## ABSTRACT

BYRON, MICHAEL JOSEPH. Immunoglobulin J Chain as a Fecal Biomarker for Understanding the Reproductive Physiology of the Female Cheetah (*Acinonyx jubatus*). (Under the direction of Dr. Paul Mozdziak).

The North American cheetah population serves as both an insurance population for their rapidly decreasing wild cohorts as well as a research population to understand the unique biology of this species. After breeding, cheetah females frequently experience a non-pregnant luteal phase, or 'pseudopregnancy,' where progesterone levels match those found in pregnant females for the first ~55 days of gestation, but parturition does not occur. This occurs in approximately 30-65% of matings. Little is known about the intrauterine physiology of the cheetah, including embryo differentiation, implantation and the development of the placenta. Relaxin and prolactin monitoring are informative for pregnancy determination in closely related species, but the requirement of urine and/or serum samples for these assays limits widespread use in the cheetah. A non-invasive method, such as fecal monitoring, allows the application of novel technologies across a large number of females at numerous holding facilities and avoids the need of anesthesia for sample collection, which is contraindicated in potentially pregnant individuals. The monitoring of excreted biomarkers in the feces may be useful for tracking pregnancy, and could provide a method for distinguishing the gravid and non-gravid states in the cheetah. Immunoglobulin J chain (IgJ) is a molecule that is involved in the activation of the secretory immune response. IgJ has been found to be indicative of pregnancy in the cheetah using fecal monitoring, and was used in this study to track pregnancy related events in the species.

Levels of IgJ in fecal samples were increased in the first two weeks post-breeding in females that were naturally bred compared with females that were exogenously stimulated to ovulate. This supports the suggestion that an immune response may be occurring to the presence

of seminal plasma in the reproductive tract. This response may act as an immune priming event, helping to promote active maternal tolerance of the paternal antigens of the fetus upon implantation. Monitoring of IgJ also revealed that the window of implantation in the cheetah occurs at 19-21 days post-breeding. In addition, increased secretory activity of the placenta appears to occur at week 8 post-breeding. The continued development of a tool for monitoring IgJ in the cheetah could help to improve husbandry conditions and management of the species in human care.

# Immunoglobulin J Chain as a Fecal Biomarker for Understanding the Reproductive Physiology of the Female Cheetah (*Acinonyx jubatus*)

by
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# CHAPTER 1

## Understanding the Reproductive Physiology of the Female Cheetah (*Acinonyx jubatus*) to Improve *ex situ* Conservation Efforts

### Introduction

The wild cheetah (*Acinonyx jubatus)* population has significantly decreased due to habitat fragmentation and human conflict with livestock and game farmers (Marker, Dickman, Jeo, Mills, & Macdonald, 2003; Durant et al., 2017). Accurate information on the species' decline, however, is difficult to determine due to their wide range and low density. Currently, the cheetah is listed by the IUCN as vulnerable, with an estimated global population of ~7100 individuals remaining in the wild (Durant et al., 2017). There is a lack of information on populations that are distributed outside of protected areas, though, possibly resulting in an underestimation of spatial threats and population decline on unprotected lands (Durant et al., 2017). Because of this, it is important to maintain a sustainable *ex situ* population that can be used as an insurance population for these wild animals. This insurance population can serve as a reservoir for the species upon potential future reintroduction efforts and can be useful as a research population to aid in understanding the unique physiology of the cheetah. However, successful breeding of cheetahs in human care has multiple challenges. The greatly reduced genetic diversity of the species has resulted in inbreeding depression, and is thought to contribute to the impairment of genes mediating immune defenses (O'Brien, Johnson, Driscoll, Dobrynin, & Marker, 2017) and the increased incidence of malformed spermatozoa (Crosier et al., 2007). The development of a sustainable population will require maintaining and improving the genetic diversity of the *ex situ*

cheetah population over future generations, ensuring that fitness-linked alleles persist, and promoting a strategy to improve the health, resiliency, and adaptability of the species *in situ* upon reintroduction (Lacy, 2013). While free-ranging cheetahs also face challenges as a result of the low genetic diversity of the species, they do not seem to face the same reproductive challenges as cheetahs in human care. Studies have shown that wild female cheetahs have excellent reproductive success and are highly fecund, with 95% of females observed producing cubs (Laurenson, Caro, & Borner, 1992; Kelly et al., 1998).

Several studies have found that semen quality is very poor among both *in situ* and *ex situ* males, with low concentrations and a high proportion of malformations of the spermatozoa (Crosier et al., 2007; Wildt et al., 1983; Wildt et al., 1987). Male cheetahs in human care begin producing sperm around 14 months of age, as determined by semen collection via transrectal electroejaculation (Crosier et al., 2007). Fecal androgen metabolite concentrations increase from 12-18 months of age, indicating puberty in the male (Maly, Edwards, Farin, Koester, & Crosier, 2018). The changes in androgen metabolites in cheetahs under human care correlate with observations of wild males, as adolescents separate from their mother during this same age range (Kelly et al., 1998). Male cheetahs appear to be reproductively active throughout their lifespan, and cheetahs as old as 15 years have been documented to produce sperm (Wildt et al., 1993). The greatly reduced genetic diversity of the cheetah is thought to contribute to the high incidence of teratospermia (Crosier et al., 2007; Wildt et al., 1993), although it is clear that there is a complex relationship between inbreeding and male ejaculate traits (Terrell et al., 2016). Examples of pleiomorphisms include abnormal acrosome development, microcephaly, sperm midpiece malformations, and flagellum coiling (Crosier et al., 2007). These malformations represent a significant hurdle to the establishment of pregnancy, and highlight the challenges that the

cheetah must overcome in order to successfully reproduce. However, it is still possible for males with low quality sperm to produce a pregnancy (Lindburg, Durrant, Millard, & Oosterhuis, 1993), and there is no significant difference in sperm quality among proven and unproven breeders (Wildt et al., 1993; Terrell et al., 2016). Several studies in the early 1990s (Wildt et al., 1993; Munson, 1993) also found no differences in reproductive anatomy, health, or genetics among adult females regardless of breeding success. There may be some aspects of management of the *ex situ* population that affect the success of reproduction, such as enclosure size, mate choice, or exposure to the public (Koester et al., 2015; Koester et al., 2017a).

Early methods for monitoring the hormone activity of cheetahs under human care were first developed using blood samples from anesthetized cheetahs to observe ovarian activity (Wildt et al., 1993). Using a radioimmunoassay (RIA), circulating progesterone concentration was successfully employed to determine luteal status. In recent years it has become an important goal to devise a non-invasive method to create a more efficient monitoring tool. As a result, fecal steroid hormone monitoring has proven to be the most successful and widely used approach for evaluating reproductive events in the cheetah. An RIA was first used to assess estradiol and progestogen metabolites in feces to determine estrus cyclicity and ovulatory events in the cheetah (Brown et al., 1996). Development of enzyme immunoassays (EIAs) to monitor fecal steroid hormones was a crucial technological advancement for the widespread use of hormone assays, now commonly used to monitor estrous, determine ovulation, and monitor pregnancy in wild species (Brown, 2006). As a result, great advances in the understanding of the reproductive physiology of the cheetah have been made (Brown et al., 1996). Mean estrogen metabolite concentrations and estrogen peaks have been found to be increased at 25-30 months of age, suggesting that the age of puberty in female cheetahs under human care is 25-30 months of age

(Maly, Edwards, Koester, Farin, & Crosier, 2019). Free-ranging cheetahs have been found to give birth to their first litter at an average age of 29 months following a three month gestation (Kelly et al., 1998), indicating a similar age of puberty in wild females. Wild females are seen to be reproductively viable as late as 12 years of age (Durant et al., 2010). If pregnancy does not occur by ~7-8 y of age, females will develop uterine pathologies, making the establishment of pregnancy difficult after 9 years of age (Crosier et al., 2011). Cheetahs are non-seasonal breeders confirmed by fecal estrogen monitoring and year-round cub production. In females under human care, the duration of estrus varies from two to six days, exhibiting a widely variable cycle length (Brown et al., 1996). It has been determined by RIA and EIA monitoring, as well as vaginal cytology, that cycle length can vary among individuals from 5 to 30 days, and variations within an individual can occur as well (Brown et al., 1996; Crosier, Comizzoli, Koester, & Wildt, 2017; Asa et al., 1992). Cheetahs in human care have also been seen to undergo periods of acyclicity, as hormone monitoring has uncovered periods of ovarian inactivity (Brown et al., 1996; Crosier et al., 2017). There appear to be no negative effects of ovarian inactivity, as females have been observed to successfully mate and give birth to litters after prolonged periods of acyclicity (Crosier et al., 2017). Cheetahs, like most domestic and non-domestic felids, are induced ovulators, and require one or more copulations during times of breeding receptivity for ovulation to occur (Brown et al., 1996). In induced ovulators, mating causes neuronal stimulation and release of luteinizing hormone (LH) (Senger, 2005). In felids, frequent matings ensure that adequate hypothalamic stimulation occurs, causing a preovulatory release of gonadotropin releasing hormone (GnRH). This stimulates a large LH surge, resulting in final follicular maturation and ovulation (Brown et al., 2006). In the domestic cat (*Felis catus*), mating induces the LH surge within minutes as determined by serum analysis, and ovulation then occurs within

24 to 48 hours as determined by ovarian observation at laparotomy (Shille, Munrot, Farmer, Papkoff, & Stabenfeld, 1983). In non-domestic felids, ovulation induction has been studied by fecal steroid analysis. Steroid hormone monitoring of 24 cycling female cheetahs without exposure to males showed no increase of fecal progestogen metabolites corresponding to increased estrogen values, an indication that ovulation did not occur (Brown et al., 1996). A laparoscopic examination of ovarian tissue also strongly suggested that the cheetah was an induced ovulator (Wildt et al., 1993). However, Brown et al. (1996) found that spontaneous ovulation occurred twice in 184 monitored estrous cycles (1.1%). Interestingly, both of these instances occurred after translocation of the female and subsequent visual and auditory contact with a male, indicating that ovulation may be triggered in some individuals by physical stimuli unrelated to mating, although this appears to be rare in the cheetah.

The administration of exogenous hormones to stimulate ovulation in the cheetah has been employed for over two decades, and artificial insemination after induced and timed ovulation has successfully produced cubs in the cheetah (Howard et al., 1992) and multiple other felid species (Howard and Wildt, 2009). In an early study, an intramuscular injection of equine chorionic gonadotropin (eCG) was given to stimulate follicular development, followed by an intramuscular injection of human chorionic gonadotropin (hCG) to induce ovulation (Howard, Roth, Byers, Swanson, & Wildt, 1997). More recent techniques include the use of an oral progestin to suppress ovarian function and subsequently stimulate follicular development, followed by gonadotrophin therapy to stimulate ovulation, resulting in oocytes and corpora lutea of high quality (Crosier et al., 2011; Crosier et al., 2017). A laparoscopic intrauterine technique is then used for semen deposition following ovulation. This laparoscopic technique is useful because of the lower number of motile sperm needed compared to intravaginal deposition (Howard and

Wildt, 2009), an advantage due to the poor semen quality that is often seen in the cheetah. Improvements in regulating ovulation have the potential to increase the viability and success of artificial insemination in the cheetah, and can help advance oocyte recovery, in vitro fertilization (IVF), and embryo transfer of genetically valuable females. A recent study assessed the quality of laparoscopically aspirated oocytes following eCG and hCG stimulation and found that high quality oocytes could be recovered from older (>8 yrs) cheetahs and successfully fertilized by IVF using cryopreserved sperm (Crosier et al., 2011). These findings present exciting possibilities for embryo transfer in future conservation efforts. Older, nulliparous females that have been unsuccessful in establishing pregnancy may have a greater genetic value to the *ex situ* population than multiparous females. Oocytes could be recovered from these older cheetahs, fertilized by IVF, and transferred to young, overrepresented fecund surrogates, increasing the genetic diversity of the population. The success of IVF using cryopreserved sperm also indicates the potential of sperm banking for gamete preservation of underrepresented or free-ranging males.

**Pregnancy and Non-Pregnant Luteal Phase in the Cheetah**

Currently there is a much greater knowledge of the uterine environment, as well as the process of implantation and maternal recognition of pregnancy, in human and livestock species than there is in non-domestic felids such as the cheetah. In humans, the molecule that is responsible for maternal recognition of pregnancy is human chorionic gonadotropin (hCG) (Senger, 2005). This molecule is luteotrophic, stimulating the corpus luteum to continue to produce progesterone until placental progesterone can adequately support the pregnancy (Fazleabas, Kim, & Strakova, 2004). Similarly, in the mouse, prolactin acts as a luteotrophic

hormone (Galosy and Talamantes, 1995). Other species have molecules for maternal recognition of pregnancy that are classified as antiluteolytic, as molecules such as estrogen in the sow and interferon tau in the ewe and cow prevent breakdown of the corpus luteum in these species (Bazer and Thatcher, 1977; Spencer, Ott, & Bazer, 1996), which is necessary to maintain pregnancy. The most relevant studies in carnivores, from which we can attempt to draw comparisons for understanding early pregnancy in the cheetah are studies conducted on domestic cats (*Felis catus*) and dogs (*Canis familiaris*). However, there remains a paucity of information on maternal recognition of pregnancy in carnivores. It appears that there is no signal from the conceptus that is required for maternal recognition of pregnancy in the cat or dog (Senger, 2005). In the domestic cat, corpora lutea form after breeding and ovulation. If fertilization is successful, the corpora lutea are sustained throughout gestation (~65 days). If fertilization is not successful, the corpora lutea and serum progesterone levels are sustained for around 35-45 days post-breeding. The result of this is the exhibition of a non-pregnant luteal phase or "pseudopregnancy" which is also common in the cheetah.

In the domestic cat, fertilization takes place in the oviduct up to 48 hours after ovulation, and implantation occurs at day 13-14 post-breeding (Denker, Eng, & Hamner, 1978). No luteotrophic signal has been discovered in the cat (Thatcher et al., 1991). Luteal activity in a pregnant cat can sustain progesterone production for around 35-45 days post-breeding (Tsutsui et al., 2009). An extragonadal source of progesterone production from the placenta can sustain the pregnancy throughout the remainder of gestation. Another study has confirmed that maternal decidual cells of the placenta act as a supplemental source of progesterone during pregnancy in queens, with progesterone production increasing with gestational age (Siemieniuch et al., 2012). There are similarities in progesterone production in wild felids and the domestic cat, with the

placenta supplementing progesterone production following day 55 post-breeding through parturition.

In the domestic cat and dog, the early conceptus is bordered by a temporary choriovitelline placenta, which is then replaced by a chorioallantoic placenta (Leiser and Koob, 1993; Aralla, Groppetti, Caldarini, Cremonesi, & Arrighi, 2013). Following implantation, the placenta can be classified as zonary endotheliochorial, consisting of a zonary girdle surrounded by two paraplacental areas consisting of many small hematomas (Leiser and Koob, 1993). This paraplacental area is also referred to as the pigmented zone, and is thought to be important in the transport of iron from the mother to the fetus (Senger, 2005). Distal to the paraplacenta is the allantochorion, a zone of transparent tissue with poor vascularity. The materno-fetal junction is the most intimate at the zonary girdle, and this is the point where most of the nutrient and gas exchange between the fetus and the mother takes place. As the syncytiotrophoblast invades the endometrial epithelium, an endotheliochorial placenta develops. The endometrial epithelium and the underlying interstitium are completely eroded, directly exposing the maternal capillaries to the chorionic epithelium (Enders and Carter, 2006). While no studies could be found regarding the mechanism of placentation in the cheetah or other non-domestic felids, the placenta in non-domestic felids is generally considered to be zonary with an endotheliochorial materno-fetal interface (Terio, St. Leger, & McAloose, 2018; Brown, 2011).

Following implantation and the development of the placenta, the cheetah has a gestation with a documented range of 89-98 days (mean length: 93 days) (Eaton, 1970). Litters usually range in size from 3-5 cubs, although litters as small as one cub and as large as 9 cubs have been recorded (Laurenson et al., 1992; Crosier, personal communication). In the wild, many cheetah cubs do not survive to adulthood, and often fall prey to larger predators such as the lion and the

hyena. Cheetah cubs are also often affected by the impairment of genes mediating the immune response due to the low genetic diversity in the species, and may succumb to disease. As a result of these issues, many cheetah cubs in the wild do not survive past three months, and average litter size at independence is only 2.1 cubs (Kelly et al., 1998). While wild cheetahs face problems with the survival of their cubs, they do not seem to encounter the reproductive issues that plague cheetahs in human care. A 1993 study of 68 female cheetahs under human care found that 16.2% had abnormalities that affected reproductive capacity, including degenerate ovaries, single ovary, and an abnormally small vaginal opening (Wildt et al., 1993). Cheetahs under human care may experience prolonged periods of estrogen fluctuation and cyclicity without being bred, leading to frequent uterine remodeling. This may have a negative effect on the uterus, and could lead to a number of pathologies as the cheetah ages known as asymmetric reproductive aging, and may decrease the chances that a female can establish a pregnancy at a later age. Free-ranging cheetahs, however, do not experience this frequent uterine remodeling that many cheetahs under human care experience, as they are usually bred during estrus and do not experience prolonged cyclicity. Incidences of uterine hyperplasia have been observed in 90% of females over the age of 9 years, and a female is more likely to develop this pathology as the time from her last litter increases (Crosier et al., 2011). Reducing the exposure of females to frequent uterine remodeling has been recommended by allowing the female to be bred to establish pregnancy at an early age, as well as regularly every two to three years to match the behavior of the animals in the wild.

One of the most challenging issues of breeding the cheetah in captivity is the common occurrence of successful copulations that fail to produce offspring. “Pseudopregnancy” or a non-pregnant luteal phase, has occurred in up to 60% of recorded matings in North American zoos in

recent years [range 30-60% 2013 – 2018] (Grisham, Lackey, & Spevak, 2013). One current method of monitoring pregnancy in the cheetah relies on fecal hormone monitoring of progestagen metabolites using EIA (Brown et al., 1996). Progesterone levels remain low until breeding or exogenous hormone administration, when ovulation is stimulated and levels rise (Brown et al., 1996; Crosier et al., 2011). While ovulation is confirmed to occur in bred females based on observation of luteal tissue (Howard et al., 1992) and progestogen metabolite monitoring (Brown et al., 1996), luteal activity maintains high levels of progestogens whether or not fertilization/embryo development/implantation have actually occurred. Luteal activity will remain elevated for approximately 55 days after breeding in non-pregnant individuals, after which progestogen levels will return to baseline (Figure 1). In pregnant females, levels will remain elevated until parturition at approximately 93 days. Radiography of non-anesthetized cheetahs has also been used as an accurate assessment of pregnancy, revealing the presence and number of cubs after 55 days post-breeding (Ware et al., 2016). Using these two methods of pregnancy determination, it is impossible to distinguish between pregnant and non-pregnant individuals until after 55 days post-breeding. Methods for the determination of pregnancy at a point earlier than 55 days post-breeding can help to improve husbandry conditions and management in the cheetah. Early pregnancy confirmation can help animal care staff prepare and mobilize resources for the birth of cubs and create a suitable environment for the mother. Females confirmed to be experiencing a non-pregnant luteal phase can be returned to the breeding population for pairing with a new mate, or can be further observed for reproductive pathologies and referred for infertility treatment.

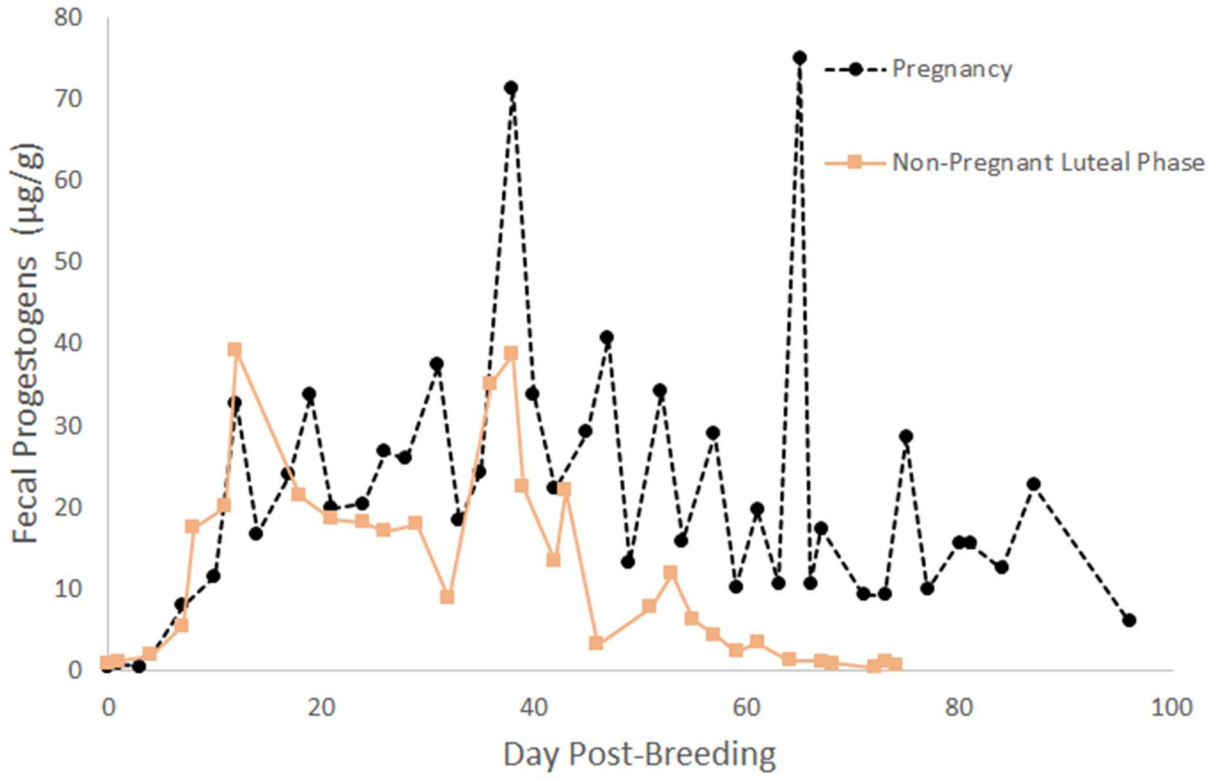


**Figure 1. Fecal progestogen metabolite comparison.** Fecal progestogens remain elevated until parturition around day ~93 during pregnancy. During a non-pregnant luteal phase, fecal progestogens remain elevated for approximately 55 days, then return to baseline levels.

**Methods of Monitoring Gestational Status**

Prolactin (PRL) increases in the domestic cat in the third trimester as measured by a canine PRL RIA, likely for the induction of mammary gland growth and lactogenesis (Banks, Paape, & Stabenfeldt, 1983; Kooistra and Okkens, 2002). An increase in PRL levels may also play an important role as a luteotropin late in gestation for both the cat and the dog (Onclin, Silva, Donnay, & Verstegen, 1993; Onclin and Verstegen, 1997; Okkens, Bevers, Dieleman, & Willemse, 1990; Okkens, Dieleman, Bevers, Lubberink, & Willemse, 1986). PRL concentrations begin to rise in mid-gestation, and increase through parturition and remain elevated during

lactation. It appears to be the main factor sustaining progesterone secretion by the corpus luteum in the dog, as PRL removal using dopamine agonists leads to luteolysis and a decrease in progesterone secretion, resulting in abortion (Okkens et al., 1986). PRL is unchanged in cats undergoing a non-pregnant luteal phase (Banks et al., 1983) and dogs (Reimers, Phemister, & Niswender, 1978) during a non-pregnant luteal phase. The pattern of PRL production has not been determined in the cheetah, but is likely similar to the domestic cat. Increased PRL levels during the third trimester may act as a luteotropin in the cheetah, but PRL monitoring does not improve upon current progestogen metabolite monitoring methods in distinguishing between a pregnant and non-pregnant luteal phase.

Urinary relaxin is a protein hormone that has been found to be indicative of pregnancy in several mammalian species such as the dog (O'Byrne and Steinetz 1976), the domestic cat (de Haas van Dorsser, Swanson, Lasano, & Steinetz, 2006), as well as the Sumatran rhinoceros and the Asian and African elephant (Steinetz, Brown, Roth, & Czekala, 2005). While there are certainly species-specific differences in the structure of relaxin and the method of its secretion, relaxin-based assays in the domestic dog and the cat have consistently distinguished between pregnancy and non-pregnancy (Steinetz, Goldsmith, & Lust, 1987; Stewart and Stabenfeldt, 1985). Relaxin was detectable by RIA by day 21 post-breeding in the urine of the domestic cat, and by day 28 in a non-domestic felid species, the Arabian leopard (*Panthera pardus nimr*). Comparatively, for both the domestic cat and the Arabian leopard, relaxin was not detected in non-pregnant mated individuals (de Haas van Dorsser et al., 2006). In the domestic cat, implantation occurs at day 13-14 post-breeding (Denker et al., 1978). In felid species, relaxin is likely produced specifically by the placenta, and plays an important role as a placental growth factor (Addiego, Tsutsui, Stewart, & Stabenfeldt, 1987). Relaxin has an effect on various other

biological functions during pregnancy, including uterine endometrial growth and development, and the softening of pelvic connective tissue in preparation for parturition (Sherwood, 2004). The timing of implantation likely varies between species and is unknown for all non-domestic felids. While the exact time of implantation in the cheetah is unknown, it can be hypothesized that the window of implantation is at a similar point proportionally in gestation as the domestic cat, which would correspond to a window of day 18-21 post breeding. Relaxin is detectable by day 18-22 post-breeding in the domestic cat, indicating the elongation of the trophectoderm and increased development of placental tissue (Addiego et al., 1987). It can also be speculated that functional placental tissue becomes established at around day 28 in the Arabian leopard, when a rise in urinary relaxin levels is seen (de Haas van Dorsser et al., 2006). The Arabian leopard has an average gestation length of 97.5 days (Cunningham and Gross, 2000), which is comparable to the 95 day gestation of the cheetah (Eaton, 1970). Harris et al. (2008) discovered a rise in urinary relaxin in the cheetah at day 34 post-breeding, the earliest sample collected. Earlier and consistent testing in the cheetah could reveal the first measurable rise in urinary relaxin, indicating the establishment of functional placental tissue.

While a diagnostic serum or urine assay to distinguish between pregnancy and a non-pregnant luteal phase at day 28 post-breeding would be a great improvement on current methods, further studies are needed to refine this promising approach. The sensitivity of de Haas van Dorsser's relaxin assay (2006) to non-domestic felids appears to be variable, indicating variations in the structure and expression of relaxin across different species (Harris, Steinetz, Bond, Lasano, & Swanson, 2008). Development of a homologous assay for feline relaxin may increase the sensitivity, and may be needed for early pregnancy detection in wild felids. Either serum or urine samples are required for use in this assay, which are rarely available from

potentially pregnant cheetah females. Limited females are trained for voluntary blood collection, and most cheetah breeding facilities lack the infrastructure to allow opportunistic urine collections. Also, factors such as pH, volume, or standardization may affect urinary testing, influencing assay sensitivity and possibly introducing matrix effect variables (Berzofsky, Epstein, & Berkower, 1989; Wood, 1993). All of these variables reveal the need for further studies to refine these methods on a species-specific basis and to develop a reliable technique using readily available and easy to collect feces, as collection and monitoring requires no special training and introduces no stress to the animal. Fecal monitoring has become a common method used in the steroid hormone analysis of wild felids, and is a logical tool for determination of pregnancy in the cheetah. A recent study found that a fecal prostaglandin $F_{2\alpha}$ metabolite (PGFM), a hormone that may play a key role in regulation of the corpora lutea, can be used as an indicator for pregnancy in multiple felid species (Denhard et al., 2012). The source of the prostaglandins could be the utero-placenta complex (Denhard et al., 2012), as PGFM was previously found to be a placental signal in the Iberian lynx (Finkenwirth, Jewgenow, Meyer, Vargas, & Denhard, 2010). An increase in PGFM levels was found to occur after 48 days post-breeding in pregnant cheetahs, with levels remaining high and peaking before parturition. PGFM levels remained at baseline in cheetahs undergoing a non-pregnant luteal phase (Denhard et al., 2012). While PGFM monitoring was successful as a tool for pregnancy diagnosis in the cheetah, it can only confirm pregnancy after around ~50 days post-breeding, and does not improve upon current methods. However, Denhard et al. (2012) importantly confirmed that biomarkers produced as a result of intrauterine events involving the placenta could be utilized to diagnose early pregnancy in the cheetah.

**Table 1.1 Pregnancy determination in the cheetah.** The most common methods currently used can diagnose pregnancy in the cheetah at around ~48-55 days post-breeding.

| Biomarker | Method of Testing | Species | Confirmation of Pregnancy (day post-breeding) |
|---|---|---|---|
| *Progestogens* | Fecal | Cheetah | ~55 |
| *Prolactin* | Serum | Domestic cat | ~45 |
| | | Cheetah | Unknown |
| *Relaxin* | Serum/Urine | Arabian Leopard | 28 |
| | | Cheetah | 34 |
| *PGFM* | Fecal | Cheetah | 48 |
| *Immunoglobulin J chain* | Fecal | Cheetah | 28 |

**Immunoglobulin J Chain as a Fecal Biomarker**

Advances in mass spectrometry and other proteomic analyses have led to the discovery of many biomarkers that are useful for monitoring physiological status, including in a clinical setting as tools for diagnosis or treatment (Burke, 2016). Recent studies utilizing serum analyses have provided a greater understanding of early events that are involved in the establishment of pregnancy and the development of preeclampsia in women (Kenny et al., 2010; Myers et al., 2013). Studies have also been conducted using circulating biomarker proteins as a determinant of pregnancy in the domestic dog (Kuribayashi et al., 2003) and several wild canid species (Bauman, Clifford, & Asa, 2008). Recently, two-dimensional gel electrophoresis and tandem mass spectroscopy have been used to identify fecal biomarkers of pregnancy in another non-domestic carnivore species, the polar bear (Curry, Stoops, & Roth, 2012). Similarly, fecal biomarkers with potential roles in early pregnancy establishment in the cheetah were identified

and isolated using commercially available antibodies (Koester, Wildt, Maly, Comizzoli, & Crosier, 2017). An increased abundance of the biomarker immunoglobulin J chain (IgJ) was found in pregnant cheetahs compared to cheetahs undergoing a non-pregnant luteal phase within four weeks after breeding. Furthermore, assessment of IgJ levels was used to successfully predict pregnancy in over 80% of females tested (Koester et al., 2017b).

IgJ is an important component of the secretory immune system, and is expressed in high levels in immunocytes of secretory tissue (Brandtzaeg, 1974; Brandtzaeg, 1983). This molecule is a small polypeptide of 15 kDa that serves to regulate polymer formation of Immunoglobulin A (IgA) and Immunoglobulin M (IgM), modulating the secretory activity of these molecules (Halpern and Koshland, 1970). IgJ links two IgA monomers, creating a dimer, and links five IgM molecules, converting it to a pentameric form (Mestecky, Zikan, & Butler, 1971). The polymerization of these immunoglobulins provides them with high avidity, allowing them to agglutinate foreign invaders such as bacteria and viruses. Incorporation of IgJ is required for these polymers to be transported through epithelial cells and into mucosal secretions, as IgJ binds the configurations to the secretory component, allowing exocrine transfer (Johansen, Braathen, & Brandtzaeg, 2001). IgJ, IgA, and IgM are expressed by plasma cells that infiltrate the lamina propria of mucosa. Upon polymerization and secretion, IgJ acts directly to mediate the secretory release of the immunoglobulins in which it is incorporated into the lumen of the mucosal surface, allowing for an immune response in the mucosal secretion (Asano and Komiyama, 2011). IgJ has been found to be a critical component in the binding process, and secretory transfer is not possible without the molecule (Vaerman et al., 1998; Johansen et al., 2001). The secretory immunoglobulins that IgJ helps to activate are the line of defense against pathogens that may enter through the mucosa, and play an integral role in responding to foreign antigens.

While this process has been demonstrated in the human intestinal tract (Asano and Komiyama, 2011; Johansen et al., 2001), more research of this mechanism needs to be conducted in the mucosal epithelium of the genitourinary tract. IgA-producing plasma cells have been found in the lamina propria regions of the female reproductive tract (McGhee and Fujihasha, 2012) and secretory IgA has been found to be present in cervico-vaginal secretions in multiple species, including the human (Tourville, Ogra, Lippes, & Tomasi Jr, 1970), and the rat (Parr and Parr, 1989), indicating the expression and activity of IgJ in the reproductive tract. In female reproductive tissue, the polymeric immunoglobulin receptor (pIgR), an essential component of exocrine transfer for IgJ-positive immunoglobulins, has been found to be influenced by sex hormones, with low expression in the vagina and high expression in the fallopian tubes and the uterus (Boyaka and Fujihashi, 2019). Several studies have also been conducted that have determined that pIgR expression in uterine epithelial cells varies at different points of the estrous cycle in the rat, with elevated levels during proestrus and estrus and low levels during diestrus (Kaushic, Richardson, & Wira, 1995; Kaushic, Frauendorf, & Wira, 1997). These results indicate the potential modulation of secretory immunity at different reproductive states, supporting the potential modulatory role of IgJ during estrus and pregnancy.

IgJ is also extremely important in preventing the activation of the complement response, which is an important part of preventing an inflammatory reaction on a mucosal surface such as the endometrium. Modulation of the immune system away from pro-inflammatory responses and towards non-inflammatory responses is thought to be an important component of the prevention of fetal and placental rejection during pregnancy (Robinson and Klein, 2012). Hexameric IgM, which IgJ is not incorporated into, is up to 20 times more efficient at activating the complement response than IgJ-positive pentameric IgM (Randall, King, & Corley, 1990; Wiersma, Collins,

Fazel, & Shulman, 1998), suggesting that hexameric IgM should be downregulated and pentameric IgM should be upregulated at mucosal surfaces during pregnancy to prevent a negative inflammatory reaction. Similarly, IgJ-positive dimers of IgA appear unable to activate the complement response through the classical pathway (Russell, Reinholdt, & Kilian, 1989). This is an important characteristic of these immunoglobulins, as only the polymers with low complement activating potential are secreted to mucosal surfaces where there is often a high amount of foreign antigens, preventing an inflammatory response and helping to limit tissue damage that could result from interactions with these antigens. IgJ is responsible for modulating the activity of the molecules that produce an inflammatory response, suggesting that it may play a role in promoting the secretion of non-inflammatory immunoglobulins to mucosal surfaces during sensitive physiological states such as pregnancy. IgJ is likely upregulated during times of immune challenge during pregnancy in the cheetah, allowing for proper immune defense at the site of fetal-maternal interaction while preventing harm to the developing embryo.

It is apparent that there are a variety of factors that are involved in the modulation of immune response as a result of pregnancy. These responses allow for the development of a fetus that is semiallogeneic, and promote the tolerance of foreign antigens present during placental invasion of the endometrium. It is clear that there are wide variations in the specific responses produced by different species. Although little research on placental recognition and tolerance in carnivores has been conducted, it is logical to theorize that immune molecules could play an important role during placental development in the cheetah. Because the uterine endometrium is covered by a mucosal lining, the secretory mucosal immune response could be modified in response to the complex physiological changes of pregnancy. IgJ is a strong candidate for tracking these modifications that are associated with pregnancy establishment in the cheetah, as

it has been found to be a biomarker of early pregnancy in the cheetah capable of distinguishing pregnant from non-pregnant females (Koester et al., 2017b). IgJ has been found to have increased abundance within four weeks after mating (Koester et al., 2017b), which significantly improves upon current methods of determining pregnancy in the cheetah by day 55 of gestation (Brown et al., 1996). IgJ levels can be successfully determined using fecal monitoring, which provides a non-invasive method that prevents physical capture or anesthesia, events that may have a negative effect on reproduction in the cheetah. Further knowledge of the role IgJ plays in pregnancy, through temporal tracking of this biomarker throughout gestation, will help researchers to better understand the physiological events that occur that result in either reproductive success or failure. IgJ abundance also has the potential to be used as a diagnostic tool for predicting pregnancy early after breeding. Understanding IgJ may improve *ex situ* husbandry and management practices for the endangered cheetah, resulting in proper resource preparation for pregnant females and allowing non-pregnant females to be returned to the breeding population.

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# CHAPTER 2

## Immunoglobulin J Chain as a Fecal Biomarker for Understanding the Reproductive Physiology of the Female Cheetah (*Acinonyx jubatus*)

### Introduction

The cheetah (acinonyx jubatus) is listed as vulnerable by the IUCN, with an estimated population of ~7100 individuals in the wild, with numbers continuously decreasing due to habitat fragmentation and human conflict (Durant et al., 2017). Because of the threats to wild cheetahs, an *ex situ* population is critical to serve as an insurance population should the wild cheetah's numbers diminish further. The *ex situ* population can serve as a reservoir for the species, and could potentially be used for reintroduction efforts in the future. This population is also invaluable for research purposes, allowing for studies that cannot be conducted on *in situ* populations due to the scarcity of the species in the wild.

The cheetah is an induced ovulator, meaning that mating or exogenous hormones are necessary for ovulation to occur (Brown et al., 1996). While the reproductive events of the domestic cat have been studied in depth (Denker, Eng, & Hamner, 1978; Leiser & Koob, 1993), little is known about the intrauterine physiology following breeding in wild felids, including the timing of events such as embryo differentiation, implantation, and placentation. Interestingly, cheetahs in human care often encounter reproductive challenges that their wild counterparts do not. Many breedings among cheetahs in human care are unsuccessful, and no offspring are produced as a result. This event is known as a non-pregnant luteal phase, or a "pseudopregnancy," and occurs in up to 30% to 60% of matings in North American zoos in

recent years (2014-2019). In these unsuccessful matings, ovulation is confirmed by detectable rises in progestogen metabolites in feces, serum or urine (Brown et al., 1996; Wildt et al., 1993). The concentration of these metabolites is elevated for approximately 55 days, and during this time the hormonal profile of a non-pregnant individual is indistinguishable from a pregnant individual. The high prevalence of a non-pregnant luteal phase after breeding in cheetahs under human care has greatly reduced the reproductive potential of the *ex situ* population, and has contributed to the challenge of reaching sustainability due to the impact on the genetic diversity of the population.

Recent advances in mass spectrometry and other proteomic analyses have led to the study of excreted biomarkers as diagnostic or treatment tools in a clinical setting (Burke, 2016). The production of some biomarkers has been shown to be affected by reproductive events, and certain biomarkers have been found to indicate physiological status such as pregnancy in the domestic dog (Kuribayashi et al., 2003) and several wild canid species (Bauman, Clifford, & Asa, 2008). Recently, methods have been developed for the identification of fecal biomarkers of pregnancy in the polar bear (Curry, Stoops, & Roth, 2012), and another study in the cheetah identified fecal biomarkers with potential roles in early pregnancy establishment using commercially available antibodies (Koester, Wildt, Maly, Comizzoli, & Crosier, 2017). This study identified a novel biomarker Immunoglobulin J chain (IgJ) with increased levels in pregnant individuals, and was able to distinguish between pregnant and non-pregnant cheetahs in the 4 weeks following breeding. IgJ is a small polypeptide that serves to regulate polymer formation of Immunoglobulin A (IgA) and Immunoglobulin M (IgM), modulating the secretory activity of these molecules (Brandtzaeg, 1983; Halpern & Koshland, 1970). IgJ functions to provide high levels of avidity to IgA and IgM, and facilitates their exocrine transfer to mucosal

surfaces (Johansen, Braathen, & Brandtzaeg, 2001). The secretory immunoglobulins that IgJ helps to activate are integral in the response to foreign antigens at surfaces such as the endometrium, and IgJ expression is likely modulated by the unique physiological status of pregnancy. Placental factors that are absent in females undergoing a non-pregnant luteal phase may act to modulate the maternal immune response and affect IgJ abundance, allowing for IgJ monitoring as a method for distinguishing between the gravid and non-gravid states in the cheetah. This current study was established to evaluate the temporal patterns of IgJ abundance over the course of pregnancy in the cheetah in order to determine the timing of intrauterine events that result in either a successful pregnancy or a non-pregnant luteal phase. Changes in IgJ abundance may indicate maternal immune modulation, and could reveal certain events such as implantation and placental development that occur during the establishment of pregnancy early after breeding.

## Methods

### *Animals*

This study was conducted according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The female cheetahs included in this study (n=19 individuals) were all housed at accredited Association of Zoos and Aquariums (AZA) institutions within the United States. All subjects were born *ex situ* and managed according to the guidelines developed by the Cheetah Species Survival Plan (SSP). The animals included in the study were female adults from 2 to 12 years of age (mean ± standard error of the mean (SEM) = 5.85 ± 0.5 y). The animals in this study were fed a diet of commercial beef or horse-based meat product (Milliken Meat Products Ltd or Central Nebraska Packing,

Inc.) five days per week, with supplements that included whole rabbit, beef and horse bone, or organ meat. Water was available *ad libitum*.

Fecal samples were collected from females that were naturally bred according to SSP breeding management recommendations and from females receiving exogenous gonadotropins to stimulate ovulation. Exogenous gonadotropin therapy was conducted according to previously published methods (Howard, Roth, Byers, Swanson, & Wildt, 1997; Pelican, Wildt, Pukazhenthi, & Howard, 2006), and included the stimulation of follicular development (with equine chorionic gonadotropin), followed by stimulation of ovulation (with human chorionic gonadotropin or porcine luteinizing hormone). Pregnancy was confirmed by the birth of offspring, and non-pregnant luteal phase was confirmed by an increase in progesterone metabolite concentration after either natural breeding with no cubs produced or exogenous gonadotropin administration.

***Sample Collection and Preparation***

All samples were collected non-invasively by fecal pickup, and did not require specific IACUC approval. Fecal samples were collected from enclosures approximately 3-4 times weekly. Only fresh samples (deposited within 24h) were chosen. Approximately 50 g of sample was bagged and immediately stored in a -20°C freezer. Individual fecal samples were then lyophilized (VirTis, 35L Ultra Super XL-70, Gardiner, NY) for four days. After drying, the samples were crushed and transferred to individually labeled tubes. Reproductive cyclicity and ovulation were confirmed by steroid hormone metabolite analysis. Fecal samples underwent a steroid hormone metabolite extraction according to Brown et al. (1994) and Koester et al. (2017). Extraction efficiency was determined by the addition of radiolabeled $^{3}$H-progesterone prior to shaking extraction. The mean extraction efficiency (±SEM) was found to be 73.9% ± 0.3% for all samples.

In order to extract total protein from fecal samples, weekly pooled samples of 0.5g were created by combining approximately 0.125g of four individual samples in a 15 mL centrifuge tube. Total protein was then extracted from pooled samples as follows. 6 mL of 0.1 M phosphate buffered saline (0.138 M NaCl, 0.0027 M KCl; pH, 7.4) with protease inhibitor (1:1000) was added to the pooled fecal sample, and the mixture was shaken for 30 minutes and centrifuged at 4600 x *g* for 30 min. The supernatant was filtered using a 0.22 µm syringe driven filter unit (Millipore Sigma), and the proteins were then precipitated from the supernatant using a 60% ammonium sulfate saturation. The ammonium sulfate solution was shaken for 30 minutes and centrifuged at 7000 x *g* for 30 min. The protein extract pellet was collected and resuspended in 400 µL of phosphate buffered saline with protease inhibitor. This protein extract solution was then desalted using a 3 kDa Millipore spin column (Amicon Ultra-0.5) and centrifugation at 7400 x *g*. All extraction steps were performed at 4°C. Extracted samples were then run on a Bradford assay (Bio-Rad Protein Assay, Hercules, CA) to determine total protein concentration. Briefly, standard samples for the assay were created by serial dilution at 0.388 mg/mL to 0.012 mg/mL. Protein samples were diluted to 1:30, and 10 µL of each sample was added to a well. 200 µL of Bio-Rad Quick Start™ Bradford Dye Reagent was added to each well, and after 5 minutes the plate was read and the protein concentration was determined using a Dynex MRX plate reader. Differences in steroid hormone and total protein concentrations between pregnant and non-pregnant groups were determined using a Student's T-test in R (version 3.3.2) (R Core Team, 2016), with differences considered significant at $P < 0.05$.

***Steroid Hormone Metabolite Analysis***

Steroid hormone neat extracts were diluted 1:20 to 1:16,000 in phosphate buffer (2.2 M $NaH_2PO_4$, 3.5 M $Na_2HPO_4$, 0.3 M NaCl, H2O; pH, 7.0) and were run for analysis on enzyme

immunoassay (EIA). Estrogen metabolites in diluted fecal extracts were used to determine reproductive cyclicity, and concentrations were determined using an estradiol EIA that has been validated for use in the cheetah (Crosier et al., 2011). Briefly, a polyclonal anti-estradiol antibody (R4972; C. Munro, University of California, Davis, CA) was added to a 96-well microtiter plate and incubated for 12 hours. Diluted samples, standards, and peroxidase-enzyme conjugated 17β-estradiol were added, and the plate was incubated for 2 hours at 23°C. Unbound components were washed off, and a TMB chromogen solution (3,3', 5,5;-tetramethylbenzidine) was added as a substrate. Optical densities of each well on the plate were determined using a microplate reader (Dynex MRX, reading filter 405 nm, reference filter 540 nm). Inter-assay variation was controlled for through the use of two internal controls, and coefficients of variation for all samples in duplicate were <10%.

Progesterone metabolites were used to determine ovulation and the presence of a luteal phase. Concentrations were determined using a progesterone EIA that has been validated for use in the cheetah (Crosier et al., 2011), using a monoclonal progesterone antibody (no. CL42, Quidel Co., San Diego, CA), and an associated peroxidase-enzyme conjugated to progesterone. Plates were prepared and run using the same procedure as the estradiol assay. Internal controls were used to control for inter-assay variation, and coefficients for samples in duplicate were <10%.

***Western Blotting and Protein Quantification***

Total protein samples were diluted to 2 mg/mL in MilliQ water to a final volume of 30 µL. Human recombinant IgJ (Abcam #140727) was used as a positive control at 16.67 µg/mL (1:30 dilution, 0.15 µg of IgJ). Samples were then separated by SDS-PAGE, transferred to a PVDF membrane, blocked with 5% milk, and incubated overnight at 4°C with a primary

antibody (Aviva Systems Biology ARP55440_P050) diluted 1:1000 in 1% milk. This antibody was developed in a rabbit against human recombinant IgJ, and was previously found to be reactive to cheetah IgJ in western blot (Koester et al., 2017). The membrane was then incubated with a secondary antibody (Cell Signaling Technology, Anti-Rabbit IgG HRP-linked antibody, #7074S) diluted 1:2500 in 1% milk, and then incubated with a chemiluminescent substrate (Bio-Rad, Clarity Max Western ECL Substrate, #1705062). Membranes were imaged on a G:Box Chemi XRQ (Syngene). Coomassie staining and image analysis of total protein were conducted in order to serve as a loading control.

Intensity of IgJ abundance was determined using GeneSys Spot Blot analysis of the band occurring within each lane at 18 kDa for each weekly pooled sample. GeneSys Total Lane analysis for each sample of the Coomassie image was used to determine the total protein in the sample as a loading control. A ratio of IgJ intensity to Coomassie intensity was calculated for each pooled sample, as well as for the positive control. Relative intensity for each pooled sample was calculated by dividing the ratio for each pooled sample by the ratio of the positive control, in order to control for inter-blot variation. A pre-breeding relative intensity value specific to each individual was then subtracted from each weekly post-breeding relative intensity value to create a comparison to baseline IgJ levels. Relative intensity values did not pass normality testing, so the data were transformed using a log transformation. After transformation, 15 of 18 groups were verified for normality using a Shapiro-Wilk test in R (Version 3.3.2) (R Core Team, 2016). Differences in IgJ intensity between pregnant and non-pregnant groups were determined using Student's T-test or Mann-Whitney U-Test in R (version 3.3.2) depending on normality of the group, with differences considered significant at $P < 0.05$, and with differences considered a tendency at $0.05 < P < 0.09$.

## Results

### *Fecal Steroid Metabolite and Total Protein Concentrations*

Fecal estrogen metabolite profiles confirmed the cyclicity of monitored females. Examples of the estrogen metabolite concentrations of cycling females prior to natural breeding can be seen in Figure 2.1a and 2.1b. Fecal progestogen metabolite concentrations were significantly higher ($P < 0.01$) during pregnancy and non-pregnant luteal phase than during a pre-breeding baseline period (Table 2.1), confirming the presence of a luteal phase after breeding. Fecal progestogen profiles of pregnant females can be distinguished from non-pregnant luteal phase females after around 55 days post-breeding, when progestogen concentrations of non-pregnant females drop (Figure 2.1c), while pregnant females have extended progestogen excretion until parturition (Figure 2.1d). Mean total protein concentration of sample extracts from pregnancies ($6.03 \pm 0.27$ mg/mL), non-pregnant luteal phases ($6.24 \pm 0.23$ mg/mL), and pre-breeding baselines ($5.72 \pm 0.56$ mg/mL) were not significantly different ($P > 0.05$).

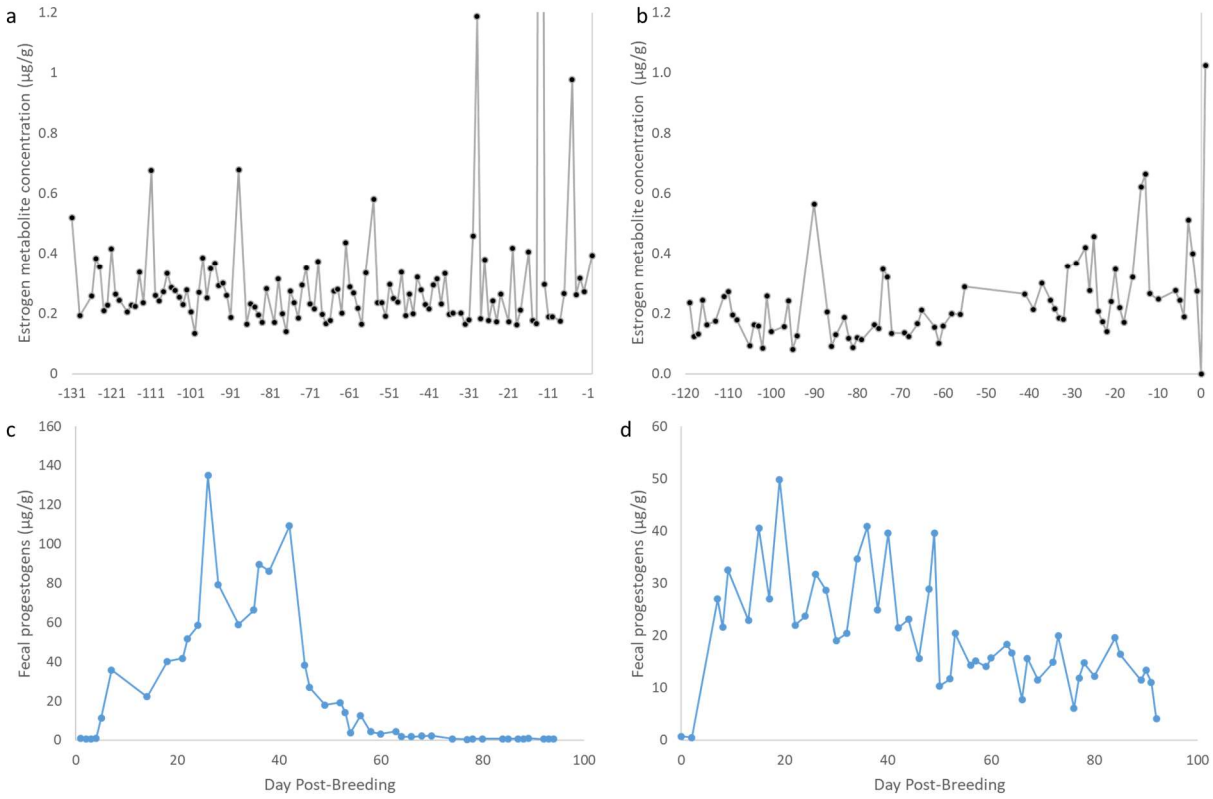


**Figure 2.1. Estrogen and progestogen metabolite profiles.** (a) Estrogen metabolite profile of a cycling female prior to natural breeding and successful pregnancy. (b) Estrogen metabolite profile of a cycling female prior to natural breeding and non-pregnant luteal phase. (c) Progestogen metabolite profile of a non-pregnant luteal phase after natural breeding. Progestogen metabolite concentrations return to baseline around ~55 days post-breeding. (d) Progestogen metabolite profile of a pregnant female after natural breeding. Progestogen metabolite concentrations remain elevated until parturition.

**Table 2.1. Mean (±SEM) estrogen and progestogen metabolite concentrations.** Values with different letters indicate statistical significance ($P < 0.01$).

| | Estrogen metabolites (µg/g) | Progestogen metabolites (µg/g) |
|---|---|---|
| *Pregnant Luteal Phase* | 0.32 ± 0.06 | 28.35 ± 6.40 [a] |
| *Non-Pregnant Luteal Phase* | 0.28 ± 0.05 | 43.92 ± 12.68 [a] |
| *Pre-Breeding Baseline* | 0.33 ± 0.07 | 0.91 ± 0.19 [b] |

***Post-Breeding IgJ Response in Females Exposed to Seminal Plasma during Natural Breeding***

Detection of IgJ by western blotting with the use of a commercially available antibody was confirmed by the use of a positive control. IgJ was confirmed in the positive control and in fecal samples at a molecular weight of ~18 kD (Figure 2.2). Females that were bred naturally with successful semen deposition, including both successful pregnancies and non-pregnant luteal phases, were found to have significantly higher IgJ levels ($P < 0.05$, mean ± SEM = 0.86±0.04) in the weeks immediately following breeding compared to exogenously stimulated females that did not have exposure to seminal plasma (0.76±0.02) (Figure 2.3). An example of a post-breeding response can be seen in Figure 2.2a, with high IgJ abundance in week 1 following the female's first breeding and exposure to seminal plasma. Another response can be seen in Figure 2.2b, with high IgJ abundance in week 2 following natural breeding and exposure to seminal plasma. In a female that was exogenously stimulated to ovulate and artificially inseminated intraoviductally with washed spermatozoa (no seminal plasma), no increase in IgJ was seen (Figure 2.2c). Similarly, no increase was seen in females that were exogenously stimulated to ovulate without subsequent artificial insemination (Figure 2.2d).

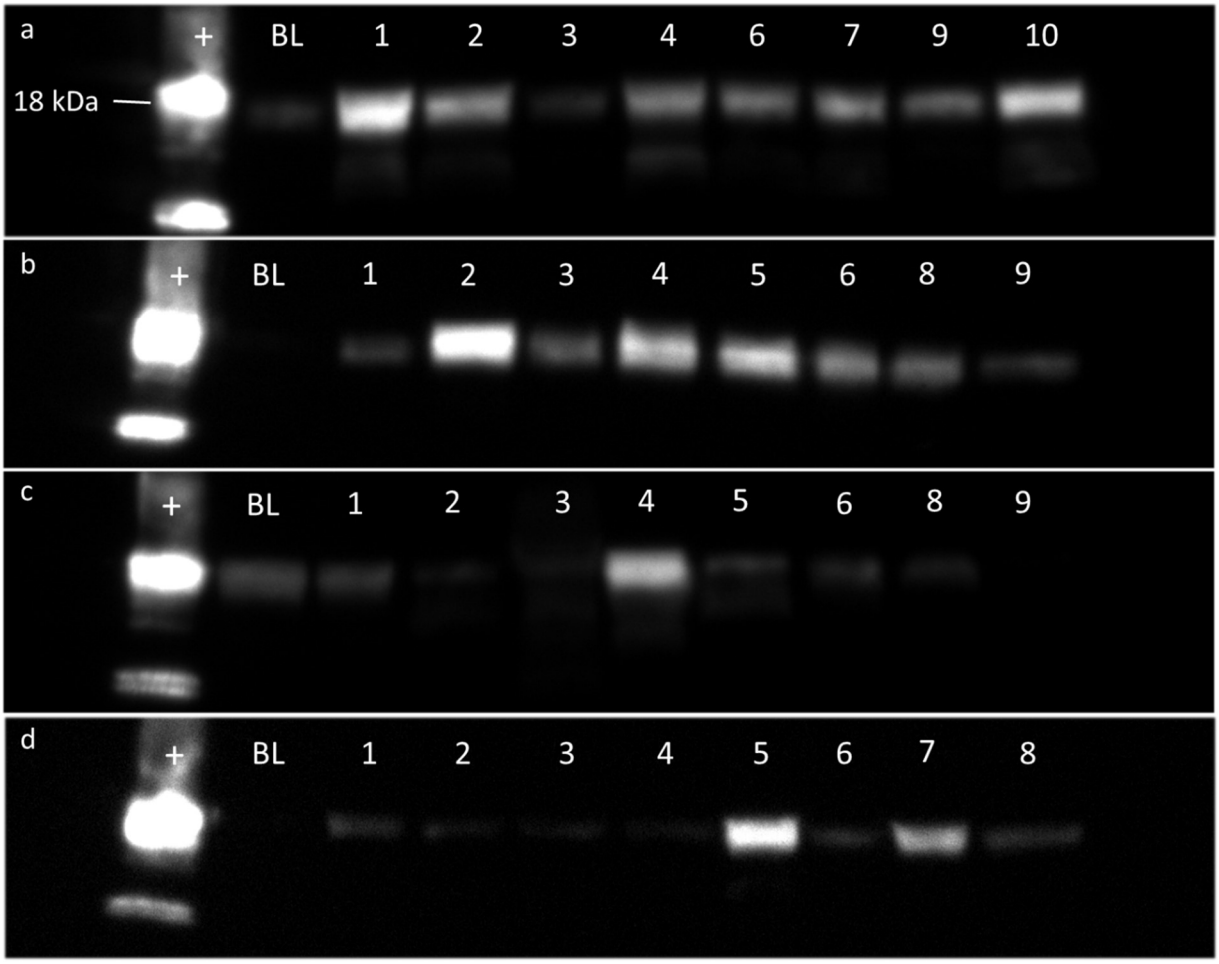


**Figure 2.2. IgJ response immediately following breeding.** Females that were bred naturally with exposure to seminal plasma had high IgJ levels in either week 1 (a) or week 2 (b) post-breeding. Females with no exposure to seminal plasma following exogenous stimulation and intraoviductal artificial insemination with washed spermatozoa (c) or exogenous stimulation without artificial insemination (d) did not experience an increase in IgJ levels immediately following breeding.

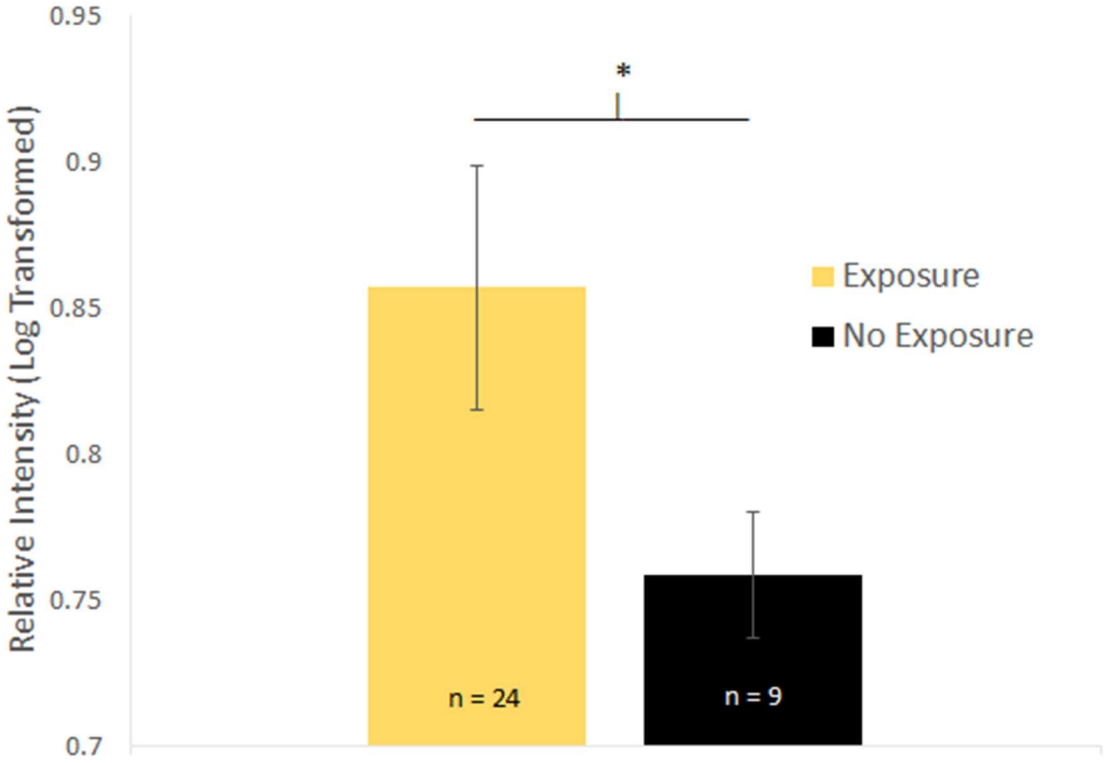


**Figure 2.3. Effect of exposure to seminal plasma on IgJ abundance.** Females that were bred naturally and exposed to seminal plasma (n = 24) were found to have higher IgJ levels immediately following breeding compared to females exogenously stimulated to ovulate with no exposure to seminal plasma (n = 9, $P < 0.05$).

***Temporal Tracking of IgJ Abundance***

Pregnant females tended to have higher IgJ levels ($P < 0.09$) in week 4 post-breeding ($0.82 \pm 0.07$) compared to females experiencing a non-pregnant luteal phase ($0.69 \pm 0.02$) (Figure 2.4). Pregnant females were also found to have significantly higher IgJ levels ($P < 0.02$) in week 8 post-breeding ($0.83 \pm 0.04$) compared to females experiencing a non-pregnant luteal phase ($0.69 \pm 0.03$). IgJ abundance was not different between the two groups in weeks 1, 2, 3, 5, 6, 7, or 9 ($P > 0.1$) (Figure 2.4). An example of a pregnancy can be seen in Figure 2.5a, with high

IgJ levels in Week 4 and following Week 7 post-breeding. An example of a non-pregnant luteal phase can be seen in Figure 2.5b, with low IgJ levels at or near baseline levels throughout the sample period.

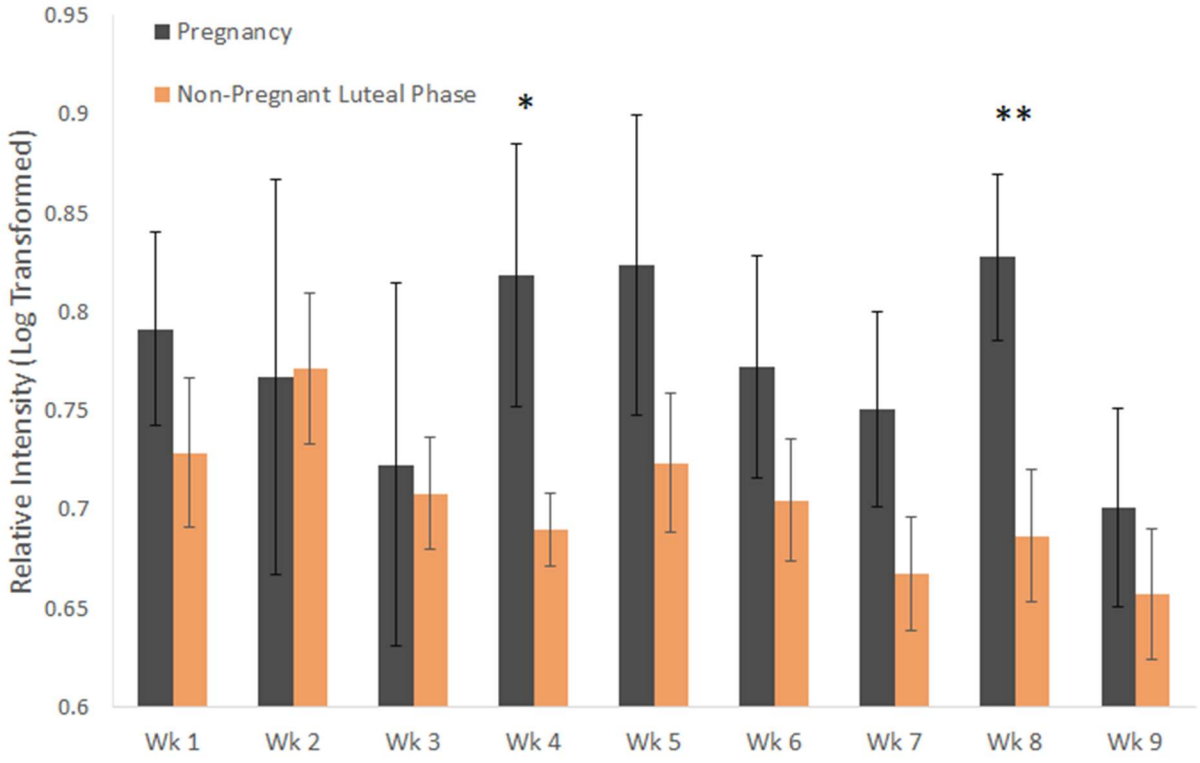


**Figure 2.4. Mean (± SEM) relative intensity of IgJ following breeding**. IgJ abundance tended to be higher (P < 0.09 *) during pregnancy (n = 15) compared to non-pregnant luteal phase (n = 19) at week 4 post-breeding. IgJ abundance was significantly higher (P < 0.02 **) during pregnancy compared to non-pregnant luteal phase at week 8 post-breeding.

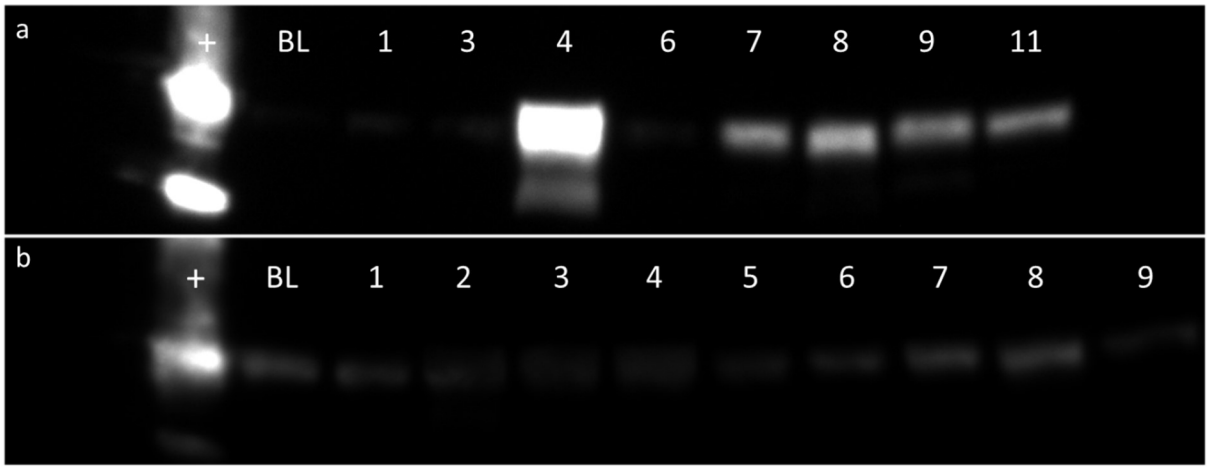


**Figure 2.5. Comparison of pregnancy and non-pregnant luteal phase.** (a) IgJ was seen to be increased in week 4 and following week 7 post-breeding during pregnancy. (b) IgJ remained at or near baseline levels throughout the 9 week sample period during a non-pregnant luteal phase.

**Discussion**

The reproductive biology of the cheetah has been studied for decades, with great advances in the understanding of this species both *in situ* and *ex situ*. However, cheetahs in the wild are in decline, as habitat fragmentation and human conflict have reduced the natural range of the species (Marker, Dickman, Jeo, Mills, & Macdonald, 2003; Durant et al., 2017). Because of the vulnerable status of the cheetah, it has become an important goal of conservationists to create an *ex situ* insurance population. However, creating a sustainable *ex situ* population with the goal of improving genetic diversity and ensuring future health and adaptability of the species has become a challenge, as many cheetahs struggle to successfully reproduce in human care. The low genetic diversity of the species as a whole, and high levels of inbreeding depression, have contributed to many health and reproductive issues that affect the cheetah, including the impairment of genes mediating immune defenses (O'Brien et al., 2017), low fecundity, and the poor semen quality of males both in captivity and in the wild (Wildt et al., 1987; Crosier et al.,

2007). While wild cheetahs appear to face similar obstacles in terms of inbreeding depression and low genetic diversity, they are able to reproduce with much more success than cheetahs in human care (Laurenson, Caro, & Borner, 1992; Kelly et al., 1998), indicating the possibility that *ex situ* environmental factors may be having a negative effect on reproductive capacity.

Temporal tracking of IgJ abundance over the first 9 weeks post-breeding in our study has provided insight into the intrauterine events that occur after the success or failure to establish pregnancy in the cheetah. Females in our study demonstrated increased elevation of IgJ, and potentially an increased immune response, after natural breeding with seminal exposure compared to exogenous stimulation with no seminal exposure. One explanation is that a secretory immune response was stimulated by the presence of seminal plasma in the reproductive tract which, as a foreign substance interacting with a mucosal surface, could induce an upregulation in IgJ. An immune response to semen has been documented previously in mice, as lymphocyte synthesis and cytokine activation is triggered in response to the constituents of seminal plasma (Johansson et al., 2004). This response may help to promote active maternal tolerance of paternal antigens of the fetus at the implantation site (Johansson, et al., 2004; Robertson & Sharkey, 2001), preventing rejection of the fetus. Upon successful breeding and exposure to semen, it is possible that the females in this study were experiencing a subsequent activation of the secretory immune response in order to promote tolerance of paternal antigens upon implantation of the fetus and invasion of the endometrium. All ten females that were exogenously stimulated to ovulate, including two females that underwent a subsequent intraoviductal artificial insemination with washed spermatozoa (e.g. no seminal fluid present) and eight females that were not artificially inseminated (e.g. no sperm interaction with their reproductive tract) demonstrated IgJ levels that were at or near baseline levels in the first weeks

post-breeding, indicating that the IgJ response is not due to ovulation, and that natural breeding and successful deposition of semen are needed to see an immune response.

The timing of early intrauterine events, including implantation and the development of the placenta, is unknown in wild felids. In the domestic cat, fertilization takes place in the oviduct up to 48 hours after ovulation, and implantation occurs at day 13-14 post-breeding (Denker, et al., 1978). It is possible that implantation in the cheetah occurs not at the same time as the domestic cat (day 13-14 of ~65 d gestation), but at a proportional point in the longer ~93 day gestation of the cheetah (day 19-21). In this study, IgJ levels tended to be elevated in pregnant cheetahs compared to non-pregnant cheetahs during the fourth week after breeding (day 22-28). Increased IgJ levels during the fourth week post-breeding could indicate an activated secretory immune response to the invasion of the endometrium by the embryo following implantation. Increased IgA secretion likely alters dendritic cell function, leading to the promotion of regulatory T cell expansion and inhibiting the release of inflammatory cytokines, preventing an inflammatory response to paternal antigens present on the fetal trophoblast (Monteiro, 2014). These findings suggest that implantation could occur directly before week 4 post-breeding (day 19-21), as a sustained secretory immune response at the endometrium likely continues and intensifies as the implanted embryo continues to develop during the fourth week.

Pregnancy in the cheetah first becomes distinguishable from a non-pregnant luteal phase using progestogen metabolite monitoring at ~day 55 post-breeding, as progestogen levels in fecal samples drop to baseline levels in non-pregnant individuals. It can be theorized that around this time in non-pregnant individuals a regression of the corpora lutea is seen, which results in a subsequent decrease in progesterone production, followed by a plateau at lower basal levels. Recent studies have suggested that the placenta is a source of progesterone production in the

domestic cat (Tsutsui et al., 2009; Siemieniuch et al., 2012), and acts to supplement luteal progesterone later in gestation. A placental source of progesterone secretion is likely in the cheetah, and may explain the observed increase in IgJ levels at week 8 (day 50-56) post-breeding. An increased and sustained secretion of progesterone from the fetal-maternal barrier at day 50-56 in gestation may modulate secretory immunity, causing IgJ levels to be increased in pregnant individuals compared to non-pregnant individuals. Local placental synthesis of progesterone may act as an immunosuppressive factor at the site of embryonic implantation during both murine and human gestation (Siiteri & Stites, 1982). Progesterone suppresses T lymphocyte proliferation and inhibits natural killer cell activity at the maternal-fetal interface (Szekeres-Bartho, 2002), decreasing the activity of these immune molecules to protect the semi-allogeneic fetus and preventing an inflammatory immune reaction. Because the endometrium is a mucosal surface, it is likely that secretory immunity is modulated as well, promoting tolerance of the fetus and allowing for non-inflammatory neutralization of foreign pathogens. Secretory immunity may also be modulated by the secretion of Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by the placenta during week 8 post-breeding, as levels of a fecal $PGF_{2\alpha}$ metabolite (PGFM) were found to be elevated in the pregnant cheetah beginning at day 48 post-breeding and increased through parturition (Denhard et al., 2012). While $PGF_{2\alpha}$ is known to have a strong luteolytic effect in many species (Senger, 2005), suggesting a possible impact on immunity, the action of this molecule is unknown in felids and the impact on IgJ levels cannot be determined.

It is possible that the presence of external immune stressors could have an adverse effect on the results of this experiment, and could serve as a limitation of this method. IgJ is a protein that is upregulated in response to an activation of the secretory immune system, which is present in all mucosal surfaces of the body in order to protect from foreign pathogens. An immune

challenge to a mucosal surface that is independent of pregnancy could increase expression of IgJ, resulting in high IgJ values in our assay that do not correspond to intrauterine events and could affect the accuracy of the assay. A future goal of our lab is the development of a reliable benchtop enzyme-linked immunosorbent assay (ELISA) for measuring fecal IgJ levels after breeding. Daily quantification using this method would improve the accuracy and efficiency of IgJ monitoring, and may be able to reveal moments of immune challenge that impact establishment of pregnancy with greater precision. The occurrence of a non-pregnant luteal phase is common in other species, indicating the potential for a felid-wide or carnivore-wide assay for determining pregnancy using IgJ monitoring.

In summary, this study detailed IgJ levels throughout the first 9 weeks of pregnancy and non-pregnant luteal phase in the cheetah. Females that were bred naturally and exposed to seminal plasma had a higher immediate IgJ response than females that were exogenously stimulated to ovulate with no seminal exposure, indicating an immune response to the constituents of seminal plasma that has the effect of promoting maternal tolerance of fetal tissue upon implantation. There was a tendency towards increased IgJ abundance at week 4 post-breeding in pregnant cheetahs compared to non-pregnant cheetahs, indicating an activation of the secretory immune system in response to implantation and the invasion of the maternal endometrium by the fetal trophoblast. A significant increase in IgJ abundance was also found in week 8 post-breeding in pregnant cheetahs compared to non-pregnant cheetahs. Taken together, these data support the suggestion that the window of implantation in the cheetah is between 19-21 days post-breeding, and that the placenta is a source of extragonadal progesterone during the third trimester. These findings will help to improve *ex situ* management of the species, and

further research will continue to advance the understanding of cheetah reproductive physiology following breeding, aiding future conservation efforts for the species.

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# CHAPTER 3

## The Modulation of Immunity during Pregnancy in the Cheetah, and the Impact of IgJ Monitoring on Cheetah Conservation

### Introduction

As a molecule involved in the regulation of a complex immune response, the role of Immunoglobulin J-chain (IgJ) has not yet been fully defined. IgJ is known to be an important component in the secretory immune system that is present in all of the mucosal surfaces of the body, and is highly expressed in immunocytes found in the lamina propria of secretory tissue (Brandtzaeg, 1974, Brandtzaeg, 1983). Because the endometrium is a mucosal surface, it can be inferred that the physiological changes brought about by pregnancy can have an effect on the secretory immune system. A successful pregnancy involves the modulation of the maternal immune response, allowing for the development of a fetus that is semiallogeneic. Tolerance of foreign antigens present on trophoblast cells must also be promoted to prevent an immune attack on the fetus. While little research on placental recognition and tolerance has been conducted in carnivore species, it is logical to suggest that an immune molecule such as IgJ could play an important role in placental development in a specialized carnivore, the cheetah. IgJ acts to regulate polymer formation of Immunoglobulin A (IgA) and Immunoglobulin M (IgM), leading to the activation of secretory immunity at mucosal surfaces (Johansen, Braathen, & Brandtzaeg, 2000). IgJ links two IgA molecules, creating a dimer, and links five IgM molecules, converting it to a pentameric form. As polymers, IgA and IgM have greatly increased avidity, allowing for the agglutination of foreign pathogens such as bacteria and viruses on mucosal surfaces (Johansen et

al., 2000). The incorporation of IgJ into dimeric IgA and pentameric IgM also prevents the activation of the complement response, helping to limit the potential damage that could result from an inflammatory response in tissue that is continuously being exposed to foreign environmental antigens. IgA dimers appear unable to activate the complement response through the classical pathway, and IgJ-positive pentameric IgM is much less efficient at activating the complement response than IgJ-negative hexameric IgM (Johansen et al., 2000). An upregulation of IgJ during pregnancy would therefore promote the formation of non-inflammatory IgA and IgM polymers, preventing an immune response that would potentially be damaging to the developing fetus.

**Activation of Secretory Immunity in Response to Seminal Plasma**

Because IgJ is an important component of the immune response, it is beneficial to analyze all potential immune challenges that a female may face during pregnancy. Multiple cheetahs, including females that carried a pregnancy to term as well as individuals that were naturally bred and underwent a non-pregnant luteal phase, were found to have an increase in IgJ abundance in the early weeks following breeding compared to cheetahs that were exogenously stimulated to ovulate (Figure 3.1). Eight of the exogenously stimulated females underwent a true non-pregnant luteal phase, with no subsequent artificial insemination or exposure to paternal antigens. Two exogenously stimulated females were artificially inseminated intraoviductally with washed (by centrifugation) spermatozoa free of seminal fluid two days following ovulation. These two females were exposed to spermatozoa but not to the constituents of the seminal fluid, and neither produced a pregnancy. One possible explanation for this increase in IgJ levels seen early after breeding is that a response was seen to the presence of semen, which as a foreign

substance interacting with a mucosal surface could induce an upregulation in IgJ. The immune response to semen as a foreign substance has been documented previously in mice, as lymphocyte synthesis and cytokine activation is triggered in response to the constituents of seminal plasma (Johansson, Bromfield, Jasper, & Robertson, 2004). Antigens found in seminal plasma are the likely trigger of this immune response, as the activation of immunity fails to occur in mice after mating in the absence of seminal vesicle secretions (Johansson et al., 2004). Sperm does not appear to have an effect on the induction of maternal immunity in mice, as the immune response triggered by mating with vasectomized males is indistinguishable from that of mating with intact males (Johansson et al., 2004). This response may help to promote active maternal tolerance of paternal antigens of the fetus at the implantation site (Johansson et al., 2004; Robertson & Sharkey, 2001). It is possible that females that are successfully bred and exposed to seminal plasma may be experiencing a subsequent activation of the secretory immune response. Transforming growth factor-β (TGF-β) is a cytokine that is found in high levels in seminal plasma, and is thought to be central to the promotion of active maternal tolerance to paternal antigens found in semen (Robertson, Ingman, O'Leary, Sharkey, & Tremellen, 2002; Moldenhauer et al., 2009). This action primes the maternal immune response upon implantation, as many of the same antigens are present on the embryo. TGF-β has a direct effect on the antigen presenting cells of the immune system (dendritic cells). TGF-β alters the maturation of dendritic cells, inhibiting development into Type 1 cells and promoting development into Type 2 cells (Robertson et al., 2002). Type 1 dendritic cells give rise to type 1 T cells, which provoke a strong immune response through the release of inflammatory cytokines (Strobl and Knapp, 1999; Kalinski, Hilkens, Wierenga, & Kapsenberg, 1999). A type 1 inflammatory response to fetal tissue would be greatly harmful to pregnancy, and the downregulation of this response may be

essential to a successful gestation (Reinhard, Noll, Schlebusch, Mallmann, & Ruecker, 1998). Type 2 dendritic cells give rise to $T_h2$ cells and $T_h3$ T cells, leading to increased tolerance of the seminal antigens. $T_h3$ cells secrete multiple suppressive cytokines, including IL-10 and TGF-β. IL-10 is an anti-inflammatorycytokine that suppresses secretion of type 1 T cell inflammatory cytokines and downregulates expression of major histocompatibility (MHC) class 2 antigens (Mittal and Roche, 2015). Secretion of TGF-β leads to a greater alteration of dendritic cell activity, promoting the increased development of $T_h3$ cells and subsequent secretion of TGF-β, creating a positive feedback loop that leads to a sustained tolerance response. High levels of TGF-β also promote the expansion of regulatory T cells, which help to suppress other immunocytes that produce inflammatory responses (Jorgenson, Persson, & Hviid, 2019). $T_h2$ cells stimulate increased antibody production, including the production of IgA and IgM (Janeway, Travers, & Walport, 2001). This promotes an increase in the non-inflammatory neutralization of pathogens, and may explain the increase in IgJ levels that was seen in this study. The increased secretion of IgA may also have an anti-inflammatory action as well, as secretory IgA (SIgA) has been found to have a similar effect on dendritic cells as TGF-β. In mice, SIgA binds the specific intercellular adhesion molecule-3-grabbing nonintegrin-receptor 1 (SIGNR1), a receptor present on dendritic cells, altering their maturation away from type 1 and towards type 2 immunity (Monteiro, 2014). Similar to TGF-β, this process promotes the expansion of regulatory T cells and the development of type 2 T cells, stimulating increased IgA production and creating another positive feedback loop, promoting increased tolerance.

Figure 3.2 shows the profile for IgJ for one female (#4568) who had a successful pregnancy by natural breeding and an unsuccessful artificial insemination attempt. Following natural breeding and exposure to semen, IgJ levels were found to be elevated during the second

week post-breeding. No increase in IgJ was seen following exogenous stimulation and intraoviductal artificial insemination with washed spermatozoa in the same female, possibly indicating the necessity of seminal exposure to activate maternal secretory immunity.

Some females only experienced an increase in IgJ levels following their first breeding and exposure to semen, with no subsequent immune activation following subsequent natural breedings. One female (#4453) had high IgJ levels during week 1 post-breeding during her first breeding, indicating an activation of secretory immunity to seminal exposure. However, IgJ abundance was near baseline levels in two subsequent natural breedings (Figure 3.3) including one pregnancy and one non-pregnant luteal phase. Similarly, another female (#6592) had high IgJ levels post-breeding during her first pregnancy and baseline IgJ levels post-breeding during her second pregnancy (Figure 3.4). However, this observation did not achieve statistical significance. Overall, females that were exposed to seminal plasma for the first time did not have higher IgJ levels than females with previous exposure. It is possible that previously bred females with subsequent seminal exposure to the same male may experience a reduced immune response compared to breeding with another male. Similarly, the antigens present in the seminal fluid of a genetically similar male to the first breeding may fail to sufficiently activate immunity, as MHC class I diversity has been shown to be greatly reduced in the cheetah (Dobrynin et al., 2015). However, more research is needed to confirm the effect of male MHC diversity on IgJ abundance in previously bred females.

For her first breeding, which did not produce cubs, one female (#6339) had elevated IgJ levels during week 2 post-breeding (Figure 3.5). In a subsequent natural breeding and pregnancy with the same male following soon after the end of the non-pregnant luteal phase, IgJ levels remained at baseline following breeding. This could indicate that a subsequent breeding with the

same male soon after an initial breeding could result in a decreased IgJ response, although more research is needed to confirm this. In another subsequent natural breeding and pregnancy with the brother of the male she was previously bred with, IgJ levels were elevated after breeding, indicating the possible activation of secretory immunity. However, IgJ levels were very high throughout this pregnancy, indicating the possible presence of an external immune challenge that resulted in elevated IgJ throughout gestation. The same female (#6339) was later housed with a vasectomized male. Estrogen metabolite monitoring confirmed cyclicity of the female, and breeding was confirmed by visual observation. Progestogen metabolite monitoring confirmed ovulation and non-pregnant luteal phase on at least five occasions during the cohabitation. IgJ remained at baseline levels following four out of five breedings with the vasectomized male in the early weeks following breeding (Fig 3.5), confirming that the act of copulation likely does not produce the immune response seen after natural breeding. In vasectomized individuals, seminal plasma is still present. Exposure to seminal plasma in the absence of sperm should still theoretically result in an increase in IgJ levels, indicating an activation of secretory immunity. However, this response was not seen in this female. Previous and frequent exposure to seminal plasma may cause a reduction of the IgJ response, although breedings of additional females with vasectomized males should be sampled to determine that this phenomenon is not specific to this individual.

In order to support this theory of a secretory immune response to the exposure to seminal plasma, it is necessary to look at IgJ levels for females that were exogenously stimulated to ovulate. This cohort includes eight females that were not artificially inseminated following stimulation (ES) and two females that received an intraoviductal artificial insemination two days following ovulation (AI). All ten of these individuals had IgJ levels that were at or near baseline

levels in the first weeks post-breeding (Figure 3.6). This supports the theory of exposure to seminal antigens, as natural breeding and the successful deposition of semen is needed to see an immune response. Intraoviductal exposure to spermatozoa in the absence of seminal fluid did not result in an activation of secretory immunity, indicating that the contents of seminal plasma likely induce the immune response that is seen during natural breeding. Only two artificially inseminated females were included in this study, so IgJ monitoring following artificial insemination should be conducted in more females in the future in order to confirm this observation. The maintenance of IgJ levels at or near a baseline value in exogenously stimulated females as well as in a female bred with a vasectomized male also indicates that the IgJ response does not occur in response to ovulation or to the act of copulation. If ovulation caused an immune response then an elevation of IgJ would be seen in all samples, as all females included in this study were confirmed to ovulate by progestogen metabolite monitoring. All of these findings suggest the exposure to semen as an indicator for an immune response after breeding in the cheetah. The immune response to the constituents of seminal plasma may be a mechanism for promoting the tolerance of the fetus, as tolerance is built to the same foreign antigens in the semen that are also present at the maternal-fetal interface. This active response, likely facilitated by TGF-β and other constituents of seminal plasma, allows for the successful growth of a semiallogeneic fetus in the endometrial environment. More research is needed to confirm the action of seminal TGF-β in the female reproductive tract of the cheetah, as well as to compare the immune response of a female to the deposition of semen from different males in subsequent breedings.

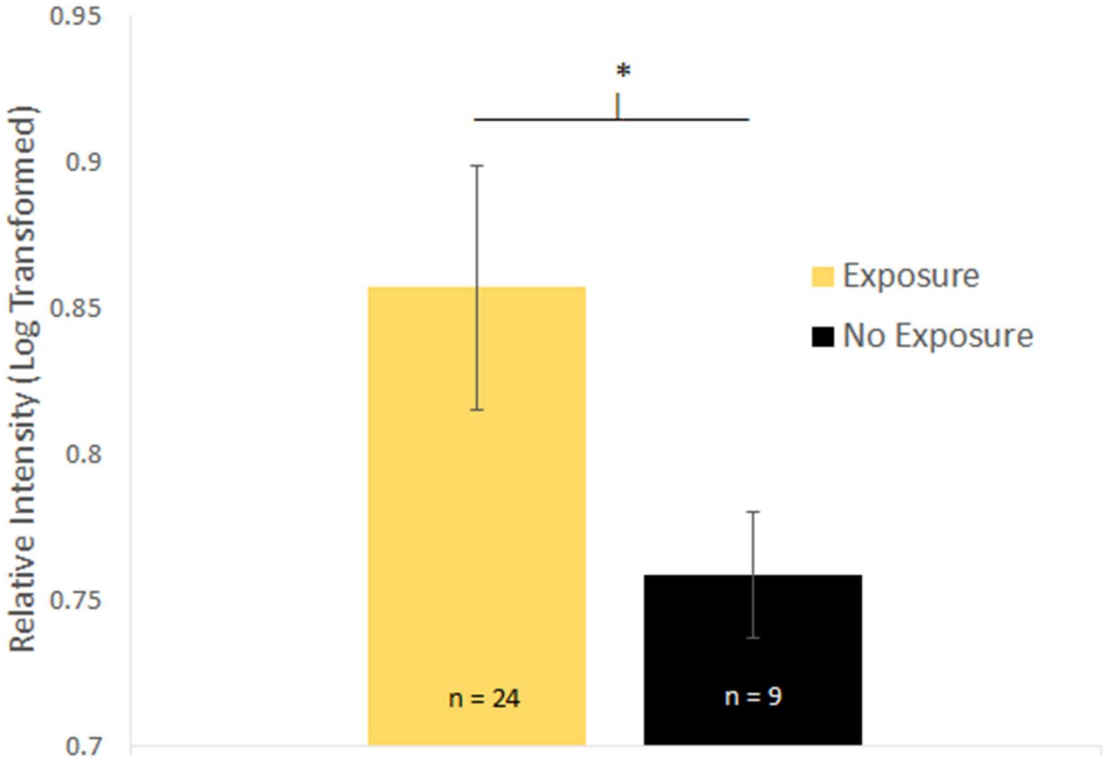


**Figure 3.1. Effect of exposure to seminal plasma on IgJ abundance.** Females that were bred naturally and exposed to semen (n = 24) had higher IgJ levels immediately following breeding compared to females exogenously stimulated to ovulate with no exposure to semen (n = 9, $P < 0.05$).

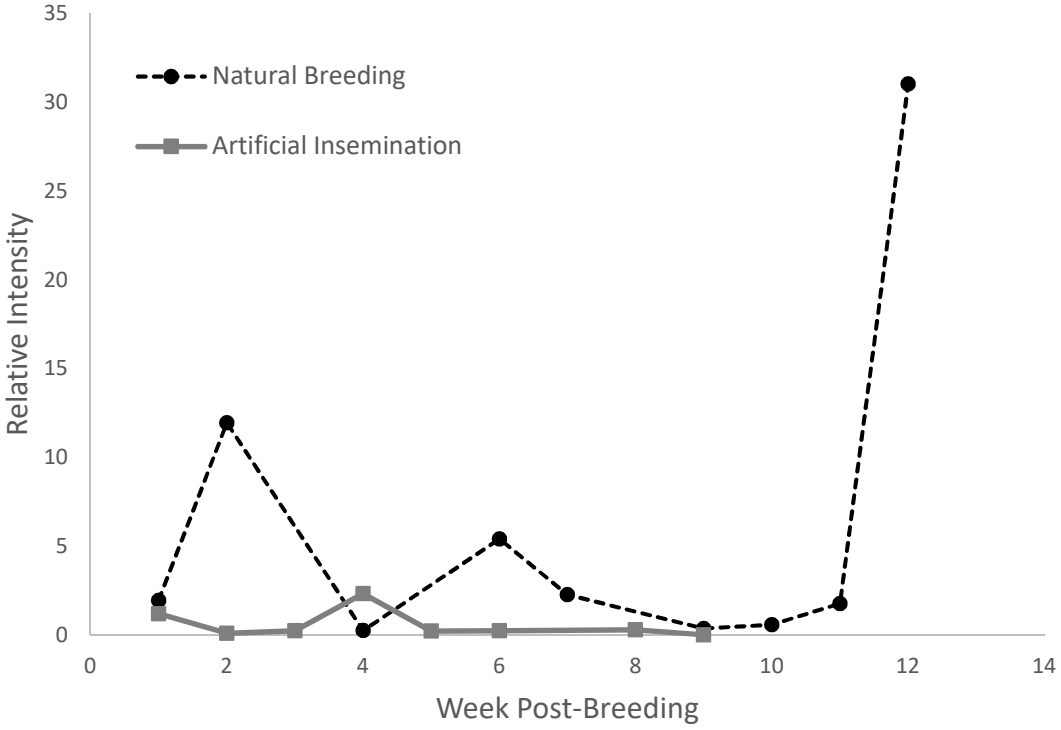


**Figure 3.2. IgJ response post-breeding upon exposure to semen in female #4568.** A peak in IgJ abundance was seen during week 2 post-breeding after natural breeding. No increase in IgJ levels were seen following exogenous stimulation and artificial insemination.

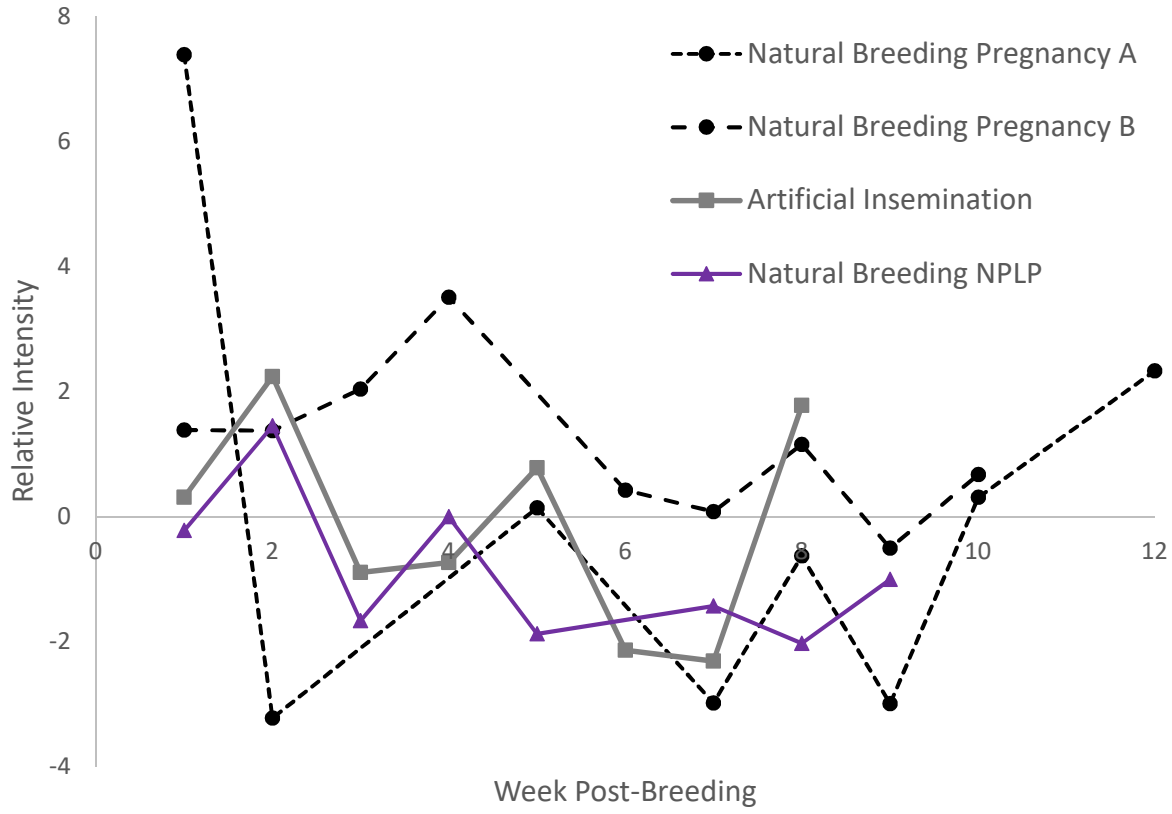


**Figure 3.3. Comparison of IgJ levels in female #4453.** Female #4453 had high IgJ levels week 1 post-breeding following her first natural breeding and exposure to semen (A). Low IgJ following breeding was seen for two subsequent natural breedings (B, NPLP) and one artificial insemination.

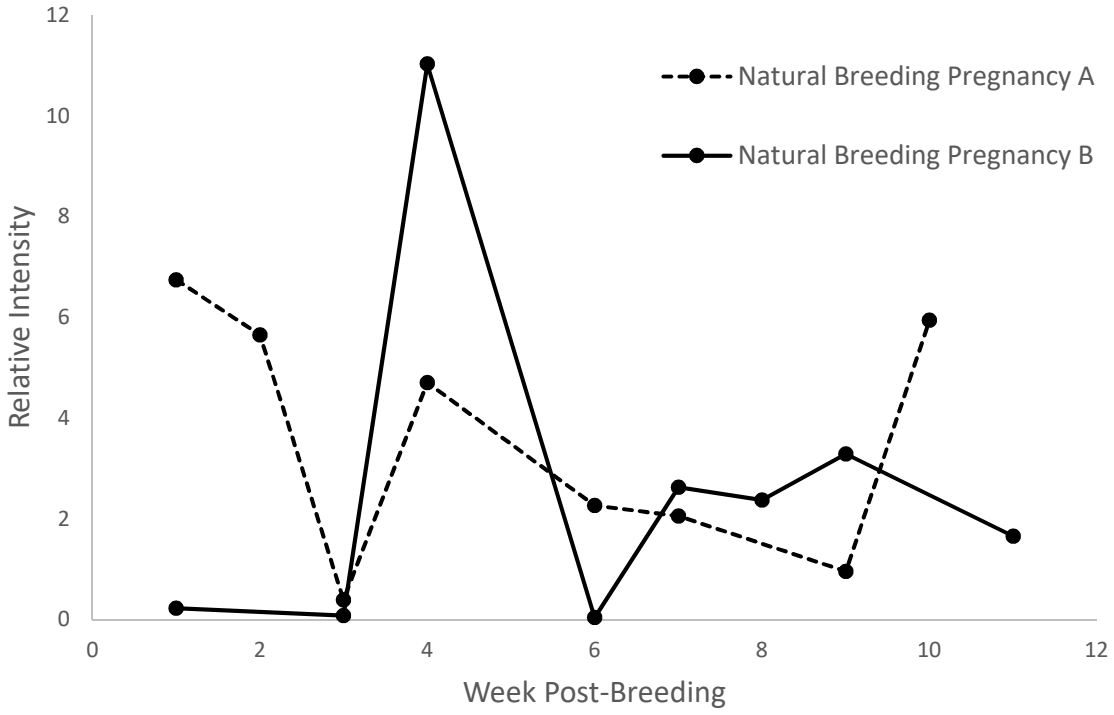


**Figure 3.4. Comparison of IgJ levels in female #6592**. High IgJ abundance was seen during week 1 post-breeding following natural breeding and pregnancy (A). Low IgJ was seen following breeding during subsequent natural breeding and pregnancy (B).

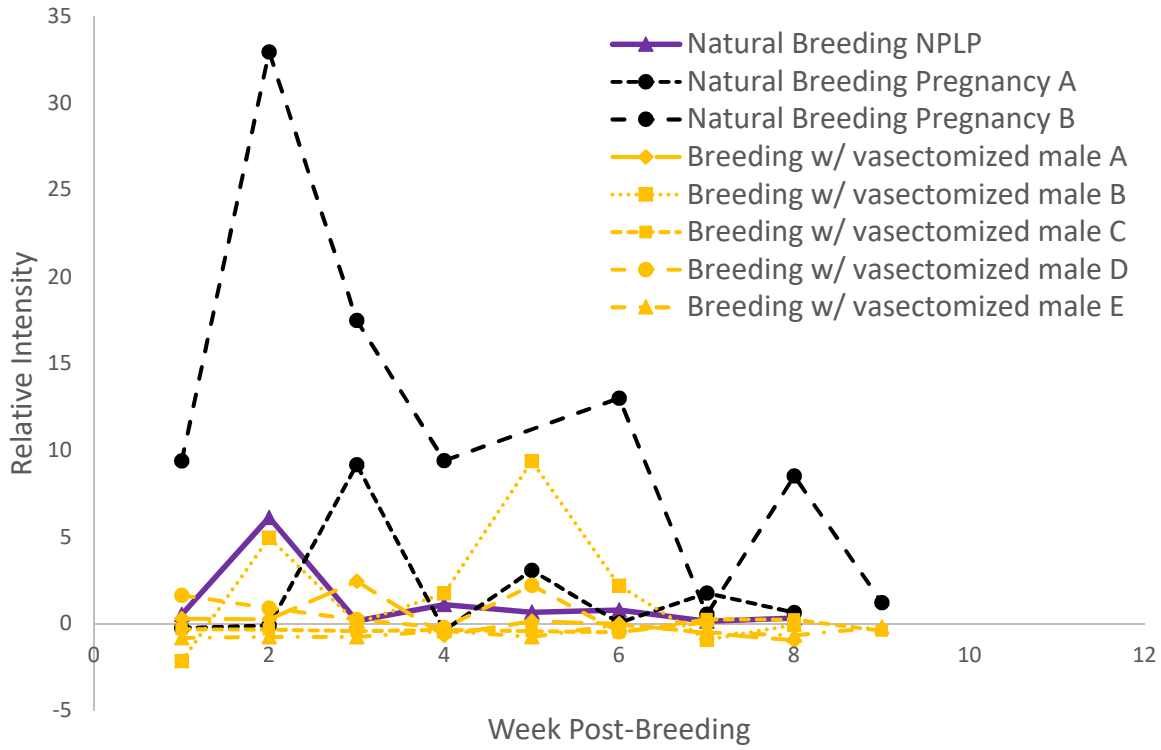


**Figure 3.5. Comparison of IgJ levels in female #6339.** High IgJ was seen following the first breeding that resulted in a non-pregnant luteal phase (NPLP). Low IgJ was seen following a subsequent breeding that resulted in a pregnancy (A). Very high IgJ was seen for a subsequent natural breeding throughout pregnancy (B). Low IgJ was seen following four out of five breedings with a vasectomized male.

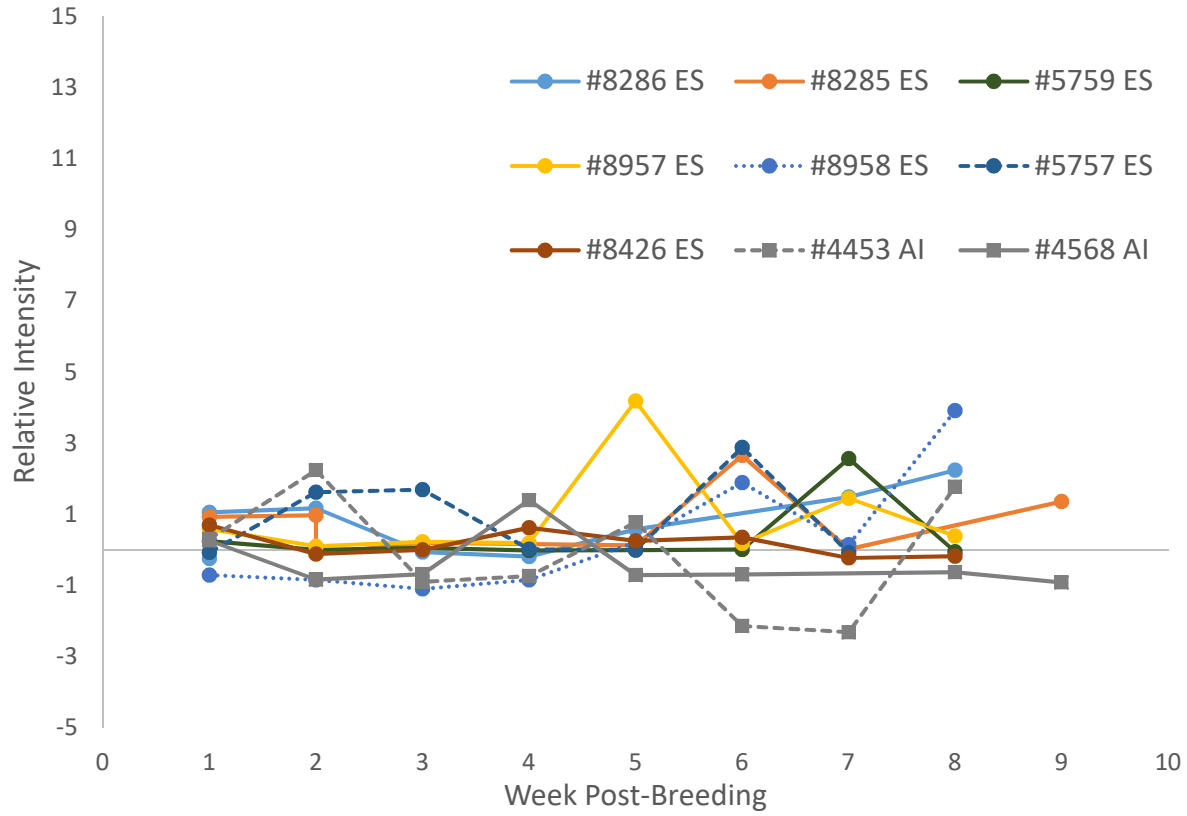


**Figure 3.6. Exogenous stimulation with no exposure to seminal plasma.** IgJ levels remain at or near baseline post-breeding in exogenously stimulated females. ES = Endogenous stimulation to ovulate without artificial insemination; AI = Endogenous stimulation followed by intraoviductal artificial insemination with washed spermatozoa.

**Activation of Secretory Immunity in Response to Embryonic Implantation**

The process of embryonic implantation in the cheetah has not been studied, and little is known of this process in non-domestic felid species. However, implantation in the cheetah likely resembles the process that has been studied more thoroughly in the domestic cat (*Felis catus*). In the cat, the syncytiotrophoblast invades the maternal endometrium to establish nutrient exchange with the mother. During this process, the endometrial epithelium and the underlying interstitium

are completely eroded, directly exposing the maternal capillaries to the chorionic epithelium (Enders and Carter, 2006). Because of this structure the placenta is classified as endotheliochorial. Embryonic implantation is clearly an invasive process, and therefore is likely to provoke an immune response due to the foreign nature of the invading tissue. Because of the role of the endometrium as a mucosal surface, it is logical that IgJ would be affected as a result of the modulation of secretory immunity. IgJ levels tended to be increased in pregnancy compared to non-pregnant luteal phase in week 4 (day 22-28, $p < 0.1$) post-breeding, indicating an increased activation of the secretory immune system in response to implantation. The timing of implantation in the cheetah has not been previously reported, so closely related felid species must be used to help understand this unknown. In the domestic cat, fertilization takes place in the oviduct up to 48 hours after ovulation and implantation occurs at day 13-14 post-breeding (Denker, Eng, & Hamner, 1978). It could therefore be theorized that implantation in the cheetah would take place at around the same time post-breeding, as the domestic cat is the closest related species in which the window of implantation is known. However, gestation in the domestic cat is shorter than the cheetah, with gestation lengths of approximately 65 days and 93 days, respectively. Because of this, it could be hypothesized that the window of implantation might occur later in the cheetah, around day 19-21, at a point in gestation that is proportional to the domestic cat. It is therefore unknown, but likely that implantation occurs at some point between 13 and 21 days post-breeding in the cheetah.

The monitoring of the hormone relaxin has been found to be a useful tool for determining pregnancy in the domestic cat as well as in a non-domestic felid species with a similar gestation length to the cheetah, the Arabian leopard (*Panthera pardus nimr*) (de Haas van Dorsser, Swanson, Lasano, & Steinetz, 2006). Relaxin is produced specifically by the placenta in felid

species, and plays an important role as a placental growth factor. In the domestic cat, relaxin begins to be detectable in urine by day 18-22 post-breeding. This rise in urinary relaxin indicates an increase in the development of placental tissue. If implantation were to occur at the same point in the Arabian leopard as it does in the domestic cat (day 13-14 post-breeding), then a rise in relaxin should be expected for the Arabian leopard at the same point as well (detectable levels observed at day 18-22 post-breeding). However, a rise in urinary relaxin levels in the Arabian leopard is not seen until day 28, indicating that functional placental tissue develops later in the leopard than in the cat. This finding seems to indicate that implantation in large non-domestic felids does not occur at the same point as the domestic cat, but later, potentially at day 19-21 post-breeding. This agrees with the findings in our study, as IgJ levels tended to be increased in pregnant cheetahs compared to cheetahs experiencing a non-pregnant luteal phase in week 4 post-breeding (day 22-28). Increased IgJ leads to the increased secretion of IgA to the mucosal endometrium, likely influencing dendritic cell function and leading to the promotion of regulatory T cell expansion (Monteiro, 2014). The increased activity of regulatory T cells at the maternal-fetal interface promotes the tolerance of fetal antigens, suppressing the activity of other lymphocytes. The altered dendritic cell function also promotes IL-10 secretion, inhibits the release of inflammatory cytokines, and prevents an inflammatory response to the paternal antigens on the fetus, creating a tolerant microenvironment for the fetal tissue (Robertson & Sharkey, 2001).

In the cheetah, the secretory immune response could be sustained for several days after implantation (day 19-21) in response to the invasion of the maternal endometrium, resulting in the increased abundance of IgJ that is seen during week 4 (day 22-28). Because of the use of fecal samples in our study, gut transit time may have an effect as well. Biomarkers that are

secreted into the intestinal lumen, such as IgJ, must travel through the small and large intestine before being excreted. This produces a small lag time between IgJ secretion and the date that the feces is collected. While gut transit time can vary between individuals and with diet consumed, the average in the cheetah is one to two days (Crosier, personal communication). The sustained immune response to embryonic implantation combined with the short delay due to gut transit time explains the tendency for increased IgJ levels in week 4 following breeding, and suggests a window of implantation between 19-21 days in the cheetah. Limited urinary relaxin testing has been done in the cheetah using a relaxin radioimmunoassay (RIA) and a bench-top kit (Harris, Steinetz, Bond, Lasano, & Swanson, 2008). Using the RIA, researchers were able to confirm the presence of urinary relaxin in the cheetah in levels comparable to the domestic cat. However, the earliest sample collected was from day 34 post-breeding, and the immunoactivity of the antibody to cheetah relaxin was unreliable. Further optimization of this assay may provide a method for tracking relaxin levels in the cheetah throughout pregnancy, and could reveal when a rise in relaxin occurs. A rise around day 28 post-breeding would agree with de Haas van Dorsser's finding in the Arabian leopard (2006) and could support a theory of implantation at day 19-21 post-breeding followed by an increased development of functional placental tissue.

**Activation of Secretory Immunity at Week 8 Post-Breeding**

Following a week 4 peak in IgJ abundance, levels appears to decrease slightly in pregnant females, with no apparent difference in pregnant and non-pregnant individuals in weeks 5, 6, and 7 post-breeding. At this point in gestation the placenta continues its development, and is beginning to increase its function as a secretory endocrine organ. No difference in IgJ abundance is seen between pregnant and non-pregnant individuals until week 8 post-breeding, when levels

are increased during pregnancy. Week 8 (day 50-56) is an important milestone in cheetah gestation, as it is currently the earliest time when pregnancy and non-pregnant luteal phase are distinguishable using progestogen metabolite monitoring. At this time progestogen metabolite levels in the non-pregnant cheetah return to baseline levels, while levels in the pregnant cheetah remain elevated until parturition. In females undergoing a non-pregnant luteal phase, the corpora lutea undergoes a regression at this time, leading to a cessation of progesterone production. The corpora lutea is possibly maintained through parturition in the pregnant cheetah, however, indicating the need for an unknown luteotropic signal from the placenta to maintain CL function. Increased IgJ levels are indicative of an activation of the secretory immune response at this time. There are several possibilities that could explain this increase in immune activity. The placenta is an endocrine organ, and around this time placental secretion of both prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and progesterone is increased. As a result of the increased secretion of these molecules and their interaction with the mucosal fetal-maternal interface, the secretory immune system could increase in activity, resulting in the increase of measurable IgJ.

***Prostaglandin $F_{2\alpha}$***

$PGF_{2\alpha}$, a molecule that has a luteolytic function in many species of subprimate mammals, has been found to be increased in pregnant cheetahs compared to non-pregnant cheetahs. This increase is first seen around day 48 post-breeding, and levels remain elevated until parturition (Denhard et al., 2012). Denhard et al. (2012) utilized fecal monitoring of a $PGF_{2\alpha}$ metabolite (PGFM), an important tool as samples are easy to collect, and it provides a non-invasive method that puts minimal stress on the animal. In species such as the mare, the ewe, and the cow, $PGF_{2\alpha}$ is produced by the endometrium during a non-pregnant luteal phase. The molecule is then transported to the ovary via a vascular countercurrent exchange mechanism and acts directly on

the CL to bring about luteolysis (Senger, 2005). However, the action of $PGF_{2\alpha}$ appears to be different in felid species. Increases in production above baseline levels are only seen in pregnant individuals, and the primary source of the prostaglandin is theorized to be the utero-placental complex (Denhard et al., 2012). Increased PGFM levels are not seen in non-pregnant individuals, which would indicate that $PGF_{2\alpha}$ does not play a luteolytic role in non-pregnant felids. This would suggest that there is a non-ovarian luteotrophic signal that likely is produced by the placenta in order to sustain luteal progesterone production past day 55 post-breeding in the pregnant cheetah, although more research is needed to uncover this potential mechanism in felids.

The physiological role of $PGF_{2\alpha}$ in the pregnant cheetah is not completely known, as it is not thought to have the drastic luteolytic action in felids that it has in other ruminant species. In ruminants, $PGF_{2\alpha}$ is theorized to bind to receptors on large luteal cells, triggering cell death and luteolysis (Senger, 2005). In felids, progesterone production by the corpora lutea decreases slightly throughout the third trimester until parturition. It is possible that $PGF_{2\alpha}$ may not act on the ovary via the countercurrent exchange mechanism. Instead, $PGF_{2\alpha}$ could be transported into normal venous circulation, where it is transported to and rapidly metabolized by the lungs into PGFM before being able to act on luteal cells. This could explain the greatly reduced luteolytic effect of $PGF_{2\alpha}$ in the cheetah. Another possibility is that the luteolytic effect of $PGF_{2\alpha}$ could be counteracted by the effect of an unknown luteotrophic molecule. This could prevent the luteolysis and subsequent decrease in progesterone levels that could result in loss of pregnancy. As gestation continues and $PGF_{2\alpha}$ levels continue to increase, the decrease in luteal progesterone production due to increased luteolysis may be supplemented by placental progesterone production until parturition. The immune system is also likely to play a role in luteolysis, as

macrophages and lymphocytes increase the production and release of cytokines that result in luteal cell apoptosis (Senger, 2005). While $PGF_{2\alpha}$ is known to trigger events in other species that result in a substantial immune response due to luteolysis, it may have a different action in felids, as luteolysis is not thought to occur immediately upon its release. In the cheetah, the action of $PGF_{2\alpha}$ may result in the activation of the secretory immune response that is seen in week 8 post-breeding. Similarly, the secretion of another, unknown molecule that has a luteotrophic effect in the cheetah may result in the observed immune response. While this mechanism has yet to be elucidated, the previous knowledge of the effect that this prostaglandin has in other species may lend credence to the theory that it has a modulating effect on immune molecules. However, further research is needed to understand the physiological role that $PGF_{2\alpha}$ has late in gestation in felids.

***Placental Progesterone***

Placental secretion of progesterone is likely to markedly increase around day 50-56 post-breeding in the cheetah. In the domestic cat undergoing a non-pregnant luteal phase, progesterone production is maintained by the corpus luteum for 35-40 days post-ovulation (Tsutsui, et al., 2009). In pregnant queens, progesterone production is sustained until parturition at day 65. Ovariectomy at day 40 or earlier results in spontaneous abortion, as progesterone levels drop precipitously and pregnancy cannot be sustained. Following ovariectomy at day 45 of gestation, however, 20-60% of cats were able to maintain pregnancy (Tsutsui et al., 2009). This finding suggests the presence of an extragonadal source of progesterone production that sustains pregnancy throughout the remainder of gestation. In the domestic cat, the maternal decidual cells of the placenta have been confirmed to be a supplemental source of progesterone (Siemieniuch et al., 2012). Progesterone production was also found to increase with gestational age, suggesting

that placental progesterone is the extragonadal source that can sustain pregnancy levels late in gestation. These findings support the theory that placental progesterone production becomes elevated later in gestation in the cheetah, acting as a non-luteal supplemental source to sustain pregnancy. Measurable levels of placental progesterone likely begin to be secreted around week 8 post-breeding in the cheetah, resulting in the possible modulation of the maternal immune response.

It has been previously theorized that local placental synthesis of progesterone may act as an immunosuppressive factor at the site of embryonic implantation in murines and in humans (Siiteri and Stites, 1982). Progesterone may suppress T lymphocyte proliferation at the maternal-fetal interface, decreasing the activity of immune molecules to protect the semiallogeneic fetus and preventing inflammatory immune reactions. Progesterone also acts to inhibit natural killer (NK) cell activity at the maternal-fetal interface, preventing the release of inflammatory cytokines that could damage fetal tissue. Because lymphocyte and NK cell activity is suppressed, secretory immune activity may be increased in compensation. Increased IgJ production increases the secretory activity of IgA, an important molecule for responding to foreign pathogens at mucosal surfaces. This allows for a non-inflammatory response to foreign pathogens at this site of immunotolerance. Upon dimerization and secretion, IgA has been found to be unable to activate the complement response. This is a very important feature of the secretory immune molecule, as an inflammatory response at any mucosal surface could be damaging to the tissue. An inflammatory response at the maternal-fetal interface could have catastrophic consequences for the fetus, so it is logical to theorize that a non-inflammatory response such as the one that IgA produces would be necessary and beneficial for a successful pregnancy. An increase in IgJ would explain this mechanism of action, as IgJ acts to dimerize IgA and is necessary for binding

to the polymeric immunoglobulin receptor (pIgR), allowing for IgA to be transported across epithelial cells and onto mucosal surfaces (Vaerman, et al., 1998; Johansen, Braathen, & Brandtzaeg, 2001). This could also allow for the promotion of the anti-inflammatory qualities of IgA, including the expansion of regulatory T cells and the altered maturation of dendritic cells at the maternal-fetal barrier (Monteiro, 2014).

**Effect of Litter Size on IgJ Abundance**

Beginning in week 7 and week 8 post-breeding, the developing fetuses begin to grow more rapidly than earlier in gestation and cubs are first able to be visualized using radiograph (Ware et al., 2016). As the fetus becomes more developed, so does the placenta, increasing the surface area of the maternal-fetal interface. This growing interface could result in a larger immune response, leading to an increase in IgJ production. It was hypothesized that a pregnancy with a larger litter size would have a larger total surface area of the maternal-fetal interface across the entire uterus because of the larger number of developing fetuses. This increased surface area across the mucosal surface of the endometrium could produce a larger IgJ response in a female with a large litter size than it would in a female with a smaller litter size. In order to test this, a linear model was created in R (version 3.3.2) (R Core Team, 2016) during week 8 post-breeding, when fetal growth begins to increase rapidly. No increase in IgJ relative intensity was seen as litter size increased (Figure 3.7), indicating that IgJ expression is affected by factors other than litter size.

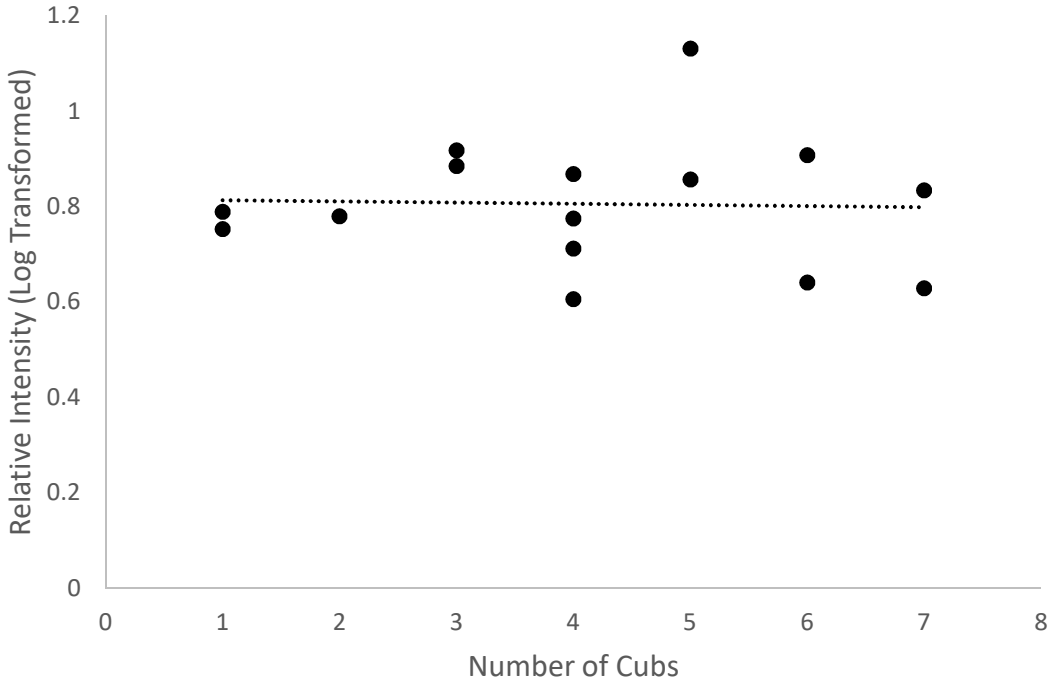


**Figure 3.7. Effect of litter size on IgJ abundance.** Litter size has no effect on IgJ levels following rapid fetal growth in week 8 post-breeding.

**Modeling IgJ Abundance for Pregnancy and Non-Pregnant Luteal Phase**

During pregnancy IgJ levels did not remain constant, but fluctuated at several points, resulting in a general profile that had two distinct peaks around week 4 and week 8 post-breeding. We attempted to create a model of IgJ abundance throughout the course of the pregnancy in order to approximate the profile of IgJ abundance during pregnancy in the cheetah. This was done using a cubic regression analysis in R (version 3.3.2) (R Core Team, 2016). Fitting this model to the data did not result in significance (Figure 3.8; $P > 0.1$). This indicates that the attempt to model the multiple peaks and valleys of IgJ abundance over the first 12 weeks of pregnancy was not successful using a cubic regression analysis. There is likely too much variability in IgJ levels between individuals to create a cubic model. Another possibility is that

the sampling windows are too wide, and smaller windows would likely increase the precision of the IgJ peaks that are seen.

A model of IgJ abundance throughout the course of non-pregnant luteal phase was created using a linear model and regression analysis in R (version 3.3.2). Fitting this model to the data resulted in significance (Figure 3.9; $P < 0.05$) and confirmed the decrease in levels of IgJ over the course of non-pregnant luteal phase. The slight negative slope of the model likely results from the initial increase in IgJ abundance in week 1 or week 2 among females that underwent a non-pregnant luteal phase following natural breeding and semen exposure.

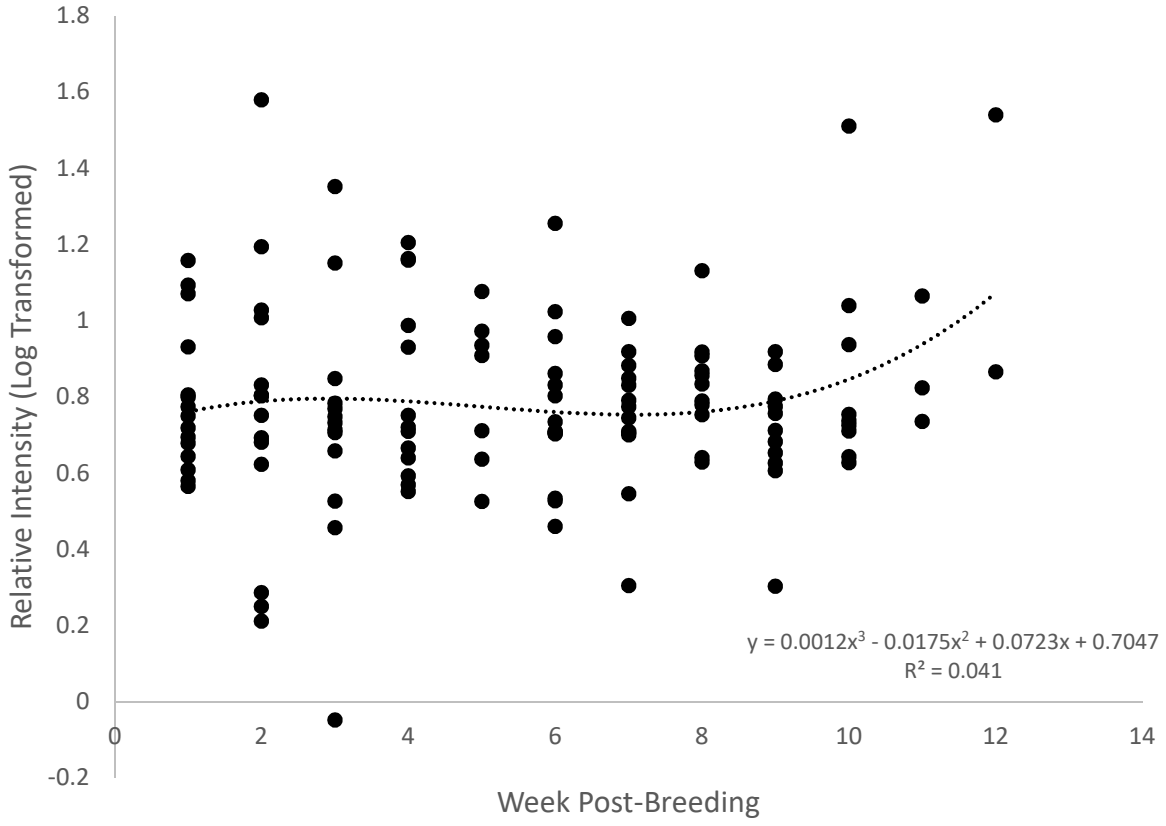


**Figure 3.8. Cubic regression analysis of IgJ abundance during pregnancy.** No significance was seen in the cubic regression model ($P > 0.1$)

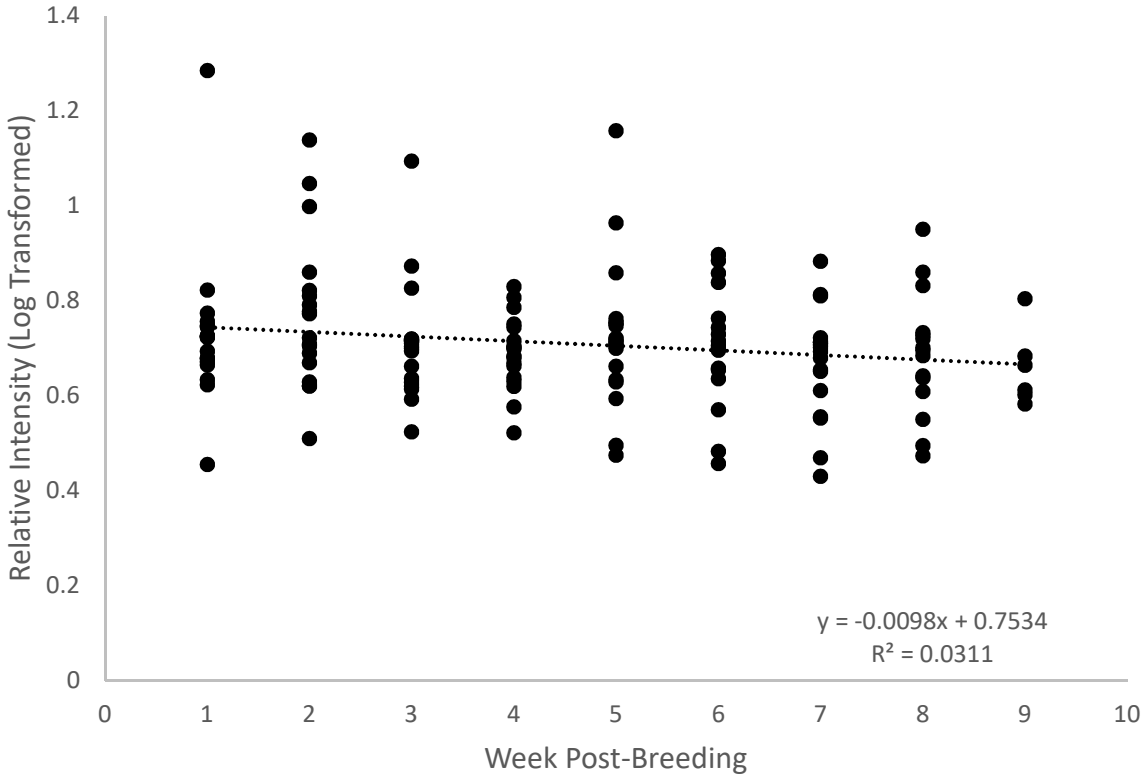


**Figure 3.9. Linear regression analysis of IgJ abundance during non-pregnant luteal phase.** IgJ levels remained near baseline throughout the non-pregnant luteal phase, with a significant decrease over time ($P < 0.05$).

**Individual Variability**

One limitation of using IgJ as a method for pregnancy diagnosis is the high level of individual variability that seems to be present. IgJ is not only regulated in response to pregnancy, but also to the immune challenges that an individual faces. IgJ is involved in secretory immunity at all mucosal surfaces, including the respiratory and intestinal tracts in addition to the genitourinary tract (Johansen et al., 2000). Because of this there is the possibility of external variables that affect levels of IgJ that are measured in the feces. There may also be genetic factors that are individual specific that affect measurable IgJ levels. For example, one female

(#6979) had three separate pregnancies that were sampled in this study. In each of the three pregnancies IgJ levels were measured that were near or below baseline levels throughout the first 9 weeks of gestation (Figure 3.10). This was an interesting finding because it indicated the possibility of a factor that could be specific to the individual rather than specific to the pregnancy. If this female experienced an immune challenge during a single pregnancy or a single baseline sampling period then only that pregnancy would have been affected. IgJ was at or below baseline levels in all three pregnancies, however, indicating the possible presence of a physiological characteristic that is unique to this female rather than a specific immune challenge. Interestingly, a daughter of #6979 was also included in this study. The female #8957 had a similar IgJ profile during pregnancy to her mother, with IgJ remaining at or below baseline levels for the first 7 weeks post-breeding (Figure 3.11). This could indicate a heritable genetic trait that is affecting secretory immunity and IgJ production during pregnancy in these individuals. Tools are now available to better determine relatedness between individuals, including between breeding pairs. Microsatellite mapping can be used to determine how well individuals match better than pedigree data alone. Similarly, the development of single nucleotide polymorphism (snp) panels that can uniquely identify the slight variations between individuals could reveal variations in IgJ production as well. Relatedness between male and female in a breeding pair may also have an effect on antigen recognition, which could subsequently affect immune response and IgJ production. More research is needed to confirm this hypothesis, however, and additional genetic testing of MHC or other immune-related genes of females from this lineage should be conducted to confirm modified IgJ levels in these individuals.

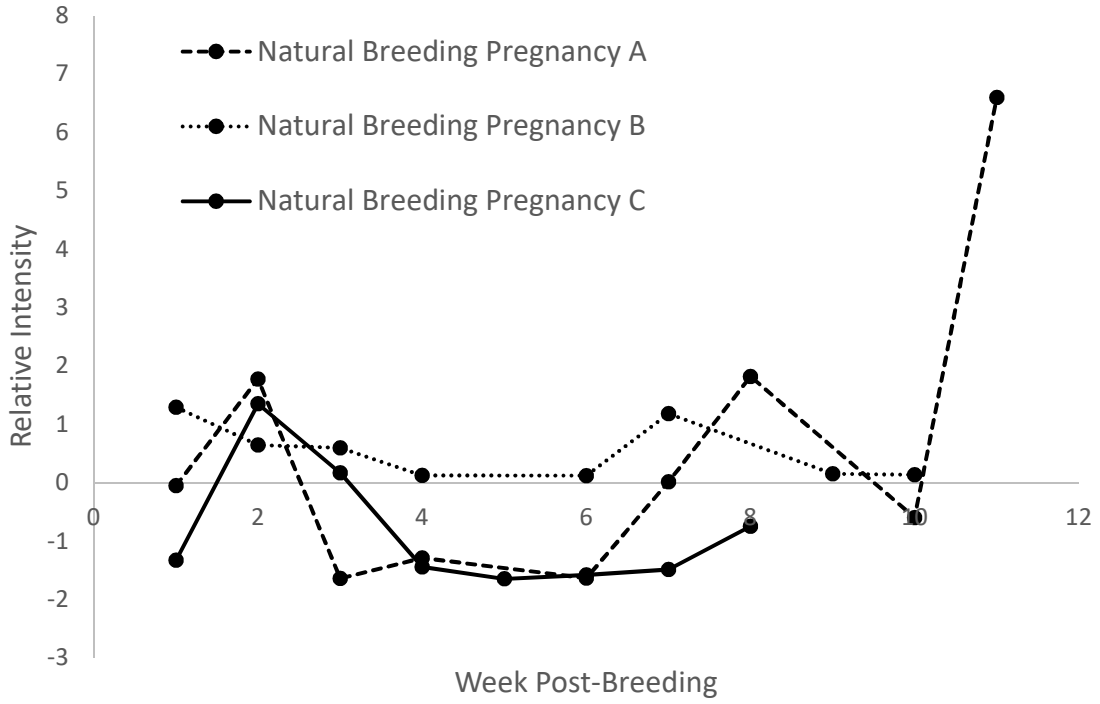


**Figure 3.10. Comparison of IgJ levels in female #6979.** IgJ remained at or near baseline levels for the majority of all three pregnancies.

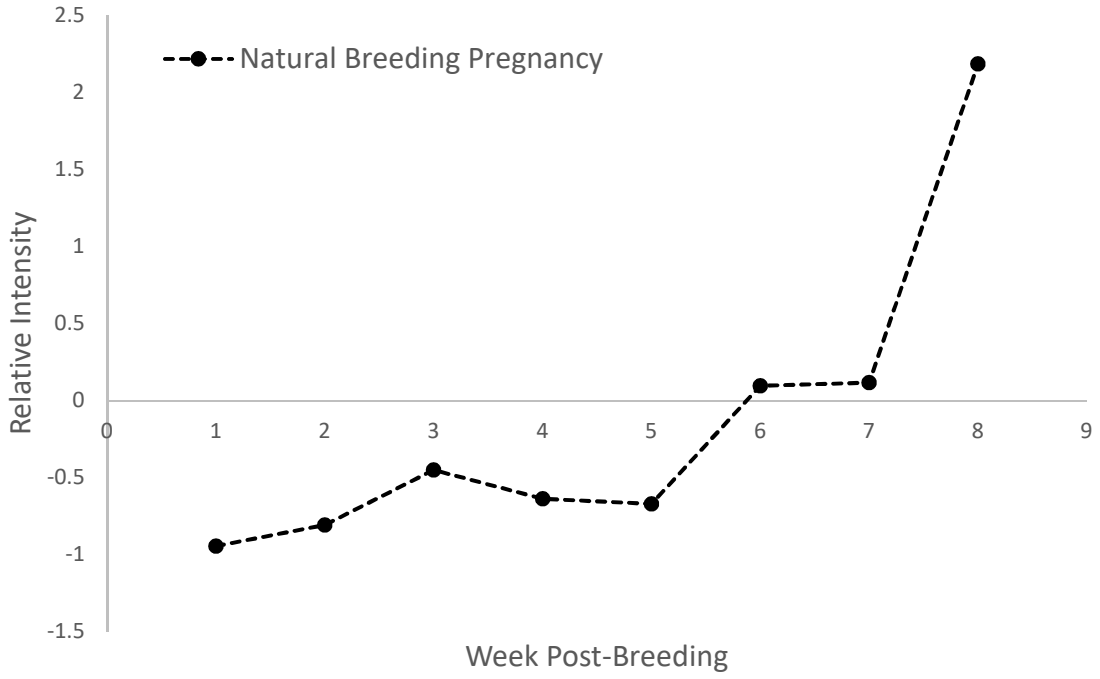


**Figure 3.11. Comparison of IgJ levels in female #8957.** IgJ levels during pregnancy remain at or below baseline levels for the majority of sampling period.

**Impact on Cheetah Conservation**

This is the first study that has elucidated the window of implantation in a non-domestic felid species. Invasive methods allowed for the discovery of the timing of implantation in the domestic cat at 13-14 days post-breeding (Denker et al., 1978). However, the value of cheetahs *ex situ*, as well as the relative rarity of pregnancy in the species, does not allow for these same methods to be used. Because of this, non-invasive sampling methods are needed to understand the physiological events such as implantation that occur in the cheetah. The results of this non-invasive fecal sampling study indicate an activation of the secretory immune response during the fourth week post-breeding. This finding, combined with the discovery that urinary relaxin levels begin to rise above baseline around day 28 in a closely related non-domestic felid species,

reveals the potential window of implantation at 19-21 days and the subsequent development of functional placental tissue based on the activation of secretory immunity that was seen as a result of these events. More research should be conducted using smaller sampling windows to better target the window of implantation in the cheetah.

The results of this study also reveal a potential method for distinguishing between pregnancy and non-pregnant luteal phase in the cheetah much earlier than current methods allow. Current methods employ fecal progesterone metabolite monitoring and radiography, and these methods allow for the diagnosis of pregnancy at around day 55 post-breeding (Brown, et al., 1996; Ware et al., 2016). Using fecal IgJ monitoring, pregnancy could be able to be distinguished from non-pregnant luteal phase at day 28 post-breeding, allowing animal care staff to provide proper care to individuals that are confirmed to be pregnant and to mobilize resources for the birth of cubs. This could also allow for non-pregnant females to be returned to the breeding pool for breeding recommendation faster, and more research could be done to understand why some breedings are unsuccessful. This could allow animal care staff to direct additional future resources or infertility treatment to females that have been bred unsuccessfully. While the western blotting methods that were utilized in this study are one method for determining IgJ abundance, the development of a reliable benchtop enzyme-linked immunosorbent assay (ELISA) and the quick quantification of IgJ levels is a goal for this lab. The ELISA method would allow for the tracking of IgJ abundance using individual daily samples collected throughout pregnancy. This individual sampling could provide more insight into the window of implantation and produce a more accurate estimation of the event. An ELISA could be used to quickly and easily diagnose pregnancy in the cheetah using non-invasive fecal sampling at a more cost-effective and less time-consuming method than western blotting.

This study helps to support the findings from previously published work in both domestic and non-domestic felid species suggesting that $PGF_{2\alpha}$ is released beginning near the third trimester of pregnancy. This molecule is only found in pregnant females and not in non-pregnant females, indicating that this molecule is placental in origin (Denhard et al., 2012). In the cheetah, measurable PGFM levels in the feces of pregnant females increase around week 8 post-breeding (Denhard et al., 2012). Similarly, at week 8 a luteotrophic mechanism is thought to help sustain luteal function until parturition, possibly helping to counteract the known luteolytic action of $PGF_{2\alpha}$ that is seen in other species. It is also known that placental progesterone production begins to increase in the late second trimester in the domestic cat, suggesting a similar course of action in the cheetah. Placental progesterone production continues to increase along with gestational age, with levels eventually becoming high enough to sustain pregnancy after ovariectomy in the third trimester in the domestic cat (Tsutsui et al., 2009). Although the role of $PGF_{2\alpha}$ in the cheetah is unknown, it could act to limit luteal steroid production, as it has been shown to have an inhibitory effect on progesterone release in luteal tissue in the domestic cat, resulting in luteolysis (Verstegen et al., 1993). Placental progesterone production may help to supplement progesterone levels in the third trimester in the cheetah. Progestogen metabolite and PGFM monitoring, as well as physical observations, seem to indicate the importance of week 8 post-breeding in the pregnant cheetah. During this time fetal growth begins to increase rapidly, placental progesterone production rises, and the utero-placenta complex begins to synthesize $PGF_{2\alpha}$. All of these actions could have substantial effects on the mother and could provoke a modulation of the immune system. Because all of this appears during week 8 post-breeding, it is logical that IgJ levels were found to be increased at this time. More research is needed to

determine the acute effect of placental progesterone and $PGF_{2\alpha}$ release on the maternal immune system, specifically on the secretory immune response.

In the future this method of pregnancy determination could be useful not only for the cheetah, but for other carnivore species as well. Using the commercially-produced IgJ antibody that was used in this study (Aviva Systems Biology ARP55440_P050), an assay could be developed to diagnose pregnancy in all wild felid species, and possibly other species of non-domestic carnivores as well. It is logical to think that an immune response to the invasion of the maternal endometrium during implantation is common among various species, as this is an invasive event from fetal tissue containing foreign antigens. As the uterine endometrium is a mucosal surface, it is understandable that there is a modulation of IgJ production and the secretory immune response. The occurrence of a non-pregnant luteal phase is common among felids, and normal methods of progesterone metabolite monitoring cannot distinguish pregnancy from non-pregnant luteal phase until later in gestation (Brown, Wasser, Wildt, & Graham, 1994). Fecal IgJ monitoring could be used in these species to determine the timing of implantation, an event that is unknown in all felid species aside from the domestic cat. This peak in IgJ abundance would be used as an indicator of pregnancy compared to non-pregnant luteal phase, allowing for the creation of a pregnancy test. The modulation of IgJ in response to pregnancy may also be present in other carnivore species. The tracking of IgJ levels could reveal intrauterine events such as implantation, placentation, and luteolysis in non-felids. A non-pregnant luteal phase is a common occurrence in other carnivore species, including canids (Bauman, Clifford, & Asa, 2008) and ursids (Curry, Stoops, & Roth, 2012). The expression of IgJ or another immune molecule may be modulated in response to the physiological events that are unique to pregnancy, indicating a method for distinguishing between pregnancy and non-pregnant luteal phase earlier

than current methods allow in various species. More research is needed to confirm the sensitivity of species-specific IgJ homologs to the commercially produced IgJ antibody that is used in our study, and to confirm the responsiveness to reproductive events. Some ursid species undergo the unique reproductive strategy of diapause, or delayed implantation (Sandell, 1990). It is thought that the window of implantation is highly variable in species with this unique adaptation, which could make normal pregnancy testing methods challenging. IgJ could be a potential target biomarker in these species, as it has been shown to be indicative of implantation in the cheetah, and its modulatory effect on secretory immunity could reveal more about diapause in other species.

This modulation of secretory immunity may also help to explain the reproductive challenges that the cheetah might face, and the knowledge from future studies could help to improve husbandry management in felids. A greater understanding of the intrauterine physiology of the cheetah early after breeding can help to determine why some cheetahs are successful breeders and why some are not. Failed breedings may either be the result of a failure of fertilization, or successful fertilization with a subsequent loss of pregnancy. The results of this study seem to indicate that implantation did not occur in naturally bred females that did not give birth, as there was no peak of IgJ abundance in week 4 post-breeding in any of these individuals. More unsuccessful breedings should be studied in order to come to a conclusion on this matter. A greater understanding of the successes and failures of these breedings could help to increase the rate of successful pregnancy in the cheetah and could alter breeding recommendations between individuals. The knowledge of individual immune responses and reproductive outcomes can be combined with genetic information to inform breeding recommendations in order to create the most genetically diverse and reproductively successful self-sustaining *ex situ*

population. A cheetah population that is genetically diverse is necessary for the maintenance of the species *ex situ* and allows for research that is essential to conservation of the species *in situ*. Improving the rate of successful breedings in cheetahs under human care will help to create the sustainable *ex situ* insurance population that is needed to prevent the extinction of this species in the wild. This population will serve as the source for future reintroduction efforts in the case of further losses to wild population numbers. Because wild cheetahs do not face the same reproductive challenges as cheetahs under human care, it is important to understand these issues before individuals are released and have the opportunity to interact with wild populations. The methods used in this study could also be utilized in a field setting with wild felids in the future. Non-invasive fecal samples from individuals that are monitored by radio telemetry could be collected, processed, and run to determine pregnancy. The profile of IgJ levels of cheetahs in the wild and in human care could be compared to better understand the similarities and differences of pregnancy and intrauterine physiology between the two populations. These findings might reveal why breedings among wild cheetahs are more successful than their counterparts in human care, and could improve felid husbandry and management techniques in a zoological setting.

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