March (1978.1 ± 222.2 ng/g feces). Fecal P4 concentrations were significantly different between C females and NM females in February ($p=0.0286$) and November ($p=0.0502$). There was a trend for significant difference in fecal P4 between P females and NM females in June ($p=0.0641$), July ($p=0.0982$), and August ($p=0.0655$), and between C females and P exposed females in November ($p=0.0902$).

4.2.5.2 Pregnant and Pseudopregnant/Pregnancy Loss Females

For pregnant and pseudopregnant/pregnancy loss females, there was no overall interaction between month and treatment ($p=0.1243$; Table A-3). Females in the C group had the highest fecal P4 concentrations in September (54106.0 ±3872.3 ng/g feces) and the lowest fecal P4 concentrations in January (710.9 ± 3473.1 ng/g feces). NM females had the highest fecal P4 concentrations in April (9798.9 ± 4260.5 ng/g feces) and the lowest fecal P4 concentrations in February (1000.5 ± 3808.8 ng/g feces). There was no

significant difference in fecal P4 concentrations between C and NM females for any month (p>0.05; Table A-3).

4.3 MALE RESULTS

4.3.1 Comparison between Treatment Groups

Males in the P treatment group had the highest mean T concentration (909.6 ± 365.3 ng/g feces), followed by males in the C treatment group (427.5 ± 353.8 ng/g feces). Males in the NM treatment group had the lowest mean T concentration (325.4 ± 283.2 ng/g feces). There was no significant difference in overall fecal T concentration between treatments (p=0.5048).

4.3.2 Urine Pheromone Treatment Group

For the urine pheromone treatment group, the overall mean fecal T concentrations were significantly different between PRE and FM (p=0.0052), PRE and W (p=0.0063), PRE and M (p<0.0001), and between FM and M (p=0.0018; Figure A-13). Mean fecal T concentration was highest during M (1122.3 ± 299.1 ng/g feces) and lowest during PRE (786.2 ± 297.7 ng/g feces). The mean fecal T concentration during FM was 905.3 ± 299.6 ng/g feces and during W was 954.5 ± 308.1 ng/g feces. There was a trend for fecal T concentrations to significantly differ for the month and trial phase interaction (p=0.0671). There was a significant interaction of season on trial phase with respect to fecal testosterone metabolite concentrations (Figure A-14; p=0.0182). During the PRE phase of the trial, T concentration was highest in the spring (959.8 ± 299.8 ng/g

feces), and lowest in the winter (738.0 ± 293.1 ng/g feces), with fall (836.8 ± 292.6 ng/g feces) and summer (747.9 ± 293.1 ng/g feces) concentrations being similar to winter, but dissimilar from one another (Figure A-14). During the M phase of the trial, T metabolite concentration was highest in fall (1261.0 ± 295.3 ng/g feces) and lowest in summer (1037.8 ± 293.4 ng/g feces).

4.3.3 Season

The interaction of season and treatment group with respect to fecal T concentrations is displayed in Figure A-15. C males had the highest fecal T concentrations in the summer (649.8 ± 452.2 ng/g feces), NM males had the highest fecal T concentrations in the spring (430.3 ± 343.3 ng/g feces), and P males had the highest fecal T concentrations in the fall (995.2 ± 454.5 ng/g feces). There was no significant interaction between treatment group and season with respect to fecal T concentration ($p>0.05$).

4.3.4 Month

With respect to fecal T concentrations, there was an overall significant interaction between month and treatment ($p<0.0001$). P males had a greater mean fecal T concentration than the other two treatment groups (Figure A-16). For P males, fecal T concentration was highest in September (1068.4 ± 369.1 ng/g feces) and fecal T was lowest in December (568.7 ± 373.7 ng/g feces). C males had the highest fecal T concentrations in August (1183.1 ± 368.6 ng/g feces) and the lowest fecal T concentrations

in May (147. 5 ± 364.4 ng/g feces). NM males had the highest fecal T concentrations in May (778.0 ± 290.8 ng/g feces) and lowest fecal T concentrations in December (197.5 ± 292.4 ng/g feces). There was very little fluctuation in fecal T concentrations in NM males, other than an increase in them in the month of May (Figure A-16).

5. DISCUSSION AND CONCLUSIONS

This study is the first to utilize urine pheromones as a mechanism to manipulate reproduction in captive RRH breeding pairs. Various changes were in response to the introduction of pheromones. In the males, T concentration significantly increased in response to pheromone exposure, with male pheromone introduction causing the highest increase. In particular, the male from Safari West had a notable increase in T concentration with greater oscillations in response to male pheromone exposure, indicative of the "boar effect" in which competitive scents stimulate reproductive function in conspecifics (Liptrap and Raeside, 1978). The female from Safari West had estrous cycles that were comparatively longer during the male pheromone introduction than during female pheromone introduction, further alluding to occurrence of the "boar effect" (Rekwot et al., 2001). In contrast, the remaining two thirds of the females in the pheromone group appeared to "shut down" and become acyclic in response to male pheromone exposure. However, it is possible that the acyclicity seen during male pheromone exposure was due seasonal anestrus as captive female RRH have been described to undergo periods of acyclicity in response to changes in photoperiod (Bryant et al., 2016; Peltoniemi et al., 2000). During female pheromone exposure, the P4 peaks of the estrous cycles for the female from Virginia Zoo appear lower, suggestive of a dampening or suppressive effect of female pheromones, whereas the female from Dallas Zoo appeared to have more normalized estrous cycles in response to female pheromones, with P4 oscillations coming closer to baseline compared to other phases of pheromone

introduction. Although only subtle variations in T and P4 concentrations occurred in response to pheromone exposure, introduction of pheromones does appear to positively modulate reproductive steroids in captive RRH breeding pairs. In addition to information about the effects of urine pheromones on fecal steroid concentrations and estrous cyclicity, vital information on baseline reproductive physiology in RRH was gained from this study through the longitudinal hormone monitoring. In particular, the role that season plays in reproduction of these animals was solidified, and the potential influence of grouped housing on reproduction in females was discovered through this comprehensive study. Lastly, this study demonstrated the potential application of urine pheromones to the modulation of reproductive parameters and potentially to the manipulation of breeding behavior in RRH breeding pairs.

Based on discussions with keepers and veterinary staff prior to the study onset, the three zoos enrolled in the pheromone trial were believed to have female RRH that were not cycling normally or were acyclic. However, females in the pheromone treatment group were cycling prior to pheromone exposure. Due to the fact that all the female RRH in this group had at least some cycles during the study period, including during the pre-pheromone exposure period, the effects of the urine pheromones in the female RRHs are subtle at best. Nonetheless, pheromone introduction had a significant effect on fecal P4 concentration during the luteal phase of the estrous cycle, specifically causing a decrease in fecal P4 concentration due to the introduction of female urine pheromones and an increase in fecal P4 concentration due to male urine pheromones. This finding is suggestive of an inhibitory effect of female urine pheromones on

ovulation and corpora lutea formation in conspecific females. Thus, lowered fecal P4 concentrations in the pheromone exposed females could be indicative of decreased corpora lutea production of P4 or decreased numbers of ovulated follicles with subsequently low P4 production. This finding is somewhat contrary to previously documented effects of estrous synchrony occurring in groups of female Suidae, and other species, a phenomenon believed to be initiated by female pheromones (Delcroix et al., 1990; Pedersen, 2007; Weissenbock et al., 2009). Although estrous synchrony is often stimulated in response to novel pheromones in domestic swine, inhibition of sexual motivation during estrous in subordinate domestic pigs can occur due to social stress from limited space and/or resources like feed and water (Pedersen, 2007). It is possible that female RRH exposed to female RRH urine pheromones resulted in stress which adversely affected ovarian function. In contrast, the increase in fecal P4 concentration when exposed to male pheromones follows suit with other studies on Suidae species where stimulus from a boar tends to induce ovulation in sows (Pedersen, 2007; Rekwot et al., 2001). In male RRH, fecal T concentration increased in response to both female and male urine pheromones, with the greatest increase occurring during male urine pheromone introduction. In domestic boars, it has been shown that sexual activity can be increased by the presence of other boars (Tanida et al., 1991) and this response is thought to be mediated by release of pheromones (Love et al., 1993). The perceived presence of another boar in the form of the male urine pheromones in the enclosure could be causing the increase in fecal T concentrations in response to male pheromone introduction. In the males, introduction of female pheromones resulted in an increase in

mean T concentration. This finding is corroborated by evidence in other species such as sheep (Illius et al., 1976) and rhesus monkeys (Rose et al., 1972), where female presence often leads to increase in circulating T concentrations.

Though not significantly different between phases of the urine pheromone trial, P female cycles were shorter when exposed to female pheromones and elongated when exposed to male pheromones, suggesting that pheromone introduction had an effect on cycle length. It is possible that had the females been acyclic before pheromone introduction, the effect would have been more pronounced. It also appears that in these pairs of RRH, the introduction of male pheromones induced a male effect on ovulation with increased P4, whereas the introduction of female pheromones may have inhibited reproductive function in these females.

Irrespective of treatment group, the mean estrous cycle length for the females in this study was significantly shorter than the published estrous cycle length of 30-37 days for this species (Berger et al., 2006; Bryant et al., 2016; Leslie and Huffman, 2015). In fact, the estrous cycle length we found in our study females (range: 12.0-18.0 days) is closer to what has been noted for the domestic pig (18-21 days) (Geisert, 1999). These shorter estrous cycle lengths seen could be due to reproductive suppression in some of the sounders of RRH with more than one female, or other environmental factors such as photoperiod, light conditions, boar exposure, or ambient temperatures, as has been noted in the European wild boar and domestic pig (Andersson et al., 1998; Love et al., 1993; Peltoniemi et al., 2000). It is also important to note that it is exceedingly difficult to accurately discern the start and end of estrous cycles for animals lacking a robust

number of samples. Therefore, the estrous cycle lengths found in this study may not be a true representation of the reproductive physiology of females of this species. Pregnant females in the study had an average gestation of 112.75 ± 5.4 days, which is similar to what is seen in the published literature for the species (~120 days)(Berger et al., 2006).

Females housed in sounders with more than one female or that were exposed to female urine pheromones showed signs of inhibition of reproductive function. Dominance hierarchies play a critical role in reproduction for cooperative breeding animals like naked mole rats (Clarke and Faulkes, 1998) and the African wild dog (Creel et al., 1997), and have been shown to affect non-cooperative breeding species such as mice (Williamson et al., 2017) and primates (Michopoulos et al., 2012). For grouped females housed at Disney Animal Kingdom Lodge and Oklahoma City Zoo, one of the two females in the group became pregnant and carried to term, whereas the other female in the sounder either did not cycle (Oklahoma) or cycled normally but became pseudopregnant (Disney). In the wild, RRH typically live in home ranges of approximately 3.8 ± 10.1 km^2 and form sounders of seven to ten individuals consisting of a single adult male, several adult females, and immature individuals of both sexes (Leslie and Huffman, 2015). The ability to maintain these larger groups without reproductive suppression occurring may be due to the increased resource availability that comes with large home ranges. Additionally, in domestic swine sexual motivation can be inhibited due to social and environmental stress (Pedersen, 2007). In a study of the behavioral strategies of domestic pigs, (Mendl et al., 1992), socially stressed pigs experienced reproductive suppression. Social stress in domestic pigs could be due to

things such as antagonistic behavior, aggression, or limited access to resources (Peltoniemi et al., 2000; Wilson and Love, 1990). The elevated cortisol levels that are associated with group-housing systems in pregnant sows are thought to disrupt reproductive success, ultimately affecting fertilization, and implantation (Salak-Johnson, 2017). Additional behavioral observations of the sounders of RRH that include more than one female would be necessary to determine if such behaviors are reason for the apparent reproductive suppression.

Pseudopregnancy is not uncommon in domestic pigs (Geisert et al., 1987; Lee et al., 1993) and has been linked to numerous causes such as aggression from companions, progressive reduction in phototropic periods, interrupted feed intake, and sudden changes in temperature leading to increased cortisol levels and decreased ovarian function (Salak-Johnson, 2017; Tarocco, 2009). Three of the females in the study displayed high levels of fecal P4 concentrations for an extended period of time but did not carry to term and were thus labeled as pseudopregnant/pregnancy failure. The female housed at the Bronx Zoo (#M07077) had a prolonged period of elevated fecal P4 concentration of approximately 54 days, characterized as pseudopregnancy, which occurred just after a long anestrous period from approximately June to December. The male she was housed with was initially not sexually mature. The resultant, longer luteal phase of her initial cycle could have been a vestige of seasonal acyclicity. RRH have previously been described as seasonally polyestrous (Berger et al., 2006; Bryant et al., 2016), a phenomenon seen in numerous other species including wild boars and domestic pigs (Love et al., 1993; Peltoniemi et al., 2000). This physiologic trait is often linked to

climate conditions that reflect food availability and energy status in the habitat and can result in decreased fertile periods or incidence of seasonal infertility (Peltoniemi et al., 2000). When estrous cycling returns after a period of anestrus under harsh or suboptimal environmental conditions for breeding, often the luteal phase of the estrous cycle is extended (Bryant et al., 2016; Peltoniemi et al., 2000).

In contrast to what would be considered a true pseudopregnancy due to CL on the ovary, the remaining two females labeled as pseudopregnant appeared to actually become pregnant but lose the fetus midway through gestation. One of the females from Disney Animal Kingdom Lodge had a prolonged rise in fecal P4 metabolites of approximately 44 days. It is possible in her case that she was actually pregnant but the pregnancy terminated due to the stress of being housed with a more dominant female. Disney Animal Kingdom Lodge always conducts pregnancy confirmation ultrasounds their females approximately 35 days post breeding. Therefore, this zoological institution had evidence of repeated pregnancy loss in this female, always in conjunction with the other female (#120138) carrying to term. Interestingly, when #120138 was contracepted, female #120137 was able to carry to term, further suggestive of reproductive suppression of the subordinate by the dominant female. Although it is uncommon in suid species, reproductive suppression by pregnancy loss has been noted in other species such as the cooperatively breeding golden lion tamarin (Henry et al., 2013), Alpine marmots (Hackländer et al., 2003), and humans (Wasser and Barash, 1983). The last pseudopregnant/pregnancy loss female (#211051) was housed singly with a male and had prolonged rises of fecal P4 that lasted 74 days. It is likely that this

female became pregnant and aborted the fetus as, according to keeper notes, blood was noted in her feces around the end of the 74 days. Reasons for the loss of this pregnancy are unknown but could include trauma, such as slipping on floors, aggression from the male companion, sudden changes in temperature between day and night (Tarocco, 2009), or merely a uterine environment that was suboptimal during the implantation window due to social and/or environmental stress weakening the immune system (Salak-Johnson and McGlone, 2007; Wilson and Anderson, 2009).

Acyclicity was seen commonly in many of the females irrespective of treatment group. We also found significant interactions between season and treatment for both males and females. Season may be an important environmental factor that is confounding the ability to make conclusions about the effects of pheromones on steroid concentrations and estrous cyclicity. RRH are seasonally polyestrous, both in the wild and in captivity (Leslie and Huffman, 2015). In the wild, RRH are seasonal breeders across their distribution with births occurring from February to July, in conjunction with the end of the dry season (Leslie and Huffman, 2015). *Ex situ* reproduction follows a similar trend with peak breeding in captive RRH in the northern hemisphere occurring from winter to summer and births occurring in the summer and early autumn (Berger et al., 2006; Leslie and Huffman, 2015). Whereas in the wild seasonality is related to seasonal rainfall and the resultant food availability, female captive RRH appear to maintain the natural ability to cue into the changing photoperiod and moderate estrous cycling accordingly (Bryant et al., 2016). In the domestic pig, seasonal changes in daylight are relayed through the endocrine system using a photoperiodic time-measuring

system which converts day-length information into hormonal signals, mediated by an increase in secretion of melatonin (Bryant et al., 2016; Peltoniemi et al., 2000). The increase in melatonin causes the hypothalamus to stimulate the GnRH pulse-generator, resulting in gonadotropin secretion from the pituitary gland leading to the onset of ovarian activity (Peltoniemi et al., 2000). It is likely that this mechanism has been evolutionarily engrained in RRH such that captive RRH experience seasonal periods of acyclicity associated with the decrease in daylight beginning in the fall. Therefore, in the pheromone exposed treatment group, effects of season, such as temperature, rainfall, and, most importantly, photoperiod, could be overshadowing the potential benefits to introduction of the female and male urine pheromones. Application of male and female pheromones from January to July would be warranted since the majority of female RRH in our study were cycling during this time frame.

In contrast, season does not appear to have the same effect on male T concentrations. Males in the NM and P treatment groups had relatively steady T concentrations throughout the year, with a slight peak seen in May, which is conducive to the optimal breeding period for females. In contrast, males in the C group had noticeable peaks in T concentrations from July to October. As these males are in the group that breeds regularly, it is interesting that the peak in T would occur during the time period when females are normally acyclic. In related Suidae species, length of the spermatogenic cycle is between 40-60 days (Costa et al., 2011). With this number in mind, it would be logical for increase in T to occur around October-November, to allow for sufficient time for spermatogensis prior to the onset of female cycling in January. In

domestic boars, steroid concentrations (Claus et al., 1983) and semen quality (Ciereszko et al., 2000) have been shown to undergo seasonal changes due to daylight fluctuations, with maximum concentrations reached from October to December, which is what we would similarly expect in RRH. Semen quality and the spermatogenic cycle has yet to be analyzed in this species, and as T concentration is linked to semen quality, analysis of semen quality throughout the year could yield further insight to the reproductive physiology of the captive male RRH.

Lastly, this study demonstrated that the currently accepted method of re-pairing animals that have not bred within three years of being paired may be detrimental to reproductive function. In general, re-pairing is stressful for the animals (Dickens et al., 2010) as it involves immobilization and translocation to a different zoo, and a quarantine period. After this stress, introduction to a new environment and social structure is rife with disruptive social interactions that may impair reproductive function, often due to attempted breeding introductions (Bryant et al., 2016; Connor and Orzechowski, 2001). Specifically, at Brookfield Zoo, the female (#3166) appeared to have her estrous cycling disrupted by removal of the old male and introduction of a new male. The re-pairing at Brookfield Zoo was conducted due to lack of breeding between the old pair, but it is apparent based on the depression of the female's fecal P4 concentrations to baseline that the introduction of the male was detrimental, rather than helpful, to her reproductive capability. As mentioned, hormone levels can be altered by a variety of social factors, including changes in group composition and housing arrangements (Bryant et al., 2016), therefore it is possible that the disruption of cycling was caused by the social stress

induced through a change in group dynamics. Furthermore, male influence should be taken into account in this case, as the level of male sexual behavior and mating competency has a large influence on reproductive performance and success (Hemsworth and Tilbrook, 2007). Interestingly, the new male's T concentration had discernable peaks during the initial introduction, yet the T concentration appeared to lower in conjunction with the development of acyclicity in the female. Therefore, behavioral observation of RRH during introduction and subsequent habituation and mating attempts is warranted.

In conclusion, it appears that urine pheromones do elicit a hormonal response in captive breeding pairs of RRH, with the introduction male urine pheromones causing the biggest changes in both male and female reproductive function. Further research on the effect of season on reproduction in RRH is warranted, particularly with the respect to the optimal time frame during the year to introduce urine pheromones to RRH. Introduction of urine pheromones to acyclic females would provide more detailed information about the physiologic effects of urine pheromones on reproductive function in male and female RRH. Lastly, the number of female RRH in a captive herd needs to be carefully considered when grouping animals so as to potentially avoid reproductive suppression in genetically valuable individuals.

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APPENDIX

Table A-1. Summary of institutions with red river hogs participating in the study.

Treatment Group	**AZA Institution**	**Location**	House #	**Sex**
Control	Cheyenne Mountain Zoo	MW	28M059	F
			28M060	M
	Columbus Zoo	N	211051	F
			209161	M
	Oklahoma City Zoo	N	775816	F
			774809	F
			774408	M
Non-Breeding New Male	Brookfield Zoo	N	3166	F
			4534	M
			2435	M
	Disney Animal Kingdom Lodge	S	120137	F
			120138	F
			120136	M
	Bronx Zoo	N	M07077	F
			M14159	M
Non-Breeding Pheromone	Safari West Zoo	W	109064	F
			108039	M
	Dallas Zoo	S	08J638	F
			10K171	M
	Virginia Zoo	S	211051	F
			212007	M

MW= Midwest, N=North, S= South, W= West

Table A-2. Dates of urine introduction for each zoo enrolled in the urine pheromone trial.

Institution	Empty Enrichment Tube Period	Female Urine Exposure Period	Washout Period	Male Urine Exposure Period
Safari West	1/31/14-2/14/14	2/15/14-5/22/14	5/23/14-6/9/14	6/10/14-9/9/14
Dallas Zoo	3/10/14-4/4/14	4/5/14-7/4/14	7/5/14-7/16/14	7/17/14-10/18/14
Virginia Zoo		2/2/13-5/2/13	5/3/13-6/4/13	6/5/13-9/30/13

Table A-3. Fecal progestogen metabolite concentrations (ng/g feces) by month for pregnant and pseudopregnant/pregnancy loss females.

Treatment	Month	**Fecal Progestogen** Metabolite Concentrations (ng/g feces)
Control	January	710.9 ± 3473.1
	February	911.4 ± 3254.4
	March	2207.3 ± 3318.7
	April	17243.0 ± 3629.5
	May	-
	June	3054.9 ± 3547.8
	July	2285.5 ± 3782.4
	August	21990.0 ± 3888.4
	September	54106.0 ± 3872.3
	October	-
	November	-
	December	-
New Male	January	2034.0 ±3223.3
	February	1000.5 ±3808.8
	March	2704.6 ± 4346.6
	April	9798.9 ± 4260.5
	May	4017.1 ± 0.0
	June	3811.0 ± 3681.7
	July	-
	August	-
	September	-
	October	-
	November	-
	December	2137.9 ± 3304.7

A – indicates no data available for this month for the treatment group. No significant difference in the treatment*month interaction term (p>0.05).

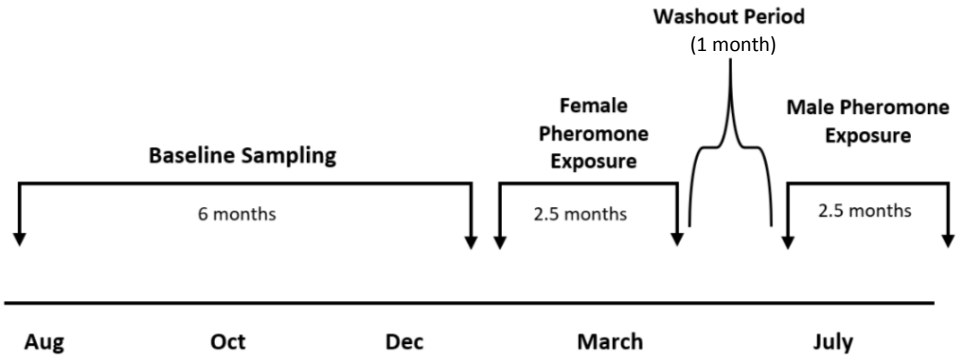


Figure A-1. Pheromone treatment group study design timeline. Approximate times for each phase of the urine pheromone trial are as indicated.

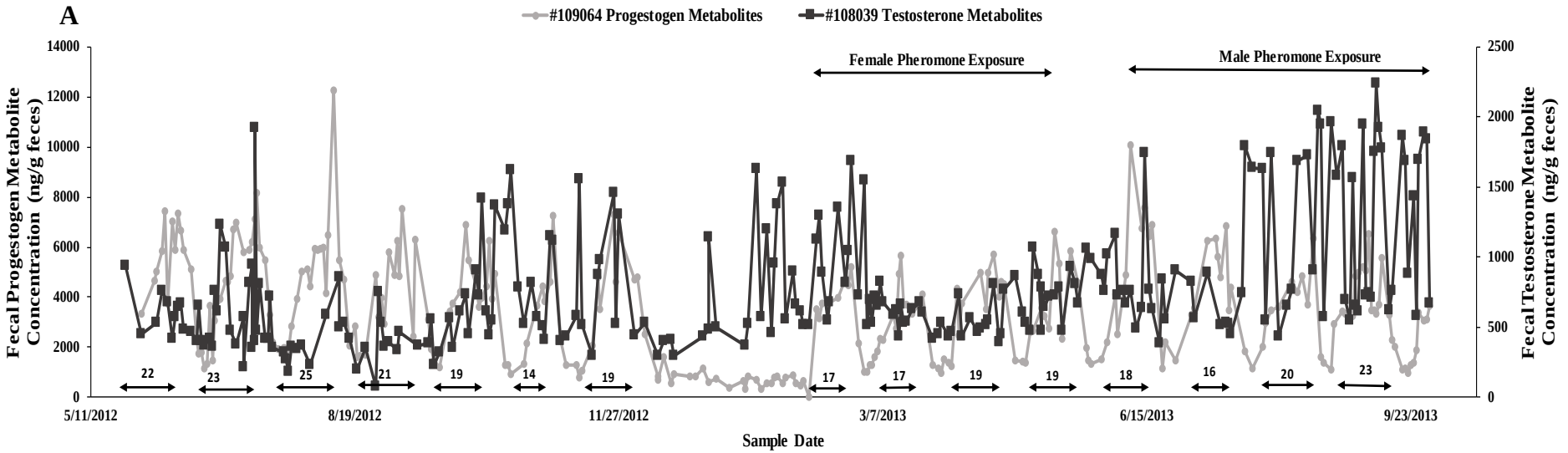


Figure A-2. Profiles of fecal progestogen and testosterone metabolite concentrations (ng/g feces) for RRH pairs in the pheromone (P) treatment group. Including: (A) #109064 (●) and #108039 (■) at Safari West Zoo in Santa Rosa, CA from May 2012 to September 2013, (B) #08J638 (●) and #10K171 (■) at Dallas Zoo in Dallas, TX from February 2013 to September 2014, and (C) #211051 (●) and #212007 (■) at Virginia Zoo in Norfolk, VA from August 2013 to October 2014. Estrous cycles are designated by arrows along the x-axis with number of days of each cycle noted above each arrow. Urine exposure period is designated by arrows above the graph.

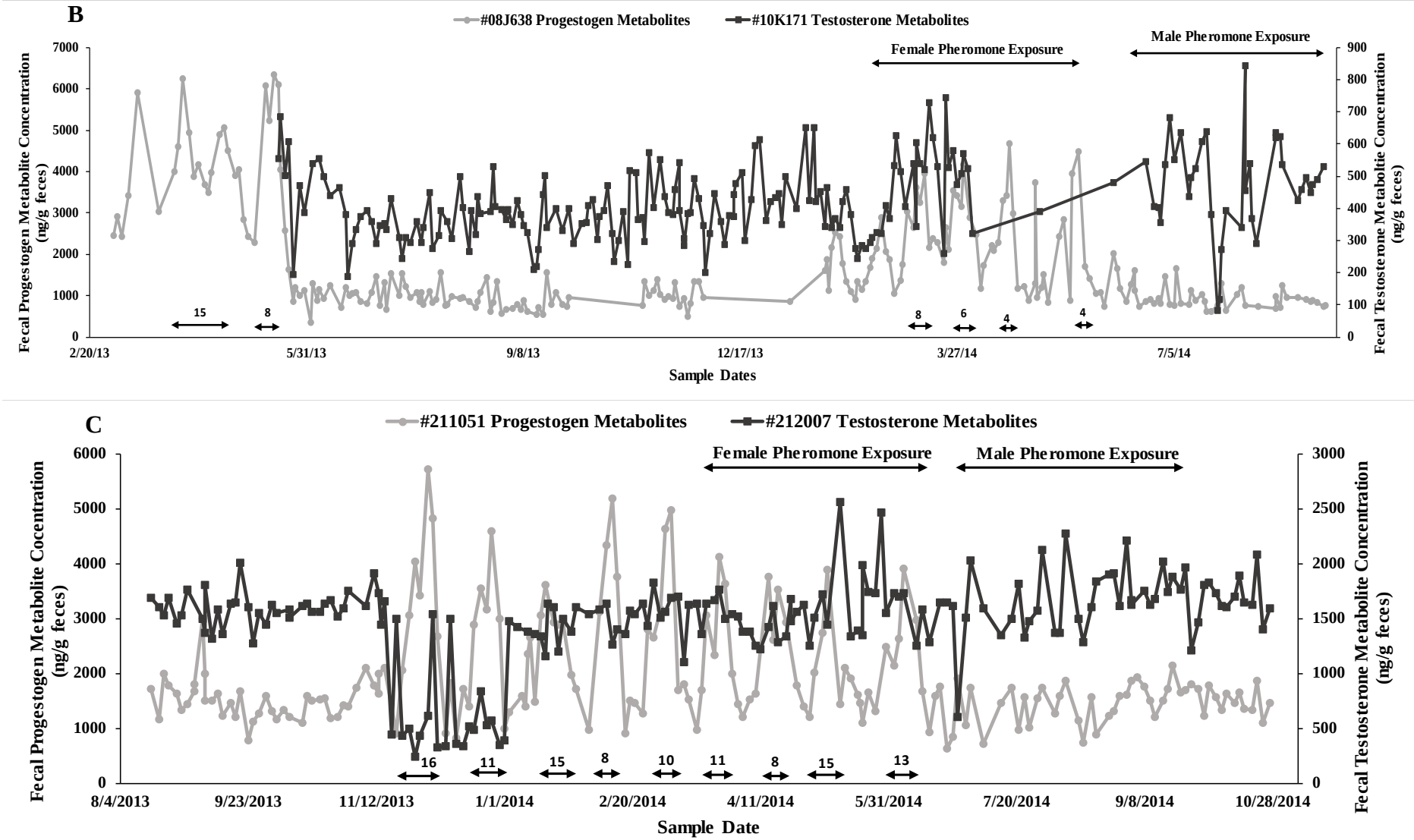


Figure A-2 continued.

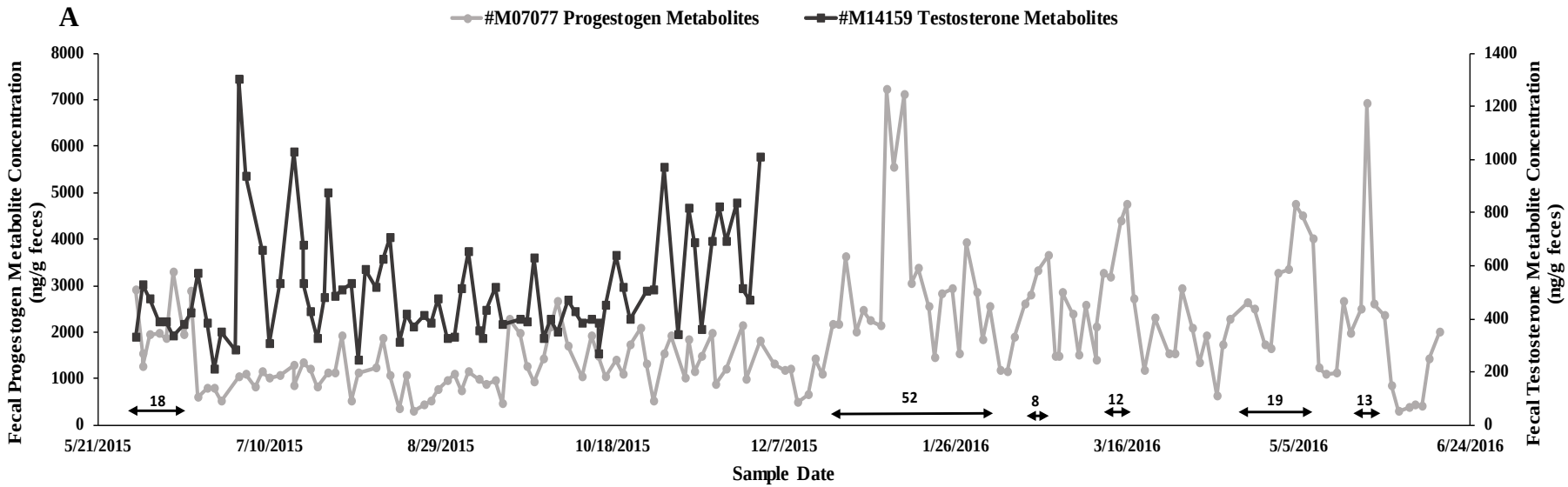


Figure A-3. Profiles of fecal progestogen and testosterone metabolite concentrations (ng/g feces) for RRH pairs/groups in the new male (NM) treatment group. Including: (A) #M07077 (●) and #M14159 (■) at the Bronx Zoo in Bronx, NY from May 2015 to June 2016, (B) #3166 (●) and #2435 (▲) and (C) #3166 (●) and #4534 (■) at Brookfield Zoo in Chicago, IL from March 2013 to April 2014, and (D) #120137 (▲) and #120136 (■) and (E) #120138 (●) and # 120136 (■) at Disney Animal Kingdom Lodge in Orlando, FL from May 2013 to June 2014. Estrous cycles are designated by arrows along the x-axis with number of days of each estrous cycle noted above each arrow. Significant events including, old male removal, new male introduction, mating events, and parturition events are designated by vertical arrows.

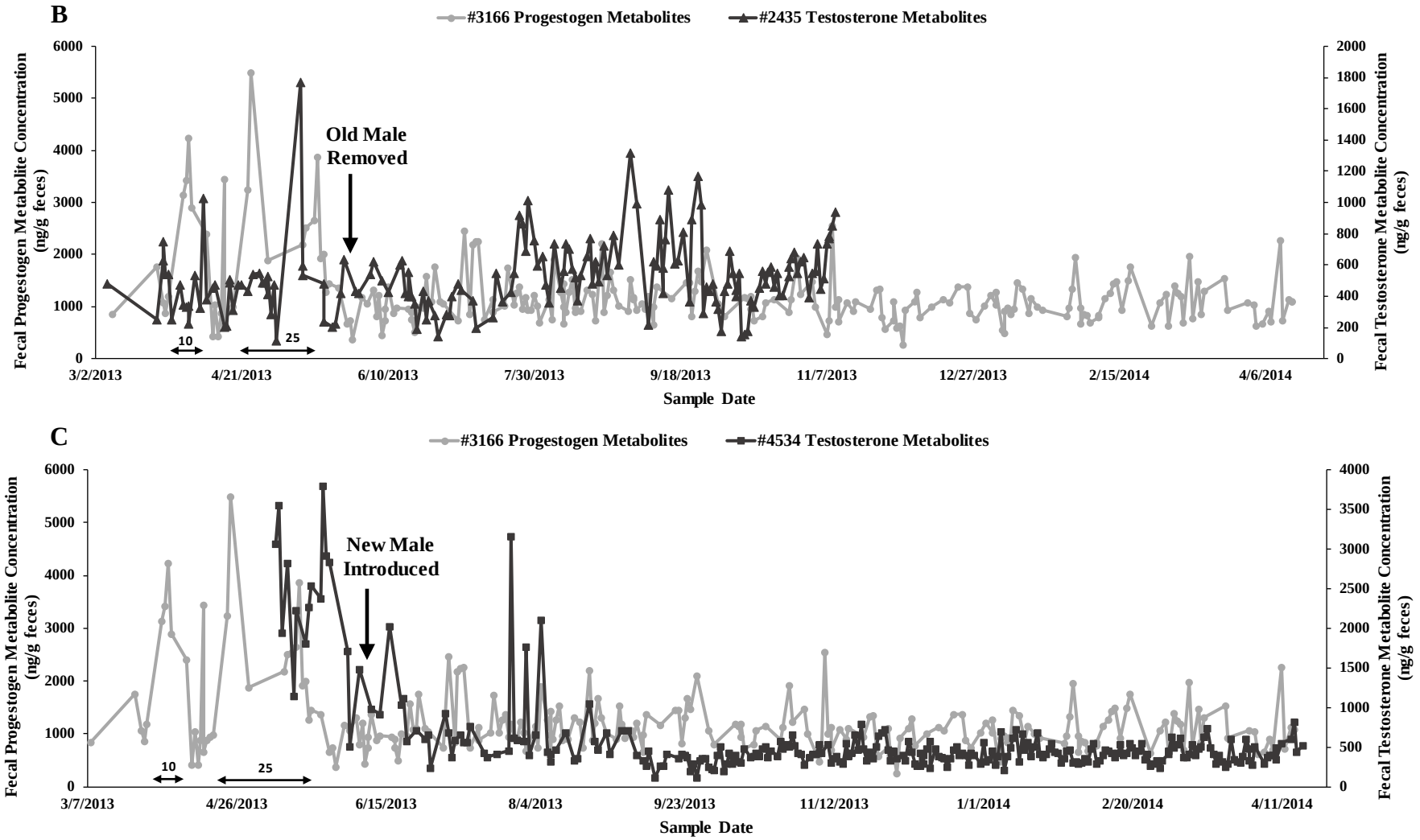


Figure A-3 continued.

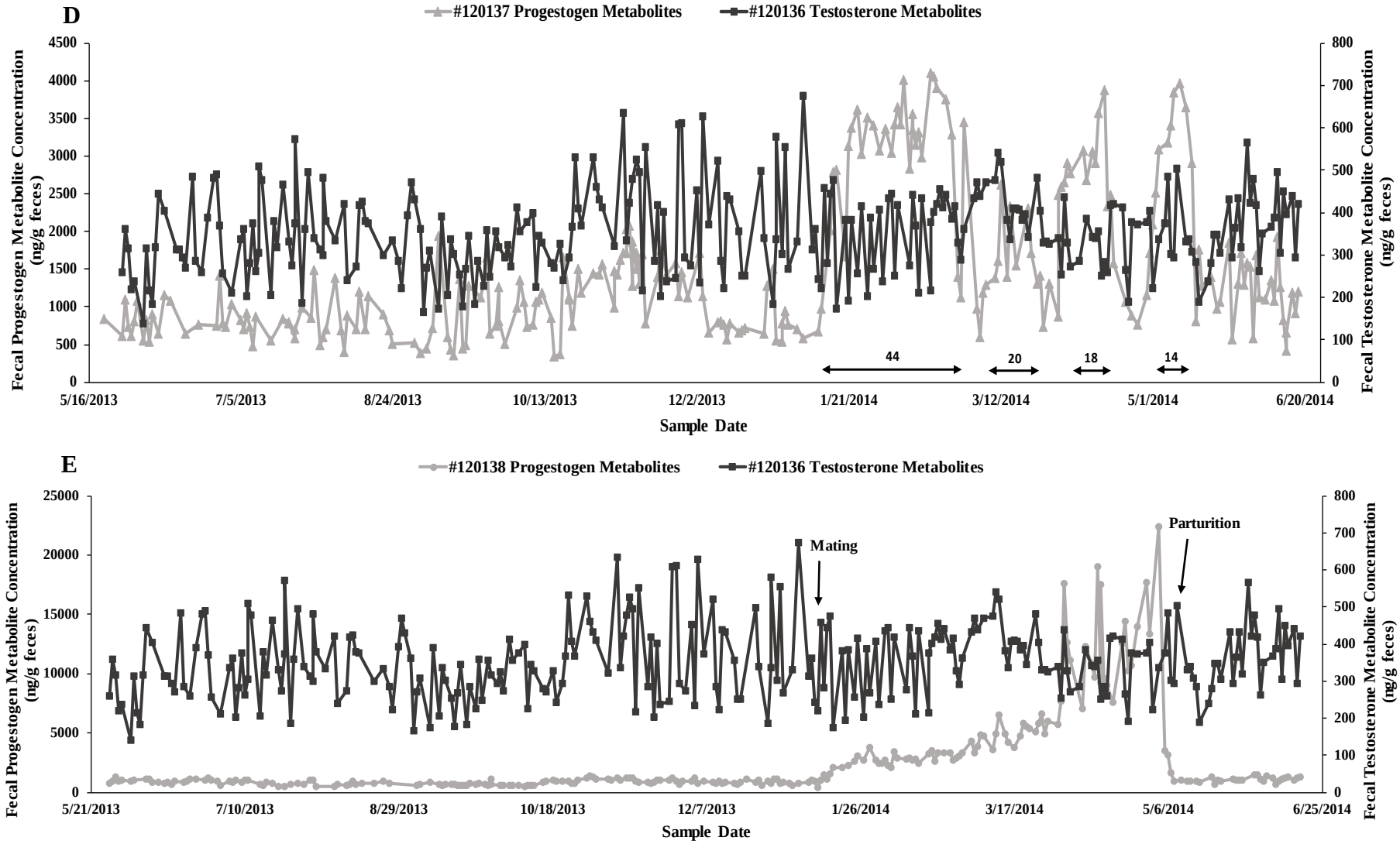


Figure A-3 continued.

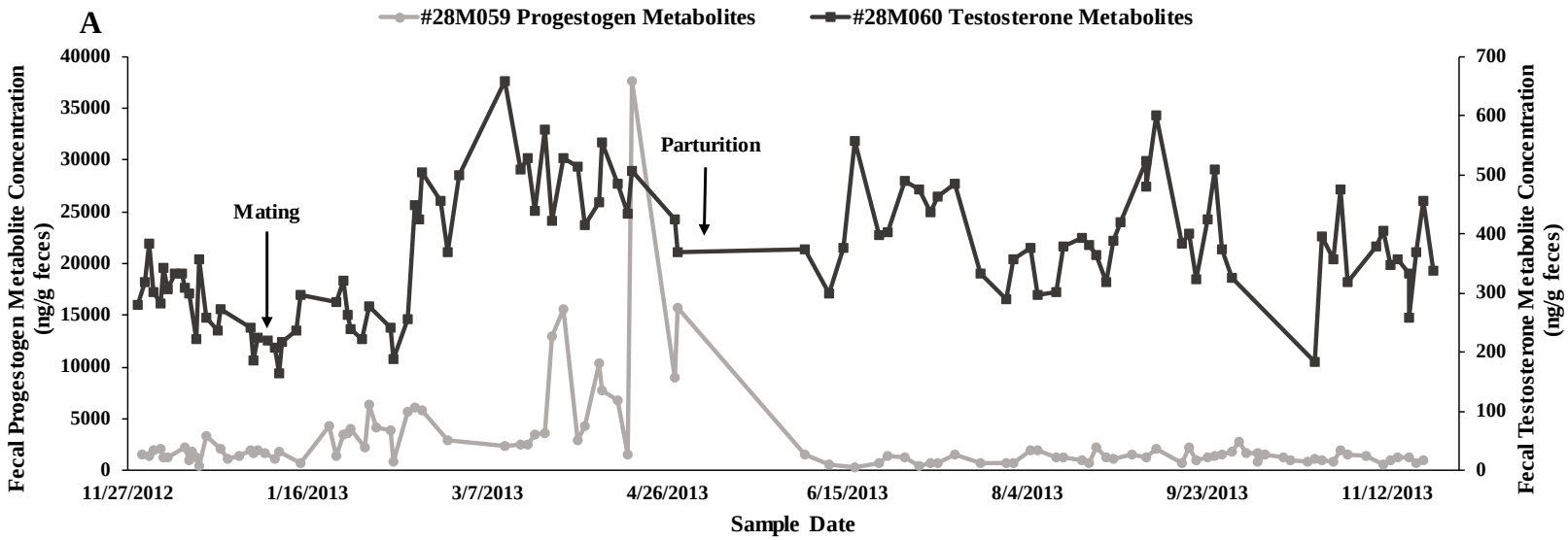
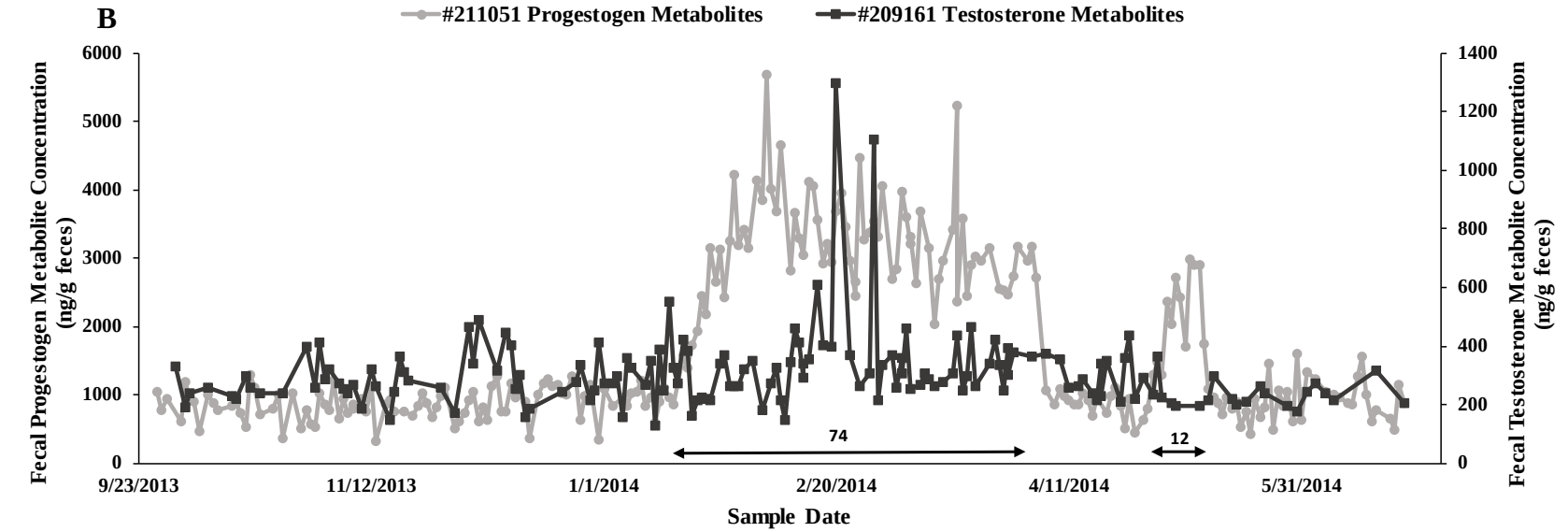


Figure A-4. Profiles of fecal progestogen and testosterone metabolite concentrations (ng/g feces) for RRH pairs/groups in the control (C) treatment group. Including: (A) #28M059 (●) and #28M060 (■) at Cheyenne Mountain Zoo in Colorado Springs, CO from May 2015 to June 2016, (B) #211051 (●) and #209161 (■) at Columbus Zoo in Columbus, OH from November 2012 to November 2013, and (C) #774809 (▲) and #774408 (■) and (D) #775816 (●) and #774408 (■) at Oklahoma City Zoo in Oklahoma City, OK from May 2013 to October 2014. Estrous cycles are designated by arrows along the x-axis with number of days of the estrous cycle noted above the arrows. Significant events including mating and parturition are designated by vertical arrows.



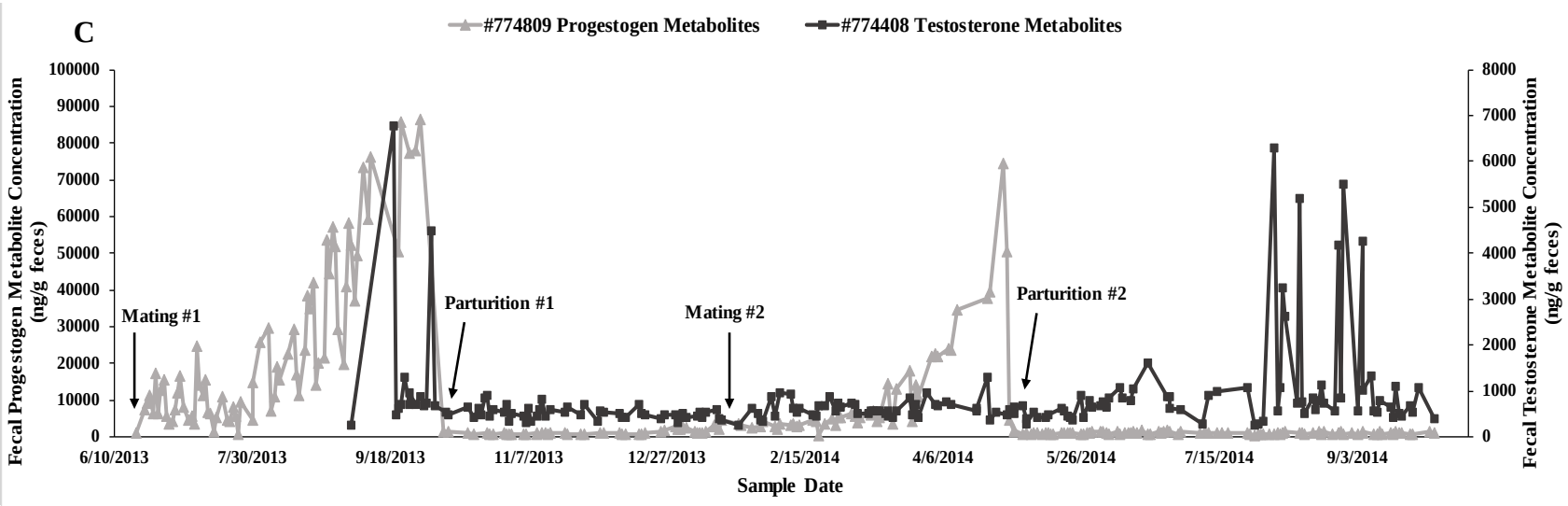


Figure A-4 continued.

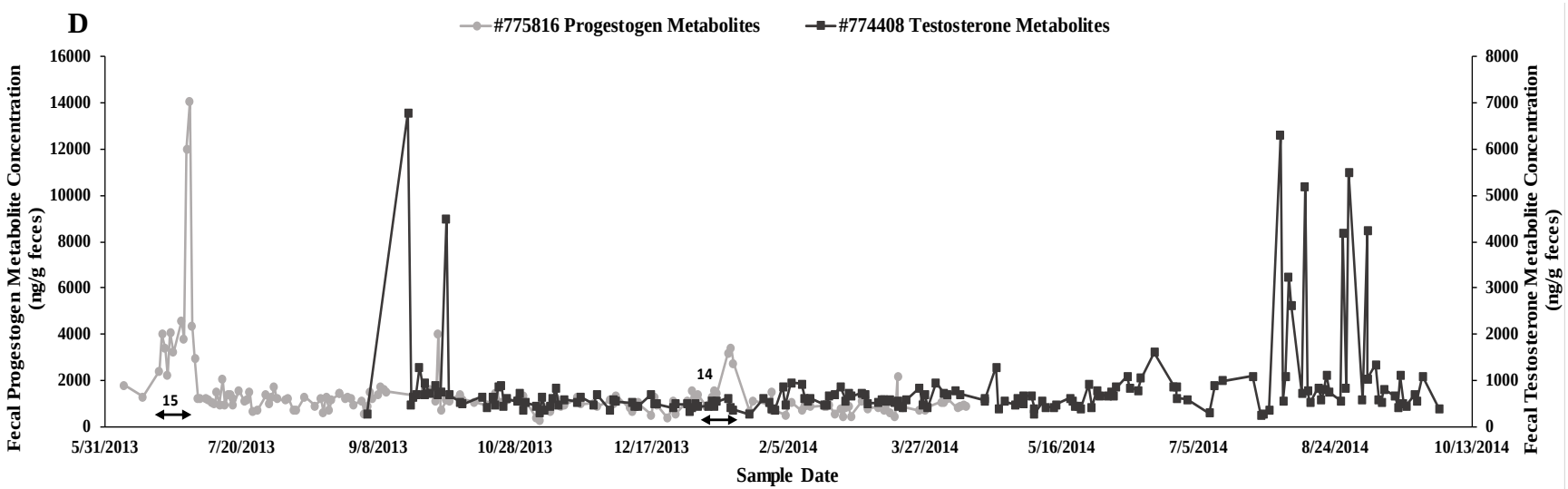


Figure A-4 continued.

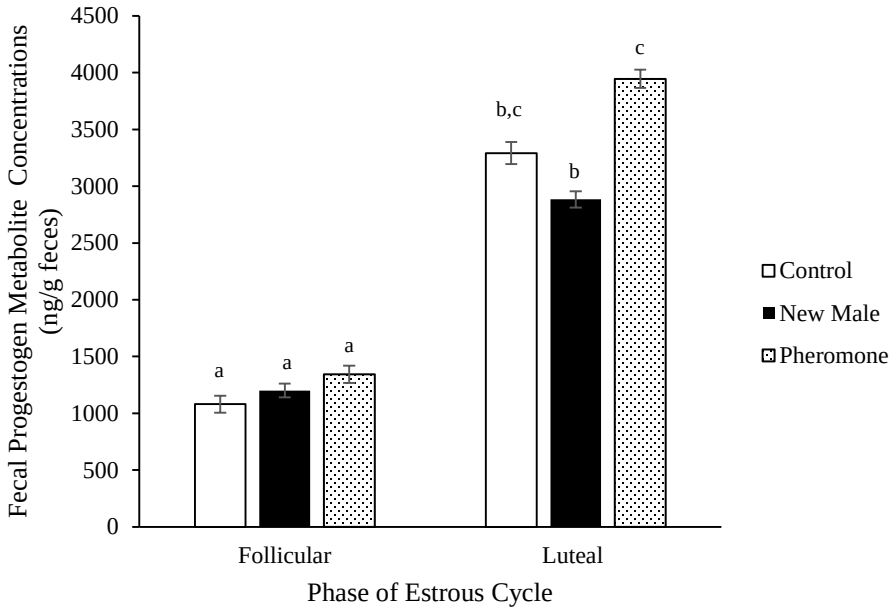


Figure A-5. Fecal progestogen metabolite concentrations (ng/g feces) for non-pregnant females during the follicular and luteal phases of the estrous cycle. There was an overall significant difference in the treatment*phase interaction term (p<0.05). Bars with no common superscript are significantly different (p<0.05).

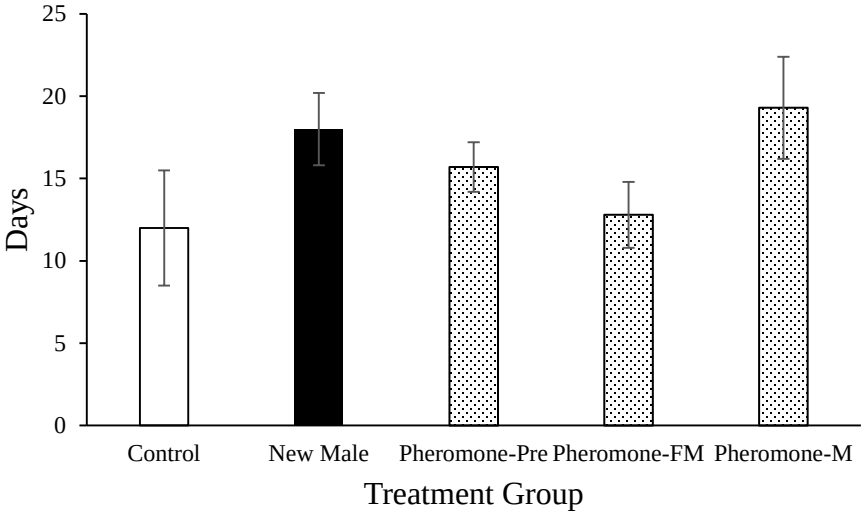


Figure A-6. Estrous cycle length in days. No significant difference in estrous cycle length amongst treatment groups (p>0.05).

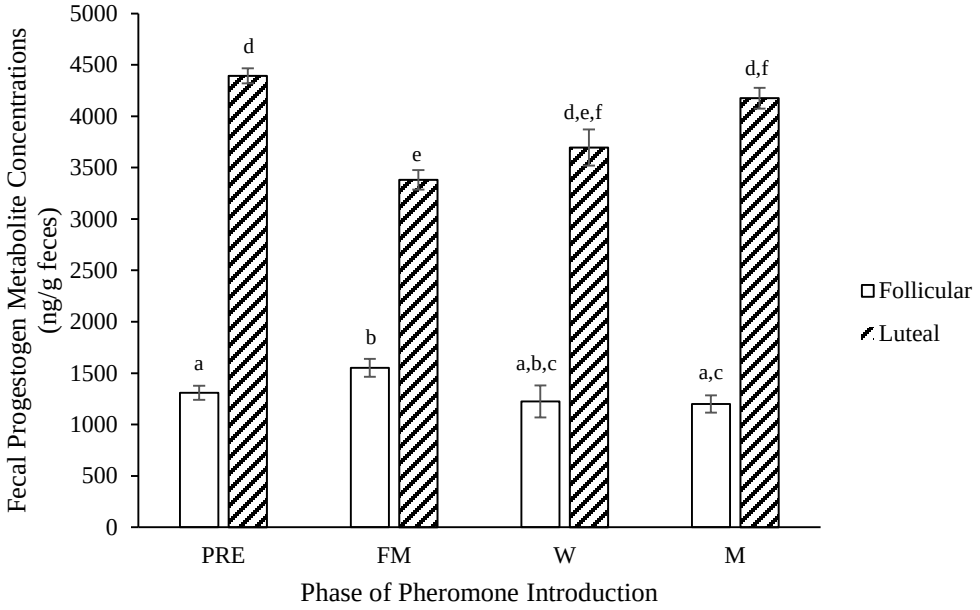


Figure A-7. Fecal progestogen metabolite concentrations (ng/g feces) for pheromone females during the follicular and luteal phases of the estrous cycle during each stage of the pheromone exposure trial. There was an overall significant difference in the treatment*phase interaction term ($p<0.0001$). Bars with no common superscript are significantly different ($p<0.05$). PRE: pre-urine pheromone introduction, FM: during female urine pheromone exposure, W: washout period with no pheromones present, M: during male urine pheromone exposure.

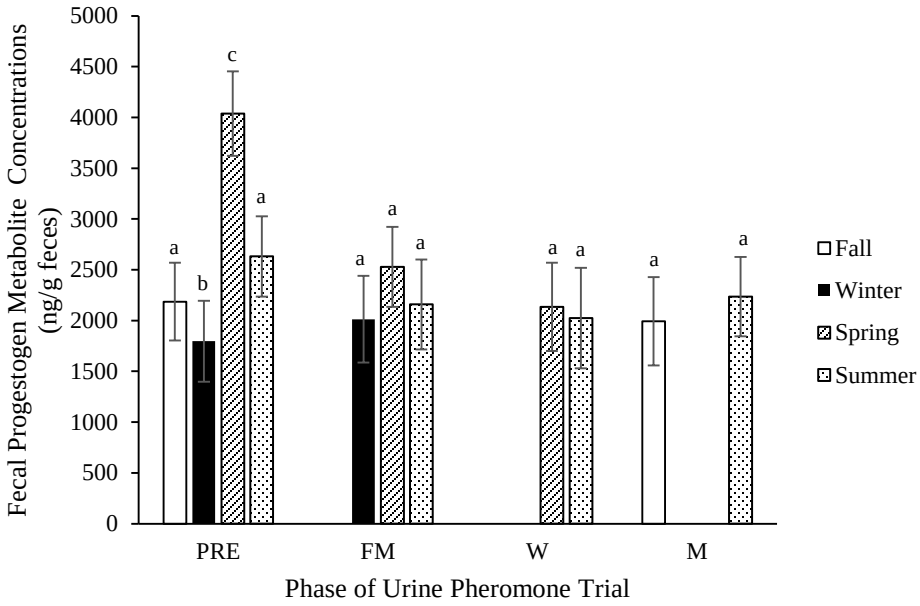


Figure A-8. Fecal progestogen metabolite concentrations (ng/g feces) for females in the pheromone treatment group for each phase of the urine pheromone trial during the four seasons. There was an overall significant difference in the treatment*season interaction term ($p<0.0001$). Within each phase, bars with no common superscript are different ($p<0.05$). PRE: pre-urine pheromone introduction, FM: during female urine pheromone exposure, W: washout period with no pheromones present, M: during male urine pheromone exposure.

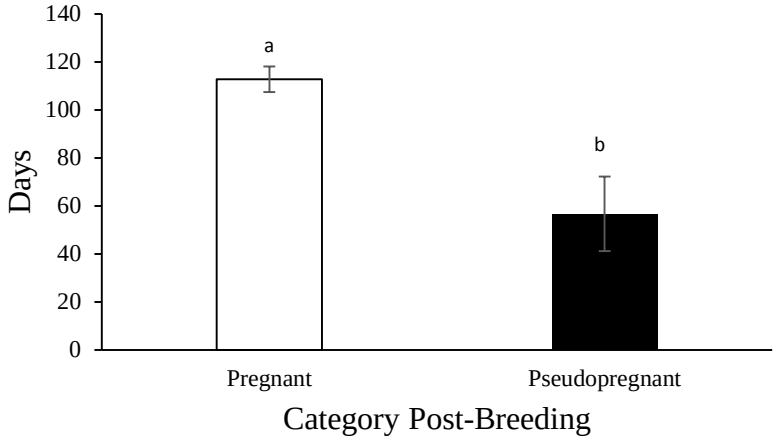


Figure A-9. Gestation length in pregnant females that carried to term (pregnant) and in females that did not (pseudopregnant/pregnancy loss). Pseudopregnant/pregnancy loss females had elevated fecal progestogen concentrations for significantly less time post-breeding than pregnant females (p<0.0009). Bars with no common superscript are significantly different (p<0.05).

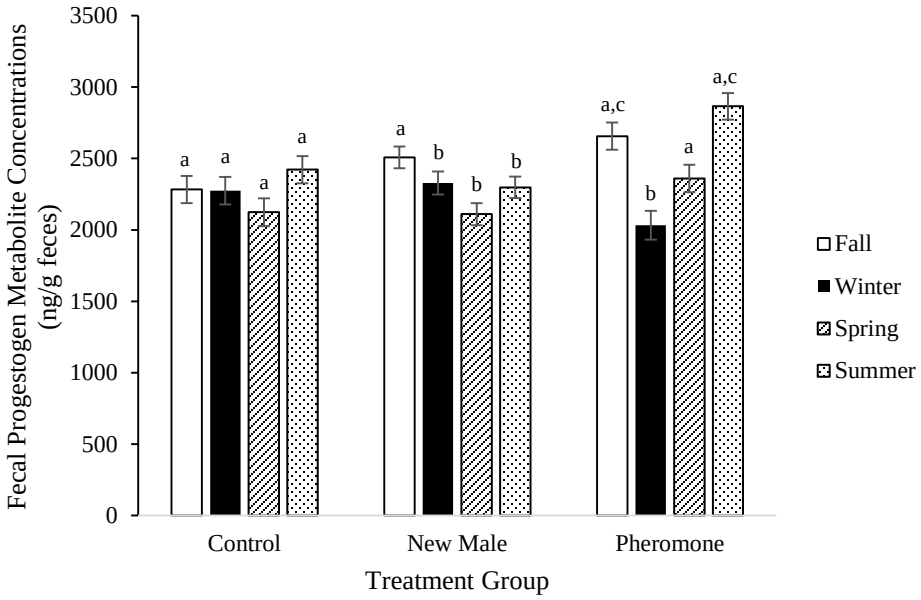


Figure A-10. Fecal progestogen metabolite concentrations (ng/g feces) for non-pregnant females during the four seasons. There was an overall significant difference in the treatment*season interaction term (p<0.05). Within treatment groups, bars with no common superscript are different (p<0.05).

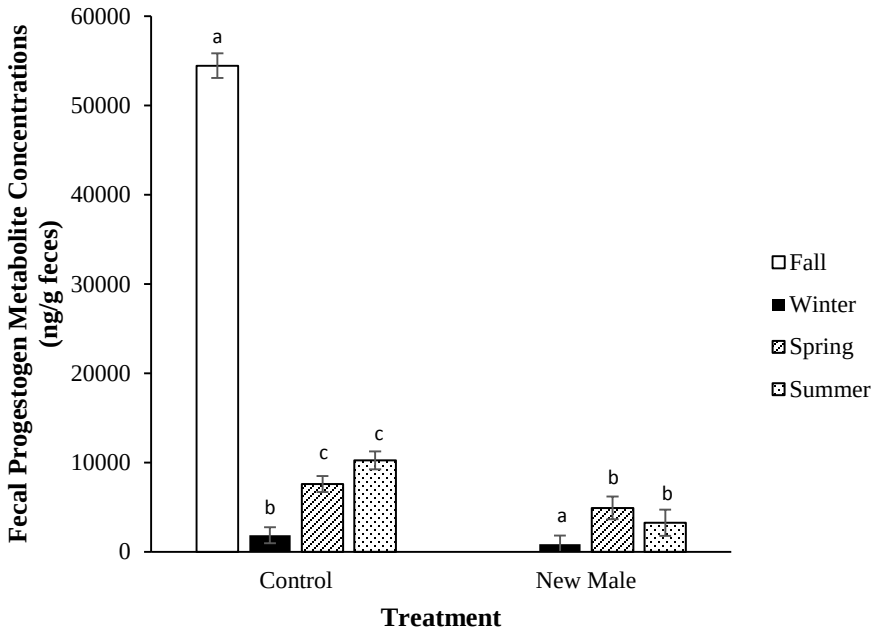


Figure A-11. Fecal progestogen metabolite concentrations (ng/g feces) for pregnant and pseudopregnant/pregnancy loss females during the four seasons. There was no significant difference in the treatment*season interaction term ($p>0.05$). Within treatment groups, bars with no common superscript are different ($p<0.05$).

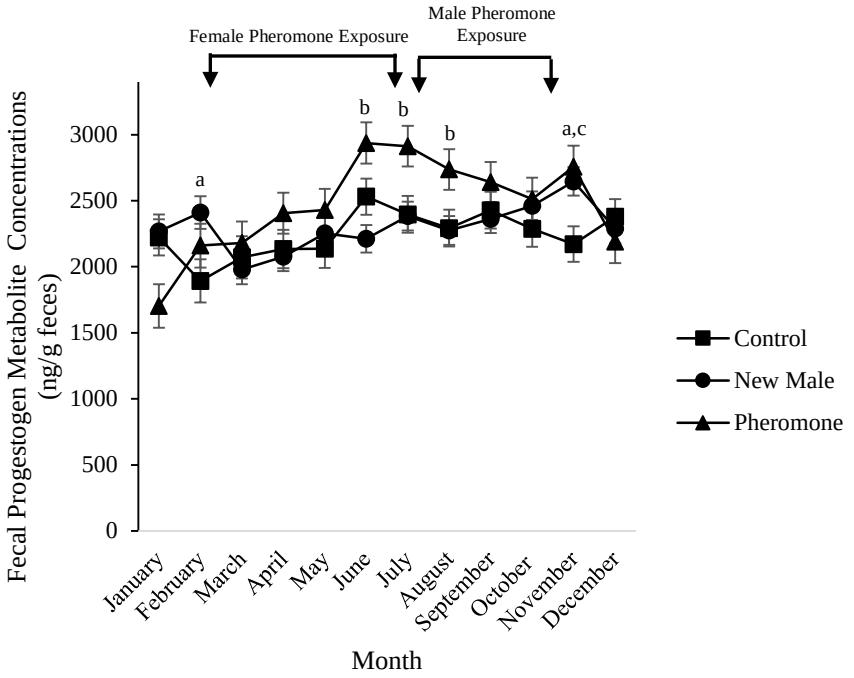


Figure A-12. Fecal progestogen metabolite concentrations (ng/g feces) by month for non-pregnant females. Approximate introduction times for female and male urine pheromone exposure for the pheromone treatment group indicated by arrows above graph. There was an overall significant difference in the treatment*month interaction term ($p<0.0001$).

[a]indicates significant difference between control and new male treatment groups ($p<0.05$).
[b]indicates a trend towards significant difference between pheromone and new male ($p<0.1$).
[c]indicates a trend towards significant difference between control and pheromone ($p<0.1$).

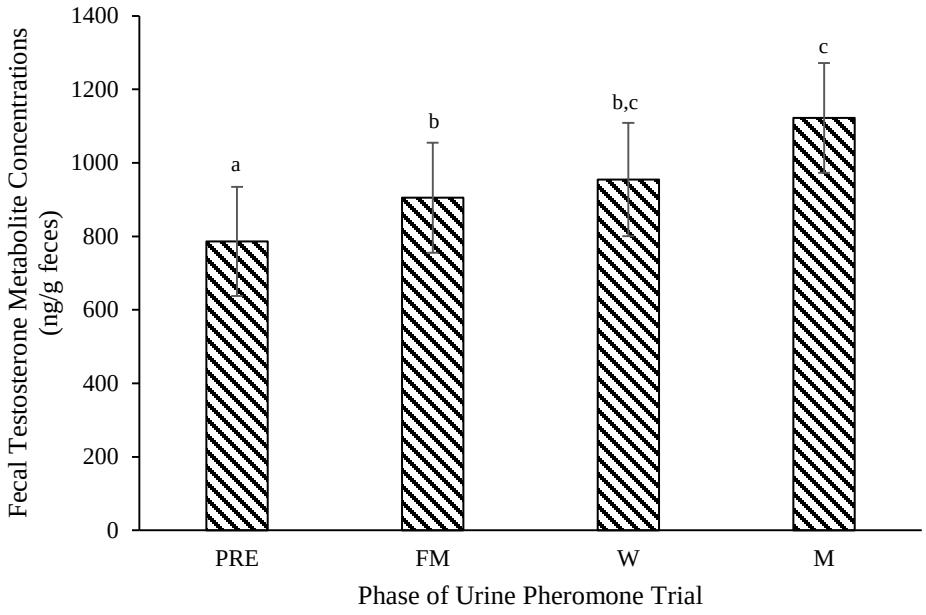


Figure A-13. Fecal testosterone metabolite concentrations (ng/g feces) for the pheromone treatment group during each phase of the urine pheromone trial. There was an overall significant difference in the treatment*phase interaction term ($p<0.0001$). Bars with no common superscript are different ($p<0.05$). PRE: pre-urine pheromone introduction, FM: during female urine pheromone exposure, W: washout period with no pheromones present, M: during male urine pheromone exposure.

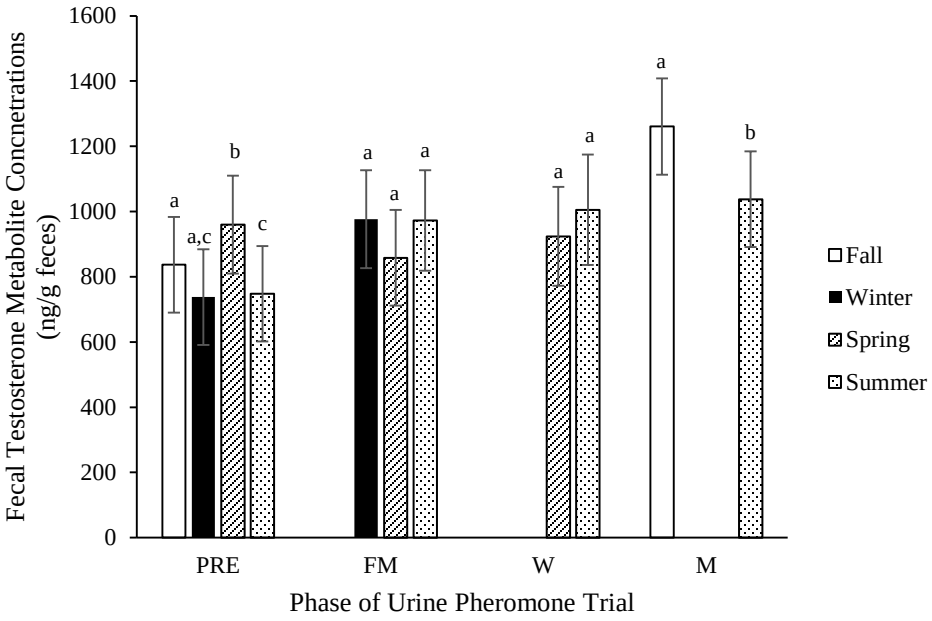


Figure A-14. Fecal testosterone metabolite concentrations (ng/g feces) for males in the pheromone treatment group for each phase of the urine pheromone trial during the four seasons. There was an overall significant difference in the treatment*season interaction term ($p<0.05$). Within each phase, bars with no common superscript are different ($p<0.05$). PRE: pre-urine pheromone introduction, FM: during female urine pheromone exposure, W: washout period with no pheromones present, M: during male urine pheromone exposure.

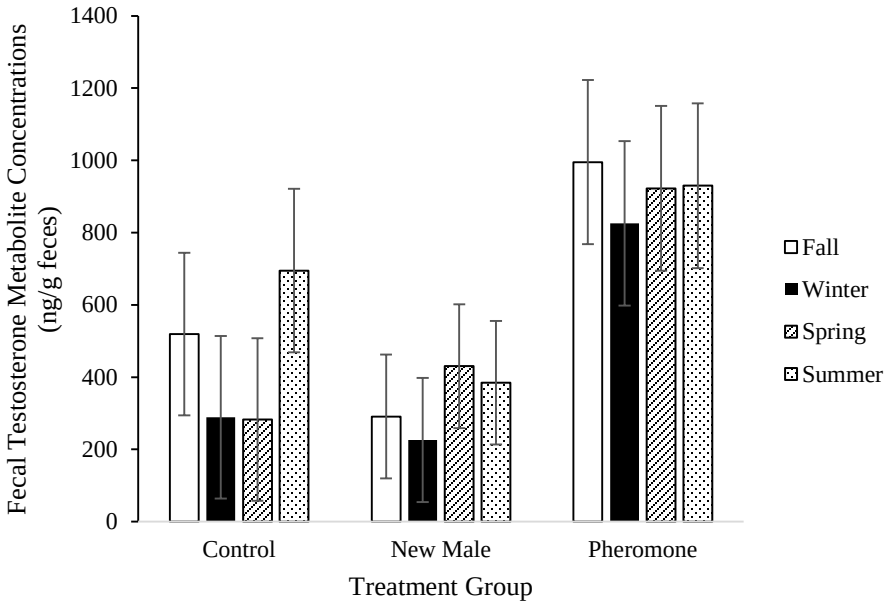


Figure A-15. Fecal testosterone metabolite concentrations (ng/g feces) for each treatment group during the four seasons. There was no significant difference in the treatment*season interaction term (p>0.05).

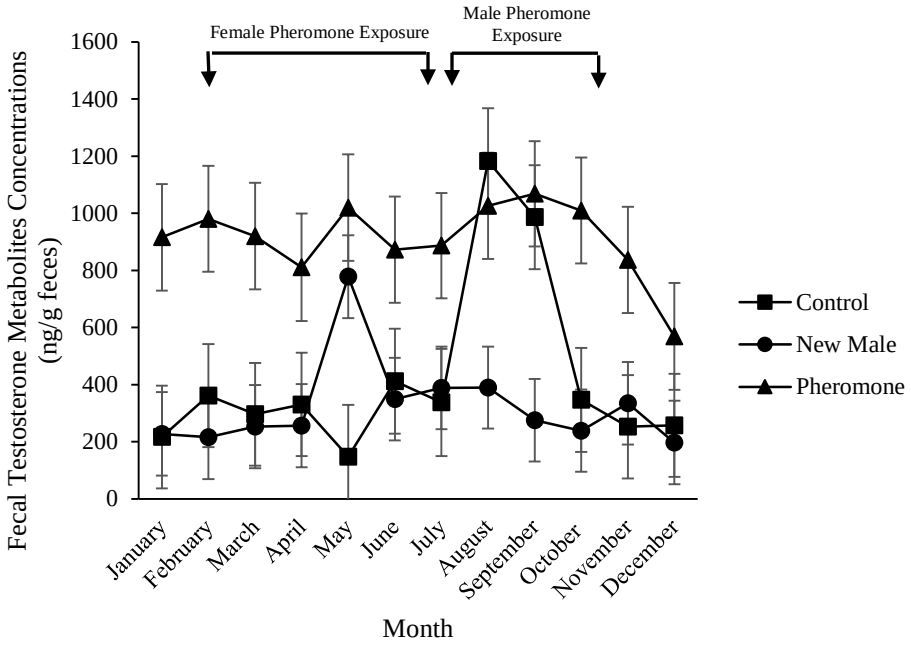


Figure A-16. Fecal testosterone metabolite concentrations (ng/g feces) for each treatment by month. Approximate introduction times for female and male urine pheromones for the pheromone treatment group indicated by arrows above graph. There was an overall significant difference in the treatment*month interaction term ($p<0.0001$).