ABSTRACT

Title of Document: CHARACTERIZATION AND CONTROL OF AGGRESSION AND REPRODUCTION IN THE MALE CLOUDED LEOPARD (*NEOFELIS NEBULOSA*)

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Clouded leopards are a striking and elusive cat species whose secretive nature has made it difficult to gather information on population statistics and behavior in the wild, where the population is in decline. While captive populations are intended as a hedge against extinction, breeding clouded leopards *ex situ* has been a challenge, primarily due to extreme male aggression toward females. Despite the importance of aggression in this species, there has as yet been no systematic study characterizing the basis of aggressive episodes. Two mechanisms seem to underlie the aggressive behavior in clouded leopards: degree of anxiety and circulating testosterone levels.

Three studies were conducted to characterize mechanisms modulating aggression in male clouded leopards. In Study 1, sixteen adult male clouded leopards were categorized as 'anxious' or 'calm' using a keeper questionnaire and fecal endocrine (androgen and glucocorticoid) profiles; these measures were correlated with behavior rates and frequencies before, during, and after a series of behavioral

reaction tests aimed at assessing an individual's response to stress-inducing situations. In Study 2, the behavioral and endocrine responses to the same tests were compared in the same clouded leopards following three treatments: 1) an anxiety-reducing psychotropic drug (clomipramine, n = 4); 2) a gonadotropin releasing hormone agonist (deslorelin, n = 5), or 3) no treatment (n = 4). In Study 3, the long-term effects of the drug treatments on spermatogenesis and hormone concentrations were compared in clouded leopards (n = 2/treatment) and domestic cats (n = 5/treatment), a model for non-domestic felid reproduction.

Studies revealed important findings about the basis of aggressive behavior in male clouded leopards. First, two of the behavioral reaction tests – 'mirror image stimulation' and 'unfamiliar people' – were effective tools for evaluating temperament and eliciting a behavioral response. Second, treatment with both clomipramine and deslorelin reduced anxious and aggressive behaviors (e.g. 'tail flicking' and 'growling') indicating multiple physiological mechanisms likely modulate aggression in this species. Finally, deslorelin temporarily suppressed hormone concentrations and reproductive function, while clomipramine had no clear effect on either. Ultimately, this information provides important tools for improving male-female pairing success and the overall management of captive clouded leopards.

CHARACTERIZATION AND CONTROL OF AGGRESSION AND REPRODUCTION IN THE MALE CLOUDED LEOPARD (*NEOFELIS NEBULOSA*)

By

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Dedication

For my beloved husband, Steven

And

In memory of Dr. JoGayle Howard

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Chapter 1: Introduction

1.1 Overview of Study

The endangered clouded leopard (*Neofelis nebulosa*) is a highly charismatic felid species. Unfortunately, breeding this species *ex situ* has been a challenge the world over, primarily due to male aggression and fatal attacks on females (Fletchall, 2003). In the North American Clouded Leopard Species Survival Plan (SSP) (a population management program guided by the Association of Zoos and Aquariums), there have been five successful breeding pairs and fewer than 25 cubs born in the past 10 years. As a result, the clouded leopard population in zoos is in crisis, with a population decrease from a high of ~120 animals to fewer than 70 animals. Despite the importance of male aggression in clouded leopards, this is the first study to have focused on characterizing and mitigating aggressive behavior.

The clouded leopard is a particularly interesting model for understanding the relative contribution of both temperament (anxiety) and physiology (testosterone) to stress-reactivity and male-female aggression. Research to date suggests that male clouded leopards have two basic temperament types: 'anxious' and 'calm'. Previous studies have correlated 'anxious' male clouded leopards with failed pairings and high excreted cortisol concentrations, whereas the converse is true for males exhibiting calm behaviors (Wielebnowski et al., 2002; MacKinnon et al., 2007). There is also evidence that testosterone plays a role in clouded leopard aggression, with juvenile pairs known to experience lower rates of failure due to hostile behavior than pairs

formed with a post-pubertal male. In a recent study of 14 clouded leopard introductions, two of four males (50%) that were paired as juveniles were successful, whereas only three of ten (30%) adult male pairings were successful (MacKinnon, 2008).

Herein, we present the characterization and modulation of aggression in the male clouded leopard, through selective treatment with drug therapies that act on the known drivers of aggression – anxiety (serotonergic system) and circulating testosterone concentrations – to better understand the relative influence of these systems on aggression in this species. The research is divided into 3 studies, discussed sequentially in the chapters that follow. Study 1 (Chapter 2) correlates the endocrine (fecal glucocorticoid and androgen metabolite concentrations) and behavioral responses to known stressors in male clouded leopards with keeper assessments of animal temperament ('anxious' versus 'calm'). This demonstrates, for the first time, the effectiveness of behavioral reaction tests at assessing temperament in male clouded leopards. Study 2 (Chapter 3) tests two drug therapies to determine the underlying causes of aggression in male clouded leopards. Behavioral and endocrine responses to the same tests applied in Study 1 are assessed following anxiety mitigation with a psychotropic drug (clomipramine) for comparison to males undergoing reversible chemical castration with a gonadotropin releasing hormone (GnRH) agonist (deslorelin). Study 3 (Chapter 4) compares the long-term effects of the two different treatments on endocrine concentrations, and their influence on spermatogenesis in the clouded leopard and the domestic cat (*Felis catus*). Finally, Chapter 5 concludes by summarizing key findings from this study, and relates these

findings to the scientific literature on aggression from an evolutionary perspective. This research provides an opportunity to improve the general understanding of the mechanisms controlling aggression in the male clouded leopard and its relationship to the reproductive axis, information that we hope will have a significant positive impact on management and breeding success for this rare species.

1.2 Hypothesis and Objectives

The overall hypothesis of this study was that aggression in the male clouded leopard is modulated by testosterone-linked serotonergic pathways. Modification of either testosterone concentrations or the serotonin system would reduce aggression and ultimately improve reproductive success in this species.

Specific objectives were to:

1. Assess the effectiveness of behavioral reaction tests in determining temperament ('anxious' versus 'calm') and eliciting anxious and aggressive behaviors; characterize male clouded leopards participating in this study as 'anxious' or 'calm';
2. Compare the effects of an anxiety-reducing psychotropic drug (clomipramine) vs. testosterone suppression with a GnRH agonist (deslorelin) on anxiety and aggression; and
3. Examine the impact of the two anti-aggression treatments on endocrine and reproductive function and determine the timing of return to full function.

1.3 Background and Significance

1.3.1 Clouded Leopard Natural History

Clouded leopards are most closely related to the Pantherine lineage of cats, which includes the five big cats (lions, tigers, leopards, jaguars, and snow leopards). The Pantherine lineage diverged from modern felidae 11 million years ago and clouded leopards diverged from Panthera 6 million years ago. There are 38 living cat species and nearly every one is listed as endangered or threatened (Davis et al., 2010). Clouded leopards are listed as 'Vulnerable' in the IUCN Red Data Book, an Appendix 1 species under CITES and 'Endangered' under the United States Endangered Species Act. The primary causes of clouded leopards' decline are rapid deforestation of their primary habitat and illegal hunting of both clouded leopards and their prey base (IUCN, 2011).

The clouded leopard primarily lives in the lowland tropical forests of the Himalayan foothills in Nepal, into China, and throughout Southeast Asia (IUCN, 2011). There are two species of clouded leopard: *Neofelis nebulosa*, found in mainland Asia, and *Neofelis diardi*, found in Borneo and Sumatra. Interestingly, the two species diverged about 1.5 million years ago and are genetically more different than any two of the five Panthera species (Buckley-Beason et al., 2006).

Clouded leopards are a medium-sized felid weighing between 11 and 20 kg, with the males significantly larger than the females. While medium in body size, they are considered a large feline due to the size of their canines – the largest in relation to their body size of any extant feline. They are extremely good climbers and have been seen hanging from their back feet (Fletchall, 2000).

Clouded leopards tend to be a very secretive and elusive species, therefore little is known about their behavior in the wild. While there have been several camera trapping studies, the most extensive knowledge comes from a study conducted in Thailand where two males and two females were radio-collared and tracked for a period of four years (Grassman et al., 2005). Through this study, it was determined that a clouded leopard's home range is between 22.9 and 51.0 km^2, with no significant size difference between male and female home ranges. Notably, male-female, female-female, and even male-male home ranges overlap. Clouded leopards were found primarily in closed forest, but to a lesser extent were seen in open forest-grassland and even abandoned orchards. They are both nocturnal and diurnal, as confirmed by both activity patterns and the presence of prey species typical of both activity patterns in their feces, but tend more often to be nocturnal (Grassman et al., 2005). Activity patterns appear determined by environmental factors – in areas where they coexist with tigers, they tend to be more diurnal and arboreal (Grassman et al., 2005; Datta et al., 2008). They eat a wide variety of prey, including hog deer (*Axiss porcinus*), slow loris (*Nycticebus coucang*), bush-tailed porcupine (*Atherurus macrourus*), Malayan pangolin (*Manis javanica*), and Indochinese ground squirrels (*Menetes herdmorei*) (Grassman et al., 2005; IUCN, 2011). Due to limited research, it is uncertain what types of family groups clouded leopards live in, but from available tracking studies and captive research it is likely that they are solitary, except when females give birth and raise 2-6 cubs (Fletchall, 2000; Grassman et al., 2005).

The *ex situ* population of clouded leopards is meant to serve as a hedge against extinction, but this is one of the most challenging felid species to manage in

breeding programs. Additional information about clouded leopard behavior in the wild would no doubt aid in management of the captive population. At the onset of this study, out of a population of 75 animals there were only two compatible pairs and no cubs had been born in over three years. Fortunately, the number of successful breeding pairs has increased slightly during the course of the study. The overall scarcity of successful breeding pairs is primarily due to male aggression. This hostile behavior is so extreme that males often kill females and often without warning or provocation. The incidence of such mortalities has been high, with as many as five female clouded leopards killed annually from aggressive attacks (Breitbeil, 2012). For this reason, zoos have become extremely cautious about initiating introductions, and all males with a history of aggressive behavior are currently not considered for breeding.

1.3.2 Neural Pathways and Aggression

Aggression has been a common focus of human and rodent behavioral and neuroendocrine research for over 40 years (Kingsbury et al., 1997; Siegel et al., 1999; Weinshenker and Siegel, 2002). In 1968, Moyer defined aggression according to the following traits: predatory, inter-male, fear-induced, irritable, territorial, maternal or instrumental (Moyer, 1968). Further refinement of this model for felids has identified two major categories: predatory attack or affective defense (Siegel et al., 1999). Predatory attack in felids involves stalking and neck biting, whereas affective defense involves retraction of the ears, arching of the back, vocalization and unsheathing of the claws. Aggressive attacks in clouded leopard pairs have included some components of each category. One incident (from video) reveals a female suddenly

running from the male, and the male chasing and breaking the female's neck. Other data indicate decreased incidence of affiliative behaviors in failed versus successful pairs (MacKinnon et al., 2007).

The neural pathways that modulate aggression primarily involve the hypothalamus, amygdala and periaqueductal gray (Berntson et al., 1976; Bandler, 1977; Bandler, 1979; Shaikh et al., 1985; Adamec, 1990; Siegel et al., 1999). The neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) plays a key role in regulating aggressive behavior (Ferris et al., 1997; Siegel et al., 1999; Summers et al., 2005). Serotonin receptors, especially the 5-HT1A receptor, are also important and especially prevalent in the areas of the brain associated with aggression (Simon et al., 1998; Summers et al., 2005; Popova, 2006). Furthermore, injection of arginine vasopressin (a hormone linked to aggression facilitation) into the hypothalamus of the golden hamster leads to increased aggression. However, subsequent treatment with fluoxetine (a stimulant of the serotonin system), leads to significant blockage of the vasopressin-mediated aggressive behaviors (Ferris et al., 1997). Mechanistically, serotonin is formed in a presynaptic neuron, released into the synaptic cleft, and either binds to a target receptor, initiating a response, or is reabsorbed by the serotonin plasma membrane transporter (SERT) in the presynaptic neuron. Thus activation of the serotonergic system can either involve increased release or decreased reabsorption of serotonin at the synaptic cleft (Squire et al., 2003).

1.3.3 Testosterone and Aggression

The endocrine system also modulates aggression. During perinatal development, the male brain becomes 'masculinized' from exposure to testosterone,

which primes the brain to become reactive to androgens during adulthood. These early effects are referred to as 'organizational'. At puberty, the effects of testosterone on the brain are referred to as 'activational' (Meek et al., 1997; Griffin and Ojeda, 2003; Browne et al., 2006). Testosterone is regulated by the release of GnRH from the hypothalamus, which stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary that, in turn, stimulate testicular Leydig cells to produce androgens (Griffin and Ojeda, 2004). Testosterone circulates throughout the body to bind to widespread testosterone-specific receptors, including those in the brain.

While strongly correlated with aggression in many species, testosterone's mechanism of action is extremely complex and not fully understood (Silverin et al., 2004; Oyegbile and Marler, 2005; Wingfield, 2005). In many species, testosterone influences frequency, intensity and persistence of aggression (Goymann et al., 2007). In the mouse, testosterone increases as males win fights and is correlated with an enhanced ability to win future fights (Oyegbile and Marler, 2005). In birds, testosterone regulates territorial aggression among males during the breeding season, when competition for mates is high (Wingfield et al., 2001). Removing testosterone's influence via castration reduces the incidence of aggressive behaviors in rodents, and subsequent testosterone administration re-establishes antagonistic behaviors (Bergvall and Hansen, 1990; Matochik et al., 1994; Hume and Wynne-Edwards, 2005). Finally, it has been suggested that the serotonergic system plays an important role in controlling testosterone-driven aggression in rats (Bonson et al., 1994).

1.3.4 Temperament and Aggression

Researchers have defined temperament as "an individual animal's distinctive pattern of behavior that is consistent across time and situations" (Phillips and Peck, 2007). Therefore, temperament is a stable and generally permanent trait, whereas behavior is a specific response to a situation or context. In clouded leopards, research to date suggests males have two basic temperament types that have been described: 'anxious' and 'calm'. Those of the former type express excessive stereotypic pacing, hiding and self-injuring behaviors, whereas those of the latter group do not exhibit these behaviors and tend to be more vocal (Wielebnowski et al., 2002; MacKinnon, 2008). A positive correlation has been demonstrated between clouded leopards that have the 'anxious' temperament and parallel elevations in excreted cortisol concentrations (Wielebnowski et al., 2002; MacKinnon et al., 2007). Additionally, males exhibiting anxious behaviors have a higher tendency to have failed male-female pairings (MacKinnon et al., 2007).

1.3.5 Stress-Sensitivity and Aggression

Aggression is closely related to an animal's stress response. During exposure to acute (e.g. predatory attack) or chronic (e.g. starvation) stressors, the brain induces a hypothalamic-pituitary-adrenal (HPA) response. The hypothalamus releases corticotrophin-releasing hormone that stimulates anterior pituitary receptors to release adrenocorticotropic hormone, which in turn triggers the release of glucocorticoids from the adrenal cortex. Corticosteroids, in turn, stimulate a cascade of metabolic and physiologic responses that stimulate conservation of metabolic resources and down-regulate non-essential functions (Griffin and Ojeda, 2004). Adrenal corticoids

also are linked to aggressive behavior. Cortisol levels increase steadily during puberty in the male golden hamster, coinciding with a change from play-fighting to adult aggression (Wommack et al., 2004; Wommack and Delville, 2007). Research has further demonstrated that injecting rodents with cortisol elicits aggressive behavior (Haller et al., 1997; Haller et al., 1998; Haller et al., 2000). Similarly, eliciting aggression through hypothalamic stimulation causes a rapid increase in corticosteroids, whereas injecting corticosterone into adrenalectomized rodents facilitates the aggressive response. Thus, there appears to be positive feedback between the adrenocortical stress response and a brain mechanism involved in aggressive behavior (Kruk et al., 2004). This connection between glucocorticoid response to stress and aggression appears to be one factor in male clouded leopard aggression, since males with higher glucocorticoid levels are associated with increased failed male-female pairings (MacKinnon, 2008).

1.3.6 Spermatogenesis and Reproductive Function

In addition to effects of treatments on aggression, the effect of treatments on male reproductive function is also investigated in this study. Male germ cells undergo spermatogenesis (the process by which they become mature spermatozoa) in the seminiferous tubules of the testes. Sertoli cells, located in the seminiferous tubules, play a critical role in sperm development. Due to the tubular structure of these cells, they are difficult to study and thus poorly understood. However, it is clear that they play a nurturing role and are critical for sperm development. Germ cells embed between sertoli cells, where they are protected from the body's immune system by the sertoli cells' blood-testis barrier (Kerr et al., 2006).

Germ cells start out as spermatogonia (Ad, A, and B) and through mitosis become primary spermatocytes. They then undergo meiosis I to become secondary spermatocytes and meiosis II to become spermatids. Finally, through spermiogenesis they are transformed from a simple round form to fully formed spermatozoa ready for spermiation. During spermiation they are released from the sertoli cells and enter into the rete testis (Kerr et al., 2006). In domestic cats (the most closely-related species to the clouded leopard that has been studied), spermatogenesis takes 46.8 days. During maturation, sperm go through a different number of structurally distinct stages depending on the species. For domestic cats, there are 8 stages (Franca and Godinho, 2003). Next, the fully formed spermatozoa enter the epididymis where they undergo further maturation and leave the male reproductive tract ready for fertilization. Due to the complexity of the process and the many factors affecting maturation, spermatozoa emerge with a wide variety of defects. In inbred endangered carnivores these defects are often in high concentration; in fact, clouded leopards often have up to 80% abnormal sperm (Pukazhenthi et al., 2006). Most head and acrosome sperm defects occur during spermatogenesis and spermiogenesis, while defects to the tail tend to develop in the epididymis (Pukazhenthi et al., 2006).

1.3.7 Testosterone and Reproductive Function

Spermatogenesis is controlled by the hypothalamus-pituitary-gonadal (HPG) axis. GnRH is a peptide hormone that is produced in the preoptic area of the hypothalamus and is released in a pulsatile manner. The arcuate is believed to be the pulse generator and produces pulses every 40-60 minutes that last about five minutes each (Herbison, 2006). GnRH travels to the pituitary where it binds to GnRH

receptors (seven-transmembrane, G-protein coupled receptor) on the gonadotrope cell. Binding causes the release of two glycoproteins, Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). LH and FSH travel through the blood stream to the testis where FSH binds to sertoli cells, in the seminiferous tubules, and LH binds to Leydig cells, adjacent to the seminiferous tubule. Through the process of steroidogenesis, the Leydig cells convert cholesterol into the steroid hormone testosterone. Once produced, testosterone binds to G-protein androgen receptors on sertoli cells to promote spermatogenesis (Griffin and Ojeda, 2004; Kerr et al., 2006). Both FSH and testosterone act in concert to stimulate the sertoli cells, affecting the many stages of sperm development (O'Donnell et al., 2006). However, by itself testosterone can maintain spermatogenesis by stimulating the proliferation of sertoli cells, signaling maturation during the spermatid and spermatozoa stages, and ultimately triggering spermiation (O'Donnell et al., 2006). Testosterone also plays a role in stimulating the epididymis to move spermatozoa through its ducts and aiding in the development of fully motile, fertile sperm (Robaire et al., 2006). In summary, the role of testosterone is critical for sperm development.

1.3.8 The Affect of Stress on Reproductive Function

In an effort to conserve resources and allocate them where most needed, chronic stress will decrease endocrine and behavioral components of reproduction. Chronic stress inhibits male sexual behavior in rats (RetanaMarquez et al., 1996; Gorzalka et al., 1998; Cordner et al., 2004). It inhibits spermatogenesis and testosterone secretion in male rats (Collu et al., 1984). In the Wistar rat, it has been shown that chronic treatment with corticosterone resulted in decreased LH levels,

testosterone, and Leydig cell LH receptor numbers (Sankar et al., 2000). An associated decrease in spermatogenesis would also occur. There is also evidence linking stress to poor semen quality, with low percentage of normal sperm and low sperm concentration in many felid species (Swanson et al., 2003; Pukazhenthi et al., 2006). It has been shown that clouded leopards, compared to other felid species, have increased basal cortisol levels that may affect overall reproduction (Wildt et al., 1986).

1.3.9 Psychotropic Drugs, Anxiety and Aggression, and Reproductive Function

Over the past 20 years, psychotropic drug therapy has been used to treat a variety of symptoms in numerous species, including aggression in the dog (*Canis lupus familiaris*) and cat (King et al., 2000; Litster, 2000). Clomipramine is a tricyclic antidepressant approved by the U.S. Food and Drug Administration for animal use in controlling anxiety-type disorders (FDA, 2007). It acts by inhibiting the re-uptake of the neurotransmitters serotonin and noradrenalin by blocking the SERT and noradrenalin transporter (NET). This, in turn, increases the availability of serotonin for binding to postsynaptic receptors, making it effective in reducing both anxiety and aggression (Dubinsky et al., 1973). In the dog and cat, this drug has been used widely for treating anxiety disorders, obsessive-compulsive behaviors, destruction, and aggression (Seksel and Lindeman, 1999; Siegel et al., 1999; King et al., 2000; Litster, 2000; Gillman, 2007).

Clomipramine has also been shown to counteract the negative effects of stress on male reproduction and to cause an overall improvement in sperm quality. In the

Syrian hamster (*Mesocricetus auratus*), males treated with clomipramine and exposed to stressors showed no decrease in sexual behavior compared to untreated males (Cordner et al., 2004). Male rabbits treated with clomipramine for 12 weeks showed a significant increase in sperm quality, including sperm concentration, total sperm per ejaculate, sperm motility, total motile sperm per ejaculate, and total live sperm. Of the traits examined, only sperm concentration per ejaculate decreased (Ahmed et al., 2008). Although clomipramine has not been tested in clouded leopards, it could have a positive effect on male reproduction.

1.3.10 GnRH Agonists, Testosterone and Aggression, and Reproductive Function

Deslorelin, a GnRH agonist, is a long-acting reversible implant used to control reproduction and male aggression in some species (Aspden et al., 1996; Bertschinger et al., 2001; Padula, 2005; Trigg et al., 2006). This hormone acts on pituitary GnRH receptors to first hyper-stimulate and then suppress gonadal function, including spermatogenesis and steroidogenesis (Aspden et al., 1996; Bertschinger et al., 2001; Padula, 2005; Trigg et al., 2006). In certain species, testosterone levels are decreased in males. Castration and subsequently reduced plasma testosterone has been used for centuries to reduce hostile behavior in many species (Bergvall and Hansen, 1990; Hagelin, 2001; Hume and Wynne-Edwards, 2005). Chemical castration with deslorelin may achieve the same result. The use of deslorelin to reduce male aggression has been reported in the lion-tailed macaque (*Macaca silenus*) and black-footed cat (*Felis nigripes*) (Norton et al., 2000; Bertschinger et al., 2001). While much deslorelin research has been conducted in the dog, this agonist is known to

decrease reproductive hormones in the cheetah (*Acinonyx jubatus*) and domestic cat (Bertschinger et al., 2006; Trigg et al., 2006; Goericke-Pesch et al., 2011). No adverse effects have been observed with deslorelin, and once the implant is absorbed (6 to 12 months, depending on the formulation), reproductive function is restored (Trigg et al., 2006).

1.4 Summary

It has been established that male clouded leopards have both 'anxious' and 'calm' temperaments and that 'anxious' males tend to be associated with failed pairings. It is also the case that pairs formed with prepubescent males tend to be more successful. Finally, it has been shown that animals participating in pairings have the highest stress levels during early stages of breeding introductions. Taken together, these findings indicate that a serotonin-modulating tricyclic antidepressant or testosterone-modulating GnRH agonist may decrease male aggression for the key period of time necessary to form breeding pairs in clouded leopards. Such a finding, as described in the following work, would represent a major breakthrough in clouded leopard management facilitating the establishment of self-sustaining *ex situ* populations.

Chapter 2: Behavioral Reaction Tests as a Measure of Stress Reactivity to Determine Temperament in the Male Clouded Leopard (*Neofelis nebulosa*)

2.1 Introduction

The clouded leopard (*Neofelis nebulosa*) is a striking and elusive cat species, whose secretive nature has made it difficult to gather information on population statistics and behavior in the wild. However, it is clear that clouded leopard populations are in severe decline throughout their historic range in Southeast Asia due to habitat loss and poaching (Grassman et al., 2005). As a result, clouded leopards are considered 'Endangered' under the United States Endangered Species Act (ESA) and as 'Vulnerable' in the IUCN Red Data Book. An *ex situ* population of clouded leopards is managed through captive breeding programs in zoological institutions worldwide; the hope is that these individuals will serve as a hedge against extinction of wild populations. However, the clouded leopard has proven to be one of the most challenging felid species to manage in captivity (Wielebnowski et al., 2002).

Strong intra-specific aggression resulting in 25 fatal attacks over 20 years has made the propagation of clouded leopards in captivity very challenging (Breitbeil, 2012). In many species, androgen levels are known to influence frequency, intensity, and persistence of aggression (Goymann et al., 2007). However, the role androgen plays in clouded leopard aggression is poorly understood. There is evidence that circulating androgen levels may play a key role in aggression in clouded leopards, with juvenile males expressing lower rates of aggression (MacKinnon, 2008). In fact,

one of the only successful strategies for breeding clouded leopards in captivity involves pairing animals as juveniles and managing them together into adulthood (Fletchall, 2000). Animals paired as adults exhibit high rates of failure and aggression (MacKinnon, 2008).

In addition to circulating androgens, aggression in clouded leopards appears to be related to anxiety and stress. Clouded leopards are known to be particularly anxious, as evidenced by captive individuals having chronically elevated glucocorticoid levels (Wildt et al., 1986) in combination with frequent anxiety-related behaviors including tail-biting, fur-plucking, excessive hiding, and pacing (Wielebnowski et al., 2002). Increased levels of anxiety may contribute to a higher frequency of aggressive behavior observed in this species in captive settings. Indeed, male clouded leopards with higher fecal glucocorticoid concentrations also experience increased anxiety-related behaviors and reduced pairing success (Wielebnowski et al., 2002; MacKinnon, 2008).

Anxious animals tend to experience increased and/or chronic stress levels, which can be detrimental to an animal's health (Leonard and Song, 1996; Shively et al., 1997; Tsigos and Chrousos, 2002). Catecholamines activate the "fight or flight" stress response, followed by elevated glucocorticoids (if a stressor remains) and ordinarily subside once a perceived or real threat is removed (Wingfield et al., 1998). If glucocorticoid concentrations remain chronically and abnormally elevated, this can lead to decreased immune response and reproductive function (Leonard and Song, 1996; Shively et al., 1997; Tsigos and Chrousos, 2002). Some species, such as the snowshoe hare, have increased stress hormone levels, which affect both the stressed

individual as well as the individual's offspring by impacting the prenatal hormonal environment of the fetus (Sheriff et al., 2010). Chronic stress in captive clouded leopards therefore has the potential for long-lasting deleterious effects.

Despite its prevalence, aggression in male clouded leopards is not always readily identifiable and if identified, the significance not always understood. Unfortunately, therefore, males are often first identified as aggressive when they injure or kill females. In order to better manage aggression and anxiety in the male clouded leopard, one important step is therefore to be able to identify aggressive males before females are introduced. Thus, the development of behavioral tools to assess an individual's temperament is critical, since certain types of temperament can be linked to an increased tendency for anxiety, aggression, and /or stress reactivity. Temperament, a term synonymous to 'personality' and 'behavioral type', can be defined as *an individual animal's distinctive pattern of behavior that is consistent across time and situations* (Phillips and Peck, 2007). Importantly, temperament is a combination of stable and generally permanent behavioral traits that may be used to predict some aspects of an individual's response to a variety of situations or contexts. Identifying a test that allows the assessment of some pertinent aspects of temperament, such as anxiety-related behavior and aggressiveness, could be an important tool for managing clouded leopards and possibly other non-domestic species as well.

For laboratory animals there are already a multitude of techniques available for assessing temperament. For example, in experiments with rodents, the 'open-field test' is used to test exploratory behavior and the 'elevated plus maze test' assesses an

animal's reaction to open spaces in order to assess anxiety-like behaviors (Pellow and File, 1986; Prut and Belzung, 2003). The 'resident-intruder test' is used to determine aggression by exposing resident males to intruding males and assessing aggressive behaviors such as latency to first attack and biting (Holmes et al., 2002). In mink and rats the 'glove test' is used to assess the degree of fearfulness to new objects (Nikulina et al., 1992; Gulevich et al., 2010; Meagher et al., 2011). In non-domestic zoo animals mechanisms have been developed for assessing individual animal temperament, including keeper surveys and monitoring glucocorticoid concentrations, but these tests require significant time and expertise to administer correctly. Furthermore, they cannot be administered by individual keepers to test individual animals as a management tool since these rely on comparisons among animals to identify the relative level of anxiety and stress in a single male.

Experienced animal keepers can become skilled at assessing the temperament of animals under their regular care and keeper survey results have been significantly correlated with glucocorticoid levels to demonstrate the biological accuracy of keepers' assessment of individuals' stress levels (Wielebnowski et al., 2002). Based on the use of these techniques in clouded leopards, the following two behavioral types were identified: cats with a more relaxed, 'calm' temperament versus cats with a more 'anxious' and stressed demeanor (Wielebnowski et al., 2002; MacKinnon, 2008). Although individual keepers appear able to gauge temperament, new tests are expected to help identify individuals with higher levels of anxiety, and thus possibly a behavioral type or temperament that predisposes that individual to increased stress reactivity and aggression.

Three behavior reaction tests that have been applied in other species may be effective tools for assessing temperament in clouded leopards: mirror image stimulation (MIS) test, airhorn test, and unfamiliar people (UFP) test. The MIS test is frequently used to test aggressiveness, shyness, and social behavior in a variety of species including fish, squirrels, and cheetahs where an animal interprets its own reflection in the mirror as an intruder and reacts accordingly (Wielebnowski, 1999; Moretz and Morris, 2003; Boon et al., 2008). A horn test was used to determine an individual's fearfulness to loud noises in order to assess temperament in domestic dogs (De Meester et al., 2011). Finally, exposure to unfamiliar people is a test to assess an individual's fearfulness of people unknown to them and has also been used to test temperament in domestic dogs (De Meester et al., 2011). Clouded leopards are sensitive to changes in their environment and exposure to novel objects, loud noises, and new people are known stressors for this species (Fletchall, 2007). Additionally, each of these tests can be standardized across zoos and thus ideally used to objectively and quantifiably assess a key aspect of clouded leopard temperament as it relates to aggression and anxiety.

Currently, clouded leopards and many captive zoo animals are managed genetically through studbooks, but there is no systematic way of determining an animal's temperament or other salient behavioral features to be taken into consideration for management decisions. Yet this information is important when making decisions about which animals might be the most compatible for breeding or for display to zoo visitors (Watters and Powell, 2011). Because chronic stress and anxiety is already known to be a serious problem in the *ex situ* management of male

clouded leopards, the aim of the present study was to develop and evaluate a simple test for assessing some salient aspects of temperament, to help categorize male clouded leopard as 'calm' or 'anxious'/aggressive. 'Anxious' animals are expected to be more susceptible to stress inducing situations and possibly more prone to aggression. In this study, three behavior reaction tests – MIS test, airhorn test, and UFP test – were administered, with concurrent behavioral observations and fecal hormone assays of androgens and glucocorticoids. This analysis was combined with keeper assessments of animal temperament through a questionnaire to assess the efficacy of the behavioral reaction tests in determining animal temperament.

2.2 Methods

2.2.1 Animals and Study Area

Sixteen singly-housed adult male clouded leopards housed at 12 North American zoos were studied for a 3 month time period. Participating zoos included: Alexandria Zoological Park, LA, USA (n = 1; 10 y of age); Audubon Nature Institute, LA, USA (n = 1; 10 y of age); Central Florida Zoo & Botanical Gardens, FL, USA (n = 1; 8 y of age); Cincinnati Zoo & Botanical Garden, OH, USA (n = 1; 7 y of age); Cleveland Metroparks Zoo, OH, USA (n = 1; 5 y of age); Houston Zoo, TX, USA (n = 1; 6 y of age); Minnesota Zoo, MN, USA (n = 1; 7 y of age); Omaha's Henry Doorly Zoo, NE, USA (n = 1; 12 y of age); San Antonio Zoo, TX, USA (n = 1; 13 y of age); Smithsonian Conservation Biology Institute, VA, USA (n = 4; 4, 11, 14, and 15 y of age); Zoo Atlanta, GA, USA (n = 1; 13 y of age); and Zoo Miami, FL, USA (n = 2; 15 and 17 y of age) (Table A1.1). These individuals represented 100% of the

singly-housed male clouded leopards in the US zoological population at the time of the study.

Animals were housed according to the Association of Zoos and Aquariums (AZA) guidelines for this species and each institution maintained their routine husbandry protocols for the duration of the study period. Nine animals were housed in areas where they spent part of the time on exhibit for public view and part of the time off-exhibit; the other seven animals were permanently housed off exhibit. All testing sessions took place in each animal's off-exhibit area.

2.2.2 Study Design

Sixteen singly-housed male clouded leopards were categorized into 'anxious' (n = 8) and 'calm' (n = 8) temperament categories using a keeper survey (Figure A1.3). A series of behavior reaction tests (MIS test, airhorn test, and UFP test) were conducted and videotaped to determine an individual's response to stress-inducing situations. Fecal samples were collected for the duration of the study to assess fecal glucocorticoid and androgen concentrations. Temperament categories were compared to fecal glucocorticoid and androgen concentrations to confirm temperament assessments. Video-based behavioral assessments were then compared to both compound survey scores and mean fecal glucocorticoid and androgen concentrations to assess the effectiveness of the three tests at eliciting behaviors used to assess male clouded leopard temperament.

The studies described were approved by the Smithsonian's National Zoological Park (#08-12) and the University of Maryland, College Park (#R-08-33)

Animal Care and Use Committees. The study was also reviewed and approved by all institutions housing clouded leopards.

2.2.3 Behavioral Questionnaire

All keepers at the participating zoos (74 total, 2-8 per cat) who had been keepers for more than six months and who had cared for an individual male for more than six months were asked to independently complete an online behavioral assessment questionnaire (through SurveyMonkey™) at the beginning of the study (Figure A1.3). Keepers were asked to rate each cat on a commonly used Likert Scale (from one, 'never' to five, 'often') on eight behavioral questions (Table 2.1) (Wielebnowski et al., 2002). Each keeper was also asked to categorize each cat overall as either 'calm' or 'anxious'.

2.2.4 Behavioral Reaction Tests

Clouded leopards were given three behavioral reaction tests over a 3-week period, each performed 1 week apart (Table A1.2). Prior to the initiation of the tests

Table 2.1 Behavioral questions presented to keepers.

Keepers were asked to rate each clouded leopard on a scale from 1 to 5 for each of these questions, those in bold showed significant inter-rater agreement across keepers and facilities.

How often does this individual hiss or growl at people or neighboring animals?
How often does this individual perform stereotypic pacing?
How often does this individual prusten?
How often does this individual appear to be tense?
How often does this individual hide from view?
How often does this individual seek out or investigate novel situations?
How often does this individual appear to be calm?
How often does this individual meow or cry?

one researcher visited all 12 zoos to help train keepers so data collection for the test could be standardized and reliable (Figure A1.1). Tests were chosen based on previous behavioral studies (Wielebnowski, 1999; Moretz and Morris, 2003; Boon et al., 2008; De Meester et al., 2011) to induce a behavior response in clouded leopards to potentially stressful situations (e.g., such as a new person, loud noise, novel objects, etc.).

Test 1: Mirror image stimulation (MIS test). An acrylic mirror (24 x 48 in, from Interstate Plastics) was secured on the fence in an animal's enclosure. The mirror remained in the enclosure for 30 min. Behavioral data collection is detailed below in Section 2.2.5.

Test 2: Noise test (airhorn test). An Orion 8 oz Safety airhorn, an unfamiliar noise to clouded leopards, was sounded a single time approximately 5 ft from the animal's enclosure.

Test 3: Exposure to unfamiliar people, both males and females (UFP test). Clouded leopards were exposed to an unfamiliar male and female person twice per day (morning and evening) on three consecutive days. The unfamiliar man and woman engaged in a conversation for 10 min at a safe distance from the animal's enclosure, but in clear visual, auditory, and olfactory proximity.

The MIS test and airhorn test were administered in a random order across zoos, while the UFP test always took place at the end of each testing period due to the longer duration of the test.

2.2.5 Videotaping and Analysis

Quantitative behavioral data were collected by video documenting (Sony Camcorder) during three different periods: a two-week pre-behavior test period (pre-behavior test), the three-week behavioral reaction test period (behavior reaction test), and a two-week post-behavior test period (post-behavior test). At all institutions videos were filmed by a keeper familiar with each cat to minimize the additional disturbance represented by the recordings themselves. During pre- and post-behavior test periods, behavioral videos were recorded in 30 min segments twice weekly for a total of 60 min/week. Observations were made during daylight hours distinct from feeding or cleaning times when individuals were expected to exhibit normal behavior patterns as identified by keepers.

For the behavioral reaction test period, video recordings were collected for each of the tests as follows: for 30 min from the time an animal was shifted into an area where the mirror was present, for 30 min including and following the sounding of the airhorn, and for 30 min including the 10 min UFP test and 20 min following the test. All videotapes were sent to the Smithsonian Conservation Biology Institute (SCBI), Front Royal, VA and all behavioral data coded by a single observer from the recordings to assure uniform data collection. Using an ethogram previously established for this species (Table 2.2, Table A1.3)) (Fletchall, 2000), the observer recorded frequencies of behavioral states (using instantaneous scan sampling at one min intervals) and the rates of behavioral events (using continuous sampling) (Figure A1.2). Several additional measures were recorded for the MIS test, including: 'latency of approach', time (sec) spent interacting with the mirror, and incidence of

Table 2.2 Ethogram of behaviors.

Behavioral Responses to Mirror (behaviors recorded once for each trial):		
Latency to approach		Amount of time in seconds for the cat to first approach the mirror and interact through any behavior.
Time spent interacting		Total time in seconds the cat spent interacting with the mirror. Time ended when the animal stepped away. Time was ended after 5 seconds of last interaction if animal did not step away.
Paw Mirror*a		Cat touches mirror with paw, may occur in rapid succession, each touch is a separate event.
Swat Mirror*a		Cat uses forepaws to tap or strike an object– occasionally this occurs without making physical contact with the object.
Behavioral States (frequency)		
Crouching*b		Cat standing with legs bent under body. Body close to ground. Often seen when cat is sniffing ground or hiding. Often a freeze position.
Lying Down*b		Cat lies in horizontal reclining position, cat may or may not be asleep
Nest Box		Cat is located in next box, may or may not be visible
Pacing*(**)c		Cat walks back and forth in a repetitive, non-directed pattern (stereotypic movement) must repeat movement 3Xs and the cat should not be performing other behaviors (i.e. sniffing, urinating etc.)
Running*c		Cat moves swiftly
Walking*c		Cat walks at a moderate pace in a directed manner towards something
Standing		Cat remains motionless while in upright position on all four feet
Behavioral Events (rate)		
Growl/hiss		Growl is a low-pitched throaty rumbling sound; hissing is a rapid expulsion of air, teeth exposed and nose wrinkled.
Tail flick		Rapid movement of the tip of the tail.
Prusten**		Soft expulsion of air through lips, similar to snorting in horses. Cat may raise muzzle while vocalizing. Often used in 'friendly' greeting or a 'reassurance' context
Meow/cry**		Either short high-pitched meow call or loud extended crying call. Both calls appear to be emitted when one cat is trying to locate another over a short or long distance

*and associated letters (a/b/c) indicate behaviors that were combined
**part of keeper survey

'swatting' (Table 2.2). Behaviors not readily distinguishable from one another (affiliated behaviors) and exhibited at a low frequency were combined for the statistical analyses (Table 2.2; combined behaviors are marked with an asterisk and associated letter). Specifically, 'crouching' was combined with 'lying down' ('lying down') and 'pawing' was combined with 'swatting' ('swatting'). 'Running', 'pacing', and 'walking' were combined to get an overall assessment of activity level ('overall activity').

2.2.6 Fecal Collection and Analysis

Fecal samples (at least 5 g) were collected daily from each animal, starting 14 days before the first video session, throughout the 7 week video behavioral testing period, and continuing 14 days after the last video session (range 51-82 samples/male). Samples were collected between September 2008 and April 2009. Once collected, samples were immediately placed in plastic bags, labeled, and frozen at -20ºC until shipping. Batches of frozen samples were then shipped on dry ice to the SCBI Endocrine Research Laboratory (Front Royal, VA) for hormonal analysis by Enzyme Immunoassay (EIA). Samples were evaluated for glucocorticoid and androgen metabolites using assays validated for the clouded leopard (Brown et al., 1996; Weinshenker and Siegel, 2002; MacKinnon, 2008). In brief, each fecal sample was lyophilized, pulverized, and 0.19–0.21 g of dry fecal powder was vortexed (5 s) and shaken (30 min) in 5 mL of 90% ethanol. After centrifugation (20 min; 2500 RPM), the supernatant was recovered, and the pellet was re-suspended in 5 mL of 90% ethanol, vortexed (5 s), shaken (30 s), and re-centrifuged (15 min, 2500 RPM). The first and second supernatants were combined, dried under air and reconstituted in

1 mL methanol. Methanol extracts were vortexed (5 s), sonicated (15 min), and dried under air. Each extract was reconstituted in 1 mL dilution buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.15 M NaCl, pH 7.0). Dilution buffer extracts were vortexed (5 s), sonicated (15 min or until completely in solution), and diluted 1:80 and 1:30 in dilution buffer for androgens and glucocorticoids, respectively. Samples were stored in polypropylene tubes at -20ºC until EIA analyses.

2.2.7 Androgen EIA

Androgen metabolite concentrations in fecal extracts were quantified with a single-antibody testosterone EIA developed for use in non-domestic species fecal hormone analyses using previously described methods (Kersey et al., 2010). The antibody cross-reacted 100% with testosterone, 57.4% with dihydrotestosterone, 0.3% with androstenedione, 0.04% with androsterone and dehydroepiandrosterone, 0.03% with cholesterol, 0.02% with estradiol and <0.02% for all other tested analytes, including cortisol (Kersey et al., 2010). Polyclonal anti-testosterone (R156/R157; 1:7,500; supplied by C.J. Munro, University of California, Davis, CA) was added (0.05 µl) to 96-well microtiter plates (Nunc-Immuno; Fisher Scientific, Pittsburgh, Pennsylvania), and allowed to incubate (12–18 h) at 4°C. Plates were washed (0.05% Tween 20 in 0.15 M NaCl solution) to remove unadsorbed antibody. Fecal extract was added in duplicate (0.05 mL) and standards in triplicate (0.05 mL; .05–12 ng/mL; 17β-hydroxy-4-androsten-3-one; Steraloids, Newport, Rhode Island), Testosterone horseradish peroxidase (HRP) (1:80,000; supplied by C.J. Munro) then was added to each well containing standard or sample and incubated (2 h; at room temperature) before unbound components were removed with wash solution. A substrate solution

(0.1 mL) consisting of buffer (citric acid), chromagen (ABTS), and catalyst (hydrogen peroxide) was added to each well and allowed to incubate (~30 min) before optical densities (ODs) were determined. ODs were read using a microplate reader (MRX, Dynex Technologies, Chantilly, VA) at 405 nm when 0 pg standard wells reached an OD of 0.9–1.1. Intra-assay variation was <10%, and inter-assay coefficients of variation (CVs) for 2 internal controls were 6.9% (mean binding, 22.2%) and 4.0% (mean binding, 67.9%) (n = 99 assays). Immunoreactivity of serially diluted fecal extracts paralleled standard binding.

2.2.8 Glucocorticoid EIA

A single-antibody cortisol EIA that cross-reacted 100% with cortisol, 9.9% with prednisolone, 6.3% with prednisone, 5% with cortisone, 0.7% with corticosterone, 0.5% with 21-deoxycortisone, 0.3% with desoxycorticosterone, 0.2% with desoxycortisol, 0.2% with progesterone, 0.2% with hydroxyprogesterone and 0.1% with androgens, including testosterone and androstenedione (Kersey et al., 2010) was used to analyze glucocorticoid metabolite concentrations in fecal samples. Procedures and assays were the same as above unless otherwise noted. Microtiter plates adsorbed cortisol antibody (R4866, supplied by C. J. Munro) before adding duplicate fecal extract (0.05 mL) and triplicate cortisol standards (0.05 mL; range 0.08–20 ng/mL; 17-hydroxycorticosterone; Sigma- Aldrich). Plates were incubated at room temperature (1 h) after adding 0.05 mL of cortisol HRP. Following incubation, unbound components were removed with a wash, and a substrate solution was added (0.1 mL) to all wells. When optimal OD (1.00) was reached, the resulting color change was quantified on the microtiter plate reader. Intra-assay variation was

<10%, and inter-assay CVs for 2 internal controls were 8.9% (mean binding, 28.8%) and 4.4% (mean binding, 68.8%) (n = 116 assays).

2.2.9 Statistical Analysis

Survey Data: In order to assess agreement among keepers responding to survey questions, Kendall's Coefficient of Concordance was applied for the comparison of survey answers obtained at zoos that had three or more keepers, while a Spearman's Rank Correlation Coefficient test was applied to survey answers at the one facility that only had two primary keepers. Due to the relatively small sample size for the survey comparison analyses per institution [i.e., each group of 2-8 keepers rated only 1-4 cats at each facility] it was not possible to obtain statistical significance unless agreement was close to perfect (95-100%) across raters and individuals. Since a level of rater agreement over 70% is generally accepted as adequate in many survey studies (Martin and Bateson, 2007), in this study agreements over 70% that also closely approached statistical significance at or below the $P \leq 0.06$ level for all tests were considered acceptable. Any survey questions that did not meet these criteria were eliminated from further analyses (Wielebnowski et al., 2002). Results from the remaining questions were then averaged across keepers at each facility and these average responses to each question were summed to obtain one final compound score of anxiety versus calmness for each clouded leopard (low score = 'calm'). Due to the binary nature of the response, keepers' overall assessments of individuals ('calm' or 'anxious') were not included in the concordance analysis. Instead these scores were averaged for each cat and this overall assessment was compared to compound scores obtained from Likert scale ratings using a Spearman Rank Correlation test.

Hormone Data: For each individual, fecal glucocorticoid and androgen metabolite concentrations were analyzed as follows: 1) Averaging all obtained hormone values per individual to obtain an overall mean concentration and Coefficient of Variation (CV) for each animal. 2) Baseline concentrations were determined using an iterative process whereby all values exceeding the mean plus 2 standard deviations (SDs) were deleted from the data set. The mean was then recalculated, and the elimination process repeated until no values exceeded the mean plus 2 SD. The final average generated using this process was considered the baseline mean for that animal, and all values removed from the data set during the iterative process were considered 'elevated' (Pelican et al., 2008; Stewart et al., 2010). 3) An average of the 'elevated' (greater than the mean plus 2 SDs) values was calculated as the peak mean for each individual. 4) Fecal glucocorticoid measures for the peak mean were divided by the baseline mean (peak/base) to obtain a potential measure of the response amplitude and general stress hormone reactivity for each individual.

Behavioral Data: The behavioral data – frequency of states and rate of events – were averaged into five time periods for each individual: 1) pre-behavior test data, 2) airhorn test data, 3) MIS test data, 4) UFP test data, and 5) post-behavior test data. Non-parametric tests were used to analyze all behavioral observation data. Mann-Whitney U tests were used to assess the degree to which behavior reaction tests elicited increased frequencies of behavioral states and the rates of behavioral events, relative to the average frequency/rate during pre- and post-behavior test periods combined. Significance was accepted at $P \leq 0.05$ level.

To examine correlations across the three data sets (survey, hormone and behavior data) Spearman Rank Correlation tests were applied for each comparison.

All average values are presented as Mean ± standard error of measurement (SEM). All analyses were performed using SAS 9.2 for Windows (SAS Institute Inc., Cary, NC).

2.3 Results

2.3.1 Survey Analysis

The Kendall's W values ranged from 0.38 to 0.86, while the *P* values ranged from <.0001 to 0.73, and the Spearman Rank R_s values ranged from 0.17 to 0.95 with *P* values of 0.03 to 0.70. Across all zoos and keepers only four questions resulted in W and R_s values of 0.70 or higher and $P \leq 0.06$ values (Table 2.1; Table A2.1)).

To calculate a compound score for each individual clouded leopard, scores on the four accepted questions were averaged across keepers and resulting average scores were then added up to reach a final "anxiety" score. To accomplish this final calculation, scores for one of the questions had to be inverted. The question on how frequently an individual shows "prusten" was actually rating an aspect of friendliness, rather than anxiety or tension. In several medium to large cat species, such as clouded leopards, lynx and tigers 'prusten' is associated with comfort and friendliness akin to the purring of a domestic cat. High ratings on 'prusten' therefore were more indicative of less anxiety, thus an inversion of these scores was needed to produce a compound score of anxiety. Individual compound scores ranged from 7.67 (most calm) to 16.00 (most anxious) (Table A1.4). Reassuringly, the compound scores

were significantly correlated with another general survey question, “Would you describe this animal as calm or anxious?” (R_s = 0.68, P = 0.004). This correlation further supports the reliability of survey results.

2.3.2 Effectiveness of Behavioral Reaction Tests

The Mann-Whitney U Test (Table 2.3) indicated significant increases in rates/frequencies for several behaviors during the MIS test as compared to the pre- and post-behavior tests combined. The behaviors ‘standing’ (U = 312.0, P < .0001, n = 126,16), ‘overall activity’ (U = 463.0, P = 0.0002, n = 126,16), ‘growl’ (U = 454.0, P < .0001, n = 126,16) , and ‘tail flick’ (U = 344, P < .0001, n = 126,16) all increased significantly whereas ‘lie down’ (U = 1274.0, P = 0.09, n = 126,16), ‘sitting’ (U = 804.5, P = 0.18, n = 126,16), and ‘nest box’ (U = 562.5, P = 0.24, n = 86,11) did not change significantly. The airhorn test did not elicit a significant change in rates or frequencies for any behaviors: ‘lie down’ (U = 978.5, P = 0.85, n = 126,16), ‘sitting’ (U = 875.5, P = 0.38, n = 126,16), ‘standing’ (U = 893.5, P = 0.41, n = 126,16), ‘nest box’ (U = 580.5, P = 0.16, n = 86,11), ‘overall activity’ (U = 857.5, P = 0.29, n = 126,16), ‘growl’ (U = 849.0 P = 0.12, n = 126,16), or ‘tail flick’ (U = 916.0, P = 0.37, n = 126,16). During the UFP test the behavior rates for ‘growl’ (U = 4234.0, P < .0001 n = 126,95) and ‘tail flick’ (U = 5325.0, P = 0.05, n = 126,95) increased significantly, while frequencies did not change significantly for ‘lie down’ (U = 6574.5, P = 0.21, n = 126,95), ‘sitting’ (U = 6572.0, P = 0.20, n = 126,95), ‘standing’ (U = 5841.0, P = 0.74, n = 126,95), ‘nest box’ (U = 2504.0, P = 0.23, n = 86,65), and ‘overall activity’ (U = 5587.5, P = 0.36, n = 126,95).

Table 2.3 Mann-Whitney U Testing for behavior incidence during behavior reaction tests (MIS, Airhorn, and UFP), relative to combined pre-and post-behavior test observations. 'Direction' columns indicate whether behavior rates/frequencies increased or decreased during behavior testing.

		N	Pr <	Direction	U
Lie Down					
MIS	**Pre/Post-test**	126	0.0856		1274.0
	Test	16			
Air	**Pre/Post-test**	126	0.8510		978.5
	Test	16			
UFP	**Pre/Post-test**	126	0.2091		6574.5
	Test	95			
Sit					
MIS	**Pre/Post-test**	126	0.1796		804.5
	Test	16			
Air	**Pre/Post-test**	126	0.3821		875.5
	Test	16			
UFP	**Pre/Post-test**	126	0.1978		6572.0
	Test	95			
Stand					
MIS	**Pre/Post-test**	126	**<.0001**	Increase	312.0
	Test	16			
Air	**Pre/Post-test**	126	0.4142		893.5
	Test	16			
UFP	**Pre/Post-test**	126	0.7373		5841.0
	Test	95			
Nest Box					
MIS	**Pre/Post-test**	86	0.2420		562.5
	Test	11			
Air	**Pre/Post-test**	86	0.1560		580.5
	Test	11			
UFP	**Pre/Post-test**	86	0.2254		2504.0
	Test	65			
Overall Activity					
MIS	**Pre/Post-test**	126	**0.0002**	Increase	463.0
	Test	16			
Air	**Pre/Post-test**	126	0.2870		857.5
	Test	16			
UFP	**Pre/Post-test**	126	0.3588		16529.1
	Test	95			
Growl					
MIS	**Pre/Post-test**	126	**<.0001**	Increase	454.0
	Test	16			
Air	**Pre/Post-test**	126	0.1172		849.0
	Test	16			
UFP	**Pre/Post-test**	126	**<.0001**	Increase	4234.0
	Test	95			
Tail Flick					
MIS	**Pre/Post-test**	126	**<.0001**	Increase	344.0
	Test	16			
Air	**Pre/Post-test**	126	0.3658		916.0
	Test	16			
UFP	**Pre/Post-test**	126	**0.0463**	Increase	5325.0
	Test	95			

2.3.3 Correlation of Compound Survey Scores and Fecal Hormone Metabolite Concentrations

With regards to individual fecal glucocorticoid summary statistics, values ranged from: overall mean (161.49 ± 6.36 to 631.66 ± 59.63 ng/g feces), CV (35.44 to 76.78 ng/g feces), baseline mean (153.13 ± 5.55 to 500.32 ± 23.54 ng/g feces), peak mean (288.58 ± 6.99 to 1218.04 ± 112.30 ng/g feces), and peak mean/baseline mean (1.88 to 3.14 ng/g feces) (Figure 2.1). In regards to individual androgen metabolite levels, values ranged from: overall mean (172.92 ± 5.47 to 748.21 ± 33.93 ng/g feces), CV (24.98 to 54.42 ng/g feces), baseline mean (170.01 ± 5.20 to 682.94 ± 25.33 ng/g feces), and peak mean (289.4 ± 11.43 to 1326.34 ± 94.28 ng/g feces) (Table A3.1).

For fecal glucocorticoids, there was a significant positive correlation between peak mean and compound survey scores ($R_s = 0.52$, $P = 0.04$, n = 16) (Figure 2.2; Table 2.4) but there was no significance between fecal glucocorticoids and the individual significant survey questions. For androgens, while there was no significant correlation between the fecal androgen concentrations and compound survey scores, there were significant correlations between individual questions on the questionnaire and androgen concentrations. Significant results include a negative correlation between the overall mean concentration and the individual questions on pacing ($R_s = -0.58$, $P = 0.02$, n = 16) and appearing tense ($R_s = -0.49$, $P = 0.05$, n = 16), between mean baseline androgen concentrations and pacing ($R_s = -0.65$, $P = 0.006$, n = 16),

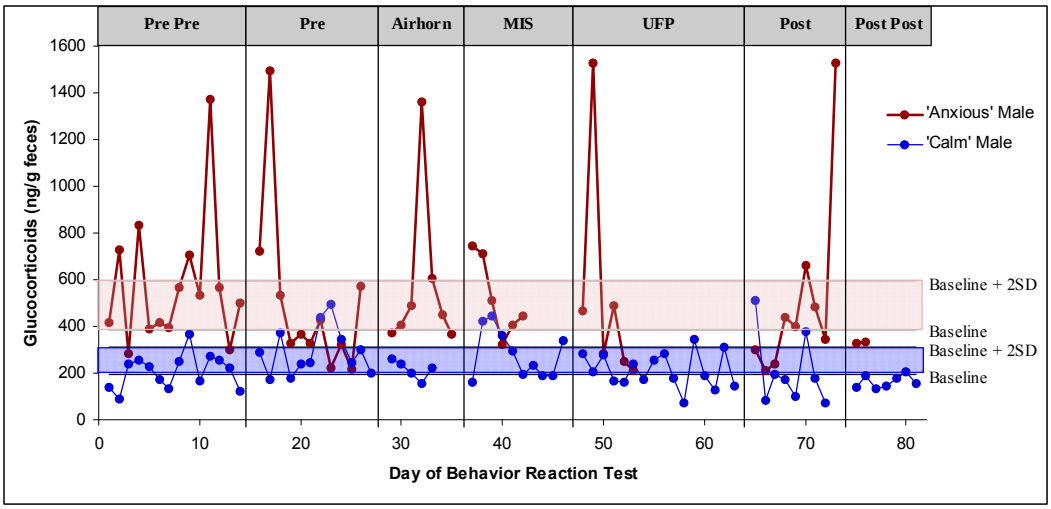


Figure 2.1 Sample endocrine profiles for two clouded leopards participating in the study. Results show fecal glucocorticoid fluctuations for a cat at either end of the 'calm-to-anxious' continuum. Includes the baseline mean and baseline mean plus 2 SDs for each cat as well as the timing of each stress test (PrePre – before video data collection began, Pre – 2 hours of normal taping, Airhorn – ½ hour airhorn test, MIS – ½ hour mirror image stimulation test, UFP – 6 hours unfamiliar person test, Post – 2 hours of normal taping, PostPost – after all taping is complete). In each case the initial test occurred at the time of the first data point within each section.

between peak mean and pacing (R_s = -0.54, P = 0.03, n = 16), and between peak mean and appearing tense (R_s = -0.51, P = 0.04, n = 16). Additionally, there was a positive correlation between overall CV and the behavioral question on frequency of hiss or growl (R_s = 0.51, P = 0.04, n = 16).

2.3.4 Correlation of Compound Survey Scores and Behavioral Observations

The survey data (compound survey scores only), based on keepers' assessments of the clouded leopards (n = 16) was correlated with the behavioral data transcribed from the videotaping. Detailed results are provided in Table 2.5. In summary, there was a significant negative correlation between survey scores and

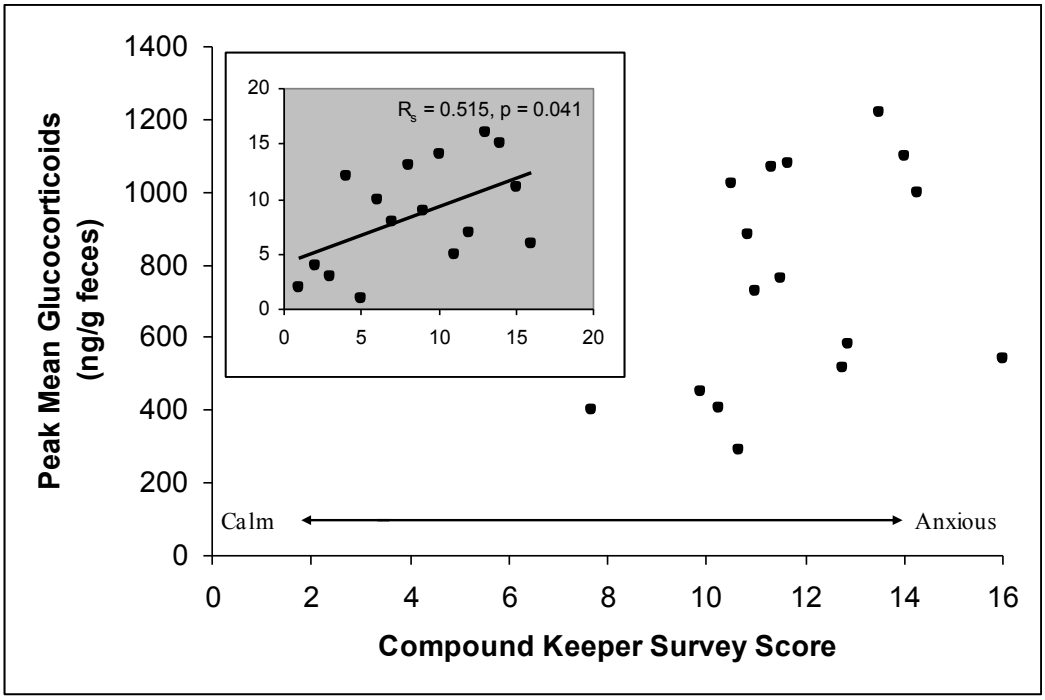


Figure 2.2 Correlation between peak mean fecal glucocorticoids and keeper survey score. Low keeper survey scores indicate 'calm' temperament and high scores indicate 'anxious' temperament. Inset shows non-parametric correlation using Spearman's Rank Correlation Coefficient, R_s. Results show significant correlation ($R_s = 0.515$, $P = 0.041$) between the two variables.

Table 2.4 Spearman-rank correlation coefficients of keepers' behavioral ratings and fecal glucocorticoid concentrations for clouded leopards (n = 16)

Average Fecal Glucocorticoids	*R* s
Overall Mean	0.441
P	0.087
Overall CV	0.429
P	0.097
Baseline Mean	0.368
P	0.161
Peak Mean	**0.515**
P	**0.041**
Peak/Base	0.475
P	0.063

Table 2.5 Spearman-rank correlation coefficients of keepers' behavioral ratings and behavior incidence during video observation for clouded leopards (n = 16)

Behavior		pre	air	MIS	UFP	post
Lie Down	**Rank**	**-0.517**	**-0.548**	0.240	**-0.682**	**-0.526**
	P	**0.041**	**0.028**	0.370	**0.004**	**0.036**
Nest Box	**Rank**	**0.803**	**0.607**	0.578	**0.724**	**0.814**
	P	**0.003**	**0.048**	0.063	**0.012**	**0.002**
Tail Flick	**Rank**	0.152	**0.649**	-0.425	0.289	-0.271
	P	0.573	**0.007**	0.101	0.278	0.311
Time Interact Mirror	**Rank**			**-0.668**		
	P			**0.005**		

frequency of 'lying down' during the pre-behavior test, airhorn test, UFP test, and post-behavior test. A significant positive correlation was found between survey scores and hiding in a 'nest box' during pre-behavior test, airhorn test, UFP test and post-behavior test. Survey scores showed a significant positive correlation with 'tail flick' during the airhorn test. Finally, there was a significant negative correlation between survey scores and time spent interacting with the mirror.

2.3.5 Correlation of Behavioral Observations and Fecal Hormone Metabolite Concentrations

Detailed results are provided in Table 2.6. There was a negative correlation between fecal glucocorticoid metabolites and 'lying down' during the pre-behavior test, airhorn test, UFP test, and post-behavior test and a significant positive correlation during the MIS test. A significant positive correlation was observed between glucocorticoid metabolites and 'standing' during pre- and post-behavior test periods only, and a significant negative correlation during the MIS test. A significant negative correlation was found between glucocorticoid metabolites and 'tail flick'

Table 2.6 Spearman-rank correlation coefficients of fecal glucocorticoid concentrations and behavior incidence during video observation for clouded leopards (n = 16)

	Lie Down					Overall Activity					Stand					Tail Flick					Mirror		
Fecal Glucocorticoid Summary Statistics	**pre**	**air**	**MIS**	**UFP**	**post**	**pre**	**air**	**MIS**	**UFP**	**post**	**pre**	**air**	**MIS**	**UFP**	**post**	**pre**	**air**	**MIS**	**UFP**	**post**	**Time to Initial Approach (sec)**	**Time spent interacting (sec)**	**Swat**
Overall Mean	**-0.662**	-0.474	-0.116	-0.471	**-0.512**	0.372	0.378	-0.111	-0.156	0.269	**0.622**	0.341	-0.323	-0.077	0.294	-0.013	0.010	-0.031	0.068	0.181	-0.164	-0.471	-0.063
p	**0.005**	0.064	0.668	0.066	**0.043**	0.156	0.149	0.683	0.563	0.314	**0.010**	0.197	0.222	0.778	0.269	0.962	0.972	0.909	0.804	0.502	0.544	0.066	0.817
Overall CV	**-0.567**	-0.238	0.374	**-0.674**	**-0.741**	0.440	0.337	-0.084	0.030	0.345	**0.571**	0.134	**-0.547**	-0.010	**0.520**	0.010	0.178	**-0.680**	-0.206	0.135	**-0.505**	**-0.556**	-0.169
p	**0.022**	0.375	0.153	**0.004**	**0.001**	0.089	0.202	0.758	0.914	0.191	**0.021**	0.621	**0.028**	0.970	**0.039**	0.972	0.510	**0.004**	0.445	0.617	**0.046**	**0.025**	0.532
Baseline Mean	**-0.561**	-0.443	-0.202	-0.309	-0.359	0.246	0.306	-0.108	-0.150	0.267	**0.526**	0.319	-0.221	-0.118	0.236	-0.055	-0.019	0.129	0.123	0.188	-0.021	-0.347	0.017
p	**0.024**	0.086	0.453	0.245	0.172	0.358	0.249	0.691	0.578	0.317	**0.037**	0.229	0.411	0.664	0.379	0.839	0.943	0.634	0.651	0.486	0.940	0.188	0.951
Peak Mean	**-0.627**	**-0.527**	0.137	-0.488	**-0.606**	0.412	0.466	-0.227	-0.024	0.342	**0.731**	0.328	**-0.502**	0.052	0.487	0.062	0.095	-0.206	0.092	0.176	-0.177	**-0.612**	-0.207
p	**0.009**	**0.036**	0.613	0.055	**0.013**	0.113	0.069	0.397	0.931	0.196	**0.001**	0.215	**0.047**	0.850	0.056	0.821	0.727	0.444	0.734	0.515	0.512	**0.012**	0.441
Peak/Base	-0.341	-0.233	**0.572**	**-0.598**	**-0.705**	0.332	0.386	-0.370	0.173	0.297	**0.513**	0.103	**-0.651**	0.260	**0.551**	0.157	0.311	**-0.738**	0.009	0.146	-0.335	**-0.689**	-0.379
p	0.196	0.386	**0.021**	**0.015**	**0.002**	0.209	0.140	0.159	0.522	0.264	**0.042**	0.703	**0.006**	0.332	**0.027**	0.561	0.241	**0.001**	0.973	0.588	0.204	**0.003**	0.148

during the MIS test. Finally, there was a significant negative correlation between glucocorticoid metabolites and the 'latency of approach to the mirror' and time spent interacting with the mirror. There was no significance found between glucocorticoid metabolites and 'overall activity' or 'swat' mirror.

In regards to androgen metabolites, there was a significant negative correlation between 'lying down' during the MIS test and androgen metabolites (overall mean R_s = -0.55, P = 0.03; baseline mean R_s = -0.52, P = 0.04; R_s = -0.61, P = 0.01). There was a significant positive correlation between 'growl' during the MIS test and androgen metabolites (overall mean R_s = 0.60, P = 0.01; baseline mean R_s = 0.64, P = 0.01; peak mean R_s = 0.57, P = 0.02). Finally, there was a significant positive correlation between androgen metabolites and 'tail flick' during both the MIS test (overall mean R_s = 0.54, P = 0.03; baseline mean R_s = 0.58, P = 0.02; peak mean R_s = 0.50, P = 0.05) and post-behavior data collection (baseline mean R_s = 0.52, P = 0.04).

Illustrative summaries of all key results are reported in Figures 2.3 and 2.4. There were significant associations between fecal glucocorticoid and fecal androgen metabolites, keeper survey ratings (i.e., compound score) and questions, and the quantitative behavioral data obtained through video recordings across the various data collection periods and tests.

Figure 2.3 Summary of behaviors correlated with compound survey scores and fecal glucocorticoid concentrations.

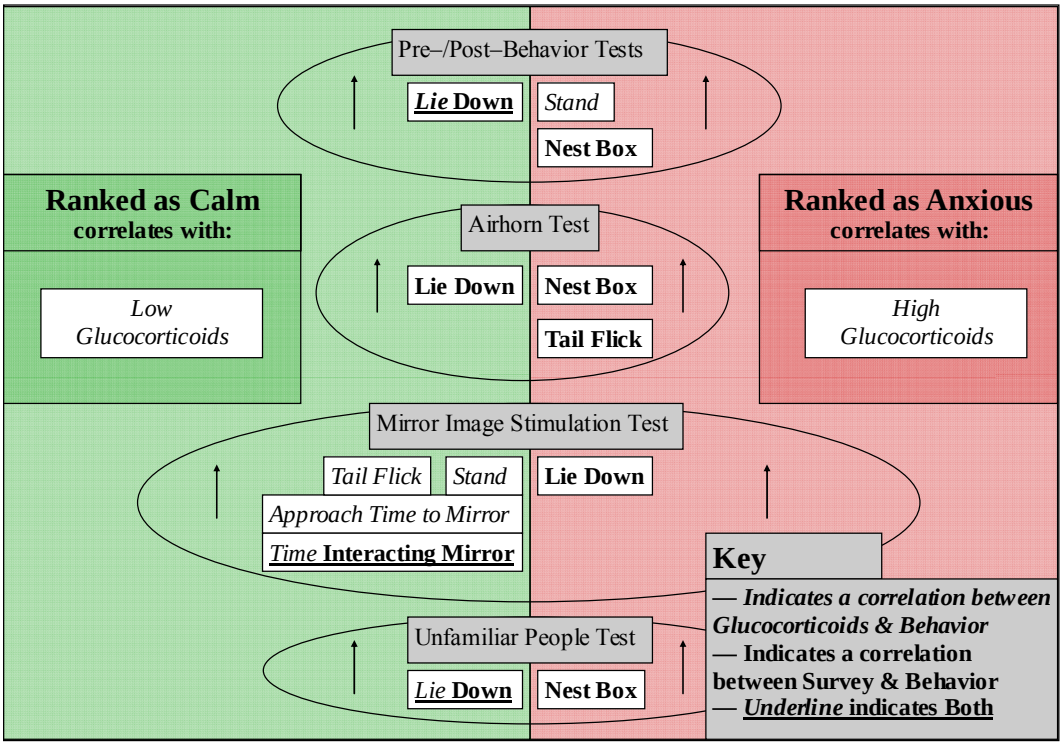
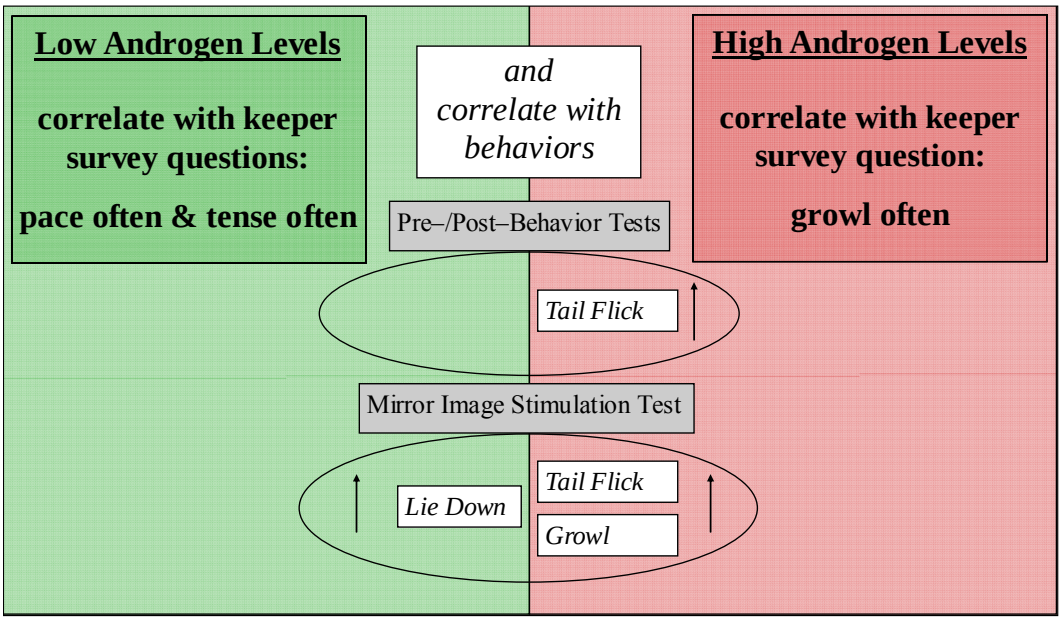


Figure 2.4 Summary of behaviors correlated with compound survey scores and fecal androgen concentrations.



2.4 Discussion

2.4.1 Summary

Results show that behavioral reaction tests in the clouded leopard are useful at differentiating reactive and potentially aggressive behavior in this species. The MIS test elicited some key behaviors such as 'overall activity', 'growl', and 'tail flick'. Additionally, individuals that showed the highest anxiety scores (compound survey) also experienced the highest peak levels of fecal glucocorticoids. These findings lend further support to the validity of regular 'ad hoc' keeper assessments of their animals. Several meaningful and significant correlations between glucocorticoid metabolite concentrations, compound survey scores, and behavioral observation data were also found. On average, 'calm' cats tend to 'lie down' more, while 'anxious' cats tend to 'stand', hide in a 'nest box' and 'tail flick' more, as shown by positive correlations with both glucocorticoids and compound survey scores. Finally, fecal androgen metabolite concentrations correlate positively with 'growl' and 'tail flick' behaviors, possibly indicating increased levels of agitation and aggression, and negatively with individual questions on the behavioral questionnaire about 'pacing' and 'appearing tense'.

2.4.2 'Calm' to 'Anxious' Continuum

Clouded leopards were evenly distributed along the 'calm' to 'anxious' continuum, rather than forming distinct 'anxious' and 'calm' groups. Similar continuums are used throughout the human and animal temperament literature, particularly with the Five-Factor Model developed to describe five broad dimensions

of personality (Costa and McCrae, 1992). In a survey of the literature on animal personality and temperament, Gosling and John (1999) used the Five-Factor Model as a framework to standardize the terminology used to assess the five dimensions of animal temperament. According to their survey, the personality/temperament dimensions of emotional stability versus neuroticism, agreeableness versus antagonism, and extraversion versus introversion showed the largest agreement across all species analyzed. The dimension of neuroticism includes facets such as anxiety and vulnerability to stress. While some key dimensions of temperament may not vary much across species, the way the individual dimensions get expressed behaviorally may vary substantially across species (Gosling and John, 1999). With regards to the current study, survey questions around 'growling', 'pacing', and appearing to be 'tense' – all behaviors associated with neuroticism – and 'prusten' – a greeting or affiliative act of reassurance (Fletchall, 2000) associated with emotional stability – were particularly effective for assessing clouded leopard temperament. Therefore, keepers' ability to identify clouded leopards along the 'calm' to 'anxious' continuum reflects the broad applicability of the stability versus neuroticism dimension to animal temperament models.

2.4.3 Effectiveness of Behavior Reaction Tests in Assessing Temperament

This study demonstrated, through the use of a simple keeper questionnaire and easy to apply behavioral tests, that male clouded leopard behavior indicative of a calmer or more anxious temperament is linked to a hormonal indicator of stress reactivity as measured by peak fecal glucocorticoid concentrations. Specifically, males with higher peak concentrations also tended to be more anxious behaviorally

than males that showed lower peak concentrations. Furthermore, increased fecal androgens correlated with increased 'growling' and 'tail flicking', two behaviors indicative of irritation and aggression (Kileyworthington, 1976; Golebiewski and Romaniuk, 1985). The results therefore show that a combination of simple keeper assessments and one or two easy to use behavioral tests can provide a valid indicator of underlying physiological differences in stress reactivity that may in turn be related to overall temperament differences, coping styles of individual animals, and aggression.

2.4.4 Dimensions of 'Calm' Temperament

Clouded leopard males exhibiting the 'calm' temperament type, as identified by lower compound keeper survey scores, lower peak fecal glucocorticoid levels, and/or low fecal glucocorticoid variability were shown in this study to have distinct behaviors compared to animals that have the 'anxious' temperament type under both 'normal'(pre-behavior test) and induced (behavior reaction test) behavioral conditions. The males categorized as 'calm' tended to lie down more than those categorized as 'anxious'; this was true during the pre- and post-behavior tests, as well as during the airhorn and UFP tests. Animals that were 'lying down' generally also appeared less tense and less agitated by all the tests; they often slept during video recording. In general the MIS test caused 'calm' individuals to interact more with the mirror. While for these males the 'latency to approach the mirror' was higher, they then spent a greater amount of time interacting, 'tail flicking', and 'standing' in proximity to the mirror. It is likely that the 'calm' cats saw the mirror and their own reflection as something novel and of interest; this curiosity may compel them to

interact rather than hide. This interaction could be akin to a novel object test that has been used in a number of species including dogs and primates (King et al., 2003; Santillan-Doherty et al., 2010). Animals that are more fearful tend to interact less with a novel object, while those that are less fearful tend to interact more. This curiosity may benefit male-female introductions if a male prefers to interact with a female rather than hide anxiously or behave aggressively as he might towards an intruder in his cage.

2.4.5 Dimensions of 'Anxious' Temperament

In contrast to the 'calm' cats, some behavioral responses were primarily observed in the more 'anxious' cats, as identified by high compound keeper survey scores, high fecal glucocorticoid levels, and/or high fecal glucocorticoid variability. They spent more time hiding in their 'next boxes' during pre- and post-behavior test collection, as well as during the airhorn and UFP tests. The tendency for an animal to hide often demonstrates fearfulness and has previously been correlated with increased fecal glucocorticoid levels in clouded leopards (Wielebnowski et al., 2002). During the airhorn test, 'anxious' cats tended to flick their tails more often than 'calm' cats. In cats the rapid motion of the tip of the tail has been described as a display of irritation when locomotion is inhibited, such as in cats stalking prey (Kileyworthington, 1976). During the MIS test 'anxious' males spent more time 'lying down', in contrast to the greater interaction with the mirror observed in 'calm' males. Finally, 'anxious' males tended to 'stand' more during pre- and post-behavior test periods, as opposed to the 'calm' cats, who spent more time 'lying down' during non-test periods. It is important to note that behavioral states in this study are

mutually exclusive – a decrease in 'lying down', for example, may simply reflect an increase in other states. Whereas 'lying down' during non-MIS testing correlated with calmer cats, the correlation between 'lying down' and 'anxious' cats during the MIS test may thus be an artifact of the increased activity seen in 'calm' cats rather than an increased tendency to 'lie down' among 'anxious' cats.

2.4.6 Correlations with Fecal Androgens

Lastly, three of the four keeper survey questions that showed significant inter-rater agreement, as well as several behaviors observed in video data collection, were significantly correlated with fecal androgen concentrations. In birds, androgens regulate territorial aggression among males during the breeding season, when competition for mates is high, and levels decrease after the breeding season when males begin to care for their young (Wingfield et al., 2001). In the mouse, androgens increase as males win fights and are correlated with an enhanced ability to win future fights (Oyegbile and Marler, 2005). Here we examined the correlation between fecal androgen concentrations and measures of temperament due to the potential influence of androgens on conspecific aggression in clouded leopards. In this study, male clouded leopards with higher androgen levels tended to demonstrate more aggressive behaviors such as 'growling' and 'tail flicking', particularly during the MIS test. These are behaviors that may be indicative of a general readiness to attack and fight. Furthermore, males with lower androgen levels were perceived, through questions in the behavioral questionnaire, to 'pace' more and 'appear tense' more often, traits linked to anxiety. These correlations indicate that anxiety in the clouded leopard may be linked to androgen levels and aggression, but that anxiety itself is not an indicator

for increased androgen levels. Further research is needed to better understand this complex relationship and is the subject of future work.

2.5 Conclusion

This is one of the first studies to utilize three behavior reaction tests – MIS test, airhorn test, and UFP test – that elicit important behaviors to allow possible categorization of some key aspects of male clouded leopard temperament along the 'calm' to 'anxious' continuum. Results from these tests were correlated with two separate measures – keeper surveys and fecal endocrine profiles. Based on the findings in this study the MIS test in particular seems appropriate for differentiating between 'calm' and 'anxious' clouded leopards – the test caused a significant change in the observed rates/frequencies of several behaviors, and calmer cats tended to spend more time interacting with the mirror. Anxiety and aggression are major issues in the management of captive male clouded leopards, and while this study hints at a connection between the two, future studies might utilize these behavioral reaction tests to further investigate various facets and dimensions of clouded leopard temperament. In addition, simple behavior reaction tests such as the ones used in this study could potentially be applied in a number of other non-domestic species as a general test of anxiety and stress reactivity to aid in the daily management of animals and in overall species management recommendations.

Chapter 3: Characterization of Multiple Pathways Modulating Aggression in the Male Clouded Leopard (*Neofelis nebulosa*)

3.1 Introduction

The clouded leopard is one of the most challenging felid species to breed in captivity due to male-female aggression. As a consequence, population numbers in captivity are too low to be self-sustaining, a key objective of zoological breeding programs (Fletchall, 2003). Captive populations are intended as a hedge against extinction in the wild, where the clouded leopard is in decline (Grassman et al., 2005) and listed as 'endangered' under the United States Endangered Species Act and 'Vulnerable' in the IUCN Red Data Book. However, despite the importance of male attacks on females in the *ex situ* management of clouded leopards, few studies have systematically focused on characterizing and mitigating aggressive behavior in this species. Two potential aggression pathways are implicated in the clouded leopard: anxiety-mediated and testosterone-mediated.

Aggression has been a common focus of behavioral and neuroendocrine research for over 40 years in a variety of species, including domestic cats, yet the underlying mechanisms remain unclear (Kingsbury et al., 1997; Siegel et al., 1999; Weinshenker and Siegel, 2002). Anxiety and aggression are closely tied to functional responses of the hypothalamic-pituitary-adrenal (HPA) axis (Haller et al., 2000; Kruk et al., 2004; Wommack and Delville, 2007). Exposure to acute (e.g. predatory attack) or chronic (e.g. starvation) stressors activates the HPA axis. Acute stressors result in increased adrenalin release for a 'fight or flight' response, possibly leading to

aggressive behaviors when a clouded leopard senses a threat. Chronic stress, meanwhile, results in hypothalamic release of corticotrophin-releasing hormone, which stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH). ACTH in turn triggers the release of glucocorticoids from the adrenal cortex, which stimulate a cascade of metabolic and physiologic responses that conserve metabolic resources and down-regulate non-essential functions (Griffin and Ojeda, 2004). A link between chronic stress and aggression has previously been reported in captive clouded leopards. Males that showed signs of anxiety resulting from chronic stress (e.g. tail-biting, fur-plucking, excessive hiding and pacing) had elevated concentrations of excreted glucocorticoids, and were also less likely to pair successfully with females due to excessive aggression (Wielebnowski et al., 2002; MacKinnon, 2008).

The serotonergic neurotransmitter system (5-hydroxytryptamine) and its associated receptor subtypes, which are especially prevalent in the areas of the brain (e.g. hypothalamus and periaqueductal gray) associated with aggression, also play a key role in regulating anxiety and aggression (Blier and Bouchard, 1994; Saudou et al., 1994; Lucki, 1998; Siegel et al., 1999; Summers et al., 2005; Popova, 2006). The role of the serotonergic system in aggression pathways has been demonstrated in a number of species, including golden hamsters in which treatment with fluoxetine (a stimulant of the serotonin system) blocked induction of aggressive behavior by vasopressin (Ferris et al., 1997) and even domestic cats where clomipramine mitigated aggressive behavior (Siegel et al., 1999). In animals experiencing stress

and anxiety – such as clouded leopards – the serotonergic system may play a key role in modulating the HPA axis (Jensen et al., 1999; Pariante et al., 2001).

The role of circulating testosterone in male aggression related to territory, courtship, and mating behaviors has been well documented across a range of species. In many species, testosterone influences the frequency, intensity and persistence of aggression (Archer, 2006; Goymann et al., 2007). For example, testosterone levels increase in male mice when they win fights and correlate with enhanced ability to win future fights (Oyegbile and Marler, 2005). In birds, testosterone regulates territorial aggression among males during the breeding season when competition for mates is high, and decreases when males begin to care for offspring (Wingfield et al., 2001; Archer, 2006). While strongly correlated with aggression in many species, testosterone's mechanism of action is extremely complex and not fully understood (Silverin et al., 2004; Oyegbile and Marler, 2005; Wingfield, 2005). There is evidence that testosterone modulates aggression in clouded leopards, with pre-pubertal males generally being easier to pair with females as they are less aggressive than adult males (MacKinnon, 2008). In fact, pairing males and females as juveniles is one of the only successful pairing strategies in this species, and is recommended by the Association of Zoos and Aquariums (AZA) Species Survival Plan (Fletchall, 2000).

Because aggression can be associated with two major divergent pathways, aggression can also be modulated through these pathways. One major mechanism of control involves moderating the serotonin pathway. This pathway can be controlled through the use of tricyclic antidepressants that act by inhibiting the re-uptake of

serotonin and noradrenalin by blocking the noradrenalin transporter and the serotonin transporter. This, in turn, increases the availability of the neurotransmitters to postsynaptic receptors (Dubinsky et al., 1973). Over the past 20 years, clomipramine (a tricyclic antidepressant) has been used to treat anxiety and aggression in numerous species. In both the domestic dog and cat, this drug has been used widely for treating anxiety disorders, obsessive-compulsive behaviors, destruction and aggression (Seksel and Lindeman, 1998; Siegel et al., 1999; King et al., 2000; Litster, 2000; Gillman, 2007).

Another major mechanism for controlling aggression involves the control of testosterone. Castration has been used for centuries to reduce hostile behavior in many species (Bergvall and Hansen, 1990; Hagelin, 2001; Hume and Wynne-Edwards, 2005). Chemical castration can also be achieved through the use of Gonadotropin Releasing Hormone (GnRH) agonists that act on pituitary GnRH receptors to first hyper-stimulate and then inhibit gonadotropin production and release, ultimately shutting down the hypothalamic-pituitary-gonadal (HPG) axis and gonadal steroidogenesis (Aspden et al., 1996; Padula, 2005). An example of a GnRH agonist is deslorelin, which is administered as a long-acting reversible implant and has effectively down-regulated GnRH, gonadotropins, reproductive steroids, and ultimately reproduction in the domestic dog, cat, and cheetah (Bertschinger et al., 2006; Trigg et al., 2006; Goericke-Pesch et al., 2011). Although control of reproduction has been the major use of this product, deslorelin has also been effectively used in a variety of species to reduce aggression, including the lion-tailed macaque and black-footed cat (Norton et al., 2000; Bertschinger et al., 2001).

In order to experimentally distinguish aggression provoked by anxiety from testosterone-related aggressive behavior in the clouded leopard, the effects of the tricyclic antidepressant, clomipramine, were compared to the effects of the GnRH agonist, deslorelin. The ability of each drug to modulate aggression was assessed by comparing their effects on behavior related to anxiety and aggression in male clouded leopards exposed to known stressors. Stressful occurrences were simulated to observe behavioral responses, with and without drug treatment, by exposing males to the following: Mirror Image Stimulation test (simulating approach by an unknown clouded leopard), Airhorn test (loud unfamiliar noise), and Unfamiliar Persons test (new person approaching enclosure where usually only keepers have access). The hypothesis was that aggression and possibly anxiety in the male clouded leopard is modulated by testosterone-linked serotonergic pathways, and that modification of either testosterone concentrations or the serotonin system would reduce aggression and anxiety and improve reproductive success in this species.

3.2 Methods

3.2.1 Animals and Study Area

Thirteen adult male clouded leopards housed at 11 North American zoos were studied over an 8 month period. Participating zoos included: Alexandria Zoological Park, LA, USA (n = 1; 10 y of age); Audubon Nature Institute, LA, USA (n = 1; 10 y of age); Central Florida Zoo & Botanical Gardens, FL, USA (n = 1; 8 y of age); Cincinnati Zoo & Botanical Garden, OH, USA (n = 1; 7 y of age); Cleveland Metroparks Zoo, OH, USA (n = 1; 5 y of age); Houston Zoo, TX, USA (n = 1; 6 y of

age); Omaha's Henry Doorly Zoo, NE, USA (n = 1; 12 y of age); San Antonio Zoo, TX, USA (n = 1; 13 y of age); Smithsonian Conservation Biology Institute (SCBI), VA, USA (n = 3; 4, 14, and 15 y of age); Zoo Atlanta, GA, USA (n = 1; 13 y of age); and Zoo Miami, FL, USA (n = 1; 15 y of age).

Although clouded leopard management practices differed among institutions, all animals were housed according to AZA guidelines for this species (Fletchall, 2000). Outside of the behavioral testing periods, each zoo was instructed to follow routine husbandry protocols for the duration of the study. Each animal was housed singly and these animals represented 100% of the single-housed male clouded leopards in the US zoological population. Seven animals were housed in areas where they spent part of the time on-exhibit for public view and part of the time off-exhibit; the other six animals were permanently housed off-exhibit. All behavioral testing sessions took place in each animal's off-exhibit area.

3.2.2 Study Design

Thirteen unpaired male clouded leopards had been previously categorized as either 'anxious' or 'calm', using a keeper questionnaire designed to assess temperament as well as through assessment of reactions to three behavioral reaction tests – Mirror Image Stimulation test (MIS), Airhorn test, and Unfamiliar People test (UFP) – and fecal hormone (glucocorticoid and androgen) concentrations (testing period 1: Figure 3.1). The clouded leopards were randomly assigned to one of three

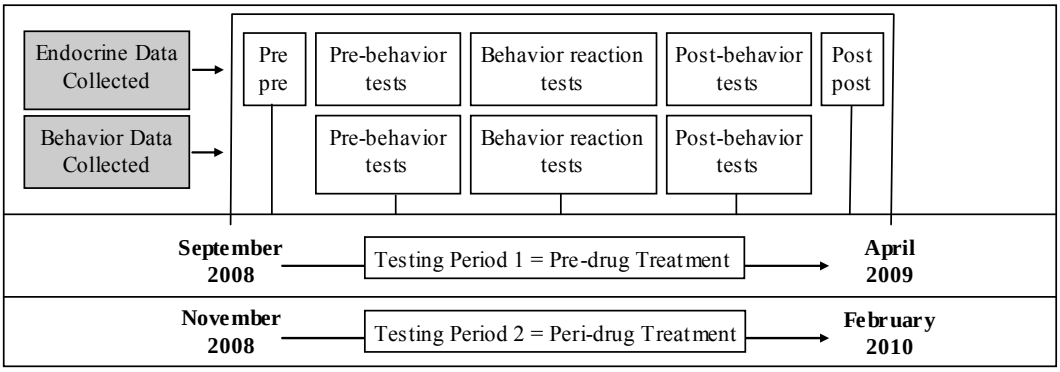


Figure 3.1 Overview of study design for Chapter 3.

Clouded leopards undergo the same series of tests, observations, and measurements twice: once pre-drug treatment and once peri-drug treatment. "Pre pre" indicates fecal collections that take place before the pre-behavior testing video recording. "Post post" refers to fecal collections after the post-behavior testing video recording.

treatments: 1) clomipramine (n = 4 cats, 2 'calm', 2 'anxious'); 2) deslorelin (n = 5 cats, 2 'calm', 3 'anxious'); or 3) no treatment (control; n = 4 cats, 2 'calm', 2 'anxious') (Table A1.4). Treatments were then administered for 60 days as each drug is estimated to take 15 to 35 days to become fully effective (King et al., 2000; Litster, 2000; El Mansari and Blier, 2005; Gaultier et al., 2005; Trigg et al., 2006). Cats were then re-tested (testing period 2: Figure 3.1) using the same behavioral reaction tests administered in testing period 1. Fecal hormone metabolite concentrations were monitored and behavioral tests were videotaped. The pre- and peri-drug treatment responses were compared to determine the effects of each drug treatment on male behavioral and hormonal responses.

The studies described were approved by the Smithsonian's National Zoological Park (#08-12) and the University of Maryland, College Park (#R-08-33)

Animal Care and Use Committees. The study was also reviewed and approved by all institutions housing clouded leopards.

3.2.3 Drug Treatment

Clomipramine (Clomicalm® Novartis Animal Health US, Inc.) was administered orally as a pill inserted in a meatball and fed to each cat daily for six months. The dosage, based on published reports in the domestic cat was 0.6 mg/kg (Litster, 2000). Deslorelin (Suprelorin®; Peptech Animal Health, Australia) implants were administered to anesthetized clouded leopards by institutional veterinarians. Briefly, animals were anesthetized using a combination of ketamine hydrochloride and sometimes midazolam. A 2 cm site was cleaned and scrubbed in the dorsal inter-scapular region and three 2.3 mm x (about) 12.5 mm length cylindrical implants were inserted through the skin using an insertion trocar. Each implant provided 4.7 mg of deslorelin for a total of 14.1 mg, dosage recommended for six months of treatment to suppress testosterone in the male clouded leopard (Asa, 2009).

3.2.4 Behavioral Reaction Tests

Clouded leopards were exposed to a series of three behavioral reaction tests over two separate 3 week periods (once in testing period 1 and once in testing period 2; Figure 3.1). Each behavior test was performed 1 week apart, resulting in a total of 6 tests over two 3 week periods. Behavioral reaction tests were designed to mimic known stressors in the clouded leopard (e.g. exposure to a new person, a loud noise, seeing another clouded leopard, etc.) (Wielebnowski, 1999; Fletchall, 2007) in order to induce a response that could be used to assess behavioral status pre- and peri-drug

treatment. The change in behavioral response to stressors following drug treatment was then compared to pre-drug treatment responses as a stand-in for aggression in this species. *Test 1: Mirror image stimulation (MIS test)*. An acrylic mirror was secured on the fence in an animal's enclosure and remained in the enclosure for 30 min. *Test 2: Noise test (airhorn test)*. An airhorn was sounded a single time approximately 5 ft from the animal's enclosure. *Test 3: Exposure to unfamiliar people, both male and female (UFP test)*. Clouded leopards were exposed to an unfamiliar human male and female twice per day (morning and evening) on three consecutive days. The unfamiliar people engaged in a conversation for 10 min at a safe distance from the animal's enclosure, but in clear visual, auditory, and olfactory proximity. (See Chapter 2 for further details.)

3.2.5 Videotaping and Analysis

Quantitative behavioral data were collected on two separate occasions (testing period 1 and testing period 2) during three different periods: a two-week pre-behavioral reaction test period, the three-week behavioral reaction test period, and a two-week post-behavioral reaction test period (Figure 3.1). For the pre- and post-behavioral reaction test periods (referred to from here on as "pre-behavior test" and "post-behavior test") behavioral data were video recorded for a total of 120 min per individual for each period. More specifically, an individual's behaviors were recorded for 60 min/week in two separate 30 min segments on different days of the week. For the behavioral reaction test period, video recordings were collected in the following manner for each of the tests: for 30 min from the time an animal was shifted into an area where the mirror was present for the MIS test, for 30 min

including and following the sounding of the airhorn, and for 30 min including the 10 min UFP test and 20 min following the test. All videotapes were sent to SCBI and all behavioral data were coded by a single observer. Using an ethogram previously established for this species (Fletchall, 2000), the frequencies of behavioral states (using instantaneous scan sampling at one min intervals) and the rates of behavioral events (using continuous sampling) were recorded (Table 2.2, Table A1.3). Several additional measures were recorded for the MIS test, including: 'latency of approach', time (sec) spent interacting with the mirror, and incidence of 'swatting' (Table 2.2). Behaviors not readily distinguishable from one another (affiliated behaviors) exhibited at a low frequency were combined for the statistical analyses (Table 2.2; combined behaviors are marked with an asterisk and associated letter). Specifically, 'crouching' was combined with 'lying down' ('lying down') and 'pawing' was combined with 'swatting' ('swatting'). 'Running', 'pacing', and 'walking' were combined to get an overall assessment of activity level ('overall activity').

3.2.6 Fecal Collection and Analysis

Fecal samples (at least 5 g) were collected daily from each cat, starting 14 days before the first behavioral reaction test session (testing period 1; see section 3.2.4) and continuing 14 days after the last behavioral reaction test session, to cover both pre- (testing period 1) and peri- (testing period 2) drug treatment time frames. Pre-drug treatment samples were collected for a total of 11 weeks per individual between September 2008 and April 2009, and for 11 weeks during drug treatment between November 2008 and February 2010, total samples collected per individual ranged from 106 to 164. Once collected, samples were placed in plastic bags, labeled

and frozen (-20ºC) and shipped on dry ice to SCBI's Endocrine Research Laboratory for hormonal analysis by Enzyme Immunoassay (EIA).

Samples were assayed for glucocorticoid and androgen metabolites using assays previously validated for the clouded leopard (Brown et al., 1996; Wielebnowski et al., 2002; MacKinnon, 2008). In brief, each fecal sample was lyophilized, pulverized, and 0.19–0.21 g of dry fecal powder was vortexed (5 s) and shaken (30 min) in 5 mL of 90% ethanol. After centrifugation (20 min; 2,500 RPM), the supernatant was recovered, and the pellet was re-suspended in 5 mL of 90% ethanol, vortexed (5 s), shaken (30 s), and re-centrifuged (15 min; 2,500 RPM). The first and second supernatants were combined, dried under air and reconstituted in 1 mL methanol. Methanol extracts were vortexed (5 s), sonicated (15 min), and dried under air. Each extract was reconstituted in 1 mL dilution buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.15 M NaCl, pH 7.0). Dilution buffer extracts were vortexed (5 s), sonicated (15 min or until completely in solution), and diluted 1:80 and 1:30 in dilution buffer for androgens and glucocorticoids, respectively. Samples were stored in polypropylene tubes at -20ºC until EIA.

3.2.7 Androgen EIA

Androgen metabolite concentrations in fecal extracts were quantified with a single-antibody testosterone EIA using previously described methods (Kersey et al., 2010). The antibody cross-reacts 100% with testosterone, 57.4% with dihydrotestosterone, 0.3% with androstenedione, 0.04% with androsterone and dehydroepiandrosterone, 0.03% with cholesterol, 0.02% with estradiol and <0.02% for all other tested analytes, including cortisol (Kersey et al., 2010). Polyclonal anti-

testosterone (R156/R157; 1:7,500; supplied by C.J. Munro, University of California, Davis, CA) was added (0.05 μL) to 96-well microtiter plates (Nunc-Immuno; Fisher Scientific, Pittsburgh, Pennsylvania), and incubated (12–18 h) at 4°C. Plates were washed (0.05% Tween 20 in 0.15 M NaCl solution) and fecal extract was added in duplicate (0.05 mL); standards were done in triplicate (0.05 mL; .05–12 ng/mL; 17β-hydroxy-4-androsten-3-one; Steraloids, Newport, Rhode Island). Testosterone horseradish peroxidase (HRP) (1:80,000; supplied by C.J. Munro) was added to each well, and plates were incubated (2 h; at room temperature) and washed. A chromagen (ABTS) and catalyst (hydrogen peroxide) was added to each well and incubated (~30 min) before optical densities (ODs) were read by microplate reader (MRX, Dynex Technologies, Chantilly, VA) at 405 nm. Intra-assay variation was <10%, and inter-assay coefficients of variation (CVs) for 2 internal controls were 6.9% (mean binding, 22.2%) and 4.0% (mean binding, 67.9%) (n = 99 assays). Immunoreactivity of serially diluted fecal extracts paralleled standard binding.

3.2.8 Glucocorticoid EIA

A single-antibody cortisol EIA was used to assay glucocorticoid metabolite concentrations in fecal samples. The antibody (R4866, supplied by C. J. Munro) cross-reacted 100% with cortisol, 9.9% with prednisolone, 6.3% with prednisone, 5% with cortisone, 0.7% with corticosterone, 0.5% with 21-deoxycortisone, 0.3% with desoxycorticosterone, 0.2% with desoxycortisol, 0.2% with progesterone, 0.2% with hydroxyprogesterone and 0.1% with androgens, including testosterone and androstenedione (Kersey et al., 2010). The concentration of cortisol standard ranged from 0.08–20 ng/mL (17-hydroxycorticosterone; Sigma- Aldrich). Intra-assay

variation was <10%, and inter-assay CVs for 2 internal controls were 8.9% (mean binding, 28.8%) and 4.4% (mean binding, 68.8%) (n = 116 assays).

3.2.9 Statistical Analysis

Hormone data: For each individual, fecal glucocorticoid and androgen metabolite concentrations were analyzed according to a number of measures both pre- and peri-drug treatment: 1. Overall mean of obtained hormone values. 2. Baseline concentrations were determined using an iterative process in which all values exceeding the mean plus 2 standard deviations (SDs) were removed from the data set. The mean was then recalculated, and the elimination process repeated until no values exceeded the mean plus 2 SD. The final average generated using this process was the baseline mean. 3. Removed values were considered 'elevated', representing a peak mean (Pelican et al. 2008; Stewart et al. 2010). After performing a Shapiro-Wilk test, data were log transformed and analyzed using a SAS general linear model ANOVA, followed by least significant means comparison tests. Finally, Pearson's correlation test was used to assess the correlation between fecal glucocorticoid and androgen concentrations.

Behavior Data: For each behavioral reaction testing period (both pre- and peri-drug treatment), behavioral data were averaged into the following five time periods: 1) pre-behavior test data, 2) airhorn test data, 3) MIS test data, 4) UFP test data, and 5) post-behavior test data. All behavior comparisons across time periods were analyzed using the non-parametric Mann–Whitney U test statistic.

All average values are presented as mean ± standard error of measurement (SEM). All analyses were performed using SAS 9.2 for Windows (SAS Institute Inc.,

Cary, NC). Significance was accepted at $P \leq 0.05$ level. For multiple comparisons, a Bonferroni correction was used to keep family-wise α levels constant.

3.3 Results

3.3.1 Androgens

Androgen levels were significantly influenced by 2-way interactions between temperament and treatment group ($F = 365.35$, $P < .0001$), temperament and drug effect ($F = 53.93$, $P < .0001$), and drug effect and treatment group ($F = 30.02$, $P < 0.0001$). No significant 3-way interaction ($F = 0.21$, $P = 0.81$) was observed, therefore 2-way interactions were examined. In regards to drug effect by treatment group interaction (Table 3.1), the peri-drug treatment overall mean in the deslorelin treatment group decreased ($P < 0.0001$) (Figure 3.2) relative to pre-drug treatment, while there was no change observed in either the clomipramine or control group (Figure 3.3). The same significant change ($P < 0.0001$) was observed for the baseline mean in the deslorelin group, while no changes were observed in the peak mean for any treatment group. For drug effect by temperament category interaction (Table 3.2), overall androgen means decreased ($P < 0.0001$), baseline means decreased ($P < 0.0001$), and peak means decreased ($P = 0.0002$) in calm clouded leopards peri-drug compared to pre-drug, but did not change in anxious animals (Figure 3.4). For temperament category by drug treatment group (Table 3.3), calm clouded leopards in the clomipramine treatment group had higher androgen levels compared to anxious males in regards to the overall mean, baseline mean, and peak mean ($P < 0.0001$). In the deslorelin treatment group, calm clouded leopards had lower androgen levels in

regards to the overall mean, baseline mean, and peak mean ($P < 0.0001$) compared to anxious males. Finally, no significant difference was observed in androgen levels for calm versus anxious males in the control treatment group.

Table 3.1 Multiple-means comparison tests based on both 3-way and 2-way ANOVA analysis of drug effect and treatment group influence on fecal glucocorticoid and androgen concentrations.
Drug effect and treatment group are included as main factors where no significant 3-way interaction was found, with temperament category also included as a main effect where there was a 3-way interaction, otherwise represented with NA = not applicable. The 'Direction' column indicates whether the means increased or decreased during drug treatment, relative to pre-drug treatment. LS Means presented as logarithm (ng/g feces).

	Glucocorticoids							Androgens						
	LS Mean Pre-drug Treatment	LS Mean Peri-drug Treatment	Std Error	Direction	Pr <	N 1	N 2	LS Mean Pre-drug Treatment	LS Mean Peri-drug Treatment	Std Error	Direction	Pr <	N 1	N 2
Clomipramine														
All														
Overall Mean	NA	NA	NA	NA	NA	NA	NA	6.21	6.28	0.046		0.7479	260	205
Base Mean	NA	NA	NA	NA	NA	NA	NA	6.16	6.20	0.045		0.9032	203	184
Peak Mean	NA	NA	NA	NA	NA	NA	NA	6.78	6.83	0.090		0.9920	57	22
Anxious														
Overall Mean	5.99	5.89	0.068		0.9181	131	121	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.71	5.76	0.062		0.9999	90	105	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.61	6.74	0.117		0.9943	41	16	NA	NA	NA	NA	NA	NA	NA
Calm														
Overall Mean	5.76	5.93	0.076		0.5270	129	85	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.64	5.89	0.063	Increase	**0.0036**	113	79	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.61	6.38	0.191		0.9880	16	6	NA	NA	NA	NA	NA	NA	NA
Deslorelin														
All														
Overall Mean	NA	NA	NA	NA	NA	NA	NA	5.59	5.35	0.038	Decrease	**< 0.0001**	341	325
Base Mean	NA	NA	NA	NA	NA	NA	NA	5.50	5.27	0.037	Decrease	**< 0.0001**	283	263
Peak Mean	NA	NA	NA	NA	NA	NA	NA	6.36	6.25	0.078		0.7394	59	62
Anxious														
Overall Mean	5.96	5.66	0.057	Decrease	**< 0.0001**	179	185	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.77	5.46	0.052	Decrease	**< 0.0001**	139	140	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.63	6.28	0.087	Decrease	**0.0040**	40	45	NA	NA	NA	NA	NA	NA	NA
Calm														
Overall Mean	5.15	4.92	0.062	Decrease	**0.0100**	161	144	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.05	4.78	0.053	Decrease	**< 0.0001**	143	124	NA	NA	NA	NA	NA	NA	NA
Peak Mean	5.96	5.78	0.129		0.9560	18	20	NA	NA	NA	NA	NA	NA	NA
Control														
All														
Overall Mean	NA	NA	NA	NA	NA	NA	NA	6.18	6.08	0.045		0.2950	273	220
Base Mean	NA	NA	NA	NA	NA	NA	NA	6.15	6.04	0.042		0.1032	229	178
Peak Mean	NA	NA	NA	NA	NA	NA	NA	6.73	6.72	0.134		1.0000	44	42
Anxious														
Overall Mean	6.05	5.93	0.070		0.8900	130	109	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.91	5.84	0.062		0.9900	111	89	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.86	6.34	0.128	Decrease	**0.0030**	19	20	NA	NA	NA	NA	NA	NA	NA
Calm														
Overall Mean	5.68	5.46	0.068		0.0580	143	111	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.53	5.26	0.061	Decrease	**0.0005**	118	89	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.41	6.29	0.116		0.9980	25	22	NA	NA	NA	NA	NA	NA	NA

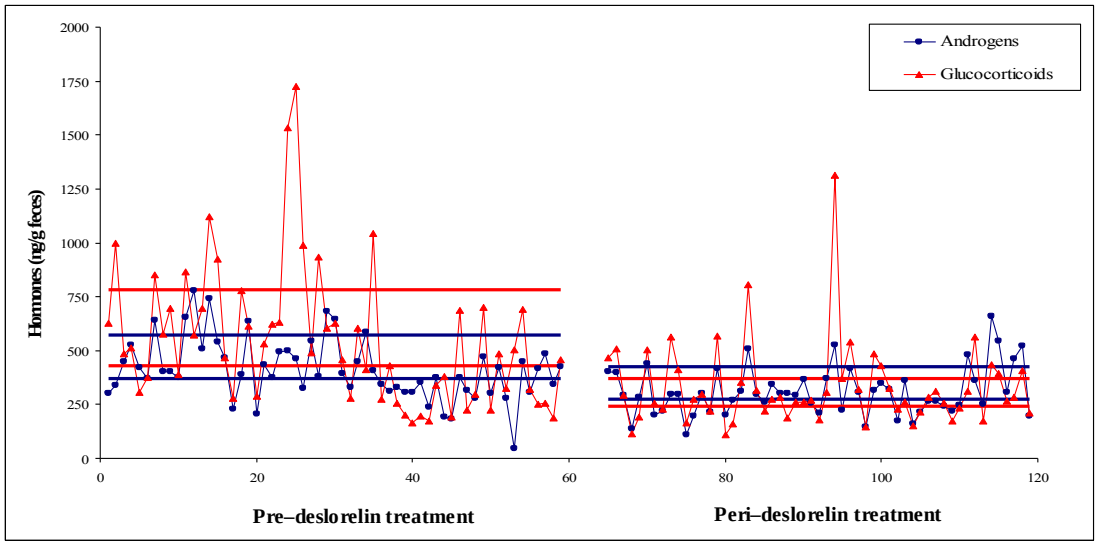


Figure 3.2 Endocrine profiles for an 'anxious' male clouded leopard in the deslorelin treatment group. Horizontal lines indicate Baseline Mean and Baseline Mean + 2 SD for fecal androgens (blue) and glucocorticoids (orange).

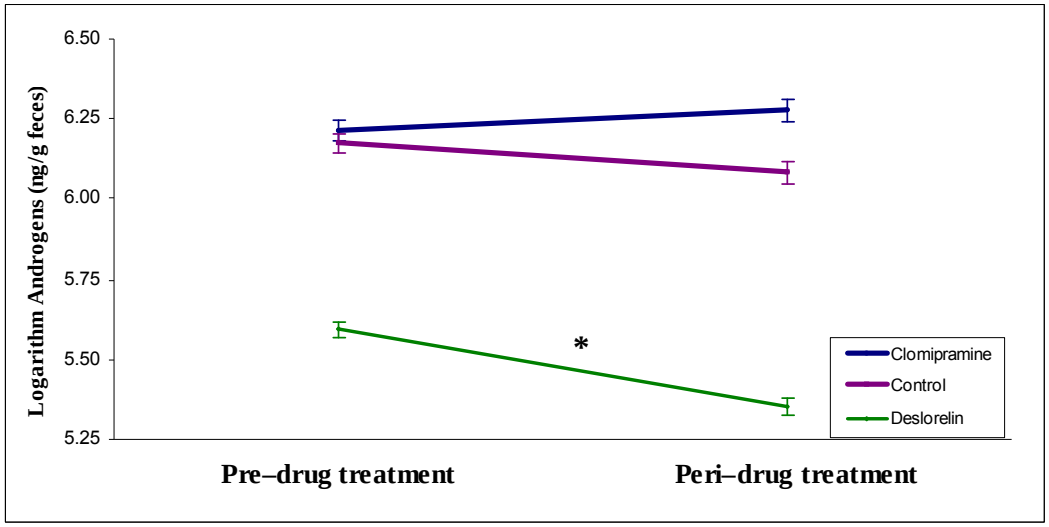


Figure 3.3 Treatment group effects on overall fecal androgen levels.

Results indicate a significant decrease in fecal androgen levels in the deslorelin treatment group from pre-drug treatment to peri-drug treatment, and no significant change in either the clomipramine or control group. Error bars indicate SEM.

Table 3.2 Multiple-means comparison tests based on both 3-way and 2-way ANOVA analysis of drug effect and temperament category influence on fecal glucocorticoid and androgen concentrations. Drug effect and temperament category are included as main factors where no significant 3-way interaction was found, with treatment group also included as a main effect where there was a 3-way interaction, otherwise represented with NA = not applicable. The 'Direction' column indicates whether the means increased or decreased during drug treatment, relative to pre-drug treatment. LS Means presented as logarithm (ng/g feces).

	Glucocorticoids							Androgens						
	LS Mean Pre-drug Treatment	LS Mean Peri-drug Treatment	Std Error	Direction	Pr <	N 1	N 2	LS Mean Pre-drug Treatment	LS Mean Peri-drug Treatment	Std Error	Direction	Pr <	N 1	N 2
Anxious														
All														
Overall Mean	NA	NA	NA	NA	NA	NA	NA	5.97	6.02	0.028		0.1885	440	411
Base Mean	NA	NA	NA	NA	NA	NA	NA	5.90	5.96	0.027		0.1878	396	369
Peak Mean	NA	NA	NA	NA	NA	NA	NA	6.55	6.64	0.046		0.1720	44	42
Clomipramine														
Overall Mean	5.99	5.89	0.068		0.9181	131	121	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.71	5.76	0.062		0.9999	90	105	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.61	6.74	0.117		0.9943	41	16	NA	NA	NA	NA	NA	NA	NA
Deslorelin														
Overall Mean	5.96	5.66	0.057	Decrease	**< 0.0001**	179	185	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.77	5.46	0.052	Decrease	**< 0.0001**	139	140	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.63	6.28	0.087	Decrease	**0.0040**	40	45	NA	NA	NA	NA	NA	NA	NA
Control														
Overall Mean	6.05	5.93	0.070		0.8858	130	109	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.91	5.84	0.062		0.9900	111	89	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.86	6.34	0.128	Decrease	**0.0030**	19	20	NA	NA	NA	NA	NA	NA	NA
Calm														
All														
Overall Mean	NA	NA	NA	NA	NA	NA	NA	6.01	5.77	0.029	Decrease	**< 0.0001**	434	339
Base Mean	NA	NA	NA	NA	NA	NA	NA	5.97	5.72	0.028	Decrease	**< 0.0001**	408	316
Peak Mean	NA	NA	NA	NA	NA	NA	NA	6.66	6.42	0.057	Decrease	**0.0002**	26	23
Clomipramine														
Overall Mean	5.76	5.93	0.076		0.5270	129	85	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.64	5.89	0.063	Increase	**0.0036**	113	79	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.61	6.38	0.191		0.9880	16	6	NA	NA	NA	NA	NA	NA	NA
Deslorelin														
Overall Mean	5.15	4.92	0.062	Decrease	**< 0.0001**	161	144	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.05	4.78	0.053	Decrease	**< 0.0001**	143	124	NA	NA	NA	NA	NA	NA	NA
Peak Mean	5.96	5.78	0.129		0.9560	18	20	NA	NA	NA	NA	NA	NA	NA
Control														
Overall Mean	5.68	5.46	0.068		0.0577	143	111	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.53	5.26	0.061	Decrease	**0.0005**	118	89	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.41	6.29	0.116		0.9980	25	22	NA	NA	NA	NA	NA	NA	NA

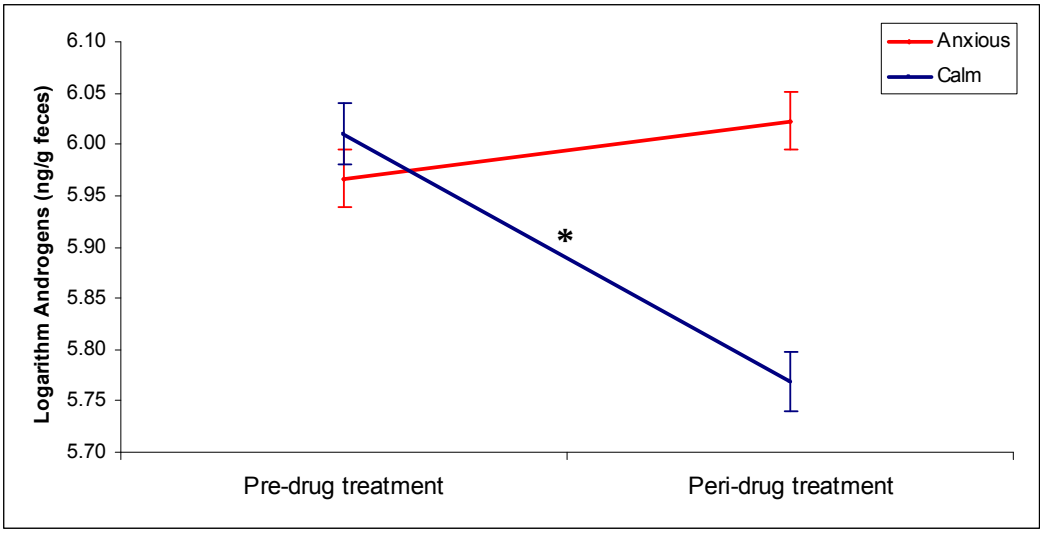


Figure 3.4 Temperament category effects on overall fecal androgen concentrations. Results indicate a significant decrease in fecal androgen concentrations in calm males from pre-drug treatment to peri-drug treatment, and no significant change in anxious males. Error bars indicate SEM.

Table 3.3 Multiple-means comparison tests based on both 3-way and 2-way ANOVA analysis of temperament category and treatment group influence on glucocorticoid and androgen concentrations. Temperament category and treatment group are included as main factors where no significant 3-way interaction was found, with drug effect also included as a main effect where there was a 3-way interaction, otherwise represented with NA = not applicable. The 'Direction' column indicates whether the means increased or decreased in calm males, relative to anxious males. LS Means presented as logarithm (ng/g feces).

	Glucocorticoids							Androgens						
	LS Mean Anxious	LS Mean Calm	Std Error	Direction	Pr <	N 1	N 2	LS Mean Anxious	LS Mean Calm	Std Error	Direction	Pr <	N 1	N 2
Clomipramine														
All														
Overall Mean	NA	NA	NA	NA	NA	NA	NA	6.01	6.52	0.038	Increase	**< 0.0001**	251	214
Base Mean	NA	NA	NA	NA	NA	NA	NA	5.94	6.46	0.036	Increase	**< 0.0001**	225	192
Peak Mean	NA	NA	NA	NA	NA	NA	NA	6.58	7.06	0.053	Increase	**< 0.0001**	26	22
Pre-Drug Treatı														
Overall Mean	5.99	5.76	0.067	Decrease	**0.0264**	131	129	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.71	5.64	0.061		0.9886	90	113	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.61	6.61	0.117		1.0000	41	16	NA	NA	NA	NA	NA	NA	NA
Peri-Drug Treat														
Overall Mean	5.89	5.93	0.077		1.0000	121	85	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.76	5.89	0.064		0.5873	105	79	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.74	6.38	0.191		0.7812	16	6	NA	NA	NA	NA	NA	NA	NA
Deslorelin														
All														
Overall Mean	NA	NA	NA	NA	NA	NA	NA	5.83	5.05	0.031	Decrease	**< 0.0001**	361	305
Base Mean	NA	NA	NA	NA	NA	NA	NA	5.73	5.00	0.030	Decrease	**< 0.0001**	313	288
Peak Mean	NA	NA	NA	NA	NA	NA	NA	6.47	5.81	0.054	Decrease	**< 0.0001**	48	17
Pre-Drug Treatı														
Overall Mean	5.96	5.15	0.059	Decrease	**< 0.0001**	179	161	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.77	5.05	0.052	Decrease	**< 0.0001**	139	143	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.63	5.96	0.113	Decrease	**< 0.0001**	40	18	NA	NA	NA	NA	NA	NA	NA
Peri-Drug Treat														
Overall Mean	5.66	4.92	0.060	Decrease	**< 0.0001**	185	144	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.46	4.78	0.053	Decrease	**< 0.0001**	140	124	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.28	5.78	0.107	Decrease	**0.0002**	45	20	NA	NA	NA	NA	NA	NA	NA
Control														
All														
Overall Mean	NA	NA	NA	NA	NA	NA	NA	6.15	6.10	0.036		0.7743	239	254
Base Mean	NA	NA	NA	NA	NA	NA	NA	5.12	6.08	0.034		0.7939	227	244
Peak Mean	NA	NA	NA	NA	NA	NA	NA	6.74	6.74	0.080		1.0000	12	10
Pre-Drug Treatı														
Overall Mean	6.05	5.68	0.066	Decrease	**< 0.0001**	130	143	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.91	5.53	0.057	Decrease	**< 0.0001**	111	118	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.86	6.41	0.121	Decrease	**0.0123**	19	25	NA	NA	NA	NA	NA	NA	NA
Peri-Drug Treat														
Overall Mean	5.93	5.46	0.073	Decrease	**< 0.0001**	109	111	NA	NA	NA	NA	NA	NA	NA
Base Mean	5.84	5.26	0.065	Decrease	**< 0.0001**	89	89	NA	NA	NA	NA	NA	NA	NA
Peak Mean	6.34	6.29	0.123		1.0000	20	22	NA	NA	NA	NA	NA	NA	NA

3.3.2 Glucocorticoids

Glucocorticoid concentrations were significantly influenced by a 3-way interaction ($F = 3.62$, $P = 0.03$) between treatment group, drug effect, and temperament. Due to the 3-way interaction, 2-way interactions between factors were examined in relation to the third factor.

In regards to the drug effect by treatment group interaction (Table 3.1; Figure 3.5) in the clomipramine treatment group, anxious cats showed no change between pre- versus peri-drug treatment glucocorticoid concentrations, while in calm cats baseline glucocorticoid concentrations increased ($P = 0.004$) peri-drug treatment. In

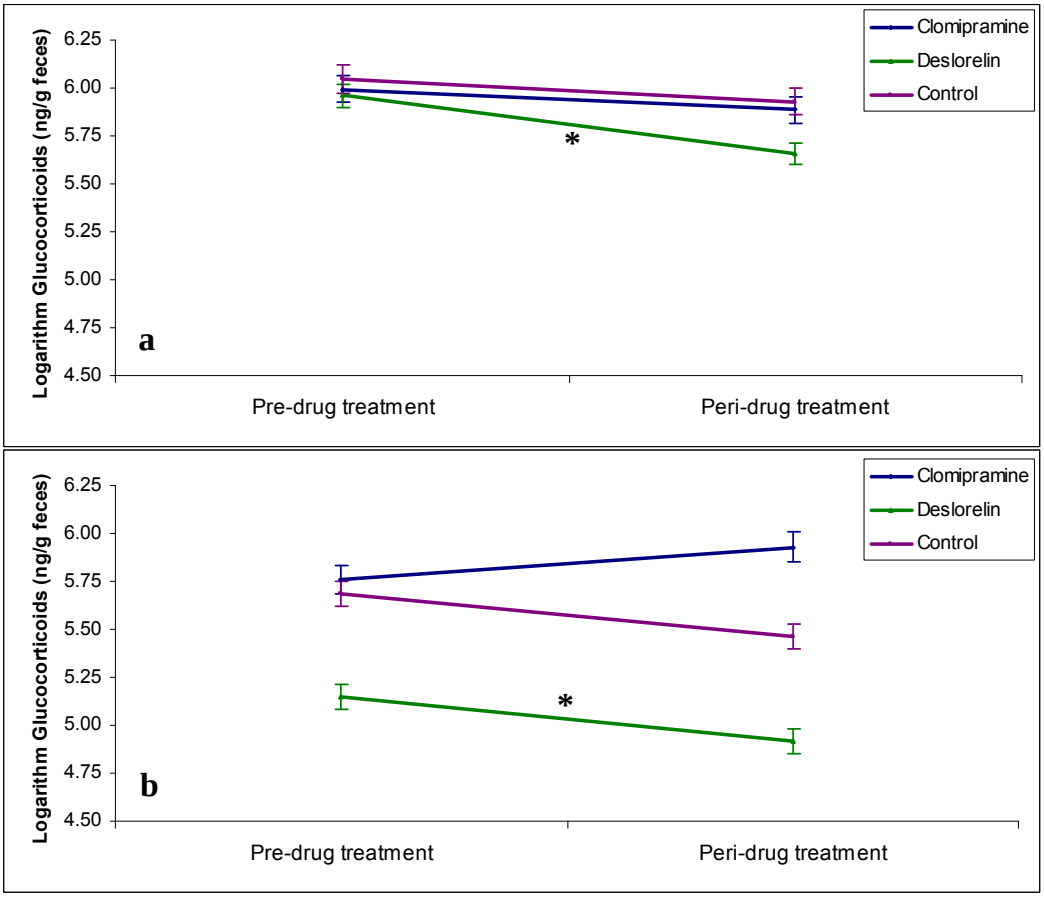


Figure 3.5 Treatment group effects on overall fecal glucocorticoid concentrations by temperament. a) 'Anxious' males and b) 'Calm' males. Results indicate a significant decrease in fecal glucocorticoid levels in the deslorelin treatment group, from pre-drug treatment to peri-drug treatment, and no significant change in either the clomipramine or control treatment group. Error bars indicate SEM.

the deslorelin treatment group there was a decrease in glucocorticoid concentrations in both calm and anxious cats peri- versus pre-drug treatment in regards to both the overall mean (calm, $P = 0.01$; anxious, $P < 0.0001$), and baseline mean (calm, $P < 0.0001$; anxious, $P < 0.0001$). Peak mean concentrations also decreased ($P = 0.004$) in anxious cats in the deslorelin treatment group, while no change was observed in the calm cats. Meanwhile, in the control treatment group there was no change in either calm or anxious males in regards to overall peri-drug versus pre-drug treatment means. For baseline means, glucocorticoid concentrations in calm cats in the control treatment group decreased ($P = 0.0005$), while no change occurred in anxious cats. Finally, for peak means glucocorticoid concentrations in anxious cats in the control treatment group decreased ($P = 0.003$), but no change was observed in calm cats.

In regards to the drug effect by temperament category interaction (Table 3.2), no change was observed in anxious cats peri-drug versus pre-drug for the clomipramine treatment group. A decrease was observed in anxious cats peri-drug versus pre-drug for the deslorelin group overall mean ($P < 0.0001$), baseline mean ($P < 0.0001$), and peak mean ($P = 0.004$). In the control group there was a decrease ($P = 0.003$) in peak mean concentrations in anxious cats while no change was observed in either the overall or baseline means. In calm males in the clomipramine treatment group, an increase ($P = 0.004$) was observed in regards to the baseline mean, but not the overall or peak means. For calm males in the deslorelin group a decrease was observed with regards to both the overall ($P < 0.0001$) and baseline ($P < 0.0001$) mean, but not the peak mean. Finally, in the control group there was a decrease ($P =$

0.0005) in baseline mean glucocorticoid concentrations in calm cats, but not in the overall or peak means.

In regards to the temperament category by treatment group interaction (Table 3.3), calm clomipramine males had lower ($P = 0.03$) overall mean fecal glucocorticoid concentrations than anxious males, pre-drug treatment. Meanwhile no difference was observed between calm and anxious glucocorticoid concentrations, neither for overall means peri-drug treatment, nor for baseline and peak means both pre- and peri-drug treatment. For the deslorelin treatment group, calm males had lower glucocorticoid concentrations than anxious males both pre- (overall, $P < 0.0001$; baseline, $P < 0.0001$; and peak, $P < 0.0001$) and peri-drug treatment (overall, $P < 0.0001$; baseline, $P < 0.0001$; and peak, $P = 0.0002$). Finally, in the control group, calm males had lower glucocorticoid concentrations than anxious males both pre- ($P < 0.0001$) and peri-drug treatment ($P < 0.0001$) for overall and baseline means. Calm males also had lower peak mean glucocorticoid concentrations than anxious males pre-drug treatment ($P = 0.01$), but there was no difference between anxious and calm males in the control group peri-drug treatment.

A Pearson correlation test between androgen levels and glucocorticoid levels revealed a significant correlation between overall mean ($r = 0.50$, $P < 0.0001$) and baseline mean ($r = 0.61$, $P = 0.03$), but not peak mean ($r = 0.44$, $P = 0.13$).

3.3.3 Behavior

There was a significant change in both rates and frequencies for several behaviors in each treatment group from pre-drug treatment to peri-drug treatment (Mann-Whitney U Test; Table 3.4). Briefly, males in the clomipramine treatment

Table 3.4 The effect of drug treatment on incidence of behavior peri- vs. pre-drug treatment, using the Mann-Whitney U test. The 'Direction' column indicates whether the behavior rates increased or decreased during drug treatment, relative to pre-drug treatment rates. For comparisons in anxious and calm categories, a Bonferoni correction is applied to keep family-wise α levels constant.

	Overall (P ≤ 0.05)											
Behavior	**Clomipramine**				**Deslorelin**				**Control**			
	N	**Pr <**	**Direction**	**U**	**N**	**Pr <**	**Direction**	**U**	**N**	**Pr <**	**Direction**	**U**
Growl	63,64	0.4522		1871.5	79,80	0.3324		2955.5	64,62	0.1814		2155.0
Tail Flick		0.5140		1905.0		**0.0409**	Decrease	2833.0		0.6173		2059.0
Lie Down		**0.0043**	Increase	2606.5		0.5431		3336.0		0.8386		1942.0
Sitting		0.1765		1745.0		**0.0020**	Decrease	2304.0		0.6217		1885.5
Standing		0.1046		1703.0		0.1691		2818.0		0.7520		2044.5
Overall Activity		**0.0540**	Decrease	1641.5		0.3037		2906.5		0.1156		2282.5
Nest Box	31,32	**0.0023**	Decrease	349.0	63, 64	0.3122		1813.0	32, 32	1.0000		512.0
Swat Mirror	4, 4	0.4286		4.0	5, 5	**0.0476**	Decrease	3.0	4, 4	0.5429		10.5

	Calm (P ≤ 0.025)											
	Clomipramine				**Deslorelin**				**Control**			
	N	**Pr <**	**Direction**	**U**	**N**	**Pr <**	**Direction**	**U**	**N**	**Pr <**	**Direction**	**U**
Growl	32,32	0.9940		511.0	32,32	1.0000		512.5	32,32	0.5453		495.0
Tail Flick		0.7722		531.0		0.5575		495.5		0.4113		555.5
Lie Down		0.9297		519.0		0.1537		618.0		0.1666		409.5
Sitting		0.3273		442.5		0.1878		415.0		0.0654		642.0
Standing		0.5250		468.5		0.5057		465.5		0.3737		572.5
Overall Activity		0.2426		437.0		0.5143		468.0		0.9427		517.5
Nest Box	NA	NA	NA	NA	16, 16	1.0000		135.0		1.0000		512.0

	Anxious (P ≤ 0.025)											
	Clomipramine				**Deslorelin**				**Control**			
	N	Pr <	Direction	U	N	Pr <	Direction	U	N	Pr <	Direction	U
Growl	31,32	0.3358		429.0	47,48	0.2812		1004.5	32,30	0.2028		550.0
Tail Flick		0.1954		423.5		**0.0475**	Decrease	958.5		0.1568		556.0
Lie Down		**<.0001**	Increase	817.0		0.4763		1033.5		0.0548		341.0
Sitting		0.3319		426.5		**0.0064**	Decrease	791.5		0.2410		562.5
Standing		0.1079		385.0		0.1689		984.5		0.1595		574.5
Overall Activity		0.0634		363.5		0.4345		1043.0		**0.0144**	Decrease	639.0
Nest Box		**0.0023**	Decrease	349.0		0.2057		963.0	NA	NA	NA	NA

group increased the amount of time spent 'lying down' and decreased time spent in a 'nest box' and engaged in 'overall activity' (Figure 3.6). Males in the deslorelin treatment group decreased 'tail flick' and 'swatting' the mirror, as well as time spent 'sitting' (Figure 3.6). No overall behavioral changes were observed in the control group.

When the behavioral data was assessed with regards to temperament category, there were significant changes in behaviors within the 'anxious' cats, but not the

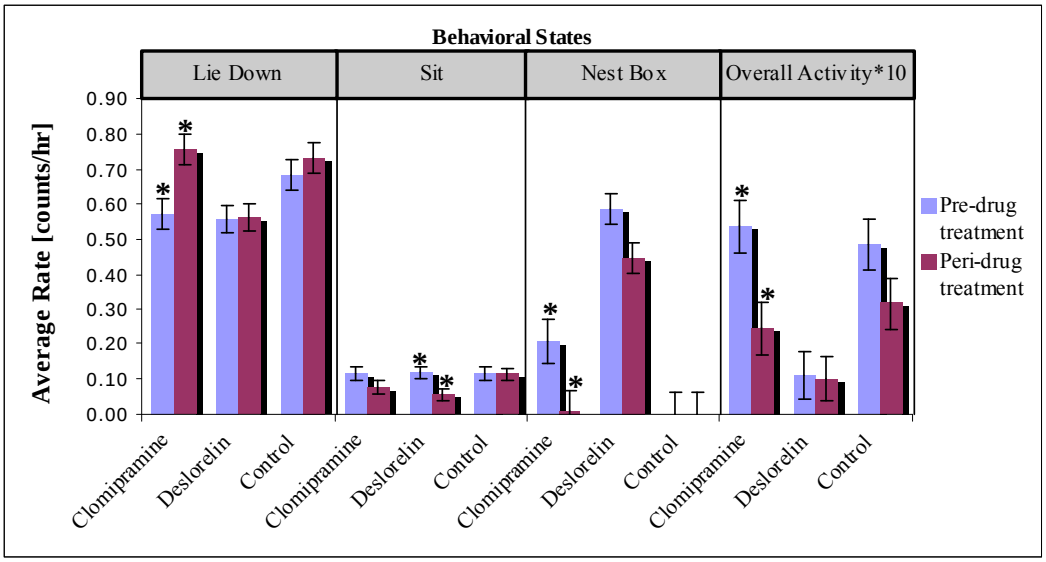


Figure 3.6 Bar chart of mean frequencies for four behavioral states both pre- and peri-drug treatment, blocked by treatment group. Asterisks indicate significant differences between pre- and peri-drug treatment within a drug treatment. 'Overall Activity' frequencies are multiplied by a factor of 10, for ease of viewing.

'calm' cats in the pre- versus peri-drug treatment periods (Table 3.4). There was a significant increase in 'lying down' and a decrease in time spent in a 'nest box' for 'anxious' cats in the clomipramine group. Deslorelin treated males showed a significant decrease in 'sitting' and a trend in increased 'tail flick' behavior that approached significance (Table 3.4). 'Anxious' control males had decreased 'overall activity'.

3.3.4 Efficacy of Drug Treatments in Altering Response in Behavioral Reaction Tests

Male clouded leopard response to MIS and UFP tests peri-drug treatment was significantly altered in all treatment groups, relative to the pre-drug treatment

response (Table 3.5). Before drug treatments were administered (pre-drug treatment period, Column 1 of Table 3.5), there were significant increases in 'standing', 'overall activity', 'growl', and 'tail flick' in animals exposed to the MIS test. Rates for 'growl' and 'tail flick' behaviors increased significantly during the UFP test (Table 3.5) in the pre-drug treatment period also. The airhorn test did not elicit significant changes to any behavior rates, pre-drug treatment (Table 3.5). Following administration of clomipramine (Column 2 of Table 3.5), analysis showed that the MIS test no longer elicited increases in 'standing', 'overall activity', or 'growling' (Figure 3.7) and the UFP test no longer elicited increased rates of 'tail flicking', indicating suppression of these behaviors during these tests, peri-drug treatment. Deslorelin treatment (Column 3 of Table 3.5) was similarly linked to a suppression of 'standing', 'growling', and 'tail flicking' during the MIS test (Figure 3.7) and both 'growling' and 'tail flicking' during the UFP test. The control group (Column 4 of Table 3.5) showed significantly decreased 'tail flick' behavior during the UFP test in peri-drug treatment observations. Two behaviors that did not significantly change during the airhorn test pre-drug treatment did change in behavior rates during treatment: 'sit' and 'growl' during clomipramine treatment and 'growl' during control treatment (Table 3.5). Finally, a number of behaviors ('standing', 'overall activity', 'growl', and 'tail flick') were elicited at significantly increased rates by behavioral reaction tests during both pre- and peri-drug treatment periods (Table 3.5). All key endocrine and behavioral results are summarized in Figure 3.8.

Table 3.5 Mann-Whitney U Testing for behavior incidence during behavior reaction tests (MIS, Airhorn, and UFP), relative to combined pre-and post-behavior test (Pre/Post-test) observations. 'Direction' columns indicate whether behavior rates/frequencies increased or decreased during behavior testing. Each drug treatment was compared to pre-drug treatment measures. Gray shading indicates a treatment effect, where a particular behavior reaction test elicited a significant change in behavior rates pre-drug treatment, but not peri-drug treatment for the given treatment group.

		Pre-drug Treatment				Clomipramine			Deslorelin			Control		
		N	Pr <	Direction	U	N	Pr <	U	N	Pr <	U	N	Pr <	U
Lie Down														
MIS	**Pre/Post-test**	126	0.0856		1274.0	32	0.1492	93.0	40	0.4742	120.0	30	0.1319	88.5
	Test	16				4			5			4		
Air	**Pre/Post-test**	126	0.8510		978.5	32	0.1966	90.0	40	0.8980	104.0	30	0.7669	66.0
	Test	16				4			5			4		
UFP	**Pre/Post-test**	126	0.2091		6574.5	32	0.8474	396.0	40	0.6947	633.0	30	0.2243	430.0
	Test	95				24			30			24		
Sit														
MIS	**Pre/Post-test**	126	0.1796		804.5	32	0.9361	62.0	40	0.4557	81.0	30	0.8033	55.0
	Test	16				4			5			4		
Air	**Pre/Post-test**	126	0.3821		875.5	32	**0.0382**	24.0	40	0.6720	89.0	30	0.4067	44.5
	Test	16				4			5			4		
UFP	**Pre/Post-test**	126	0.1978		6572.0	32	0.9719	381.5	40	0.3141	522.0	30	0.2301	292.5
	Test	95				24			30			24		
Stand														
MIS	**Pre/Post-test**	126	**<.0001**	Increase	312.0	32	0.4317	49.0	40	0.3041	74.5	30	**0.0172**	17.5
	Test	16				4			5			4		
Air	**Pre/Post-test**	126	0.4142		893.5	32	0.4342	49.0	40	0.2673	72.5	30	0.3154	42.0
	Test	16				4			5			4		
UFP	**Pre/Post-test**	126	0.7373		5841.0	32	0.1818	456.5	40	0.0607	726.0	30	0.5243	393.0
	Test	95				24			30			24		
Nest Box														
MIS	**Pre/Post-test**	86	0.2420		562.5	16	0.8597	17.0	32	0.6707	55.5	16	1.0000	16.0
	Test	11				2			4			2		
Air	**Pre/Post-test**	86	0.1560		580.5	16	0.8597	17.0	32	0.8935	67.0	16	1.0000	16.0
	Test	11				2			4			2		
UFP	**Pre/Post-test**	86	0.2254		2504.0	16	0.4273	102.0	32	0.1171	293.0	16	1.0000	96.0
	Test	65				12			24			12		
Overall Activity														
MIS	**Pre/Post-test**	126	**0.0002**	Increase	463.0	32	0.0884	32.0	40	**0.0247**	48.5	30	**0.0159**	18.0
	Test	16				4			5			4		
Air	**Pre/Post-test**	126	0.2870		857.5	32	0.8678	60.5	40	0.1902	70.5	30	0.4616	72.5
	Test	16				4			5			4		
UFP	**Pre/Post-test**	126	0.3588		16529.1	32	0.8878	376.0	40	0.7224	623.0	30	0.9604	357.0
	Test	95				24			30			24		
Growl														
MIS	**Pre/Post-test**	126	**<.0001**	Increase	454.0	32	0.2420	44.0	40	0.7098	93.0	30	**<.0001**	15.0
	Test	16				4			5			4		
Air	**Pre/Post-test**	126	0.1172		849.0	32	**0.0326**	27.0	40	0.8412	96.0	30	**0.0081**	45.0
	Test	16				4			5			4		
UFP	**Pre/Post-test**	126	**<.0001**	Increase	4234.0	32	**0.0116**	246.0	40	0.2666	536.0	30	**0.0510**	315.0
	Test	95				24			30			24		
Tail Flick														
MIS	**Pre/Post-test**	126	**<.0001**	Increase	344.0	32	**0.0157**	27.0	40	0.7773	102.5	30	**0.0065**	20.0
	Test	16				4			5			4		
Air	**Pre/Post-test**	126	0.3658		916.0	32	0.2536	47.0	40	0.7773	102.5	30	0.3542	72.0
	Test	16				4			5			4		
UFP	**Pre/Post-test**	126	**0.0463**	Increase	5325.0	32	0.3706	343.5	40	0.0967	537.0	30	0.8673	367.0
	Test	95				24			30			24		

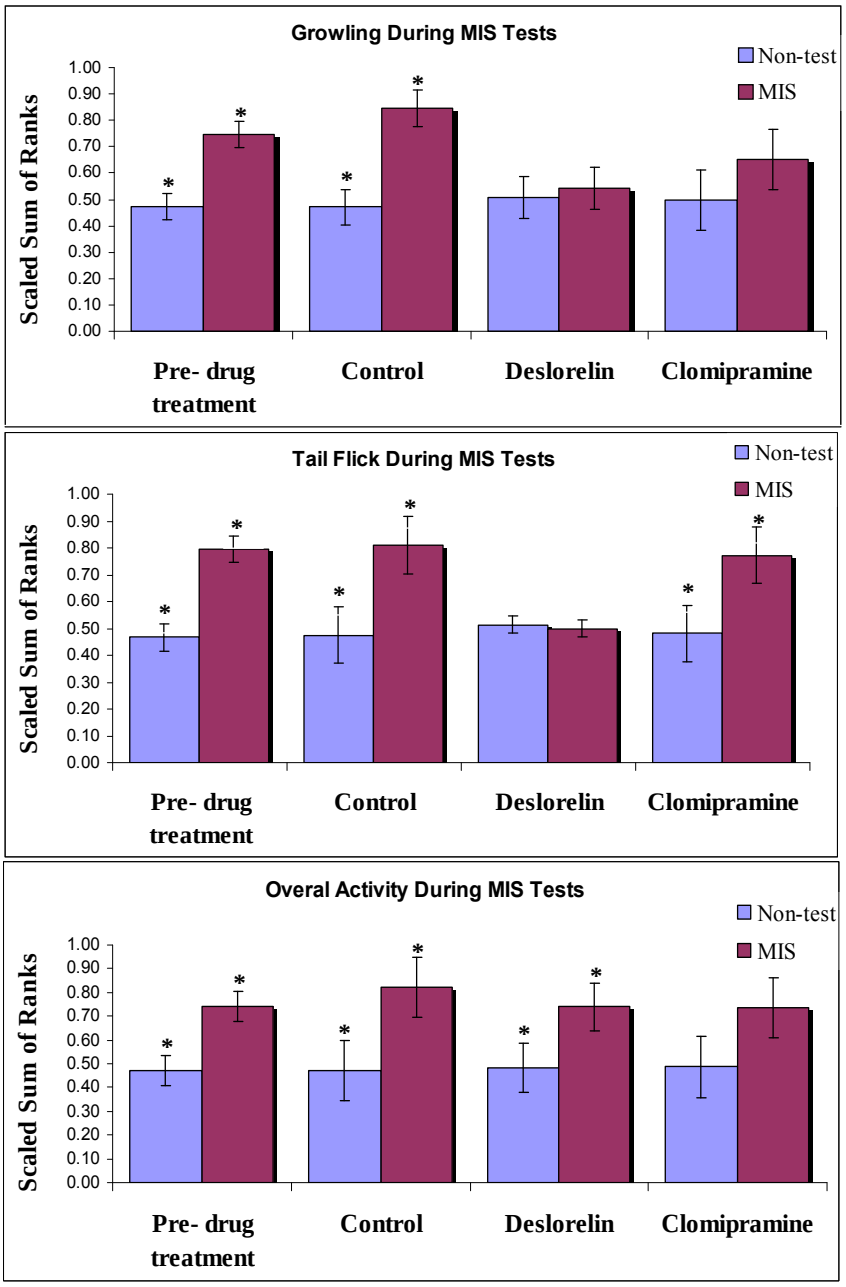


Figure 3.7 Bar charts demonstrating effectiveness of the mirror test (MIS) in eliciting select behaviors compared to behavior rates from combined pre- and post-behavioral reaction test observations (Non-test) and the effect of drug treatment groups on suppressing these behaviors during MIS testing. Asterisks indicate a significant difference between MIS and non-test behavior rates/frequencies. Results show the effectiveness of deslorelin and clomipramine in suppressing some behaviors linked to aggression and anxiety during MIS tests. Since data are not normally distributed, behavior rates are represented by the scaled sum of ranks calculated for Mann-Whitney U tests.

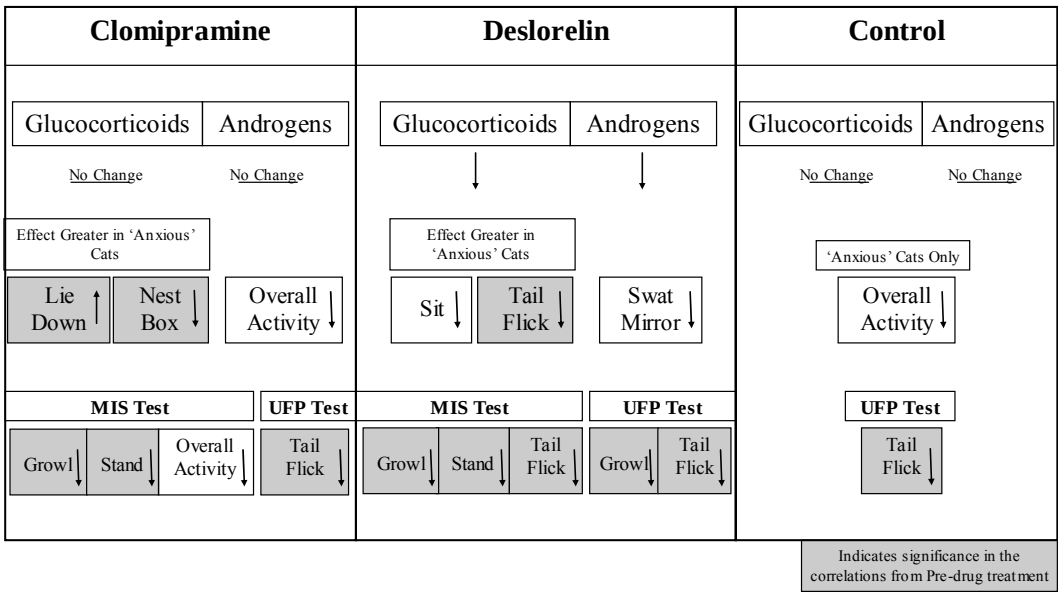


Figure 3.8 Summary diagram illustrating the overall effects of each treatment group on endocrine and behavioral data. Arrows indicate whether endocrine levels and behavior rates increased (arrow up) or decreased (arrow down). Shaded boxes indicate behaviors that were significantly correlated with clouded leopard temperaments in chapter 2.

3.4 Discussion

3.4.1 Summary

These data conclusively show, for the first time, that aggressive clouded leopard behavior is modulated through both neural and endocrine pathways. Clomipramine had a direct impact on the neural serotonergic pathways modulating 'anxious' behaviors, such as activity and hiding, without altering glucocorticoid levels. Meanwhile, deslorelin treatment resulted in a significant decrease in aggressive behaviors such as 'swatting', 'growling', and 'tail flicking,' which therefore appear to be modulated by testosterone. This study also demonstrated that the two drug treatments were effective in suppressing aggressive behaviors elicited by

the MIS and UFP tests, such as 'growling' and 'tail flicking'. Overall, it is encouraging that both pharmaceutical treatments were effective in mitigating anxiety and aggression in male clouded leopards, suggesting that pharmacological therapeutics might be a viable management approach to form reproductive pairings.

3.4.2 The Use of Behavioral Reaction Tests to Evaluate Levels of Anxiety and Aggression

No previous studies in male clouded leopards have used a structured panel of successive behavioral tests to assess aggression and anxiety. Using behavioral tests to elicit aggressive and/or anxiety related behaviors and then comparing behavioral responses after drug treatment provides a strong direct evaluation of treatment efficacy on an individual basis. This is particularly important in zoo settings, where interactive behaviors may not be routinely stimulated in isolated animals. Indeed, in isolated male clouded leopards, behaviors induced by the MIS and UFP tests ('growling', 'tail flicking', and to a lesser extent 'overall activity') have not been routinely demonstrated unless in response to a stimulus, such as a mirror where a male may perceive his reflection as a rival animal (Wielebnowski, 1999). Based on these results, the behavior reaction tests provided excellent assessment tools for individual anxious and aggressive behavior patterns. As such, the MIS and UFP tests offer a promising tool to rapidly evaluate some aspects of temperament, such as inherent pre-disposition to aggression and anxiety, as well as potential changes in such behavior patterns in response to drug and/or medical treatments.

Importantly, behavioral reaction tests can provide insight into individual behavioral responses prior to attempting male-female pairing. Males often kill

females during breeding introductions, making many zoological institutions reluctant to attempt clouded leopard breeding (Fletchall, 2007). Furthermore, the logistics of introductions, including the transport of breeding pairs to a common facility and numerous keeper hours to oversee the pairings, are extensive. The use of behavior reaction tests to identify animals prone to aggression could provide important insight into the likelihood of pairing success. Ultimately, the goal would be to target pharmaceutical interventions to moderate aggressive behaviors in those animals identified as high risk for aggression.

3.4.3 Clomipramine Treatment

3.4.3.a Endocrine Effects

The tricyclic antidepressant clomipramine is a serotonin–norepinephrine reuptake inhibitor (SNRI), and is active on both the serotonin and norepinephrine systems. However, it is commonly believed that its ability to decrease anxious behaviors is more the result of its ability to increase the availability of serotonin, rather than its effects on the norepinephrine system (Dubinsky et al., 1973; Zohar and Westenberg, 2000). Mice lacking 5-HT1A receptors, which bind serotonin, have increased anxiety and stress response (Parks et al., 1998; Ramboz et al., 1998). In cats, serotonin decreases affective defensive behaviors such as growling (Golebiewski and Romaniuk, 1985). Research has also supported a linkage between the serotonin system and the HPA axis in humans (Jensen et al., 1999; Pariante et al., 2001). The behavioral changes observed in clouded leopards indicate that clomipramine was efficacious, presumably by blocking serotonin reuptake and increasing the availability of serotonin to postsynaptic receptors. However, no overall change was seen in fecal

glucocorticoid concentrations with clomipramine treatment. In fact, with regards to 'calm' cats there was actually an increase in baseline concentrations, despite the decrease in anxious and aggressive behaviors. This could indicate a lack of connectivity between the two systems in the clouded leopard, or perhaps that assessment of glucocorticoid levels through fecal samples was not sensitive enough to detect changes resulting from the behavioral reaction tests in the way that serum analysis might (Sheriff et al., 2011). Another possibility is that the behavior reaction tests presented a transient stressor, inducing release of adrenal catecholamines, and was insufficient to release cortisol (Jansen et al., 1995).

3.4.3.b Behavioral Effects

The effect of increased serotonin on anxiety-related behaviors was evident, with changes primarily decreasing activity levels (increased 'lying down', decreased 'overall activity') and resulting in less hiding behavior. Captive clouded leopards are often categorized as either 'anxious' or 'calm'. Similar distinct behavioral characteristics have been observed in other species, resulting in the definition of two temperament types: the proactive type (aggressive males) and the reactive type (non-aggressive) males, which are more adaptive and respond with aggression only as a last option. These temperaments are regarded as coping styles, which have been studied in a wide variety of species including mice, cattle, chicken, and monkeys. Both types of temperaments are important for survival, and adaptive for different situations that the animal encounters. The proactive individual adapts well in a stable colony, while the reactive individual adapts well to variable conditions (Koolhaas et al., 1999; Sih et al., 2004). Interestingly, in our study the more 'anxious' cats

appeared to be more strongly affected by clomipramine treatment and spent significantly more time 'lying down' and less time hiding in their 'nest boxes' relative to pre-drug treatment. Yet neither behavioral change was observed in the cats identified as 'calm', probably because 'calm' cats already spent more time lying down. The tendency for an animal to hide often demonstrates increased fearfulness and has been correlated with increased fecal glucocorticoid concentrations in clouded leopards (Wielebnowski et al., 2002). Thus, there is good indication that serotonin mitigates stress-induced behavioral responses in this species.

Clomipramine treatment in male clouded leopards also decreased aggressive behaviors such as 'growling' and 'tail flicking' in response to the MIS and UFP behavioral reaction tests. Both 'growling' and 'tail flicking' have been described as aggressive behaviors indicating an irritated state (Kileyworthington, 1976; Gregg and Siegel, 2001). Increased serotonin levels decrease aggression in a number of species, including cats (Olivier et al., 1995; Gregg and Siegel, 2001; Summers et al., 2005). Furthermore, serotonin levels have been shown to affect both types of aggression exhibited by felids: predatory attack, which can include 'tail flicking' (Kileyworthington, 1976) and affective defense which would include 'growling' (Golebiewski and Romaniuk, 1985; Olivier et al., 1995; Lucki, 1998). These data in combination with our observations support the key role of the serotonergic system in modulating aggressive behavior, especially related to anxiety in clouded leopards.

3.4.4 Deslorelin Treatment

3.4.4.a Endocrine Effects

The GnRH agonist deslorelin significantly decreased fecal androgen levels in the male clouded leopard, associated with inhibition of the HPG Axis. A significant decrease was observed in both the overall and baseline mean. In addition, overall fecal glucocorticoid levels decreased significantly relative to the control group. While the suppression of fecal androgens was expected as a direct effect of the GnRH agonist, the mechanism behind the observed decrease in fecal glucocorticoids is less clear.

Although the two axes that facilitate the production of fecal androgens and fecal glucocorticoids (HPG and HPA, respectively) are linked and fluctuate together seasonally (Kersey et al., 2010), there tends to be an inverse relationship. Glucocorticoids are known to suppress the HPG axis, while androgens are known to suppress the HPA axis (Viau, 2002; Seale et al., 2004; Hardy et al., 2005). However, both androgens and glucocorticoids are known to increase together as a result of acute stress, due to production of both glucocorticoids and the androgen dehydroepiandrosterone (DHEA) in the adrenal glands (Newman et al., 2008). Therefore, while the two axes are inextricably linked, the linkage is still poorly understood and there is no currently known mechanism by which androgen suppression would affect a decrease in glucocorticoids. While minor assay cross-reactivity between fecal androgen and glucocorticoid metabolites could have occurred, this would not account for the significant results observed. Cross-reactivity is further discounted by the lack of correlation between peak means of the two

hormones. As discussed below, there was a decrease in both anxious and aggressive behaviors as a result of deslorelin treatment in male clouded leopards. Since it is well know that glucocorticoids are linked to a stress response (Griffin and Ojeda, 2004), the simultaneous decrease in fecal androgen and glucocorticoid levels therefore suggests that the suppression of testosterone led to a decrease in overall anxiety and stress-sensitivity in clouded leopards. We speculate that these changes, in turn, led to the decreases in anxious and aggressive behaviors, as well as excreted fecal glucocorticoids, observed in this treatment group.

3.4.4.b Behavioral Effects

It is widely accepted that increased androgen levels are linked to aggressive behaviors. According to the "challenge hypothesis," androgen levels vary with reproductive competition such as territorial defense or male-male interactions that involve aggression (Wingfield et al., 1990). In deslorelin-treated male clouded leopards, aggression associated behaviors were the primary behaviors that were altered, both overall ('tail flicking'), and in response to the MIS and UFP tests ('swatting', 'growling', and 'tail flicking'). The mirror-image response is consistent with a territorial response by males when they see their reflection, whereas the unfamiliar person more likely stimulates a defensive response.

3.5 Conclusion

The goal of this study was to investigate two probable mechanisms underlying aggressive behavior in male clouded leopards, using a series of behavioral reaction tests before and after treatment with either a tricyclic antidepressant, clomipramine,

or a GnRH agonist, deslorelin. Results demonstrated that aggression in the male clouded leopard is modulated by both the serotonergic system and circulating androgens. The MIS and UFP behavior reaction tests were useful in eliciting behaviors indicative of anxiety and aggression; both clomipramine and deslorelin were in turn effective in modulating these behaviors. Further, clomipramine effectively reduced the incidence of behaviors associated with anxiety and aggression while not affecting adrenal glucocorticoid levels. Deslorelin was also effective at reducing aggressive and anxious behaviors, and also decreased excreted fecal androgens and glucocorticoids. Overall, it is encouraging that both pharmaceutical treatments were effective in mitigating anxiety and aggression in male clouded leopards, suggesting that pharmacological therapeutics might be a viable management approach to form reproductive pairings.

Chapter 4: Physiological Effects of Behavior Modification Treatment in Clouded Leopards (*Neofelis nebulosa*) and Domestic Cats (*Felis catus*)

4.1 Introduction

The clouded leopard (*Neofelis nebulosa*) is one of the most challenging felid species to breed in captivity, due to male aggression that often results in fatal attacks on females. As a consequence, population numbers in captivity are too low to be self-sustaining and risk compromising genetic diversity, key objectives of zoological breeding programs (Fletchall, 2003). Captive populations are intended as a hedge against extinction in the wild, where the clouded leopard is in decline (Grassman et al., 2005). They are listed as 'endangered' under the United States Endangered Species Act and 'Vulnerable' in the IUCN Red Data Book. As a temporary 'rescue strategy,' breeding management similar to line-breeding is currently practiced to maintain stable demographics. Meanwhile, researchers continue to examine factors that may contribute to poor reproduction in adult clouded leopards managed in captivity.

Previous studies indicate that aggression in clouded leopards is correlated with both anxious temperament and post-pubertal testosterone. These studies have correlated anxious male clouded leopards with failed male-female pairings, high excreted cortisol concentrations, and aberrant behaviors such as excessive pacing, fur plucking, and hiding (Wielebnowski et al., 2002; MacKinnon et al., 2007). There is also evidence that testosterone plays a role in clouded leopard aggression, with

juvenile pairs known to experience lower rates of failure due to hostile behavior than pairs formed with a post-pubertal male (MacKinnon, 2008). Furthermore, reduced circulating testosterone is correlated with a reduction in behaviors associated with aggression (e.g. 'growling', 'swatting', and 'tail flicking') (Chapter 3). This positive correlation between testosterone and aggression is well documented in many species, including birds, mice, and more, recently, clouded leopards (Wingfield et al., 2001; Oyegbile and Marler, 2005; Archer, 2006, Chapter 3). These two underlying pathways to aggression – anxiety and increased circulating testosterone levels – point to two potential pharmaceutical treatments: 1) clomipramine (a tricyclic antidepressant) and 2) deslorelin (a gonadotropin releasing hormone agonist; GnRH agonist) as effective strategies to control aggression in male clouded leopards (Chapter 3). However, little is known about the effects of these drugs on male clouded leopard reproductive function including sperm production. The ultimate goal of aggression mitigation is the formation of successful breeding pairs; therefore a thorough investigation of each drug's effect on reproductive physiology is merited.

Deslorelin is known to reduce aggression in a variety of species, including the lion-tailed macaque (*Macaca silenus*), black-footed cat (*Felis nigripes*), and clouded leopard (Norton et al., 2000; Bertschinger et al., 2001, Chapter 3). It is a long-acting reversible GnRH agonist that acts on pituitary GnRH receptors to first hyper-stimulate and then down-regulate testosterone steroidogenesis (Aspden et al., 1996; Bertschinger et al., 2001; Padula, 2005; Trigg et al., 2006). While deslorelin is used to control aggression, it is primarily used as a contraceptive, most extensively in the domestic dog (Trigg et al., 2006). By down-regulating the hypothalamic-pituitary-

gonadal (HPG) axis, deslorelin suppresses endocrine and germinal testicular function. Deslorelin has been effective in down-regulating the HPG axis in several species including the boar (Kauffold et al., 2010), cheetah (*Acinonyx jubatus*) (Bertschinger et al., 2006), and black flying-fox (*Pteropus alecto*) (Melville et al., 2012), but failed to exert any beneficial effect in the tammar wallaby (*Macropus eugenii*) (Herbert et al., 2004) or antelope (Penfold et al., 2002). Furthermore, while the timing and effectiveness of deslorelin treatment on testosterone and secondary male sex characteristics has recently been documented in the male domestic cat (*Felis catus*) (Goericke-Pesch et al., 2011), there is currently no information on the duration of reproductive suppression, re-establishment of spermatogenesis, or overall effects on ejaculate quality in felids.

Clomipramine is widely used for treating anxiety disorders, obsessive-compulsive behaviors, destruction, and aggression in the domestic dog (*Canis lupus familiaris*) and cat (Seksel and Lindeman, 1999; Siegel et al., 1999; King et al., 2000; Litster, 2000; Gillman, 2007). It inhibits the re-uptake of the neurotransmitters serotonin and noradrenalin by blocking the serotonin transporter and noradrenalin transporter. This, in turn, increases the availability of serotonin for binding to postsynaptic receptors, making it effective in reducing both anxiety and aggression (Dubinsky et al., 1973). Although clomipramine treatment is not expected to act directly on reproductive function, chronic stress can inhibit male reproductive function by altering sexual behavior, testosterone secretion, and spermatogenesis (Collu et al., 1984; RetanaMarquez et al., 1996; Gorzalka et al., 1998; Cordner et al., 2004). Furthermore, there is evidence linking stress to poor sperm quality, with low

percentage of normal sperm and low sperm concentration in many felid species (Swanson et al., 2003; Pukazhenthi et al., 2006). Clouded leopards, compared to other felids, have increased basal cortisol levels (Wildt et al., 1986), indicating high stress levels that may impact spermatozoa and overall reproductive function. Thus, it is possible that decreasing anxiety in the male clouded leopard will also improve sperm quality. In fact, clomipramine has been shown to counteract the negative effects of stress on male reproduction and ejaculate traits in rabbits. Male rabbits treated with clomipramine for 12 weeks showed a significant improvement in sperm traits, including sperm concentration, total sperm per ejaculate, percent sperm motility, total motile sperm per ejaculate, and total live sperm (Ahmed et al., 2008). Although clomipramine has not been tested in clouded leopards, it may have a similar positive effect.

The domestic cat has been used extensively as a model for their non-domestic felid counterparts (Pelican et al., 2006; Swanson, 2006; Harris et al., 2008; Pelican et al., 2008). Furthermore, domestic cats are an important model species for laboratory research, and have been used to model human reproductive function (Travis et al., 2009; Wildt et al., 2010). It has previously been shown that deslorelin and clomipramine are effective in suppressing reproductive hormones and mitigating anxiety-related disorders, respectively, in the domestic cat (Siegel et al., 1999; Litster, 2000; Goericke-Pesch et al., 2011). Domestic cats therefore serve as an important model for the effects of these drug treatments on reproductive function in a species in which they are known to be effective in mitigating negative behaviors.

The goal of this study was to examine the effects of both a GnRH agonist and a psychotropic drug on the reproductive physiology of male clouded leopards and domestic cats. In the present study, the effects of each drug on cortisol and testosterone steroidogenesis, and their subsequent impact on testicular function were investigated. The objective was to identify a safe and effective treatment to modify behavior with minimal impact on long-term reproductive function. In so doing, this study adds significant new knowledge to the long-term effects of these drug classes on male feline reproductive function with an ultimate goal of using them to aid in the formation of successful breeding pairs.

4.2 Materials and Methods

4.2.1 Animals and Study Area

Eight adult male domestic cats housed at the Smithsonian Conservation Biology Institute (SCBI), VA, USA (1.5 to 8 y of age) were studied from June 2009 to February 2012. Males were housed individually in 2.7-m^3 indoor cages, maintained on a 14L:10D cycle, and provided dry, commercial cat food (Purina Cat Chow; Ralston Purina Co., St. Louis, MO) and water *ad libitum*.

Five adult male clouded leopards housed at four North American zoos were studied from April 2009 to May 2011. Participating zoos included: Central Florida Zoo & Botanical Gardens, FL, USA (n = 1; 8 y of age); Houston Zoo, TX, USA (n = 1; 6 y of age); Omaha's Henry Doorly Zoo, NE, USA (n = 1; 13 y of age); and SCBI, (n = 2; 5, and 15 y of age). Although management practices differed among institutions, all animals were housed according to the Association of Zoos and

Aquariums (AZA) guidelines for this species (Fletchall, 2000). Zoos were instructed to follow routine husbandry protocols for the duration of the study. Each clouded leopard was housed singly: one animal spent time on-exhibit for public view (~ 9 hr per day) and the remaining time off-exhibit; the other four animals were permanently housed off-exhibit. One animal was housed permanently indoors and maintained on a 10L:14D cycle.

4.2.2 Study Design

Domestic cat males were randomly assigned to one of two treatment groups: deslorelin or clomipramine. Of the 8 domestic cats, 6 males were randomly assigned to one treatment group only, while 2 males were assigned to the clomipramine treatment first, followed 8 months later by deslorelin treatment (total n = 5 cats/treatment). Full sperm production (>100 million spermatozoa/mL) was confirmed prior to initiation of deslorelin treatment. For the deslorelin group, semen was collected immediately before deslorelin implantation and then collected at 2, 4, 6, 8, 10, and 12 months post-implantation. After 12 months semen was collected at varying intervals (up to 28 months post-implantation) until each animal produced a sperm concentration greater than 100 million spermatozoa/mL. For the clomipramine group, semen was collected immediately before the start of drug treatment to establish baseline sperm traits. Drugs were administered for 4 months, and semen was collected at 2, 4, 6, and 8 months after the start of treatment. Fecal samples were collected at least 3 days/week on each animal for the duration of the study to assess fecal glucocorticoid and androgen metabolite concentrations.

Clouded leopard males were randomly assigned to either the deslorelin (n = 3) or clomipramine (n = 2) treatment groups. Clomipramine was administered for 6 months. For both treatment groups, semen was collected immediately before the start of drug treatment and at 6 and 12 months following the start of treatment. One clouded leopard in the deslorelin treatment group became aspermic at the 12 month collection interval; therefore two additional collections were performed on this individual at 18 and 24 months. This male was still aspermic at 24 months and collections were discontinued for health reasons. This male is considered an outlier and was excluded from all analysis (addressed further in the Discussion). Fecal samples were collected at least 3 days/week on each animal for the duration of the study to assess fecal glucocorticoid and androgen concentrations.

The studies described were approved by the Smithsonian's National Zoological Park (#08-12) and the University of Maryland, College Park (#R-08-33) Animal Care and Use Committees. The study was also reviewed and approved by all institutions housing clouded leopards.

4.2.3 Drug Treatment

Deslorelin (Suprelorin®; Peptech Animal Health, Australia), was administered as either three (clouded leopard) or one (domestic cat) subcutaneous implants (~ 2.3 mm x 12.5 mm length) each containing 4.7 mg of deslorelin for a total dosage of 14.1 mg deslorelin in the clouded leopard and 4.7 mg in the domestic cat. Specifically, clouded leopards were anesthetized with ketamine hydrochloride and sometimes midazolam by institutional veterinarians and a 2 cm site was cleaned and scrubbed in the dorsal inter-scapular region. The three microchip sized

cylindrical implants were inserted through the skin using an insertion trocar. Domestic cats were physically restrained and one implant inserted in the inter-scapular region using an insertion trocar. Each represented the recommended dosage for six months of testosterone suppression (Asa, 2009; Goericke-Pesch et al., 2011). Clomipramine (Clomicalm® Novartis Animal Health US, Inc.) was administered orally at a dosage of 0.6 mg/kg in both clouded leopards and domestic cats as a pill inserted in a meatball and fed to each cat daily for four months (domestic cats) or six months (clouded leopards). The dosage was based on published reports in the domestic cat (Litster, 2000).

4.2.4 Semen Collection

Animals were anesthetized according to protocols determined by institutional veterinarians and similar to those previously used for semen collection in clouded leopards and domestic cats (Neubauer et al., 2004; Pukazhenthi et al., 2006). Drugs included ketamine hydrochloride and sometimes midazolam for clouded leopards and a combination of ketamine hydrochloride, xylazine hydrochloride, butorphanol tartrate, and/or diazepam in domestic cats. Upon reaching a surgical plane of anesthesia, animals were weighed and semen was collected as previously described (Howard et al., 1990; Howard, 1993). Briefly, testes were measured for total volume and each male was electroejaculated into a warm sterile cup using a 60-Hz sine-wave stimulator and a 1.9 cm diameter (clouded leopard) or 1.0 cm diameter (domestic cat) rectal probe with three longitudinal electrodes (P.T. Electronics, Boring, OR). A total of 80 low-voltage (2–6 V) stimuli were delivered in three series over the course of 30 min. Each ejaculate was diluted 1:1 in Hams F10 Hepes Basal Medium containing 25

mM hepes (Ham's F10; Irvine Scientific, Santa Ana, CA, USA) supplemented with 5% (v/v) fetal calf serum (domestic cats; Irvine Scientific) or 5% (v/v) heat inactivated clouded leopard serum (clouded leopards), pyruvate (1 mM), L-glutamine (2 mM) and 10,000 IU 1/mL penicillin, 10 mg/mL streptomycin and 20 mg/mL neomycin (Sigma Chemical Co., St. Louis, MO, USA). Semen was then evaluated for pH, volume, sperm concentration, percent sperm motility, and forward progressive status (FPS; i.e., speed of forward progress; scale, 0–5; 0 = no forward movement, 5 = rapid linear forward movement) using a phase contrast microscope (Howard et al., 1990). A sperm motility index (SMI) was calculated using the formula [(percent motility + (FPS x 20)) ÷ 2] (Howard, 1993). For morphological assessment, a 10-µL aliquot of fresh semen was fixed in 0.3% (v/v) glutaraldehyde in PBS (pH 7.4; 340 mOsm) and later evaluated (100 sperm/aliquot) by phase-contrast microscopy (1000x) (Neubauer et al., 2004).

4.2.5 Fecal Collection and Analysis

Fecal samples (at least 5 g) were collected a minimum of 3 days/week on each animal, starting 14 days before the first semen collection and ending on the day of the last semen collection. Total samples collected per individual ranged from 106 to 164 (clouded leopards), 211 to 317 (domestic cats in the deslorelin treatment group), and 99 to 109 (domestic cats in the clomipramine treatment group). Once collected, samples were placed in plastic bags, labeled and frozen (-20ºC), and shipped (if applicable) on dry ice to the SCBI Endocrine Research Laboratory for hormonal analysis by Enzyme Immunoassay (EIA). Samples were evaluated for glucocorticoid and androgen metabolites using assays previously validated for the clouded leopard

and domestic cat (Brown et al., 1996; Graham and Brown, 1996; Wielebnowski et al., 2002). In brief, each fecal sample was lyophilized, pulverized, and 0.19–0.21 g of dry fecal powder was vortexed (5 s) and shaken (30 min) in 5 mL of 90% ethanol. After centrifugation (20 min; 2,500 RPM), the supernatant was recovered, and the pellet was re-suspended in 5 mL of 90% ethanol, vortexed (5 s), shaken (30 s), and re-centrifuged (15 min; 2,500 RPM). The first and second supernatants were combined, dried under air and reconstituted in 1 mL methanol. Methanol extracts were vortexed (5 s), sonicated (15 min), and dried under air. Each extract was reconstituted in 1 mL dilution buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.15 M NaCl, pH 7.0). Dilution buffer extracts were vortexed (5 s), sonicated (15 min or until completely in solution), and diluted 1:80 (androgens) and 1:30 (glucocorticoids) for clouded leopards. For the domestic cat, samples were diluted 1:800 (androgens, for clomipramine treatment alone), 1:200 (deslorelin treatment) or 1:64 (glucocorticoids) in dilution buffer. Samples were stored in polypropylene tubes at -20ºC until EIA analyses.

4.2.6 Androgen EIA

Androgen metabolite concentrations in fecal extracts were quantified with a single-antibody testosterone EIA, as recently detailed in our lab (Kersey et al., 2010). The antibody cross-reacted 100% with testosterone, 57.4% with dihydrotestosterone, 0.3% with androstenedione, 0.04% with androsterone and dehydroepiandrosterone, 0.03% with cholesterol, 0.02% with estradiol and <0.02% for all other tested analytes, including cortisol (Kersey et al., 2010). Polyclonal anti-testosterone (R156/R157; 1:7,500; supplied by C.J. Munro, University of California, Davis, CA) was added

(0.05 µl) to 96-well microtiter plates (Nunc-Immuno; Fisher Scientific, Pittsburgh, Pennsylvania), and allowed to incubate (12–18 h) at 4°C. Plates were washed (0.05% Tween 20 in 0.15 M NaCl solution) to remove unadsorbed antibody. Fecal extract was added in duplicate (0.05 mL) and standards in triplicate (0.05 mL; 0.05–12 ng/mL; 17β17b-hydroxy-4-androsten-3-one; Steraloids, Newport, Rhode Island), Testosterone horseradish peroxidase (HRP) (1:80,000; supplied by C.J. Munro) was then added to each well containing standard or sample and incubated (2 h; at room temperature) before unbound components were removed with wash solution. A substrate solution (0.1 mL) (0.04 M ABTS, 0.5 M H_2O_2 in a 0.05 M citric acid solution) was added to each well and allowed to incubate (~30 min) before optical densities (ODs) were determined. ODs were read using a microplate reader (MRX, Dynex Technologies, Chantilly, VA) at 405 nm when 0 pg standard wells reached an OD of 0.9–1.1. Intra-assay variation was < 10%, and inter-assay coefficients of variation (CVs) for 2 internal controls were 10.7% (mean binding, 23.9%) and 4.6% (mean binding, 68.87%) (n = 196 assays). Immunoreactivity of serially diluted fecal extracts paralleled standard binding.

4.2.7 Glucocorticoid EIA

A single-antibody cortisol EIA that cross-reacted 100% with cortisol, 9.9% with prednisolone, 6.3% with prednisone, 5% with cortisone, 0.7% with corticosterone, 0.5% with 21-deoxycortisone, 0.3% with desoxycorticosterone, 0.2% with desoxycortisol, 0.2% with progesterone, 0.2% with hydroxyprogesterone and 0.1% with androgens, including testosterone and androstenedione (Kersey et al., 2010), was used to analyze glucocorticoid metabolite concentrations in fecal samples.

Procedures and assays were the same as above unless otherwise noted. Microtiter plates adsorbed cortisol antibody (R4866, supplied by C. J. Munro) and were washed to remove unadsorbed antibody before adding duplicate fecal extract (0.05 mL) and triplicate cortisol standards (0.05 mL; range 0.08–20 ng/mL; 17-hydroxycorticosterone; Sigma- Aldrich). Plates were incubated at room temperature (1 h) after adding cortisol HRP (0.05 mL). Following incubation, unbound components were removed with a wash, and a substrate solution was added (0.1 mL) to all wells. When optimal OD (1.00) was reached, the resulting color change was quantified on the microtiter plate reader as described earlier. Intra-assay variation was <10%, and inter-assay CVs for 2 internal controls were 8.9% (mean binding, 28.4%) and 4.3% (mean binding, 68.8%) (n = 209 assays).

4.2.8 Statistical Analysis

After performing a Shapiro-Wilk test, all endocrine data were log transformed and divided into time periods that satisfied the study design and best captured treatment effects: deslorelin (pre-drug treatment, interim period for treatment to become effective, followed by repeated 90 day periods) and clomipramine (pre-drug treatment, interim period for treatment to become effective, followed by repeated 30 day periods for domestic cats; clouded leopards were grouped every 50 days during treatment and then every 30 days after treatment, for comparison to domestic cats). When a time period included only one cat (due to varying length of the study for domestic cats) the remaining observations were grouped into a single, final time period (longest range: days 639 – 847; domestic cats in deslorelin treatment group). For domestic cats, means across time and treatments were compared using mixed

model repeated measures analysis. When a significant ($P \leq 0.05$) F-statistic was measured it was followed by a least squares (LS) means comparison test. For clouded leopards, means were calculated, but statistical analysis was not performed due to the small sample size (n = 2 animals per treatment group).

For spermatozoa data, all percentage data were arcsine-transformed before evaluation. Data were divided into time periods that satisfied the study design and best captured treatment effects: deslorelin (pre-drug treatment followed by every six months) and clomipramine (pre-drug treatment and then monthly). For domestic cats means across time and treatments were compared using mixed model repeated measures analysis. When a significant ($P \leq 0.05$) F-statistic was measured it was followed by a least squares (LS) means comparison test. For clouded leopards, means were calculated, but statistical analysis was not performed due to the small sample size (n = 2 animals per treatment group). For domestic cats, a paired Student t-test was used to determine treatment effects on morphology by comparing the sperm collected pre-drug treatment and the first spermatozoa visible post-drug treatment.

All average values are presented as mean ± standard error of measurement (SEM). All analyses were performed using SAS 9.2 for Windows (SAS Institute Inc., Cary, NC). Significance was accepted at $P \leq 0.05$ level.

4.3 Results

4.3.1 Effects of Deslorelin

4.3.1.a Effect of Deslorelin on Fecal Androgens and Glucocorticoids

Deslorelin treatment had a significant ($P \leq 0.05$) longitudinal effect on fecal androgen concentrations (Figures 4.1a, Figure 4.2a). Within domestic cats, mean fecal androgens initially increased by ~ 45% over an 8-day period ($P \leq 0.05$) and then decreased ($P \leq 0.05$) by ~80% from the pre-drug mean. Mean androgen levels remained depressed relative to the pre-drug mean from day 9 to 368, increasing

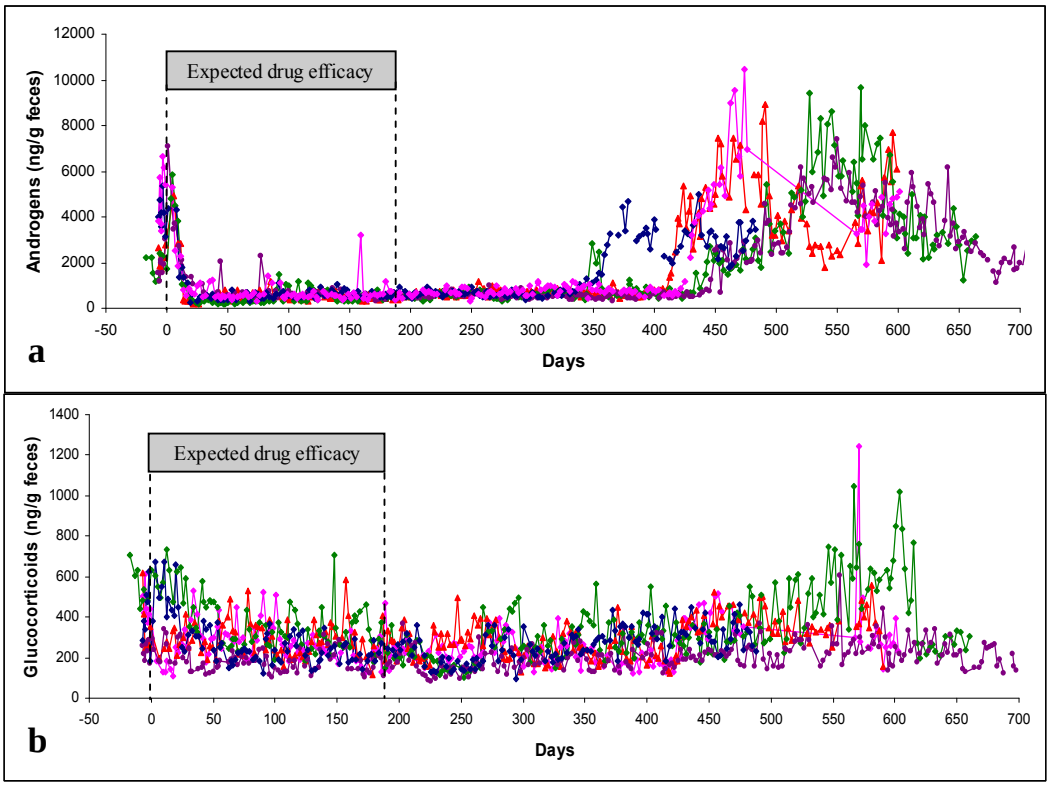


Figure 4.1 Endocrine profiles demonstrating the effect of deslorelin treatment on a) fecal androgens and b) fecal glucocorticoids in five male domestic cats. Day 0 indicates the day of implant insertion (treatment).

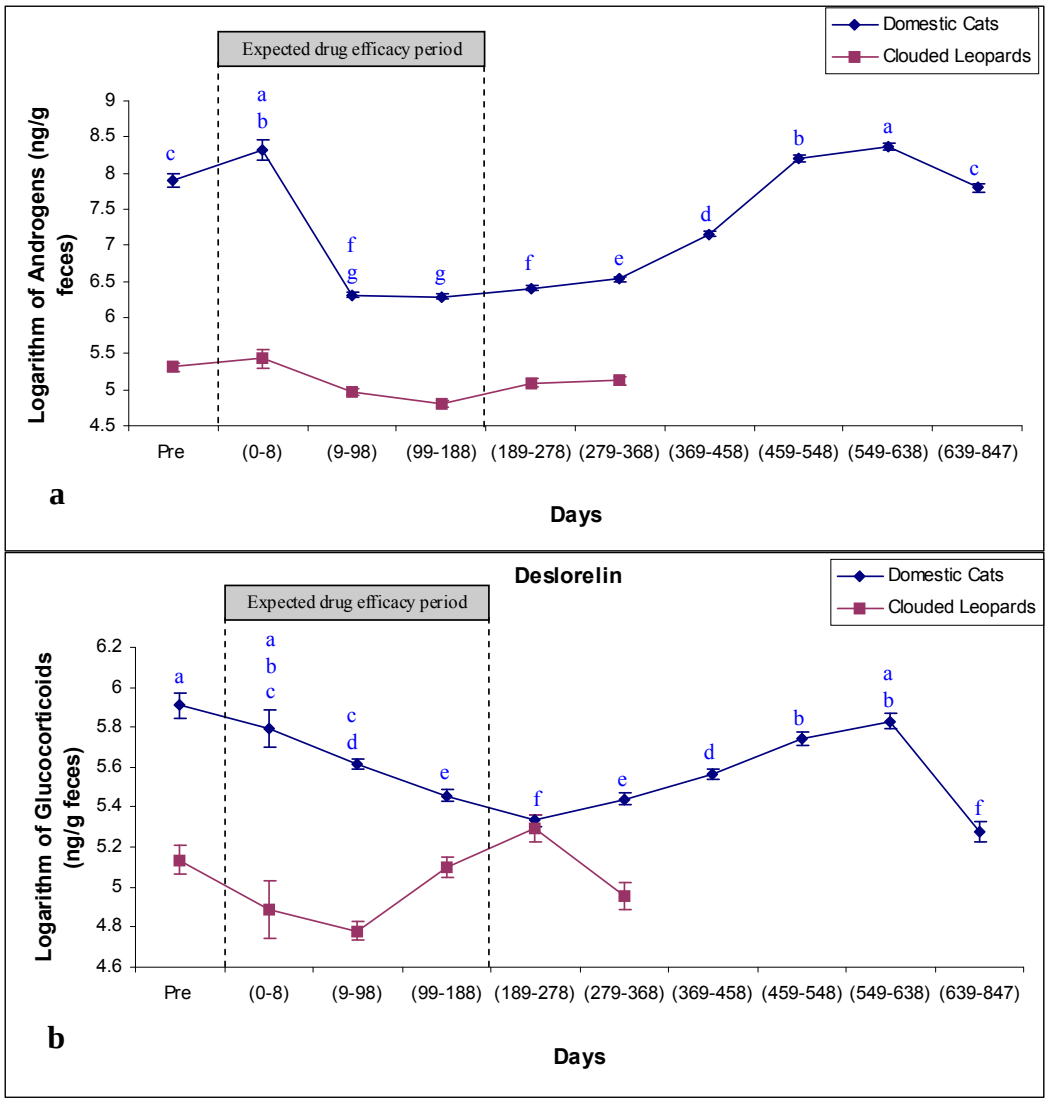


Figure 4.2 The effect of deslorelin treatment. a) fecal androgens and b) fecal glucocorticoids over time in male domestic cats and clouded leopards. Males were implanted on day 0; 'pre' indicates samples collected before treatment began. Error bars indicate standard error and for domestic cats are accompanied by multiple mean comparison test groups. While means for clouded leopards are presented for qualitative purposes, due to n = 2 statistical analysis was not performed.

($P \leq 0.05$) only slightly, at which point concentrations increased to an intermediate value ($P \leq 0.05$) between days 369 and 458, and then to elevated values ($P \leq 0.05$) ~ 45% greater than the pre-drug mean from day 459 to 638. Mean androgen levels then

decreased ($P \leq 0.05$), returning to pre-drug levels ($P > 0.05$) between days 639 to 847. Clouded leopard fecal androgens demonstrated a similar longitudinal pattern, but decreased by less than 40%, relative to pre-drug levels (Figure 4.2a, Figure A4.1).

Deslorelin treatment also had a significant ($P \leq 0.05$) longitudinal effect on fecal glucocorticoid concentrations (Figures 4.1b, Figure 4.2b). Within domestic cats mean glucocorticoids steadily decreased ($P \leq 0.05$) between days 0 and 278, with a maximum decrease of ~ 44%, after which mean concentrations increased steadily ($P \leq 0.05$) and returned to pre-drug treatment levels ($P > 0.05$) between days 549 and 638, before again decreasing ($P \leq 0.05$) between days 639 and 847 by ~48%. In clouded leopards, glucocorticoids showed more frequent fluctuations. Similar to the trend observed in domestic cats, mean levels initially decreased, with a maximum decrease of ~21%, but mean concentrations then increased by ~21% between 99 and 278 days, before again decreasing (Figure 4.2b, Figure A4.1).

4.3.1.b Effect of Deslorelin on Ejaculate Traits and Body Weight

Deslorelin treatment completely suppressed spermatogenesis in domestic cats (Table 4.1). Mean seminal volume and total sperm (Figure 4.3) decreased significantly ($P \leq 0.05$) in collections between 0 and 6 months after implantation and remained suppressed until 13 to 18 months after initial treatment. Similarly, mean % sperm motility, FPS, sperm concentration (spermatozoa/mL; Figure 4.3), and SMI (Figure 4.3) all decreased ($P \leq 0.05$) between months 0 and 18, before increasing ($P \leq 0.05$) between months 19 and 28, returning to pre-treatment levels ($P > 0.05$). The means for these traits were all equal to 0.0 (to within SEM) from 7 to 12 months (Figure 4.3). Testes volume also decreased between months 0 and 12 ($P \leq 0.05$).

Table 4.1 The effect of deslorelin treatment on ejaculate traits and body weight over time. Means ± standard errors are represented along with associated multiple mean comparison test groups for domestic cats. Within Rows, values with different superscripts differ significantly ($P \leq 0.05$). While means for clouded leopards are presented for qualitative purposes, due to n = 2, statistical analysis was not performed.

Sperm Traits	Pre Implant	0 - 6 Months	7 - 12 Months	13 - 18 Months	19 - 28 Months
Domestic Cats					
Arcsin Motility (%)	0.94 ± 0.14^{a}	$0.19 \pm 0.08^{b,c}$	0.00 ± 0.08^{c}	0.38 ± 0.10^{b}	0.88 ± 0.12^{a}
Forward Progressive Status	3.70 ± 0.56^{a}	$0.87 \pm 0.32^{b,c}$	0.00 ± 0.32^{c}	1.85 ± 0.39^{b}	3.75 ± 0.51^{a}
Concentration (10^6/mL)	237.48 ± 61.25^{a}	76.35 ± 35.36^{b}	0.02 ± 35.36^{b}	79.62 ± 43.31^{b}	$101.46 \pm 55.91^{a,b}$
Seminal Volume (mL)	0.10 ± 0.02^{a}	0.03 ± 0.01^{b}	0.01 ± 0.01^{b}	0.07 ± 0.01^{a}	0.09 ± 0.02^{a}
Total Sperm (10^6)	20.40 ± 4.95^{a}	4.25 ± 2.86^{b}	0.00 ± 2.86^{b}	8.48 ± 3.50^{ab}	9.13 ± 4.52^{ab}
Arcsin Sperm Motility Index	0.99 ± 0.14^{a}	$0.22 \pm 0.08^{b,c}$	0.00 ± 0.08^{c}	0.45 ± 0.10^{b}	0.97 ± 0.13^{a}
Testes Volume (mm^3)	3.00 ± 0.33^{a}	1.83 ± 0.19^{b}	1.69 ± 0.19^{b}	2.81 ± 0.23^{a}	3.35 ± 0.30^{a}
Normal Morphology (%)	41.80 ± 11.34^{a}	29.00 ± 12.68^{a}	N/A	19.20 ± 11.34^{a}	27.67 ± 10.35^{a}
Weight (kg)	4.43 ± 0.31^{c}	5.60 ± 0.18^{a}	5.90 ± 0.18^{a}	$5.44 \pm 0.22^{a,b}$	$4.78 \pm 0.28^{b,c}$
Clouded Leopards					
Arcsin Motility (%)	0.97 ± 0.10	0.91 ± 0.10	0.79 ± 0.10	-	-
Forward Progressive Status	3.25 ± 0.25	3.25 ± 0.25	3.25 ± 0.25	-	-
Concentration (10^6/mL)	27.25 ± 15.39	20.75 ± 15.39	26.75 ± 15.39	-	-
Seminal Volume (mL)	1.01 ± 0.25	0.47 ± 0.25	0.46 ± 0.25	-	-
Total Sperm (10^6)	32.84 ± 15.06	8.17 ± 15.06	12.00 ± 15.06	-	-
Arcsin Sperm Motility Index	0.95 ± 0.07	0.93 ± 0.07	0.86 ± 0.07	-	-
Testes Volume (mm^3)	10.01 ± 1.17	8.12 ± 1.17	9.34 ± 1.17	-	-
Normal Morphology (%)	17.00 ± 5.17	21.00 ± 5.17	8.50 ± 5.17	-	-
Weight (kg)	20.70 ± 0.47	21.90 ± 0.47	22.80 ± 0.47	-	-

Deslorelin treatment did not have a longitudinal effect ($P > 0.05$) on the mean proportion of normal spermatozoa observed in domestic cats (Table 4.1). In clouded leopards, mean % sperm motility, semen volume, and SMI all appeared to decrease incrementally between each time period. When changes were examined in the two males individually, SMI appeared to increase between 0 and 6 months in one male and decrease in the other (Figure 4.4). Meanwhile, mean sperm concentration per mL of ejaculate (Figure 4.4), total number of sperm (Figure 4.4), and testes volume also

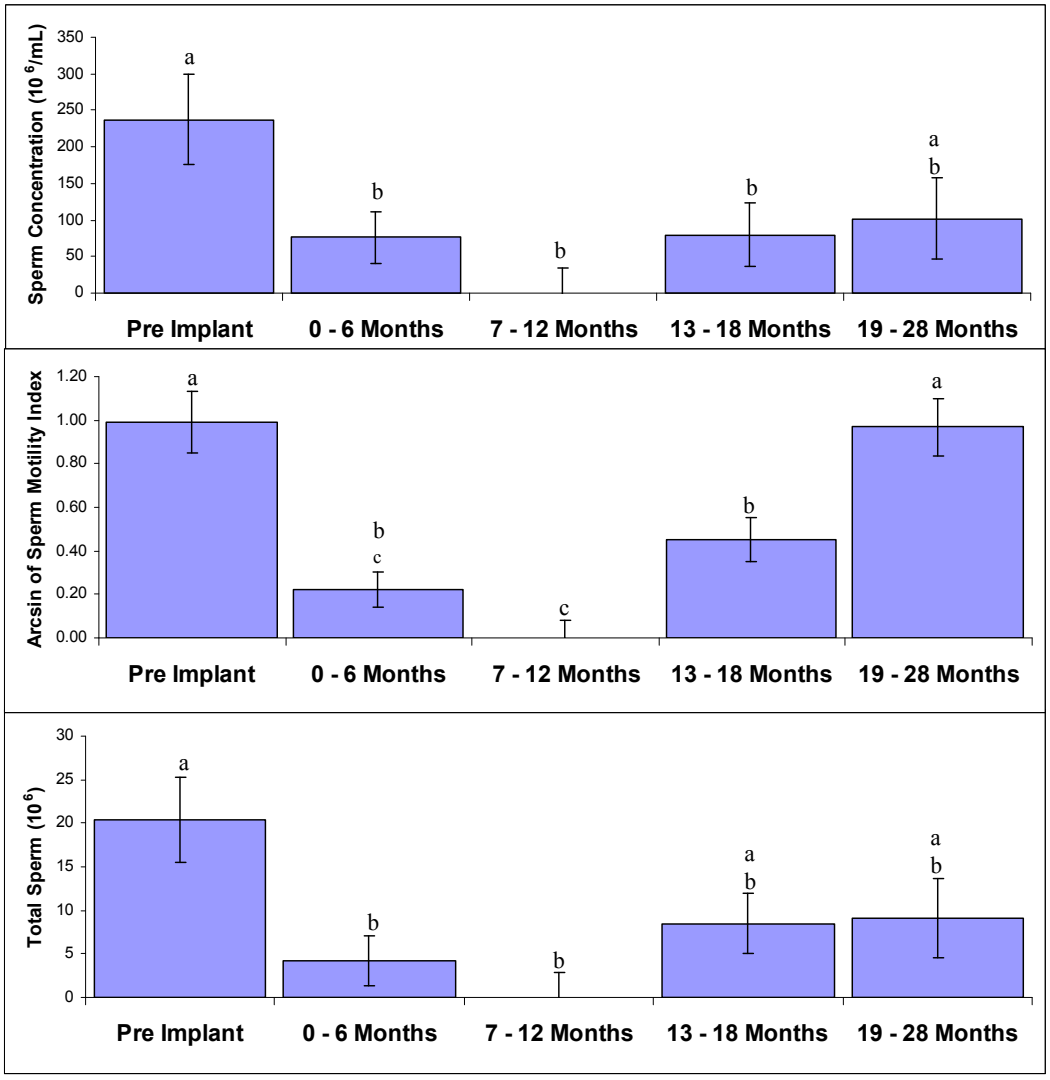


Figure 4.3 Effect of deslorelin treatment on ejaculate traits in male domestic cats over time. 'Pre implant' indicates the collection before drug treatment began. Error bars indicate standard error and are accompanied by multiple mean comparison test groups.

tended to decrease between 0 and 6 months and then increase between 7 and 12 months. There was no change in FPS. However, the proportion of spermatozoa with normal morphology appeared to increase marginally between 0 and 6 months and then decrease between 7 and 12 (Table 4.1). Mean domestic cat body weight increased ($P \leq 0.05$) between 0 and 18 months, and returned to pre-drug treatment

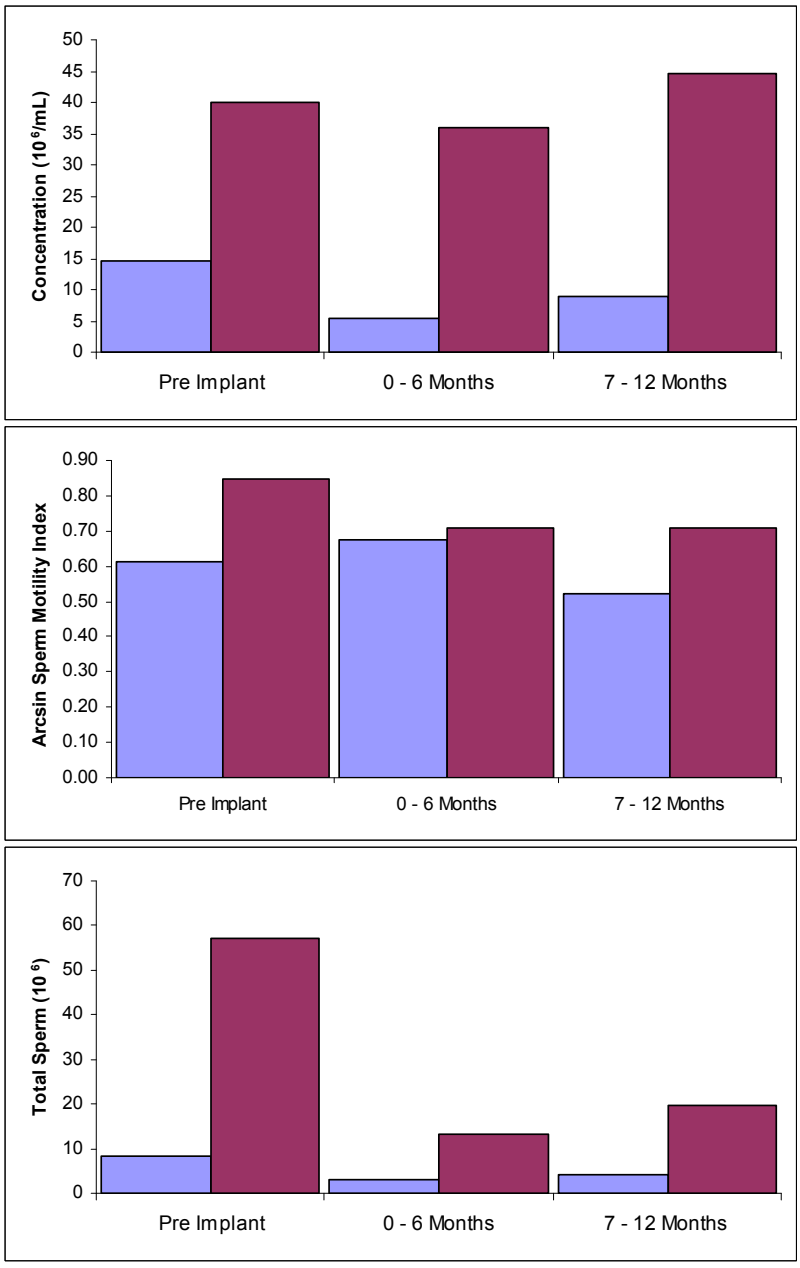


Figure 4.4 Effect of deslorelin treatment on sperm traits in two male clouded leopards over time. 'Pre implant' indicates the collection before drug treatment began.

levels ($P > 0.05$) between months 19 and 28. Mean clouded leopard weights also appeared to increase incrementally from 0 to 12 months (Table 4.1).

Finally, after the resumption of spermatogenesis, there was a significant increase in the mean proportion of spermatozoa with abnormal acrosomes ($P = 0.04$)

and proximal cytoplasmic droplets ($P = 0.03$) in domestic cats treated with deslorelin. There was no change ($P > 0.05$) in the proportion of spermatozoa with macrocephalic, microcephalic, bicephalic, abnormal midpiece, tightly coiled tail, spermatid, bent midpiece with cytoplasmic droplet, bent midpiece without cytoplasmic droplet, bent tail with cytoplasmic droplet, bent tail without cytoplasmic droplet, distal cytoplasmic droplet, or bent neck (Figure A4.2, Table A4.1).

4.3.1.c Parameters of Suppression

In the domestic cat, full suppression of androgens was accomplished in 20.8 ± 3 days after deslorelin treatment. Gonadal function was suppressed, including spermatogenesis, for an average of 525.2 ± 17 days, and androgen concentrations for an average of 396 ± 15 days. Once androgen concentrations reached pre-drug treatment levels, it required an average of 108.2 ± 14 days for spermatogenesis to resume (Table 4.2). These data are not available in clouded leopards because deslorelin was unable to effect complete suppression of gonadal function.

Table 4.2 Summary of the effect of deslorelin treatment on androgen concentrations and spermatozoa in the domestic cat over time. Means ± standard errors are represented.

Events	**Average (days)**	**Range (days)**
Implant to significant decrease in androgens	20.8 ± 3	14 - 32
Androgens suppressed	396.2 ± 15	340 - 418
Interval between implant and spermic ejaculate	525.2 ± 17	480 - 552
Interval between androgen increase and spermic ejaculate	108.2 ± 14	55 - 135
Interval between implant and sperm over $100x10^6$	638.2 ± 63	488 - 852
Interval between androgen increase and sperm over $100x10^6$	220.6 ± 53	119 - 402

4.3.2 Effect of Clomipramine on Fecal Androgens, Glucocorticoids, Ejaculate Traits and Body Weight

In regards to the domestic cat, there were significant ($P \leq 0.05$) mean longitudinal changes in both fecal androgen and glucocorticoid concentrations with clomipramine treatment. Mean fecal androgen concentrations did not change from pre-drug treatment until between 90 and 120 days of drug treatment, at which point they increased ($P \leq 0.05$) and remained elevated ($P \leq 0.05$) for 120 days (90 days following the end of drug treatment), with a maximum increase of ~65%, before decreasing ($P \leq 0.05$) to pre-drug treatment levels (Figure 4.5a and Figure 4.6a).

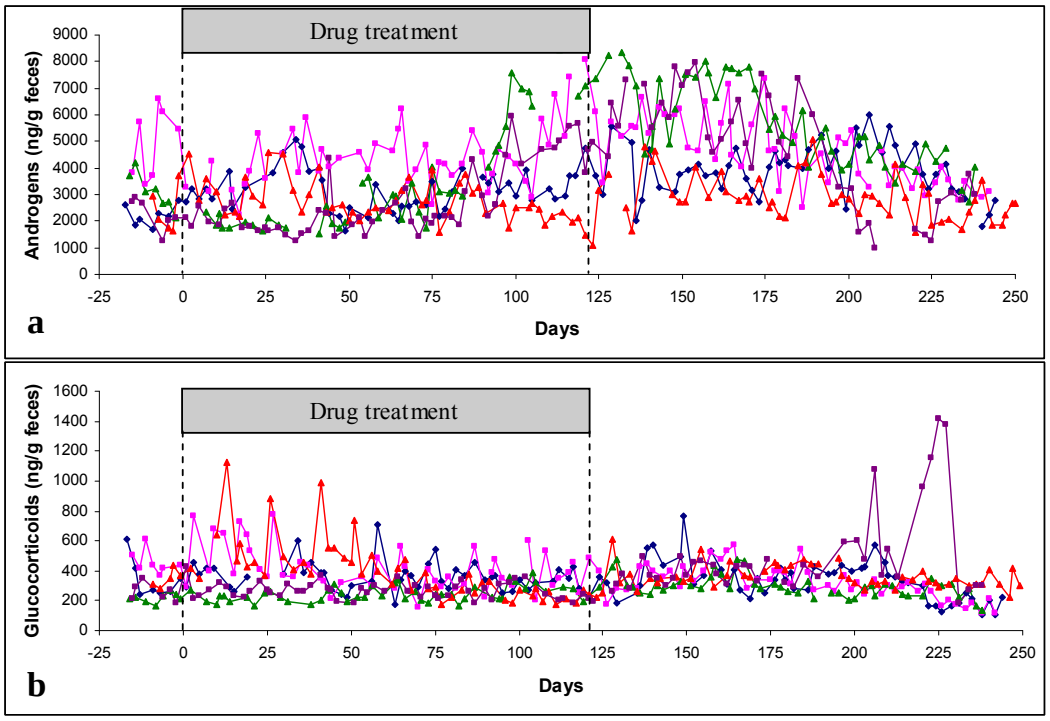


Figure 4.5 Endocrine profiles demonstrating the effect of clomipramine treatment a) fecal androgens and b) fecal glucocorticoids in five male domestic cats. Clomipramine was administered from day 0 to day 120.

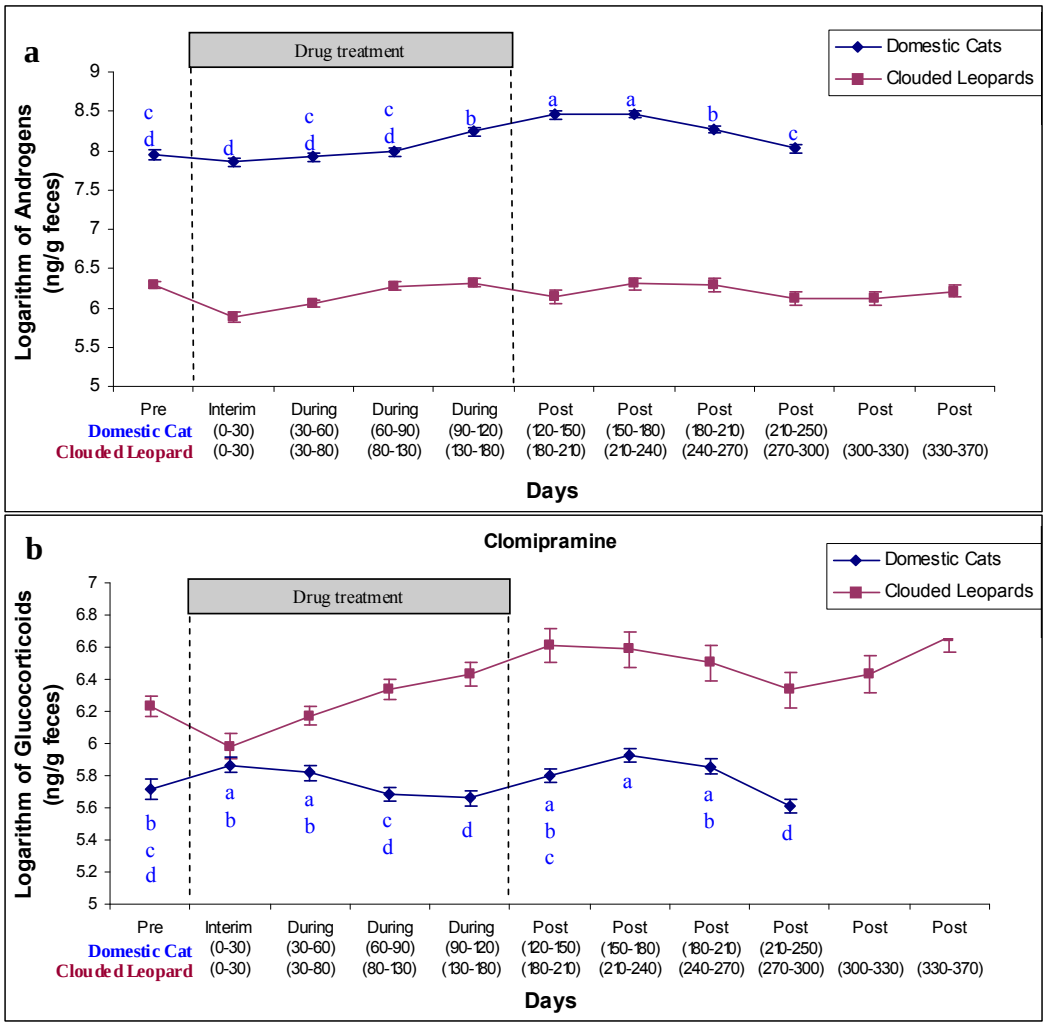


Figure 4.6 The effect of clomipramine treatment on a) fecal androgens and b) fecal glucocorticoids over time in male domestic cats and clouded leopards. Males were treated starting on day 0; 'pre' indicates samples collected before treatment began; 'interim' indicates samples that were collected while the drug took effect; 'during' indicates samples that were collected during treatment; 'post' indicates samples that were collected after drug treatment ended. Error bars indicate standard error and for domestic cats are accompanied by multiple mean comparison test groups. While means for clouded leopards are presented for qualitative purposes, due to n = 2 statistical analysis was not performed.

With regards to mean fecal glucocorticoid concentrations, data collected between days 0 and 60 showed no significant difference relative to the pre-drug treatment concentrations. While there were significant temporal fluctuations during the drug treatment period, none of these means varied significantly from the pre-treatment mean. There was a decrease in glucocorticoids ($P \leq 0.05$) between the 30 to 60 day time period and the 60 to 90 day period. There was a rise ($P \leq 0.05$) in mean glucocorticoid concentrations between days 120 to 210 by ~20% relative to pre-drug treatment concentrations. Finally, glucocorticoids decreased ($P \leq 0.05$) between 210 and 250 days to pre-drug treatment concentrations (Figure 4.5b and Figure 4.6b). In the clouded leopards, longitudinal patterns differed from those seen in domestic cats (Figure 4.6a and b, Figure A4.1). In regards to all sperm traits and morphology described above, clomipramine treatment had no effect (data not shown).

4.4 Discussion

4.4.1 Summary

For the first time, the duration of deslorelin's down-regulation of gonadal hormones (340 to 418 days) and testicular function (480 to 552 days) in the domestic cat was confirmed. All aspects of male reproductive function were either suppressed or completely ceased. Conversely, while some parameters were affected by drug treatment in male clouded leopards, spermatogenesis never ceased. Androgen concentrations were suppressed, but not to the degree seen in the domestic cat. Clomipramine treatment had no negative effect on reproductive function in either species. Due to the small sample size in clouded leopards and the current findings in

the domestic cat (a model for endangered felids), the drug effect seen in domestic cats should be considered when interpreting clouded leopard results. As such, since both drugs were effective at controlling aggression in male clouded leopards (Chapter 3), clomipramine may be the preferred option for mitigating aggression and forming breeding pairs.

4.4.2 Deslorelin Treatment

4.4.2.a Effect on Androgen Levels

The GnRH agonist deslorelin is commonly known to cause an initial 7- to 14-day increase in testosterone concentrations, known as the "flare effect", due to the agonist's stimulation of the pituitary (Herbst, 2003; Melville et al., 2012). This occurs before causing a down-regulation of GnRH receptors and effectively shutting down the HPG axis, including testosterone production (Herbst, 2003). This pattern was observed in the domestic cat, with the "flare effect" occurring within 8 days of deslorelin treatment. An associated increase in aggression would be expected due to the "flare effect," and in fact aggression in a clouded leopard, lasting approximately two weeks, was reported by one institution. When considering deslorelin treatment for the formation of breeding pairs it would be important to wait until after the "flare effect" and to carefully observe a pair around the time androgen levels begin to rise (range: 340 to 418 days post treatment).

4.4.2.b Effect on Glucocorticoid Levels

A parallel decrease in glucocorticoid concentrations resulting from deslorelin drug treatment was observed in the domestic cats and less conclusively in the clouded leopards. While a decrease in glucocorticoid levels was also observed in domestic

cats during the final time period (between 639 and 847 days), this time period included only two animals and one of these males had the lowest glucocorticoid concentrations relative to other males, likely skewing the mean for this time period. In clouded leopards, glucocorticoid concentrations initially decreased with drug treatment, but then increased 180 days earlier than their domestic cat counterparts. Due to the limited number of clouded leopards examined in the present study, it is difficult to determine if the initial decrease was in fact related to a drug effect. However, after reviewing institutional daily animal records the increase between days 99 and 278 appears to be related to nearby construction, which has been previously correlated with increased glucocorticoids in carnivores (Young et al., 2004). A similar parallel relationship between glucocorticoids and androgens, as that observed in domestic cats, was recently observed in clouded leopards (Chapter 3). The HPG and HPA axes are linked and have been shown to fluctuate together in a variety of manners. The two hormones generally vary inversely, with glucocorticoids known to suppress the HPG axis and androgens to suppress the HPA axis (Viau, 2002; Seale et al., 2004; Hardy et al., 2005). However, they do fluctuate together in a seasonal pattern (Kersey et al., 2010) and can increase together as a result of acute stress and adrenal production of both glucocorticoids and the androgen dehydroepiandrosterone (DHEA) (Newman et al., 2008), and it was recently shown that a decrease in androgens can lead to decreases in associated stress and anxiety, thereby decreasing glucocorticoid levels (Chapter 3).

4.4.2.c Effect on Ejaculate Traits and Body Weight

As expected from prior studies (Bertschinger et al., 2001; Goericke-Pesch et al., 2009; Melville et al., 2012), treatment with the GnRH agonist deslorelin caused complete suppression of spermatogenesis in domestic cats. The length of suppression is known to vary by species, and until now had not been measured in domestic cats. Spermatogenesis abated from month 7 to month 12, post-drug treatment. The suppression was evident in decreased spermatozoa concentration and seminal volume, which resulted in a sharp decline in total sperm, motility, FPS, and SMI values. Testes volume, an indicator of seminiferous activity, also decreased significantly. If deslorelin were to be used as a tool for decreasing aggression while forming breeding pairs, the re-establishment of spermatogenesis would be necessary (up to 552 days post-drug treatment) before successful breeding could occur. However, due to the longer than expected (~1.5 years rather than 6 months) and complete suppression observed in domestic cats, deslorelin may be a useful contraceptive in feral cats, for example.

Reproductive function in clouded leopards, compared to domestic cats, was only partially down-regulated, with decreases in several sperm traits (% sperm motility, seminal volume, and total sperm) and lowered androgen (~40%) production. The dosage of deslorelin administered to clouded leopards was 0.68 mg/kg, while the dosage for domestic cats was 1.00-1.21 mg/kg. This may suggest that the dosage in clouded leopards was too low. However, previous research in other species has demonstrated effective treatment at lower dosages, proportional to body weight. An average dosage of 0.13 mg/kg was determined to be effective for suppression of

endocrine and germinal testicular function in cheetahs (Bertschinger et al., 2006) and a dosage as low as 0.11 mg/kg was effective in domestic dogs (Trigg et al., 2006). Additionally, ovulation in female clouded leopards has been stimulated with the same exogenous gonadotropin dose per animal used in the domestic cat (Howard et al., 1996; Howard et al., 1997), a species approximately 20% smaller in size, indicating that clouded leopards may actually be among the most sensitive species to the effects of exogenous hormones. Therefore, based on weight this dosage should have been effective for male clouded leopards and the lack of effectiveness suggests species-specific differences in responsiveness to deslorelin treatment. Such differences have previously been observed, with deslorelin reported to be completely ineffective in the wallaby and antelope (Penfold et al., 2002; Herbert et al., 2004).

In one male clouded leopard, spermatogenesis ceased at 12 months post-deslorelin treatment and remained suppressed until collections were discontinued due to health reasons at 24 months post-drug treatment. This male was 16 years old at 12 months post-drug treatment and 17 years old at 24 months post-drug treatment. Data is limited, but according to the Smithsonian Institution's database, at the time of necropsy a 16-year-old male was still producing sperm, while both a 17 and a 19 year-old male exhibited no spermatogenic activity in their testes. Furthermore, according to AZA Studbook records, the oldest male to sire offspring was 15 years of age. Therefore, we speculate that the male in the current study may have prematurely crossed a physiological age threshold due to drug treatment. This male continued to produce androgens at the time semen collections stopped, 24 months post-drug treatment. Similar results have been found in older ferrets and hamsters where sperm

viability and Leydig cell numbers decreased, but testosterone concentrations remained unaffected (Horn et al., 1996; Wolf et al., 2000). One explanation for a decrease in spermatogenesis concurrent with sustained androgen production would be a testicular tumor such as a seminoma, which are common in aging domestic dogs, for example (Peters et al., 2000; Peters et al., 2000).

It is commonly known that castration leads to muscle loss, adipose tissue gain, and overall weight gain (Kriegsfeld and Nelson, 1996; Kanchuk et al., 2003; Martin et al., 2006). Therefore, it is not surprising that in both domestic cats and clouded leopards there was an increase in weight while testosterone levels were suppressed. Also, similar to a previous report in the domestic dog (Trigg et al., 2006), with the exception of one domestic cat, there was an inverse relationship between the length of drug effectiveness and initial body weight.

4.4.2.d Effect on Sperm Morphology

An increased number of spermatozoa with proximal cytoplasmic droplets and abnormal acrosomes were observed immediately after spermatogenesis resumed in domestic cats. Sperm defects are quite common in domestic cats and clouded leopards, with proximal cytoplasmic droplets and abnormal acrosomes being two of the more common abnormalities (Pukazhenthi et al., 2006). The presence of a proximal cytoplasmic droplet may indicate impaired spermiation, (Neubauer et al., 2004; O'Donnell et al., 2006) and acrosome deficiencies tend to occur during spermatogenesis and spermiogenesis (Pukazhenthi et al., 2006), processes that are both testosterone dependent (O'Donnell et al., 2006). Furthermore, research on bulls demonstrates that proximal droplets and knobbed acrosomes are some of the last

abnormalities to decrease as testosterone levels increase post-puberty (Evans et al., 1995). In the domestic cat spermatogenesis takes 46.8 days to complete a full cycle (Franca and Godinho, 2003). Therefore, it is highly likely that when the presence of spermatozoa was first detected post-drug treatment – in one case just 55 days after androgen concentrations increased – spermatogenesis had not yet returned to full function and so sperm cells had a higher incidence of abnormalities.

4.4.3 Clomipramine Treatment – Effect on Androgens, Glucocorticoids, and Ejaculate Traits

Clomipramine treatment did not appear to have an effect on fecal androgen concentrations, glucocorticoid concentrations, or sperm traits in either the domestic cat or clouded leopard. It was initially hypothesized that by blocking the serotonin transporter and increasing serotonin levels, anxiety and circulating glucocorticoids would decrease, thereby improving reproductive function. Increases in both androgen and glucocorticoid levels were observed in clomipramine-treated domestic cats. The timing of the rise in androgen levels, shortly before the end of clomipramine treatment, makes it an unlikely result of drug treatment. However, it is possible that the increase in androgen levels was a latent response. Studies have shown that stressors have the effect of suppressing testicular steroidogenesis in rats, baboons, and humans (Ferin, 2006). Therefore, clomipramine may have the converse effect, increasing androgen levels. It is unlikely that the increase in glucocorticoid levels, 30 days after the end of clomipramine treatment, was due to a drug effect. However, at one zoological institution a male clouded leopard was reported to be uncharacteristically aggressive (e.g. hissing and growling at people) approximately 14

days post-clomipramine treatment. This increased aggression could be explained by an increase in glucocorticoid concentrations, but further investigation is warranted.

4.5 Conclusions

This study demonstrated for the first time the duration and effectiveness of deslorelin treatment on male reproductive function in the clouded leopard and domestic cat (a model for felid species). Deslorelin temporarily suppressed endocrine and testicular function in the domestic cat, but not in the clouded leopard. Conversely, clomipramine treatment appeared to have no negative effects on reproductive function in either species. Both drugs are effective at suppressing anxious and aggressive behaviors in male clouded leopards, making clomipramine a more appealing option for aiding in the formation of genetically valuable breeding pairs.

Chapter 5: Conclusions

5.1 Overview

The preceding work presents three studies related to the characterization and modulation of aggression in male clouded leopards. Ultimately, the goal of this work has been to produce important tools for the management of a self-sustaining *ex situ* clouded leopard population, which acts as a hedge against extinction in the wild. Part of the difficulty in managing this species in captivity stems from the limited knowledge of clouded leopard behavior in the wild, as described in the Introduction. To conclude, I provide below a brief review on aggression and animal domestication from an evolutionary perspective, and speculate how these two subjects impact how the lessons from this study are applied. Finally, I provide a summary of key findings and comment on future work regarding the control of aggression in male clouded leopards.

5.2 Aggression and This Study from an Evolutionary Perspective

Previous research in clouded leopards and other carnivore species indicates two potential causes of male aggression. Defensive aggression tends to include hissing, growling, and increased heart rate in defense of a perceived threat, and is stimulated by the HPA axis, where an increase in glucocorticoids is correlated with an increase in aggression and an increase in serotonin correlates with inhibited aggressive behavior. Meanwhile, offensive or predatory aggression is seen as an

attack on a prey or other individual. This type of aggression appears linked to testosterone (Wingfield et al., 2001; Albert et al., 2008).

Genetic studies have determined aggression to be a quantitative trait (linked to multiple genes) that would cause a continuum of behavior patterns. In rats, two strains (tame and aggressive) were selected for over 60 generations. The lines were then backcrossed and the classic bell-shaped curve (tame, moderate, and aggressive) emerged in the F2 generation, indicating that aggression is a quantitative trait. Three specific genes were discovered that control aggression - two affected adrenal size and one led to white markings on the pelt (detracts from camouflage) (Albert et al., 2009). In a similar study involving the selection of aggressive male lines of *Drosophila melanogaster*, it appears that there may be 19 different genes controlling aggression (Edwards et al., 2006). Due to the small sample size, the clouded leopards in this study were ultimately divided into 'calm' versus 'anxious' categories, but initial results did fall along a behavioral continuum from 'calm' to 'anxious', in agreement with previous research.

Aggression in clouded leopards is prominent and appears to be driven by both anxiety and circulating testosterone. It is important to consider the reason behind and purpose for the aggression. Is there a need for male aggression in the wild? Is this a trait that aids in survival? With an ultimate goal of zoo populations serving to re-populate wild populations that have gone extinct, it is extremely important that captive management decisions not affect the genetic make-up of species so as to render them maladapted to their natural environment.

The captive US clouded leopard population is inbred, with a founder equivalent of 2.57 individuals (Fletchall, 2007). Given this low number, it is possible that aggression is the result of a founder effect – the population has been influenced by a few extremely aggressive individuals while varying strains of less aggressive animals may have been lost, leading to an abnormally aggressive population. It would be extremely difficult to test this due to the limited information on clouded leopards in the wild. However, knowing that male-female, female-female, and even male-male home ranges overlap, there is some indication that aggression may not be as prominent in the wild.

Anxiety-driven aggression (defensive) may be amplified by captive conditions, and aggressive captive animals released into the wild may not demonstrate the same aggressive tendencies. This theory is supported by research demonstrating that clouded leopards have relatively high glucocorticoid levels in captivity compared to other felids (Wildt et al., 1986) and that changing captive management practices such as increasing enclosure height, having fewer keepers caring for an individual, and not housing animals near predators can alleviate some anxiety and decrease glucocorticoid levels (Wielebnowski et al., 2002). Through management practices, and now through administration of clomipramine, more extreme cases of aggression can be mitigated.

However, as this study demonstrates, testosterone driven territorial aggression is also prominent in male clouded leopards, and is thus likely important for survival in the wild. The following brief review of the literature on domestication

demonstrates that it is important to neither intentionally nor unintentionally select for calm individuals.

There are three types of selection: natural selection to the wild, natural selection to captivity, and artificial selection to captivity (Richter, 1952). Natural selection in the wild is necessary for a species to survive in a constantly changing ecological environment, limited natural selection to captivity is unavoidable (but should be limited wherever possible), and artificial selection must be avoided.

Domestication of the dog is an example of artificial selection that may have begun as many as 100,000 years ago, but occurred most intensely over the past 8,500 years (Diamond, 2002). Initially, wolves that lacked an intense fear of humans likely spent time around human camps and stole scraps of food. Over time this fear decreased until the domestic dog became tame – tameness was selected for over aggression. Interestingly, with tameness came a host of other phenotypic and physiological traits such as floppy ears, white spots, decreased adrenal gland size, and year-round, rather than seasonal, breeding (Trut et al., 2009).

An important experiment that started 60 years ago, called the fox farm experiment, set out to duplicate the domestication of dogs with the Silver Fox (*Vulpes vulpes*) (Trut et al., 2009). Two lines of fox have been selected for over many generations, one completely domesticated and one extremely aggressive. With the domestic line of fox came the same visual traits mentioned above: floppy ears and white spots. Interestingly, the domestic foxes also have smaller adrenal glands, decreased glucocorticoid production, increased serotonin production, and the ability to breed year-round. The adrenal glands are important for maintaining an animal's

homeostasis and preparing an animal to face threats. With a chronic threat, glucocorticoids levels rise and this is correlated with a rise in aggression; such aggression is necessary for an animal's basic survival, enabling it to defend its territory, mate, and food resources. However, in captivity this response is less important and can become muted, making the adrenal gland less important and ultimately allowing for shrinkage. These same factors lead to increases in serotonin concentration, which is known to decrease defensive aggression. Finally, without the costly drain of the adrenal system on metabolic resources, the reproductive system has amplified, allowing the foxes to breed year-round.

Similar findings have been demonstrated in rats and guinea pigs. In rats two strains were selected for – tameness and aggression (Richter, 1952). Here again the tame rats had decreased adrenal size and when adrenalectomized they could survive with just a salt supplement, unlike the more wild/aggressive counterparts that could not survive without an adrenal gland. Similar studies have been conducted in guinea pigs, where an important observation was made: there are behavioral shifts in 'domestic' lines in response to thresholds compared to 'wild' lines, but behaviors were never fully extinguished, nor were completely new behaviors learned; animals simply modified known behaviors to fit new conditions (Kunzl and Sachser, 1999).

This final point is an important one when considering the adaptations of male clouded leopards to a captive environment. If they are like the guinea pigs, important behaviors may become muted, but it is unlikely they will ever be extinguished or that new maladaptive behaviors will be learned, even if more extreme artificial selection were performed. Regardless, natural selection to a captive environment could still be

devastating to the survival of a species or individual released from captivity into the wild. Over time, captive zoo animals (like the wolf) may become tamer as they adjust to living in close proximity to humans and the aggressiveness needed for survival may become muted. This decrease in alertness, defensiveness, and fear of the unknown could be detrimental in the wild. However, previous research suggests that adaptation to captivity may not be detrimental to captive species re-introduced to the wild. For example, while Golden Lion Tamarins reintroduced into the jungles of Brazil were unable to adapt to the novel environment, their offspring were able to thrive (J. Dietz, personal communication). This indicates that despite the likelihood of male clouded leopards naturally adapting to a captive environment, if reintroduced to the wild the likelihood is high that they may re-establish a viable population.

In conclusion, aggressive behavior may be vital for clouded leopard survival in the wild and should be maintained in captivity. It is important that 'calm' males not be selected for over aggressive males, potentially leading to a calm line of male clouded leopards. The current practice is to form breeding pairs with males that have not yet reached sexual maturity, and so pairs are selected based on age and genetic compatibility. However, highly aggressive males rarely breed, which could lead to decreased aggression in the population. Therefore, the results of this study offer an additional pairing solution that might be particularly effective for the more aggressive males. The majority of aggression is demonstrated early in a male-female pairing, so by controlling aggression with either clomipramine or deslorelin, aggression will be muted temporarily, allowing for an introduction to occur without permanently

altering what might be an important trait in the long-term survival of clouded leopards.

5.3 Summary of Findings

Male aggression in the endangered clouded leopard is a serious problem, one that prevents critical male-female pairings due to often fatal attacks of males on females. It is unlikely that this behavior occurs in the wild, where solitary animals likely cross paths on only a limited basis and remain in proximity for extended periods of time only when the female is in estrous. However, captivity puts unique pressures on animals which may elicit unique behavioral syndromes such as the extreme aggression seen in male clouded leopards.

Given our limited general knowledge of clouded leopards and the general understanding that aggression may be important for the overall survival of the species, a novel mechanism for mitigating aggression was sought. Previous research in felids has indicated two possible causes of male aggression: defensive (related to anxiety) and offensive (related to territorial) aggression. With the ultimate goal of forming breeding pairs to propagate the captive population meant to serve as a hedge against species extinction – and knowing that aggression might be critical for the survival of potentially reintroduced individuals – pharmaceutical treatments to mitigate aggression were sought with mechanisms that target both types of male aggression. In order to fully assess the effectiveness of each pharmaceutical mechanism, it was necessary to 1) identify a behavior reaction test or tests that allowed us to assess male behavior and temperament, 2) find a drug treatment

effective at controlling male anxiety and aggression, and 3) assess the effect of each drug treatment on reproductive function. As discussed below, the results of this study suggest two potential mechanisms for mitigating aggression in male clouded leopards.

5.3.1 Behavior Reaction Tests

The first major goal of this study was to determine if any of the three behavior reaction tests – airhorn test, MIS test, and UFP test – were effective for differentiating between 'calm' versus 'anxious' clouded leopards and for determining the effects of clomipramine and deslorelin treatments on stimulated behaviors. For differentiating between 'calm' versus 'anxious' males, the MIS test was the most effective. During the MIS test rates and frequencies of several behaviors – 'overall activity', 'growling', 'tail flicking', and 'standing' – increased significantly compared to the pre- and post-behavior test data. Furthermore, several behaviors elicited during the MIS test correlated positively with fecal glucocorticoid levels and keeper survey assessments of animal temperament. For assessing treatment efficacy on aggressive behaviors, both the MIS test and UFP tests were effective. The tests elicited increased rates of 'overall activity', 'growling', 'tail flicking' and 'standing,' behaviors, which were then significantly suppressed by both clomipramine and deslorelin treatment. Moreover, the behaviors elicited by these tests (with the exception of 'standing') were indicative of anxiety and aggression and were not behaviors readily demonstrated without provocation.

Unlike in domestic animals, standard temperament assessment tests have not yet been developed for zoo animals. Currently available tools are time-consuming

and often expensive. This is one of the first studies to investigate and demonstrate the effectiveness of such behavior reaction tests. Simple behavior reaction tests such as those used in this study could potentially be applied in a number of other zoo species as a general test of anxiety, to aid in the daily management of the species and in overall management recommendations.

5.3.2 Clomipramine versus Deslorelin

The second major goal of this study was to determine the effectiveness of clomipramine, a tricyclic antidepressant, and deslorelin, a GnRH agonist, at changing the rates and frequencies of anxious and aggressive behaviors in male clouded leopards. It was determined that both drug treatments were effective, indicating that more than one mechanism is responsible for modulation of aggressive behavior in the male clouded leopard. Furthermore, decreases in activity (decreased 'overall activity', increased 'lying down') and hiding associated with clomipramine treatment indicates that serotonergic neural pathways modulate anxiety in this species. It was originally hypothesized that increased serotonin levels would decrease glucocorticoid levels, thereby leading to a decrease in anxious behaviors. However, there was no significant change in circulating glucocorticoid levels as a result of clomipramine treatment. This suggests a direct impact of the neural serotonergic system in modulating anxious behaviors. Deslorelin, on the other hand, resulted in a significant decrease in both androgen and glucocorticoid levels, and also a decrease in more behaviors associated with aggression, such as 'swatting' a mirror, 'growling', and 'tail flicking'. Overall, it is encouraging that both pharmaceutical treatments were effective in mitigating anxious and aggressive behaviors in male clouded leopards,

suggesting that pharmacological therapeutics might be a viable management approach to form reproductive pairings.

5.3.3 Keeper Statements about Drug Treatment

Following drug treatment, all keepers were asked to complete a follow-up survey. This data was ultimately not analyzed because it was not readily comparable to the first survey. For anecdotal purposes, all keeper statements related to drug treatments are included below. Such comments were an optional field, so not all cats are represented while others are represented more than once. Overall, keepers for six of the cats provided commentary, providing an anecdotal view of the day-to-day effects of drug treatment. Spelling and typographical errors have been corrected for readability, and names have been replaced with 'male'.

Clomipramine

> **Zoo 1, Keeper 1:** Many positives on Clomicalm for exhibit purposes. Took the edge off. More visible, less anxious about going out. Just all around, totally mellow cat. Downside, no stress burning calories. Really started to pack on the pounds. Cut his diet by 50% and he did not react. As far as being hungry. But if something startled him he would still react very quickly from an "assumed" relaxed state. So the edge was still there just not always on High Alert. I think the Clomicalm is a great treatment for tense exhibit animals but would never trust in a breeding situation for Cloudeds, perhaps something less aggressive.
>
> **Zoo 1, Keeper 2:** After being taken off meds 1 week after significantly more tense and sizing up little kids. Had to be moved to hospital holding as a safety measure.
>
> **Zoo 1, Keeper 3:** During the study there seemed to be no change in male's behavior, especially regarding his response to people. Once he got over any initial starts, he would return to his "normal self". There seemed to be more of a change in him one he completed his rounds of Clomicalm.

***Note: The male at Zoo 1 escaped once and almost a second time approximately two weeks after clomipramine treatment was discontinued.*

Zoo 2, Keeper 1: I did see a change in male's behavior, but often he seemed "spaced out" during training sessions. Or, sometimes he wouldn't come over to train. This does happen, but he's usually hiding. In this case, he would just sit and stare at the shift door and not enter.

Zoo 2, Keeper 2: There was a period of four months that I had left the country during this study. Before I left he had not been receiving the daily dose of Clomicalm, but during the time I was gone he started to receive it. The first time that I had seen him when I returned I noticed a large shift in behavior especially in the morning. Before I had left he normally was alert and active. When I returned he appeared to be "drugged". He did not realize that I had approached this holding, and remained that way even after calling him. His movement was also lethargic when he shifted onto exhibit. This difference was not seen in the late evening, though this could have just been the anticipation of receiving his diet for the day.

Zoo 2, Keeper 3: Male's behavior on exhibit seems calmer overall, however his behavior when interacting with keepers did not seem significantly different to me.

Zoo 2, Keeper 4: Much calmer. Easier to shift. Great at stressful training such as squeeze and crate. Even watched us crate train one of the tigers. He had never seen that tiger before and male sat calmly by us and watched the whole training session!

Zoo 2, Keeper 5: Male reverted back to his normal behavior once he was taken off the medication.

Deslorelin

Zoo 3, Keeper 1: We are in the deslorelin group and it was noticed that when the implant was initially put in, overnight security guards (all males) commented that he was aggressive towards them for a couple weeks (which was unusual), but the keepers (all females) did not.

Zoo 3, Keeper 2: I have not really observed a change in the calmness of our male. However, beginning approximately 2 to 3 weeks after the implant, I did notice an increase in prusten toward me.

Zoo 4, Keeper 1: There was a significant increase in his aggression towards keepers after the implant. Prior to that he was very calm and relaxed.

Zoo 4, Keeper 2: After the implant we did see a slight increase in his aggression in certain situations and towards certain keepers. This mainly involved hissing, growling, and lunging at times when being shifted out onto exhibit. Prior to this, the aggression was limited to hissing and not usually at anyone in particular. Also after the implant he began to hiss at the male keepers while out on exhibit which is something he never did before. All of this behavior was slight compared to prior behavior but noticeable.

Zoo 4, Keeper 3: Male used to be very calm around me and a reliable shifter. He now hisses and occasionally refuses to shift for me. The most significant change has been after he received his implant.

Zoo 5, Keeper 1: He seems to be a lot calmer and exploring his display a lot more. He still at times is anxious but for the most part a lot calmer and really moving around on display and shifting great. No real nerves around things and a lot more playful.

Zoo 6, Keeper 1: His aggression towards me lessened to a slight degree after the implant. Most the aggression towards me is when I am trying to get male out and on exhibit or after giving him access to the inside. He has charged the exhibit glass when I walk past and I have seen him do this towards visitors occasionally as well. I did notice after the implant for the study that male was a bit calmer even towards me.

Zoo 6, Keeper 2: Since drug treatment began he has started sleeping, in the afternoon, on the ground pretty frequently. When the guest are banging and beating on the glass he gets tense very easily and will hiss at the people harassing him. I believe that he is harder to shift on exhibit now since doing the study. I rarely saw any aggression before and now there are times where he won't shift and lets it know (hisses and sometimes lunges).

Zoo 6, Keeper 3: For the most part he acts calm, but he is always watching you. If you happen to do something unfamiliar or startle him, he lets you know he doesn't like it. On most days he'll be sleeping up top or just watching. Other days he is very aggressive and will run the length of the exhibit and charge the window at you and especially visitors who find it entertaining to tease him.

5.3.4 Drug effect on reproductive function

The third major goal of this study was to determine the effect of each drug treatment on reproductive function. In this study, due to the low sample size of clouded leopards, domestic cats were also used as a model species. Deslorelin

treatment suppressed gonadal function in the domestic cat, including spermatogenesis for an average of 525.2 ± 17 days, and androgen concentrations for an average of 396 ± 15 days. Conversely, while some reproductive parameters were affected by deslorelin treatment in male clouded leopards, spermatogenesis never ceased. Androgen concentrations were suppressed, but not to the degree seen in the domestic cat. Clomipramine treatment had no negative effects on reproductive function in either species. Due to the small sample size in clouded leopards and the importance of domestic cats as a model species, the drug effect seen in domestic cats should be considered when interpreting clouded leopard results. Notably, this study demonstrated for the first time the duration and effectiveness of deslorelin and clomipramine treatment on male reproductive function in both the domestic cat and clouded leopard.

5.3.5 Keeper Survey

The keeper survey given at the beginning of the study, similar to previous research, demonstrated the effectiveness of keepers in assessing the temperaments of animals under their care. Specifically, the male clouded leopards categorized by keepers on a scale from 'calm' to 'anxious' were significantly and positively correlated with peak mean fecal glucocorticoid concentrations. Thus, individuals with the highest anxiety scores on keeper surveys also had the highest peak levels of fecal glucocorticoids. These findings lend further support to the validity of regular 'ad hoc' keeper assessments of their animals.

5.3.6 Clouded Leopard Temperament

By comparing fecal metabolite concentrations, compound keeper survey scores, and behavioral observation data, we found several meaningful and significant correlations indicating that clouded leopards can be divided into at least two temperament categories and possibly a continuum. On average, 'calm' cats tended to 'lie down' more, while 'anxious' cats tended to 'stand', hide in a 'nest box' and 'tail flick' more. Meanwhile, fecal androgen metabolite concentrations correlate positively with 'growling' and 'tail flicking' behaviors, possibly indicating increased levels of agitation and aggression, and negatively with individual questions on the behavioral questionnaire about 'pacing' and 'appearing tense'.

5.4 Future Work

Given the low sample size per treatment group (n = 4 clomipramine, n = 4 control, and n = 5 deslorelin) it would be advisable to repeat this study or a similar study with a larger sample size of clouded leopards. This would certainly increase the statistical power and thus the ability to detect treatment effects. However, in implementing the current study, all zoos with singly-housed male clouded leopards were contacted and agreed to participate. Therefore, it would be extremely difficult to perform a similar study with a larger sample size in U.S. zoological institutions. While it is conceivable to increase the sample size by focusing on one drug at a time, it should be noted that repeating the study design using the same institutions would put considerable stress on the animals and greatly impact the animal care staff. Thus

the benefit versus costs of such a study would need to be fully assessed before being conducted.

A logical extension of this work might instead be to assess the effectiveness of clomipramine and deslorelin during a male-female introduction. A possible study design would include the following. Twelve adult males (6 'anxious' and 6 'calm') from Study 1 and 2 would be moved to enclosures adjacent to twelve adult females to assess male behavior toward females in a 'howdy' situation (clouded leopards spend 2 months in adjacent enclosures separated by chain-link fence in clear visual and olfactory proximity). This initial step would be conducted for 2 months without drugs, with the animals acting as their own controls, and 90 days after initiation of drug therapy (n = 6 clomipramine treatment and n = 6 deslorelin treatment). The order of these two test periods would be randomized, to assess whether any changes in response are due to drug treatment or due to repeated exposure. Endocrine and behavioral responses would be compared with and without drug treatment. If clomipramine and/or deslorelin are effective during this initial step in the introduction, the next step would be to allow males and females access to the same enclosure and to monitor their actions.

5.5 Overall Summary

In conclusion, both the MIS and UFP tests were effective for assessing clouded leopard behavior and should be considered for future use. Deslorelin was effective at suppressing androgen and glucocorticoid concentrations as well as anxious and aggressive behaviors. However, deslorelin fully suppressed reproductive

function in the domestic cat and partially in the clouded leopard. Meanwhile, clomipramine did not suppress hormone concentrations, but did positively affect anxious and aggressive behaviors. Clomipramine had no negative effect on male reproductive function. As both drugs are similarly effective at suppressing anxiety and aggression in male clouded leopards, clomipramine may be a better option for aiding in the formation of genetically valuable breeding pairs.

Appendix – Additional Figures and Tables

Table A1.1 Clouded leopards and domestic cats participating in the study.

Clouded Leopards			
Location	**Studbook Number**	**Date of Birth**	**House Name**
Alexandria Zoological Park	1110	14-Aug-99	Risha
Audubon Nature Institute	1099	7-Apr-99	Joe
Central Florida Zoo	1134	7-Apr-01	Max
Cincinnati Zoo	1181	12-Jun-02	Kiri
Cleveland Metropark Zoo	1227	21-Feb-04	Luke
Houston Zoo	1211	3-Apr-03	Rama
Minnesota Zoo	1178	22-Feb-02	Noy
Omaha's Henry Doorly Zoo	1065	21-Mar-97	Jing
San Antonio Zoo	1059	23-Apr-96	Ghost
SCBI	1186	25-Feb-04	Dao
SCBI	681	13-Aug-97	Brandon
SCBI	750	9-Aug-94	Xing Xing
SCBI	931	31-Mar-94	Junior
Zoo Atlanta	1060	23-Apr-96	Moby
Zoo Miami	1013	5-Apr-94	Jhansi
Zoo Miami	920	4-Aug-92	Iago

Domestic Cats			
Location	**ID-Tattoo**	**Date of Birth**	**House Name**
SCBI	H 06A238	23-Jan-06	Neo
SCBI	C224	20-Mar-01	Thumbprint
SCBI	20	25-Jan-08	Loverboy
SCBI	H 06A236	23-Jan-06	Aslan
SCBI	19	25-Jan-08	Calvin
SCBI	G234	18-Jul-02	Mr. Thang
SCBI	C29-4801	24-Jan-09	Woody
SCBI	C29-4802	24-Jan-09	Lumos

Figure A1.1 All of the zoological institutions visited (in order) over a two week period in order to standardize research across all facilities.

Table A1.2 Schedule Provided to Zoos

Timeline: Pre-drug Treatment (3 months)							
Done Initial	Date	Time 1	Time 2	Week	Drugs	Fecals	Video
				Keeper Survey			
				Week 1		daily fecals	
				Week 2		daily fecals	
				Week 3		daily fecals	1 hour of video data (two 30 min)
				Week 4		daily fecals	1 hour of video data (two 30 min)
				Week 5		daily fecals	Air Horn Test, video for 30 min or MIS Test, video for 30 min
				Week 6		daily fecals	MIS Test, video for 30 min or Air Horn Test, video for 30 min
				Week 7		daily fecals	Unknown Human Test - twice a day, three days in a row, video for 3 hours (total)
				Week 8		daily fecals	1 hour of video data (two 30 min)
				Week 9		daily fecals	1 hour of video data (two 30 min)
				Week 10		daily fecals	
				Week 11		daily fecals	
Timeline: Peri-drug Treatment (5 months) - implant Deslorelin or begin administering Clomipramine (daily), or No Drug (control)							
				Week 12	drug therapy	daily fecals	
				Week 13	drug therapy	daily fecals	
				Week 14	drug therapy	daily fecals	
				Week 15	drug therapy	daily fecals	
				Week 16	drug therapy	daily fecals	
				Week 17	drug therapy	daily fecals	
				Week 18	drug therapy	daily fecals	
				Week 19	drug therapy	daily fecals	
				Week 20	drug therapy	daily fecals	
				Week 21	drug therapy	daily fecals	
				Week 22	drug therapy	daily fecals	1 hour of video data (two 30 min)
				Week 23	drug therapy	daily fecals	1 hour of video data (two 30 min)
				Week 24	drug therapy	daily fecals	Air Horn Test, video for 30 min or MIS Test, video for 30 min
				Week 25	drug therapy	daily fecals	MIS Test, video for 30 min or Air Horn Test, video for 30 min
				Week 26	drug therapy	daily fecals	Unknown Human Test - twice a day, three days in a row, video for 3 hours (total)
				Week 27	drug therapy	daily fecals	1 hour of video data (two 30 min)
				Week 28	drug therapy	daily fecals	1 hour of video data (two 30 min)
				Week 29	drug therapy	daily fecals	
				Week 30	drug therapy	daily fecals	
				Keeper Survey			

Table A1.3 Full Clouded Leopard (*Neofelis nebulosa*) Behavior Ethogram

STATES

SOLITARY BEHAVIORS

Sitting	cat sitting on hind quarters, in upright position with forelegs braced
Standing	cat remains motionless while in an upright posture on all four feet
Crouching	cat standing with legs bent under body. Body close to ground. Often seen when cat is sniffing ground or hiding
Lying	cat lies in a horizontal reclining position, cat may or may not be sleeping
Walking	cat walks at a moderate pace in a directed manner
Running	cat moves swiftly
Pacing	cat walks or runs back and forth across an area in a repetitive, non-directed pattern
Auto Groom	cat grooms self
Out of Sight	cat is out of view for a portion of the observation time

EVENTS

SOLITARY BEHAVIORS

Cheek Mark	cat rubs cheek or head against object
Urine Mark	cat squats and sprays surface with urine. Marking may be accompanied by scraping substrate with hind feet.
Claw Mark	cat scratches object, often wood, with front claws
Object sniff	cat smells an object
Flehmen	open mouthed grimace, often curling back top lip, tongue may extend outside of mouth. Usually seen after cat sniffs object or another cat
Meow	short meow, high pitched, close contact call
Cry	loud extended call, can be heard from distance, often used when one cat is attempting to locate another.
Watching	one cat watches another with great concentration, remains focused on other cat with a non-threatening stare. Follows other cat's movements with head motion. Behavior often observed in males watching females

SOCIAL BEHAVIORS

Approach	one cat moves towards another within 0-3 body lengths. No aggression is shown
Retreat	one cat avoids approaching cat by walking or running away for at least 3 body lengths
Follow	one cat walks or chases after retreating cat within 0-3 body lengths for at least 3 seconds.
Rub	one cat rubs another with cheek or head, and may continue rubbing along entire length of body
Aggression	one cat approaches another within 0-3 body lengths in aggressive posture, head lowered, growling
Hit	cat strikes at another with paw
Bite	one cat bites another
Fight	two cats hit and bite, or attempt to bite, each other repeatedly, may include vocalizations (hissing, growls)
Prusten	soft expulsion of air through lips, similar to snorting in a horse, may raise muzzle as vocalizing. Often used in greeting or as reassurance.
Growl	low pitched, throaty rumble sound
Hiss	rapid expulsion of air, teeth shown, nose wrinkled
Anogenital Sniff	one cat sniffs anogeital region of another

Observer: _____ Date: _____ Time: start: _____ end: _____ Test start: _____ end: _____ Comments: _____

Animal(s): _____ Enclosure(s): _____ Test: _____ Treatment: _____ Filmer: _____ Date: _____ Video ID _____

Jump time (airhorn resp.) _____ Time to initial approach _____ Total # of approaches _____ Total time spent interacting with mirror _____

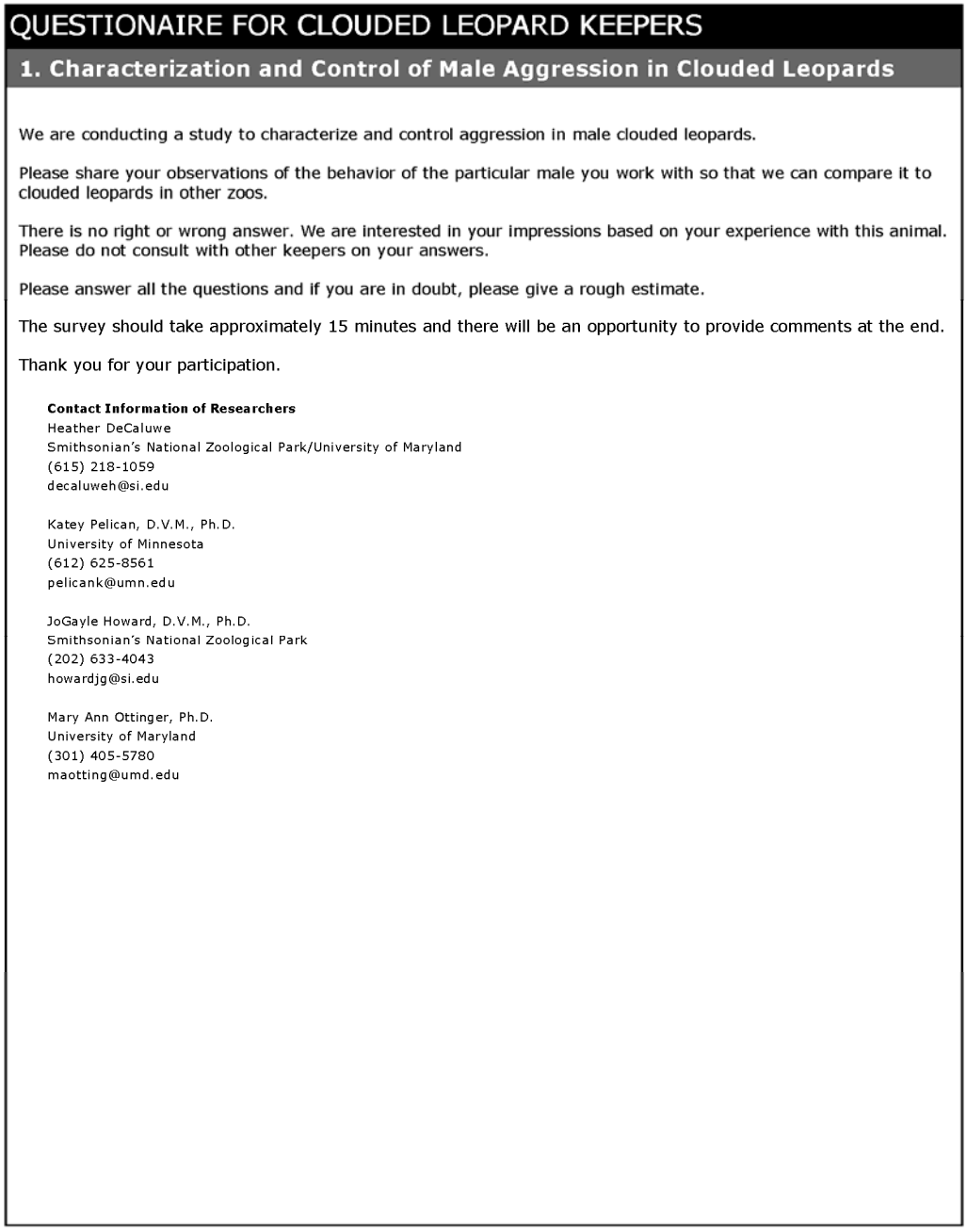
Behavioral States - recorded at the end of every minute from the time of initial response

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	Total
# body lengths mir																																
Crouching																																
Lying Down																																
Nest Box																																
Out of Sight																																
Pacing - Walk																																
Pacing - Run																																
Patrolling																																
Running																																
Sitting																																
Standing																																
Walking																																

Behavioral Events - recorded continuously from the time of initial response

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	Total
Claw Other																																
Defecate																																
Flehmen																																
Groom self																																
Growl/hiss																																
Lick Object																																
Meow/cry																																
Prusten																																
Roll																																
Rub Object																																
Sniff Object																																
Tail flick																																
Urine Scrape																																
Urine walk																																
Yawn																																
Approach Trial																																
Bite Mirror																																
Flinch Trial																																
Follow in Mirror																																
Lick Mirror																																
Paw Mirror																																
Retreat Trial																																
Rub Mirror																																
Sniff Mirror																																
Swat Mirror																																

Figure A1.2 Video behavior observation sheet



QUESTIONAIRE FOR CLOUDED LEOPARD KEEPERS

1. Characterization and Control of Male Aggression in Clouded Leopards

We are conducting a study to characterize and control aggression in male clouded leopards.

Please share your observations of the behavior of the particular male you work with so that we can compare it to clouded leopards in other zoos.

There is no right or wrong answer. We are interested in your impressions based on your experience with this animal. Please do not consult with other keepers on your answers.

Please answer all the questions and if you are in doubt, please give a rough estimate.

The survey should take approximately 15 minutes and there will be an opportunity to provide comments at the end.

Thank you for your participation.

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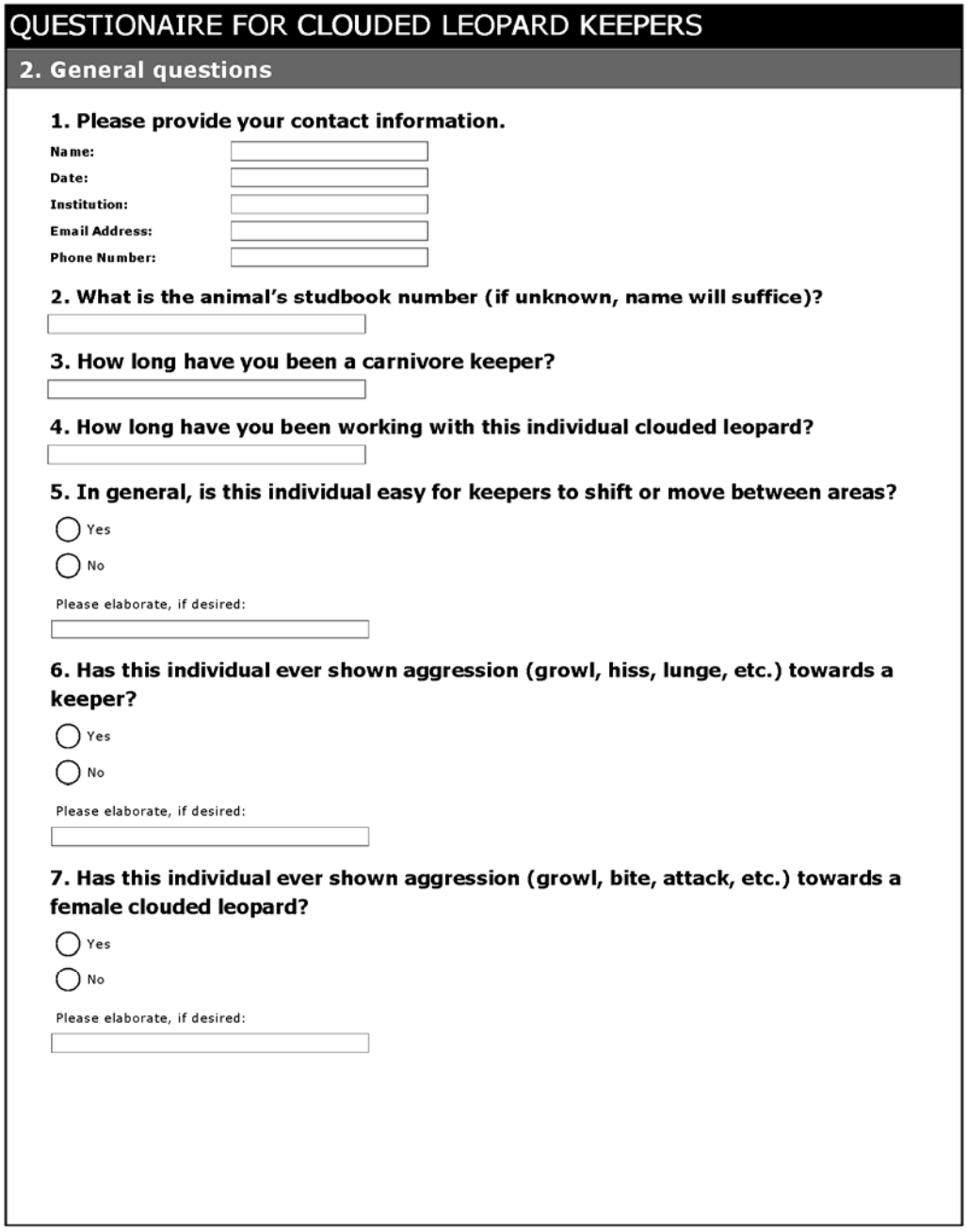
Page 1

Figure A1.3 Survey administered online via SurveyMonkey to keepers at the 12 participating zoological institutions (6 Pages, total).



QUESTIONAIRE FOR CLOUDED LEOPARD KEEPERS

2. General questions

1. Please provide your contact information.

Name:

Date:

Institution:

Email Address:

Phone Number:

2. What is the animal's studbook number (if unknown, name will suffice)?

3. How long have you been a carnivore keeper?

4. How long have you been working with this individual clouded leopard?

5. In general, is this individual easy for keepers to shift or move between areas?

Yes

No

Please elaborate, if desired:

6. Has this individual ever shown aggression (growl, hiss, lunge, etc.) towards a keeper?

Yes

No

Please elaborate, if desired:

7. Has this individual ever shown aggression (growl, bite, attack, etc.) towards a female clouded leopard?

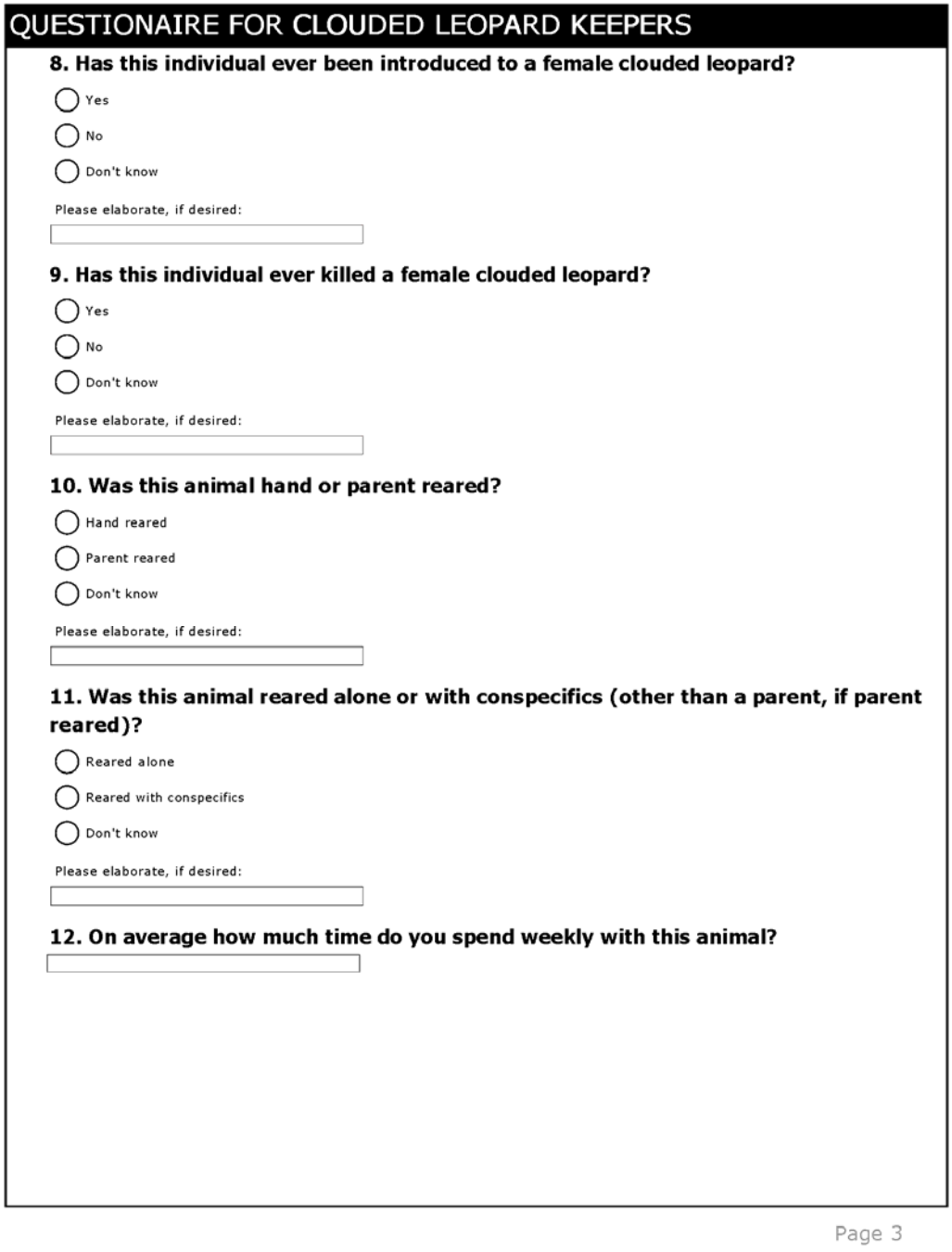
Yes

No

Please elaborate, if desired:

Page 2

Figure A1.3 (Continued)


QUESTIONAIRE FOR CLOUDED LEOPARD KEEPERS

8. Has this individual ever been introduced to a female clouded leopard?

- ◯ Yes
- ◯ No
- ◯ Don't know

Please elaborate, if desired:

9. Has this individual ever killed a female clouded leopard?

- ◯ Yes
- ◯ No
- ◯ Don't know

Please elaborate, if desired:

10. Was this animal hand or parent reared?

- ◯ Hand reared
- ◯ Parent reared
- ◯ Don't know

Please elaborate, if desired:

11. Was this animal reared alone or with conspecifics (other than a parent, if parent reared)?

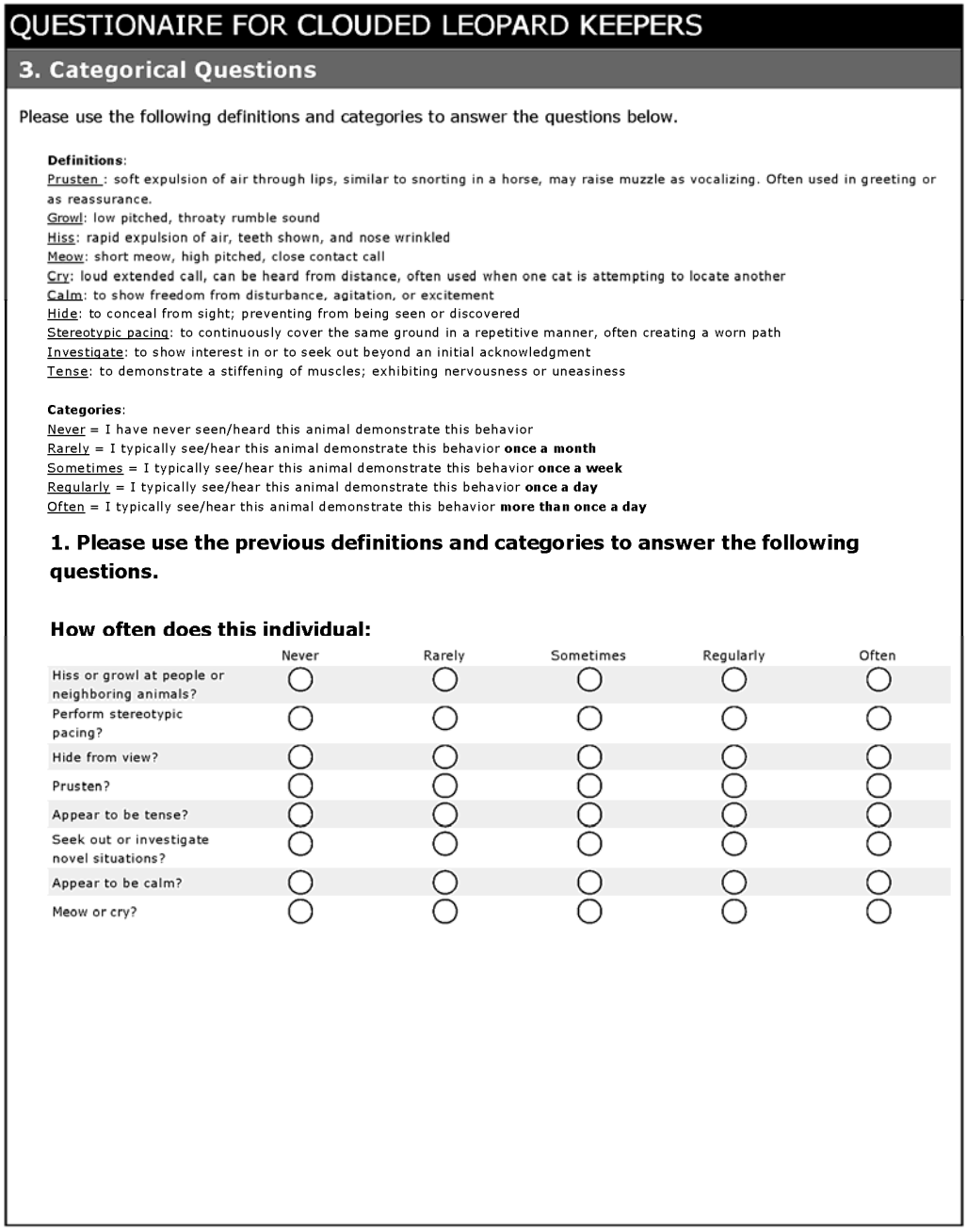
- ◯ Reared alone
- ◯ Reared with conspecifics
- ◯ Don't know

Please elaborate, if desired:

12. On average how much time do you spend weekly with this animal?

Page 3

Figure A1.3 (Continued)



QUESTIONAIRE FOR CLOUDED LEOPARD KEEPERS

3. Categorical Questions

Please use the following definitions and categories to answer the questions below.

Definitions:
Prusten : soft expulsion of air through lips, similar to snorting in a horse, may raise muzzle as vocalizing. Often used in greeting or as reassurance.
Growl: low pitched, throaty rumble sound
Hiss: rapid expulsion of air, teeth shown, and nose wrinkled
Meow: short meow, high pitched, close contact call
Cry: loud extended call, can be heard from distance, often used when one cat is attempting to locate another
Calm: to show freedom from disturbance, agitation, or excitement
Hide: to conceal from sight; preventing from being seen or discovered
Stereotypic pacing: to continuously cover the same ground in a repetitive manner, often creating a worn path
Investigate: to show interest in or to seek out beyond an initial acknowledgment
Tense: to demonstrate a stiffening of muscles; exhibiting nervousness or uneasiness

Categories:
Never = I have never seen/heard this animal demonstrate this behavior
Rarely = I typically see/hear this animal demonstrate this behavior **once a month**
Sometimes = I typically see/hear this animal demonstrate this behavior **once a week**
Regularly = I typically see/hear this animal demonstrate this behavior **once a day**
Often = I typically see/hear this animal demonstrate this behavior **more than once a day**

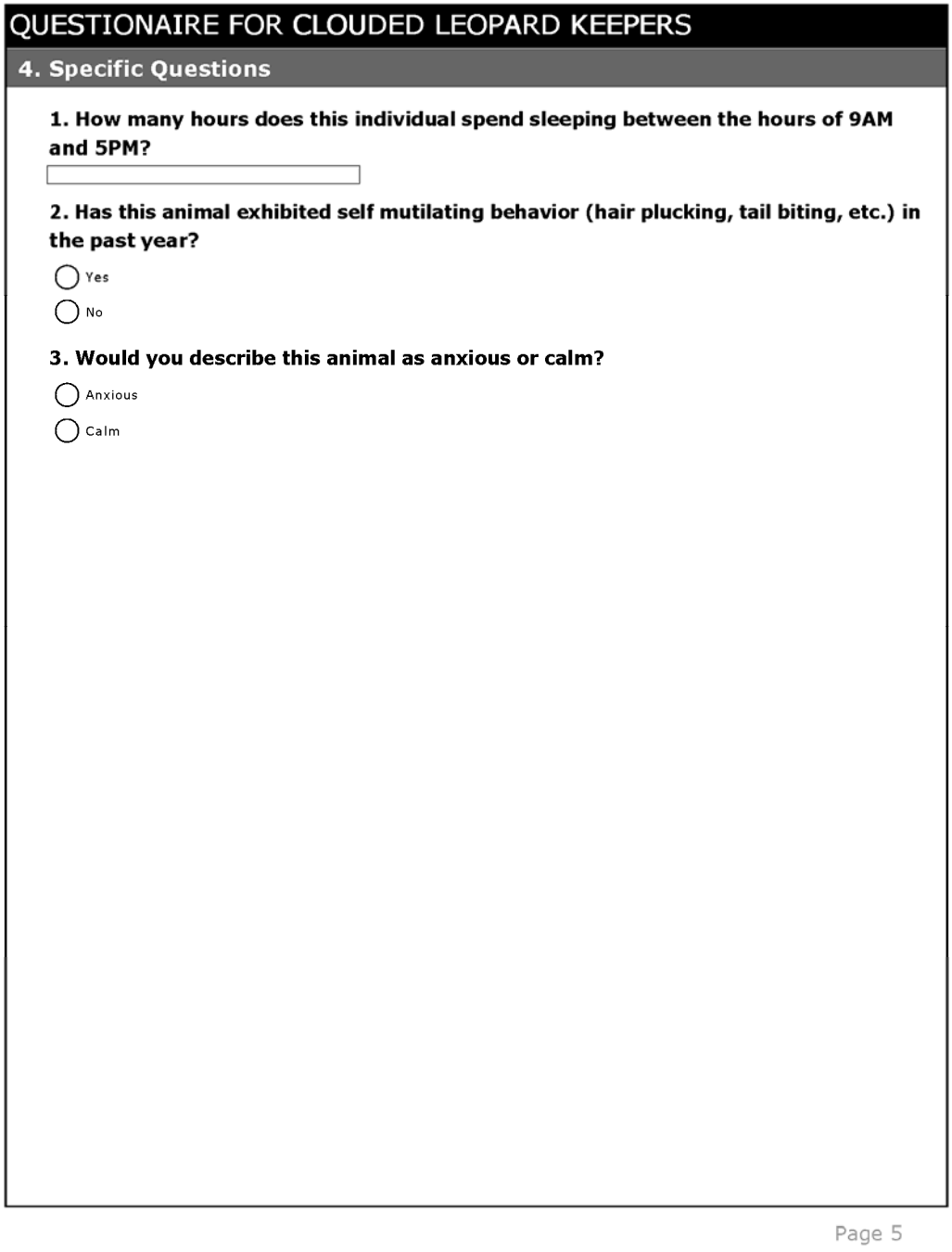
1. Please use the previous definitions and categories to answer the following questions.

How often does this individual:

	Never	Rarely	Sometimes	Regularly	Often
Hiss or growl at people or neighboring animals?	◯	◯	◯	◯	◯
Perform stereotypic pacing?	◯	◯	◯	◯	◯
Hide from view?	◯	◯	◯	◯	◯
Prusten?	◯	◯	◯	◯	◯
Appear to be tense?	◯	◯	◯	◯	◯
Seek out or investigate novel situations?	◯	◯	◯	◯	◯
Appear to be calm?	◯	◯	◯	◯	◯
Meow or cry?	◯	◯	◯	◯	◯

Page 4

Figure A1.3 (Continued)


QUESTIONAIRE FOR CLOUDED LEOPARD KEEPERS

4. Specific Questions

1. How many hours does this individual spend sleeping between the hours of 9AM and 5PM?

2. Has this animal exhibited self mutilating behavior (hair plucking, tail biting, etc.) in the past year?

- Yes
- No

3. Would you describe this animal as anxious or calm?

- Anxious
- Calm

Page 5

Figure A1.3 (Continued)

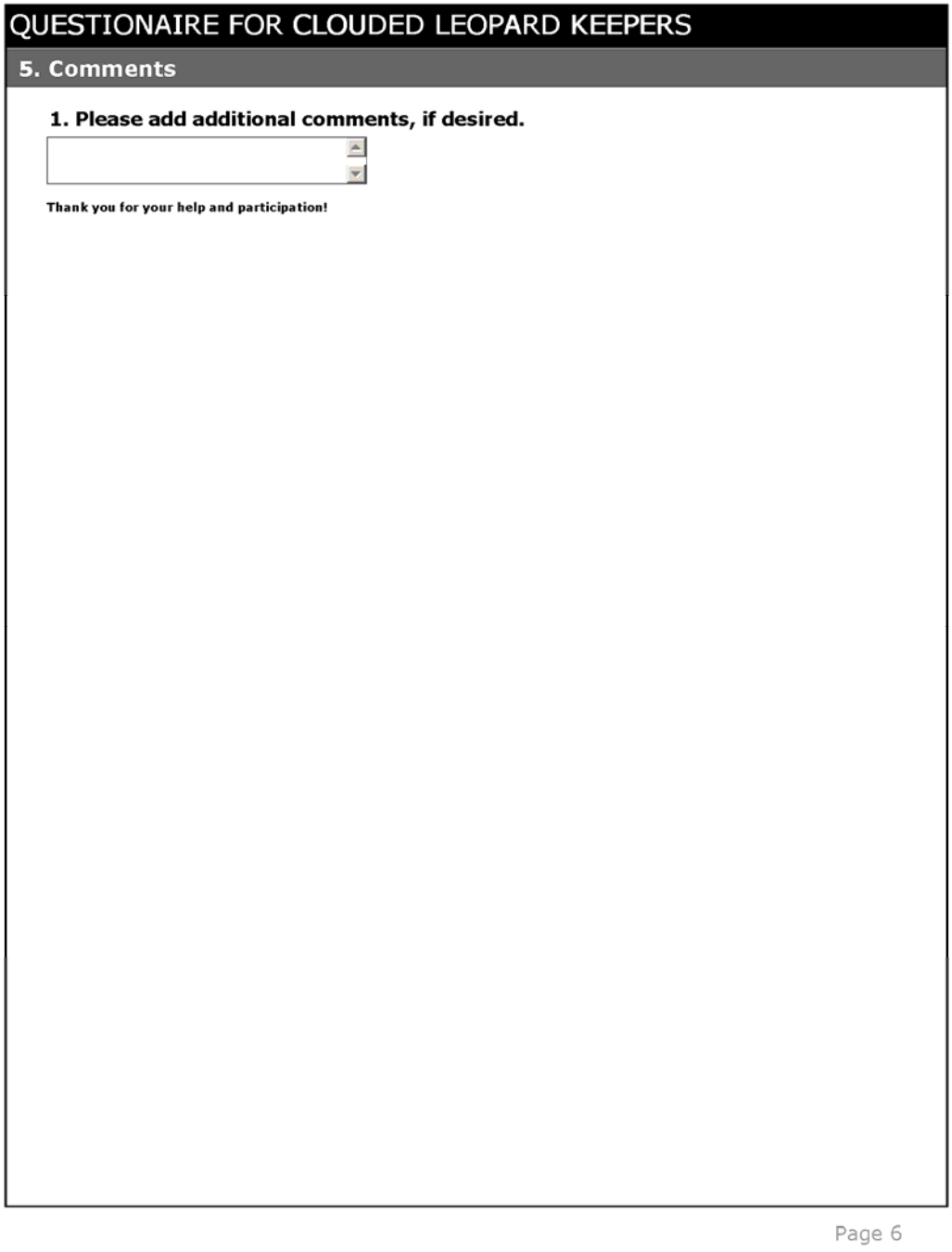


Figure A1.3 (Continued)

Figure A1.4 Proc Cluster statistical analysis, which helped inform temperament categories.

Table A1.4 Clouded leopards participating in the study

Includes compound survey scores used for analysis in chapter 2 (pre-drug treatment) and temperament categories ('calm' = green and 'anxious' = red) used for analysis in chapter 3 (peri-drug treatment).

Treatment	Zoological Institution	Clouded Leopard Name	Compound Survey Score
Control	Audubon Nature Institute	Joe	7.67
Deslorelin	Houston Zoo, Inc	Rama	9.88
Clomipramine	Cleveland Metropark Zoo	Luke	10.25
Control	SCBI	Junior	10.50
Deslorelin	Central Florida Zoo	Max	10.67
Clomipramine	Henry Doorly Zoo	Jing	10.83
Control	Cincinnati Zoo	Kiri	11.00
Control	Alexandria Zoological Park	Risha	11.33
Clomipramine	Zoo Atlanta	Moby	12.88
Clomipramine	SCBI	Dao	13.50
Deslorelin	SCBI	Xing	14.00
Deslorelin	Miami Metrozoo	Jhansi	14.25
Deslorelin	San Antonio Zoo	Ghost	16.00
Pre-drug treatment only, due to death or relocation			
Pre-drug only	SCBI	Brandon	11.50
Pre-drug only	Miami Metrozoo	Iago	11.67
Pre-drug only	Minnesota Zoo	Noy	12.75

Table A2.1 Raw Survey Statistical Analysis

Significant p <.06

Test	Zoo	Questions to Omit	Keepers to Omit	Chi-Square	# questions - 1	# keepers	K or S	p value
Kendall	Alexandria	6,8	none	11.1673	5	3	0.74	0.048
Kendall	Audubon	none	none	16.1304	7	3	0.77	0.024
Kendall	Central Florida	6,8	3	10.7386	5	3	0.72	0.057
Kendall	Cincinnati	6,8	2	11.4737	5	3	0.76	0.043
Kendall	Cleveland	6,8	3	15.3571	5	4	0.77	0.009
Kendall	Henry Doorly Zoo	6,8	3,4,6	22.3232	5	6	0.74	0.001
Kendall	Houston Zoo, Inc	6,8	none	28.1984	5	8	0.70	<.0001
Kendall	Miami Metrozoo: Iago	6,8	none	10.8523	5	3	0.72	0.054
Kendall	Miami Metrozoo: Jhansi	8	none	17.2268	6	4	0.72	0.009
Kendall	Minnesota Zoo	3	2,3,6,8	19.0606	6	4	0.79	0.004
Kendall	San Antonio Zoo	none	none	33.5744	7	6	0.80	<.0001
Kendall	Zoo Atlanta	6,8	3	28.2016	5	8	0.71	<.0001
Spearman	SCBI - Brandon	3,7,8	none	NA	NA	NA	0.72	0.0003
Spearman	SCBI - Dao	3,7,8	none	NA	NA	NA	0.72	0.0003
Spearman	SCBI - Junior	3,7,8	none	NA	NA	NA	0.72	0.0003
Spearman	SCBI - Xing	3,7,8	none	NA	NA	NA	0.72	0.0003

Question #	How often does this individual:
1	**Hiss or growl at people or neighboring animals?**
2	**Perform stereotypic pacing?**
3	Hide from view?
4	**Prusten?**
5	**Appear to be tense?**
6	Seek out or investigate novel situations?
7	Appear to be calm?
8	Meow or cry?

**Bold indicates questions with significant interrater agreement across keepers and facilities

Table A3.1 Mean Hormone Concentrations for Each Male Both Pre- and Peri-drug treatment.

Fecal Androgens			Study 1													Study 2											
			Mean				Baseline				Peak Mean				Summary	Mean				Baseline				Peak Mean				Summary
Final Compound Score	Name	Treatment	Mean	CV	STD	SE	Mean	CV	STD	SE	Mean	CV	STD	SE	peak/base	Mean	CV	STD	SE	Mean	CV	STD	SE	Mean	CV	STD	SE	peak/base
13.50	Dao	Clomipramine	398.87	36.93	147.30	19.51	359.09	28.83	103.52	14.64	683.05	12.76	87.14	32.94	1.90	478.72	29.844	142.87	20.62	462.93	26.452	122.45	18.05	841.80	11.5535	97.2574	68.77	1.82
10.83	Jing	Clomipramine	748.21	37.67	281.87	33.93	682.94	29.20	199.41	25.33	1326.34	18.81	249.43	94.28	1.94	667.20	34.789	232.11	37.17	571.15	24.367	139.17	25.00	1059.97	9.76258	101.47	37.75	1.86
10.25	Luke	Clomipramine	706.30	27.44	193.80	25.02	673.69	22.98	154.84	20.69	1162.87	3.14	36.49	18.24	1.73	738.00	34.682	255.96	37.74	701.50	31.634	221.91	33.84	1261.18	2.0933	26.4004	15.24	1.80
12.88	Moby	Clomipramine	368.64	28.37	104.59	12.16	346.43	21.41	74.18	9.00	620.36	9.15	56.76	23.17	1.79	479.37	39.172	187.78	22.13	418.52	23.57	98.644	12.63	816.85	25.7083	209.998	63.32	1.95
7.67	Joe	Control	501.90	38.74	194.41	22.30	492.78	36.24	178.60	20.62	1186.05	NA	NA	1 Value	2.41	424.46	35.595	151.08	22.78	424.46	35.595	151.08	22.78	NA	NA	NA	NA	NA
10.50	Junior	Control	576.29	25.94	149.51	18.27	561.92	24.35	136.82	17.10	882.95	1.78	15.71	9.07	1.57	431.05	35.518	153.1	18.70	399.76	29.686	118.67	15.19	749.15	11.1965	83.8782	34.24	1.87
11.00	Kiri	Control	470.69	31.00	145.91	17.57	446.16	26.17	116.76	14.60	784.55	15.32	120.22	53.76	1.76	471.30	22.074	104.04	13.21	467.66	21.563	100.84	12.91	693.24	NA		1 Value	1.48
11.33	Risha	Control	478.65	24.98	119.55	15.31	474.37	24.40	115.74	14.94	735.59	NA	NA	1 Value	1.55	554.65	38.744	214.89	31.35	502.44	29.859	150.02	23.15	993.17	17.8343	177.125	79.21	1.98
16.00	Ghost	Deslorelin	380.43	47.05	178.99	22.20	309.56	35.90	111.14	15.41	663.90	14.72	97.69	27.10	2.14	397.50	40.846	162.36	19.13	359.76	33.059	118.93	14.75	747.90	6.53031	48.8404	18.46	2.08
14.25	Jhansi	Deslorelin	387.53	39.89	154.57	20.84	355.78	28.34	100.82	14.12	792.47	19.90	157.66	78.83	2.23	323.45	47.403	153.33	20.67	272.85	27.21	74.244	10.83	620.75	26.4357	164.099	58.02	2.28
10.67	Max	Deslorelin	172.92	28.67	49.57	5.47	170.01	27.36	46.52	5.20	289.40	5.59	16.16	11.43	1.70	133.26	43.793	58.357	6.44	131.70	43.269	56.987	6.33	259.15	NA	NA	1 Value	1.97
9.88	Rama	Deslorelin	249.17	43.06	107.29	12.00	223.59	25.48	56.98	6.76	450.92	40.69	183.49	61.16	2.02	155.67	65.41		8.38	144.06	37.853	54.532	7.29	285.60	6.16	NA	7.86	1.98
14.00	Xing	Deslorelin	410.38	34.32	140.83	18.34	369.69	27.10	100.19	14.03	669.72	9.11	61.00	21.57	1.81	310.06	36.479	113.11	15.25	274.77	27.175	74.669	10.89	517.42	13.0334	67.4378	23.84	1.88

Fecal Glucocorticoids			Study 1													Study 2											
			Mean				Baseline				Peak Mean				Summary	Mean				Baseline				Peak Mean				Summary
Final Compound Score	Name	Treatment	Mean	STD	CV	SE	Mean	STD	CV	SE	Mean	STD	CV	SE	peak/base	Mean	CV	SE	Mean	CV	SE	Mean	CV	SE	peak/base			
13.50	Dao	Clomipramine	631.66	450.16	71.27	59.63	402.83	129.73	32.20	20.26	1218.04	449.19	36.88	112.30	3.02	533.28	63.344	48.26	392.56	38.144	23.98	1082.10	28.2506	96.67	2.76			
10.83	Jing	Clomipramine	530.98	222.07	41.82	26.73	448.71	108.86	24.26	14.55	885.37	239.75	27.08	66.50	1.97	769.50	33.229	40.94	753.72	31.724	38.79	1369.17		1 Value	1.82			
10.25	Luke	Clomipramine	202.19	72.61	35.91	9.37	191.49	53.40	27.89	7.07	405.47	102.12	25.19	58.96	2.12	237.87	49.198	17.26	204.82	31.152	9.96	508.90	19.9936	45.50	2.48			
12.88	Moby	Clomipramine	366.95	189.89	51.75	22.07	258.23	61.90	23.97	8.84	580.05	174.86	30.14	34.97	2.25	326.84	35.01	13.49	302.43	26.451	9.85	595.38	15.338	37.28	1.97			
7.67	Joe	Control	230.65	97.26	42.17	11.16	195.56	59.09	30.22	7.45	400.69	57.34	14.31	15.90	2.05	181.43	32.72	8.95	167.02	27.345	7.31	293.89	5.21678	6.86	1.76			
10.50	Junior	Control	486.61	311.31	63.98	38.03	369.50	90.50	24.49	12.20	1023.38	398.00	38.89	114.89	2.77	353.03	75.107	32.39	233.77	31.745	10.50	703.81	44.4725	75.91	3.01			
11.00	Kiri	Control	351.36	146.56	41.71	17.64	322.10	100.72	31.27	12.59	725.83	128.09	17.65	57.28	2.25	326.52	35.315	14.64	275.49	17.21	6.92	486.41	24.6828	31.00	1.77			
11.33	Risha	Control	630.83	292.25	46.33	37.42	500.32	161.36	32.25	23.54	1068.95	183.81	17.20	49.13	2.14	517.32	38.44	29.01	465.73	27.55	19.80	950.71	16.0549	68.26	2.04			
16.00	Ghost	Deslorelin	315.10	160.81	51.04	19.95	235.53	63.78	27.08	9.21	539.77	137.37	25.45	33.32	2.29	316.27	56.472	20.76	221.92	34.06	10.48	539.30	28.0379	32.24	2.43			
14.25	Jhansi	Deslorelin	531.00	331.13	62.36	44.65	385.99	103.72	26.87	16.00	999.50	381.17	38.14	105.72	2.59	325.55	48.564	21.13	277.76	23.157	9.28	612.29	39.6671	85.87	2.20			
10.67	Max	Deslorelin	161.49	57.24	35.44	6.36	153.13	48.36	31.58	5.55	288.58	12.57	4.36	6.99	1.88	113.28	40.561	5.07	104.70	32.156	3.86	222.01	19.6048	17.77	2.12			
9.88	Rama	Deslorelin	222.87	124.39	55.81	13.91	178.81	58.89	32.94	7.19	449.95	126.86	28.19	35.18	2.52	219.26	54.711	15.23	167.36	25.662	6.20	397.18	32.6502	34.66	2.37			
14.00	Xing	Deslorelin	544.19	321.07	59.00	41.80	430.95	176.28	40.91	25.18	1099.07	294.41	26.79	93.10	2.55	329.76	58.362	25.95	243.69	26.307	10.14	559.30	41.4711	59.89	2.30			

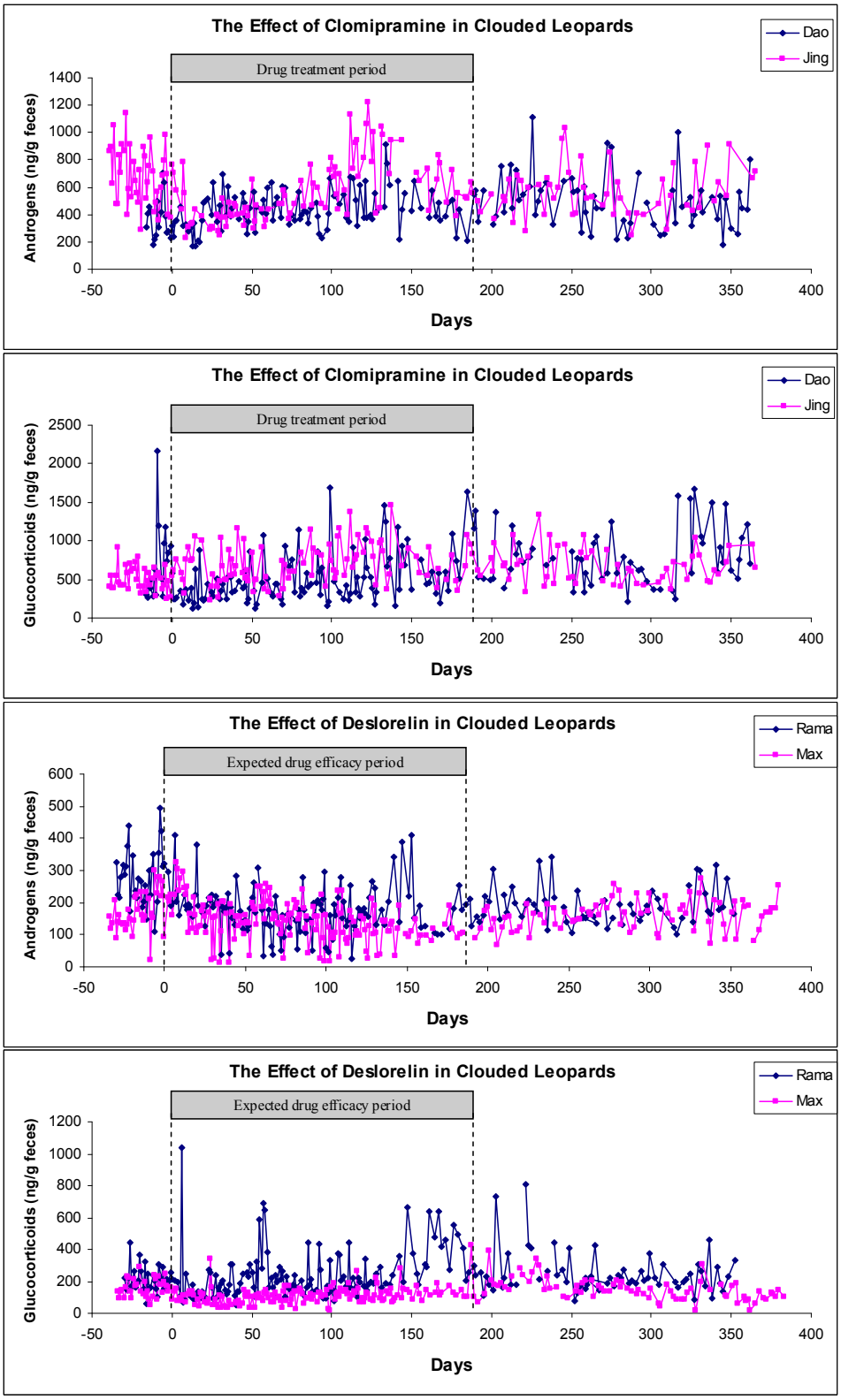
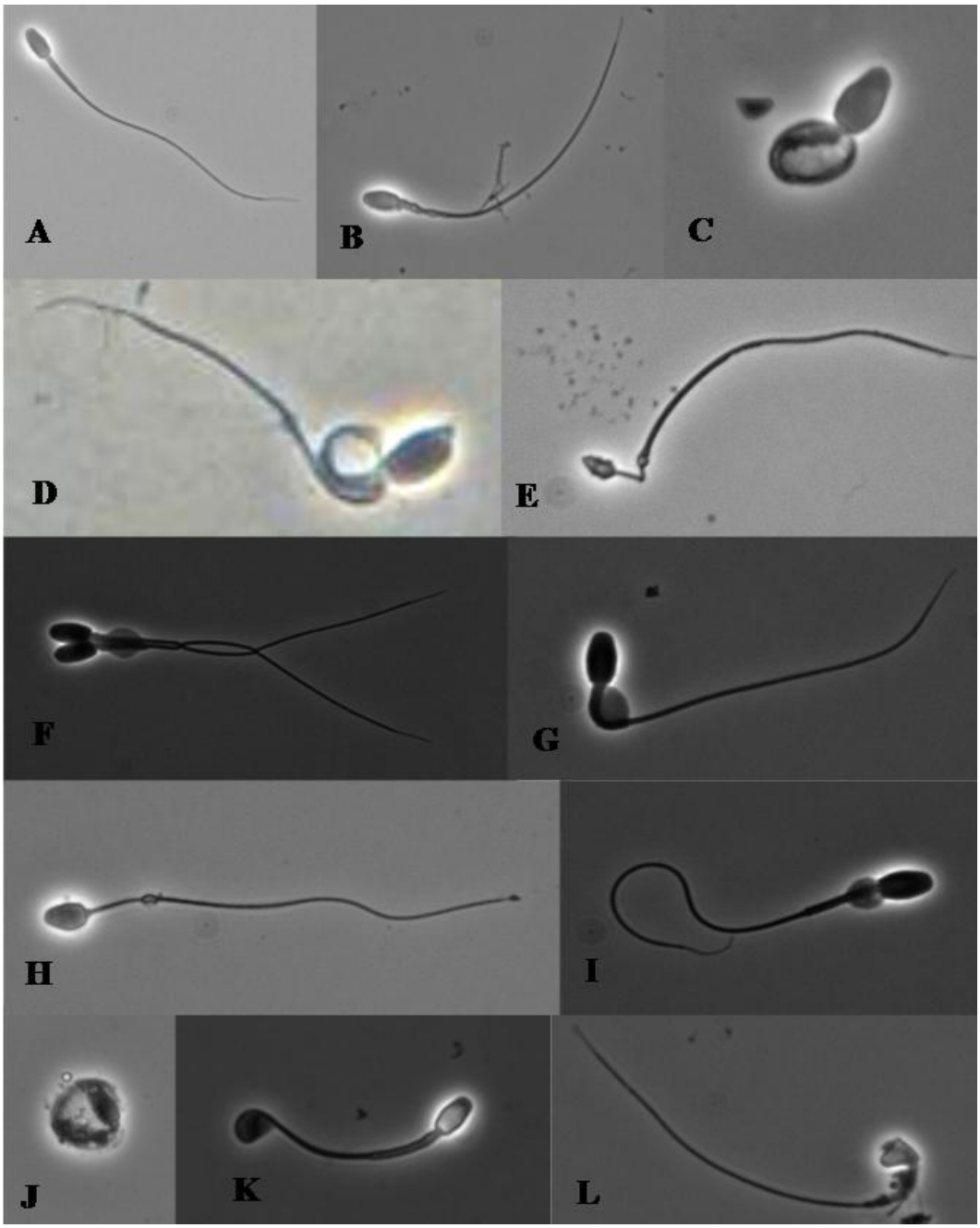


Figure A4.1 Clouded Leopard Endocrine Profiles with Drug Treatment



A. Normal
B. Abnormal Midpiece
C. Tightly Coiled Tail
D. Abnormal Acrosome
E. Microcephalic
F. Bicephalic
G. Bent Midpiece with Cytoplasmic Droplet
H. No Midpiece
I. Proximal Cytoplasmic Droplet
J. Spermatid
K. Bent Tail with Cytoplasmic Droplet
L. Abnormal Acrosome, Abnormal Midpiece

Figure A4.2 Morphology of Clouded Leopard Spermatozoa

Table A4.1 Table of Sperm Morphology

Paired t-test results for the effect of deslorelin treatment on sperm traits in domestic cats. Means represent the incidence of a given morphology (per 100 counts) immediately following the return of spermatogenesis, minus the incidence prior to the start of drug treatment. Significant results are indicated by bold probability (Pr) values.

Sperm Morphology	N	Mean	Std Err	DF	t Value	Pr > |t|
Normal	5	22.6	9.65	4	2.34	0.0791
Macrocephalic	5	-0.6	0.24	4	-2.45	0.0705
Microcephalic	5	-1.4	0.93	4	-1.51	0.2056
Bicephalic	5	-0.4	0.81	4	-0.49	0.6483
Acrosome	5	-2.8	0.97	4	-2.89	**0.0447**
Abnormal Midpiece	5	-2.8	1.77	4	-1.58	0.1892
Coiled Tail	5	-14.8	11.34	4	-1.30	0.2619
Spermatid	5	0.8	2.85	4	0.28	0.7931
Bent Midpiece w/ Droplet	5	-0.6	10.11	4	-0.06	0.9555
Bent Midpiece w/out Droplet	5	0.8	2.56	4	0.31	0.7700
Bent Tail w/ Droplet	5	2.4	5.33	4	0.45	0.6761
Bent Tail w/out Droplet	5	1.6	2.25	4	0.71	0.5162
Proximal Droplet	5	-9.6	2.91	4	-3.30	**0.0299**
Distal Droplet	5	4.0	4.92	4	0.81	0.4618
Bent Neck	5	0.8	0.66	4	1.21	0.2943

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