PAIRING CLOUDED LEOPARDS (*NEOFELIS NEBULOSA*) IN A

CAPTIVE BREEDING PROGRAM

By

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To the Faculty of Washington State University:

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accepted.

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Abstract

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Pairing the endangered clouded leopard (*Neofelis nebulosa*) for reproduction in captivity is risky

due to unpredictable male aggression toward females, which can result in lethal attacks or a need

to intervene to prevent injury. To assess potential early indicators for successful pairings (i.e.,

pairings producing cubs), behavioral observations and fecal hormones were evaluated on four

juvenile-male (< 1 y) and nine adult-male ($\geq$ 1 y) pairs of clouded leopards in a breeding

program in the Thailand Clouded Leopard Consortium at the Khao Kheow Open Zoo. Fecal

cortisol concentrations, stereotyped pacing behavior and fighting were predicted to be lower in

successful than failed pairs, and animals in successful pairs were predicted to display more

affiliative behaviors than those in failed pairs. It also was predicted that pairs are more likely to

be successful when females are paired with a juvenile male, rather than an adult male.

Behavioral observations were conducted on paired males and females 2-5 times weekly. Daily

fecal samples were collected for hormonal analysis of cortisol by enzyme immunoassay. Data

were analyzed to identify predictors of successful (n = 5) versus failed (n = 8) pairings using

generalized linear models. As predicted, animals in successful pairs exhibited higher ($p < 0.05$)

levels of affiliative behaviors (grooming, rubbing on other cat) than animals in failed pairs. Neither pacing nor exploratory behaviors were significantly different between animals in successful versus failed pairs. Male fecal cortisol baseline, mean and peak mean were higher in failed than successful pairs ($p < 0.01$). No differences were observed in female fecal cortisol mean, baseline or peak mean concentrations ($p > 0.05$). However, fecal cortisol coefficient of variation around the mean was higher ($p < 0.05$) in successful than failed females. Although pairing females with juvenile versus adult males did not directly predict success ($p > 0.05$), specific behaviors (e.g., groom other cat, female rub on other cat) did predict success ($p < 0.05$) when the juvenile/adult-male variable was included in the model. These results suggest that behavior and hormone levels could be used to predict whether a pair of clouded leopards will reproduce successfully.

TABLE OF CONTENTS

LIST OF TABLES   Page

LIST OF FIGURES   Page

DEDICATION

This thesis is dedicated to all my family and friends, who have supported,

loved, and encouraged me throughout this experience.

INTRODUCTION

Male aggression toward females has been the primary obstacle in captive breeding of clouded leopards (*Neofelis nebulosa*) throughout the world. Males in captivity over one year old are typically aggressive toward, and have even killed females (Yamada and Durrant 1989; Law and Tatner 1998; Kitchener 1999; Fletchall 2000; Wielebnowski et al. 2002). To my knowledge there is no other species of mammal in which this type of aggression is so common. Though this is a recurrent difficulty in zoos attempting to breed clouded leopards, very few studies have focused on the complexities of pairing this species (Law and Tatner 1998; Zimmermann 2002).

This literature review will lay the groundwork for the study described within this thesis by characterizing pertinent aspects of the literature and identifying unanswered questions relating to aggression during pairing of clouded leopards. A brief introduction to aggression and stress and their sources will be followed by a look at the causes of stress and aggression in captive animals, a review of the current methods for monitoring and managing aggression and stress in captive wildlife, methods for hormone monitoring and behavioral observation, the theories and purposes behind captive breeding, the social system of cats (felids), as well as what is currently known about clouded leopards.

Aggression: Overview

For decades, scientists have studied the physiological and behavioral aspects of aggression. As a result, several types of aggression are identified in the literature. Brain (1979) described five primary categories of aggression: 1) self-defensive behavior; 2) social conflict; 3) predatory attack; 4) parental aggression; and 5) reproductive termination (infanticide).

1

Behavioral scientists commonly use the term "agonistic behavior" to refer to offensive, defensive, and parental aggression (Maxson and Canastar 2006); but this term typically does not include predatory aggression or infanticide. Offensive aggression involves perception of opportunity for resource acquisition, while defensive aggression is associated with protection of resources from intruders (Wingfield et al. 2006). Aggression in any category can be conditioned when an animal learns from aggressive action (any type) and the aggressive behavior is reinforced (Brain 1979). The aggression occurring between captive clouded leopard breeding pairs may be explained by the interaction of these categories brought about by the captive environment. For example, clouded leopards in the wild are believed to be primarily solitary but, in the captive environment, they are housed in close proximity (social conflict) and "flight is difficult or precluded" (Brain 1994) generating a need for self defense. There is one video of a clouded leopard attack that resulted in the death of the female (K. Pelican, personal communication). In it, the female ran, and the male responded as predator to prey, chasing her and biting her on the neck. After she was dead, he released her and wandered away, returning shortly thereafter and vocalizing to her in an apparent attempt to interact with her (K. Pelican, personal communication). The female's impulse to run may have been triggered by an aspect of social conflict, but resulted in predatory attack.

In addition to categories of aggression, there are several key elements to aggressive behavior. These elements are described by Brain (1994) as essential in understanding aggression, though none considered individually qualify the behavior as aggressive per se. First, aggression involves the "potential for harm or damage," whether emotional or physical aggression. The second element is intentionality. Though the determination of intent is reliant

upon interpretation, the one receiving aggression may be the only one for which perception is relevant. Arousal is described as the third element, in that some measure of alertness and physiological response (e.g., increased heart rate and respiration, change in posture) is altered. Lastly, the target of aggression would act in avoidance of the behavior. For captive clouded leopards, where the female is generally the target of male aggression, the female's interpretation of the male's intention is likely an essential element for pairing success.

Sexual Aggression

Within and between-sex aggression varies between species. For example, intra-sexual aggression is more common in bonobos (*Pan paniscus*) than inter-sexual aggression, and aggression is highest on mating days and in groups with greater numbers of females in estrus (Hohmann and Fruth 2003). Conversely, males in species such as impala (*Aepyceros melampus*) compete for breeding rights with females and, thus, aggression is common between males during the breeding season (Greene et al. 1998). Sands and Creel (2004) found that both aggression and glucocortocoid levels increased during mating periods for wolves (*Canis lupus*) within all social ranks. Evidence of reduced breeding as a result of aggression has been seen in many species. Ways that reproduction potential could be reduced for individuals include: fewer copulations by defeated males (inter-male aggression), inter-female aggression reducing the number of breeding females, inter-sex aggression causing injury or repulsion, and the influence of inter-male aggression on females (stress, or perhaps fewer matings from combating males) (King 1973).

Aggression in Felids

Most felids are solitary and territorial, both male and female. Male territories often overlap multiple female territories and both sexes scent mark their territory with glands on their faces and feet, as well as with feces and urine, and they also use claw marks. Territorial aggression can occur when animals compete for resources – whether males are competing for females in estrous or either sex is searching for better hunting grounds (Maxson and Canastar 2006). When this occurs, the aggression is defensive for the territory's occupant, while it is offensive for the intruder. Though male clouded leopards in captivity do not compete for females, aggression between males and females has occurred over food resources (K. Lang, personal communication, 2006).

Neural Pathways of Aggression

Few studies have focused on the neural mechanisms of offensive aggression in felids (Maxson and Canastar 2006). However, a study of the brain centers for defensive rage and predatory attack in the cat (*Felis catus*) and rat (*Rattus norvegicus*) by Seigel and Shaikh (1997) revealed that for predatory attack to be expressed, the lateral perifornical hypothalamus is vital, and for defensive rage expression, both the periaqueductal gray (PAG) and hypothalamus are crucial. The PAG signals areas in the brain stem involved in defensive motor patterns. Serotonin, acetylcholine, gamma-aminobutyric acid (GABA) and neurokinin are believed to act as neurotransmitters sending input from the cortical, hypothalamic, and limbic areas to the PAG to control the intensity of defensive behaviors (Maxson and Canastar 2006). Summers and Winberg (2006) explained that serotonin (5-HT), a neurotransmitter involved in stress response, has a strong role in curtailing aggressive interactions in adults, but 5-HT has low efficacy in

puberty. Dopamine in the limbic areas appears to be involved in regulation of learning, motivation and reward, social status, motor activity, and aggression (Summers and Winberg 2006). Defensive and predatory attack response expressions, such as those produced by territorial species, are modulated by input to the PAG from the limbic and cortical areas (Siegel and Shaikh 1997; Maxson and Canastar 2006).

Steroids and Aggression

Aggression is influenced by many factors, including genetics, hormones, environment, and experience. Steroid hormones involved in aggression include the gonadal hormones (including testosterone, estrogens, and progestins), as well as glucocortocoids. These hormones are constructed from a base of cholesterol and are lipids that can pass between cells through cell membranes (Senger 1999).

Many studies have established a relationship between level of testosterone (T) and levels of aggression in male birds and some other vertebrates during the breeding season (Wingfield et al. 1990; Hirschenhauser et al. 2004; Smith et al. 2005; Soto-Gamboa et al. 2005). Contextual variations in circulating T levels, described in the "challenge hypothesis," fall into 3 primary levels: a) a low, non-breeding baseline; b) a mid-level breeding baseline; and c) a maximum physiological level of T. In each of these states there are bell-shaped curves of physiologic response, dependent on whether they are measured in animals during maturation, breeding, or seasonal regression. As male T increases from breeding baseline in several species of vertebrates including 20 species of birds, it incites and is stimulated by a related increase in aggression to maximum physiological level (Wingfield et al. 1990).

However, the connection between T and aggression is not consistent across all vertebrate species. In Lapland longspurs (*Calcarius lapponicus*), Hunt et al. (1997) found that high dose T implants resulted in increased incidence of song but not aggression. Male northern fence lizards (*Sceloporus undulates hyacinthinus*) are aggressive during the breeding season, but territorial intrusion challenges do not increase their T levels (Klukowski and Nelson 1998). Creel et al. (1993) found that male wild dwarf mongooses (*Helogale parvula*), which show increased aggression during mating periods, do not have correlated increases in T. Rufous-collared sparrows (*Zonotrichia capensis*) implanted with T were not more aggressive than those with blank implants (Moore et al. 2004). No studies have been focused on the relationship between T and aggression in clouded leopards.

Estrogens have also been identified as contributors to aggression for some species (Kendrick and Schlinger 1996). Female mountain spiny lizards (*Sceloporus jarrovi*) have higher levels of both testosterone and estradiol during seasonal display of territorial aggression, but neither hormone was found to have a direct relationship to aggression (Woodley and Moore 1999). Soma et al. (2000) found that estrogens control territorial behaviors of the male song sparrow (*Melospiza melodia morphna*) in the non-breeding season when testosterone levels are low.

As Summers and Winberg (2006) explained, neurotransmitters involved in aggression are also associated with the stress response. Wingfield et al. (2006) also described both hormonal (corticoids) and situational links (territorial or dominance aggression leading to "fight or flight" and modification of submissive behaviors) between stressors and aggression. Watts and Meder

(1996) outlined methods to reduce stressors involved in pair or socially housing solitary species that help to reduce the likelihood of aggression in these situations.

The Stress Response

Originally described as the "fight or flight" syndrome by Walter B. Cannon (1923), the stress response is a physiological mechanism that makes it possible for animals to adjust to changing situations, events, or other stimuli and react quickly. The effect that a stressor has on the animal is contingent on the animal's capacity for coping with that stressor (Baer 1998; Canoine et al. 2002). Though this is the generally accepted description of the stress response, in that it indicates a natural system for adapting to changes in life, the term "stress" is generally thought of as negative, corresponding to the term "distress," accepted to refer to the harmful effects of some stressors and stress responses (Wielebnowski 2003). Fundamentally, the stress response is positive to the extent that it is necessary for survival.

The sympathetic nervous system (SNS) works as an acute, emergency response within animals to defuse threats to homeostasis. Pervasive innervation of the SNS allows for selective activation of the adrenal medulla, producing adrenaline (epinephrine) and noadreniline (norepinephrine) (Archer 1979). Adrenaline and noradrenaline are involved in many physiological aspects of the flight or flight response, including dilation of the bronchioles, increase in heart rate and blood pressure, constriction of blood vessels, and increase in metabolism (Broom and Johnson 1993).

The hypothalamic-pituitary-adrenal (HPA) axis is another important component of the stress response (Archer 1979). Stressors come in several forms detectable by the Central Nervous System (CNS), including "high intensity stimulation, pain, suddenly-appearing and novel stimulation, and frustrating events" (Archer 1979). Once the animal senses a stressor, stimulation of the hypothalamus causes it to synthesize and release corticotrophin-releasing hormone (CRH). The pituitary is then activated by CRH and secretes adrenocorticotropic hormone (ACTH) which travels to the adrenal gland (Selye 1956). Glucocortocoid hormones, predominantly corticosterone or cortisol depending on the species, are then secreted by the adrenal cortex (Archer 1979; Wielebnowski 2003). The stress response defuses threats to homeostasis, facilitating efficient use of an animal's physiology to get the animal away from or allow the animal to deal with stressors.

The Negative Impact of Chronic Stress

The stress response is critical to survival, and maintains normal body function in times of deprivation or physiologic challenge. However, when the stress response becomes chronically stimulated, resulting in excessive and prolonged elevations in glucocorticoids, there can be profound physiologic and behavioral impacts. Chronic stress involving consistent high levels of corticosteroids hinders the body's response to infection and influences several aspects of reproduction (Archer 1979; Janeway and Travers 1996; Wingfield et al. 2006). Gross and Siegel (1985) found that chickens under stress that respond with higher corticosterone levels were more likely to develop virus-induced tumors and coccidiosis and this was associated with lower immunocompetence (Apanius 1998). Stress has also been shown to decrease the size of testes

(Selye1956; Archer 1979) and cause fewer embryos to complete development (Archer 1979), as well as impede lactation in females experiencing intense stress (Selye 1959).

Chronic Stress in the Captive Context

Koolhaas et al. (1999) described stress coping style as when particular groups of individuals have distinctive physiological as well as behavioral proactive and reactive responses that are stable through time. Interactions with humans, captive environments, and novel stimuli influence the behavioral and physiological coping mechanisms of animals. Broom and Johnson (1993) outlined a graded stress response accompanied by 3 levels of coping and tolerance: 1) requires extra responses or higher levels of stress response, and the animal should be able to tolerate the stressor indefinitely; 2) the animal is able to cope, but does so with difficulty and the stressor is barely tolerable; 3) the animal is unable to cope, tolerance is constrained, and continued exposure to the stressor is likely to be fatal. Lack of (or minimal) stimulus can also act as a stressor, evoking the various levels of coping (Broom and Johnson 1993).

There are many potential sources of stressors for captive animals, including sounds, lighting conditions, scents, temperature, substrate, constrained space or movement, feeding restrictions, and forced, abnormal social groupings (e.g.,socially-housed solitary species, or isolate-housed social species) and lack of mate choice (Morgan and Tromberg 2007). Research focused on human-livestock interactions has revealed that positive interactions like talking while moving toward an animal and patting animals, as well as negative interactions like kicking or slapping, influence the levels of corticosteroids, adverse behaviors, and productivity levels of livestock (Hemsworth and Coleman 1998). Captive black (*Diceros bicornis*) and white

(*Ceratotherium simum*) rhinoceros are more susceptible to chronic stress responses (sustained high corticoid concentrations) when they are housed with breeding partners or more exposed to the public (Carlstead and Brown 2005). Carlstead et al. (1993b) found that for laboratory cats, erratic feeding and cleaning schedules and a lack of attention (petting and talking) from caretakers resulted in higher cortisol levels in the urine, sensitivity to the lutenizing hormone-releasing hormone (LHRH) and increased time spent hiding and alert. Clouded leopards have higher corticoid concentrations when cared for by a greater number of keepers (Wielebnowski 2002). Housing is also important, with clouded leopards in the sightline of larger carnivores having higher cortisol levels compared to those with no nearby large carnivores (Wielebnowski 2002).

Stimulus opportunities or lack thereof, in regards to stressors will influence animals' ability to cope with captivity (Baer 1998). The natural environment provides constant stimulation that elicits continuous responses from individuals. In captive environments, natural activities and stimulation (such as foraging, exploring territory or competing for mates) are controlled and in many cases restricted. Continuous understimulation produces one of two potential responses (Broom and Johnson 1993): 1) apathy or 2) self-generated stimulation that materializes as pointless repetitive behaviors referred to as abnormal repetitive behaviors (ARBs) including stereotypic behaviors.

Stereotypic behaviors and other ARBs are caused mainly by "motivational frustration and/or brain dysfunction, with possible contributory roles also being played by habit formation and 'coping' effects" (Mason et al. 2007). The motivational frustration is often food related, as

Mallapur and Chellam (2002) found with Indian leopards (*Panthera pardus*), where the approach of feeding time is associated with increased stereotypic pacing. Frustration associated with confinement can also stimulate the development of stereotypy over time, as it did in Montaudouin and Le Pape's study (2005) in which adult brown bears exhibited more stereotypy than young bears when housed indoors overnight. Pacing is a common stereotypic behavior exhibited by carnivores (Clubb and Mason 2007), including clouded leopards (Wielebnowski et al., 2002), that may develop in attempts to cope with stressors.

How are Aggression and Stress Managed in Captive Animals?

Aggressive behaviors of captive animals, like other behaviors, are influenced by elements of captivity including environmental, territorial and social restrictions, human and various other species' presence, as well as the animal's ability to cope with these stressors (Carlstead 1996). Most zoos have strict, species-specific protocols for managing aggression and stress responses in captive wildlife with the goal of maintaining the safety of the public and the keepers, and the well-being of the animals. For some species, especially those susceptible to stress or prone to aggressive behaviors, there are husbandry manuals generated by Association of Zoos and Aquariums (AZA) members (e.g. Fletchall 2000). Basic husbandry usually combines animal training (Laule and Desmond 1998) with keeper and caretaker methodology protocols (Bush 1996; Flanagan and Tsipis 1996; Mellen and Ellis 1996), and it is through this husbandry that stress and aggression are typically managed.

Enrichment refers to changes made to the captive environments or management strategies for animals, with the explicit goal of improving the individual animal's biological responses to

11

their environment. Changes are true enrichment only when the animals benefit biologically from them, such as in improved inclusive fitness, more surviving offspring, or related elements like improved health and well-being (Newberry 1995). Enrichments such as adjustment to enclosures (e.g., climbing structures, novel objects) and husbandry changes (e.g. clicker training, novel foods) have been shown to reduce, though not abolish undesirable behaviors like stereotypic pacing (Clubb and Mason 2007). Providing naturalistic enclosures and live prey as enrichment prior to release of captive-raised animals has increased their survival rate in the wild (Castro et al. 1998; Miller et al. 1998). Carlstead et al. (1993a) found that providing enrichment, in the form of branches and hiding places, facilitated coping with a changes to housing conditions and reduced levels of urinary cortisol in leopard cats (*Felis bengalensis*).

Many studies have been completed in recent years to assess the influence of enrichment on behavior and hormones of mammals and birds. McAfee et al. (2002) found that horses provided with mirrors angled to provide an outdoor view performed less stereotypic weaving while in their box stalls. Conures (*Pyrrhura perlata perlata*) provided with enrichment showed increased activity and reduced preening, and a stabilization of plumage problems (van Hoek and King 1997). Novel items such as spices and frozen fish, provided as enrichment to several felid species, have resulted in a significant increase in active behaviors (including swimming, playing alone, social interactions, rolling, and vocalizing) and a significant reduction in time spent pacing (Skibiel et al. 2007). Shepherdson et al. (1993) found that fishing cats (*Prionailurus viverrinus*) offered the opportunity to hunt for live fish showed increased activity, used more of their enclosure space, and slept less (Maple and Perkins 1996). Moreira et al. (2007) discovered that moving some female tigrinas (*Leopardus tigrinus*) and margays (*Leopardus wiedii*) from large

enriched enclosures into smaller barren enclosures increased corticoid concentrations and decreased ovarian follicular activity, suggesting that enrichment in the captive environment may be essential for breeding success. Monitoring the physiology, including hormone levels, of the felids in these studies facilitated improved understanding of the impact of housing and husbandry factors on reproductive fitness. For captive clouded leopards, enrichment items such as nestboxes and climbing structures have become key components of husbandry (Fletchall 2000).

In instances where training and basic husbandry methods have not reduced incidence of aggression or stress, some research has revealed effectiveness of chemical remedies. Gage (2005) found that a female American badger (*Taxidea taxus*) who intermittently displayed anxiety and self mutilation, did not display any of the abnormal behaviors during treatment with buspirone, an anti-anxiety medication. An aggressive male Grant's zebra was treated with altrenogest, and Zehnder et al. (2006) found that though an initial low dose seemed to have no effect, a higher dose, later lowered, appeared to resolve the aggression. Though pharmacological treatments can be quite effective in reduction of adverse behaviors, they are usually not a permanent solution (e.g. when drug treatment stops, the detrimental behaviors return).

Hormone Monitoring of Wildlife

Historically, blood hormone levels have been used to monitor health and stress, and to understand the reproductive physiology of animals. Many hormones are found in blood, but it is only with repeated sampling that patterns become visible. Collection of serial blood samples from tractable animals allows plotting of hormonal patterns over time. In some animals, such as laboratory rats and zoo elephants, blood samples are an easy and reliable source for hormone

monitoring. However, it is not practical (and could be quite dangerous) to attempt collection of sequential blood samples from most wildlife (Pukazhenthi and Wildt 2004). In addition, repeated sampling can itself induce a stress response and confound blood glucocorticoid results (Reburn et al. 2000).

Saliva, hair, urine, and feces also retain hormones frequently monitored in research, such as corticosteroids and reproductive hormone metabolites. The use of urine or fecal samples for hormone analysis minimizes stress on the animal, is safer for personnel, and provides biologically accurate and robust endocrine data to researchers (Carlstead et al. 1992; Brown et al. 1994; Brown et al. 1995; Terio et al. 1999; Möstl and Palme 2002; Robeck et al. 2004; Palme et al. 2005; Pelican et al. 2006) when suitably validated (Millspaugh and Washburn 2004; Palme 2005; Touma and Palme 2005). Felid hormone metabolites are almost entirely excreted in feces, with limited amounts in the urine (Brown 2006). Non-invasive fecal hormone monitoring has been used successfully in many free ranging (Creel et al. 1997; Berger et al. 1999) and captive (Young et al. 2004; Pelican et al 2006) carnivore species, including felids, to assess stress hormone levels associated with management changes (Wielebnowski et al. 2002), determine cyclicity and seasonality in male and female cats (Brown et al. 2002) and assess hormone protocols for assisted reproduction (Brown et al. 1995; Pelican et al 2006). It has become a valuable asset to researchers studying free-ranging and captive wildlife.

Behavioral Observation: How are Behavior Data Collected?

Behavioral monitoring is also invaluable in the management of captive wildlife. There are different phases to the study of behavior. Martin and Bateson (1993) indicate eight steps,

including: 1) generating a question and making initial observations; 2) generating hypotheses and associated predictions; 3) selecting behavioral measures and the research design; 4) generating definitions for the measures; 5) determining suitable methods for recording behavior; 6) rehearsing recording behavior with those methods; 7) collecting the data; and 8) analyzing the data. All of these elements are essential when planning and implementing a behavior study.

Behavior can be described in multiple dimensions, including latency, frequency, duration, and intensity. Behavior is often categorized into states and events. Behavioral states are recorded when the behavior occurs over a relatively long period of time, such as sitting, lying, or walking. Behaviors classified as states are often measured for duration. Behavioral events occur for a brief period of time, such as vocalizations or distinct actions such as clawing and jumping, and are usually recorded as frequencies (Martin and Bateson 1993).

Methods for sampling behavior include *ad libitum*, focal, scan and behavior sampling. *Ad libitum* sampling is where the observer notes what is visible and what is deemed appropriate at the time, and there are no limits or expectations for time or behavior recording. Focal sampling is a common method in behavioral studies, where all occurrences of one individual's behaviors are observed for a set period of time. Scan sampling is a rapid survey technique where each animal's current behavior is recorded at set intervals. Behavior sampling is done by one or more observer for a group of individuals, where the observer records each instance of behavior. Behaviors are generally recorded in three ways, (1) with a check-sheet, either on-site by direct observation, or by observing behavior recorded on video; (2) using a computer event recorder, either where the observer uses computer keys to denote behaviors, or the animal is in an

apparatus which automatically sends an electrical signal to a computer when a specific event has occurred (e.g. pressing a lever or entering a corridor); and (3) by verbal description, involving hand-written notes or voice recordings on a tape recorder (Martin and Bateson 1993). Methods selected for use must fit the objectives of the study.

Monitoring Behavior of Captive Wildlife

Behavior monitoring has been used for a variety of purposes in zoo animals. In most zoos, keepers keep daily notes on each animal (usually in verbal description or notes) that are essential for revealing changes in health or behavior in individuals or groups. In addition to these daily notes, ethologists have used behavior monitoring to reveal species-specific stressors (Wielebnowski et al. 2002; Wielebnowski 2003; Carlstead and Brown 2005), the effectiveness of enrichment (Carlstead et al. 1993a; Skibiel et al. 2007), and reproductive problems or potential for problems (Lindburg and Fitch-Snyder 1994; Wielebnowski and Brown 1998; Carlstead et al. 1999; Wielebnowski 1999; Carlstead and Brown 2005). In every case, behavioral monitoring is also essential for understanding, often for the first time, normal and abnormal behavior in rare and under-studied wildlife species.

Felid Social Systems

Most felids are solitary and territorial, with offspring dispersing after weaning. Lions (*Panthera leo*) are the only known wild felid to live in multi-generational matrilineal groups with changeable patriarchal dominance by a single male or a coalition of males (Yamaguchi et al. 2004), although feral cats (*Felis domesticus*) can also be found in extended family groups. Anecdotal accounts suggest that in some other species, pairs will occasionally travel and hunt

together, including mating pairs or related, same-sex pairs. Young cheetahs often stay together after weaning, but eventually the females become solitary. Male cheetahs, however, are frequently seen in bachelor pairs or groups (Nowak 1999). Monogamy is very uncommon in mammals and does not exist in felids where males have low reproductive investment in the offspring (Alcock 2005). Only when ecological factors facilitate females' ability to live in small, defendable territories will the cost-benefit ratio lean toward male monogamy (Alcock 2005).

Berger and Stevens (1996) provide definitions for mating systems that apply to felids. They describe "promiscuity" as "the absence of any pair bond or long-term relationship between the male and female." If a male occupies a large territory, it is likely that there is more than one female with an overlapping territory, and he would mate with each female in estrus that he encountered. The common term used for the felid mating system is "polygyny." This mating system is characterized by males mating with more than one female during the breeding season, and males having virtually no parental responsibility (Berger and Stevens 1996). Females make considerable investment in their young and, thus, female felids are generally choosier about their mates than male felids (Estep and Dewsbury 1996).

In theory, when males have nothing to offer but sperm, the natural mate choice mechanism for females would be to select for males with traits such as a healthy, strong appearance, brighter colors, or complex displays (Alcock 2005). Felids do not have bright coloring (most likely an adaptation to maintain ability to surprise prey) or complex displays and, with most species being solitary, opportunities for breeding are rarer than for social species. Thus, female felid mate selection likely occurs primarily through sexual selection in the

reproductive tract (Lariviere and Ferguson 2003). This may be influenced by dominance of the male, including which male is dominant in the territory or which male won a fight for the female in her presence (Alcock 2005).

Captive breeding currently focuses on maintaining genetically diverse populations and not on mate choice (Grahn et al. 1998). In captivity, due to limited capacity for housing numerous animals in most facilities, providing each animal with mate choice is not practicable (Grahn et al. 1998). Animals in the wild will naturally disperse, thereby avoiding inbreeding (Alcock 2005), but in captivity, especially with limitations of facilities and difficulties with pairing some species, inbreeding can be the only option for some captive animals (Grahn et al. 1998). The current clouded leopard captive population faces this dilemma (Fletchall 2007).

Captive Breeding: Why is it Needed? Why Difficult for Some Species?

Estimation by Magin et al. (1994) indicates that only 34% of species listed as threatened are maintained in the world's zoos. Since the early 1970s, the primary objective of zoos has changed from simply maintaining a collection for public display, to becoming an essential tool in conservation through fundraising support for both *in situ* and *ex situ* research. In following the policy of the International Union for the Conservation of Nature, zoos work to establish self-sustaining captive populations (needing no additional wild-caught individuals to add to the gene pool), with the ultimate goal being reparation or reinstatement of strong wild populations (Curio 1998). Currently, only about 23% of captive populations managed by Species Survival Plans (SSP) in North America are self-sustaining (Wielebnowski 1998). Two primary reasons for this low percentage are a high infant mortality and irregular or no reproductive success

(Wielebnowski 1998).   In most species, therefore, research is desperately needed to improve

breeding success (Snyder et al, 1997; Clubb and Mason 2007).

Clouded Leopards

Smallest of the large cat species, clouded leopards have, until recently, been widely

accepted as the only members of their genus, *Neofelis*, with four sub-species, including two on

mainland Asia and two on Asian islands (Buckley-Beason et al. 2006). However, recent

deoxyribonucleic acid (DNA) and morphological research has revealed differences between the

mainland species (*Neofelis nebulosa*) and the clouded leopard species existing on the islands of

Sumatra and Borneo (*Neofelis diardii*: Buckley-Beason et al. 2006; Kitchener et al. 2006).

Mainland clouded leopards (*Neofelis nebulosa*) are a lighter, tawny color, have small, faint spots

within several large "clouds" (from which they draw their common name) that stretch the full

height of the side with a fore to aft angle, and a partial double stripe along their backbone

(Kitchener et al. 2006). Members of the island species (*Neofelis diardii*), however, have darker,

more grey fur with distinct spots inside smaller clouds and a distinct double stripe along the

backbone (Kitchener et al. 2006). DNA analysis revealed greater genetic distances between the

two species of clouded leopard than between several *Panthera* species, including jaguar, lion and

tiger (Buckley-Beason et al 2006). It is the mainland species (*Neofelis nebulosa*) that is the

focus of this thesis.

The mainland clouded leopard is listed as Endangered by the U.S. Fish and Wildlife

Service, and is listed as an Appendix I species under CITES. Clouded leopards are officially

protected in most countries within their natural range, but enforcement in many areas is weak. It

is estimated that the mature, breeding population of clouded leopards (*Neofelis nebulosa*) in the wild is below 10,000 animals (Cat Specialist Group 2002). The reduced number of pelts encountered at fur markets and sightings of live clouded leopards by resident peoples within their range suggest that the species is in decline (Shoemaker 1998). Clouded leopards are frequent victims of habitat destruction and illegal hunting. Clear cutting of forests for use as agricultural lands is the primary threat, as the clouded leopard requires large tracts of forest for hunting. They are widely hunted for their teeth, decorative pelt, and bones for the traditional Asian medicinal trade (Kitchener 2006). Urban markets in Myanmar, Laos, Vietnam Cambodia, Nepal and Thailand continue to sell large numbers of pelts (Cat Specialist Group 1996). Resident peoples within the natural range of the clouded leopard have reported a decreasing number of sightings, which is the basis for the estimate of decline for the species (Fletchall 2000).

Natural History and Biology

Native to the forests of Southeast Asia, ranging from Nepal, Bangladesh, and Assam (eastern India) through Indochina, and northeastward to southern China and formerly Taiwan, clouded leopards live a combined terrestrial and arboreal life (Shoemaker 1998). Adaptations for life in the trees include short legs, large paws, and a long tail used for balance as they leap from tree to tree. Their arboreal talents include running headfirst down trees, scrambling across horizontal branches upside down, and even hanging by their back feet to reach down from branches. Though their hunting methods have not been recorded, it is believed that clouded leopards stalk their prey along the ground as well as ambush prey from the trees (Fletchall 2000). Clouded leopards have the longest canine teeth in proportion to their size of all cats (Brakefield

1993), of which the posterior edge is very sharp (Guggisberg 1975). Males weigh between 35 and 50 pounds (16 to 23 kg), and males are often twice the size of females (Nowak 1999).

Radio-tracking Studies

Only seven clouded leopards have been radio-collared in the wild. The first clouded leopard that was radio-collared was a sub-adult male captured in Nepal invading a chicken pen, transported to the Sauraha, Royal Chitwan National Park, released, and tracked for just over ten days. During the first nine days he stayed in a one square kilometer area around the release site. On the tenth day he was radio-located eight kilometers from the release site, and observed continuing for the next few days to move in the direction of the capture site, about 350 kilometers away (Dinerstein and Mehta 1989).

Research by Austin (2002) and Grassman et al. (2005) was focused on clouded leopards in Thailand, in the Khao Yai National Park and Phu Khieo Wildlife Sanctuary, respectively. Two males and two females were tracked in the Grassman et al. (2005) study for seven to seventeen months, totaling between 62 and 133 locations for each cat. The Austin (2002) study included one male and one female, tracked for three and five months respectively, with locations collected three to five times per week. The male was then located once per week for three and a half more months until found dead, while the female was also located once per week for the next twelve months (Austin 2002). These studies revealed that territories of males and females in Thailand overlap and range in size from 22.9 to 45.1 km^2. Movements within territories were interpreted to be associated with the hunting of hog deer (*Axis porcinus*) and muntjak (*Muntiacus*

muntjak) (Austin 2002, Grassman 2005). Austin's (2002) work also revealed that clouded leopards seem least active between 10 am and 2 pm.

Stress in Captive Clouded Leopards

More than most other felids, clouded leopards are believed to be susceptible to captivity stress, as evidenced by a variety of health and behavior problems (Fletchall 2000; Wielebnowski et al. 2002). Some of these behavior problems include stereotypic pacing, fur-plucking, and hiding. The "cloudie stare" is another abnormal behavior considered by the clouded leopard caretakers and researchers to be a sign of stress, usually involving a tense, crouched stance, with a direct, highly-alert stare that does not seem focused on any specific thing. Wielebnowski et al. (2002) reported that the number of keepers was positively correlated with fecal corticoid concentrations, while enclosure height and number of hours the keepers spent with study animals were negatively correlated with fecal corticoid concentrations. Self-injuring clouded leopards, those who displayed fur-plucking or bit/chewed at their tails, also had higher concentrations of fecal corticoids (Wielebnowski et al. 2002).

Challenges to Breeding Clouded Leopards in Captivity

Very little is known about wild clouded leopard breeding behavior. In several species of felids in the wild, males stay with the female only during estrus, mate-guarding and mating with the female for a few days until estrus ends, after which the male moves off (Wolff and Macdonald 2004). In captivity, many zoos structure most felid breeding, such as with tigers and cheetahs, in a manner similar to the wild scenario, where the animals are introduced for breeding, and then separated.

There are 223 clouded leopards presently in captivity at 69 facilities worldwide, with 72 animals in the North American SSP at 31 institutions, including 32 males and 40 females (Fletchall 2007). The only animals considered potential or proven breeding animals in the population amount to eight males and 18 females (Fletchall 2007). Only four percent of the SSP animal pedigrees can be followed back to the thirteen founder animals represented in the current captive population, but most of the animals in the current SSP are descended from 16 siblings born to one male and three females (Fletchall 2007). For the current population to be maintained at the present level, 10-12 kittens need to be born each year within the SSP, based on genetic analysis of the population. To attain 85% genetic diversity, every 5 years for the next 20 years the SSP will need to import 4 unrelated animals from outside the North American SSP (Fletchall 2007).

Captive clouded leopard pair formation has rarely been successful with males older than 12 months of age. Formation of adult pairs, at least in captivity, often results in injury or death of the female through attacks by the male (Fletchall 2000; Law 1996; Law and Tatner 1998; Kitchener 1999; Yamada and Durrant 1989). Between June 1999 and December 2002, out of 22 adult female deaths reported in the International Studbook for the Clouded Leopard, over 15% were adult female mortalities as a result of male aggression (Fletchall 2002). A 1997 survey of international institutions found that 18 of 28 respondents had pairings resulting in female injuries and mortalities due to male attack (Fletchall 2000). When males have injured or killed a female, they are unlikely to be paired again as it is assumed that future pairing will lead to further injuries. Clouded leopard specialists have observed that pairing attempts within the North American SSP appear to be more likely to succeed when males paired are juvenile males

(Fletchall 2000). Although based on low numbers and not subjected to statistical analysis, experienced managers have used this tactic for over a decade and testify to its utility in pairing this difficult-to-breed species (JG. Howard and K. Pelican, personal communication).

Another challenge to pairing clouded leopards is variability in estrous cycle length. Although many solitary cat species are induced ovulators, some cat species, including clouded leopards, can be both induced and spontaneous ovulators (Howard et al. 1996). Pelican et al. (2006) found that 3 of 7 females in their study were spontaneous ovulators. At the same time, estrus is difficult to identify based on behavior alone. Many females don't show obvious behavior changes. Some females, however, will display lordosis and treading as well as rolling which are seen in both domestic and wild cats (Leyhausen 1979). Combining male aggression with this difficulty in determining the optimal time to put animals together based on estrus, makes a slow introduction process for breeding clouded leopards necessary. This allows time for the cats to become acquainted and the caretakers to closely monitor behavior and make sure the female is not injured.

When successful mating has occurred, females will usually bear two to four young after a gestation of about 90 days. The young reach independence in less than one year, though 41% of cubs born die within the first year (Fletchall 2002). Hand-raised cubs have a higher survival rate (84% >1yr) than mother-raised cubs (59% >1yr), so most zoos hand-raise cubs (Fletchall 2002). March is the most common month for cub births (21%), though mating and birth happen year round (Fletchall 2002).

Assisted reproductive techniques, like artificial insemination, would be especially useful to the captive clouded leopard population. With hormone treatments to induce ovulation, intrauterine artificial insemination (AI) has been attempted using fresh electroejaculated sperm with no fewer than 24 female clouded leopards, but only one female has produced cubs (Howard et al. 1996; Howard et al. 1997). Research continues to identify efficient methods for freezing sperm (Pukazhenthi et al. 2006) and appropriate hormones and dosages to obtain essential responses for successful AI (Pelican et al. 2006), as well as to understand pairing behaviors, causes for the inter-sexual aggression, and methods to improve pairing for natural breeding.

Only two clouded leopard pairing studies have been conducted, involving a total of 12 animals in 10 pairing attempts (Law and Tatner 1998; Zimmermann, unpub.), where one study had one successful pair, and the other had a successful pair at one zoo and two successful pairs (one female, two males) at a different zoo. Though some information in Zimmermann's (unpub.) study indicated a correlation between corticoid concentration and pacing for one female and a correlation between corticoid concentration and hissing for another female, very little information has been compiled to explain the difficulty in pairing this species or provide reliable predictors for success. None of the pairs in these studies were juvenile-male pairs and, thus, no comparison has previously been made between juvenile-male and adult-male pairings.

The Thailand Breeding Program

In 2001, the Smithsonian Institution in collaboration with the Clouded Leopard SSP, the Nashville Zoo and the Thailand Zoological Park Association took the lead in forming a consortium to develop a self-sustaining clouded leopard breeding program in Thailand. The

Khao Kheow Open Zoo (KKOZ) in Chonburi was identified as an ideal location for this project. KKOZ had multiple large enclosures, originally built as flight cages for hornbills, which provided suitable housing for pairing clouded leopards. Thailand was also an excellent site for a breeding program since, as a range country, it had a large number of wild-born clouded leopards, a key genetic resource for the captive population. Twenty-three of the original 27 animals in the program were wild-born. This provided a unique opportunity to study, for the first time, multiple pairs of primarily wild-born cats, and compare successful to failed pairs sharing identical housing conditions and husbandry methods that were recommended by the SSP (Fletchall 2000; Wielebnowski et al. 2002).

OBJECTIVE AND HYPOTHESES

The objective of this study was to identify potential behavioral and hormonal indicators of successful pairing during early stages of introductions of clouded leopard pairs. For the purpose of this study, successful pairing of clouded leopards was defined by copulation of the male and female, resulting in cubs born, while a failed pairing was one in which the male attacked and injured the female, or when the program manager deemed further introduction attempts would not result in a successful pairing. Also, in this study a juvenile male is defined as less than one year old, and a juvenile male pair involved a pairing started when the male was juvenile, with a female of variable age. I compared successful to failed adult-male and juvenile-male pairs of these cats housed in the Khao Kheow Open Zoo in Thailand. Due to the combination of the captive setting (Morgan and Tromberg 2007), the forced interactions between potential breeding pairs (Carlstead and Brown 2005), the difference in cat behavior when presented with environmental enrichment (Carlstead et al. 1993a), and this species' historic

difficulties in captivity (Wielebnowski et al. 2002), I made the following hypotheses and associated predictions.

First, I hypothesized that *animals in failed breeding pairs exhibit more behavioral and physiological signs of stress during early stages of introduction than animals in successful breeding pairs (H_1)*. Predictions arising from this hypothesis were:

P1: Males and females in failed pairs have higher overall pairing baseline, mean and peak mean fecal cortisol metabolite levels than those in successful pairs.

P2: Both sexes in failed pairs spend more time pacing than animals in successful pairs.

P3: Both sexes in successful pairs display more exploratory behaviors (rub on object, clawing, sniff object, flehmen, urine-spraying, rolling) than animals in failed pairs.

Secondly, I hypothesized that *interactions are less positive in failed pairs than successful pairs during the early stages of pairing (H_2)*, and the predictions were as follows:

P1: Both sexes display more aggression (growl/hissing or fighting) in failed pairs than successful pairs.

P2: Both sexes display more affiliative behavior (sniff other cat, groom other cat, rub on other cat) and more positive vocalizations (prusten) in successful pairs than failed pairs.

Due to the common difficulty of pairing, and females often being injured during pairing attempts (Fletchall 2002), as well as Wielebnowski et al.'s (2002) findings that there was a significant between-sex difference in corticoid concentrations within feces of clouded leopards, I

hypothesized that *pairing is more stressful for females than males and, thus, that females show more physiological and behavioral signs of stress than males (H₃)*, with the following prediction:

P1: Females spend more time pacing, display more growl/hissing, and have higher baseline, mean, and peak fecal cortisol metabolite levels than males during early stages of pairing.

Finally, following the observations of clouded leopard specialists, I hypothesized that the variable *juvenile-male positively influences pairing success (H₄)*, with the associated prediction:

P1: Successful pairs are more likely than failed pairs to comprise juvenile than adult males.

RESEARCH DESIGN AND METHODOLOGY

Animals, Housing and Husbandry

All clouded leopards included in this study (Table 1) were housed at the Khao Kheow Open Zoo in Thailand. Each animal had a covered, cement-floored, individual holding area (Figure 1, mean area 14.5 m^2) including one or more nest boxes and nest boards. Males and females within pairs alternated in large, vegetation-rich enclosures (23 m long x 7.5 m wide x 9 m high. Figures 2 to 4). The large enclosures and holding areas had logs for climbing and perching, and the trees in the large enclosure provided the same opportunities. These large enclosures also had water pools (Figure 4) filled intermittently, and cats were occasionally given cargo nets, ladders, and boomer balls for enrichment. When animals alternated in the large enclosure, one or the other was given access to it at night or during the day. Animals with access to the large enclosure maintained access to their holding areas. To minimize stress,

28

determination of which animal had access was based on the neighboring animals that had access to their own adjacent large enclosures (Figure 3). For example, males were given access when there were females in the large enclosures on either side of the male, so that two males could not fight through the fence.

The Clouded Leopard Breeding Consortium supported an on-site breeding program manager and interim managers (to allow vacations for the program manager) from the USA with extensive experience in breeding clouded leopards. The Thai staff included an assistant program manager and three keepers. Water was made available *ad libitum*. Early in the project, pairs were fed raw (gutted) chicken. Live quail were also provided as enrichment. After a high pathogenic H5N1 avian influenza outbreak in the population in January 2004 (when two older, non-breeding clouded leopards died and a few others got sick as a result of eating infected quail), the diet was changed to beef with a vitamin/mineral supplement and the cats were occasionally provided with beef bones and bloodsicles (or blood "popsicles") as food enrichment. Animal health and body weight were monitored by keepers and the program manager, their diets and care adjusted accordingly, and they also received yearly physicals.

There were thirteen pairings involving 21 clouded leopards within my study (Table 2). The pairings all occurred between the years 2002 and 2006. They included ten males and eleven females in a total of five successful pairs and eight failed pairs. Two successful pairs and two failed pairs were juvenile-male pairings (Table 2). For this reason, a comparison of juvenile male to adult male pairings was made in addition to the comparison of successful to failed pairs. The influence of sex differences on success was also examined.

29

Introduction Protocol

There were five stages to the pairing process, and length of stage was dependent upon the KKOZ breeding program manager's assessment of how the pairing was progressing. When animals appeared to be consistently chiefly agreeable, or tolerant of each other, pairing attempts at the next stage would begin. By definition, successful pairs completed all stages, while failed pairs generally failed during stages one to three of pairing. All stages involving interactions occurred within the holding areas. Total days per stage for each pair are reported in Table 2. Following are the five stages:

1. Neighboring Enclosures/Protected Contact – Animals were moved into adjacent holding areas primarily separated by chain-link fence, and could see each other and interact through the fence. Determination of which animal was moved was dependent upon neighboring animals inhabiting adjacent enclosures. Paired animals began to alternate in the adjoining large enclosure. With some pairs, protected contact introductions took place – the female was locked in her nestbox (protected) and the male was allowed access to her holding area to investigate.

2. Face-to-Face – Under observation animals were allowed to meet and interact directly. As this progressed and animals were getting along, time spent together was gradually increased. At the beginning of this stage the animals were together only when being directly observed. As time together increased beyond 1 hour, animals were together when keeper or breeding manager was nearby but not necessarily having the pair under direct observation.

3. Together all Day – Animals were left together for eight or more hours each day. Some of this time was under observation and some not.

4. Together Overnight – Animals were allowed together overnight or late into the evening when the female was displaying estrous behaviors, the manager was confident of an agreeable pairing, and keepers, assistant or manager were available to monitor the pair.

5. Breeding – animals copulated, resulting in pregnancy, and were considered a successful pair. Successful pairs had different breeding habits: some bred at night only, or only out of view of keepers and program manager, while others bred during daylight hours and within sight of keepers or program manager. Copulation usually occurred multiple times over a few days, and these breeding periods all resulted in cubs. For this study, stage 5 ended 7 days post first copulation.

Fecal Sample Collection, Steroid Extraction and Enzyme Immunoassays

For fecal cortisol metabolite analysis, daily fecal samples were collected throughout the entire introduction process (Stages 1-5). Six individuals also had daily samples collected for at least a month prior to Stage 1 of introduction. For collection, fecal samples between 6 and 10 cm in length from each animal in the study were collected daily by the keepers resulting in samples every 1 to 5 days, as clouded leopards did not defecate daily. Females were fed green or blue food coloring in their daily diet, so fecal samples from each sex were easily identified. Samples were collected into 3 x 5 inch zip lock bags, name of the animal and the date were written on each bag, and samples were stored in freezers at -20°C. In preparation for hormone extraction, samples were dried within their open zip lock bags in a lyophilizer. Once dried, they were pounded with a mallet to a powder consistency. Hair and other debris were removed (when possible) using a sifting technique and the powder sample was stored in labeled 12 x 55mm plastic tubes.

The dry weight fecal boiling extraction protocol then was followed according to the *Endocrine Manual for the Reproductive Assessment of Domestic and Non-Domestic Species* (Brown et al. 2004). In short, 0.18 to 0.2 grams were weighed out into numbered 16 x 125 mm glass tubes and boiled in a 90% ethanol (EtOH) solution (with 10 % H_2O) for 20 minutes in a hot water bath. Additional EtOH was added to each sample during the boiling process to prevent samples from boiling dry. The samples then were centrifuged for 20 minutes at 2000 rpm and the supernatant was poured into identically-labeled tubes. The pellet was vortexed for 30 seconds in 90% EtOH, centrifuged for an additional 20 minutes, and the supernatant added to the first pour-off. The combined supernatant was dried completely under air, redissolved in 1 mL of methanol and sonicated for 20 minutes to free particles adhering to the vessel wall. Each extractant was then diluted 1:2 in dilution buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.15 M NaCl, pH 7.0) and stored at -20°C. Thawed extracts were re-diluted as needed (1:2-1:192) prior to analysis. Extraction efficiency was determined by adding radioactive tracer (^{3}H-cortisol) to the fecal samples prior to boiling, and recovery was calculated from methanol extractions as percentage of total recovered tracer following extraction/total added. Sample recovery was measured by a liquid scintillation counter, which detects beta levels of radiation. Samples had a mean extraction efficiency of 84.2%. After extraction recovery revealed consistent efficiency (> 80% recovery in 10 extractions), tracer was no longer added to samples. Sample concentrations with known extraction efficiency were corrected to reflect their specific recovery levels.

Fecal samples were analyzed for corticoid metabolites using a cortisol enzyme immuno assay (EIA) previously validated for this use in clouded leopards (Iseman 2005). Pooled extracts from males and females exhibited parallelism with the cortisol EIA standard curve and accuracy

was demonstrated (Iseman 2005). All hormone assays run for this project were single antibody

assays on micro titer plates and used 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

(ABTS) in the substrate to react with horseradish peroxidase (HRP) and hydrogen peroxide

(H_2O_2). Resulting color changes were recorded using a light spectrometer. The cortisol antibody

had reported 100% specificity for cortisol and 0.7 % for corticosterone. This antibody cross-

reacted with numerous polar metabolites of cortisol in clouded leopard feces (Iseman 2005). The

light spectrometer reported results in picograms per milliliter, so concentrations of hormone per

fecal sample were converted to nanograms per gram of feces to account for different sample

weights prior to statistical analysis. The EIA was performed in 96-well microtiter plates (Nunc-

Immuno™, Maxisorp™ Surface; Fisher Scientific, Pittsburgh, PA). The antibody was produced

against cortisol-3-carboxymethyloxime:bovine serum albumin in the New Zealand white rabbit

at the University of California at Davis by Coralie Munro. The antibody is used at a 1:8500

dilution. The cortisol assay sensitivity at 90% binding was 4.7 pg/well. Pooled urine hormones

historically used to monitor inter-assay variability were included in each assay as controls. Intra-

assay coefficient of variation (CV) was < 10%, while inter-assay CV was < 15%.

Behavior Observations

The ethogram providing definitions for the behavioral states and events recorded is

presented in Table 3. A sample of each behavior in the ethogram was captured on video for

reference. The behavior datasheet used for recording observations, originating from

Wielebnowski's research (a version of which was also used in Zimmermann 2002, unpub.) is

shown in Appendix 1. One behavior datasheet comprised 30 min of observation, during which

each behavioral state was recorded using instantaneous scan sampling every 2 min (total of 15

states recorded per animal for the observation period) and behavioral events were recorded from both animals continuously as they happened.

Behavior was recorded by the experienced Clouded Leopard Breeding Consortium on-site managers, the Thai program assistant and the keepers in 30 min periods. They conducted a focal observation on 9 of the 13 pairs 2 to 5 days per week, once or twice per day as time allowed. Observations were carried out at various times during the day, generally morning and afternoon observations, again as time allowed. Observers sat quietly to avoid disrupting pair behavior. Over 6 months of behavior observations were collected on most pairs. Behavior datasheets were compiled according to animals/pairs in original paper copies and entered into electronic files by pair, stage of pairing, and date. Inter-observer reliability was determined by having all observers score a video of a pairing introduction at KKOZ, and using Kendall's tau-b to measure the overall agreement between them.

Statistical Analysis

Available data for each pair by stage are indicated in Table 4, and number of behavior datasheets and fecal samples per stage in Table 5. One failed pair reached stage 4, but the female was killed the first night by the male, and no failed pair achieved stage 5, so cortisol and behavior results for failed pairs are missing for those stages. Due to unforeseeable events, some data were not collected consistently for each pair in each stage while other data varied due to differences in length of stage. There were more cortisol than behavioral data collected in each stage, so behavioral data analyses were based on stage 1-3 results only whereas all available cortisol data from stages 1-5 were analyzed.

For each animal, baseline values for cortisol metabolites were calculated using an iterative process in which values that exceeded the mean plus two standard deviations (SD) were excluded (Brown et al. 1994). The average was then recalculated and the elimination process repeated until no values exceeded the mean plus two SD. The average of the remaining values was considered baseline mean for that animal and the average of all excluded values was considered peak mean (greater than two SD above baseline). Overall (stages 1-5) and by stage means, coefficient of variation around the mean (CV), baseline mean, and peak mean were calculated for each animal.

For analysis of behavior, overall (stages 1-3) and by stage means for each behavioral state and behavioral event in the ethogram were calculated for each animal within each pair per 30 minute observation period. To determine the proportion of time spent in each behavioral state, each state mean was divided by 15 (since scans were made at 2 min intervals for 30 min), while means of events were analyzed as frequencies per 30 min period. In addition to the individual behaviors, some behaviors were summed to create combined variables for analysis as follows: Affiliative behavior = (rub on other cat + sniff other cat + groom other cat); Exploratory behavior = (rub on object + claw + sniff object + flehmen + urine-spray + roll); Active behavior = (stand + walk + run + pace).

Using Type III generalized linear models (Proc GenMod in the Statistical Analysis Service (SAS) software program, SAS Institute Inc. 2004) the likelihood ratios were determined for successful pairing as explained by: juvenile/adult-male pairings; the overall (Stage 1-3) means for behavioral states and events; and overall (Stage 1-5) and by-stage fecal cortisol

metabolite concentration mean, baseline, peak mean, and coefficient of variation (CV). Because data were not available consistently from each pair in each stage, repeated measures analyses could not be performed, so analyses by stage were conducted separately for each stage. Models were fitted comprising one or combinations of two of these explanatory variables. There were insufficient data to investigate the contributions of more than two explanatory variables simultaneously in these models. Generalized linear models were used due to the categorical nature of the response variable (successful versus failed) and some of the explanatory variables (e.g. juvenile versus adult male), and because the residuals of most of the behavior and cortisol variables were not normally distributed as determined from the Shapiro-Wilk (W) statistic in output from Proc Univariate (SAS Institute Inc. 2004). For reporting results, when the juvenile/adult male variable or other categorical variable was included in the model along with a behavioral or cortisol variable and this inclusion generated a stronger effect of the behavioral or cortisol variable, results for that variable were reported for the combined model. Otherwise, results for that variable were reported based on the model where it was tested alone.

Using the Wilcoxon Signed Rank test, differences in behavior and cortisol between the male and female of each pair were investigated by analyzing the value for (male-female) for each variable in the Univariate procedure (SAS Institute Inc. 2004) and identifying which differences produced a significant S statistic. Where significant, the sex difference variables were also investigated in Proc GenMod (SAS Institute Inc. 2004) to ascertain whether they predicted pair success or failure. Statistical tests were considered significant at $p < 0.05$.

RESULTS

The means and standard errors for all data by sex and pair success versus failure are presented in Tables 6 to 9, and statistics for significant effects are presented in Tables 10 and 11.

H_1:Prediction 1 Males and females in failed pairs have higher overall pairing baseline, mean and peak mean cortisol levels than those in successful pairs. In agreement with the prediction, males in failed pairs had higher overall (Stages 1-5 combined) pairing baseline, mean and peak mean cortisol levels than those in successful pairs, and these variables significantly predicted pair failure ($p < 0.01$; Table 10 and Figure 5). Failed males also had higher fecal cortisol metabolite levels than successful males for stage 1 baseline and stage 1 and 3 mean and peak mean ($p < 0.05$; Table 10 and Figure 6). Elevated male CVs for cortisol also predicted success in stage 2, and failure in stage 3 ($p < 0.05$; Table 10, Figure 7). In females, elevated cortisol CVs were significant predictors of success in stages 1 and 2 (Table 10, Figure 7).

Examination of longitudinal fecal cortisol metabolite pair profiles demonstrate these differences between male and female response to stressors. Examples of fecal cortisol metabolite patterns over time for successful pairs are shown in Figures 8 and 9, and for failed pairs in Figures 10 and 11. A successful female's responsiveness to stressors can be seen in Figure 8, where the female was attacked by another male through the fence and her fecal cortisol metabolite concentration increased. Male differences are also reflected in the greater number and intensity (higher fecal cortisol metabolite values) of peaks in failed males (Figures 10 and 11) than in successful males (Figures 8 and 9).

H₁:Prediction 2 Both sexes in failed pairs spend more time pacing than animals in successful pairs. There was no difference in percent of time spent pacing between successful versus failed males and females ($p > 0.05$; Table 7). Likewise, when pacing was combined in the active variable with standing, walking and running, activity did not predict success or failure ($p > 0.05$; Table 7). In males (Tables 7 and 11) and females (Tables 7 and 10), time out-of-sight significantly predicted success with successful animals spending more time out-of-sight than failed animals ($p < 0.05$; Figure 12). There was an interaction between the juvenile/adult-male variable and time spent out of sight, with successful males out of sight more than failed males in adult-male pairs but not in juvenile-male pairs and successful females out of sight more than failed females in adult-male and juvenile-male pairs (Figure 12).

H₁:Prediction 3 Both sexes in successful pairs display more exploratory behavior (rub on object, clawing, sniff object, flehmen, urine-spraying, rolling) than animals in failed pairs. The frequency of exploratory behavior was not different between successful and failed males or females when combined in one variable ($p > 0.05$; Table 8). Nor was any individual behavior in the exploration category different between successful and failed pairs ($p > 0.05$; Table 8). There was an interaction between the sex difference in rolling and juvenile/adult male pairing, with females rolling more than males and females in juvenile-male pairings rolling more than females in adult-male pairings in both successful and failed pairs (Tables 9 and 11).

H₂:Prediction 1 Both sexes display more aggression (growl/hissing, fighting) in failed pairs than successful pairs. Contrary to my prediction, fighting between the two sexes significantly

38

predicted success. When analyzed in a model with juvenile/adult-male pairing (Table 11), both males and females in juvenile, but not adult-male pairs exhibited more fighting in successful versus failed pairs (Figure 13). Also contrary to prediction, male growl/hiss predicted success whereas male meow/cry predicted failure ($p < 0.05$; Table 8, Figure 14).

H₂:Prediction 2 Both sexes display more affiliative behavior (sniff other cat, groom other cat, rub on other cat) and more positive vocalizations (prusten) in successful pairs than failed pairs. Male "groom other cat" predicted pair success when combined in a model with the juvenile/adult-male pairing variable (frequency per 30 min – successful adult-male pair: 0.82 ± 0.67; failed adult-male pair: 0.02 ± 0.01; successful juvenile-male pair: 0.86; failed juvenile-male pair: 0.32 ± 0.23; $p < 0.05$; Tables 9 and 11, Figure 15) . The male "affiliative" combined variable predicted pair success when combined in models with the juvenile/adult-male pairing variable (frequency per 30 min – successful adult-male pair: 1.2 ± 1.02; failed adult-male pair: 0.18 ± 0.05; successful juvenile-male pair: 1.16; failed juvenile-male pair: 0.48 ± 0.34; $p < 0.05$; Tables 9 and 11). Pair success was also predicted when including the juvenile/adult-male pairing variable in models with female "rub on other cat" (frequency per 30 min – successful adult-male pair: 0.05 ± 0.01; failed adult-male pair: 0; successful juvenile-male pair: 0.22; failed juvenile-male pair: 0.09 ± 0.09; $p < 0.05$; Tables 9 and 11) or female "groom other cat" (frequency per 30 min – successful adult-male pair: 0.26 ± 0.11; failed adult-male pair: 0.02 ± 0.01; successful juvenile-male pair: 0.33; failed juvenile-male pair: 0.22 ± 0.02; $p < 0.05$; Tables 9 and 11, Figure 15). Males and females in juvenile-male pairs performed these affiliative behaviors more frequently than animals in adult-male pairs, as well as animals in successful pairs performing affiliative behaviors more than animals in failed pairs (Tables 8 and 11). Female "prusten"

predicted failure when combined in a model with juvenile/adult-male pairing, with females in failed adult-male pairings performing prusten more frequently than females in failed juvenile-male pairings (frequency per 30 min – 1.06 ± 0.98 vs. 0.16 ± 0.01) and females in failed pairings performing prusten more than females in successful pairings (frequency per 30 min – 0.7 ± 0.58 vs. 0.04 ± 0.02; $p < 0.05$; Tables 8, 9 and 11).

H₃:Prediction 1 Females spend more time pacing, display more growl/hissing, and have higher baseline, mean, and peak cortisol levels than males during early stages of pairing. There was no significant difference between males and females in time spent pacing ($p > 0.05$; Table 7). The difference in frequency of growling/hissing between males and females was not significant ($p > 0.05$; Table 8). No significant differences ($p > 0.05$) in overall (stages 1-5) fecal cortisol metabolite levels were found between males and females in the Wilcoxon Signed Rank test (Table 6).

H₄: Prediction 1 Successful pairs are more likely to comprise juvenile than adult males than failed pairs. Forming pairs with juvenile versus adult males did not significantly affect success ($\chi^2 = 0.32$, $p > 0.05$; Table 2), but when combined in models with male "out of sight," female "rub on other cat," or male "groom other cat", this variable made a significant contribution to the prediction of success (Tables 9 and 11). More adult than juvenile males were out of sight in successful pairs (proportion of time spent – 0.11 ± 0.07 vs. 0.02) whereas adult and juvenile males spent a similar amount of time out of sight in failed pairs (proportion of time spent – 0.01 ± 0.01 and 0.01 ± 0.01; Figure 12). Also, the animals in the successful juvenile-male pairing were observed fighting more than animals than any other pairing combinations

(sucessful juvenile-male pairing – Male: 0.22, Female: 0.24 vs. Successful adult-male pairing – Male and Female each: 0.03 ± 0.02, Failed adult-male and juvenile-male pairings – Male and Female each: 0.01 ± 0.01; Table 9).

DISCUSSION

This was the first study to document hormonal and behavioral correlates of pairing success and failure in the endangered clouded leopard.   Three important findings were documented. First, this study establishes unequivocally that stress responses play an important role in pairing success and failure. Non-invasive fecal corticoid monitoring facilitated assessment of daily changes in stress hormone levels without the confounding influence of blood collection and thus effectively elucidated physiologic changes associated with pair introductions, a period of high male-female aggression. Results indicate that males with high cortisol levels, indicating sensitivity to pairing stress, were more likely to show aggression toward the female resulting in pair failure. Females, on the other hand, with strong stress-responses that had high variability (CV) in their cortisol levels but not greater levels overall, were more likely to pair successfully. Second, this study shows that interactions between males and females are critical to pairing success. Affiliative behaviors, particularly, play a role but even seemingly negative interactions like growling and hissing were associated with an improved chance of pairing success. Finally, this study shows that stress-related behaviors in clouded leopards are difficult to characterize. Pacing and other active behaviors were not different between physiologically stressed animals compared to animals with lower stress hormone levels.

As predicted, fecal cortisol metabolite concentration is a valuable measure to monitor during pairing in clouded leopards. This confirms previous work in domestic cats and leopard cats showing that changing management and husbandry practices increases corticoids in felids (Carlstead et al. 1993b). Contrary to prediction, however, only male fecal cortisol metabolite levels showed significant differences between successful and failed pairings. This result is surprising due to the stressful aspects of pairing on females. The finding in this study of higher female mean and peak mean values of fecal cortisol than males in successful pairs supports previous research (Wielebnowski et al. 2002) of higher fecal corticoid concentrations in females than males, but the lower female mean and peak mean than males in failed pairs does not. Many animals included in the Wielebnowski et al. (2002) study were on public display. The pairs housed at KKOZ are in a secluded facility and not open to viewing by the public. This allowed for a more natural physiological response to pairing in captivity, without the added stressor of the public, and may explain the change in sex differences between successful and failed pairs.

Results from the present study indicate that male stress response is a significant factor to consider in the success or failure of clouded leopard pairings. Whereas in some cases, male attacks upon females may have been predatory in nature, in other cases, pairing failure may have been due to male motivation for dominance over the females, such that some males may have viewed females simply as smaller conspecifics rather than as potential mates. If this was the case, it might have parallels with the physiological effects of dominance hierarchy in wild wolves (*Canis lupus*), where dominant animals had higher levels of fecal cortisol metabolites than less dominant individuals (Sands and Creel 2004). Social groups of cats have been shown to develop dominance hierarchies (Natoli et al. 2001; Crowell-Davis et al. 2004). The single

42

video of a male clouded leopard predatory attack on a female and the observation by keepers of different types of attacks (K. Pelican, personal communication) suggests involvement of different social, behavioral and physiological variables in male clouded leopard aggression towards females.

Clouded leopards are prone to stress in captivity (Wielebnowski et al. 2002; Fletchall 2000), so stress sensitivity could be involved in pairing aggression. The successful females' high coefficient of variance (CV) in cortisol metabolite levels in early stages of pairing appears to indicate their ability to respond to the stressful aspects of pairing, while the lower variance in failed females may be due to a blunting of the stress response from continuous stimulation. Since successful females also interacted more with males than failed females, perhaps the failed females' minimal interactions resulted in reduced hormonal response. The contradictory results for male CV in stage 2 versus 3 may have been skewed by differences in available data from failed males: data in stage 2 included four adult males and 2 juvenile males, while stage 3 only had one adult male (not included in stage 2) and the same 2 juvenile males. It is likely that the males that failed prior to stage 3 of pairing had different levels of stress, aggression, and stress-response compared to males that were successful in stage 2 but then failed in stage 3, so the loss of these males could be skewing the stress-response data.

Also contrary to prediction, though pacing is common in SSP clouded leopards (Wielebnowski et al. 2002), it was not statistically significant in any of the analyses for this project and was generally low (< 20%). Wielebnowski et al. (2002) explained that pacing by

clouded leopards can be an indicator of chronic stress. The apparent stress of pairing, often

involving female injury by the male, led to the initial prediction of high pacing in failed females.

When the breeding program was started at KKOZ, renovation of the empty enclosures to convert

the facility into a clouded leopard breeding center followed the recommendations laid out in

Wielebnowski et al. (2002), with the intent to reduce stress for this species. It is possible that

these clouded leopard-specific enclosures reduced stress sufficiently to minimize stereotypic

pacing in these cats. It is apparent from the data that pacing is not a sensitive indicator of

currently occurring stress in this species, supported by the fact that males with higher fecal

cortisol responses did not pace more than males with low cortisol. Many of the animals in this

study came from various locations in Thailand with different husbandry and housing. As a

result, the pacing seen in only some of the animals may have been induced by stressors earlier in

life and become emancipated from the original cause as a result of habit formation.

Though not a variable in my predictions, the proportion of time animals spent out of sight

was statistically one of the strongest predictors for success in this study. The clouded leopard

has a natural inclination to hide from human view while in captivity (Fletchall 2000;

Wielebnowski et al. 2002). With animals in successful pairs having lower anxiety associated

pairing, they may have been responding more to captivity stress. Failed pairs, on the other hand,

may have been more influenced by fear due to pairing stress and, as the animals had direct access

to each other's holding areas and nestboxes in stages 2 and 3, hiding may have been less

effective as a coping mechanism for pairing stress thus staying in sight to monitor the other

animal. It is possible that successful pairs spent more time out-of-sight together, though

comments by the observers did not directly indicate this and unequal proportions of time spent

out-of-sight between males and females make that unlikely. In future studies, novel stress-associated behaviors previously noted by clouded leopard managers such as the 'cloudie stare' may be better indicators of stress in clouded leopards than the behaviors captured on the data sheet used in this study.

As predicted, aggression plays a key role in success, though contrary to prediction, aggression statistically predicted success, not failure. That fighting, as recorded, was almost entirely found in successful juvenile-male pairs indicates that the fighting episodes may not have been serious fights, but rather play-fights between a young male and the female. This is supported by informal comments on several datasheets indicating that the fighting being recorded might be play. Play-fighting is most common in juvenile animals, and is well known to be common in juvenile felids (West 1974; Crowell-Davis et al. 2004), though we know little of play in clouded leopards. The finding that hissing/growling were also more commonly given by males in successful than failed pairs suggests that these vocalizations may occur not only during serious fighting but also during positive interactions. Results for meowing/crying were, however, consistent with the interpretation that they were indicators of a negative interaction.

Affiliative behaviors, as predicted, occurred more in successful than failed pairs, and male "groom other cat" may be an especially useful behavioral indicator for observers of future pairings in determining likelihood of success. Contrary to prediction, the prusten vocalization was not associated with pairing success. Comprehension of the function of the prusten vocalization at the start of this project might have led to predictions more in line with results.

45

While the vocalization sounds much like the purr of the domestic cat, which is a sign of contentment or encouragement (Leyhausen, 1979), a more plausible explanation for clouded leopards (following personal communication with K. Lang) is that prusten may, in fact, be used more like reassurance from the male to build the female's trust, or as appeasement by the female during pairing to avoid confrontation. This finding, in conjunction with the greater amount of play fighting and affiliative behavior in successful pairs, demonstrates that the quality of male-female interactions plays a key role in the success of pairing in clouded leopards and that finer grained analysis of vocalizations could be revealing.

In between-sex comparisons, pacing was not a strong marker for understanding the complexities of pairing, likely due to pacing not being closely tied to physiological stress in this study. Though the difference in growling/hissing between males and females was not statistically significant, further study may further clarify this difference. Fecal cortisol metabolite measures revealed no sex differences.

The breeding program was started at KKOZ in 2002. Fecal collection started at the inception of the program, and the behavior datasheet was translated into Thai so that behavior data collection could start shortly thereafter. There were numerous pairs in this study, all at one location with the same housing and husbandry variables – a luxury rarely afforded those who research captive wildlife. There were many challenges to creating a successful breeding program in Thailand, involving unforeseen and uncontrollable variables. These included escaping animals, avian influenza, and tainted meat resulting in sickness and mortality of some animals.

There was much hard work involved, with project managers and keepers having responsibility for all aspects of the program, including all keeper duties, husbandry, long, laborious introductions of dangerous animals, while at the same time providing round-the-clock care to hand-raise cubs. Breeding program staff worked seven days per week, often 24 hours a day and staff members were not always able to adhere to optimal experimental procedures during this study. As a result, fecal collections and behavioral observations were sometimes uneven. This was, after all, primarily a breeding program and this research emerged from that focus. However, all the hard work paid off, not only in producing 35 new clouded leopards in captivity (offspring of the successful pairs in this study), but also in providing the ground-breaking data presented in this thesis. This is the first time in the history of clouded leopard breeding that enough clouded leopard pairs were formed in one place to be able to study the dynamics of pair success and failure. This is especially critical now, since no new clouded leopard pairs have been formed in North America in over 3 years. The results presented here will be critical to creating a sustainable captive clouded leopard population as a hedge against future extinction of this beautiful species. It would not have happened without the dedicated and passionate commitment of all the people involved in the Thailand Clouded Leopard Breeding Consortium.

That said, the power of hindsight provides us with opportunities to recommend some practical changes to future projects to improve data collection and study design to better fit the vagaries and difficulties that projects such as these present. Given the challenges to the staff's time, future researchers may want to fund an independent person to focus on data collection, thereby reducing the burden on the program staff and improving the ability to collect data according to experimental protocols. In designing a future pairing study, I would also

recommend obtaining behavior and hormone data from each cat before and after pairing attempts to provide a control for levels observed during pairing. Extra staff would facilitate this additional work. I would also recommend a comparison of morning and afternoon activity levels, or conducting a keeper survey involving questions with that objective, prior to determining time of day for behavior watches during pairing attempts, because personal communication indicates that there was more activity in the afternoon. Attention to location of the animals when they are out of sight may provide a stronger assessment of time spent interacting. This would likely clarify the importance, or lack thereof, of the out-of-sight, activity and exploration variables in predicting successful pairing. Altering the behavior datasheet (suggestion concurred in personal communication with N. Wielebnowski) to accommodate play fighting and perhaps including the "cloudie stare" (the abnormal behavior discussed in the introduction that is considered by the clouded leopard community to indicate a stressed clouded leopard) is also recommended. A temperament assessment, as well, may be useful for predicting pairing success and failure, as it was for cheetahs (Wielebnowski 1999). In all, despite the stress and difficulty of developing a pairing study around an active and understaffed breeding program for this challenging species, the importance of the resulting information and production of 35 clouded leopard cubs speaks to the value of this effort.

I shall be running additional analyses to investigate a possible correlation between time of day for observations and proportion of time spent out of sight. Analyses will also be run that correct for time out of sight to further clarify the significance of activity during pairing. Finally, I shall be running supplementary analyses to investigate the influence of the mother/hand-raised and wild/captive-born variables on success.

In conclusion, there are clear behavioral and endocrine correlates of pairing success in clouded leopards. This study shows that affiliative interactions between male and female clouded leopards are important for pairing success. Monitoring of fecal cortisol metabolite concentrations revealed male sensitivity to pairing stressors and female responsiveness to stressors during pairing. This study also clearly showed that both sexes influence pairing success or failure in this challenging species.

CONCLUSIONS

1. Stress-sensitive or anxious (high cortisol) males are more likely to fail.

2. Females with strong stress responses (high cortisol CV) are more likely to be successful.

3. Interactive behaviors (including affiliative behaviors and play fighting) predict success, especially male grooming of females.

4. Successful pairs spend more time out-of-sight.

5. Pacing is not an indicator of success or failure.

6. There are behavior differences between juvenile- and adult-male pairs, but these differences do not consistently predict success of one more than the other.

7. Males and females both play a part in pair success or failure.

Table 1. Animal Information

Names	Wild-Born or Captive-Born	Mother-Reared or Hand-Reared	Age at 1st Pairing in Program	Age at 2nd and 3rd Pairing in Program
Males				
Songkhla	Unknown	Unknown	~ 3 yrs	-
Cheang	WB	MR	1 yr	1 yr 2 mo
Sompon	WB	MR	8 yrs 3 mo	-
NoName	WB	HR	4 mo	3 yrs 7 mo
Wanchai	CB	HR	10 mo	-
Mei	CB	MR	10 mo	-
Ken	WB	MR	6 yrs	9 yrs
Arawan	WB	MR	2 yrs 1 mo	-
Rick	CB	HR	1 yr 6 mo	1 yr 9 mo; 2 yrs 9 mo
Sakdaa	CB	HR	10 mo	-
Females				
Numfun	CB	MR	4 yrs 5 mo	-
Lamoon	WB	MR	2 yrs 6 mo	2 yrs 8 mo
Somwang	WB	MR	5 yrs	-
Juujii	CB	HR	4 mo	-
Mini	CB	MR	7 mo	1 yr 9 mo
Nok	CB	HR	2 yrs	-
Gaint	WB	MR	2 yrs	5 yrs
Numphung	CB	MR	2 yrs 11 mo	-
Somsri	WB	MR	3 yrs 6 mo	-
ThapThim	CB	HR	1 yr 3 mo	-
Maesa	CB	HR	1 yr 8 mo	-

Table 2. Experimental Groups

Pairs	Juvenile or Adult Male Pairing	Stages: Number of Days for Each				
Successful: Male, Female		*1*	*2*	*3*	*4*	*5*
Songkhla, Numfun	Adult	144	158	331	0	7
Cheang, Lamoon	Adult	118	240	52	0	7
Sompon, Somwang	Adult	0	44	50	101	7
NoName, Juujii	Juvenile	0	0	0	507	7
Wanchai, Mini	Juvenile	34	48	265	68	7
Failed: Male, Female						
Cheang, Gaint	Adult	27	18	0	0	0
Ken, Numphung	Adult	0	0	16	66	0
Arawan, Lamoon	Adult	61	1	0	0	0
Rick, Somsri	Adult	229	0	0	0	0
NoName, ThapThim	Adult	41	182	0	0	0
Rick, Maesa	Adult	9	122	0	0	0
Mei, Nok	Juvenile	42	22	97	0	0
Sakdaa, Gaint	Juvenile	5	26	481	0	0

Table 3. Ethogram

Behavior Terms	Definitions
Behavioral States	**Instantaneous scan sample every 2 min for 30 min**
Lie	Cat lies in horizontal reclining position, may or may not be asleep
Sit	Cat sitting on hindquarters in upright position with forelegs braced
Stand	Cat remains motionless while in upright position on all four feet
Walk	Cat walks at a moderate pace in a directed manner
Run	Cat moves swiftly
Pace	Cat walks or runs back and forth in a repetitive, non-directed pattern (stereotypic movement)
Out of Sight	Cat is out of view from observer
Behavioral Events	**All occurrences of vocalizations and flehmen recorded for 30 min; each bout recorded for all other behaviors**
Rub on Object	Cat rubs on an object (e.g., fence, log, etc.) with cheek or head and may continue rubbing along entire length of body
Rub on Other Cat	Same as above except that cat rubs on another cat not on object
Roll	Cat rolls on back and rubs back against ground, rolling back and forth
Urine Spray	Cat sprays urine in standing or squatting position
Claw	Cat scratches object, often wood, with front claws
Meow/Cry	Either short high-pitched meow call, or loud extended crying call
Prusten	Soft expulsion of air through lips, similar to snorting in horses. Cat may raise muzzle while vocalizing. Often used in 'friendly' greeting or reassurance context
Sniff Other Cat	Cat examines an object with its nose
Sniff Object	Cat examines another cat with nose
Flehmen	Open-mouthed grimace, often with curled upper lip, tongue may or may not protrude out of mouth. This behavior is usually seen after cat sniffs urine, feces or body of another cat
Groom Self	Cat cleans itself by licking
Groom Other Cat	Same as above except performed on other cats
Fight	Cat growls, hisses, hits, attempts to bite or bites other cat
Growl/Hiss	Growl is a low-pitched throaty rumbling sound; hissing is a rapid expulsion of air, teeth exposed and nose wrinkled. Both vocalizations are usually performed in an aggressive context
Lordosis	A behavior usually only seen in females and in the context of mating. Female raises her hindquarters while lowering frontquarters, tail maybe positioned to one side. This position usually indicates her willingness to mate.
Mount	Male straddles over female's back with his front and hind legs. Successful intromission may or may not occur.

Table 4. Available Data. Endo=Endocrinology. Within "Endo," M=Male, F=Female, indicate > 10 fecal samples collected. O=1-10 samples/cat. Within "Behavior," X=Pairing behavior collected for pair. Totals are numbers of pairings represented for analysis. Empty cells indicate no data collected.

Pair (M/F)	Successful	Failed	Stage 1 Endo	Stage 1 Behavior	Stage 2 Endo	Stage 2 Behavior	Stage 3 Endo	Stage 3 Behavior	Stage 4 Endo	Stage 4 Behavior	Stage 5 Endo	Stage 5 Behavior
Songkhla/Numfun	X		F	X	MF	X	MF	X			MF	
Cheung/Lamoon	X		MF		O	X	O	X			O	X
Sompon/Somwang	X				MF	X	MF		MF		MF	
NoName/Jujii	X		F		MF	X		X	MF	X	MF	X
Wanchai/Mini	X		F	X	MF	X	MF	X	MF	X	MF	X
Mei/Nok		X	MF		MF	X	MF	X				
Cheung/Giant		X	MF		MF							
Ken/Numphung		X			MF		MF		MF			
Arawan/Lamoon		X	MF		MF							
Rick/Somsri		X	MF	X								
Sakdaa/Giant		X	MF	X	MF	X	MF	X				
NoName/ThapThim		X	MF		MF	X						
Rick/Maesa		X	MF		MF	X						
Totals	5	10	13	4	11	7	7	5	4	2	5	3
Total Succesful	5		4	2	4	3	3	3	3	2	5	3
Total Failed		10	9	2	7	4	3	2	1	0	0	0
Total Succ Juv. Male Pair	2		1	1	1	1	1	1	2	2	2	2
Total Failed Juv. Male Pair		3	3	1	3	2	2	2	0	0	0	0

Table 5. Number of Samples for Each Pair. Number of fecal samples and behavior observation datasheets analyzed by pair and stage of introduction.

Pairs	Total Behavior Pair	Total Fecals Male	Total Fecals Female	Stage 1 Pair	Stage 1 Male	Stage 1 Female	Stage 2 Pair	Stage 2 Male	Stage 2 Female	Stage 3 Pair	Stage 3 Male	Stage 3 Female	Stage 4 Pair	Stage 4 Male	Stage 4 Female	Stage 5 Pair	Stage 5 Male	Stage 5 Female
Successful: Male, Female																		
Songkhla, Numfun	478	213	230	81	0	26	140	35	40	257	174	161	0	0	0	0	4	3
Cheang, Lamoon	465	206	197	0	94	95	342	109	96	118	2	3	0	0	0	5	1	3
Sompon, Somwang	0	91	92	0	0	0	0	40	33	0	6	5	0	40	46	0	5	8
NoName, Juujii	63	110	102	0	0	0	0	0	0	0	0	0	61	107	100	2	3	2
Wanchai, Mini	190	243	174	21	0	21	33	18	20	90	188	104	44	32	26	2	5	3
Failed: Male, Female																		
Cheang, Giant	0	33	39	0	20	22	0	13	17	0	0	0	0	0	0	0	0	0
Ken, Numphung	0	70	62	0	0	0	0	0	0	0	70	62	0	0	0	0	0	0
Arawan, Lamoon	0	60	42	0	55	40	0	5	2	0	0	0	0	0	0	0	0	0
Rick, Somsri	125	138	162	125	138	162	0	0	0	0	0	0	0	0	0	0	0	0
NoName, ThapThim	128	100	131	0	15	18	128	85	113	0	0	0	0	0	0	0	0	0
Rick, Maesa	75	90	72	0	7	5	75	83	67	0	0	0	0	0	0	0	0	0
Mei, Nok	103	119	110	0	26	33	36	20	15	67	73	62	0	0	0	0	0	0
Sakdaa, Giant	264	286	280	6	2	4	4	7	10	254	277	266	0	0	0	0	0	0

Totals by Stage: Pair = # Behavior Datasheets; Male/Female = # Fecal Samples

Table 6. Fecal Cortisol Metabolite Concentrations (Mean ± SE).

Stage	Sex	Pairing Outcome	Baseline ng/g feces	Mean ng/g feces	Peak Mean ng/g feces	Coefficient of Variance %
Overall (Stages 1-5)	Male	Successful	68.27 ± 9.11	96.97 ± 13.61	203.46 ± 20.58	82 ± 7
		Failed	100.45 ± 4.66	153.34 ± 12.29	389.95 ± 47.38	115 ± 16
	Female	Successful	88.76 ± 13.15	150.54 ± 29.91	341.94 ± 54 85	120 ± 21
		Failed	100.67 ± 10.89	140.2 ± 18.05	328.39 ± 54.87	87 ± 14
Stage 1	Male	Successful	55.69	91.23	194.87	111
		Failed	156.2 ± 28.34	196.71 ± 22.32	519.48 ± 148.05	93 ± 24
	Female	Successful	115.3 ± 30.86	175.18 ± 50.52	523.64 ± 239.89	114 ± 8
		Failed	102.05 ± 12.01	127.54 ± 18.95	301.65 ± 73.35	60 ± 12
Stage 2	Male	Successful	113.86 ± 33.88	123.59 ± 29.98	209.8 ± 78.51	66 ± 13
		Failed	135.82 ± 18.24	146.85 ± 19.08	280.97 ± 24.88	35 ± 7
	Female	Successful	95.96 ± 9.19	175.2 ± 18.63	382.68 ± 44.53	120 ± 24
		Failed	114.68 ± 12.62	130.12 ± 16.27	356.97 ± 96.21	57 ± 14
Stage 3	Male	Successful	93.98 ± 25.14	88.87 ± 16.41	342.59 ± 70.57	54 ± 16
		Failed	125.28 ± 9.44	202.92 ± 19.2	533.71 ± 32.41	133 ± 18
	Female	Successful	237.5 ± 87.95	246.13 ± 83.61	231.56 ± 39.09	92 ± 21
		Failed	112.96 ± 16.68	176.38 ± 21.61	395.85 ± 78.07	109 ± 31
Stage 4	Male	Successful	87.54 ± 37.25	120.4 ± 45.84	339.13 ± 179.05	77 ± 2
	Female	Successful	146.29 ± 64.23	197.54 ± 70.28	804.41 ± 352.65	120 ± 47
Stage 5	Male	Successful	101.67 ± 17.55	101.67 ± 17.55	.	49 ± 5
	Female	Successful	188.67 ± 40.6	188.67 ± 40.6	.	46 ± 10

Table 7. Proportion of Time Spent in Different Behavioral States (Mean ± SE) Per 30 Min Observation Session.

Behavioral States Variables	Overall (Stages 1-3)			
	Male		**Female**	
	Successful	**Failed**	**Successful**	**Failed**
Lie	0.62 ± 0.09	0.76 ±0.04	0.51 ± 0.17	0.6 ± 0.06
Sit	0.08 ± 0.03	0.06 ± 0.02	0.05 ± 0.01	0.06 ± 0.0045
Stand	0.09 ± 0.05	0.09 ± 0.02	0.05 ± 0.02	0.09 ± 0.01
Walk	10.0 ± 0.05	0.06 ± 0.01	0.03 ± 0.0015	0.09 ± 0.04
Run	0.002 ± 0.0007	0.0009 ± 0.0006	0.002 ± 0.007	0.004 ± 0.001
Pace	0.03 ± 0.01	0.02 ± 0.01	0.19 ± 0.18	0.13 ± 0.06
Out-of-Sight	0.11 ± 0.07	0.15 ± 0.01	0.01 ± 0.01	0.01 ± 0.00
Active	0.23 ± 0.11	0.17 ± 0.04	0.28 ± 0.2	0.32 ± 0.07

Table 8. Frequency (n/30 min) of Behavioral Events (Mean ± SE).

Behavioral Events Variables	Overall (Stages 1-3)			
	Male		Female	
	Successful	Failed	Successful	Failed
Rub on Object	0.9 ± 0.63	0.65 ± 0.25	0.18 ± 0.08	0.19 ± 0.09
Rub on Other Cat	0.06 ± 0.04	0.1 ± 0.06	0.1 ± 0.06	0.03 ± 0.03
Roll	0.05 ± 0.03	0.01 ± 0.01	0.02 ± 0.01	0.12 ± 0.05
Urine Spray	0.18 ± 0.15	0.1 ± 0.06	0.02 ± 0.01	0.08 ± 0.04
Claw	0.14 ± 0.07	0.07 ± 0.02	0.05 ± 0.02	0.04 ± 0.03
Meow/Cry	0.09 ± 0.06	0.55 ± 0.09	0.08 ± 0.08	0.17 ± 0.07
Prusten	2.27 ± 2.19	1.96 ± 0.91	0.04 ± 0.02	0.7 ± 0.58
Sniff Other Cat	0.28 ± 0.16	0.14 ± 0.04	0.05 ± 0.01	0.15 ± 0.09
Sniff Object	2.1 ± 1.34	1.28 ± 0.56	0.23 ± 0.07	0.82 ± 0.36
Flehmen	0.33 ± 0.21	0.44 ± 0.33	0.01 ± 0.01	0.03 ± 0.01
Groom Self	1.57 ± 0.79	0.91 ± 0.16	0.57 ± 0.2	0.87 ± 0.24
Groom Other Cat	0.84 ± 0.39	0.14 ± 0.1	0.28 ± 0.07	0.1 ± 0.08
Fight	0.09 ± 0.06	0.1 ± 0.07	0.01 ± 0.01	0.01 ± 0.01
Growl/Hiss	0.05 ± 0.01	0.00 ± 0.00	0.2 ± 0.02	0.84 ± 0.67
Lordosis	.	.	0.19 ± 0.18	0.06 ± 0.03
Mount	0.02 ± 0.01	0.02 ± 0.01	.	.
Affiliative	1.18 ± 0.59	0.3 ± 0.13	0.44 ± 0.11	0.28 ± 0.14
Exploratory	3.7 ± 2.41	2.55 ± 1.17	0.52 ± 0.2	1.3 ± 0.52

Table 9. Variables Influenced by Pairing Type (Mean ± SE).

Variables Influenced by Pairing Type	Male				Female			
	Successful		Failed		Successful		Failed	
	Adult-Male Pair	Juvenile-Male Pair	Adult-Male Pair	Juvenile-Male Pair	Adult-Male Pair	Juvenile-Male Pair	Adult-Male Pair	Juvenile-Male Pair
Overall (Stages 1-5) fecal cortisol mean (ng/g feces)	89.69 ± 13.75	107.88 ± 32.96	149.88 ± 16.49	163.74 ± 2.59	186.85 ± 33.16	96.08 ± 26.52	122.34 ± 18.78	193.8 ± 1.44
Fecal cortisol Stage 2 Mean (ng/g feces)	104.6 ± 32.8	180.56	130.39 ± 24.81	179.78 ± 11.93	182.41 ± 24.28	153.54	112.82 ± 18.21	164.72 ± 13.59
Out-of-Sight (proportion of time spent per 30 min)	0.11 ± 0.07	0.02	0.01 ± 0.01	0.01 ± 0.01	0.15 ± 0.01	0.19	0.01 ± 0.0046	0.06 ± 0.06
Rub on Other Cat	0.07 ± 0.07	0.06	0.01 ± 0.0046	0.05 ± 0.03	0.05 ± 0.01	0.22	0	0.09 ± 0.09
Roll (n/30 min)	0.06 ± 0.06	0.03	0	0.02 ± 0.003	0.01 ± 0.01	0.05	0.08 ± 0.08	0.19 ± 0.02
Prusten (n/30 min)	0.08 ± 0.05	6.64	2.14 ± 1.57	1.66 ± 0.9	0.03 ± 0.02	0.08	1.06 ± 0.98	0.16 ± 0.01
Groom Other Cat (n/30 min)	0.82 ± 0.67	0.86	0.02 ± 0.01	0.32 ± 0.23	0.26 ± 0.11	0.33	0.02 ± 0.01	0.22 ± .02
Fight (n/30 min)	0.03 ± 0.02	0.22	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.02	0.24	0.01 ± 0.01	0.01 ± 0.01
Affiliative (n/30 min)	1.2 ± 1.02	1.16	0.18 ± 0.05	0.48 ± 0.34	0.34 ± 0.09	0.64	0.22 ± 0.16	0.36 ± 0.34

Table 10. Individual Variables Predicting Success/Failure. Significant ($p < 0.05$) results of

generalized linear model involving individual variables.

Variable	Direction (S=Successful, F=Failed)	χ^2	p
Male Stage 1-5 Cortisol Baseline	S < F	8.3	0.0040
Male Stage 1-5 Cortisol Mean	S < F	8.18	0.0042
Male Stage 1-5 Cortisol Peak Mean	S < F	9.77	0.0018
Male Stage 1 Cortisol Baseline	S < F	6.03	0.0141
Male Stage 1 Cortisol Mean	S < F	6.03	0.0141
Male Stage 1 Cortisol Peak Mean	S < F	4.5	0.0339
Male Stage 2 Cortisol CVs	S > F	5.02	0.0251
Male Stage 3 Cortisol Mean	S < F	9.56	0.0020
Male Stage 3 Cortisol Peak Mean	S < F	6.73	0.0095
Male Stage 3 Cortisol CVs	S < F	9.56	0.0020
Female Stage 1 Cortisol CVs	S > F	12.22	0.0005
Female Stage 2 Cortisol CVs	S > F	4.89	0.0270
Female Stage 1-3 Out-of-Sight	S > F	10.59	0.0011
Male Stage 1-3 Meowing/Crying	S < F	10.59	0.0110
Sex Difference Stage 1-3 Fighting	S = ♂-♀ = - F = ♂-♀ = 0	5.18	0.0229
Male Stage 1-3 Growl/Hissing	S > F	10.59	0.0011
Male Stage 1-3 Aggression	S > F	10.59	0.0011

Table 11. Combined Variables Predicting Success/Failure ($p<0.05$ in generalized linear model incorporating two explanatory variables). Affiliative = Groom Other Cat + Sniff Other Cat + Rub on Other Cat.

Variable	Direction (S=Successful, F=Failed)	χ^2	p
Male Stage 1-3 Out-of-Sight + Juvenile/Adult (J/A)	S (A>J) > F (A=J)	10.55 5.11	0.0012 0.0238
Sex Difference Stage 1-3 Out of Sight + Juvenile/Adult (J/A)	S = ♂-♀ = - (♂, A>J; ♀ A<J) F = ♂-♀ = + (♂, A=J; ♀ A<J)	4.96 2.97	0.0259 0.0848
Female Stage 1-3 Rub on Other Cat + Juvenile/Adult (J/A)	S (A<J) > F (A<J)	10.55 9.25	0.0012 0.0024
Sex Difference Stage 1-3 Rolling + Juvenile/Adult (J/A)	S = ♂-♀ = - (♂, A>J; ♀ A<J) F = ♂-♀ = + (A<J)	5.02 2.97	0.0251 0.0848
Female Stage 1-3 Prusten + Juvenile/Adult (J/A)	S (A<J) < F (A>J)	3.94 0.49	0.0470 0.4840
Male Stage 1-3 Groom Other Cat + Juvenile/Adult (J/A)	S (A<J) > F (A<J)	10.55 6.46	0.0012 0.0110
Female Stage 1-3 Groom Other Cat + Juvenile/Adult (J/A)	S (A<J) > F (A<J)	4.11 1.7	0.0427 0.1919
Male Stage 1-3 Fight + Juvenile/Adult (J/A)	S (A<J) > F (A=J)	6.17 1.62	0.0130 0.2035
Female Stage 1-3 Fight + Juvenile/Adult (J/A)	S (A<J) > F (A=J)	6.25 1.51	0.0124 0.2188
Male Stage 1-3 Affiliative + Juvenile/Adult (J/A)	S (A>J) > F (A<J)	5.1 1.73	0.0239 0.1880

Figure 1. Holding Areas. Images of the holding areas with nestboxes (yellow arrow) and climbing structures (pink arrow). Female is locked in nestbox (a) while male investigates the holding area for protected contact introduction. When pairs are more established (Stages 3 to 5), they are allowed access to each other and nestboxes throughout the day (b).

a)

b)

Figure 2. Large Enclosures: Vegetation. The large enclosures (a, b) are maintained with climbing logs (yellow arrow) and naturally growing plants and trees (pink arrow).

a)

b)

Figure 3. Large Enclosures: Access and Climbing. Animal access to large enclosure was based on neighbors with access (a) to reduce likelihood of fights through the fence. Both man-made (yellow arrow) and natural (pink arrow) climbing structures were provided in the large enclosures (b).

a)

b)
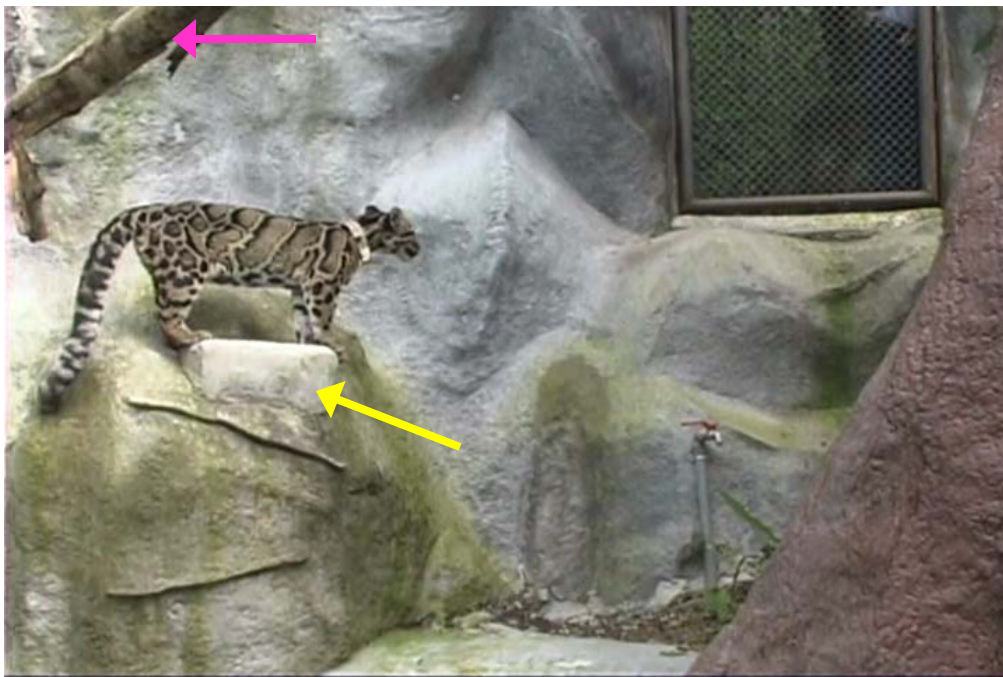
Figure 4. Large Enclosures: Pool. Man-made pools in the large enclosures were occasionally filled with water. This image is of an empty pool.

Figure 5. Comparison of Overall Male Fecal Cortisol Metabolite Levels. Mean, baseline, and peak mean male fecal cortisol metabolites throughout introduction (stages 1-5 combined) were significantly higher in failed (solid bar) than in successful (open bar) pairings (mean ± SE). Within a category means with stars differ ($p < 0.01$).
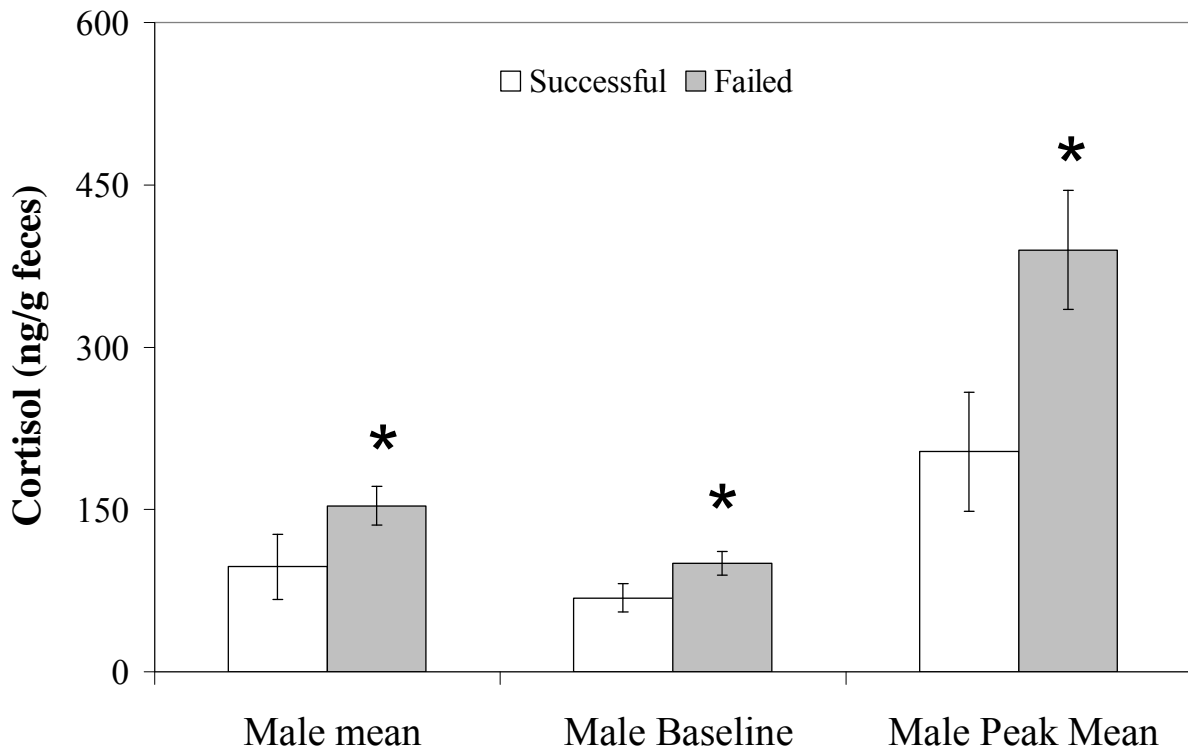
Figure 6. Comparison of Male Fecal Cortisol by Stage. Changes in fecal cortisol metabolite baseline (a), mean (b), and peak mean (c) throughout introduction (stages 1-5 individually) in male clouded leopards in successful (open triangle) versus failed (solid dot) pairings (mean ± SE). Within a stage means with stars differ (*$p < 0.05$, **$p < 0.01$).
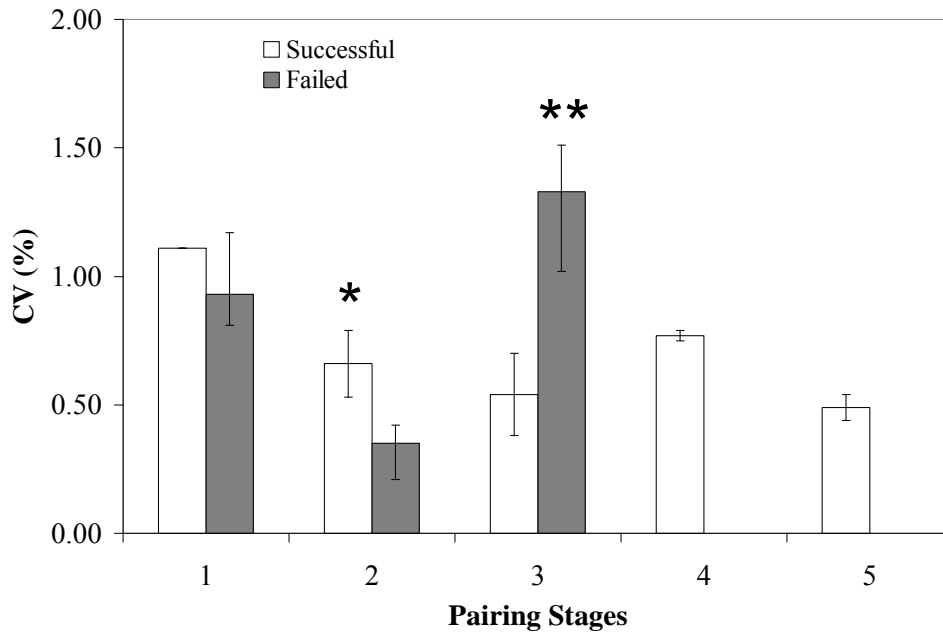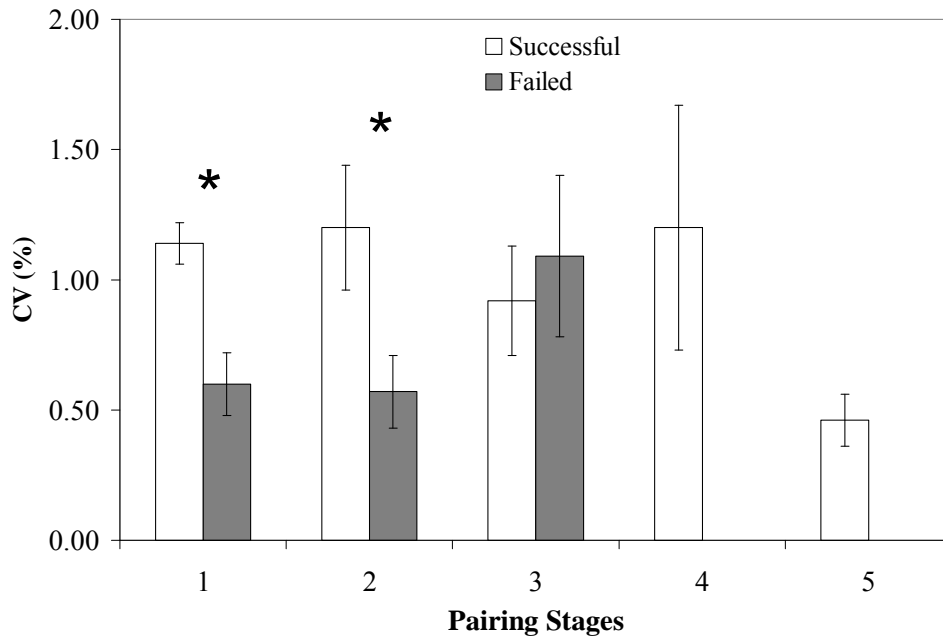
a)
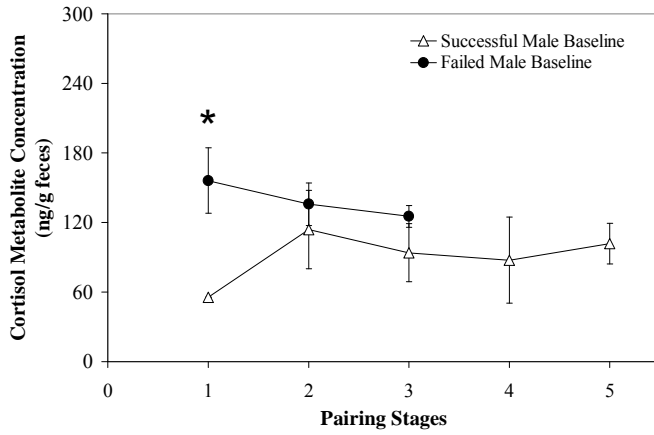
b)
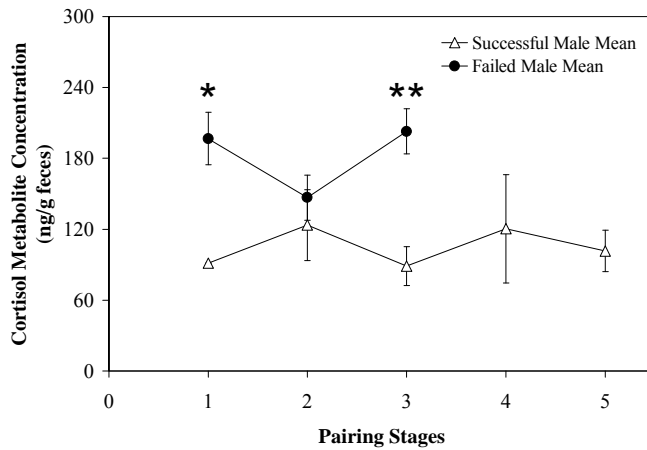
c)
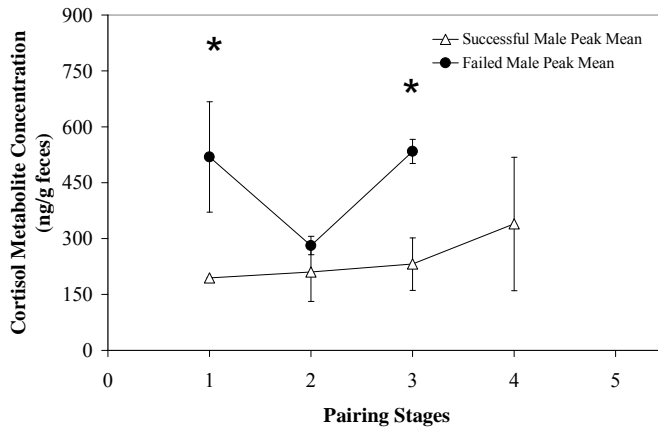
Figure 7. Coefficient of Variation (CV) in Fecal Cortisol Metabolites. Fecal cortisol metabolite CV around the mean in stages 1-5 of introduction of male (a) and female (b) clouded leopards in successful (open bar) versus failed (solid bar) pairings (mean $\pm$ SE). Within a stage, means with stars differ (*p < 0.05, **p < 0.01).
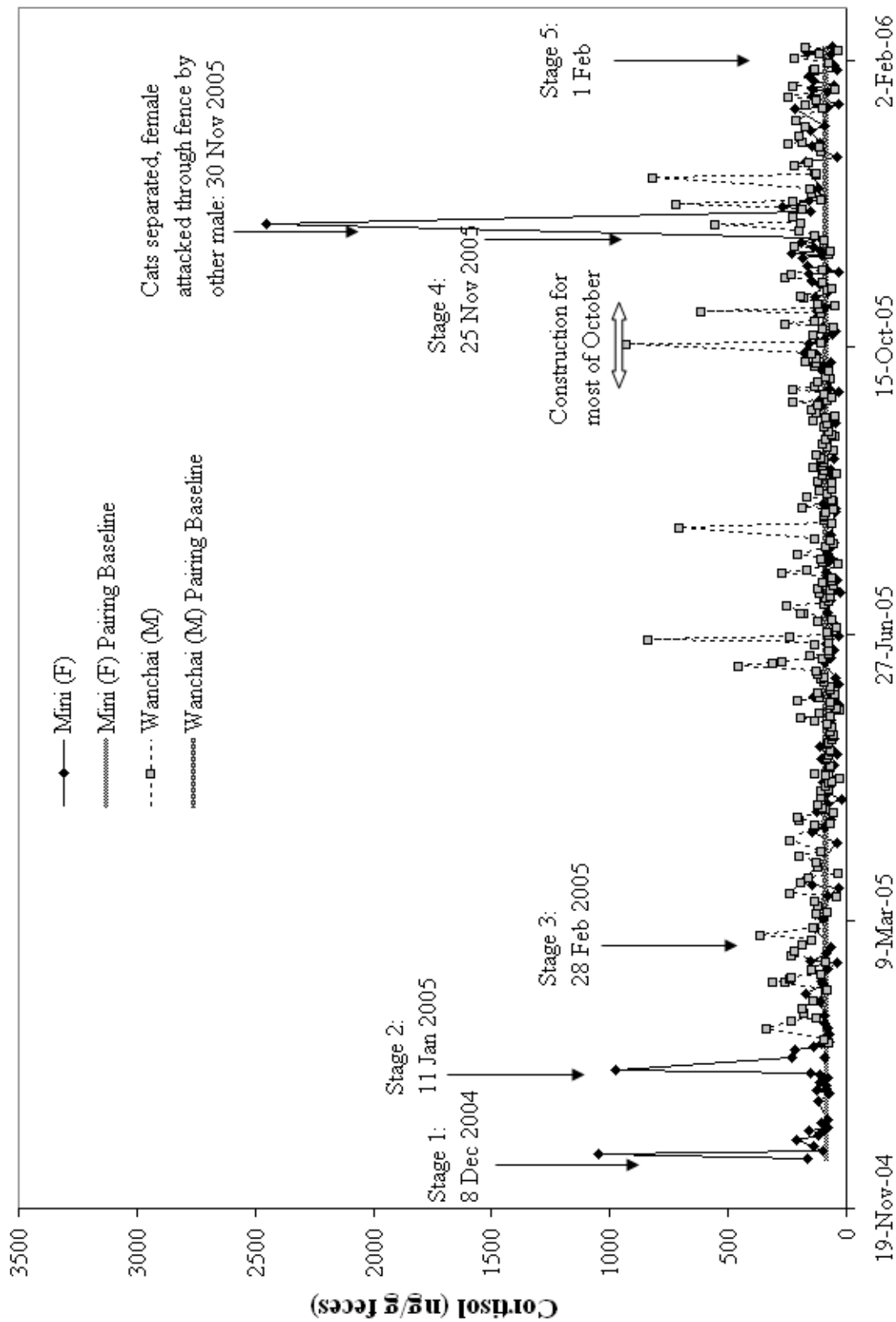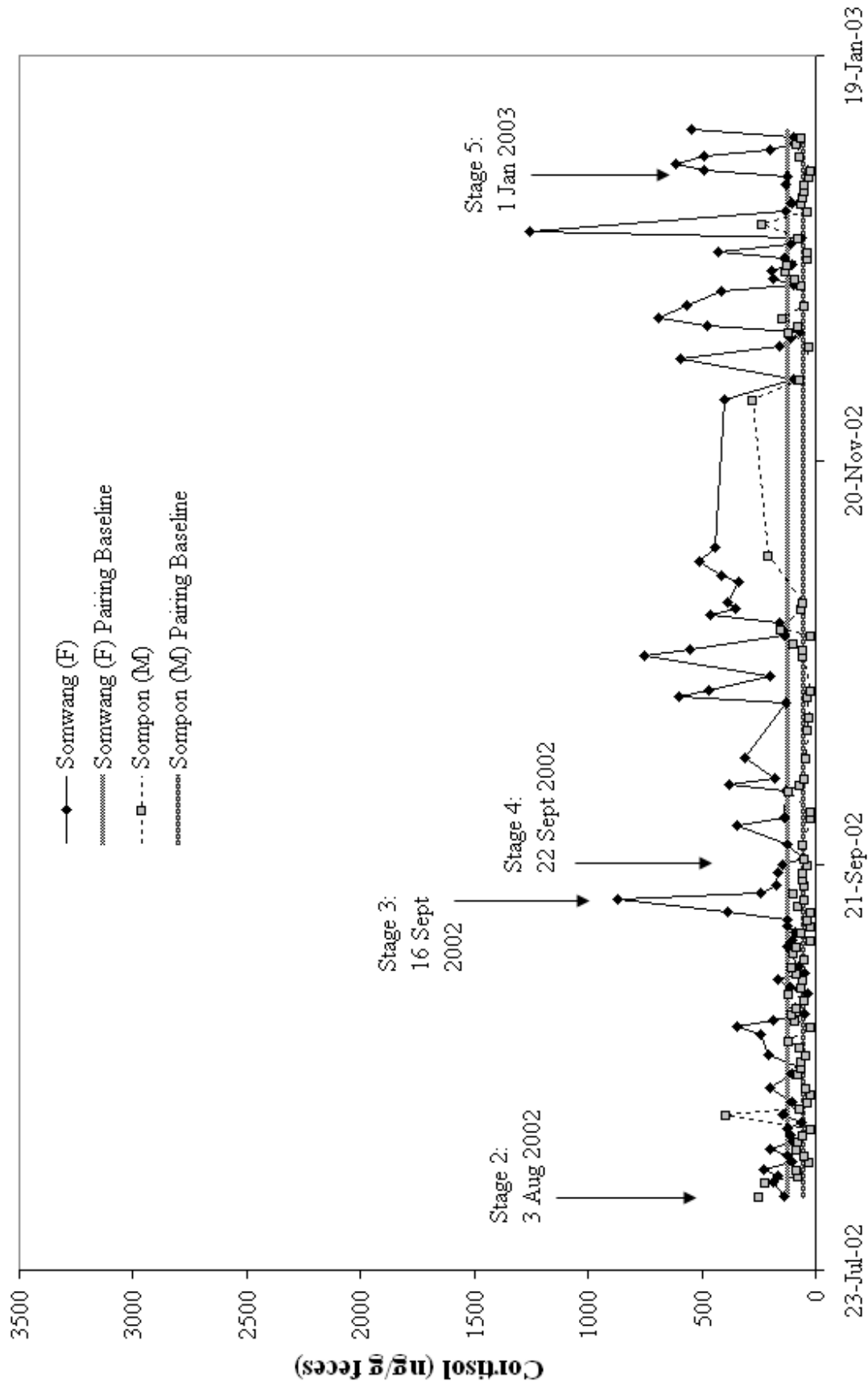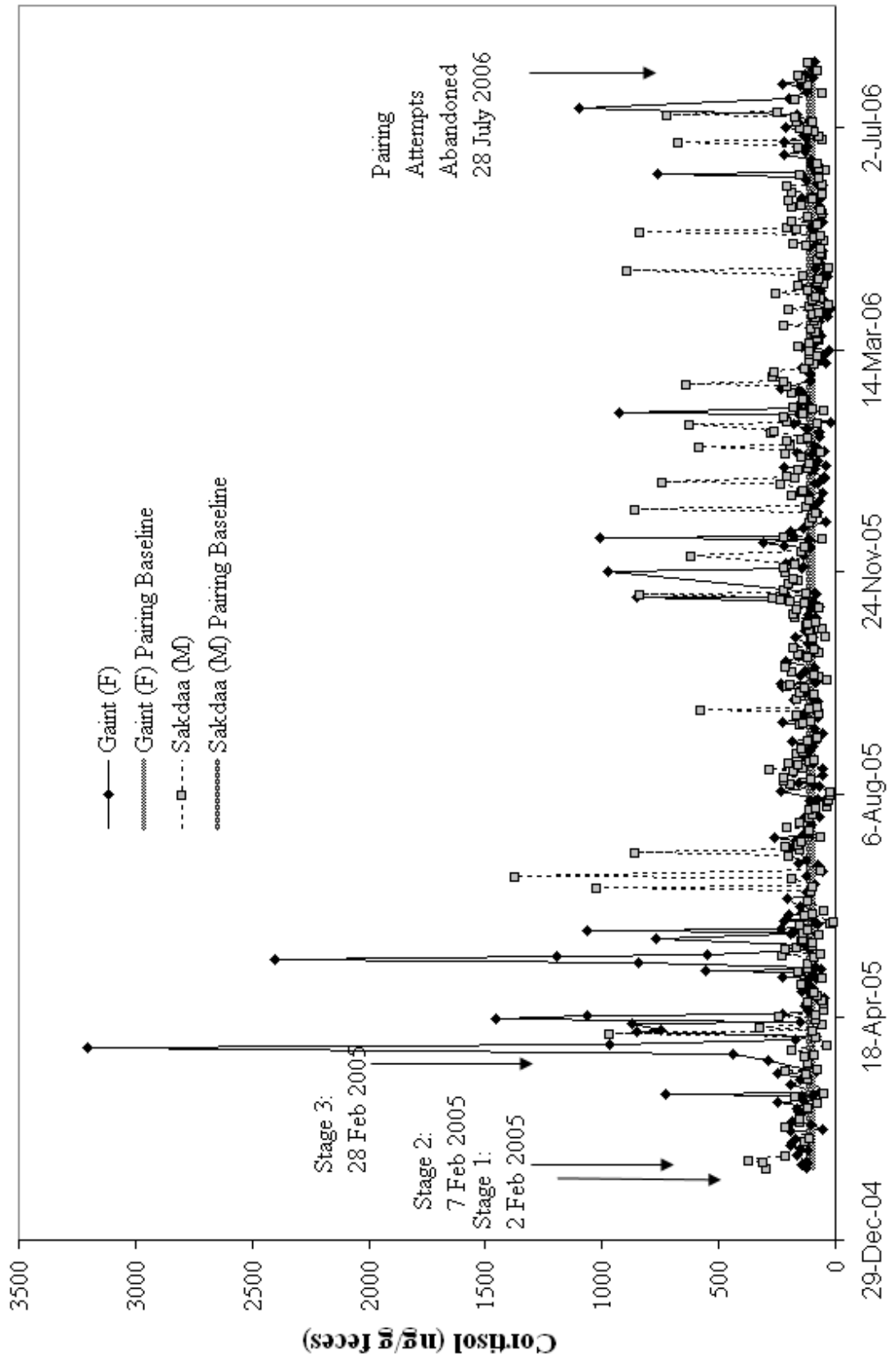
a)

b)

Figure 8. Successful Juvenile-Male Pairing: Wanchai (M) and Mini (F). Representative longitudinal fecal cortisol metabolite profiles in a female (black diamond) and male (grey box) clouded leopard during the five stages of pairing (marked). Note marked elevations in female cortisol metabolites following an attack by a male (not her mate) through the fence.

Figure 9. Successful Adult-Male Pairing: Sompon (M) and Somwang (F). Representative longitudinal fecal cortisol metabolite profiles in a female (black diamond) and male (grey box) clouded leopard during the five stages of pairing (marked). Note the high variation of the female (black diamond) fecal cortisol metabolite concentration as well as infrequent and relatively low peaks and baseline in male (gray box) fecal cortisol metabolite concentration.

Figure 10. Failed Juvenile-Male Pairing: Sakdaa (M) and Gaint (F). Representative longitudinal fecal cortisol metabolite profiles in a female (black diamond) and male (grey box) clouded leopard during the first three stages of pairing (marked). Note the higher and more frequent peaks and higher baseline in male fecal cortisol metabolite concentration.

70

Figure 11. Failed Adult-Male Pairing: Arawan (M) and Lamoon (F). Representative longitudinal fecal cortisol metabolite profiles in a female (black diamond) and male (grey box) clouded leopard during the first two stages of pairing (marked). Note the markedly elevated peak and higher baseline in male fecal cortisol metabolite concentration than was seen in the successful adult male, as well as the low variation in female (black diamond) fecal cortisol metabolite concentration.
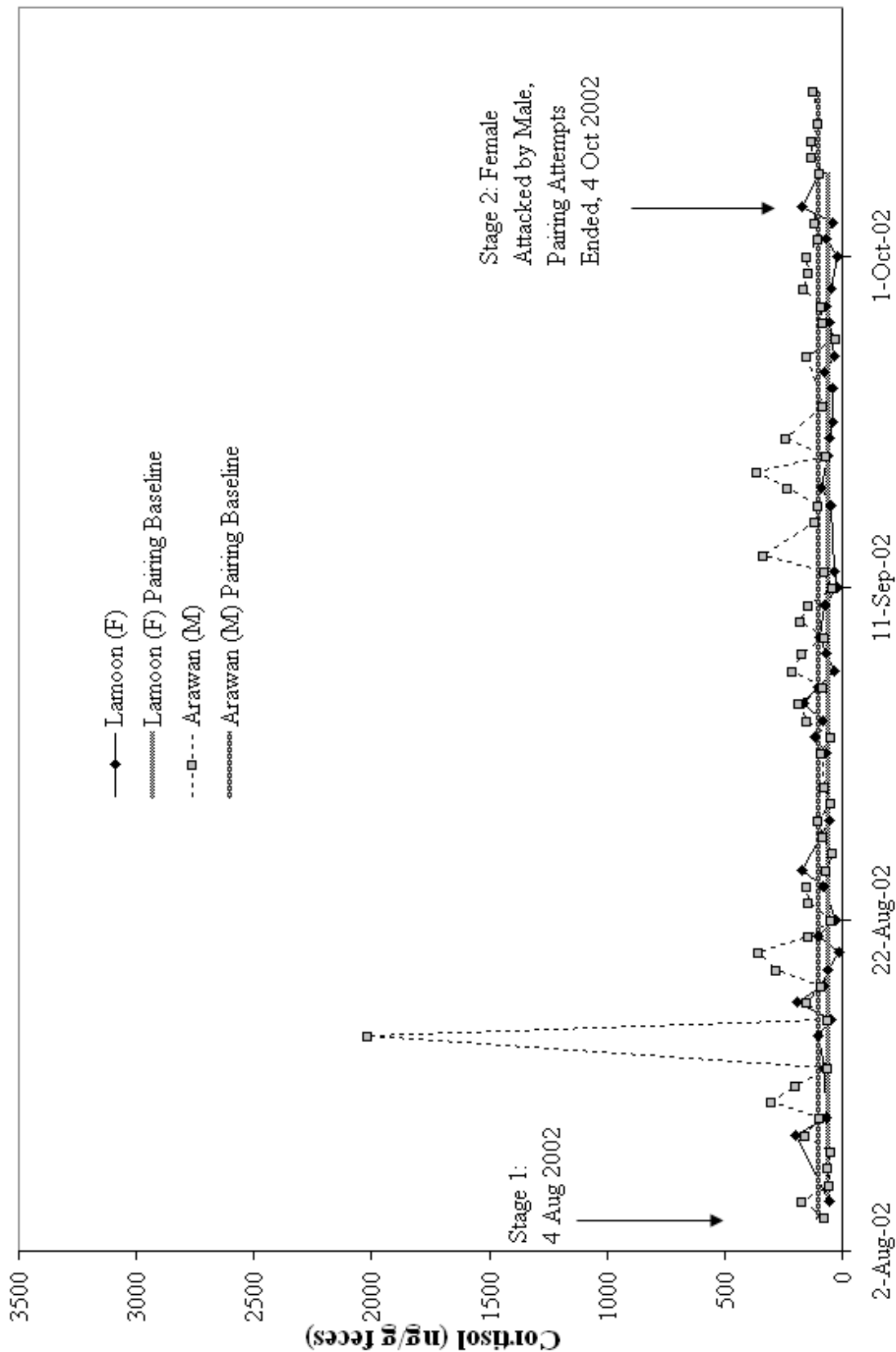
Figure 12. Out-of-Sight: Successful vs. Failed. Comparison of proportion of time spent out-of-sight in successful (unhatched) versus failed (hatched) male (open) and female (solid) clouded leopards in all pairs (overall), adult-male pairs, and juvenile-male pairs. Within pairing and sex category, means with stars differ ($p < 0.05$).
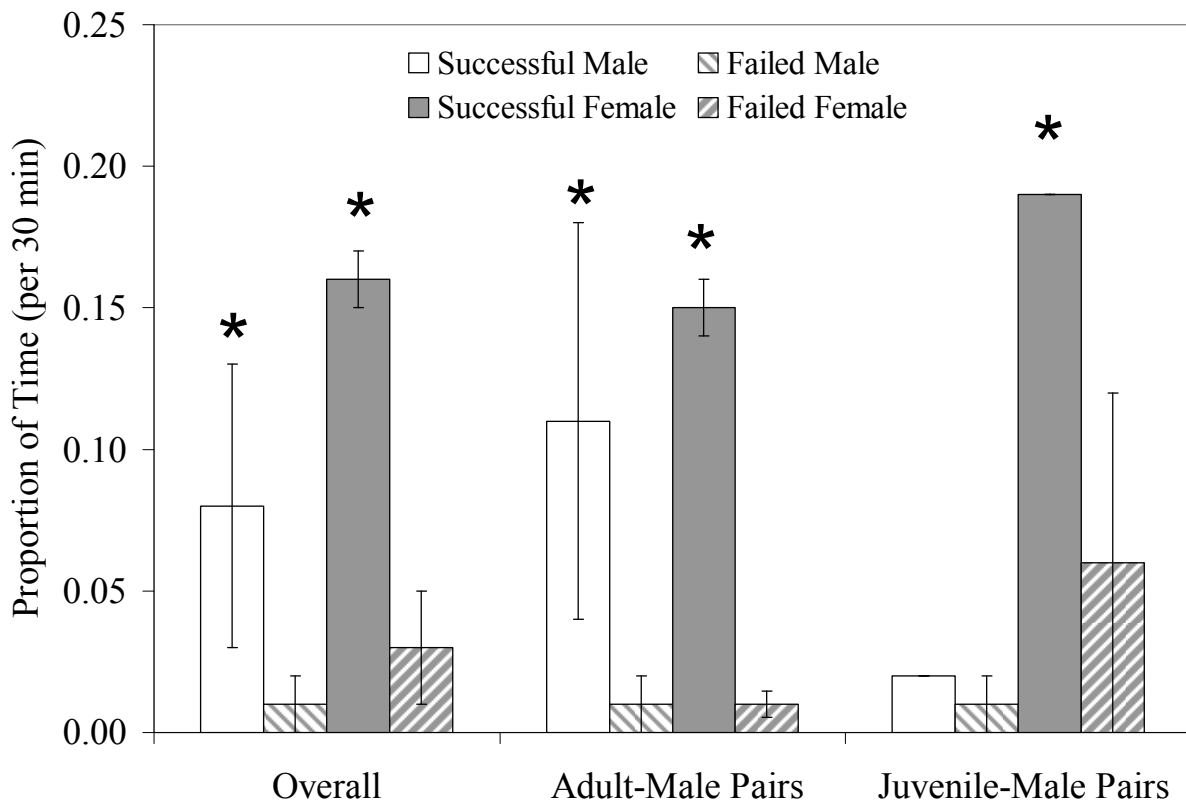
Figure 13. Fighting: Successful vs. Failed. Comparison of fighting frequency in successful (unhatched) versus failed (hatched) male (open) and female (solid) clouded leopards in all pairs (overall), adult-male pairs, and juvenile-male pairs. Within pairing and sex category, means with stars differ ($p < 0.05$).
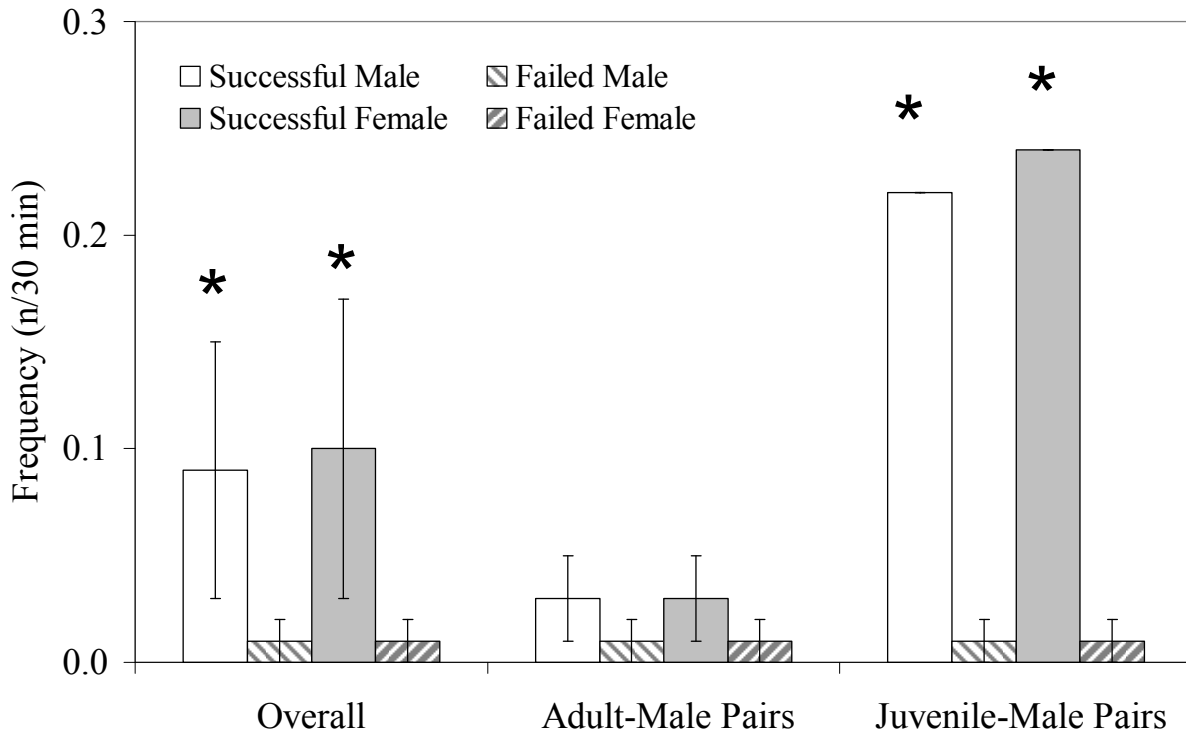
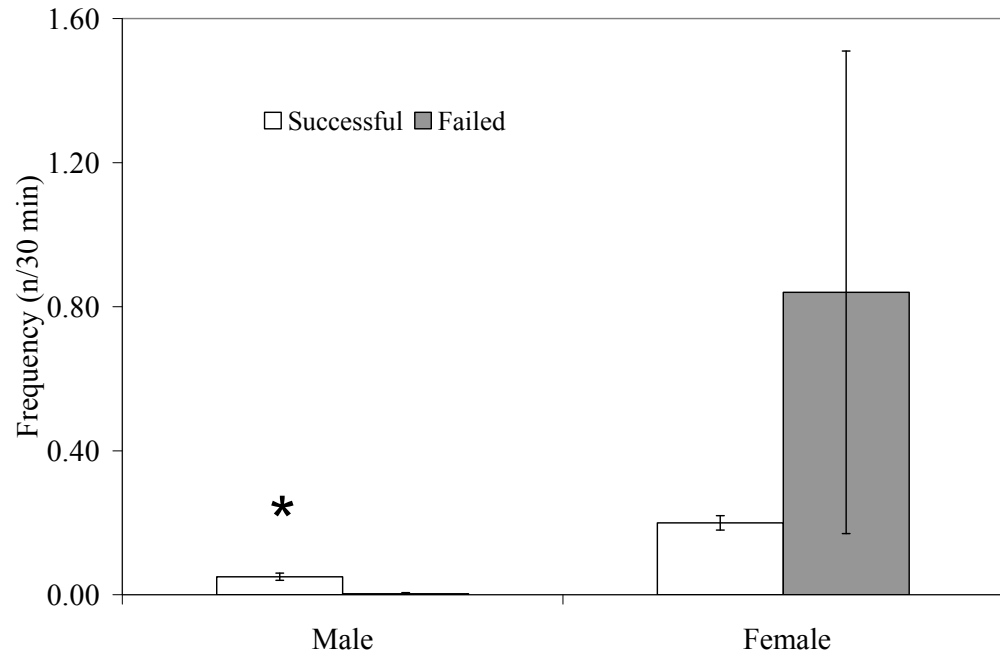**Figure 14. Vocalizations: Successful vs. Failed.** Comparison of growl/hiss (a) and meow/cry (b) frequencies in successful (open) versus failed (solid) male and female clouded leopards. Within a pairing and sex category means with stars differ ($p < 0.05$).
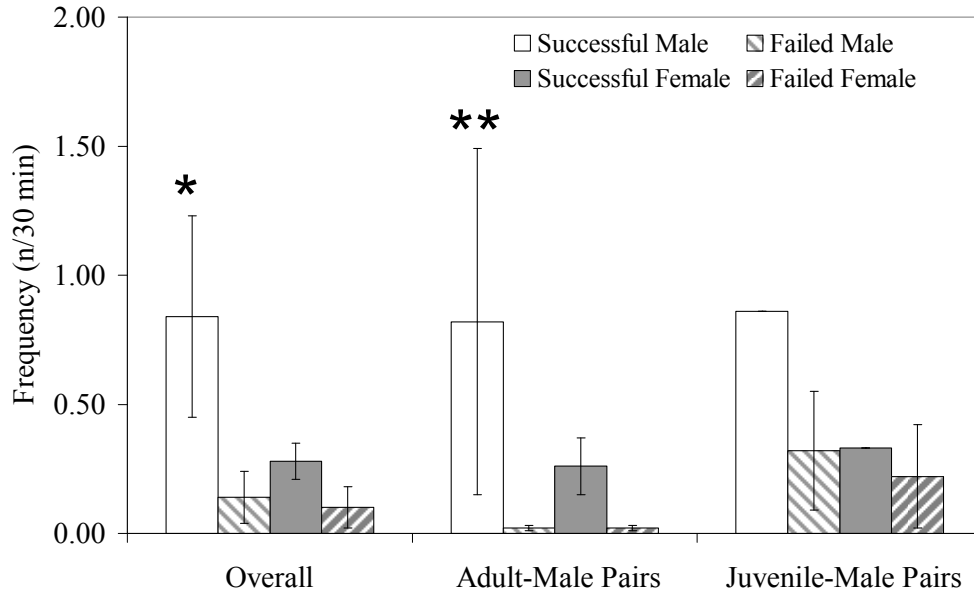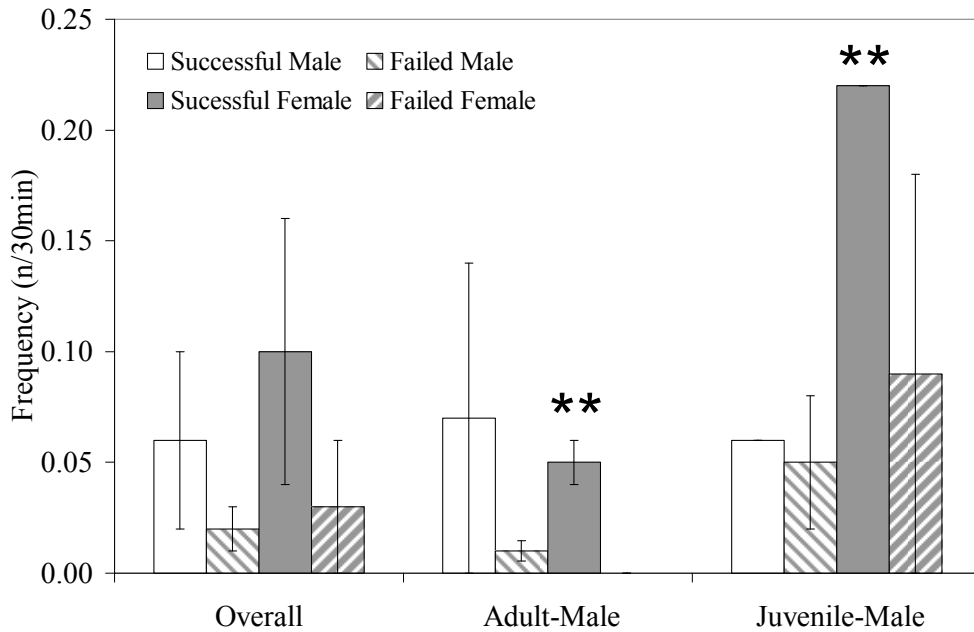
a)

b)
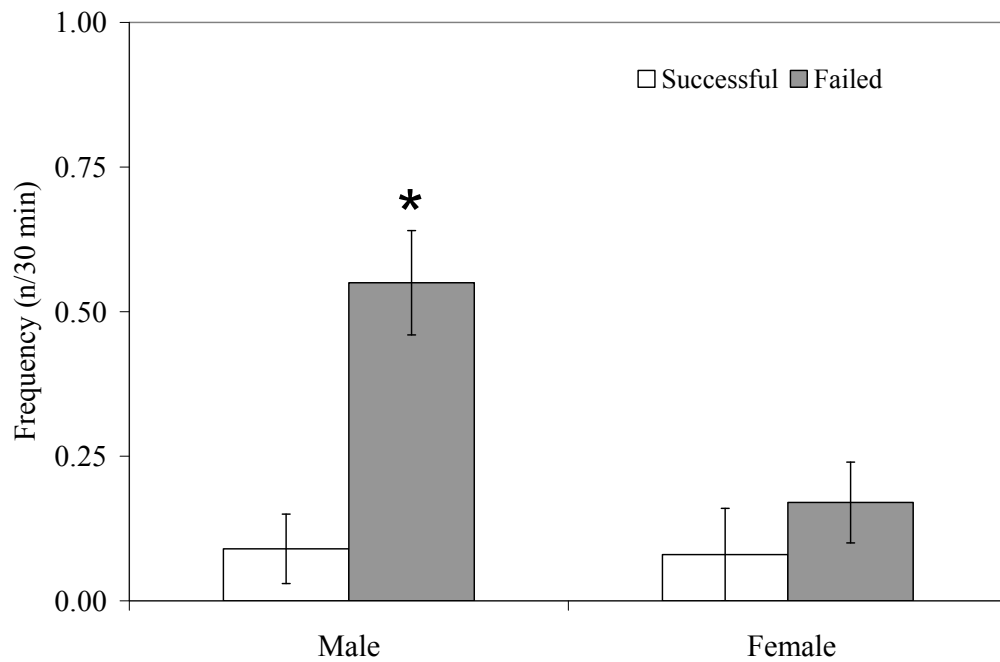
Figure 15. Affiliative Behaviors: Successful vs. Failed. Comparison of groom other cat (a) and rub on other cat (b) frequencies in successful (unhatched) versus failed (hatched) male (open) and female (solid) clouded leopards. Within a pairing and sex category means with stars differ (*$p < 0.05$, **$p < 0.01$).

a)

b)

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Observer: Date: Time: start:

                                                      end:

Animal(s):   Enclosure(s):

Behavioral States - recorded at the end of every 2 minutes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total F	Total M
Lying																	
Sitting																	
Standing																	
Walking																	
Running																	
Pacing																	
Out of Sight																	

Behavioral Events - recorded continuously																Total F	Total M
rub on object																	
rub on other cat																	
rolling																	
urine spraying																	
clawing																	
meowing/crying																	
prusten																	
sniff other cat																	
sniff object																	
flehmen																	
groom self																	
groom other cat																	
fighting																	
growl/hissing																	
lordosis																	
mounting																	

Comments: