Relationships of Social Behavior and the Captive Environment to Reproduction in Female Southern White Rhinoceros (*Ceratotherium simum simum*)

DISSERTATION

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By

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Abstract

Poor reproductive success among captive female southern white rhinoceros (*Ceratotherium simum simum*) might be related to social behavior, social status, or the captive environment. This study examined reproductive success; estrous cyclicity, as evidenced in progesterone profiles; and corticosterone concentrations and their relationships to social behavior, the social environment, and the captive housing environment for 45 female white rhinos located at 16 institutions (13 parous, captive-born; 13 nulliparous, captive-born; 6 parous, wild-caught; 7 nulliparous, wild-caught; and 6 adolescent). Behavioral observations (80-240 hrs) of aggression, dominance relationships, sexual behavior, and sexual play interactions were recorded for 36 females at each of 12 institutions. Social group size and composition, enclosure size, and other housing characteristics were assessed for all the females in the project through review of historical and institution records. Fecal and serum samples from 38 females at 15 AZA institutions were collected for 4 months for adults and for 1-2 years for adolescents. Progesterone and corticosterone concentrations in serum and their metabolites in feces were measured by enzyme immunoassay.

Progestagen profiles provided clear evidence (luteal phases) of ovulatory cycles in 22 of 35 non-pregnant females, 12 of which were nulliparous. Four of the 6 adolescents showed evidence of estrous cycle activity at 29 to 42 months of age. Of the behaviors

examined, average daily frequency of sexual play behavior was different between groups of females: Nulliparous, captive-born females and wild-caught, parous females engaged in sexual play less often than adolescents ($p < 0.05$), and acyclic ($p = 0.097$) and pregnant ($p = 0.051$) females tended to engage in sexual play less often than adolescents. Subordinates engaged in sexual play more often than dominant females ($p < 0.05$).

The proportion of females that had given birth (hereinafter, parity) was larger for females housed in large enclosures (>0.01 km^2 or 2.5 acres; $p = 0.001$) and in groups with >2 females/adolescents ($p = 0.003$) than in smaller enclosures or groups. Ovulatory cycles were observed in a larger proportion of females held in large enclosures than in smaller enclosures ($p = 0.032$), and more of the females housed with a novel male showed ovulatory cycles than those housed with a familiar male ($p = 0.038$). Parity and the proportion of females having ovulatory cycles were not influenced ($p > 0.05$) by dominance.

Average fecal corticosterone metabolite (hereinafter, corticosterone) concentration differed ($p < 0.05$) across institutions in almost every analysis. Corticosterone concentration did not differ ($p > 0.05$) between dominant and subordinate females or between acyclic and cycling females. Corticosterone concentrations were not consistently elevated ($p > 0.05$) for females housed in any of the environmental conditions assessed with the exception that housing with a female companion known from adolescence was associated ($p = 0.057$) with lower mean corticosterone than housing with a female companion introduced during adulthood, or no female companion. Wild-caught females had a higher ($p < 0.05$) average corticosterone concentration than

captive-born females. Corticosterone concentration did not differ ($p > 0.05$) between nulliparous and parous females, but, at institutions housing both types of females, 8/10 nulliparous females had higher mean corticosterone than parous females ($n = 9$) at their institution.

This study provides evidence that a captive environment that provides elements of conditions in the wild is most conducive to successful reproduction and estrous cyclicity in white rhinos. A larger proportion of females were parous when housed among a group of females and adolescents in a large enclosure, and ovulatory cycles were more prevalent in females housed in large enclosures with males that were not known during early adolescence. In addition, housing females with another female known from adolescence might help to minimize their perception of stressful stimuli. Documentation of cyclicity in nulliparous females suggests that reproductive failure in white rhinos occurs primarily during conception or early pregnancy, but acyclicity does not appear to be associated with elevated corticosterone. Lower frequencies of sexual play behavior among nulliparous, captive-born females and acyclic females compared to adolescents suggest that observations of reduced sexual play behavior as females mature could be used to identify those who might be prone to reproductive difficulties. Because wild-caught, parous females engaged in less sexual play behavior than adolescents, and wild-caught females had higher corticosterone than captive-born females, both of which indicate a stress response, the numerically elevated corticosterone in nulliparous females might have biological relevance in light of their reduced sexual play behavior.

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Fields of Study

Major Field: Evolution, Ecology and Organismal Biology

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Introduction

The order Perissodactyla includes 3 extant families, Equidae, Tapiridae, and Rhinocerotidae. While Equidae and Tapiridae each contain only 1 extant genus (*Equus* and *Tapirus*, respectively), there are 4 extant genera in Rhinocerotidae, *Ceratotherium* and *Diceros* on the African continent, and *Rhinoceros* and *Dicerorhinus* in Asia. The African genera and *Rhinoceros* diverged approximately 26-27 million years ago (Xu and Arnason, 1997; Tougard et al., 2001). *Dicerorhinus sumatrensis*, the hairy Sumatran rhinoceros, is geographically closest to the singly-horned Javan (*Rhinoceros sondaicus*) and greater one-horned or Indian (*Rhinoceros unicornis*) rhinoceros, but it has 2 horns like the black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros, a situation that causes debate about its phylogenetic relationship with the other genera. Even molecular techniques yield conflicting results, suggesting that *Dicerorhinus* is more closely related to the African genera (Morales and Melnick, 1994); that it is more closely related to *Rhinoceros* (Tougard et al., 2001); and that there is not significant molecular support for either the morphological or the geographic classifications, or for a tree in which the African genera and *Rhinoceros* are more closely related to each other than to *Dicerorhinus* (Willerslev et al., 2009). Alas, some rhino species may go extinct before science unravels these and other questions about this unique family.

Poaching and habitat loss threaten all wild rhinoceros. The Sumatran rhino population is currently estimated at 160-300 in the wild, and the Javan rhino is even more critically endangered with 38-49 remaining in the wild (IUCN and TRAFFIC, 2009). Indian rhino number is estimated at 2,800, and black rhino populations are thought to total about 4,230 (IUCN and TRAFFIC, 2009). The subject of this paper, the white rhino, includes 2 subspecies. The northern white rhino (*C. simum cottoni*) is nearly extinct (8 individuals), while the southern white rhino (*C. simum simum*) is a conservation success story, rebounding from a poaching-decimated population of approximately 50 to a current population of 17,475 (IUCN and TRAFFIC, 2009). This large, growing population enabled the establishment of a captive white rhino population in zoos around the world. Currently, the transfer of wild rhinos into captivity also is required to maintain the size and genetic diversity of the captive population.

In general, reproduction is particularly poor among captive-born, female white rhinoceros (Swaisgood et al., 2006). Approximately 50% of all captive female white rhinos in North America have reproduced [Association of Zoos and Aquariums (AZA), 2009], but only 39% of captive-born females have produced young, in some cases despite being consistently mated by a male. A population growth rate of ~5% per year is desirable in the wild for population sustainability and recovery from periodic losses (Hutchins and Kreger, 2006), but an assessment in 2006 indicated that the captive white rhino population was not growing ($\lambda = 1.001$; Foose and Wiese, 2006). The long generation time of the rhinoceros is advantageous genetically because drift is minimal and diversity persists as long as the animals are living (Foose and Wiese, 2006).

However, growth and recovery of small populations in the wild or in captivity is slow because of long gestation and maturation times (Foose and Wiese, 2006). As the wild-caught founders of the captive population age, fewer reproductive females are left to sustain the genetic integrity of the captive population, which has already been supplemented by new imports from Africa (AZA, 2005; Swaisgood et al., 2006). In order to maintain the goal of 90% gene diversity for 100 years (Foose and Wiese, 2006; AZA, 2009), one of the objectives of the White Rhinoceros Species Survival Plan is to increase the genetic contribution of the captive rhinos that have not yet successfully reproduced to the population (AZA, 2005).

Social Behavior in Wild and Captive White Rhinoceros

The behavioral ecology of the social white rhinoceros differs from that of the other rhino species, which are largely solitary despite overlapping home ranges (Owen-Smith, 1988b; Hutchins and Kreger, 2006), and that of the horse (*Equus caballus*), which forms harem groups led by a dominant stallion (McCort, 1984). Wild female and subadult black rhinos might form temporary, small groups on occasion (Owen-Smith, 1988b; Hutchins and Kreger, 2006), but pairs or small groups of white rhino females and adolescents are the norm (Owen-Smith, 1973, 1975; Pienaar, 1994; Shrader and Owen-Smith, 2002). These pairs or groups occupy large home ranges (7.2-45.2 km^2; Pienaar, 1994), which overlap extensively with those of other pairs and groups, but the majority of activity may be in smaller core areas (Owen-Smith, 1973, 1975; Pienaar, 1994). Close companions are typically 2 animals, such as mother and calf, 2 adolescents, or an adolescent and an adult female (Owen-Smith, 1973; Hillman-Smith, 1987; Shrader and

Owen-Smith, 2002). The space between companions is usually less than 5 m and rarely more than 25 m (Owen-Smith, 1973). In captivity, companion subgroups also usually include 2 or 3 socially-bonded adult females and/or adolescents and may be composed of only adult females (Kuneš and Bičík, 2002; Metrione, 2005; Metrione et al., 2007).

Associations between free-ranging individuals, particularly those that involve adolescents, frequently last less than a month, but some may persist for 5 or more months (Owen-Smith, 1973, 1975; Shrader and Owen-Smith, 2002). Companionships in captivity can be long-lasting (years) and might change only when a calf is born to one of the companions (Metrione, 2005; Metrione et al., 2007). While other rhinoceros may accompany adult females with older calves, females do not tolerate companions when they are accompanied by a young calf (Owen-Smith, 1973; Shrader and Owen-Smith, 2002; Metrione, 2005; Metrione et al., 2007). In fact, females seek dense brush in areas not frequented by other rhinoceros to give birth in the wild (Owen-Smith, 1973) and will separate from the rest of the group approximately 20 hours prior to parturition in captivity (Metrione, 2005; Metrione et al., 2007).

Female home ranges overlap the territories of 4-15 territorial males (White et al., 2007), providing an opportunity for mate choice in white rhinos. Mate choice by female black rhinos appears to be based on criteria that result in the preference of different males by different females (Garnier et al., 2001). White rhinos might choose mates based on the vegetative quality of a male's territory or his ability to defend a desirable territory. For example, preferential use by females of male territories was correlated with the total area of grassland in those territories, and time spent by females in male territories was a

significant predictor of mating (White et al., 2007). Variation in the number of offspring per male and in the frequency of occurrence of females in male territories might be related to territory size, vegetation structure, and tree species (Kretzschmar et al., 2002).

Kolar et al. (2002) found that female white rhinos identified males with the highest reproductive potential (older, territorial males as opposed to younger, subordinate males) based on urine. Although the number of male territories visited by females when they were acyclic did not differ from that when they were cycling (White et al., 2007), anestrous females might, during daily grazing activities, evaluate males based on chemical cues in their excrement and use this information for mate selection when in estrus. Estrous females might be testing the physical fitness of a male when she attempts to leave his territory and pass into that of another male, an action that the males strenuously attempt to prevent (Owen-Smith, 1971, 1973).

White rhino males mate with more than 1 female in both the wild (Owen-Smith, 1973) and in captivity, and Christensen et al. (2009) suggested that the positive relationship between serum testosterone concentrations in males and the number of females in their enclosure supports the observation that white rhino males are polygynous. Reproductive white rhino males, which are only 33 to 67% of all adult males, establish and defend non-overlapping territories, while non-reproductive, subordinate males live within a dominant male's territory (Owen-Smith, 1971, 1973, 1975; Rachlow, 1997; Rachlow et al., 1999). Territory boundary areas are narrow (50-100 m wide) and are visited by neighboring territorial males that strictly observe the

borders (Owen-Smith, 1971, 1973, 1975). Interestingly, black rhino males occupy and defend mutually exclusive territories in southern Africa, but they occupy undefended, overlapping home ranges in East Africa (Owen-Smith, 1988b).

Territory boundaries are delineated by scent marks left only by territorial males during spray-urination, dung-kicking, scraping with the fore- and/or hindlegs, and horn-scraping (Owen-Smith, 1973, 1975). These behaviors also are expressed by captive white rhino males throughout their enclosures (Metrione, 2005) and by wild black, Indian, and Sumatran rhino males (Owen-Smith, 1988b). Similar to male rhinos, only dominant, sexually mature, male white-tailed deer (*Odocoileus virginianus*) perform significant scraping and subsequent urination, and these actions might, in addition to advertising a male's presence to females, act to maintain spacing between males (Miller et al., 1987).

Both in the wild and in captivity, male and female rhinos defecate almost exclusively at communal dung piles (88% of the time; Laurie, 1982) and often spend time investigating excrement at the dung piles (Owen-Smith, 1973; Metrione, 2005). Female rhinos often urinate at dung piles as well (Metrione, personal observation). It has been suggested that the dung piles serve as a "bulletin board" (Owen-Smith, 1973), signaling the identity and, perhaps, the social and reproductive status of individuals. Odors may then be tracked across the ground as animals travel away from the dung pile with the excrement on their feet. Odors also might remain on the ground in places where a rhino has rested, particularly if it did so just after rolling in a mud wallow, another favorite place for female urination (Metrione, personal observation). Unfortunately, accurate

quantification of olfactory behavior in white rhinos is particularly challenging because they graze constantly and often walk with the head low to the ground wherein pheromones and other olfactory chemicals are likely encountered.

Grevy's zebra (*Equus grevyi*) are similar to white rhinos in that males maintain and defend distinct territories. Chaudhuri and Ginsberg (1990) reported higher concentrations of urinary androgens in territorial (19 ng/mg Cr) than in non-territorial (10.7 ng/mg Cr) male zebras, but this difference was not maintained when a territorial male was temporarily removed from his territory (12 ng/mg Cr, a 37% decrease from on-territory concentrations). When territorial male rhinos temporarily move off of their territory in search of water during drought, they adopt the behavior of a subordinate male, even in the presence of a female (Owen-Smith, 1973). Perhaps this behavioral change in territorial males and the behavioral differences between territorial and subordinate males are associated with differences in androgen levels. If androgen levels also are highly influential on semen quality, however, reproductive success of territorial males could be compromised by temporary changes in androgens and behavior. Subordinate rams (*Ovis aries*) had significantly lower semen volume and sperm concentration than dominant males, but subordinate males' average plasma testosterone concentration was only 15% lower than that of dominant males (Aguirre et al., 2007). Reduced semen quality does not necessarily render a male infertile or even subfertile, however, so it is possible that there is no reproductive disadvantage to a possible correlation between behavior, androgen concentrations, and semen quality in male white rhinos.

White rhino males (particularly younger, smaller individuals) can be killed in fights, which may select for delayed puberty or suppressed reproductive activity in subordinate males (Rachlow, 1997; Rachlow et al., 1998). Observations of territorial males strenuously preventing females from leaving their territories, but not entering the adjacent male's territory beyond 200 m if she should escape (Owen-Smith, 1971, 1973), suggest that territory defense is risky. Highly ritualized confrontations, involving advancements, retreats, horn-scrapings, horn-clashes directed mainly at the opponent's horn, and checked jabbing gestures as opposed to injury-causing movements, probably reduce the risk of injury (Owen-Smith, 1973, 1975). Some confrontations, however, particularly during a territory take-over, might be prolonged and bloody (Owen-Smith, 1973, 1975). Most males, therefore, will not become territory holders until they are 12 years of age or older (Owen-Smith, 1973). The average duration of occupation of the same territory by a male was 5.4 years, after which he might continue to be a territorial male in a different territory (Owen-Smith, 1973).

Reproductive Behavior in Wild and Captive White Rhinoceros

White rhino males appear to have the ability to identify reproductively valuable females. Males have been observed following a female as much as a week prior to the onset of estrus (Owen-Smith, 1973; Metrione, personal observation). Subordinate males rarely associate with estrous females for any significant length of time, while territorial males associate with a significantly larger number of such females (Rachlow, 1997; Rachlow et al., 1998). Similarly, subadult males and less preferred mates do not associate with female black rhinos during their fertile period, when fecal progesterone

metabolite concentrations are lowest (Garnier et al., 2002). Because females are not clumped in their distribution, have large home ranges, and are in estrus for only 1 day (Owen-Smith, 1973) every 2-3 years (if they become pregnant each time they mate; Owen-Smith, 1988c), successful males must maintain territorial status for a long period of time (Rachlow, 1997), especially as a male might only secure 1 or 2 copulations each year (Owen-Smith, 1973). Thus, there is an advantage to males being able to detect, perhaps through olfaction, a female coming into estrus in advance of its actual onset; the male can then work to maintain her in his territory, ensuring that he will mate that year (Owen-Smith, 1973).

Anestrous white rhinos will not tolerate a male within 10 m in the wild (Owen-Smith, 1973, 1975). Anestrous, captive females generally are also intolerant of the male's presence, but the tolerable male-female inter-individual distance varies from rhino to rhino (Metrione, personal observation). Mating is a long, slow process. The consort period lasts up to 20 days, during which the territorial male is obedient to the space-maintenance threats of the female, unless she approaches the border of his territory (Owen-Smith, 1973). Estrus typically lasts for 24 hours (Owen-Smith, 1973, 1975; Metrione, 2005), during which the male makes regular advances and "hiccing" vocalizations (Owen-Smith, 1973) and unfailingly makes olfactory investigations of the squirts of urine released by the female (Owen-Smith, 1973, 1975; Kuneš and Bičík, 2002; Metrione, 2005). Eventually, the female tolerates chin-resting by the male, usually on her hindquarters or back, mounting attempts, and finally copulation (Owen-Smith, 1973, 1975; Metrione, personal observation). Copulation lasts 15-30 minutes and might

include multiple ejaculations (Owen-Smith, 1973, 1975; Metrione, personal observation), which are preceded by rapid thrusting and characterized by quivering hindquarters in the male (Metrione, personal observation). This behavioral sequence differs from that of wild black rhinos in which reproductive behavior lasts up to 4 days (Garnier et al., 2002).

Reproductive Biology of Female Rhinoceros and Other Perissodactylids

Most research on the reproductive biology of white rhinos has been on captive animals and has utilized hormone analysis and ultrasound technologies. The corpus luteum, which forms after ovulation, produces progesterone, and thus, the luteal phase of the estrous cycle is characterized by elevated progesterone concentrations (Senger, 2003). Comparatively lower progesterone concentrations are observed during the follicular phase, which lasts from the regression of the corpus luteum to the next ovulation (Senger, 2003). Therefore, a sustained rise in progesterone (in blood) or progesterone metabolite (in feces) concentrations that is followed by baseline concentrations is recognized as evidence of the luteal phase of an estrous cycle and, indirectly, ovulation and formation of a corpus luteum, which has been documented by ultrasound (Radcliffe et al., 1997). The cyclic rise and fall of progesterone concentrations over time can be used to determine the presence, regularity, and length of estrous cycles (Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001).

Onset of ovarian activity, detected using fecal progesterone metabolites (hereinafter, progestagens), occurred at approximately 2.5 years of age in 1 captive female (Patton et al., 1999). Puberty, including the initiation of estrous cycles and display of appropriate sexual behaviors during estrus, in wild white rhinos is suspected to

occur between 3.8 and 4.5 years of age, although calves are not produced until 6.5 years (Owen-Smith, 1988c). Black rhinos in the wild mate for the first time at 4.5 years of age and produce their first calves at about 6 years (Owen-Smith, 1988c). Indian rhinos produce their first offspring in the wild at 6-8 years of age, but in captivity calves are born to females as young as 3 years of age (Owen-Smith, 1988c). A captive Grevy's zebra copulated for the first time at 1.3 years of age but did not conceive her first successfully-born foal until 2.2 years (Asa et al., 2001).

Estrous cycle lengths of both 1 month (30-35 days) and approximately 2 months (65-70 days) have been reported in white rhinos in several studies (Radcliffe et al., 1997; Patton et al., 1999; Brown et al., 2001; Graham et al., 2001; Roth, 2006). Additional complexity was recognized by Schwarzenberger et al. (1998) who described 4 categories of cycles based on duration and luteal phase progestagen concentrations: 1) regular, 10-week estrous cycles with luteal phase progestagens >800 ng/g of feces, 2) cycles ranging from 4-10 weeks with progestagens 250-750 ng/g, 3) no cycle regularity but some luteal activity (100-200 ng/g), and 4) acyclic, no luteal activity (<100 ng/g). This categorization scheme is challenging to apply unless baseline progestagen concentrations are consistent, but it does highlight the difficulty in defining the temporal features of the white rhino estrous cycles based on blood progesterone or fecal progestagen profiles. Indeed, even the same female can have more than 1 type of cycle (Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001). It is still uncertain which cycle is "normal," but an unpublished study on wild white rhinos suggests the cycle should be ~35 days (Swaisgood, personal communication).

In any form, the estrous cycle of the white rhino and some other members of Rhinocerotidae is considerably longer than that of the domestic horse (21 days; Blanchard et al., 2003a), a species used as a model for rhinoceros reproductive management. Estrous cycles in other rhinoceros species are 36-39 days or 61-86 days (average 43 days; Schwarzenberger et al., 2000; Stoops et al., 2004) in the Indian rhino (Roth, 2006), 26 days in the black rhino (Berkeley et al., 1997; Brown et al., 2001; Radcliffe et al., 2001), and 21-25 days, depending on when ovulation is induced, in the Sumatran rhino (Roth et al., 2001). The estrous cycle of Grevy's zebra (28-35 days; Asa et al., 2001) is similar in length to the short cycle of white rhinos, as are those of Baird's tapir (*Tapirus bairdii*) (30.8 days; Brown et al., 1994) and Malayan tapir (*Tapirus indicus*) (43.6 days; Kusuda et al., 2007). Not very different from the white rhino, a long cycle approximately twice as long as the shorter cycle also is documented in Malayan tapir (Kusuda et al., 2007).

Evident in the estrous cycle categories described by Schwarzenberger et al. (1998), anestrus or acyclicity and irregular cycles are common among adult white rhinos (Radcliffe et al., 1997; Patton et al., 1999; Brown et al., 2001; Hermes et al., 2006). Reproductive seasonality is not evident in captive white rhino females (Patton et al., 1999; Brown et al., 2001), and androgen and sperm concentrations remain constant throughout the year in captive males (Brown et al., 2001; Hermes et al., 2005; Roth, 2006). However, male androgen metabolite concentrations and the frequency of intersexual conflicts were positively correlated with the months of highest rainfall in the wild (Kretzschmar et al., 2000, 2004). Mating (Owen-Smith, 1988d) and conception

(Kretzschmar et al., 2004) peaks also occur in the wet season, and birth peaks occur in the dry season in the wild (Owen-Smith, 1988d). Wild female black rhinos also have peak fertility in the early rainy season (Owen-Smith, 1988d; Garnier et al., 2002). Black rhinos might have a period of anestrus during short days in the wild (Garnier et al., 2002), and fewer fertile ovulations were observed during the fall and winter in captivity (Radcliffe et al., 2001).

Progestagen profiles are far more useful than estrogen concentrations for monitoring reproductive cycles of white rhinos (Brown et al., 2001). This is not the case in other members of Perissodactyla: Urinary estrogen conjugate concentrations increase above baseline once the preovulatory follicle reaches 8 cm, and they return to baseline 3.9 days after their peak during behavioral estrus in Indian rhinos (Stoops et al., 2004). A surge in circulating estradiol precedes increasing concentrations of progesterone in Baird's tapir (Brown et al., 1994), and copulation occurs just before peaks in estrogen metabolites in Grevy's zebra (Asa et al., 2001). The follicular phase in white rhinos, during which preovulatory follicles grow 0.2 cm/day (Hildebrandt et al., 2007), corresponds to declining fecal progestagen concentrations, lasting anywhere from 2-21 days in one study (Brown et al., 2001) and 2-18 days in another study, with little difference in the average length depending on whether the cycle was of the shorter or longer type (9.7 and 10.5 days, respectively; Patton et al., 1999). The follicular phase was 9 days in a female in a third study (Radcliffe et al., 1997). The follicular phase among Indian rhinos is 14-15.9 days (Schwarzenberger et al., 2000; Stoops et al., 2004).

Low progestagen concentrations coincide with mating behavior, including mounting attempts and copulations, in white rhinos (Radcliffe et al., 1997; Patton et al., 1999), black rhinos (Garnier et al., 2002), and a Sumatran rhino (Roth et al., 2001), suggesting that these low hormone levels indicate the fertile period. Low fecal progestagens at copulation also are observed in Grevy's zebra (Asa et al., 2001), and copulation precedes a rise in serum progesterone in Malayan tapir (Kusuda et al., 2007). Though the estrous cycle of the white rhino is longer than that of the horse, estrus is longer in the horse (4-7 days; Blanchard et al., 2003a) and zebra (1-9 days; Asa et al., 2001) than in the white rhino (1-2 days; Owen-Smith, 1973, 1975; Radcliffe et al., 1997; Patton et al., 1999; Metrione, 2005).

Prior to ovulation in white rhinos, which occurs within 24 hours after breeding, the dominant follicle increases to 30 mm in diameter and changes from spherical to pear-shaped (Radcliffe et al., 1997). In horses, which usually ovulate within 48 hours prior to the end of estrus, the follicle also is 30-70 mm at ovulation, usually 40-45 mm (Blanchard et al., 2003a). The preovulatory follicle reaches 120 mm in Indian rhinos before releasing an ovum 48 hours following estrus (Stoops et al., 2004), and it reaches 30-50 mm in black rhinos (Berkeley et al., 1997; Radcliffe et al., 2001) before releasing an ovum 24-72 hours following estrus (Radcliffe et al., 2001). Two preovulatory follicles reach 21-22 mm 24 hours before mating in Sumatran rhinos, and both ovulate 50 hours after mating (Roth et al., 2004). A spike in luteinizing hormone, lasting less than 22 hours, was 30-fold higher after mating than at any other time during the estrous cycle in a

Sumatran rhino (Roth et al., 2001). Since ovulation is induced by mating in Sumatran rhinos, the dominant follicles will continue to expand to 79.5 mm if mating does not occur (Roth et al., 2001).

Fecal progestagens begin to rise 7-10 days following ovulation, documented by ultrasound or mating in white rhinos (Radcliffe et al., 1997; Schwarzenberger et al., 1998), and elevated luteal progestagen concentrations were observed to last 19-34 days (average ~25 days) for short cycles (Radcliffe et al., 1997; Patton et al., 1999) and 44-66 days for long cycles (Patton et al., 1999). Similarly, progestagens increased after day 5-12 post-mating in black rhinos (Garnier et al., 2002), and the luteal phase lasts 17-19.1 days in Indian rhinos (Schwarzenberger et al., 2000; Stoops et al., 2004). Diestrus lasts only 14-15 days in the horse (Blanchard et al., 2003a), but, as in the short cycle for white rhinos, this is approximately 71% of the estrous cycle. In contrast to the relatively long luteal phase and short follicular phase of white rhinos and horses, the interluteal period of Baird's tapir occupies approximately 40% of their estrous cycle (Brown et al., 1994). A technical note: 5α-reduced pregnanes are the predominant progestagens in African rhino species while 5β-reduced pregnanes are dominant in Indian and Sumatran rhinos (Schwarzenberger et al., 2000).

Normal gestation in the white rhinoceros is approximately 16-17 months (490-525 days; Patton et al., 1999), which is the longest among rhino species (425-487 days, Indian rhino; Schwarzenberger et al., 2000; 475 days, Sumatran rhino; Roth et al., 2004; 465-475 days, black rhino; Berkeley et al., 1997; Radcliffe et al., 2001). Gestation in Grevy's zebra is 391-406 days (Asa et al., 2001) and in Baird's tapir is 392 days (Brown et al.,

1994). Chorionic gonadotropin, produced by the endometrial cups that form early in the second month of gestation, is detectable in horses by day 38-42 and disappears by day 150 (Noakes, 1996), and it also is detectable in Grevy's zebra by day 38-40 after mating up to day 195 (Asa et al., 2001). Chorionic gonadotropin is not, however, detected in rhino species (Roth et al., 2001; Roth, 2006). Increasing and sustained progesterone concentrations elevated above luteal levels by 3-5 months post-mating are currently the best method for pregnancy diagnosis in African and Indian rhinos (Berkeley et al., 1997; Patton et al., 1999; Schwarzenberger et al., 2000; Brown et al., 2001; Radcliffe et al., 2001; Roth, 2006), and estradiol is consistently below detection in Sumatran rhinos as well (Roth et al., 2004).

In contrast, fecal estrogen metabolites increased between days 71 and 104 of gestation in Grevy's zebra and did not decline until the last weeks before parturition (Asa et al., 2001), and estrogen metabolites were 10-100 times higher during pregnancy than postpartum or in non-pregnant animals in Przewalski's horses (*Equus przewalskii*; Bamberg et al., 1991). Estrogens rise during gestation in the domestic horse until day 210, after which they gradually decline (Noakes, 1996). Estradiol also appears in late pregnancy in Baird's tapir (Brown et al., 1994), and estrogen metabolites were approximately 10 times higher during pregnancy than postpartum in Malayan tapir (Bamberg et al., 1991).

Progesterone and its metabolites are highly elevated throughout pregnancy in white rhinos (Patton et al., 1999; Metrione, 2005; Oliva, personal communication). Circulating progesterone from the corpus luteum is high only at the beginning of

pregnancy among zebra species (*Equus burchelli*, *E. zebra hartmannae*, and *E. grevyi*), and 5α-dihydroprogesterone from the placenta becomes predominant after the fifth month of gestation (Klima et al., 1999; Asa et al., 2001). The corpus luteum, supplemented by accessory corpora lutea between days 40 and 140 (Noakes, 1996), also is the main source of progesterone during the first 3 months of gestation in the horse (Blanchard et al., 2003c), after which the placenta takes over progesterone production (Noakes, 1996). Progesterone in the placental tissue then remains high while circulating progesterone is low in the mare during mid- to late gestation (Noakes, 1996). Decreased progesterone between days 120 and 150 in Sumatran rhinos is believed to be equivalent to the same decrease in the horse during the transition from ovarian progesterone to those of placental origin (Roth et al., 2004). Relaxin increases in Sumatran rhinos during late pregnancy, peaking 2 weeks before parturition, and prolactin increases after day 458 (Roth et al., 2004).

Interbirth intervals in wild white rhinos range from 2-3.4 years and tend to be shorter for younger females, increasing as females age (Owen-Smith, 1988c). Reports of 17, 18, and 21.5-month interbirth intervals in captivity suggest that postpartum estrus is possible in this species (Patton et al., 1999; Steele, personal communication). Ovulation was successfully induced 30 days postpartum for an artificial insemination procedure (Hildebrandt et al., 2007). Baird's tapirs resume cycling 16 days following parturition and can become pregnant during the first postpartum estrus (Brown et al., 1994). Wild black rhinos might have a 4-8-month postpartum anestrus (Garnier et al., 2002), and postpartum anestrus also occurs in captive black rhinos (Brown et al., 2001). Mean

calving interval among wild black rhino is 1.7-4 years depending on where in Africa the animals are located, the shortest intervals occurring in South Africa and the longest intervals occurring in Tanzania and in Amboseli in Kenya (Owen-Smith, 1988c). Wild Indian rhinos have a 2.8-year calving interval (Owen-Smith, 1988c).

Early embryo development in white, black, and Sumatran rhinos is similar to that of the horse model (Roth, 2006). The embryonic vesicle enters the uterine lumen of the horse approximately 6 days after ovulation (Arthur, 1996; Blanchard et al., 2003c). The white rhino embryonic vesicle can be visualized using ultrasonography by day 15 post-ovulation (Radcliffe et al., 1997). Two embryonic vesicles form in Sumatran rhino (visible 17 days post-mating), but the smaller one eventually regresses and dies (Roth et al., 2004). The surviving vesicle grows 2.5 mm/day from days 14-21, after which growth pauses and the embryo ceases to migrate through the uterus (Roth et al., 2004). The embryo proper (the forerunner of the fetus as opposed to the entire conceptus) forms at this time (Roth et al. 2004), similar to the timing in white rhinos (day 23; Radcliffe et al., 1997). Vesicle fixation (cessation of motility) occurs in the Sumatran rhino between days 21 and 31 (Roth et al., 2004), and growth of the vesicle resumes at day 28 and grows 3.0 mm/day until at least day 63 (after which it was too large to measure accurately; Roth et al., 2004). Fixation occurs between days 16-18 in the horse (Arthur, 1996), and the conceptus does not begin attaching to the endometrium until days 40-45 of gestation (Blanchard et al., 2003c). Frequent movement of the equine conceptus throughout the

uterine horns and body prior to fixation is critical to maternal recognition of pregnancy, as the corpus luteum will regress spontaneously when embryo mobility is restricted (Noakes, 1996).

Rhino biologists have given much attention to the absence and irregularity of estrous cycles as the primary source of reproductive failure in white rhinos, but there is no reason to exclude the involvement of post-copulatory and postovulatory events, namely during conception and early pregnancy, in reproductive failure. Indeed, early embryonic death (EED) has been identified by ultrasound in white (Radcliffe et al., 1997), black (Radcliffe et al., 2001), and Sumatran rhinos (Roth et al., 2001), and 6 cases have been confirmed in white rhino alone (AZA, 2009). Extended luteal phases (related to the longer cycle) might be correlated with endometritis and pyometra, which might cause EED (Patton et al., 1999; Roth, 2006; AZA, 2009). During 2 white rhino pregnancies, irregular embryo mobility and orientation was accompanied by inflammatory exudates and was followed by EED (Radcliffe et. al, 1997). Reduced uterine contraction, associated with aging in horses, can lead to reduced clearance of foreign material, including bacteria that cause inflammation (Carnevale and Ginther, 1992). Inflammation and the resulting fluid production probably have spermicidal and embryocidal effects (Carnevale and Ginther, 1992; Blanchard et al., 2003d).

Luteal insufficiency or genetic incompatibility also might contribute to EED (Blanchard et al., 2003d; Roth, 2006; AZA, 2009). The extensive transuterine movement of the equine conceptus 14-16 days after ovulation prevents premature luteolysis of the primary corpus luteum, which would result in pregnancy loss, by preventing the uterus

from producing prostaglandin $F_{2\alpha}$ (Noakes, 1996; Blanchard et al., 2003c). Supplementing the pregnancy with a synthetic progestin, altrenogest, was successful in avoiding embryo loss in a Sumatran rhino (Roth et al., 2004). Embryo loss might not be limited to females in captivity, as it also was probable in 2 wild black rhinos in which progestagen concentrations sustained above 2,000 ng/g declined abruptly after day 70 and 100 of presumed gestation (Garnier et al., 2002). In addition to EED, vaginal and cervical prolapse can lead to abortion in mid-pregnancy (Vahala, 1993).

Other problems among captive southern and northern white rhino include cystic hyperplasia; cervical, ovarian, and uterine tumors, polyps, and cysts (Hermes et al., 2002, 2006). Hermes et al. (2006) reported a lower incidence of pathological lesions in parous compared with nulliparous females and suggested that the reproductive pathology and ovarian inactivity in adult white rhinos is an age-related consequence of long periods without pregnancy. For example, among females with absent or erratic luteal hormone profiles, ovarian activity was still present in females 3-19 years of age but absent in females 15-38 years of age (Hermes et al., 2006). What, then, is the cause of these long non-reproductive periods?

"When breeding does not occur, something is wrong with the methods of keeping the animals; if breeding does occur, it is a guarantee that the conditions are essentially right, since regular breeding presupposes, at least among the higher animals, a certain measure of well-being in the parents."—H. Hediger (1964a)

Because white rhinos reproduce well in the wild and many of the founders of the captive population reproduce when appropriate husbandry is practiced, the lack of reproduction in the captive-born females and some wild-caught, nulliparous females might be due to factors in the captive environment. Behavioral comparisons between captive-born and wild-caught, adult females implicated effects of the captive environment on development as the cause for failed reproduction among captive-born females (Swaisgood et al., 2006). Whether it compromises reproductive development or reproductive function in adulthood, one of the first culprits that comes to mind is "stress" in the captive environment.

Stress and Its Potential Effects on Reproduction

The stress response was first described by Selye (1936) as a "general adaptation syndrome" to physical "nocuous agents". This definition was later expanded to include responses to psychological stimuli, including perceived environmental deficiencies, lack of control over the environment, and lack of adequate social interaction (Engel, 1967; Dantzer and Mormède, 1983). The stress response, therefore, can be defined as the physiological and behavioral response elicited when the brain perceives a significant disturbance of homeostasis, caused by a marked or unpredictable environmental change (Wingfield and Raminofsky, 1999; Moberg, 2000; Nelson, 2005b). The first step in the biological response to a stressor might be behavioral, followed by the short-duration effects of epinephrine release from the adrenal medulla and activation of the "fight or flight" response (Dantzer and Mormède, 1983; Moberg, 2000; Nelson, 2005b). Lastly, the hypothalamic-pituitary-adrenal (HPA) axis is activated, resulting in increased

secretion of corticotropin-releasing hormone (CRH) from the hypothalamus, adrenocorticotropic hormone (ACTH) from the anterior pituitary, and glucocorticoids (e.g., corticosterone and cortisol) from the adrenal cortex (Dantzer and Mormède, 1983; Moberg, 2000; Nelson, 2005b). Glucocorticoid secretion affects carbohydrate metabolism to liberate stored energy (Nelson, 2005b). These responses are essential for the animal to adapt to new conditions (Selye, 1936) and restore homeostasis (Moberg, 2000). For example, salivary cortisol was elevated in 2 Indian rhinos and 6 Asian elephants (*Elephas maximus*) in the month the zoo opened to the public compared to that in the months before and after the opening (Menargues et al., 2008), suggesting these animals responded to the environmental change with activation of the adrenal cortex, followed by a return to baseline adrenal activity. Restraint and translocation are stressful for white and black rhinos based on elevated levels of cortisol and corticosterone, but recovery was observed in 4-6 weeks in the black rhinos that were monitored long-term (Turner et al., 2002).

While declining glucocorticoid levels were viewed as recovery or acclimation in the previous 2 examples, it could be argued that suppressed glucocorticoids are indicative of chronic stress, as suggested by Linklater et al. (2010) in their recent study of wild white and black rhinos that were captured and then held in small enclosures prior to transport to other reserves. Fecal corticoid concentrations remained 2-3 times higher than pre-capture concentrations for 17 days in black rhino males and females and remained 3-5 times higher than pre-capture concentrations for 75 days in white rhino males (Linklater et al., 2010). Fecal corticoid concentrations in these animals then gradually

declined to below pre-capture concentrations (Linklater et al., 2010). In contrast, fecal corticoid concentrations in white rhino females continued to increase throughout their time in captivity (Linklater et al., 2010). Sex hormone (progestagens and androgen metabolites) concentrations declined to below pre-capture levels, and this decline was most immediate in white rhino females (Linklater et al., 2010). Based on the fact that gonadal hormones were suppressed in all rhinos and that they continued to show aggressive and flight responses throughout their time in captivity, Linklater et al. (2010) argued that the suppressed corticoid levels in black rhinos and in white rhino males were a result of intrinsic negative-feedback control mechanisms, not acclimation, and that those animals were, in fact, experiencing chronic stress. Chronic stress in white rhino females, however, appears to result from a slightly different set of conditions, namely sustained, elevated corticoid levels in conjunction with suppressed reproductive hormones. It is worth discussing the mechanisms by which hormones involved in the stress response might suppress reproduction.

When the initially-adaptive response to acute or chronic stress shifts sufficient resources away from other biological functions, deleterious effects can occur (Moberg, 2000). For example, mortality of captive black rhinos was positively correlated with variability in fecal corticoid metabolites (Carlstead and Brown, 2005). A single significant stressor is not necessarily required; combinations of low-level stressors (exercise, diet) can synergize to compromise reproduction by increasing the length of estrous cycles in *Macaca fascicularis* (70% of individuals in one study; Williams et al., 2007). Even chronic psychological stress can cause infertility in mammals (Boonstra et

al., 1998) because an increase in any or all of the stress hormones (CRH, ACTH, glucocorticoids) can suppress reproductive function by interfering with the normal functioning of the hypothalamic-pituitary-gonadal (HPG) axis (Moberg, 1991) and, for example, disrupting follicle development and ovulation. Increases in ACTH and glucocorticoids can suppress release of luteinizing hormone (LH) in sheep (reviewed by Dobson et al., 2003), and CRH abolishes plasma LH pulses in rats (*Rattus norvegicus*; Rivier et al., 1986). In addition, higher CRH might decrease gonadotropin-releasing hormone (GnRH) production in stress-sensitive primates (*Macaca fascicularis*; Centeno et al., 2007b). Neuronal transport of GnRH peptides might be impeded in stress-sensitive primates (Centeno et al., 2007a), and serotonin, which normally enhances GnRH secretion (Dobson et al., 2003), also is compromised due to lower expression of serotonin transporter messenger RNA compared to that in stress-resistant primates (Bethea et al., 2005).

Increasingly elevated corticoid concentrations concomitant with suppression of progestagens in wild female white rhinos (Linklater et al., 2010) and higher fecal corticosterone metabolite variability in non-cycling compared to cycling captive white rhinos (Carlstead and Brown, 2005) are compelling reasons to investigate the effects of stress hormones on reproduction in this species. Factors that might stimulate the stress response in white rhinos also should be explored. As Linklater et al. (2010) point out, however, elevated or suppressed glucocorticoid concentrations on their own should not

be interpreted as having a negative impact on the animal. Moreover, analysis of data on behavior, reproductive hormones, and glucocorticoids collected simultaneously is likely to provide the most complete picture of an animal's health and welfare.

A Preview of the Chapters That Follow

Swaisgood et al. (2006) suggested that multi-institutional research should be utilized to determine which aspects of the captive environment might lead to reproductive failure. Brown et al. (2001) noted that appropriate social groupings probably are essential for stimulating and sustaining reproductive behavior in white rhinos. Thus, the overall goal of the project described in this dissertation was to examine social behavior, the social environment (dominance status), and characteristics of the captive environment that might affect normal sexual development in captive-born females and/or limit reproductive success in adults. This was accomplished by examining behavior and hormone (progesterone and corticosterone) concentrations in females housed in specific captive conditions throughout the United States. This study focused on captive-born females, but parous, wild-caught females were included for comparative purposes if they were housed with the observed captive-born females, and samples were collected from nulliparous, wild-caught females for use in hormone analyses. The following hypotheses (parenthetically referenced throughout Chapters 2 and 3) were examined:

Hypothesis 1: Social interactions in captivity influence reproductive success (i.e., parity), estrous cyclicity, and onset of puberty.

1A. Aggression and dominance are associated with reproductive success, estrous cyclicity, and earlier onset of puberty.

1B. More frequent sexual advances by males are associated with dominance, reproductive success, and estrous cyclicity in females.

1C. More frequent sexual play interactions among females are associated with reproductive success, estrous cyclicity, and onset of puberty.

Hypothesis 2: Characteristics of the captive environment influence reproductive success and estrous cyclicity.

2A. More spacious enclosures are associated with reproductive success and estrous cyclicity.

2B. A social group of females/adolescents is associated with reproductive success and estrous cyclicity.

2C. The presence of a familiar conspecific known from adolescence is associated with reproductive success and estrous cyclicity.

2D. Access to more than 1 male is associated with reproductive success and estrous cyclicity.

2E. Access to a novel male is associated with reproductive success and estrous cyclicity.

Hypothesis 3: Wild-caught females who have not reproduced in captivity have progestagen profiles similar to nulliparous, captive-born females.

Hypothesis 4: High corticosterone concentrations are associated with subordinate social status, particular captive housing conditions, poor reproductive success, and anestrus.

Chapter 2: The Effects of Social Behavior, the Social Environment, and the Captive Environment on Reproductive Success and Estrous Cyclicity in Female White Rhinoceros

Introduction

With 61% of captive-born, female white rhinoceros failing to reproduce, only ~50% of all the captive females sustain the North American zoo population, most of whom were wild-caught (AZA, 2009). As these wild-caught females age, fewer reproductive females are left to sustain the captive population, both in terms of numbers and in terms of genetic variability. One of the objectives of the White Rhinoceros Species Survival Plan is to increase the genetic contribution of the captive rhinos that have not yet successfully reproduced to the population (AZA, 2005). In the meantime, the captive population continues to be supplemented by the costly, and sometimes risky, importation of animals from Africa (Swaisgood et al., 2006). Poor reproductive success in captive rhinos has been attributed to a number of factors, including: anestrus and irregular estrous cycles (Radcliffe et al., 1997; Patton et al., 1999; Brown et al., 2001; Hermes et al., 2006); pathologies of the reproductive tract (Hermes et al., 2002, 2006); and the loss of early embryos (Radcliffe et al., 1997; Patton et al., 1999; Roth, 2006; AZA, 2009). In turn, social behavior, social status, and the captive environment might affect these aspects of reproduction.

The white rhinoceros is the most social of the 5 species in Rhinocerotidae. Wild females live in pairs or small groups of up to 6 individuals (Owen-Smith, 1973, 1975; Pienaar, 1994; Shrader and Owen-Smith, 2002), and it is widely assumed that successful reproduction in white rhinos requires the maintenance of social groupings similar to those in the wild (AZA, 2005). However, not all female rhinos are housed with female companions, and at least some of the non-reproductive females in the captive population are housed in moderately-sized groups. In these instances, perhaps the non-reproductive females are at the bottom of dominance hierarchies. In a study of 2 captive white rhino groups, the most subordinate female rhino in each group was the only nulliparous female, suggesting that reproduction might be suppressed in low-ranking captive females (Metrione, 2005; Metrione et al., 2007). Some researchers have speculated that social hierarchies in group-housed white rhinos might be associated with the suppression of estrous cycles (Hermes et al., 2006), while others have found only limited evidence for the dominance of wild-caught, founding females over captive-born females and no evidence for the suppression of sexual behavior in captive-born females by wild-caught females (Swaisgood et al., 2006). If reproduction is suppressed in low-ranking females, irrespective of their country of origin, then more aggressive and dominant females should tend to be parous while those that are less aggressive and subordinate should tend to be nulliparous (hypothesis 1a). However, subordinate females might show some evidence of estrous cyclicity. The latter prediction is based on previous observations of behavioral estrus occurring at approximately 1-month intervals in the lowest-ranked female in one captive group (Metrione, 2005; Metrione et al., 2007). If dominance plays a role in

reproductive success, it also might be possible that a correlation exists between aggression and the onset of puberty, in which more aggressive female adolescents show first signs of luteal activity sooner than those who are less aggressive (hypothesis 1a).

Swaisgood et al. (2006) observed that wild-caught and captive-born females did not differ in their proficiency of displaying appropriate sociosexual behavior: Males were equally likely to direct courtship behaviors toward wild-caught and captive-born females during peak estrus, and copulatory behavior was not compromised in captive-born females. Data from a questionnaire sent to institutions housing captive-born females, however, indicated that wild-caught females might have been more likely to copulate than captive-born females (Swaisgood et al., 2006). Given that there also might have been a tendency for wild-caught females to dominate captive-born females (Swaisgood et al., 2006), it is worthwhile to investigate if differences in copulatory behaviors and estrous consort relationships exist among females based on dominance or aggression. For example, there was a significant negative correlation between dominance rank (dominant = rank 1) among female gelada baboons (*Theropithecus gelada*) and the mean number of offspring per female even though there was no significant interference of sexual behavior by the dominant females (Dunbar, 1980). Greater amounts of aggressiveness and assertiveness by a female were found to contribute positively to female black rhinos' chances of breeding (Carlstead et al., 1999b). It has previously been noted that the male in 1 group of white rhinos had a greater tendency to engage the least dominant female in a confrontation while he generally behaved submissively to the other females (Metrione, 2005; Metrione et al., 2007). Based on these observations, females

that are dominant might be predicted to be approached and mated by the male more frequently than subordinate females, and a higher frequency of non-threatening, sexual advances by males may be associated with greater reproductive success and estrous cyclicity (hypothesis 1b). Differences in sexual play behavior among and between adult and female adolescents also should be examined for relationships to reproductive success, estrous cyclicity, and onset of puberty (hypothesis 1c).

Because of poaching pressures in the wild, rhinos must be protected on small reserves, which limit natural dispersal, and as density increases on such preserves, the recruitment rate of calves decreases (Rachlow, 1997; Rachlow and Berger, 1998). The age at first reproduction was delayed and birth intervals were longer in female white rhinos living at high densities (Whovi Game Park within Matobo National Park, Zimbabwe) compared to those living in lower densities (Hazelside Area of Matobo National Park) (Rachlow, 1997; Rachlow and Berger, 1998). Such negative effects on reproduction in high-density populations might be due to competition for reduced food resources because reductions of fat deposits and muscle mass observed by the end of the dry season were more pronounced in the high-density population compared to the low-density population (Rachlow, 1997; Rachlow and Berger, 1998). Interestingly, age at first reproduction also was delayed in bighorn ewes (*Ovis canadensis*) living at high densities even though young ewes exceeded the threshold body mass for reproduction (Jorgenson et al., 1993). With relatively small numbers of rhinos at each institution (rarely more than 10 even at the largest institutions), density might be less influential than it is in wild populations, but the response of captive rhinos to enclosure size might be

similar to that of wild rhinos living in preserves at high densities. Though more than enough food is provided for all the rhinos in a captive group to receive adequate nutrition, dominant females do appear to gain access to food sooner and feed for longer durations than subordinate females when the group is fed in specific and spatially restricted areas (Metrione, 2005; Metrione et al., 2007). The prediction, therefore, is that a greater proportion of adult females housed in spacious enclosures will exhibit estrous cyclicity and produce calves compared to females housed in more restricted enclosures (hypothesis 2a).

Adolescent dispersal is instigated by the mother's aggression at the birth of her next calf (Owen-Smith, 1973). Owen-Smith (1973) observed that male adolescents were sometimes chased by territorial males, but there was no apparent social pressure for female dispersal out of the home range. Regardless of sex, excursions from home ranges usually occur in pairs or small groups, either with another adolescent(s) or an unrelated adult female (Owen-Smith, 1973, 1975; Shrader and Owen-Smith, 2002). After they are weaned from their mother (~2-3 years of age), if captive female adolescents are transferred to different institutions, they might not be sent with other rhinos. Interestingly, the only 2 second-generation captive-born females (Julie and Maggie; Metrione, 2005; Metrione et al., 2007) who reproduced prior to the start of this study were both born at the same institution, lived together there for 2 years, and later were together at a different institution where their first calves were conceived and born. Furthermore, Swaisgood et al. (2006) found that captive-born females living with their wild-caught mothers produced significantly more calves than captive-born females living

without their mothers. This lends support to the idea that perhaps successful reproduction in this social species is aided not only by natural groupings of conspecifics (AZA, 2005; Swaisgood et al., 2006) (hypothesis 2b), but, more specifically, by the presence of familiar conspecifics, possibly including the mother (hypothesis 2c). It also is possible that housing at the natal vs. non-natal institution is associated with calf production and cyclicity.

Not all male-female pairings are compatible (Steele, personal communication), and it has been suggested that estrous cycling and breeding require the presence of a novel, sexually mature male (Reece, 1993; Bertschinger, 1994; Pienaar, 1994; Patton et al., 1999; Brown et al., 2001). Home ranges of wild females overlap with the territories of several males (White et al., 2007), and each male might hold his territory for only about 5 years before moving to a different territory (Owen-Smith, 1973). Females at some institutions might be housed with the same male with which they were raised since birth, or they might have access to only 1 male. In the absence of the opportunity to choose a genetically and phenotypically desirable or compatible mate, the frequency of reproductive females in a population and the rate of reproduction in those females are likely to be reduced (Møller and Legendre, 2001). Female reproductive decisions (i.e., failure to choose a mate) can, therefore, dramatically influence the population growth of small populations (Møller and Legendre, 2001). One might predict that cycling and parous females would have access to more than 1 male (hypothesis 2d) and that those males are not the same ones with which the females were raised (hypothesis 2e).

Some wild-caught females, including those that are housed in moderately-sized groups, fail to reproduce. Whether these females are anestrous, have infertile cycles, experience early embryo loss, or have other problems that prevent successful reproduction is usually unknown. It is presumed that wild-caught, adult rhinos experienced normal puberty and normal reproductive activity as adults prior to capture. This study includes 2 wild-caught females that produced calves in the wild but have not done so in captivity. Thus, although the development of wild-caught females would not have been impacted by captivity, the captive environment might have a suppressive effect on their reproduction as adults. Comparisons of progestagen profiles of wild-caught females who have not reproduced to those of nulliparous, captive-born females might reveal similar patterns. Acyclicity among both wild-caught and captive-born females would suggest that anestrus in captive-born females might not be due to an inadequate developmental environment prior to puberty, but rather from a suppressive effect of the captive environment during adulthood (hypothesis 3).

The goal of this project was to examine social behavior, the social environment (dominance status), and characteristics of the captive environment that might affect normal sexual development in captive-born females and/or limit reproductive success in adults. Aggressive, sexual, and sexual play behaviors of captive-born adolescents and adults and wild-caught adults were compared among the females grouped by parity (parous vs. nulliparous), estrous cyclicity (acyclic vs. cycling), onset of puberty (defined as the first appearance of luteal phases of estrous cycles), and dominance (hypotheses 1a-c). The influence of the social environment, as described by dominance status, also was

evaluated relative to behavior and the proportion of females that was parous or that had ovulatory cycles (hypotheses 1a-c). Relationships between features of the captive environment and females' reproductive activity, as evidenced in the proportion that was parous or that had ovulatory cycles, were examined (hypotheses 2a-e). Estrous cyclicity and pregnancy were determined by examination of profiles of progesterone in serum or progesterone metabolites (hereinafter, progestagens) in fecal samples. Estrous cycle lengths of both 1 month (30-35 days) and approximately 2 months (65-70 days) have been reported in captive white rhinos in several studies (Radcliffe et al., 1997; Patton et al., 1999; Brown et al., 2001; Graham et al., 2001; Roth, 2006), and the same female can have more than one type of cycle (Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001). Nulliparous, wild-caught females also were included in the progestagen analyses to determine if the captive environment might impact reproduction in adulthood despite presumed normal development in the wild (hypothesis 3).

Methods

Study population.

Sixteen member institutions of the AZA housing a total of 45 female white rhinos participated in this project (Table 1). Sample sizes for each group were as follows: captive-born, parous; n = 13; captive-born, nulliparous; n = 13; wild-caught, parous; n = 6; wild-caught, nulliparous; n = 7; and captive-born, adolescent; n = 6 (Table 1). Sample sizes for components of the study were: behavioral analysis, n = 36; analysis of reproductive and housing history, n = 40; progesterone and progestagen analysis, n = 37. Two of the females in the wild-caught, nulliparous group (Bertha and Mashile, captured

Table 1. Study population of captive female southern white rhinoceros involved in behavioral observations (Sept. 2007-Dec. 2008), fecal and serum sample collection (Oct. 2007-Aug. 2009), and analysis of reproductive and housing history.

Rhino	Institution‡	Behavior	History	Samples	Notes
Captive-born parous					
Maggie	WOCC	X	X	X	Serum
Gabby	Jacksonville	X	X	X	Fecal. Access to adolescent male only.
Julie	Wilds	X	X	X	Fecal
Maggie	Wilds	X	X	X	Fecal
Zenzele	Wilds	X	X	X	Fecal
Bloom	LCS	X	X	X	Fecal
Eliza	LCS	X	X	X	Fecal. Pregnant.
Lissa	LCS	X	X	X	Fecal
Taraja	LCS	X	X	X	Fecal. Pregnant.
Holly	SDWAP	X	X	X	Fecal
Yvonne	Audubon	X	X	X	Fecal
Laptop	Birmingham	X	X	X	Fecal. No access to male.
Kendi	DAK		X	X	Fecal
TOTALS		12	13	13	
Captive-born nulliparous					
Lucy	WOCC	X	X	X	Serum
Bonnie	LCS	X	X	X	Fecal
Kiangazi	LCS	X	X	X	Fecal
Paddy	LCS	X	X	X	Fecal
Yebonga	Reid Park	X	X	X	Serum
Dumisha	SDWAP	X	X	X	Fecal
Kiazi	SDWAP	X	X	X	Fecal
Utamu	SDWAP	X	X	X	Fecal
Taryn	WS	X	X	X	Fecal
Jeannie	Tulsa	X	X	X	Fecal
Ajabu	Birmingham	X		X	Fecal. No access to male.
Lulu	Louisville	X	X		No access to male.
Sindi	Louisville	X	X		No access to male.
TOTALS		13	12	11	

Continued.

Table 1. Continued.

Rhino	Institution‡	Behavior	History	Samples	Notes
Wild-caught parous					
Kathy	WOCC	X	X	X	Serum. Corticosterone.
Alice	LCS	X	X		
Kisiri	Busch	X	X		
Mlaleni	Busch	X	X		
Nthombi	SDWAP	X	X		
Macite	Audubon	X	X		
TOTALS		6	6	1	
Wild-caught nulliparous					
Bertha	Albuquerque		X	X	Fecal. Calf in wild.
Emalah	Albuquerque		X	X	Fecal
Helen	DAK		X	X	Fecal
Jao	DAK		X	X	Fecal
Mashile	Omaha		X	X	Fecal. Calf in wild.
Marina	Omaha		X	X	Fecal
Mambo	Indianapolis		X	X	Fecal
TOTALS		0	7	7	
Adolescent					
Kelly	WOCC	X	X	X	Serum. Parous in 2010.
Evey	Wilds	X		X	Fecal
Sally	Wilds	X		X	Fecal
Dakari	Busch	X		X	Serum
Lucy	Busch	X	X	X	Serum. Parous in 2010.
Kayla	DAK			X	Fecal
TOTALS		5	2	6	
GRAND TOTALS		36	40	38	45*

*45 females were involved in at least 1 aspect of the study.

‡Albuquerque Biological Park = Albuquerque; Audubon Zoo (LA) = Audubon; Birmingham Zoo = Birmingham; Busch Gardens (FL) = Busch; Disney's Animal Kingdom = DAK; Henry Doorly Zoo = Omaha; Indianapolis Zoo = Indianapolis; Jacksonville Zoo = Jacksonville; Lion Country Safari (FL) = LCS; Louisville Zoo = Louisville; Reid Park Zoo (AZ) = Reid Park; San Diego Wild Animal Park = SDWAP; Tulsa Zoo & Living Museum = Tulsa; White Oak Conservation Center (FL) = WOCC; Wildlife Safari (OR) = WS; the Wilds (OH) = Wilds

in 1998 and 1999, respectively) reproduced in the wild but have not reproduced in captivity. To be considered an adolescent for this project, females needed to be nulliparous throughout sample collection, show no evidence of mating during and for at least 3 months after behavioral observations were concluded at their institution, and be 4 years of age or younger for the majority of 2008. Diets for study animals are described in Appendix A. In general, diets included once or twice daily rations of a concentrated feed and access to hay and/or grass throughout the day and night.

Behavioral observations.

Between 10 days (at least 80 hours) to 1 month (~240 hours) was spent observing 36 selected females at each of 12 institutions (Table 1). Less time (<30 days) was spent at institutions that did not house mature males with the females (Birmingham, Jacksonville, Louisville) or where captive-born adolescents were the primary focus (Busch Gardens). One month was spent at the other institutions to increase the probability of observing estrous consort between captive-born females and mature males. The total number of observation hours per day was, whenever possible, held constant across institutions. All observations were recorded by L. C. Metrione during daytime hours. In addition, video recordings were made of rhinos in the barn at the Wilds during 5 nights. Keepers also recorded estrus and mating for the entire duration of fecal and serum sample collection.

Specific behaviors were identified according to a wild white rhino ethogram (Appendix B) provided by Owen-Smith (1973) and used in other studies (Metrione, 2005; Swaisgood et al., 2006; Metrione et al., 2007). The frequency, context, and

particular details of the behaviors were recorded as they occurred using continuous focal-animal and critical incident sampling (Altmann, 1974). Behaviors that were assessed in the final analyses included aggressive, sexual, and sexual play behaviors. Other behaviors and interactions of note were recorded on the field data sheets.

Detailed behavioral definitions and the ways in which particular interactions were handled are described in Appendix C. Behavioral estrus was defined (Owen-Smith, 1973, 1975; Metrione, 2005, Metrione et al., 2007) as the period of consort during which: 1) the male approaches the female approximately every 15 minutes making a "hiccing" vocalization, 2) the male smells the vaginal opening, and the female squirts urine, 3) the male chin-rests, and 4) the male attempts mounting, intromission, and ejaculation. To be considered a sexual advance by a male, however, it was not necessary for every male behavior listed in the above definition to occur; 1 of the 4 was sufficient. Chin-resting and mounting are sexual behaviors for males, but they are also sexual play behaviors scored for females. The majority of aggressive behaviors were expected to occur during feeding times, particularly when a group of rhinos was fed in close-quarters (Metrione, 2005; Metrione et al., 2007). Daily frequencies of aggression calculated for this project therefore include feeding times that occurred during the observation period, but observation of full feeding times at every institution was not always permitted.

Behavioral data analyses and determination of dominance status.

Behavioral frequencies were recorded and summed for 30-minute intervals from beginning to end of observation each day, providing half-hourly behavioral frequencies for each female (Metrione et al., 2007). If breaks in observations were absolutely

necessary, they were timed to coincide with when the rhinos were resting (recumbent on the ground), and behavioral frequencies were recorded as a "0" for the first half-hour and as "No Data" for any additional half-hour increments. Half-hours with "No Data" were not included in the final analysis. Additional details on exceptions and contingencies in recording behavioral observations are presented in Appendix D.

To ensure the validity of using average daily frequencies for subsequent analyses, the half-hourly sums of the behavioral frequencies recorded per female during the first half of the observation period per institution (5-15 days) were compared with those recorded during the second half of the observation period. A Spearman correlation test was used as opposed to a T-test because of the large frequency of zeros in the data set. The correlation coefficient was significant for aggression ($p < 0.0001$, $r = 0.86$) and sexual play behaviors ($p < 0.0001$, $r = 0.61$), indicating no effect of time across the observation period on the recorded frequencies of those behaviors. Sexual advances by mature males ($p = 0.24$, $r = 0.23$) and sexual advances by mature and adolescent males ($p = 0.09$, $r = 0.32$), however, were not correlated across observation days, as should be expected when males change their association with females according to their receptivity. An average daily frequency of sexual advances made by males to each female accurately reflects which females were courted during the observation period and which were not. As a result, average daily frequencies for each type of behavior were calculated for each female (Appendix E) and used in subsequent statistical analyses.

Dominance was determined by calculating the percentage of the total antagonistic interactions that resulted in a "win" for each female in every possible dyad, which was

then organized using dominance matrices (Appendix F; Thompson, 1993; Metrione et al., 2007) and dominance diagrams (Kuneš and Bičík, 2002; Metrione et al., 2007), which depicted the number of females that dominated and were dominated by the focal female. All antagonistic interactions were used for this analysis, regardless of visibility for a full 30 minutes (refer to Appendix D). Though the presence of a calf probably increases aggression, if that mother subsequently wins her interactions, she is in fact more dominant at that time than the rhinos that lose those interactions. Thus, determination of dominance includes all the rhinos in the housing group, regardless of age or accompaniment by a calf, as this represents the true group social dynamic at that time.

Assessment of reproductive history and the captive environment.

The reproductive and housing history of adult females involved in any aspect of the project (Table 1) was evaluated. Two females that began the study as adolescents gave birth in the summer of 2010 (after behavioral data were recorded and samples were collected); their data were included in the captive-born, parous group only for the analyses of parity relative to the captive environment. One nulliparous adult, Ajabu, was excluded because she did not have sufficient access to a male for breeding at her previous institution or currently at Birmingham. Other contingencies relative to analysis of historical data are described in Appendix D. Information for each female was obtained from the institutions' written records and the *Ceratotherium simum simum* North American Regional Studbook (Christman, 2007, 2009). The assessed environmental conditions included: 1) outdoor pasture/enclosure size; 2) group size including females and adolescents; 3) number of and duration of access (year-round or seasonal) to mature

males available at the institution; 4) if the female had access to a novel male, i.e., the male was unknown during early adolescence; 5) if the female was housed at her natal institution; 6) if the female was housed with at least 1 of the female rhinos with which she lived as an adolescent; 7) if the female was housed with her mother; and 8) if the institution was open to the public. Environmental conditions in place at the time of conception (estimated as 16 months prior to the birth of a calf) were used in analyses for parous females, and those in place when behavioral observations were recorded were used for nulliparous females. For questions of access to males and previously-known companions for analysis with parity, however, affirmative counts were scored if the nulliparous female ever during her adult life (≥5 years of age) had access to >1 male at an institution, to a novel male, or to a female companion known during adolescence. Conveniently, enclosure sizes of participating institutions did correspond directly to rhino density: Enclosures <0.01 km^2 had <0.002 km^2 per rhino; enclosures 0.01-0.1 km^2 had 0.004-0.007 km^2 per rhino; and enclosures >0.1 km^2 had 0.016-0.14 km^2 per rhino.

Statistical analyses of behavioral, dominance, and environmental data.

To account for differences in average daily behavioral frequencies [given as average events per day ± standard error of the mean (SEM)] that might be due to housing at different institutions rather than to an effect of the four groups (captive-born, parous; captive-born, nulliparous; wild-caught, parous; and adolescent), 2-way analyses of variance (ANOVA) were used after applying the square-root transformation to the data. Not all institutions housed females belonging to all 4 groups, so comparisons of average daily behavioral frequencies between the groups were analyzed as incomplete,

unbalanced, randomized block designs with sub-sampling. In Proc Mixed (SAS Institute, 2002-2003), *institution* was designated as the random effect, *group* as the fixed effect, and *institution x group* as the random effect of the interaction. The interaction was confirmed to be non-significant in every test, and it was subsequently dropped from the model (refer to Appendix D for details and contingencies). The Tukey-Kramer adjustment was used to differentiate which of the 4 groups' least squares means were different when a significant group effect was found. Spearman correlation coefficients (Carlstead et al., 1999a,b; Carlstead and Brown, 2005) were used with the untransformed data to determine if correlations existed between behavioral frequencies, and logistic regression (Wald test) was used to determine if parity (parous vs. nulliparous) was affected by behavioral frequencies. For analyses of correlation between average daily sexual advances by mature males, the total sample size was only 26 (or 28 where non-nursing adolescent females are included in the correlation with average daily sexual play). This is because the adolescent females were not included in the analysis and 5 females did not have access to mature males during the behavioral observations (1 of these did have access to an adolescent male). Thus, for ANOVA analysis of average daily sexual advances by males including adolescent males and non-nursing adolescent females, the sample size was 29. Statistics of average daily frequencies of sexual advances including those made by both mature and adolescent males are given in Appendix G. Adolescent females were excluded from analyses that included parity.

Two-way ANOVA for randomized block designs with sub-sampling was conducted as described above to test for differences in average daily behavioral

frequencies between dominant and subordinate rhinos, which included all 32 females (adult and adolescent) observed in a group with at least 1 other female. For these analyses, after the dominance hierarchy was determined and the females were ordered linearly, the group was divided in half, and the winning-most females were called dominant. If a group could not be divided evenly, the middle-ranked female was designated as subordinate, and thus, there was 1 fewer dominant female than subordinate females in such groups. The comparison of average daily frequency of sexual advances between dominant and subordinate females (n = 23), however, does not include the adolescent females and considers only advances made by mature males.

Tests for independence used Fisher's 2-sided exact chi-square (or Pearson's exact chi-square for comparisons >2x2) to calculate if the proportion of parous females was influenced by dominance or a particular environmental factor. Tests for a difference in the proportion of parous females between dominants and subordinates were conducted in 2 ways. The first method considered the reproductive status of only the uppermost- and bottommost-ranked females in each housing group (n = 8) or companion subgroup (n = 7), including adolescents. For this analysis of companion subgroup, only females at institutions where the subgroup was different from the housing group were considered. The second method considered all the adult females (n = 26) in each housing group or companion subgroup with dominant/subordinate designations consistent with the behavioral analyses. Thus, adolescents were considered when determining dominance ranks among the females, but they were not part of the analysis of dominance and parity, nor was the nulliparous female that did not have access to a male. For this analysis of

subgroup, companion subgroups that also were the housing group were included. Mean differences and correlations were considered statistically significant when $p \leq 0.05$.

Fecal and serum sample collection for hormone analysis.

Fecal or serum samples were collected from 37 female white rhinos (Table 1), once weekly from each adolescent for 1-2 years and approximately every other day (3/week) from each adult for 4 months. Although cycle irregularity is common in white rhinos (Patton et al., 1999; Brown et al., 2001), it was deemed likely that reproductively active females would show evidence of luteal cycle activity in samples collected during a 4-month period. Fecal samples were collected at institutions where defecations could be identified to individual rhinos, and samples were stored at -20°C until analyzed. Location within the fecal pile or fecal ball from which the sample (≥ 50 g) was collected was not considered a confounding factor because Schwarzenberger et al. (1998) found that progesterone metabolite concentrations did not differ between the outer layer and central portion of white rhino fecal balls. At institutions where defecations could not be identified to individual rhinos (6 females at Busch, Reid Park, White Oak), blood samples were collected (ear or leg venipuncture) from females trained to stand for blood collection without restraint. Serum samples (3 ml) were decanted after centrifugation (10 min at 1,211×g) and stored at -20°C until assayed. Additional details regarding sample collection for individual animals are described in Appendix D.

Fecal extraction.

To extract steroid metabolites from feces, 0.5 ml of deionized water and 4.5 ml of anhydrous ethanol were added to 16×100 mm culture tubes (Fisher Scientific, Pittsburg,

PA, USA) containing 0.48 to 0.52 g of crushed feces. Samples were then vortex-mixed for 30 seconds and shaken in a horizontal position for 1 hour before centrifuging for 20 minutes at 786×g (Metrione et al., 2008). Fecal samples were not boiled prior to extraction because Wasser et al. (2000) found that the boiling and vortexing extractions produced similar recoveries (90-100%) of radioactive labeled steroids (progesterone, corticosterone, and testosterone) and their immunoreactive metabolites. Aliquots (500 µl) of extract supernatant were dried in 12×75 mm glass culture tubes (Fisher Scientific, Pittsburg, PA, USA). A second aliquot (3 ml) of extract supernatant was held undiluted in reserve. Neat ethanol extracts were diluted in enzyme immunoassay (EIA) buffer (0.2 M phosphate buffered saline containing 1.0 g/L bovine serum albumin; see Appendix H) for a working dilution of 1:128 for the progesterone assay. In addition, 2 large pools of fecal extract (1 with extract from pregnant females and 1 with extract from non-pregnant females) were made from randomly selected extract samples from every female. The serum pool was made from randomly selected serum aliquots from every female. Aliquots from these pools were serially diluted to test for parallelism with standard curves and diluted to provide reference (control) solutions for evaluation of intra- and interassay variability. All extracts were stored at -20ºC.

Progesterone and progestagen immunoassay.

Progesterone has been evaluated previously for white rhinos in both feces and serum using radio- and enzyme immunoassay techniques (Radcliffe et al., 1997; Patton et al., 1999; Brown et al., 2001; Graham et al., 2001; Metrione, 2005). The assay protocol was adapted from Munro and Stabenfeldt (1984) and Graham et al. (2001). In brief,

assay plates (Nunc MaxiSorp^{TM}, Roskilde, Denmark) were coated with 50 µl of monoclonal antibody (1:5,000; Quidel clone number 425 produced against 4-pregnen-11-ol-3,20-dione hemisuccinate:bovine serum albumin; provided by C. Munro, University of California, Davis, CA, USA; see Appendix I for cross-reactivity) and refrigerated overnight. After washing, 50 µl of EIA buffer was added to each well and incubated at room temperature for 2-4.5 hours. Standard, sample, or control (50 µl) was then added to each well, followed by 50 µl of progesterone:horseradish peroxidase conjugate (HRP; 1:70,000; U.C., Davis). After shaking for 2 hours at room temperature, plates were washed, and 100 µl of color-changing substrate solution (Appendix H) was added to each well. Light absorbance in the wells was measured with a 405 nm filter.

Dose-response displacement curves based on serial dilutions of pooled extract for pregnant (1:512 to 1:16,384) and non-pregnant females (1:2 to 1:1,024) were parallel (Pearson correlation, $p < 0.0001$, $r > 0.96$ for both comparisons) to the standard curve (serially diluted standard progesterone preparations). Fecal extract diluted 1:128 displaced approximately 50% of the progesterone-HRP conjugate and was used as the sample dilution in the assay. Another dose-response displacement curve based on the serially diluted (1:1 to 1:4) serum pool for non-pregnant females also was parallel (Pearson correlation, $p = 0.003$, $r = 0.99$) to the standard curve. A dilution of 1:2 displaced approximately 50% of the progesterone-HRP conjugate, but this concentration was too dilute for serum from most females. Thus, serum samples (50 µl) were assayed directly, undiluted. Recovery of known amounts of progesterone (0.078-10.0 ng/ml) added to pools of diluted fecal extract (1:128) was 77.8% (regression equation: y =

0.876x – 5.6364, r^2 = 0.99) and added to pools of neat serum was 54.8% (regression equation: y = 0.6122x – 3.2609, r^2 = 0.99). Assay sensitivity was 0.08-0.156 ng/ml of standard (31.2-62.4 ng/g feces), determined as the value obtained at 90 to 95% binding of the progesterone-HRP conjugate. When estimated progesterone concentrations in serum samples were below assay sensitivity, the value was recorded as calculated, or it was recorded as "0" if the computer was unable to calculate any value.

Controls of high and low hormone mass were made from pools of either non-pregnant fecal extract [average percent binding (average percentage of displaced progesterone-HRP conjugate) = 33.5% for high and 67.2% for low controls] or serum (average percent binding = 45.4% for high and 68.2% for low controls) diluted in EIA buffer. The fecal extract and serum high and low controls were assayed in 4 wells per control (2 wells on each side of the plate for each control) on every plate containing fecal or serum samples, respectively. Fecal extract high and low controls (2 wells per control per plate) also were assayed on plates containing serum samples for consistency in the calculation of interassay variation (n = 70 plates). Average interassay variation, calculated as a coefficient of variation (C.V.) in control hormone masses across all the plates, was 8.3% for the high control and 11.0% for the low control. Calculated as a maximum range above and below the average control mass for all plates, interassay variation was <18% for the high and low controls. Intra-assay variation in hormone mass was determined using the fecal extract controls for plates containing fecal samples and using the serum controls for plates containing serum samples. Intra-assay variation was calculated using the C.V. among the hormone masses in all 4 wells containing the control

on every plate. Average intra-assay variation was 3.7% (maximum 9%) for high controls and 6.2% (maximum 14%) for low controls. The C.V. in hormone masses between duplicate wells for all samples was $\leq$10%.

Evaluation of progesterone and progestagen profiles.

Assessment of the females' reproductive status (acyclic, cycling, or pregnant) was based on temporal and quantitative features of the progesterone and progestagen profiles (concentrations in samples plotted over time). A sustained rise in progesterone or progestagen concentrations followed by baseline concentrations was taken as evidence of the luteal phase of an estrous cycle and, indirectly, ovulation and formation of a corpus luteum.

Baseline concentrations in progesterone and progestagen profiles were calculated using an iterative process (Brown et al., 2001; Graham et al., 2002; North and Harder, 2008) in which values that exceeded the mean +1.3 standard deviations (SD) were excluded. The average was then recalculated and the elimination process was repeated until no values exceeded the mean +1.3 SD. Luteal values were defined as the baseline +1.3 SD. The luteal phase of a cycle was identified as intervals in which progestagen concentrations remained above baseline at luteal values for a minimum of 12 days, and the end of a luteal phase of the cycle was identified by a minimum of 1 or 2 samples with baseline concentrations, depending on whether samples were collected approximately once weekly or every other day, respectively (modified from Brown et al., 2001). Cycle length was determined by the number of days between the last baseline value before the first luteal value and the first or second (depending on sample frequency) baseline value

after the last luteal value or the nadir value if there were more than 2 baseline values between consecutive cycles. Behavioral data, including estrous behaviors and mating, also were used in determining cycles. Retention time of food in white rhinos is 48-60 hours (Owen-Smith, 1988a; Brown et al., 2001), and this was considered when comparing the date of a behavioral observation with hormone levels. As samples were collected every other day, 1-point peaks and nadirs were ignored when they were inconsistent with surrounding values.

The start of ovulatory cycles (onset of puberty) in adolescents was characterized by the first appearance of consistent luteal peaks in their progesterone and progestagen profiles and observations of estrous behavior (Patton et al., 1999). Adolescents began estrous cycling just at or after behavioral observations were completed, and adolescents for which full cycles were documented (Lucy—Busch, Kayla, Kelly) were included with cycling adults for statistical analyses involving cyclicity alone. They were considered adolescents for all behavioral analyses and were not considered in analyses of parity.

Statistical analyses of progesterone and progestagen data.

Two-way ANOVA was used to account for differences in baseline and average luteal progesterone and progestagen concentrations that might be due to housing at different institutions rather than to an effect of reproductive status. The natural log transformation was applied to the fecal progestagen concentrations, but the serum progesterone concentrations were not transformed. Again, not all institutions housed females belonging to all groups (nulliparous, parous, acyclic, cyclic, adolescent), so comparisons of baseline and average luteal concentrations between the groups were

analyzed as incomplete, unbalanced, randomized block designs with sub-sampling using Proc Mixed (SAS Institute, 2002-2003) as described for the behavioral data (refer to Appendix D for contingencies). A completely randomized design with sub-sampling was used for the comparison of baseline serum progesterone concentration between acyclic and cycling females because each institution had only 1 type of female. In Proc Mixed (SAS Institute, 2002-2003), *cyclicity* was designated as the fixed effect, and *institution within cyclicity* was designated as the random effect.

Females were assigned to acyclic, cycling, pregnant, or adolescent based on their progesterone or progestagen profiles at or near the time of behavioral observations for assessment of differences in behavioral frequencies relative to ovarian activity. Square-root transformed average daily behavioral frequencies were analyzed as incomplete, unbalanced, randomized block designs with sub-sampling using Proc Mixed (SAS Institute, 2002-2003) as described previously. Comparisons of aggression and sexual play behavior included 28 females (not all females with samples were observed), whereas comparisons of sexual advances made by mature males included only 22 females (not all females had access to mature males, and nursing adolescents were excluded). Logistic regression (Wald test) was used to determine if cyclicity [including only acyclic (n = 7) and cycling (n = 9) females with behavioral observations] was affected by behavioral frequencies.

Tests of independence used Fisher's 2-sided exact chi-square to test if the proportion of females with cycles was influenced by dominance or a particular environmental factor. Dominance tests of independence included all applicable females

(i.e., acyclic and cycling, n = 12; reproductively inactive and active, n = 19) with designations of dominant or subordinate consistent with the previous analyses of behavior. Tests of independence for characteristics of the captive environment considered the females' (n = 30) housing conditions at the time of collection. The 2 females that potentially lost their pregnancies, 3 adolescents with incomplete or absent cycles before the end of sample collection, and 2 females that were pregnant throughout sample collection were not included in the statistics for environmental influences on cyclicity. Means are presented ± SEM, and mean differences and correlations are considered statistically significant when $p \leq 0.05$. For all analyses, unless otherwise stated, there was no effect of housing institution (as a separate random effect or considered within each condition of the second variable).

Results

Aggressive, sexual, and play behavior and parity.

Average daily frequency of sexual play behavior differed among captive-born, nulliparous females; captive-born, parous females; wild-caught, parous females; and adolescents ($p = 0.025$; Table 2): Adolescents engaged in sexual play behavior at a higher frequency than did captive-born, nulliparous females ($p = 0.024$) and wild-caught, parous females ($p = 0.039$) (Fig. 1), but not more often ($p > 0.05$) than captive-born, parous females. However, frequency of sexual play behavior, treated as the independent variable, was not related to whether a female was parous or nulliparous ($p > 0.05$). Together, these results suggest that reductions in the frequency of sexual play behavior in adults from that observed during adolescence might be indicative of nulliparity and

Table 2. Comparison of average (± SEM) daily behavioral frequencies among groups of female rhinos observed between September 2007 and December 2008. Females are grouped according to parity and origin (sample size as shown in Table 1) and according to dominance within the full housing group and companion subgroup.

Group	**Aggressive Behavior**	**Sexual Advances (Mature Males)**	**Sexual Play Behavior**
Captive-born parous	6.03 ± 1.25	1.23 ± 0.41	0.57 ± 0.15
Captive-born nulliparous	5.33 ± 1.51	1.67 ± 0.51	0.30 ± 0.08
Wild-caught parous	7.40 ± 4.13	0.87 ± 0.38	0.29 ± 0.13
Adolescent	3.32 ± 1.10	0.12 ± 0.04	1.51 ± 0.53
p-value	0.09	0.55	0.03
Housing group status			
Dominant (n = 15*)	7.55 ± 1.95	1.01 ± 0.23	0.34 ± 0.08
Subordinate (n = 17*)	4.63 ± 0.93	1.38 ± 0.39	0.85 ± 0.21
p-value	0.09	0.14	0.01
Companion subgroup status			
Dominant (n = 14*)	7.92 ± 2.05	0.93 ± 0.21	0.33 ± 0.08
Subordinate (n = 18*)	4.50 ± 0.86	1.41 ± 0.37	0.82 ± 0.20
p-value	0.07	0.15	0.02

* Sample sizes for the comparison of sexual advances by mature males were 12 and 11 for dominant and subordinate animals, respectively, in housing groups and were 11 and 12 for dominant and subordinate animals, respectively, in companion subgroups.

acclimation to captivity from the wild, but comparison of frequencies of sexual play behavior between adults is not useful for distinguishing nulliparous from parous females. Average daily frequencies of sexual play behavior were not correlated ($p > 0.05$) with average daily frequencies of aggression among all the females ($r = -0.21$) or among only

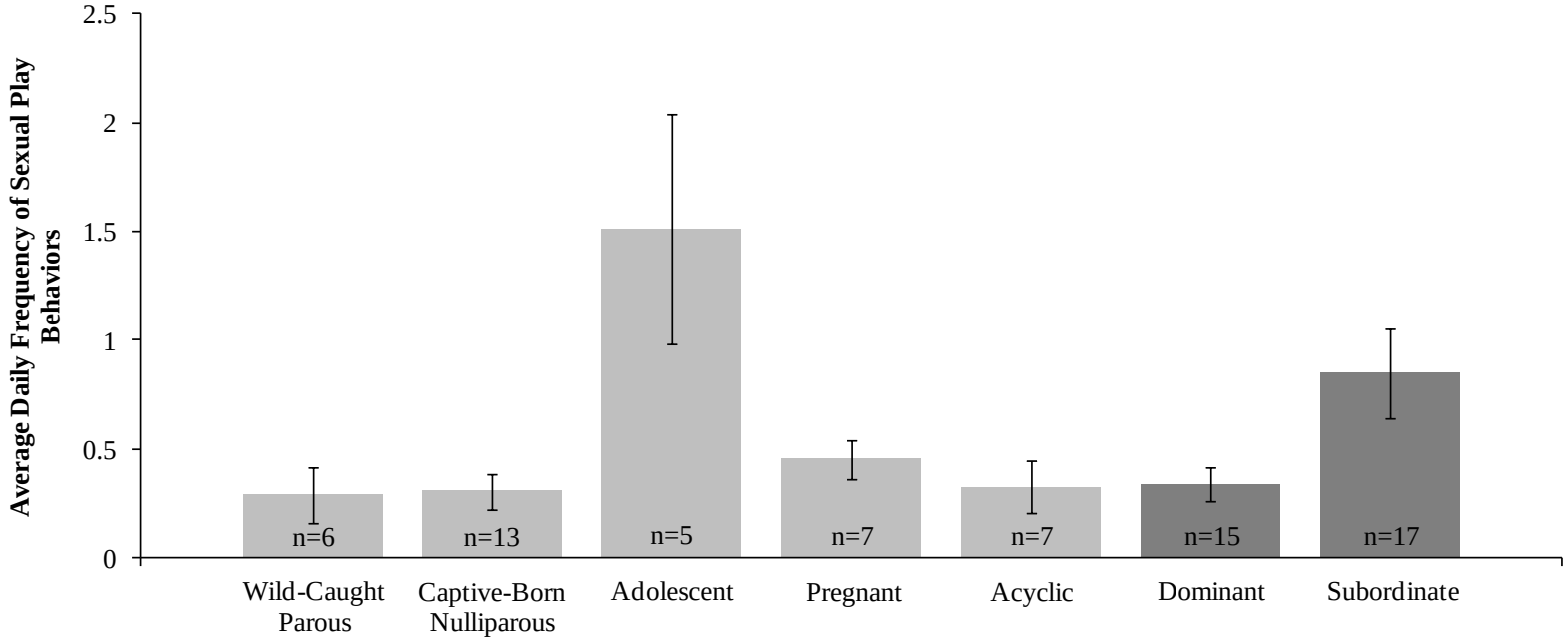

Fig. 1. Average daily frequency of sexual play behaviors among observed groups of female rhinos. Wild-caught, parous; captive-born, nulliparous; and pregnant females engaged in sexual play behavior less often than adolescents ($p < 0.05$), while acyclic females tended to engage in sexual play behavior less often than adolescents ($p = 0.1$). Subordinate females engaged in sexual play behavior more often than dominant females ($p < 0.05$).

the adolescents ($r = 0.10$). Average daily frequencies of sexual advances made by mature males toward all non-nursing females also were not correlated with those females' average daily frequencies of sexual play behavior ($p > 0.05$, $r = -0.29$).

Average daily frequency of aggressive behavior differed ($p = 0.028$) across institutions but not ($p > 0.05$) between the female groups (Table 2). Average daily frequency of sexual advances made by mature males toward all non-nursing females did not differ ($p > 0.05$) among the female groups (Table 2), nor were sexual advances by mature males correlated with adult females' average daily frequencies of aggression ($p > 0.05$, $r = 0.23$). Parity (parous vs. nulliparous) was not related ($p > 0.05$) to average daily frequency of female aggression or sexual advances by mature males.

Average daily frequency of sexual play behavior was different between dominant and subordinate females (Table 2): Subordinate females engaged in sexual play behavior more frequently (housing groups, $p = 0.013$; companion subgroups, $p = 0.021$) than dominant females (Fig. 1). When adolescent females were excluded from this analysis, the trend persisted within housing groups ($p = 0.08$; 0.59 ± 0.14, subordinate; 0.30 ± 0.08, dominant), but there was an interaction ($p = 0.035$) between dominance within companion subgroups and housing institution, in which sexual play differed between dominant and subordinate females depending on the institution at which they were housed. Though average daily frequency of aggressive behavior tended to differ ($p = 0.06$) across institutions, it did not differ ($p > 0.05$) between dominant and subordinate females (Table 2). Dominant and subordinate females also did not differ ($p > 0.05$) in average daily frequency of sexual advances made by mature males to adults (Table 2).

Social environment and parity.

The proportion of females that gave birth (hereinafter, parity) did not differ ($p > 0.05$) between the uppermost dominant and the bottommost subordinate females or between all dominant and subordinate adult females in housing groups (Fig. 2) or in companion subgroups (Table 3). Males are typically subordinate to females, but in 2 cases of long-term, exclusive pairing, the females were subordinate and nulliparous (Yebonga and Jeannie). Copulatory behavior, including repeated mounting in all cases and intromission and ejaculation in 2 cases, was observed in males involving 4 females, 2 of which were subordinate within their housing groups (Maggie—White Oak; Kiazi), 3 of which were subordinate within their subgroups (Maggie—White Oak; Kiazi; Paddy), and 1 of which was housed with only the male (Taryn). The behavior of these females and the males appeared consistent with previous observations of copulation with dominant females (Metrione, personal observation).

Captive environment and parity.

A larger proportion of females were parous (Table 4; Fig. 2) when housed in large enclosures >0.01 km^2 or 2.5 acres ($p = 0.001$) and when housed in groups totaling >2 females/adolescents ($p = 0.003$). Enclosure size and group size are obviously related, however, so these factors probably interact in their effects on parity, which is supported by observation of higher parity ($p = 0.0006$) when data are pooled for females housed in >0.01 km^2 with >2 females/adolescents than in the other combinations of those factors (Table 4). In addition, larger enclosures are often mixed-species exhibits, and parity was

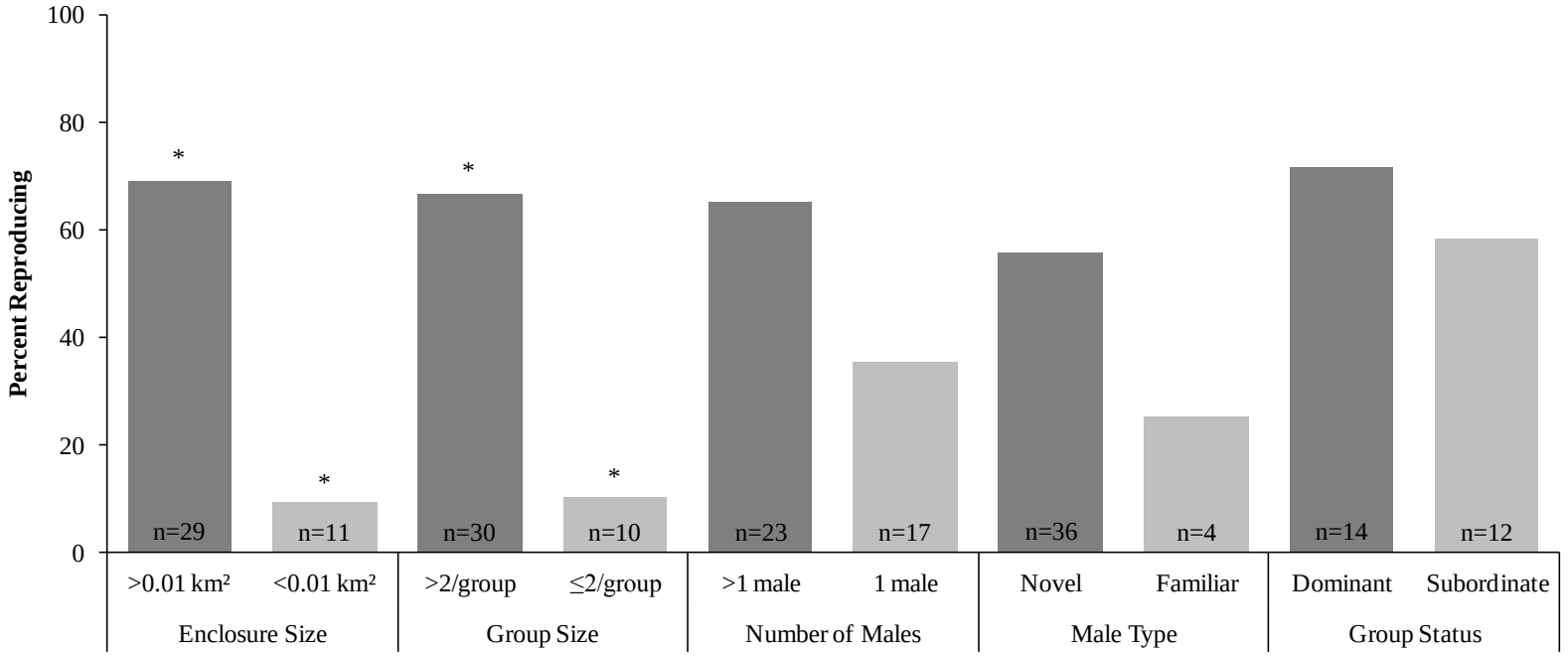

Fig. 2. The percentage of female rhinos producing calves compared by enclosure size, group size, number of males at the institution, familiarity of the male, and group status. Parity is higher (*, $p < 0.05$) among females living in large enclosures and large groups but only tends to be higher ($p = 0.109$) in groups with more than 1 male.

Table 3. On the left, parity in female rhinos, including adolescents, did not differ ($p > 0.05$) between the uppermost dominant and the bottommost subordinate females within housing groups or companion subgroups. On the right, parity did not differ ($p > 0.05$) between dominant and subordinate adult (only) females within housing groups or companion subgroups. The total numbers of females in each group are given in parentheses.

Housing Group (with adolescents)	**Parous (9)**	**Non-reproductive (7)**	**Housing Group (adults only)**	**Parous (17)**	**Nulliparous (9)**
Dominant	6	2	Dominant	10	4
Subordinate	3	5	Subordinate	7	5
Companion Subgroup	**(7)**	**(7)**	**Companion Subgroup**	**(17)**	**(9)**
Dominant	5	2	Dominant	10	3
Subordinate	2	5	Subordinate	7	6

higher for females housed in large, mixed-species exhibits ($p = 0.001$; $p = 0.046$ if one considers species number alone) than in the other combinations of those factors (Table 4). Parity did not differ ($p > 0.05$) whether or not a female was housed at her natal institution, with her mother, with a companion that was known from some point during the first 5 years of life, at a public or private/seasonal institution, with a male year-round or only seasonally, with 1 or more than 1 male, or with novel versus familiar males (Table 4). Although 95% of parous females reproduced with a novel male (a male unknown during early adolescence), 84% of nulliparous females also were housed with a novel male (Table 4).

Table 4. The number of parous (n = 21) and nulliparous (n = 19) female rhinos living in different housing conditions. Parity was influenced ($p \leq 0.05$) by the environmental characteristics listed on the left side of the table.

Significant Characteristics	**Parous (21)**	**Nulliparous (19)**	**Non-significant Characteristics**	**Parous (21)**	**Nulliparous (19)**
>0.01 km^2	20	9	>1 male	15	8
<0.01 km^2	1	10	≤1 male	6	11
>2 females/adolescents	20	10	Novel male	20	16
≤2 females/adolescents	1	9	Familiar male	1	3
>0.01 km^2 and >2 females/adolescents	20	8	Year-round access to male	18	17
>0.01 km^2 and ≤2 females/adolescents	0	1	Seasonal access to male	3	2
<0.01 km^2 and >2 females/adolescents	0	2	Familiar female companion	14	13
<0.01 km^2 and ≤2 females/adolescents	1	8	No familiar female companion	7	6
Mixed species	17	9	Mother present	5	4
Single species	4	10	Mother absent	16	15
>0.01 km^2 and mixed species	16	8	Natal institution	9	6
>0.01 km^2 and single species	4	1	Non-natal institution	12	13
<0.01 km^2 and mixed species	1	1	Public	15	17
<0.01 km^2 and single species	0	9	Private/seasonal	6	2

Estrous cyclicity.

Based on the assumption that periodic elevation of progesterone or progestagens in profiles (Appendix J) indicates formation of an active corpus luteum following ovulation, elevated progestagen patterns indicative of luteal phases provided evidence of

ovulatory cycles in 22 of 35 non-pregnant females, 12 of which were nulliparous (Table 5). Eight non-pregnant adult females did not show evidence of ovulatory cycles (Table 5), based on the absence of sustained (12-day minimum) luteal progesterone or progestagen concentrations, 2 of whom were wild-caught and have not reproduced since their capture. Two other females might have been in the early stages of pregnancy before losing the pregnancy (Table 5): Thirteen days after keepers observed copulation, the progestagen levels for 1 female (Lissa) remained at luteal concentrations for 78 days before gradually returning to baseline. For the second female (Yvonne), progestagen concentrations gradually rose over 25 days after keepers observed mounting, and they were then sustained at luteal levels for 60 days before gradually returning to baseline. Progestagen profiles provided evidence of luteal activity in 4 of the 6 adolescents (Table 5) between the ages of 29 to 42 months of age. One young, adult female (Zenzele) was cycling at least by 43 months of age and became pregnant at 44.5 months, and another (Maggie—White Oak) became pregnant at 60 months of age. After sample collection was completed, 1 adolescent (Kelly) became pregnant at ~39 months of age and another (Lucy—Busch) became pregnant at ~53 months of age.

Consistent with previous studies (Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001; Graham et al., 2001; Roth, 2006), short and long estrous cycles (n = 37 total) were observed, but the majority were short cycles (Fig. 3). Based on the somewhat ($p > 0.05$) bimodal frequency distribution of cycle lengths (Fig. 3), short cycles were characterized as those ≤ 44 days in length, and long cycles were characterized as those ≥ 45 days. Thus, with this demarcation, the average cycle

Table 5. Number of female rhinos categorized by reproductive activity, as determined by progestagen profiles and reproductive history.

Reproductive Activity	**N**
Acyclic, nulliparous	6
Acyclic, parous	2
Acyclic, adolescent	2
Cyclic, nulliparous	12
Cyclic, parous	7
Cyclic, adolescent	4 (3 with full cycles)
Pregnant throughout sample collection	2
Pregnant during part of sample collection	4 (cyclic otherwise)
Possible lost pregnancy	2
Total	41

lengths were 30.8 ± 1.5 days (range = 18-44 days) for short cycles and 59.2 ± 3.2 days (range 46-89 days; the 89-day cycle was the first cycle of an adolescent) for long cycles (Table 6). Twelve females had short cycles, 6 females had long cycles, and 4 females exhibited both short cycles and long cycles during sample collection (Table 6). Interestingly, 3 females with both short and long cycles had equal numbers of each, and the other female, an adolescent, had 2 short cycles and 1 long cycle. Two females that cycled initially became pregnant, one having 1 long cycle during 3 months of sample collection before her pregnancy began and the other having 1 short cycle during 2.5 months of sample collection before becoming pregnant. One adult from whom serum was obtained for a full year had 4 cycles, 2 short and 2 long. For the remaining adults with samples collected for 4 months, 13 had only 1 cycle (4 of those females had a long cycle), 2 females had 2 cycles (1 of those females had both a long and a short cycle), and only 1 female had 3 cycles (Table 6). Thus, cycles for most of the adult females could be

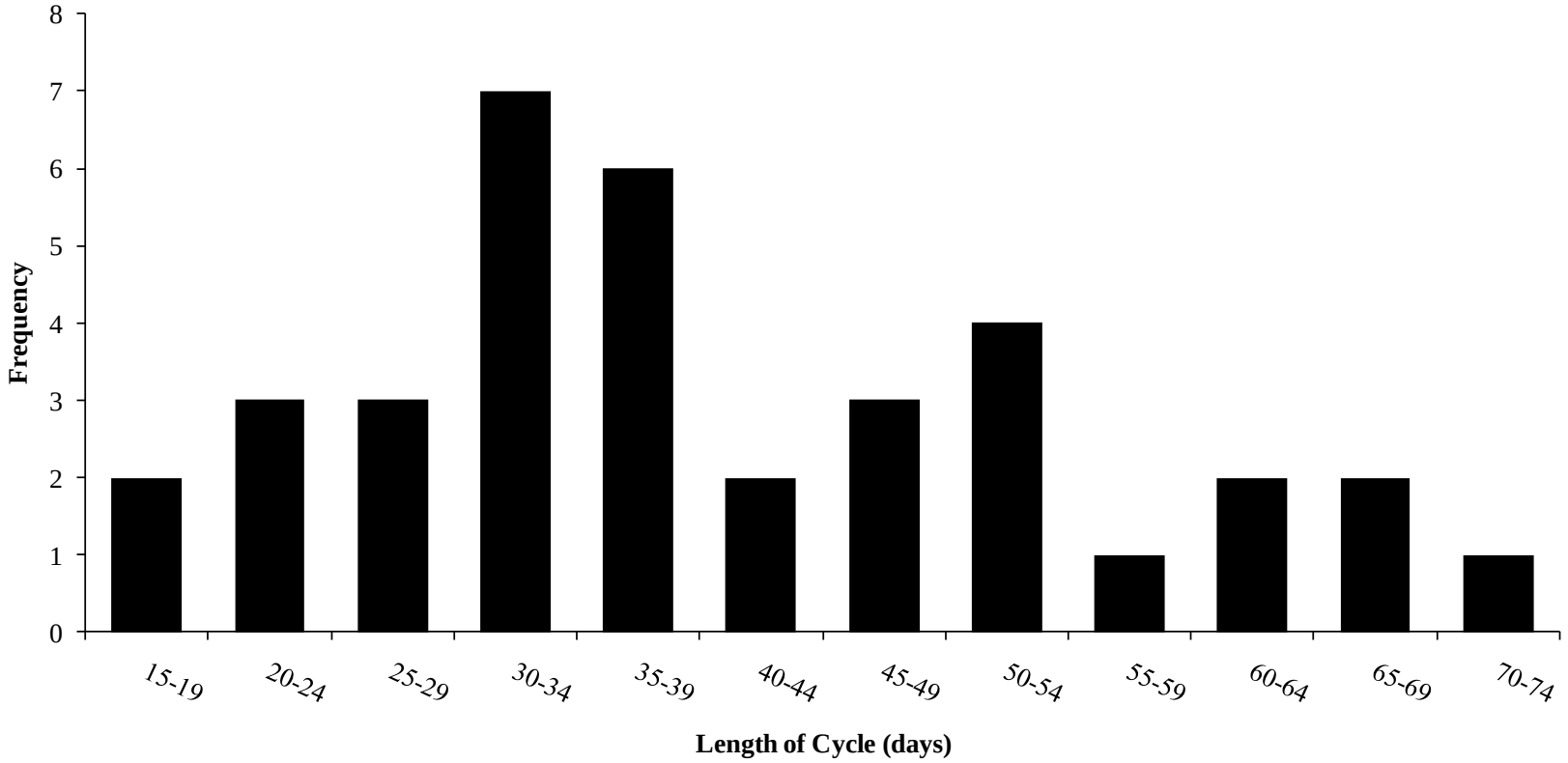

Fig. 3. The frequency of estrous cycle lengths observed among female rhinos in this study.

Table 6. Length of ovarian cycles (in days) among female rhinos. Short cycles (≤44 days) and long cycles (≥45 days) are averaged separately for all females with each type of cycle in the last row and for individual females in the far right column.

Rhino	**Short Cycles**	**Long Cycles**	**Average**
Ajabu	38		38
Bloom		47	47
Bonnie	30		30
Dumisha	36		36
Emalah	24		24
Gabby		56	56
Helen	37		37
Jao	18		18
Julie	30		30
Kayla (adolescent)		89, 50	69.5
Kelly (adolescent)	37, 33, 28	51, 49, 54	32.7, 51.3
Kendi	26, 21, 19		22
Kiazi		67	67
Lucy—Busch (adolescent)	32, 38	46	35, 46
Lucy—WOCC*	30, 44	63, 69	37, 66
Maggie—WOCC*		72	72
Maggie—Wilds		53	53
Mambo	30, 22		26
Marina	25		25
Paddy	32		32
Taryn	43	63	43, 63
Zenzele	35		35
AVERAGE ± SEM	30.8 ± 1.5	59.2 ± 3.2	41.5 ± 2.7

*White Oak Conservation Center

described as irregular. Adolescents were slightly more regular: Once cycling began, 3 adolescents had, respectively, 2 long cycles in 5 months, 3 cycles (1 of which was long) in 9 months, or 6 cycles (3 were long) in 10.5 months (Table 6).

Increased progesterone or progestagen concentrations lasting more than 3 days but not long enough (12 days) to be considered luteal phases appeared in the profiles of 15 adults, 6 of whom were considered acyclic (2 parous, 4 nulliparous). An average of 10.5 days (mode = 9 days; range = 4-17 days) elapsed between observations of behavioral estrus (n = 16 events, 11 females) and the first luteal value. The average duration of the luteal phase of short and long cycles was 19.9 ± 1.5 and 38 ± 3.8 days, respectively (Table 7), slightly shorter than durations (range 19-34 and 44-66 days, respectively) reported by Patton et al. (1999).

The parous female with serum samples had a higher ($p = 0.001$) baseline progesterone concentration than the 2 nulliparous females with serum samples, but this might be an artifact of the small sample size (Table 8). Otherwise, baseline progestagen or progesterone concentration (in fecal or serum samples, respectively) did not differ ($p > 0.05$) between any of the groups (Table 8): parous vs. nulliparous; parous vs. nulliparous vs. adolescent; acyclic vs. cyclic; acyclic vs. cyclic vs. adolescent (only those without full cycles). Average progestagen concentration during luteal phases among cycling females did not differ ($p > 0.05$) between parous (1,382.8 ± 57.9 ng/g) and nulliparous (1,231.3 ± 67.5 ng/g) females, and average progesterone concentration during luteal phases between the 2 cycling females from which serum was collected did not appear to differ (1.71 ng/ml, parous female; 1.98 ng/ml, nulliparous female).

Table 7. Lengths of luteal phases (in days) of estrous cycles characterized as short (≤44 days) and long (≥45 days) among female rhinos. Luteal phase lengths are averaged separately for short and long cycles for all females with each type of cycle in the last row and for individual females in the far right column.

Rhino	**Luteal Phase For Short Cycles**	**Luteal Phase For Long Cycles**	**Average**
Ajabu	30		30
Bloom		23	23
Bonnie	24		24
Dumisha	32		32
Emalah	16		16
Gabby		49	49
Helen	30		30
Jao	12		12
Julie	16		16
Kayla (adolescent)		61, 31	46
Kelly (adolescent)	22, 21, 12	14, 28, 32	18.3, 24.7
Kendi	14, 12, 12		12.7
Kiazi		53	53
Lucy—Busch (adolescent)	13, 14	20	13.5, 20
Lucy—WOCC*	28	39, 54	28, 46.5
Maggie—WOCC*		49	49
Maggie—Wilds		44	44
Mambo	18, 14		16
Marina	19		19
Paddy	21		21
Taryn	28	35	28, 35
Zenzele	30		30
AVERAGE ± SEM	19.9 ± 1.5	38 ± 3.8	26.9 ± 2.3

*White Oak Conservation Center

Table 8. Comparison of baseline fecal progestagen (ng/g) and serum progesterone (ng/ml) among groups of female rhinos.

Group	Fecal progestagen	N (fecal)	Serum progesterone	N (serum)
Parous	1004.6 ± 54.7	10	1.16	1
Nulliparous	985.8 ± 50.1	16	0.05 ± 0.001	2
Adolescent	1055.0 ± 105.6	3	1.25 ± 0.63	3
p-value	0.96		0.18	
Acyclic	1000.2 ± 98.3	7	0.05	1
Cyclic	983.0 ± 39.3	18	0.74 ± 0.42	4
Adolescent	1160.3 ± 15.6	2	1.75	1
p-value	0.48		0.73	

Social behavior and estrous cyclicity.

Average daily frequency of sexual play behavior tended to differ ($p = 0.059$) between adolescents, acyclic, cyclic, and pregnant/lost pregnancy females (Table 9), in which pregnant/lost pregnancy ($p = 0.051$) and, to a lesser extent, acyclic ($p = 0.097$) females tended to engage in sexual play less than adolescent females (Fig. 1). However, average daily frequency of sexual play behavior was not different ($p > 0.05$) between acyclic and cycling females or between all non-cycling (including acyclic, pregnant/lost pregnancy, and adolescent females together as a single group) and cycling females. Furthermore, whether a female was acyclic or cyclic was not related to average daily frequency of sexual play ($p > 0.05$). Together, these results suggest that reductions in the frequency of sexual play behavior in adults from that observed during adolescence might be indicative of acyclicity or pregnancy, but comparison of frequencies of sexual play

behavior between adults is not useful for distinguishing acyclic from cyclic females. Higher average daily frequency of sexual play behavior did not appear to correlate with earlier onset of puberty (Table 10).

Table 9. Comparison of average (± SEM) daily behavioral frequencies among female rhinos observed between September 2007 and December 2008 and grouped by reproductive activity.

Group	**N***	**Aggressive Behavior**	**Sexual Advances (Mature Males)**	**Sexual Play Behavior**
Acyclic	7	5.28 ± 2.52	0.73 ± 0.12	0.32 ± 0.12
Cycling	9	4.93 ± 1.50	2.60 ± 0.76	0.50 ± 0.20
Pregnant	7	5.57 ± 1.15	0.91 ± 0.16	0.45 ± 0.09
Adolescent	5	3.32 ± 1.10	0.12 ± 0.04	1.51 ± 0.53
p-value		0.16	0.02	0.06

* Sample sizes for the comparison of sexual advances by mature males were 6 (acyclic), 7 (cyclic), 7 (pregnant), and 2 (adolescent).

Table 10. Onset of puberty in female rhinos shows a relationship to average daily frequency of aggressive behavior but not average daily frequency of sexual play behavior.

Rhino	**Onset of Puberty (months)**	**Aggressive Behavior**	**Sexual Play Behavior**
Kelly	29	5.60	0.16
Sally	34	6.310	2.517
Lucy (Busch)	41	1.154	1.077
Dakari	Not as of 29	1.308	0.846
Evey	Not as of 32	2.207	2.966

Average daily frequency of sexual advances made by mature males to females differed ($p = 0.023$) between acyclic, cyclic, pregnant/lost pregnancy, and non-nursing adolescent females (Table 9), in which cycling females were approached more frequently ($p = 0.038$) than acyclic females. Average daily frequency of sexual advances by mature males also were directed toward cyclic females more often ($p = 0.004$) than toward all non-cycling females (0.73 ± 0.11). Whether a female was acyclic or cycling was not related to average daily frequency of sexual advances by mature males ($p > 0.05$). However, this finding was likely due to the small number of females (4) that were observed in an estrous consort relationship with a male. In fact, even using a different calculation method within logistic regression analysis (a likelihood ratio test instead of the Wald test) suggests that the frequency of sexual advances made by mature males was associated with cyclicity ($p = 0.010$).

Average daily frequency of aggressive behavior differed across institutions ($p \leq 0.05$) but did not differ ($p > 0.05$) between acyclic, cyclic, pregnant/lost pregnancy, and adolescent females (Table 9); acyclic and cyclic females; or all non-cycling and cycling females. Whether a female was acyclic or cyclic was not related to average daily frequency of aggression ($p > 0.05$), but average daily frequencies of aggressive behavior did appear to be associated with onset of puberty (Table 10): Of the 5 adolescents for which there were behavioral data, the 2 females beginning luteal activity at 29 and 34 months of age had relatively high average daily frequencies of aggression. The third adolescent that was observed and that began luteal activity did not do so until 41 months of age and had a lower average daily frequency of aggression. The remaining 2

adolescents that were observed had not yet begun luteal activity at 29 and 32 months of age, and they also had lower average daily frequencies of aggression compared to the youngest females to attain puberty. The sixth adolescent, who started cycling at 41.5 months of age, was not observed.

Social environment and estrous cyclicity.

Dominant and subordinate adults did not differ ($p > 0.05$) in the proportion showing evidence of estrous cyclicity or in the proportion showing evidence of any reproductive activity, including gestation, either within housing groups or within companion subgroups (Table 11).

Captive environment and estrous cyclicity.

Ovulatory cycles were observed in a larger proportion of females held in large enclosures (>0.01 km^2) than in smaller enclosures ($p = 0.032$) and in those housed with a novel male than with a familiar male ($p = 0.038$) (Table 12, Fig. 4). The proportion of females with ovulatory cycles tended to be larger for females housed with a companion known at some point during adolescence ($p = 0.078$) but not ($p > 0.05$) if they were in groups with >2 females/adolescents, with >1 male, with their mother, or at their natal institution (Table 12; Fig. 4). All adolescents housed in large enclosures with novel males and companions started cycling ($n = 4$) or were young and might yet begin cycling ($n = 1$), and the single adolescent without a novel male also was young and might still begin cycling.

Table 11. The proportion of female rhinos cycling (having ovulatory cycles) or showing any reproductive activity (pregnancy included) did not differ ($p > 0.05$) between dominant and subordinate females within housing groups or companion subgroups. Total numbers of females in each group are given in parentheses.

Housing Group	**Cycling (7)**	**Acyclic (5)**	**Housing Group**	**Reproductively Active (14)**	**Reproductively Inactive (5)**
Dominant	2	2	Dominant	6	2
Subordinate	5	3	Subordinate	8	3
Companion Subgroup			**Companion Subgroup**		
Dominant	1	2	Dominant	5	2
Subordinate	6	3	Subordinate	9	3

Table 12. The proportion of female rhinos showing ovulatory cycles while living in different housing conditions. A larger proportion of females housed in large enclosures and with novel males had ovulatory cycles than those in small enclosures and with familiar males (*, $p \leq 0.05$). Total numbers of females in each group are given in parentheses.

Characteristic	**Cycling (22)**	**Acyclic (8)**	**Characteristic**	**Cycling (22)**	**Acyclic (8)**
>0.01 km^{2*}	18	3	Novel male*	20	4
<0.01 km^{2*}	4	5	Familiar male*	1	3
>2 females/adolescents	18	4	>1 male	14	2
≤2 females/adolescents	4	4	≤1 male	8	6
Well-known female companion	17	3	Mother present	9	1
No well-known female companion	5	5	Mother absent	13	7
Natal institution	10	2			
Non-natal institution	12	6			

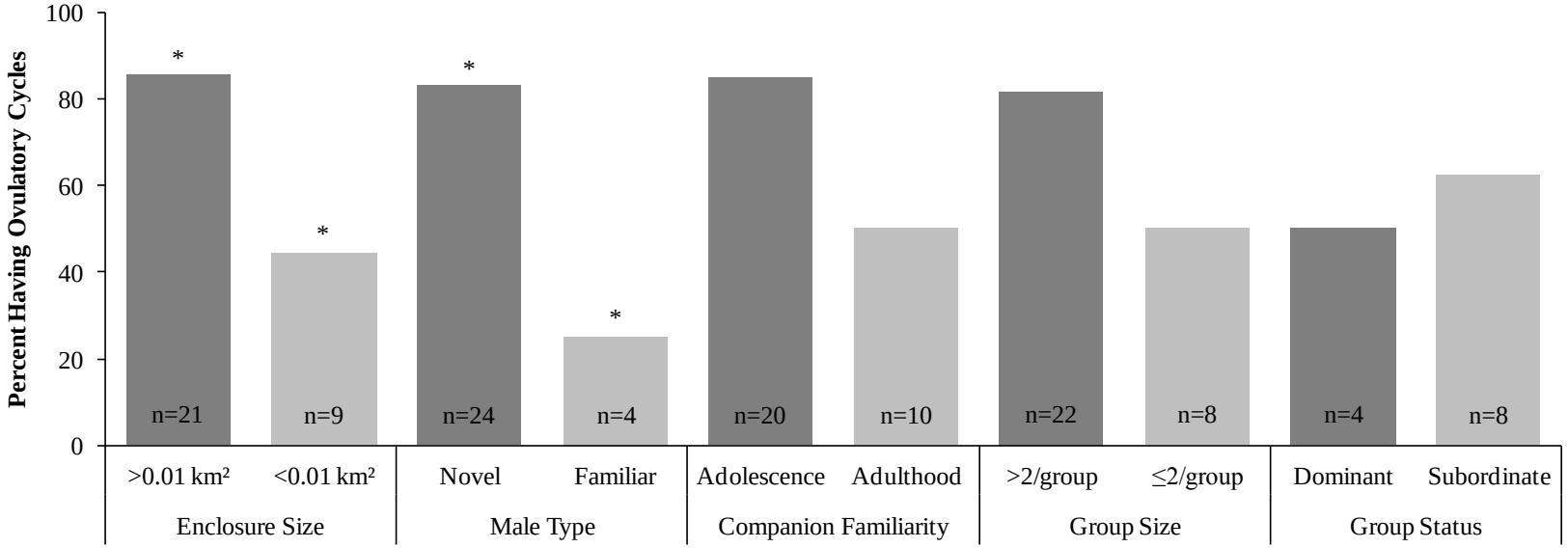

Fig. 4. The percentage of female rhinos having ovulatory cycles compared by enclosure size, familiarity of the male, length of association (since adolescence or adulthood) with the most familiar female, group size, and status in the housing group. The percentage of females having ovulatory cycles was higher (*, $p < 0.05$) among females living in large enclosures and with novel males. A trend ($p = 0.078$) to a higher percentage was observed in females living with a companion known from adolescence.

Discussion

The results of this study contribute to our understanding of the relationships between reproduction and social behavior, the social environment, and the captive environment in captive, female white rhinoceros. Sexual play interactions among captive-born, nulliparous; wild-caught, parous; pregnant; and acyclic females were fewer than among adolescents, however parity and the proportion of females having ovulatory cycles were not influenced by aggression or dominance. Parity was higher in housing groups with >2 females/adolescents, and parity and the proportion of females having ovulatory cycles was greater in females housed in enclosures >0.01 km^2. The proportion of females having ovulatory cycles also was larger when females were housed with a novel male. These findings suggest that an environment similar to what would be experienced in the wild (a social group of females inhabiting a large home range and a mature male unknown during early adolescence) and with adequate stimulation to encourage play behavior is most conducive to reproductive success in female white rhinos. Twenty-two females, 12 of which were nulliparous, had ovulatory cycles, and 2 wild-caught females, who reproduced in the wild but have not reproduced in captivity, were acyclic. These findings suggest that postovulatory problems are a substantial cause of nulliparity and that reproduction can be compromised in captivity during adulthood, even after a presumably normal adolescence. It is highly unlikely that these factors act in isolation or to the exclusion of other factors, but rather, they are major components of an integrated suite of social and environmental conditions that act on reproduction in white rhinos.

Sexual play behavior, enclosure size, and reproduction.

Captive-born, nulliparous females, but not captive-born, parous females, engaged in less sexual play behavior than adolescents (hypothesis 1c). This is consistent with the tendency for acyclic, but not cyclic, females to engage in less sexual play behavior than adolescents since 4 of the 8 acyclic females were captive-born and nulliparous (hypothesis 1c). Baldwin and Baldwin (1971) found that larger troops of squirrel monkeys (*Saimiri spp.*) tended to engage in higher frequencies of social interaction, but small group sizes (≤2 females/adolescents) cannot adequately account for the reduction in play behavior among nulliparous and acyclic white rhinos. Only 6 of 13 nulliparous, captive-born female rhinos were housed in small groups, and, of the 6 acyclic females observed in this study, only 3 were in a small group. Although quantification of olfactory rates in a grazing species with low head carriage is subject to underestimation and error, Carlstead and Brown (2005) found that non-cycling female white rhinos had lower rates of olfactory behaviors than cycling females. Decreased interaction with conspecifics (e.g., play interactions) or with objects in the environment (e.g., olfactory investigation) can be viewed as a stress response to environmental stimuli (Engel, 1967). Further exploration of the possibility for stress-induced reproductive failure is warranted and will be explored in Chapter 3. A wild-caught rhino also might be more sensitive to stressful stimuli in the captive environment than a captive-born rhino, and this might explain why wild-caught, parous females, but not captive-born, parous females, engaged in sexual play less often than adolescents.

As with white rhinos in this study, reproductive success was higher in black rhinos held in larger enclosures (Carlstead et al., 1999a) (hypothesis 2a). Lack of space for the performance of normal activities and the inability to control the timing of certain activities contributes to the expression of stereotypical movements (e.g., pacing, circling) by captive animals (Hediger, 1964b). The higher rates of stereotypy reported for non-cycling female white rhinos compared with cycling females (Carlstead and Brown, 2005) might have been a consequence of holding females in small enclosures, although enclosure size was not reported in that study. If so, their findings would be consistent with the results of this study, i.e., lower cyclicity in females held in small enclosures (hypothesis 2a). Similarly, periods of acyclicity in captive African elephants (*Loxodonta africana*) tended to occur in the winter when they spent less time outside (Schulte et al., 2000).

Two parallel relationships appeared in the results of this study: 1) lower parity and acyclicity are associated with reduced space, and 2) reduced frequency of sexual play behaviors is associated with the nulliparous condition in captive-born females and with acyclicity. Taken together, this set of relationships suggests that the reduction in sexual play behavior might be a symptom of a spatially inadequate captive environment, which also might lead to poor reproduction. Play behavior is generally more frequent in captive animals than in free-ranging animals, perhaps because animals do not need to be as vigilant and/or because it provides one of the few opportunities for physical activity (Thompson, 1996). However, play also is sensitive to social and environmental factors, and so, the presence or absence of play among captive animals can indicate the adequacy

of the captive environment (Thompson, 1996). Thus, combining evaluations of reproductive success with assessments of play behavior can be useful in determining which female rhinos are thriving in captivity and which are merely surviving. Monitoring changes in behavioral frequencies (chasing, mouthing, and stereotypical behaviors) as an indicator for reproductive performance also was suggested for black rhinos (Carlstead et al., 1999b). It is important to recognize, however, that the frequency of sexual play behaviors among pregnant females also was reduced compared to adolescents. Aside from the differences in circulating hormones that might contribute to this behavioral difference, it might be expected that pregnant females could be unwilling to engage in vigorous play in general, which often includes or leads to sexual play behaviors.

Aggressive behavior, the social environment, and reproduction.

It was hypothesized (1a) that subordinate females have lower reproductive success compared to dominant females, an idea supported by 2 lines of evidence: 1) subordinate female white rhinos in 2 groups at separate locations appeared to experience reproductive suppression (Metrione, 2005; Metrione et al., 2007), and 2) higher ratings of aggressiveness and assertiveness in female black rhinos were associated with higher breeding success (Carlstead et al., 1999b). The current study of white rhinos, however, found no evidence that parity or estrous cyclicity was influenced by social dominance. Furthermore, aggressive females did not experience higher rates of sexual advances by mature males (hypothesis 1b) or better reproductive success than less aggressive females (hypothesis 1a). In fact, almost all observed copulatory behavior involved subordinate

females. Recall also that nulliparous, captive-born females and acyclic females exhibited lower frequencies of sexual play behavior than adolescents. Thus, if subordinate females tended to be nulliparous or acyclic, then they would be expected to engage in less sexual play behavior. On the contrary, subordinate females exhibited a higher frequency of sexual play behavior than dominant females.

Subordinate status might contribute to the lack of reproduction observed in 2 situations where a female was kept with only 1 male rhino to whom she was subordinate and with whom she was housed since birth or early adolescence. Reduced reproductive success also was found in black rhino females that were not more dominant than the male (Carlstead et al., 1999b). Alternatively, the lack of estrous cycles in these 2 females could be attributed to the fact that they were not exposed to stimuli of a novel male. Also, these females lacked female companions, a condition associated with low parity.

Female/adolescent composition of the captive environment and reproduction.

Higher parity among white rhino females housed with 2 or more other females/adolescents (hypothesis 2b) contrasts sharply with findings in the more solitary black rhino, in which the mean age at first reproduction is lower when they are the only female at a given zoo, and reproductive rate is negatively associated with the number of females at a zoo (Carlstead et al., 1999a). Contrary to the findings of Swaisgood et al. (2006), the presence of the mother within the group of females in this study did not improve parity or cyclicity (hypothesis 2c), which might be understood with reference to behavior of white rhinos in the wild. Mothers drive their older adolescents away at the birth of the next calf, and dispersal movements out of the home range are made by both

sexes (Owen-Smith, 1973). Similar behavior is seen in red deer (*Cervus elaphus*) in which the degree of proximity between mothers and daughters declined as daughters grew older and home ranges became progressively distinct (Albon et al., 1992). There was a slight tendency for more female rhinos to cycle when housed with a companion known from early adolescence (hypothesis 2c), and perhaps this is due to an increased likelihood to engage in play interactions.

Sexual behavior, male number, male novelty, and reproduction.

As might be expected, males made more sexual advances toward cycling females than acyclic females, though sexual advances were not associated with parity (hypothesis 1b). Females are aggressive to males unless they are in estrus (and aggression might still continue at this time), and so, males should be expected to approach females that might be receptive more often than those that are not. Contrary to prediction, however, having more than 1 male available at an institution did not increase parity or the proportion of females with ovulatory cycles (hypothesis 2d). Though home ranges of wild females typically overlap with 4 to 15 male territories, females mated with the male in whose territory they spent the most time, and this was correlated with the total grassland area in those territories (White et al., 2007). This important grassland area factor does not vary in zoo enclosures relative to the male housed therein, which possibly accounts for male number having little influence on reproductive success in captivity. Though captive females might not have or need the opportunity to select one mate over another, they might require exposure to olfactory, visual, or tactile stimuli from a novel male.

Results of this study suggest that the presence of a novel male might have a stimulatory effect on female cyclicity or might help to maintain it (hypothesis 2e), similar to the way in which a male mouse (*Mus musculus*) will synchronize estrous cycles when introduced into a previously anestrous, all-female mouse group (Whitten, 1957) or in which a male goat (*Capra hircus*) will stimulate estrus and ovulation in seasonally anestrous does (Shelton, 1960; Delgadillo et al., 2009; Bedos et al., 2010). Previous isolation from all male goats might not be necessary if the stimulus male is novel and sexually active (Delgadillo et al., 2009). This is particularly interesting since 1 female rhino (Lucy—White Oak), who was acyclic for at least 4 months while housed with a male for at least 2 years, commenced estrous cyclicity 3 days after a new male was first allowed into the pasture with the females overnight (see Appendix J). A significantly greater proportion of daughters (3/3) ovulated in families in which the father had been replaced by an unrelated male than in intact natal families (19/41 daughters) in common marmosets (*Callithrix jacchus*; Saltzman et al., 1997), and, as with white rhinos in this study (hypothesis 2b), the number of females in the family did not influence the proportion of common marmoset daughters having ovulatory cycles (Saltzman et al., 1997).

Why is a novel male rhino important for female ovulatory cycles? Rhinos are particularly attentive to excrement at dung piles, and both males and females display flehmen when investigating urine in particular (Metrione, personal observation). It is likely that olfactory cues are the stimulus for reproductive activity, both physiological and behavioral. In support of this notion, the onset of estrus in mares might be detected

by stallions through excrement investigation, especially since flehmen is highest during peak breeding season (McCort, 1984). Over long periods of time or with continued repetition, stimuli in all media might be vulnerable to habituation, and evolution therefore favors exaggeration, rearrangement, or replacement of stimuli (Moynihan, 1998). Female rhinos might be habituated to the pheromones of a male experienced since birth or early adolescence, and thus, pheromones of that male are inadequate to initiate ovulatory cycles. This could be a mechanism for inbreeding avoidance. Wild males are able to maintain a given territory for only ~5 years (Owen-Smith, 1973). So, even if an adolescent remained in her natal home range, it is likely that she would be exposed to the pheromones of a novel male shortly after weaning. Periodic entry of novel territory holders also would expose adult females to new pheromones. The introduction of a male into the captive group might have induced estrous cycles and mating in 1 northern white rhino female (Kuneš and Bičík, 2002) and erratic ovarian activity in 1 southern white rhino female (Patton et al., 1999). It should be noted, however, that while 95% of parous females reproduced with a novel male, 84% of nulliparous females also were housed with a novel male. Thus, a novel male might provide necessary stimulation of ovulatory cycles, but such stimuli are not sufficient for successful reproduction. A final note, while olfactory stimuli can induce the initial increase in secretion of luteinizing hormone, male sexual behavior enhances and might even be necessary for the maintenance of that response and induction of ovulation in some species (i.e., small ruminants; Delgadillo et al., 2009; Bedos et al., 2010).

Adolescent development and the potential for post-pubertal reproductive failure.

Initiation of follicular activity in white rhinos has been reported at 36 to 48 months of age (Hermes et al., 2006) and at ~30 months of age (Patton et al., 1999), which is consistent with the age of first luteal peaks observed in adolescents in this study (29-42 months). Onset of cyclicity in Nile hippopotamus (*Hippopotamus amphibius*), a similar-sized African megaherbivore, also occurred between 36 and 48 months of age in captivity (Graham et al., 2002). It appeared that adolescents in this study with a higher average daily frequency of aggression demonstrated luteal activity, as measured by progesterone and progestagen, sooner than those with a lower frequency of aggression (hypothesis 1a), but a larger sample size is needed to confirm this finding. Average daily frequency of sexual play did not appear to be related to the onset of luteal activity (hypothesis 1c), but, since sexual play was different between adolescent and captive-born, nulliparous (but not parous) as well as acyclic (but not cyclic) females, changes in frequencies of sexual play behavior in maturing adolescents should be monitored.

Since the commencement of luteal activity was documented for most of the adolescents in this study, it appears that the captive environment was adequate for the sexual development of captive-born, female adolescents. It is important to note that all of these adolescents were group-housed with companions in large enclosures, and most had access to more than 1 male, including a novel male. The 2 adolescents that did not begin to cycle before the end of the study were still in the early to mid-age range (29-32 months) for onset of puberty, based on the age of puberty of the other 4 adolescents.

Though group-housing among female rodents tends to delay the onset of estrus, the presence of the male or his pheromones still accelerates the onset of first estrus (Vandenbergh, 1974). Since housing among a social group increases parity in female white rhinos and encourages social interactions, it is recommended that adolescents should be raised in social groups and either a new male should be introduced shortly after weaning, or the adolescent female should be relocated to another institution housing a mature male and a group of females.

While at least 4 of the adolescents in this study experienced what appears to be normal sexual development, it should not be assumed that they will continue to cycle or reproduce successfully. No evidence of ovulatory cycles was found in 8 females, 2 of which were wild-caught and have not reproduced in captivity. Both had calves in the wild but have not had calves in captivity. Though this is a small sample, these data do suggest that cyclicity is vulnerable to disruption in captivity at any life stage, regardless of normal reproductive development and prior reproductive success (hypothesis 3).

Nulliparous, cyclic white rhinos?

A number of factors that negatively influence fertility could be considered for the nulliparous, female white rhinos, including anovulatory follicles, elevated estrogen:progesterone ratios, cycle irregularity, influences of prolactin, and early embryonic death. Persistent anovulatory follicles (PAF, also called hemorrhagic anovulatory follicles) in horses appear at first to develop as normal follicles but then become filled with blood and fibrin strands and fail to ovulate (McCue and Squires, 2002; Nunes et al., 2002; Blanchard et al., 2003b; Ellenberger et al., 2009). Though

ovulation does not occur, progesterone levels indicate the presence of active luteal tissue in 85.7% of the mares (McCue and Squires, 2002). PAF are associated with extended periods of anestrus (Bosu et al., 1982; McCue and Squires, 2002; Blanchard et al., 2003b), often recur in subsequent estrous cycles, and their incidence increases with age (McCue and Squires, 2002). In white rhinos, despite absent or erratic luteal phases, active ovaries had 4 to 10 follicles >10 mm in diameter and were found in females 3 to 19 years of age, while inactive ovaries had <1 to 2 follicles <4 mm in diameter and were found in females 15 to 38 years of age (Hermes et al., 2006). An anovulatory follicle believed to be analogous to PAF in horses also was documented by Radcliffe et al. (1997) in a white rhino, but progestagens were at non-luteal concentrations. Increases in serum progesterone were associated with anovulatory follicles in a Sumatran rhino, but the observed concentrations varied considerably and were not consistently at luteal levels (Roth et al., 2001). Similarly, urinary progesterone metabolites associated with anovulatory follicles in an Indian rhino were erratic and markedly lower than those associated with successful ovulations (Stoops et al., 2004). Recurring irregular follicular development without ovulation and associated with extended periods of anestrus appears to differ in horses and rhinos primarily in the consistent appearance of luteal tissue in the horse but not the rhino. Most of the females in this study showed evidence of active luteal tissue in sustained, elevated progestagen levels. These luteal cycles were generally consistent with the 35-day or 65- day cycles described in other studies (Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001; Graham et al., 2001; Roth, 2006) and were thus considered ovulatory cycles.

Hermes et al. (2006) suggested that follicular development without ovulation was associated with persistent elevation of estrogen, which could cause the cystic hyperplasia and endometrial cysts observed in older animals (Hermes et al., 2006). Other authors also have suggested that elevated baseline estrogen or estrogen:progesterone ratios might prevent follicular development and ovulation (Creel et al., 1997). Though estrogen was not measured, it is unlikely that it was elevated as most of the nulliparous females in this study were able to ovulate. Low fecal progesterone levels in wild elk (*Cervus canadensis*) herds were associated with low calf recruitment the following year (Creel et al., 2007). In this study, baseline progestagen concentrations did not differ between nulliparous and parous females or between acyclic and cycling females, and luteal progestagen levels were not lower in cycling, nulliparous females compared with cycling, parous females.

Cycle irregularity also might be associated with sub-optimal fertility. Females that experience normal ovulatory cycles and have access to males should mate and conceive. When successful mating and/or conception do not occur, timely return to estrus should increase chances for success. The irregularity of cycles in most of the females in this study undoubtedly limits their chances for successful reproduction and probably influences the fact that 12 of the 22 cycling females remain nulliparous. Extended estrous cycles (those ≥45 days in this study) also minimize the frequency that the females are sexually receptive.

In rats, apoptosis of regressing luteal cells from previous cycles is triggered by a preovulatory surge in prolactin after which the remaining luteal cells become refractory

to the lytic effects of prolactin, but not the luteotropic effects, until after the cycle has again progressed though diestrus (Gaytán et al., 2001). Prolactin concentrations are cyclic in African elephants, increasing during the follicular phase, but elevated mean prolactin was observed in 37% (11/30) of acyclic African elephants (Brown et al., 2004). More frequent or higher amplitude prolactin pulses could contribute to higher mean prolactin concentration in those acyclic elephants. Prolactin responses to psychological stress in humans are associated with passive coping strategies (Sobrinho, 2003). The same type of strategy might be expressed in captive animals whose ability to engage in natural activities and choices or in a response that would neutralize stressful stimuli is strictly limited (Hediger, 1964a).

If a luteolytic preovulatory prolactin surge occurs in rhinos as it does in rats, perhaps cycle irregularities arise from the improper timing or absence of that surge or the presence of additional prolactin surges. The absence of a preovulatory prolactin surge or the continued refractoriness of luteal cells from the previous cycle could result in the maintenance of luteal cells and extended luteal periods. Refractoriness of newly-formed luteal cells will not occur until after the next preovulatory surge, and since no luteotropic effects of prolactin on functional luteal cells are known in rhinos, it is possible that a sudden spike in prolactin during diestrus could induce premature apoptosis of new luteal cells, causing abbreviated luteal cycles. Luteal spikes that were too brief to be considered full cycles were present in the profiles of a number of females. Hermes et al. (2006) also documented abbreviated luteal spikes in white rhinos that they were able to associate with the formation of luteal structures on the ovary with ultrasound examinations. Luteal

insufficiency during early pregnancy also could be related to a lack of refractoriness in newly-formed luteal cells if they are exposed to a surge in prolactin. A luteolytic role for prolactin in rhinos might be unlikely, however, because it does not appear to be involved in the initiation of luteolysis in horses (Shand et al., 2000). Periovulatory prolactin surges occur during the reproductive season in mares (King et al., 2008b), but they probably facilitate follicular development (Shand et al., 2000).

Documentation of ovulatory cycles in the majority (12/18) of nulliparous females in this study suggests that reproductive failure might be related to problems during conception or early pregnancy rather than to anovulatory or abnormal cycles. The 2 failed pregnancies observed in this study might have resulted from early embryonic death (EED), which can be caused by endometritis, pyometra, luteal insufficiency, improper timing of oviductal transport, or genetic incompatibility (Carnevale and Ginther, 1992; Radcliffe et al., 1997; Patton et al., 1999; Blanchard et al., 2003d; Roth, 2006; AZA, 2009). Six confirmed cases of EED have been reported in white rhinos (AZA, 2009), 2 before day 28 postovulation (Radcliffe et al., 1997). Embryo loss occurred by day 30, 42, and 90 in a Sumatran rhino (Roth et al., 2001). Estimates for the rate of EED in horses average 20% from conception to day 40 of gestation (Blanchard et al., 2003d), and it is possible that EED is equally as common in rhinos. Not surprisingly, the incidence of EED before day 14 of gestation is 7 to 8 times greater for aged, subfertile mares (Blanchard et al., 2003d). Pregnancy cannot be diagnosed in rhinos by ultrasound until day 15, and so, it is unlikely that embryo loss before day 15 would be detected.

Conclusions.

Large enclosures and housing groups with more than 2 females/adolescents are conducive to reproduction in white rhinos. Large enclosures and the presence of a novel male are associated with estrous cyclicity in captive rhinos. Therefore, young, genetically valuable females should be moved to institutions where these conditions exist. Females should not be kept as an exclusive pair with a male, particularly a familiar one. While dominance interactions do not appear to affect reproduction, managers should continue to exercise good judgment regarding situations in which aggression is excessive and prevents a rhino from engaging in activities such as feeding, wallowing, and interacting with the group. Sexual play interactions should be encouraged, perhaps by adding mud wallows, dirt mounds, or other objects that instigate play in general. Monitoring females for reductions in their frequency of sexual play behavior could be useful in determining which females might require particularly careful management in order to promote successful reproduction. Nulliparous females do experience estrous cycles and, presumably, normal ovulation. Consequently, future studies might benefit from attention to conception and early pregnancy while management continues to encourage cyclicity through environmental modifications. Adolescents that have not commenced estrous cycles by 43 months of age but are group-housed in large enclosures with a novel male should be evaluated for reproductive pathologies.

Chapter 3: Relationships Between Corticosterone Concentrations and Social Behavior, the Social Environment, the Captive Environment, and Reproduction in Female White Rhinoceros

Introduction

The stress response is the physiological and behavioral response elicited when the brain perceives a significant disturbance of homeostasis, caused by a marked or unpredictable environmental change (Wingfield and Raminofsky, 1999; Moberg, 2000; Nelson, 2005b). While the stress response is normally adaptive, if the response to acute or chronic stress shifts sufficient resources away from other biological functions, deleterious effects might occur (Moberg, 2000). Activation of the hypothalamic-pituitary-adrenal (HPA) axis during a stress response results in increased secretion of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and glucocorticoids (corticosterone and cortisol). An increase in any or all of these stress hormones has the potential to suppress reproductive function by interfering with the reproductive axis (see Chapter 1; Rivier et al., 1986; Moberg, 1991; Dobson et al., 2003; Centeno et al., 2007a,b).

Response to various minor stressors, such as those that occur in confinement and are associated with husbandry practices, may incur measurable biological costs (Moberg, 2000). For example, stress in captive cheetahs (*Acinonyx jubatus*), evidenced by higher

fecal cortisol metabolites and higher corticomedullary ratios of adrenal glands relative to free-ranging cheetahs, might be associated with the high prevalence of diseases and poor reproduction in captive individuals (Terio et al., 2004). Stressors in the environment of captive female white rhinos might include lack of space, lack of companions, competition for clumped food resources (Metrione, 2005; Metrione et al., 2007), or social subordination.

The relationship between social status and stress is complex and varies considerably among species of mammals. Although chronic stress is a cost of social dominance rather than of subordination in some species, e.g., African wild dogs (*Lycaon pictus*; Creel et al., 1996, 1997) and dwarf mongooses (*Helogale parvula*; Creel et al., 1996), reproduction is compromised or delayed in subordinates of other mammalian species, famously exemplified by reproductive inactivity in all but a single, dominant queen in the naked-mole rat (*Heterocephalus glaber*; Jarvis, 1981). Inter-birth intervals are longer in subordinate than dominant gelada baboons, which might be explained by infertile cycles due to social stress in subordinates (Dunbar, 1980). A less obvious example is the positive correlation between dominance status and circulating progesterone levels following ovulation in red deer, which might be caused by interference of luteal function by stress in subordinate females (Flint et al., 1997). Black rhino females that scored higher on dominance behavior than their mate tended to be more successful breeders (Carlstead et al., 1999b), and reproduction might be suppressed in low-ranking female white rhinos (Metrione, 2005; Metrione et al., 2007). Interestingly, Carlstead and Brown (2005) found higher fecal corticosterone metabolite

variability in non-cycling compared to cycling white rhino females. If glucocorticoids are higher in subordinates than in dominant females, then chronic social stress could be considered as a factor contributing to their reduced reproductive success. Based on this possibility, this study was designed to examine possible relationships between serum corticosterone and fecal immunoreactive glucocorticoid metabolite (hereinafter, corticosterone) concentrations of captive female white rhinos and social behavior, the social environment (dominance status), aspects of the captive environment, and reproductive success (hypothesis 4). Mean corticosterone concentration was compared between females grouped according to dominance, reproductive history (parous vs. nulliparous), estrous cyclicity, institution, enclosure and group size, number and novelty of males, the presence of a well-known companion or their mother, and residence in a natal or non-natal institution, or at an institution with year-round or limited public access.

Methods

Behavioral analyses, assessment of reproductive history and the captive environment, and progesterone/progestagen analyses.

Results of the behavioral investigations, dominance analyses, and progesterone/progestagen analyses conducted in Chapter 2 were applied to analyses of the same animals in this corticosterone investigation. The housing environment was assessed for conditions (listed in Chapter 2) at the time of sample collection. Also considered for each institution was the extent of public access to rhinos, i.e., seasonal or by appointment, or year-round.

Collection of fecal and serum samples for hormone analysis.

Fecal or serum samples were collected from 38 females (Table 1) as described in Chapter 2. Serum samples were collected from 7 females (Busch, Reid Park, White Oak), and fecal samples were collected from the remaining 31 females. Also, fecal samples were collected from females at the Wilds while they were housed in a barn and while they were out at pasture.

Brown et al. (2001) found that fecal corticoid concentrations did not differ between seasons, and so, differences in corticoid concentrations based on the time of year in which samples were collected was not considered a confounding factor in this study. However, circulating glucocorticoid concentrations exhibit a circadian rhythm in many mammals (Nelson, 2005a), and so, serum samples were collected in late morning to mid-day to minimize any potential effects of circadian variation in corticosterone secretion. Such variation in fecal corticosterone was not expected in rhinos. In these hind-gut fermenters, steroid metabolites accumulate in the digesta over a period of about 48-60 hours (Owen-Smith, 1988a; Brown et al., 2001) before being released in feces, thus representing an average of circulating levels during the day. To test this assumption, corticosterone was measured in fecal samples collected 2 to 3 times per day for 7 days from 6 females at Lion Country Safari (LCS) and for 8 days from 4 females at San Diego Wild Animal Park (SDWAP).

ACTH challenge.

Elevated corticosterone in serum or fecal samples is widely accepted as evidence of activation of a stress response in mammals, including rhinos (Turner et al., 2002). A minimal test of this assumption is demonstration of a timely rise and fall of corticosterone following injection of ACTH, i.e., an ACTH challenge (Wasser et al., 2000). This response was previously documented in feces and serum of black rhinos (Brown et al., 2001). In this study, an ACTH challenge was evaluated in serum with serial blood sampling of a wild-caught, parous white rhino (Kathy). Following the protocol used by Brown et al. (2001) with black rhinos, an initial baseline blood sample was collected after which an intramuscular injection of slow-release ACTH gel (2,000 IU; Wedgewood Pharmacy, Swedesboro, NJ, USA) was administered. Blood samples were drawn at 1, 1.5, 2, 3, 4, 5, and 6 hours after injection. An increase in serum corticosterone concentrations at intervals following injection of ACTH was expected.

Fecal extraction and sample reconstitution.

Pools of reconstituted fecal extract (extraction procedure described in Chapter 2) that were serially diluted to test for parallelism with standard curves and diluted to provide reference (control) solutions for evaluation of intra- and interassay variation were prepared by dissolving the original dried fecal extracts in 500 µl of EIA buffer. For the samples, 10 µl-aliquots of fresh ethanol fecal extracts were dried overnight at room temperature. For the 1:40 dilution used in the assay, 400 µl of EIA buffer was added to

each dried sample and allowed to sit at room temperature for ~25.5 hours. Samples were then vortex-mixed for 20 seconds, and they were briefly vortex-mixed again immediately before assay (1-2.5 hours after the initial vortex).

Corticosterone immunoassay.

Turner et al. (2002) found approximately twice as much corticosterone as cortisol in the serum, urine, and feces of white rhinos; therefore, we measured corticosterone concentrations in serum and fecal samples. High-performance liquid chromatography analysis of eluates of black and white rhino fecal extracts revealed retention times of corticoid immunoreactive peaks that were associated with the corticosterone reference tracer and other unidentified metabolites (Brown et al., 2001). Brown et al. (2001) developed a glucocorticoid radioimmunoassay for serum of black rhinos and for fecal extracts from both white and black rhinos. However, this is among the first EIAs developed and validated for estimation of corticosterone in serum and feces of white rhinos. The protocol is the same as described for the progesterone assay (Chapter 2) except for the antibody (1:20,000; polyclonal antibody CJM006 produced against corticosterone-3-carboxymethyloxime, provided by C. Munro, U.C., Davis; see Appendix I for cross-reactivity) and corticosterone-HRP conjugate (1:90,000; U.C., Davis). After the first washing step and addition of EIA buffer, plates were incubated at room temperature for 2-3.5 hours.

Dose-response displacement curves based on serial dilutions of pooled serum (1:1 to 1:64) or fecal extract (1:2 to 1:1,024) from non-pregnant and pregnant females were parallel (Pearson correlation, $p < 0.0001$, $r > 0.99$ for all comparisons) to the standard

curve. The displacement curves of the pregnant and non-pregnant females were almost identical, indicating samples from both types of females could be similarly diluted. A dose-response displacement curve of pooled, reconstituted fecal extract from pregnant and non-pregnant females together (1:1 to 1:1,024) also was parallel (Pearson correlation, $p < 0.0001$, $r = 0.99$) to the standard curve. Reconstituted extract of feces diluted 1:40 and serum diluted 1:2 displaced approximately 50% of the corticosterone-HRP conjugate and was used as the sample dilutions in the assay. Recovery of known amounts of corticosterone (standard concentrations 0.078-10.0 ng/ml) added to pools of reconstituted fecal extract (1:40) was 132.8% (regression equation: $y = 1.4542x + 1.5096$, $r^2 = 0.99$) and added to pools of serum (1:2) was 99.5% (regression equation: $y = 1.2781x – 13.024$, $r^2 = 0.99$). Assay sensitivity was 0.08 ng/ml of standard (31.2 ng/g feces).

Pools of serum and reconstituted fecal extract were diluted in EIA buffer to provide controls of relatively high and low hormone concentrations. The average percent of conjugate binding was 34.0% for high and 64.6% for low fecal extract controls and 41.8% for high and 70.5% for low serum controls. Once made, fecal controls were frozen immediately and were subsequently thawed only once before use. Serum controls were thawed and used a maximum of 3 times. The reconstituted fecal extract controls were assayed in 4 wells per control (2 wells on each end of a column for each control) on every plate containing fecal samples. Serum controls were assayed in 4 wells per control (2 wells on each end of a column for each control) on every plate containing serum samples. Reconstituted fecal extract controls (2 wells per control per plate) also were assayed on plates containing serum samples for consistency when calculating interassay

variation, which was calculated as a C.V. of high and low reconstituted fecal extract controls assayed on every plate. Interassay variation was 8.9% for the high extract control and 10.5% for the low extract control (n = 51 plates). Calculated as a maximum range above and below the average control mass for all plates, interassay variation was <18% for the high and low controls. Intra-assay variation in hormone mass was determined using the reconstituted fecal extract controls for plates containing fecal samples and serum controls for plates containing serum samples. Intra-assay variation was calculated using the coefficient of variation among the hormone masses in all 4 wells containing the control on every plate. Average intra-assay variation was 11.0% (maximum 36%) for fecal and 12.2% (maximum 17%) for serum high controls and 13.2% (maximum 30%) for fecal and 15.9% (maximum 20%) for serum low controls.

During assay development, it was found that hormone mass in reconstituted fecal extracts decreased with successive freeze-thaw cycles and over time. Thus, all new samples were made and used immediately for each assay. Fecal sample extracts were assayed in 1 plate per female (the females from the Wilds each were assayed on 2 plates, one for samples collected during 2008 and one for samples collected during 2009, or one for samples collected during pregnancy and one for samples collected when the female was not pregnant); 28 samples spanning the entire sampling period for each female were assayed, and only those samples with a C.V. $\leq$15% between duplicate wells were used in calculating the average corticosterone concentrations. No decrease in hormone mass was observed for serum samples. Serum samples were not limited to fit on 1 plate, and they were assayed a maximum of 3 times.

Statistical analyses.

Average corticosterone concentration (ng/g or ng/ml ± SEM) per female was calculated from samples (n = 12-53) collected throughout the entire sampling period because no significant differences were found between means of samples collected from females during pregnancy compared to when they were not pregnant or between means of samples collected before or after the start of luteal peaks in adolescents (see Results). However, in the comparison of mean corticosterone concentration among acyclic, cycling, adolescent, and pregnant/lost pregnancy females, females for which there were samples collected during and outside of pregnancy were included in both the cycling and pregnant groups but with different average corticosterone concentrations calculated only from samples obtained during each condition. This ensured accuracy in the findings since there was a tendency for corticosterone to be higher during pregnancy (see Results). Serum corticosterone concentrations were extremely high in 2 adolescents, and so, they were excluded from analysis, which resulted in a sample size (n = 5) too small for valid statistical analysis in most cases. Fecal samples collected throughout the day at LCS and SDWAP were grouped into 3-hour intervals (4 intervals from 600-1800 at LCS, and 3 intervals from 600-1500 at SDWAP) for Kruskal-Wallis analysis to confirm that there was no circadian pattern in fecal corticosterone metabolites.

To account for differences in average fecal corticosterone that might be due to housing at different institutions rather than to an effect of the variables being tested, 2-way ANOVA was used after applying the natural log transformation to the data. Comparisons of average fecal corticosterone between variables (e.g., parity) for which

some but not all institutions housed females in each condition of that variable (e.g., parous and nulliparous) were analyzed as incomplete, unbalanced, randomized block designs with sub-sampling. In Proc Mixed (SAS Institute, 2002-2003), *institution* was designated as the random effect, the variable of interest as the fixed effect, and *institution x variable* as the random effect of the interaction. The interaction was confirmed to be non-significant in every test, and it was subsequently dropped from the model (see Appendix D). Variables tested in this manner included dominance (within housing groups and within subgroups); place of origin (wild-caught or captive-born); housing with a novel or familiar male, with or without the mother, with or without a familiar companion, or at the natal or non-natal institution; reproductive activity (cyclicity and/or pregnancy); and parity. Ajabu, the nulliparous female that did not have sufficient access to a male for breeding, was excluded from analyses of parity. Proc Mixed (SAS Institute, 2002-2003) with repeated measures was used to test for a difference in average fecal corticosterone before vs. after the start of luteal peaks in adolescents, during vs. before/after pregnancy among females that were pregnant, and while housed inside vs. outside for females at the Wilds. Only 2 institutions were involved in the tests for luteal peaks and pregnancy, so *institution* was treated as a fixed effect, and the interaction was excluded when testing for luteal peaks since there was only 1 female at each institution.

Comparisons of average fecal corticosterone between variables (e.g., enclosure size) for which all the rhinos at the institution could fall into only one condition of that variable (e.g., $>0.01 km^2$ or $<0.01\ km^2$) were analyzed as completely randomized designs with sub-sampling. In Proc Mixed (SAS Institute, 2002-2003), the variable of interest

was designated as the fixed effect, and *institution within variable* was designated as the random effect. Variables tested in this manner included enclosure and group size, amount of public access, or housing with 0-1 or >1 male.

Spearman correlation coefficients (Carlstead et al., 1999a,b; Carlstead and Brown, 2005) were used to determine whether correlations existed between rhino density and average fecal corticosterone concentrations per institution ($n = 12$) and between behavioral patterns and average fecal corticosterone concentrations ($n = 22$ as not all females with samples were observed). For analyses of correlation between corticosterone concentrations and average daily frequency of sexual advances made by mature males, the total sample size was only 17 because nursing, adolescent females were not included in the analysis and some females did not have access to mature males. Statistics including sexual advances made by both mature and adolescent males are provided in Appendix G. Differences between mean values and correlations were considered statistically significant when $p \leq 0.05$.

Results

ACTH challenge and circadian corticosterone variation.

The ACTH challenge demonstrated a greater than 20-fold increase in serum corticosterone concentrations following injection of exogenous ACTH, thus confirming the predicted activation of an adrenal response in white rhinos and confirming biological relevance of serum concentrations from the EIA used in this study to assess stress (Fig. 5). In agreement with the findings of Turner et al. (2002), no circadian pattern in corticosterone metabolites was observed ($p > 0.05$) at LCS or SDWAP (Fig. 6).

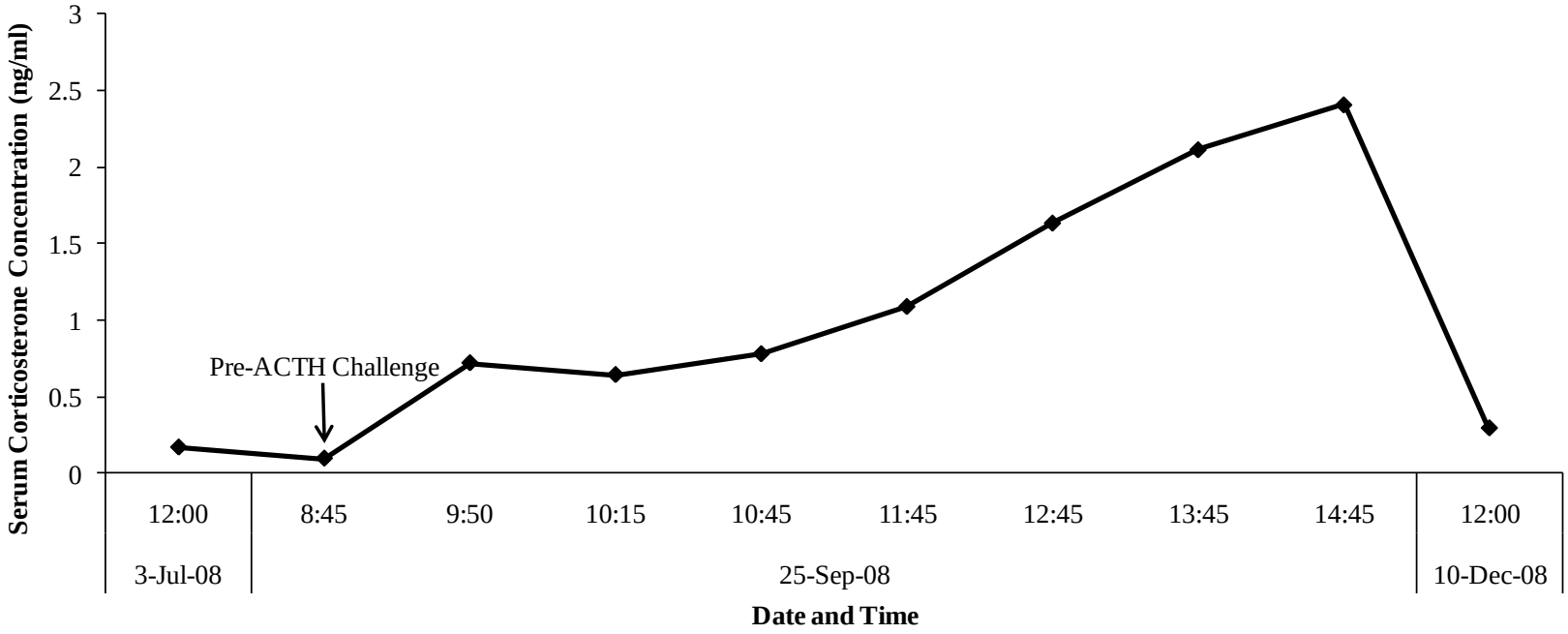

Fig. 5. Increase in corticosterone concentrations in serum samples collected from a captive female rhino before and at 1-hour intervals for 6 hours following intramuscular injection of ACTH on 25 September 2008. Baseline corticosterone concentrations are plotted for samples collected on 3 July, at pre-challenge (8:45 AM, 25 September), and on 10 December.

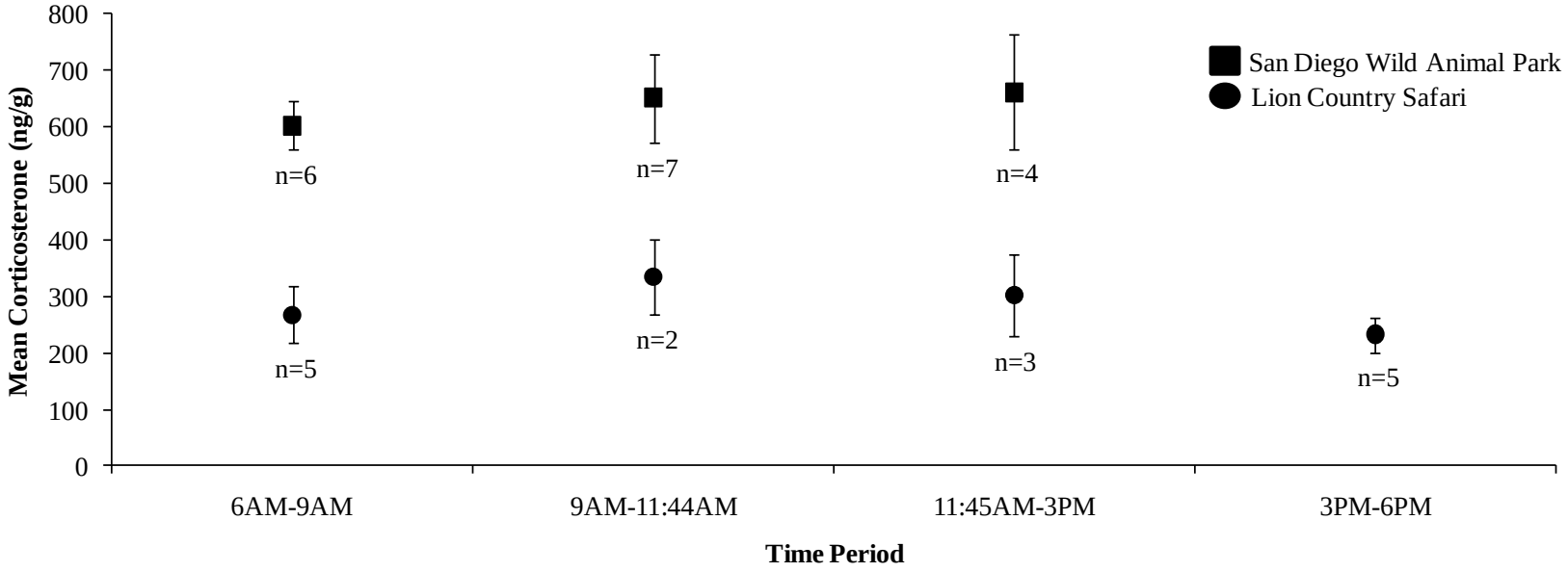

Fig. 6. Comparison of mean (± SEM) corticosterone metabolite concentrations in fecal samples collected at 3 intervals during the day for 8 days from 4 female rhinos at San Diego Wild Animal Park and at 4 intervals for 7 days from 6 female rhinos at Lion Country Safari. Differences in concentrations in samples collected over time were not significant ($p > 0.05$). Sample sizes on the graph indicate the number of fecal samples contributing to each mean corticosterone concentration.

Social behavior, the social environment, and corticosterone.

Average fecal corticosterone concentration did not differ ($p > 0.05$) across the institutions at which the females involved in the dominance analyses were housed. Average fecal corticosterone concentration did not differ ($p > 0.05$) between dominant and subordinate females within housing groups (Fig. 7) or within companion subgroups (Table 13), nor was there a correlation ($p > 0.05$) between average corticosterone concentrations and average daily frequencies of aggression ($r = 0.13$), sexual advances made by mature males to non-nursing females ($r = -0.07$), or sexual play behaviors ($r = 0.28$). Average daily behavioral frequencies and average serum corticosterone concentrations per female ($n = 5$) were not correlated ($p > 0.05$).

The captive environment and corticosterone.

The housing institution (as a separate random effect or considered within each condition of the second variable) had a significant ($p < 0.05$) effect on average fecal corticosterone concentrations in all analyses of environmental factors, but average fecal corticosterone concentration per institution was not correlated ($p > 0.05$, $r = 0.02$) with the density of rhinos at each institution. Wild-caught females had a higher ($p = 0.034$) average fecal corticosterone concentration than captive-born females (Fig. 7; Table 13). Average fecal corticosterone concentration tended ($p = 0.057$) to be lower when females were housed with a companion known from adolescence compared to housing with no female companion or a female companion that was introduced sometime during adulthood (Fig. 7; Table 13). Average fecal corticosterone concentration was not affected ($p > 0.05$) by enclosure size >0.01 km^2 (Fig. 7), by year-round public access, or

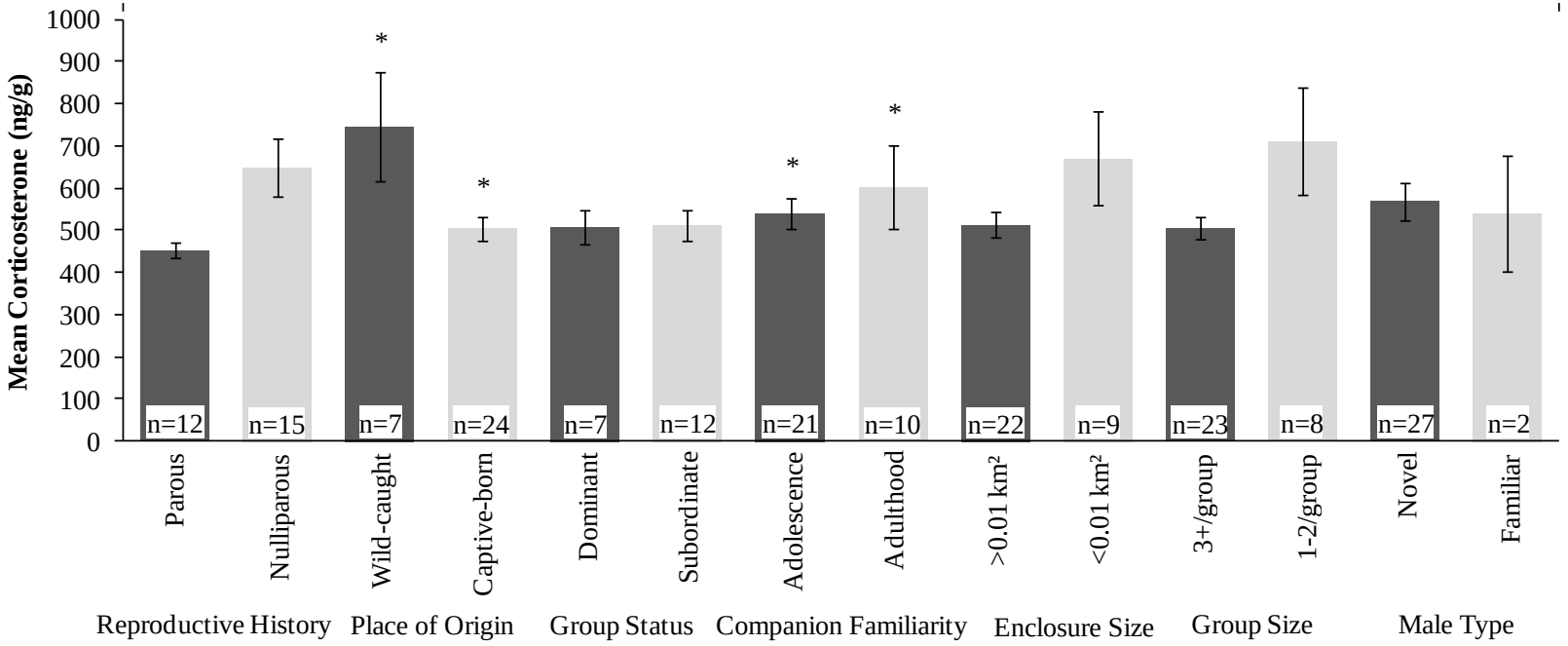

Fig. 7. Mean (± SEM) fecal corticosterone concentrations in female white rhinos compared relative to reproductive history, place of origin, social status, companion familiarity, enclosure size, group size, and male type. Corticosterone concentrations were significantly higher (*, $p < 0.05$) in wild-caught females and in females housed with a female companion known only since adulthood or no female companion.

Table 13. Average (± SEM) fecal corticosterone concentrations (ng/g) for female rhinos grouped according to housing institution, social status, particular characteristics of the housing environment, and reproductive activity.

Institution*	**N**	**Corticosterone Concentration**	**Institution***	**N**	**Corticosterone Concentration**
Audubon	1	309.8	Albuquerque	2	600.5 ± 5.4
Birmingham	2	454.0 ± 8.4	Omaha	2	1180.9 ± 124.4
DAK	4	372.2 ± 46.0	Indianapolis	1	850.7
Jacksonville	1	440.7	SDWAP	4	604.1 ± 54.8
LCS	7	441.9 ± 9.5	Wildlife Safari	1	855.4
Tulsa	1	404.7	Wilds	5	598.6 ± 47.0
Social Status			**Social Status**		
Dominant (housing group)	7	509.1 ± 40.0	Subordinate (housing group)	12	513.1 ± 35.9
Dominant (subgroup)	6	518.4 ± 46.0	Subordinate (subgroup)	13	508.5 ± 33.4
Housing Variable			**Housing Variable**		
Wild-caught	7	747.2 ± 128.6	Captive-born	24	503.9 ± 28.5
Familiar female companion	21	538.4 ± 36.4	No familiar female companion	10	601.8 ± 98.9
Enclosure >0.01km^2	22	513.1 ± 30.0	Enclosure <0.01km^2	9	670.7 ± 111.1
>2 females/adolescents	23	505.8 ± 24.9	≤2 females/adolescents	8	711.3 ± 126.8
Novel male	27	567.9 ± 44.7	Familiar male	2	541.8 ± 137.0
>1 male	18	552.1 ± 60.5	≤1 male	13	568.2 ± 46.8
Mother present	11	553.4 ± 63.9	Mother absent	20	561.8 ± 51.7
Natal institution	14	504.6 ± 38.0	Non-natal institution	17	603.5 ± 64.3
Year-round public access	25	539.0 ± 46.8	Limited public access	6	641.4 ± 57.5
Inside (the Wilds)	5	537.4 ± 38.4	Outside (the Wilds)	5	614.2 ± 110.1
Reproductive Activity			**Reproductive Activity**		
Cycling	18	544.1 ± 47.8	Acyclic	7	628.6 ± 118.5
Pregnant	7	476.6 ± 35.8	Adolescent	2	707.0 ± 31.0
During pregnancy	5	512.8 ± 31.0	When not pregnant	4	467.1 ± 36.3
Before luteal peaks	3	559.7 ± 145.9	After luteal peaks	2	506.4 ± 278.0

* Disney's Animal Kingdom (DAK), Lion Country Safari (LCS), San Diego Wild Animal Park (SDWAP)

by housing females in groups totaling >2 females/adolescents (Fig. 7), with novel males (Fig. 7), with >1 male, with their mother, or at their natal institution (Table 13). The average corticosterone concentration in fecal samples collected from females while living in a barn at the Wilds during December-April did not differ ($p > 0.05$) from that in samples collected from the same females on pasture during May-November (Table 13).

Reproductive activity and corticosterone.

Average fecal corticosterone concentration varied among housing institutions ($p < 0.05$) and affected all analyses of reproductive activity and parity, except for the comparisons of concentrations before and after the start of luteal peaks in adolescents, and during and before/after pregnancy in females that were pregnant. Average fecal corticosterone concentration did not differ ($p > 0.05$) between acyclic and cycling females; acyclic, cycling, pregnant/lost pregnancy, and adolescent females (Table 13); all non-cycling (acyclic, pregnant/lost pregnancy, adolescent) and cycling females; or all non-pregnant and pregnant/lost pregnancy females. Average serum corticosterone concentration did not appear to differ between acyclic (n = 1; 0.31 ng/ml), cycling (n = 3; 0.37 ± 0.14 ng/ml), and pregnant (n = 1; 0.47 ng/ml) females, but 2 adolescent females had very high average serum corticosterone concentrations (3.5 and 4.3 ng/ml). Average fecal corticosterone concentration did not differ ($p > 0.05$) before and after the start of luteal peaks in adolescent females (Table 13).

Average fecal corticosterone concentration did not differ ($p > 0.05$) between nulliparous (650.6 ± 69.2 ng/g, n = 15) and parous (453.4 ± 18.6 ng/g, n = 12) females (Fig. 7), which was also the case if the female without adequate access to a male (Ajabu)

was included in the nulliparous group (Table 14). It is interesting that average fecal corticosterone was numerically higher in nulliparous compared to parous females in spite of the inclusion of pregnant females in the parous group. Average fecal corticosterone was numerically higher ($p > 0.05$) in samples collected from females during pregnancy compared to when they were not pregnant (Table 13). Among all institutions ($n = 5$) housing both parous and nulliparous females, 8/10 nulliparous females had higher average serum or fecal corticosterone concentrations than all of the parous females ($n = 9$) at the same institution. Also, average serum corticosterone concentration in nulliparous females ($n = 2$; 0.48 ± 0.18 ng/ml) appeared to be higher than that in parous females ($n = 2$; 0.32 ± 0.08 ng/ml). It could be argued that wild-caught females had higher corticosterone than captive-born females (nulliparous, parous, and adolescent) because all the wild-caught females were nulliparous. This might be true, and it adds more support to the notion that nulliparous females could, in fact, have higher corticosterone than parous females. Indeed, most of the nulliparous females, irrespective of where they were born, have higher corticosterone than parous females (Table 15), and only 2 wild-caught, nulliparous females had corticosterone concentrations higher than any captive-born, nulliparous females. This perspective, however, does not account for important differences in corticosterone that are attributable to the housing institution. Individual corticosterone profiles for all females are provided in Appendix K.

Table 14. Average (± SEM) fecal corticosterone concentrations (ng/g) (n = 12–53 samples per female) for individual parous and nulliparous female rhinos.

PAROUS			NULLIPAROUS		
Rhino	**Institution***	**Corticosterone Concentration**	**Rhino**	**Institution***	**Corticosterone Concentration**
Bloom	LCS	454.7 ± 22.0	Ajabu	Birmingham	462.4 ± 23.6
Eliza	LCS	426.7 ± 21.3	Bertha	Albuquerque	605.9 ± 32.9
Gabby	Jacksonville	440.7 ± 15.0	Bonnie	LCS	482.1 ± 28.7
Holly	SDWAP	501.9 ± 15.7	Dumisha	SDWAP	717.0 ± 25.8
Julie	Wilds	567.9 ± 16.3	Helen	DAK	457.2 ± 24.2
Kendi	DAK	423.5 ± 23.9	Jao	DAK	359.8 ± 18.8
Laptop	Birmingham	445.7 ± 23.6	Jeannie	Tulsa	404.7 ± 16.5
Lissa	LCS	414.9 ± 28.7	Kiangazi	LCS	458.3 ± 23.7
Maggie	Wilds	521.9 ± 14.5	Kiazi	SDWAP	518.8 ± 21.1
Taraja	LCS	443.3 ± 26.1	Emalah	Albuquerque	595.1 ± 31.3
Yvonne	Audubon	309.8 ± 13.8	Mambo	Indianapolis	850.7 ± 51.0
Zenzele	Wilds	489.3 ± 18.8	Marina	Omaha	1056.5 ± 53.8
			Mashile	Omaha	1305.2 ± 78.3
			Paddy	LCS	413.4 ± 19.6
			Taryn	WS	855.4 ± 31.4
			Utamu	SDWAP	678.8 ± 25.0
N = 12			N = 16		
Average		453.4 ± 18.6	Average		638.8 ± 65.8

*Lion Country Safari (LCS), San Diego Wild Animal Park (SDWAP), Disney's Animal Kingdom (DAK), Wildlife Safari (WS)

Table 15. Average (± SEM) fecal corticosterone concentrations (ng/g) arranged by row from lowest to highest with females categorized in columns as captive-born, parous; captive-born, nulliparous; and wild-caught, nulliparous.

Captive-Born, Parous		Captive-Born, Nulliparous		Wild-Caught, Nulliparous	
Rhino ID	**Corticosterone Concentration**	**Rhino ID**	**Corticosterone Concentration**	**Rhino ID**	**Corticosterone Concentration**
Yvonne	309.8 ± 13.8				
				Jao	359.8 ± 18.8
		Jeannie	404.7 ± 16.5		
		Paddy	413.4 ± 19.6		
Lissa	414.9 ± 28.7				
Kendi	423.5 ± 23.9				
Eliza	426.7 ± 21.3				
Gabby	440.7 ± 15.0				
Taraja	443.3 ± 26.1				
Laptop	445.7 ± 23.6				
Bloom	454.7 ± 22.0				
				Helen	457.2 ± 24.2
		Kiangazi	458.3 ± 23.7		
		Ajabu	462.4 ± 23.6		
		Bonnie	482.1 ± 28.7		
Zenzele	489.3 ± 18.8				
Holly	501.9 ± 15.7				
		Kiazi	518.8 ± 21.1		
Maggie	521.9 ± 14.5				
Julie	567.9 ± 16.3				
				Emalah	595.1 ± 31.3
				Bertha	605.9 ± 32.9
		Utamu	678.8 ± 25.0		
		Dumisha	717.0 ± 25.8		
				Mambo	850.7 ± 51.0
		Taryn	855.4 ± 31.4		
				Marina	1056.5 ± 53.8
				Mashile	1305.2 ± 78.3

Discussion

The hypothesis that chronic social stress might contribute to reduced reproduction in subordinate females compared to dominant females was not supported by the results of this study. Nulliparous and acyclic females were not more likely to be subordinate (Chapter 2), and subordinate females did not have a higher average corticosterone concentration than dominant females. Also surprising, while average corticosterone concentration was different across institutions, place of origin and the presence of a female companion known from adolescence were the only environmental variables that were associated with differences in corticosterone concentration. Finally, average fecal corticosterone concentration did not differ between acyclic and cyclic females or between nulliparous and parous females.

A previous study of white rhinos at 2 institutions suggested that subordinate female white rhinos might not reproduce (Metrione, 2005; Metrione et al., 2007), and thus, subordinates might be expected to experience chronic stress and have elevated corticosterone related to their low social status. However, the results of this study, based on a larger and more diverse sample size, revealed that the proportions of parous and cyclic females were not different between subordinate and dominant females (Chapter 2). Furthermore, mean corticosterone concentration did not differ between dominant and subordinate females. Similarly, no relationship was found between cortisol concentrations and dominance status or ovarian acyclicity in group-housed, mature African elephants (Proctor et al., 2010). In that species, prolactin-induced ovarian dysfunction was suggested as a cause of acyclicity (Proctor et al., 2010). One

explanation for the similarity in corticosterone concentrations in dominant and subordinate females in this study is that they were members of long-established groups or the females within them were familiar with each other. Long-established groups tend to have stable social hierarchies that can minimize aggression (DeVries et al., 2003). Indeed, the only behavioral difference between dominant and subordinate females observed in this study was increased sexual play behavior in the latter; the frequency of aggression did not differ (Chapter 2). Similarly, in African wild dogs, the rate of initiation of aggressive encounters by females during the nonmating period was not affected by dominance (Creel et al., 1997). Subordinate female rhinos lost more interactions than dominants, but they did not necessarily endure more aggressive attacks than dominants, and this might explain why the subordinate females did not have higher corticosterone than the dominant females (DeVries et al., 2003). Moreover, average daily frequencies of aggression were not correlated with average fecal corticosterone concentrations.

Social connectedness and positive social contact can reduce the costs (high levels of stress hormones) associated with aggression and competition when living in a group (DeVries et al., 2003). For example, adult, female bongos (*Taurotragus euryceros*) had a significantly higher frequency of sociopositive interactions when feeding in clumped situations than when feeding in dispersed feeding situations (Ganslosser and Brunner, 1997). Because bongos are "concentrate selectors" (Ganslosser and Brunner, 1997), they encounter "contest" conditions during feeding in which access to resources is determined by rank (VanSchaik, 1989). Under clumped feeding conditions, sociopositive behaviors

are a mechanism for reducing tension (VanSchaik, 1989; Ganslosser and Brunner, 1997). Although white rhinos are grazers or "dispersed feeders," food in captivity is often clumped in space and time. Thus, while aggression is higher during feeding times than at other times during the day (Metrione, 2005; Metrione et al., 2007), the dominance hierarchy within housing groups and companion subgroups might reduce some of the aggression during feeding. Positive contact behaviors, such as sexual play, especially among companions, could further mitigate the stress caused by interactions with the group as a whole. It is also possible that the increased sexual play behavior on the part of the subordinate females (Chapter 2) is a means of reducing tensions between themselves and the other rhinos with which they are housed.

In accord with the likely benefits of group stability and social connectedness, it appears that housing female white rhinos with a female companion known from adolescence (even if there was a period of separation between adolescence and the present) is associated with lower average fecal corticosterone concentration than that in females housed with a female companion that was introduced sometime during adulthood or with no female companion. The familiar, positive social contact of the female companion known from adolescence might reduce the likelihood of a given stressor to elicit a stress response in the focus female, or the companion's presence might reduce the duration and/or amplitude of the stress response in the focus female. The well-known female companion does not need to be the mother, as evidenced in the similarity of corticosterone concentrations in females housed with or apart from their mothers. This result lends additional support to the finding that parity was not improved by the presence

of the mother (Chapter 2), as originally suggested by Swaisgood et al. (2006). Instead, the percentage of females having ovulatory cycles tended ($p = 0.08$) to be higher among those housed with a companion known from adolescence. Adolescent dispersal in the wild is instigated by the mother's aggression at the birth of her next calf (Owen-Smith, 1973), and the adolescent subsequently forms a companionship with another adolescent(s) or an unrelated adult female (Owen-Smith, 1973, 1975; Shrader and Owen-Smith, 2002). Based on these observations of wild white rhino behavior and on the tendency for corticosterone to be lower among females housed with a companion known from adolescence, managers should transport females to new institutions in pairs whenever possible, especially when relocating adolescents, as this might promote overall psychological and physiological well-being in the rhinos.

Two adolescents (Lucy and Dakari) had very high serum corticosterone concentrations compared with all the other females in the study. The first samples collected from these females had the highest corticosterone concentrations; one female was 3 years of age, and the other female was just over 1 year old. While their corticosterone concentrations decreased and then leveled during the study, they were still higher than those in any other female and often were higher than those recorded during the ACTH challenge. The cause for these high corticosterone concentrations is unknown. However, coincidentally, 2/6 calves and the only yearling in a study of North Atlantic right whales (*Eubalaena glacialis*) also had the highest fecal corticosterone metabolite concentrations while the other calves had much lower concentrations, similar to most of the other animals in the study (Hunt et al., 2006). Those authors suggested the high

glucocorticoids might have been associated with weaning stress, or variable, high concentrations might be a normal part of calf development in that species (Hunt et al., 2006). The weaning stress hypothesis is less consistent with this study since the corticosterone concentrations in the younger adolescent gradually decreased, though they were still very high, as she neared the time at which she was weaned from her mother with the birth of the next calf (May 2008). It is possible that high glucocorticoids are a normal characteristic of development, but this begs the question, why were glucocorticoids not elevated in all of the adolescents?

Cortisol concentration was higher in male Père David's deer (*Elaphurus davidianus*) housed in small, high-density enclosures with public exposure than in free-ranging stags (Li et al., 2007). Similarly, cortisol was higher in clouded leopards (*Neofelis nebulosa*) who had less vertical climbing space and in those on public display (Wielebnowski et al., 2002). In contrast, though there was a difference in average fecal corticosterone concentration between institutions, higher corticosterone concentrations were not associated with any particular environmental characteristics other than place of origin and the absence of a female companion known from adolescence. This suggests that environmental factors not tested in this study might account for differences between institutions. Also, females with elevated corticosterone might be individuals who are particularly sensitive to stimuli that are perceived as aversive, either environmental or social. Considerable variability in salivary cortisol between individual Indian rhinos and Asian elephants, possibly reflecting individual animals' abilities to cope with changes in their captive environment, was observed when a zoo opened to the public for the first

time (Menargues et al., 2008). Individually stress-sensitive and behaviorally withdrawn animals also were found among socially-housed rhesus macaques (*Macaca mulatta*): Elevations in cortisol were more sustained over time after separation events in animals characterized as highly withdrawn compared with less withdrawn animals (Erickson et al., 2005).

Both reduced behavioral interactions (e.g., sexual play behaviors) and increased production of glucocorticoids are characteristics of the "conservation-withdrawal" response to stressful stimuli (Carlstead, 1996). With the conservation-withdrawal response, the active fight or flight response is ineffective, and so, the behavioral response shifts to energy conservation, reducing contact with conspecifics and objects in the environment, and minimizing detection (Engel, 1967). That nulliparous and wild-caught white rhinos might be stress-sensitive animals is supported by the fact that captive-born, nulliparous and wild-caught, parous females, but not captive-born, parous females, exhibited less sexual play behavior than adolescents (Chapter 2). A higher average fecal corticosterone concentration in wild-caught females compared to that in captive-born females also supports the notion that wild-caught females might be stress-sensitive. The presence of zoo visitors and strange noises, reduced space, and other aspects of the captive environment might be perceived as threatening or aversive, thus eliciting a stress response in wild-caught females. Based on the relationship between infrequent sexual play behavior and high corticosterone in wild-caught females, the statistically insignificant higher average fecal corticosterone concentration in nulliparous compared to parous females might be biologically relevant, having an association with infrequent

sexual play behavior and possible stress-sensitivity in nulliparous females. Carlstead and Brown (2005) found that increased pacing and decreased olfactory behaviors were characteristics of acyclic white rhino females, and both decreased olfactory behavior and acyclicity were associated with higher variation in corticosterone concentrations.

That all the wild-caught females contributing fecal samples to the corticosterone analyses were nulliparous leaves room to debate which characteristic is responsible for their higher corticosterone compared to captive-born females. If, instead of the association being between high corticosterone and wild-caught, their nulliparous condition is associated with high corticosterone, then this would support the relationship between low sexual play behavior and nulliparity, and it would support the possibility that corticosterone is actually higher in nulliparous than in parous females.

For best management, individual rhinos with elevated corticosterone, especially young, genetically valuable, nulliparous females, should be considered for relocation to institutions with larger enclosures, social groups, and perhaps less public exposure, as these factors might be expected to be associated with stress in captive animals in general (Carlstead, 1996). Relocations also should aim to move rhinos to institutions where a female companion known from adolescence already resides or to move such companions together. The ability to exert control over the termination of stressful stimuli was associated with reduced plasma ACTH in female rats (Anderson et al., 1996). Another option for the management of stress-sensitive rhinos is providing structures within their current enclosures, such as a berm, that allow rhinos to separate themselves, at least visually, from disturbing stimuli (Hediger, 1964c). Adequate grazing or access to

abundant and widely dispersed grass hay also could give rhinos more control over their daily feeding schedule. Managers should provide logs and boulders for rubbing and manipulating as well as other enrichment activities that distract the rhinos from disturbing stimuli (Moberg, 2000) or allow them to cope with stressful stimuli through displacement behaviors (Dantzer and Mormède, 1983). A particularly good enrichment suggestion, offered by Carlstead (1996), is to hang a swinging boxing bag in rhino enclosures because it will "respond" to the prodding of the rhino with unpredictable movements.

That average fecal corticosterone was not significantly higher during pregnancy compared to when the same females were not pregnant is perhaps not surprising when one considers that among wild white and black rhinos, pre-capture fecal corticoid concentrations in pregnant animals did not differ from pre-capture concentrations in non-pregnant animals (Linklater et al., 2010). Fecal corticoid concentrations also were stable throughout gestation except for a slight increase near parturition in a captive black rhino, and her concentrations during gestation were not different from those of non-pregnant females (Brown et al., 2001). In Baird's tapir, serum cortisol concentration also was unchanged during pregnancy and parturition (Brown et al., 1994).

Corticosterone and the other hormones of the HPA axis can impact reproduction by affecting secretion or binding of gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), and luteinizing hormone (LH), perhaps preventing ovulation (Rivier et al., 1986; Moberg, 1991; Dobson et al., 2003; Centeno et al., 2007a,b). If the unusually high corticosterone concentrations in the adolescents at Busch Gardens, particularly in late 2007 and early 2008, were high enough to interfere with normal

functioning of the hypothalamic-pituitary-gonadal (HPG) axis, it might explain why 1 of the females did not have luteal cycles until late in the age range for puberty (41 months, Chapter 2). Nevertheless, glucocorticoid inhibition of cyclicity is an unlikely explanation for nulliparity and acyclicity in this study because 12/18 nulliparous females exhibited estrous cycles (Chapter 2), and average fecal corticosterone concentration did not differ between acyclic and cycling females. These findings are in accord with those of Brown et al. (2001) who found that corticosterone concentrations were not different between rhinos without ovarian activity and those that showed at least some ovarian activity. The negative effects of elevated glucocorticoids, ACTH, and CRH on the HPG axis are not evident in all species (Moberg, 1991), and it seems that elevated corticosterone cannot account for acyclicity in white rhinos.

Statistically, average fecal corticosterone concentration was not higher in nulliparous females compared to that in parous females, but there was evidence of subtle differences across and within institutions housing both types of females. Repeating and expanding this part of the study to include females outside of the United States would be useful for clarifying any relationship that might exist between corticosterone concentration and parity. A larger sample size that includes more institutions housing both nulliparous and parous females together will improve statistical power. A Wilcoxon exact test, which did not account for institutional differences, indicated that nulliparous females had a higher ($p = 0.037$) average fecal corticosterone concentration than parous females. If further study demonstrated a significant difference in corticosterone concentration, those results, based on a comparison between discrete categories (parous

and nulliparous), would not necessarily conflict with Carlstead and Brown (2005) who found that there was no correlation between corticosterone and lifetime reproductive rate, treated as a continuous variable, which would require assurance that females had uninterrupted access to males and that lactational anestrus was consistently absent or occurred for approximately equal durations in all of the females.

Recall that most nulliparous females apparently experienced ovulatory cycles, and so, if further study reveals that average corticosterone concentration is higher in nulliparous than in parous females, those results would be consistent with the notion that activation of the HPA axis and chronic elevation of glucocorticoids might interfere with conception or early pregnancy. Elevated glucocorticoid secretion mobilizes glucose, and thus, elevated corticosterone might lead to toxic levels of oxygen free radicals in embryos when that glucose is metabolized. Development of 8-cell bovine (*Bos taurus*) embryos was compromised by the addition of 4mM glucose due to the activity of glucose 6-phosphate dehydrogenase (G6PD; Kimura et al., 2005). Glucose 6-phosphate dehydrogenase is the rate-limiting enzyme in the pentose phosphate pathway, which generates oxygen free radicals. This might be particularly problematic for female embryos (Larson et al., 2001; Kimura et al., 2005) because the gene for G6PD is X-linked, and the inactivation of 1 of the X chromosomes might not be completed quickly enough (Kimura et al., 2005; Gutiérrez-Adán et al., 2006). Toxic by-products from glucose metabolism might be the cause of greater female than male embryo death during early gestation among translocated white, black, and Indian rhinos (Linklater, 2007). Another possibility is that in monotocous animals, asynchrony between the sensitivity of

the uterus, influenced by progesterone, and the implantation signal of the blastocyst could result in reduced fertility (Krackow, 1995). Linklater (2007) suggested that this asynchrony might occur or be exacerbated by cortisol blocking uterine progesterone receptors, inhibiting uterine blood flow that would normally facilitate implantation. Elevated glucocorticoids also could interfere with estrogen or progesterone acting on the oviduct, slowing the rate of ovum or blastocyst transport (Krackow, 1997). Improper timing of oviductal transport is a cause of pregnancy loss in mares (Blanchard et al., 2003d).

This study has demonstrated that most nulliparous females do have estrous cycles (Chapter 2) and that glucocorticoids do not appear to be responsible for acyclicity when it does occur. Therefore, if further study confirms that corticosterone is not elevated in nulliparous compared to parous females, other mechanisms for embryo loss, such as endometritis and pyometra (Carnevale and Ginther, 1992; Radcliffe et al., 1997; Patton et al., 1999; Blanchard et al., 2003d; Roth, 2006; AZA, 2009), genetic incompatibility (Blanchard et al., 2003d; Roth, 2006; AZA, 2009), and premature luteolysis of the primary corpus luteum (Noakes, 1996; Blanchard et al., 2003c) need to be explored in detail. Certainly, other explanations for acyclicity are needed. Studying the endocrine changes that ultimately lead to seasonal anestrus in mares might provide helpful insights. For example, the absence of the early diestrous FSH surge during autumn estrous cycles could lead to suboptimal follicular development (Irvine et al., 2000). The magnitudes of estrogen and LH surges are positively correlated, and this suggests that the preovulatory LH surge cannot commence when underdeveloped follicles secrete insufficient estrogen

(Irvine et al., 2000). Follicle stimulating hormone pulse magnitude and frequency might be altered in acyclic compared to cyclic rhinos. The signal for uterine release of prostaglandin $F_{2\alpha}$ is compromised in cases of spontaneously prolonged corpus luteum activity in mares (King et al., 2010), but administration of a dopamine receptor (D2) antagonist reduced the incidence of spontaneously prolonged corpus luteum in autumn-transition mares, suggesting that dopamine might be involved in luteolytic failure (King et al., 2008a). Thus, dopamine might influence the occurrence of long estrous cycles in rhinos.

In conclusion, subordinate females did not have a higher average fecal corticosterone concentration than dominant females, suggesting they probably are not experiencing any more chronic social stress than dominant females. Housing females with another female companion known from adolescence is associated with lower average fecal corticosterone than housing with a female companion introduced in adulthood or with no female companion. Environmental factors other than those considered in this study need to be explored in order to better explain why rhinos at some institutions have higher corticosterone than those at other institutions. Although elevated glucocorticoid levels probably are not responsible for acyclicity in white rhinos, it appears that some females, including wild-caught and possibly nulliparous females, are individuals in which the HPA axis responds more strongly to stressful stimuli. Adequate space and social interactions, particularly opportunities for sexual play interactions, and other stress-reducing mechanisms might improve the well-being and, potentially, the reproductive performance of females with elevated corticosterone.

Chapter 4: Summary of Key Findings and Conclusions

Progesterone/Progestagen Findings

- Ovulatory cycles occurred in 12 of 18 nulliparous females.
- Two wild-caught females, who were parous in the wild but have not reproduced in captivity, were acyclic.
- Puberty, characterized as the onset of luteal activity evidenced from progestagen profiles, occurred between 29 and 42 months of age.

Social Behavior, Reproductive Success, and Estrous Cyclicity

- Captive-born, nulliparous and wild-caught, parous females engaged in sexual play less often than adolescents, and the frequency of sexual play was similar in adolescents and captive-born, parous females.
- Pregnant and acyclic females tended to engage in sexual play less often than adolescents, and the frequency of sexual play was similar in adolescents and cyclic females.
- The frequency of sexual advances by males was greater toward cycling than acyclic females.

The Social Environment, Reproductive Success, and Estrous Cyclicity

- Subordinate females engaged in sexual play more often than dominant females.

- Subordinate and dominant females did not differ in average daily aggression or in the frequency of advances made by mature males toward them.
- Parity and the proportion of cyclic females did not differ between dominant and subordinate females.

The Captive Environment, Reproductive Success, and Estrous Cyclicity

- Parity was higher in females that were housed in large enclosures (>0.01 km^2) and in large groups (>2 females/adolescents).
- A larger proportion of females had ovulatory cycles when housed in large enclosures (>0.01 km^2) and with males that were unknown during early adolescence.
- Housing with more than 1 male, with a companion that was known during adolescence, with the mother, or at the natal institution did not affect the proportion of females that were parous or experiencing ovulatory cycles.

Corticosterone Concentrations, Reproductive Success, and Estrous Cyclicity

- The corticosterone EIA was validated for use with white rhino serum and feces.
- Average fecal corticosterone concentration did not differ between subordinate and dominant females.
- Average fecal corticosterone concentration was different across the housing institutions.
- Average fecal corticosterone concentration was higher in wild-caught females than in captive-born females.

- Average fecal corticosterone concentration tended to be lower in females housed with a female companion known from adolescence than in those housed with a female companion introduced during adulthood or no female companion.
- Average fecal corticosterone concentration did not differ between acyclic and cycling females or between nulliparous and parous females.

Final Comments

Reproduction is not only poor in captive-born females, but it also can be compromised in captive, wild-caught females, despite their presumed normal development in the wild. The presence of estrous cycles in nulliparous females suggests that future studies of reproduction in white rhinos should focus on problems during conception and early pregnancy. Similar corticosterone concentrations among acyclic and cycling females suggest that activation of the adrenal stress response is not primarily responsible for the inhibition of estrous cycles in those females that are acyclic.

The trend for elevated fecal corticosterone concentrations in nulliparous compared to that in parous females deserves further study with samples collected from additional females, including captive rhinos outside of the United States. Some females, such as wild-caught females, are particularly sensitive to stimuli that elicit an adrenal stress response, and infrequent sexual play behavior among captive-born, nulliparous females indicates that they also may be stress-sensitive. Elevated corticosterone concentrations in such females might subsequently affect proper function of the female reproductive tract (Krackow, 1997; Linklater, 2007) or embryo viability (Larson et al., 2001; Kimura et al., 2005; Gutiérrez-Adán et al., 2006; Linklater, 2007).

Environmental conditions that are likely to influence reproduction in most females include enclosure size, group size, and access to a novel male. Every effort should be made to ensure that females intended for reproduction are housed in enclosures >0.01 km^2, with 2 or more other females/adolescents, and with a male that was unknown during early adolescence. This might require moving weaned adolescent females to a suitable institution. Care should be exercised when choosing which adolescents will be moved, since housing with a female companion known from adolescence is associated with lower corticosterone levels than housing with a female companion that is introduced in adulthood or with no female companion. Enclosures and management should be designed to encourage play behaviors as a positive social interaction. In addition, the frequency of sexual play behavior should be monitored as females mature in order to identify those exhibiting a lower frequency and who might, therefore, be prone to reproductive difficulties. By following these guidelines, it should be possible to increase the reproductive success of the captive, female white rhinos, resulting in a self-sustaining population.

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Appendix A: Table 16. Daily Feed and Approximate Daily Hay Ration for Female White Rhinos During the Study

Institution	Concentrate Per Female	Hay Per Female
Albuquerque Biological Park	2.3 kg Mazuri Wild Herbivore[1]	12 kg timothy, 11 kg sudan
Audubon Zoo	11.3 kg Mazuri ADF-#16[2], 1.4 kg Quality Blend Classic Finish[3]	2 kg alfalfa, 15 kg timothy
Birmingham Zoo	7.7 kg Mazuri ADF-#16[2]	4 kg alfalfa, 14 kg coastal
Busch Gardens	None	23 kg timothy
Disney's Animal Kingdom	5-11.8 kg Mazuri Wild Herbivore[1] (varies/female)	Free-choice coastal, 1 kg alfalfa
Henry Doorly Zoo	None	20 kg timothy, 18 kg prairie
Indianapolis Zoo	4.5 kg Mazuri Wild Herbivore[1]	17 kg timothy/orchard grass
Jacksonville Zoo	11.3 kg Mazuri ADF-#16[2]	9 kg coastal
Lion Country Safari	4.5 kg Elephant Pelleted[4]	Free-choice coastal
Louisville Zoo	7.3 kg Mazuri ADF-#16[2]	6 kg alfalfa, 4 kg timothy
Reid Park Zoo	4.8 kg Fancy Race Horse Crimped Oats[5], 4.2 kg Mazuri Wild Herbivore[1]	15 kg bermuda
San Diego Wild Animal Park	Free-choice Mazuri Wild Herbivore[1]	Free-choice bermuda and sudan
Tulsa Zoo	4.5 kg Mazuri ADF-#16[2]	21 kg prairie
White Oak Conservation Center	7.3 kg Mazuri ADF-#16[2]	6 kg coastal bermuda
Wildlife Safari	2.3 kg Grainland Select sweetfeed[6], 4.1 kg DC Farmers Alfalfa Pellets[7], 5.4 kg Mazuri Elephant Supplement-Regional[8]	2.5 kg alfalfa, 2 kg local grass hay
the Wilds	5 kg Mazuri ADF-#16[2]	1-14 kg local grass hay (summer vs. winter)

[1]Mazuri Wild Herbivore Hi Fiber: 12% protein, 3% fat, 30% fiber; contains soy
[2]Mazuri ADF-#16 Herbivore: 17% protein, 3% fat, 15% fiber; contains soy
[3]Quality Blend Classic Finish: 12% protein, 8% fat, 12% fiber; contains soy
[4]Elephant Pelleted: 24% protein, 4% fat, 9% fiber; contains soy
[5]Fancy Race Horse Crimped Oats: 11% protein, 4% fat, 13% fiber
[6]Grainland Select C.O.B. W/MOL: 8% protein, 2.5% fat, 7% fiber
[7]DC Farmers Co-op Alfalfa Pellets: 15% protein, 0.01% fat, 30% fiber
[8]Mazuri Elephant Supplement-Regional: 24% protein, 4% fat, 12% fiber; contains soy

Appendix B: Table 17. Wild White Rhino Ethogram (Owen-Smith, 1973)

Behavior or Vocalization	Purpose	Description
Snort (vocalization)	Mild "keep-away" warning	Nasal ex- or inhalation
Snarl (vocalization)	More powerful distance-increasing tool	A gruff roar, brief or rumbling, made with the mouth open, head thrust back, and ears laid back
Pant (vocalization)	Contact seeking or maintaining call	A chesty exhalation or inhalation
Hic (vocalization)	Signifies bull's intent to court	Repetitive wheezy exhalations with a throb produced at the beginning of each inhalation
Squeal (vocalization)	Signifies the actions of the bull (towards a cow) are in the context of territory boundary blocking	High pitched then falling off; may become a singing wail
Shriek (vocalization)	Attack inhibiting	Intense/shrill; ears thrust back, head thrust forward
Whine (vocalization)	Calf seeking udder or adolescents moving back toward companions	A thin, mewing tone that rises and falls in pitch
Squeak (vocalization)	Calf distress signal	Abrupt and high pitched
Gruff squeal (vocalization)	Emphasizes bull's presence	Throaty, rumbling squeal
Gasp-puff (vocalization)	Response to sudden fright	Sudden in- or exhalation
Pinning ears back	Distance increasing display	Ears laid back, usually coupled with head thrust and snort or snarl
Advancing steps	More powerful distance-increasing effect than a snarl or snort alone	Actor steps quickly toward the recipient and simultaneously gives a snarl, snort, or shriek
Horn prod	Ritualized attack movement	Head lowered followed by upward jabbing movement
Horn clash	Gesture to repel encroachment	Horn lowered parallel to the ground then hit sideways against horn of the recipient

Continued.

Appendix B: Table 17. Continued.

Behavior or Vocalization	Purpose	Description
Charge	Intimidation display	Rapid advance
Head flings	Play invitation and indication of excitement	Head swung up and down rapidly
Presenting the side	Act of appeasement	Turning head away from other rhino
Horn against horn stare	Intimidation display	Horns of 2 bulls pressed together with heads raised and ears forward
Horn wiping	Assertion of presence/status	Sideways, twisting movements of the horn on the ground
Scraping	May be related to the deposition of scent marks	Hindlegs or forelegs dragged with nail pressed against the ground
Tail curled	Associated with situations of general autonomic stimulation	Curling of tail may be held or repeated
Nasonasal meeting	Potentially for individual identification	Movements slow and relaxed eventually allowing noses to meet
Attack	To drive recipient away	Horn jabbing movements directed toward body of recipient
Fight	Opponents attempting to drive each other away	Attack gestures made by both opponents
Acceptance of tactile contact	To strengthen bonds	Expression of a close bond through non-aggressive physical contact
Urine/Dung Smelling	Identification	Smelling of urine or dung; may be followed by flehmen
Smelling of vagina	Estrus identification, courtship	Bull smells cow's vaginal area; may be followed by flehmen
Chin Resting	Courtship	Bull rests his head on the rump or back of the cow
Mounting	Breeding	Bull straddles cow's back with forelegs while standing on hindlegs; may or may not be preceded by erection

Appendix C: Behavioral Definitions

Aggression

1. Acts of aggression were counted even if asserted against a non-rhino.
2. An aggressive act included a physical aggressive movement such as advancing steps, a prod (contact unnecessary), a fast and direct turn, direct movement (not wandering or gradual) on a rhino or its food pile, a clash, a full charge, a sideways horn-swipe, a shove with the horn or head, or quickly moving to a full standing position when in close proximity to the approaching/close-standing rhino.
    a. A weak horn gesture that could not possibly reach its target and was only generally directed toward a specific rhino was a warning (not scored).
    b. If 2 rhinos lowered their horns at each other (usually while eating) but no further aggressive action was executed, then this was a warning (not scored), but it was still counted as a draw if neither backed.
    c. If 1 rhino lowered its horn as another approached but no further action was taken, then this was a preparatory act for defense, not an aggressive act, and the approach of the rhino was aggressive only if it was accompanied by obvious characteristics (snarls/snorts). If the approach was aggressive, then the result was a tie.
    d. If a rhino moved exactly onto another's place and put its nose in the other's face but did not take the other's space or food once the other moved (if it did), then it could not be assumed that that was an aggressive act. The oncoming rhino might have been seeking contact only. The other rhino acted in subordination, though, if it yielded.
3. Subordination occurred when ground, food, shade, or some resource was yielded to the aggressing/approaching rhino.
    a. A subordinate gesture could occur even if the dominating rhino did not make an aggressive action (e.g., was merely approaching).
    b. If only the front or back foot stepped back and the overall body position did not change, then that was a draw.
    c. If the head was pushed out of the way or moved over but the rhino continued eating and/or held its ground otherwise, then it was a draw.

    d. If a prod or a clash occurred and the receiver responded only by standing its ground, then that was a draw since there was physical contact against which some resistance must have been used in order to remain immobile.
    e. If a rhino stood up from a resting position in response to a snort, prod, etc., but did not subsequently initiate its own aggressive act (the standing process was not directed at the encroaching rhino nor did it facilitate an advance on the encroaching rhino), then it was a loss for the previously resting rhino.
    f. If a rhino joined another at a food pile (no necessarily an aggressive advance) and the rhino already at the food pile did not immediately yield the pile but did eventually move away, it could not be counted as a subordinate act because it was not a direct response to the approach.
4. If it was not seen, the part that was unseen was not scored.
    a. If the aggressive action was seen but not the result, then the action was recorded but not the result (and vice versa).
    b. If the result of an interaction could not be determined, then neither rhino received winning/losing points.
    c. If, as far as could be determined, a clash was simultaneously initiated, then both rhinos received the aggression initiation point.
5. The number of aggressive interactions scored was the number initiated by that rhino, not the number in which the rhino was engaged.
    a. If 1 rhino advanced on another and clashed, the second did not get a point for its defensive actions during the clash because it was defending.
    b. If 1 rhino advanced on another but the second initiated the actual clash or further aggression, then aggression was scored for both rhinos.
6. Snort and snarl were not necessarily signs of aggression.
    a. A snort was often a soft warning, and a snarl alone could be defensive.
    b. A snort or snarl was part of an aggressive act if it accompanied a physical aggressive movement.
    c. Regardless of whether a snort was accompanied by aggression, if a rhino responded to a snort by stopping, backing from food, yielding ground, or altering its trajectory, then that rhino acted in subordination.
    d. If a snort was given and the receiving rhino did not back, come forward, or alter overall activity but did show evidence of having received the signal (ears turned, looked up, etc.), then that was a draw.
        i. If a female snarled at a male who was trying to initiate play and he did not back and continued to show intent to play but he did

hesitate from coming forward and "danced" around her, he was reacting to her snarls but was not acting in subordination.

ii. If the receiver flinched or hesitated mid-stride only, then that was a reaction and was a draw. Prolonged hesitation (full cessation of motion) was a loss.

e. A snort met by no reaction was not a win, loss, or draw. If the receiver continued to behave as it was before the snort occurred and neither indicated signal reception nor retaliated, then there was no response.

f. If an exchange of snorts occurred in which the rhinos stopped what they were doing and stood horn-to-horn with neither yielding, or in which 1 or both continued their activity, then that was a draw.

7. If an aggressive interaction was initiated (no question that it could have been play) and the receiver tried to wrestle (no question that it was not a defensive clash), then it counted as an aggressive interaction for the first but did not count as a play initiation for the second because it was done as a response, and the result was a draw.
    a. If the actions of the first could have been play, then it was counted as a play initiation.
    b. If an interaction was play but 1 rhino clearly tired of the play and made an aggressive action, then this was counted as aggression, and the response of the playful rhino was considered in that context.

Play

1. Play interactions were counted only when they were with another rhino, which is the only context in which sexual play behaviors were observed.
    a. Non-sexual play interactions with rhinos were recorded and counted separately.
    b. Play interactions with non-rhinos were noted on field data sheets.
2. If a female chin-rested or mounted, it was scored as play only for that female (not the receiving female).
    a. If a mounting attempt followed a chin-rest, it was scored separately as individual play actions.
    b. If a female initiated a wrestle and the second female initiated a chin-rest during the interaction, each received a point for initiating play because it was a different type of play. If the first continued to wrestle, then it was still part of the same initiation for the first.

    c. If a female initiated a wrestle, then chin-rested, then wrestled again, even though it was the same female, each was counted because a different type of play was initiated.
3. Trying to initiate play without succeeding still counted as initiation of a play interaction.
4. Frolicking did not count as a play interaction unless it was accompanied by body-bouncing.
5. A new play initiation was scored after every full stop in movement with each rhino standing apart.
    a. A horn-wrestle followed by sustained pressure or horn-horn lock followed by more wrestling movement was part of the same interaction.
    b. If 1 rhino in a play interaction was still in pursuit (following closely or still with "intent to engage," meaning forward leaning, "dancing" in place, head wagging or tossing) of the other after breaking actual physical contact, then it was still the same interaction.
6. Backing or turning away from the attempted initiator in a play interaction was not an act of subordination. The rhino simply declined to play.
    a. Backing during a wrestle was not an act of subordination. It could have been a tactic to gain better footing, or 1 rhino was stronger than the other.
    b. If a rhino tried to initiate play, was snorted at, and backed before play was successfully initiated, then that was counted as a loss.
7. If a male initiated a wrestle and then chin-rested as the female moved, then that was 1 play initiation and 1 sexual advance.
8. Chin-resting by calves on their mothers before nursing was observed for the first time (no previous documentation), thus the purpose of chin-resting should be broadened to "a behavior that prepares recipients for physical manipulation"

Sexual Advances/Mounting

1. The number of sexual advances by the male included approaches with hiccing, chin-resting, and mounting, each counted separately.
    a. These were still scored as advances when performed by a male adolescent.
    b. Hiccing while mounted did not count as a subsequent sexual advance.
    c. Mounting attempts were scored even if he was unsuccessful.
2. Hiccing at the group of females counted for all of them, except nursing calves, unless it was clear which member of the group the male intended.

Appendix D: Details and Contingencies for Recording and Analyzing Behavioral Data, Assessing the Historical Record, and Sample Collection and Hormone Analysis

Details and Contingencies for Recording and Analyzing Behavioral Data

Visibility was a challenge at Busch Gardens, Lion Country Safari, San Diego Wild Animal Park, and the Wilds. In order to keep the number of observation hours for each rhino within an institution as consistent as possible (and approximately equivalent between morning and afternoon hours), decisions were made as necessary to move to a vantage point that allowed observation of some rhinos and not others. The decision was based exclusively on which rhinos were lacking in the number of hours of visibility each day and was not based on behavior. The only time that behavior determined which rhinos were observed was if there was estrous consort between a female and male. If a rhino was not visible for more than 15 minutes during a 30-minute increment, "No Data" was recorded, and that half-hour was not counted in the final analysis. If the rhino was visible for at least 15 minutes, the 30-minute increment was counted, but a * was used in the spreadsheet to indicate that a full half-hour was not observed.

Later in analysis, if 2 or more partial half-hours could be combined to make a full half-hour, this was done if the activity (grazing, resting, etc.) of the rhinos was similar. If the activity was not similar but "0" counts were being added, data from partial half-hours

was still combined because, even without moving "0" counts, the remaining time in the half-hour would have been assumed to include "0" instances of the behavior. Thus, combining the times to create full half-hours allowed for the most robust estimates of behavior in 30-minute increments. If time was combined and there was an interaction with another rhino during those times, it was necessary that the counts for that time be combined for both rhinos. Often, counts during those times already needed to be combined for both rhinos because they were in the same companion subgroup and had similar periods of non-visibility.

Due to the unbalanced, incomplete experimental design, it was not always possible to estimate the interaction term using the mixed-model (both random and fixed effects), 2-way ANOVA. In such cases, all variables, including the interaction term, were treated as fixed effects. After confirming that the interaction was not significant ($p > 0.05$), the interaction term was dropped, and the mixed-model was applied as described in Chapter 2. In the comparison of average daily sexual play behavior between dominant and subordinate females within subgroups when adolescents were excluded, the interaction term was significant ($p = 0.03$) in the completely fixed model, and thus, interpretation of this effect is limited to the institutions and animals in this study.

Details and Contingencies for Assessing the Historical Record

For the assessment of the housing environment relative to parity, as Laptop and Macite reproduced at previous institutions (Fossil Rim, and San Diego Wild Animal Park and Fossil Rim, respectively) but not yet at their current locations (Birmingham and Audubon, respectively), the analysis considered the captive environment at the former

institutions. Jao had recently moved to Disney's Animal Kingdom at the time of analysis in early 2009, so the captive environment evaluated for her was that at Indianapolis.

Details and Contingencies of Sample Collection and Hormone Analysis

Two females were pregnant during the entire sample collection period (Eliza and Taraja) and gave birth just before assays started, thus it was not necessary to run their samples in the progesterone assay. Two females were pregnant during the original collection period (Julie and Maggie—Wilds), so additional samples were collected 6 months following parturition. Two other females were pregnant during part of the sample collection period (Zenzele, Maggie—White Oak), and all the samples collected while they were not pregnant were assayed as well as most of the samples collected during pregnancy. The collection period for 1 female (Bloom) began 1 month after delivering a stillborn calf, so she was not lactating.

Samples collected during the entire collection period were used to calculate baseline progesterone and progestagens for the 3 adolescents included in the cycling group for the baseline comparison between cycling, acyclic, and adolescent females. However, for the comparison of adolescents' baselines with those of nulliparous and parous females, only progesterone and progestagen values determined from samples collected before the start of estrous cycles were used. For comparisons involving baseline and luteal serum progesterone concentrations, and of fecal corticosterone concentrations between wild-caught and captive-born females, the interaction term was dropped from the mixed-model, 2-way ANOVA when only 1 institution had females in each level of either parity, cyclicity (acyclic, cyclic, and adolescent), or place of origin.

Appendix E: Table 18. Average Daily Behavioral Frequencies for Female White Rhinos Observed Between September 2007 and December 2008

Rhino	Aggressive Behavior	Sexual Advances (by mature males)	Sexual Play Behavior
Captive-born, parous			
Gabby	4.083	(adolescent male)	0.333
Maggie (WOCC[1])	16.24	4.80	0.44
Julie	6.207	0.448	0.655
Maggie (Wilds)	9.448	0.690	0.207
Zenzele	2.483	0.759	1.966
Taraja	3.133	1.033	0.367
Lissa	2.167	1.533	0.567
Bloom	3.033	0.80	0.80
Eliza	5.467	1.367	0.40
Holly	1.552	0.379	0.897
Yvonne	9.567	0.50	0.167
Laptop	9.0	(no male)	0.083
Captive-born, nulliparous			
Lucy (WOCC[1])	18.96	1.12	0.04
Bonnie	5.067	1.60	0.733
Kiangazi	2.033	0.933	0.533
Paddy	1.50	2.567	0.133
Yebonga	0.28	0.72	0.36
Dumisha	3.966	0.414	0.207
Utamu	3.241	0.828	0.345
Kiazi	1.241	2.276	0.207
Taryn	4.50	5.792	0.0
Jeannie	1.923	0.423	0.0
Ajabu	5.25	(no male)	0.50
Sindi	6.9	(no male)	0.0
Lulu	14.4	(no male)	0.90
Wild-caught, parous			
Kathy	27.32	2.72	0.12
Alice	2.033	0.767	0.167
Mlaleni	8.692	0.538	0.308
Kisiri	2.154	0.154	0.923
Nthombi	1.517	0.379	0.103
Macite	2.70	0.633	0.10
Adolescent			
Kelly	5.60	(nursing)	0.16
Sally	6.310	(nursing)	2.517
Evey	2.207	(nursing)	2.966
Lucy (Busch)	1.154	0.077	1.077
Dakari	1.308	0.154	0.846

[1]White Oak Conservation Center

Appendix F: Dominance Matrices for Housing Groups (Fig. 8) and Companion Subgroups (Fig. 9) of Observed Female White Rhinoceros

A.	Kathy	Lucy	Kelly	Maggie
Kathy	0	94	NA	100
Lucy	0	0	47	46
Kelly	NA	24	0	54
Maggie	0	14	26	0

B.	Mlaleni	Dakari	Kisiri	Lucy
Mlaleni	0	98	100	100
Dakari	0	0	86	86
Kisiri	0	14	0	76
Lucy	0	14	21	0

C.	Julie	Maggie	Sally	Evey	Zenzele
Julie	0	52	NA	NA	94
Maggie	22	0	NA	NA	88
Sally	NA	NA	0	89	69
Evey	NA	NA	0	0	75
Zenzele	0	5	14	13	0

D.	Dumisha	Nthombi	Utamu	Holly	Kiazi
Dumisha	0	38	90	85	85
Nthombi	31	0	60	67	95
Utamu	2	20	0	58	44
Holly	12	22	25	0	75
Kiazi	15	0	22	17	0

E.	Taraja	Alice	Bloom	Paddy	Eliza	Bonnie	Lissa	Kiangazi
Taraja	0	67	44	67	63	74	79	69
Alice	33	0	63	44	50	73	60	95
Bloom	50	25	0	47	50	66	50	77
Paddy	33	56	40	0	48	42	60	50
Eliza	40	34	27	38	0	71	54	78
Bonnie	18	24	24	47	23	0	22	70
Lissa	14	27	28	27	46	75	0	46
Kiangazi	31	5	16	50	12	16	46	0

F.	Macite	Yvonne
Macite	0	58
Yvonne	18	0
G.	Laptop	Ajabu
Laptop	0	50
Ajabu	26	0
H.	Lulu	Sindi
Lulu	0	60
Sindi	29	0

Fig. 8. Dominance matrices for full housing groups of female white rhinos at White Oak Conservation Center (A), Busch Gardens (B), the Wilds (C), San Diego Wild Animal Park (D), Lion Country Safari (E), Audubon (F), Birmingham (G), and Louisville (H). Interactions between nursing calves and adults were not included since either the adults always won or the mother of the calf interfered with the interaction (or was the opponent). Values indicate the percentage of times that the rhino listed on the left of the row won an interaction with the rhino listed above the column.

A.	Maggie	Sally	Zenzele
Maggie	0	NA	88
Sally	NA	0	69
Zenzele	5	14	0

B.	Dakari	Kisiri	Lucy
Dakari	0	86	86
Kisiri	14	0	76
Lucy	14	21	0

C1.	Dumisha	Utamu	Holly
Dumisha	0	90	85
Utamu	2	0	58
Holly	12	25	0

C2.	Nthombi	Kiazi
Nthombi	0	95
Kiazi	0	0

D1.	Lissa	Eliza	Bonnie
Lissa	0	46	75
Eliza	54	0	71
Bonnie	22	23	0

D2.	Alice	Bloom	Kiangazi
Alice	0	63	95
Bloom	25	0	77
Kiangazi	5	16	0

D3.	Taraja	Paddy
Taraja	0	67
Paddy	33	0

Fig. 9. Dominance matrices for companion subgroups of female white rhinos at White Oak Conservation Center (A), Busch Gardens (B), San Diego Wild Animal Park (C1, C2), and Lion Country Safari (D1, D2, D3). Interactions between a nursing calf and her mother were not included. Values indicate the percentage of times that the rhino listed on the left of the row won an interaction with the rhino listed above the column. While group D1 can be correctly ordered numerically with Eliza dominant over Lissa, the latter was nonetheless believed to be dominant based on following behaviors of Eliza and Bonnie in response to movements by Lissa.

Appendix G: Statistical Analysis of Sexual Advances Including those Made by Adolescent Males

- Average daily frequency of sexual advances by mature and adolescent males toward all non-nursing females was not different ($p = 0.52$) between captive-born, nulliparous females; captive-born, parous females; wild-caught, parous females; and adolescents.
- Average daily frequencies of sexual advances by mature and adolescent males were not correlated with all non-nursing females' daily frequencies of sexual play behaviors ($p = 0.23$, $r = -0.23$).
- Parity (nulliparous vs. parous) was not affected by average daily frequencies of sexual advances made by mature and adolescent males ($p = 0.43$).
- Average daily frequency of sexual advances by mature and adolescent males was different ($p = 0.035$) between acyclic, cycling, pregnant/lost pregnancy, and non-nursing adolescent ($n = 2$) females, in which cycling females (2.52 ± 0.66) tended ($p = 0.057$) to be approached more than adolescents (0.12 ± 0.04). Sexual advances made by mature and adolescent males were directed toward cycling females more often ($p = 0.011$) than toward all non-cycling (acyclic, pregnant,

and adolescent) females (0.85 ± 0.14). Mature and adolescent males also made sexual advances toward cycling females more often ($p = 0.044$) than acyclic females (0.73 ± 0.12).

- Cyclicity had no relationship with average daily frequencies of sexual advances made by mature and adolescent males ($p = 0.095$). This insignificant finding might be due to the small number of females (4) that were observed in estrous consort relationships with a male, however, since even the use of a different calculation method within logistic regression analysis (a likelihood ratio test instead of the Wald test) suggests that the frequency of sexual advances by mature and adolescent males was associated with cyclicity ($p = 0.006$).
- There was no correlation between average fecal corticosterone concentrations and average daily frequencies of sexual advances made by mature and adolescent males to non-nursing females ($p = 0.82$, $r = 0.06$).

Appendix H: Table 19. EIA Reagent Recipes

Reagent	Cat#	Chemical	Amount	pH
Coating Buffer			For 500 ml	Bring to 9.6
	Sigma S-2127	Na_2CO_3	0.795 g	
	Sigma S-8875	$NaHCO_3$	1.465 g	
		Ultra-purified water	fill to 500 ml	
EIA Buffer			For 1 L	Bring to 7.0
		Stock A	195 ml	
		Stock B	305 ml	
	Fisher S671-3	NaCl	8.7 g	
	Sigma A-7906	BSA	1.0 g	
		Ultra-purified water	fill to 1000 ml	
Stock A (0.2 M NaH_2PO_4)			For 1 L	
	Sigma S-0751	NaH_2PO_4	27.8 g	
		Ultra-purified water	fill to 1000 ml	
Stock B (0.2 M Na_2HPO_4)			For 1 L	
	Sigma S-0876	Na_2HPO_4	28.4 g	
		Ultra-purified water	fill to 1000 ml	
Substrate Buffer			For 1 L	Bring to 4.0
	Sigma C-0759	Citric Acid (anhydrous)	9.61 g	
		Ultra-purified water	fill to 1000 ml	
Peroxide			For 8.5 ml	
	Sigma H-1009	H_2O_2 (30% solution)	500 µl	
		Ultra-purified water	8.0 ml	
ABTS			For 25 ml	Bring to 6.0
	Sigma A-1888	ABTS	0.55 g	
		Ultra-purified water	25 ml	
Wash Solution Concentrate			For 4 L	
	Sigma P-1379	Tween 20	0.4 ml	
		Ultra-purified water	4000 ml	

Appendix I: Table 20. Cross-Reactivity for Progesterone and Corticosterone Antibodies

Antibody	Steroid	% Cross-Reactivity
Progesterone (CL425)	4-pregnen-3,20-dione (progesterone)	100
	4-pregnen-3β-ol-20-one	172
	4-pregnen-3α-ol-20-one	188
	4-pregnen-11α-ol-3,20-dione	147
	4-pregnen-11β-ol-3,20-dione	2.7
	5α-pregnan-3α,20β-diol	<0.1
	5α-pregnan-3α-ol,20-one	64
	5α-pregnan-3β-ol-20-one	94
	5α-pregnan-3,20-dione	55
	5β-pregnan-3α,20α-diol (pregnandiol)	<0.1
	5β-pregnan-3,20-dione	8
	5β-pregnan-3α-ol-20-one	2.5
	5β-pregnan-3β-ol-20-one	12.5
	5β-pregnan-3,17-dione (androstendione)	<0.1
	5β-pregnan-11β,21-diol-3,20-dione (corticosterone)	<0.1
Corticosterone (CJM006)	Corticosterone	100.0
	Desoxycorticosterone	14.25
	Tetrahydrocorticosterone	0.90
	11-deoxycortisol	0.03
	Prednisone	<0.01
	Prednisolone	0.07
	Cortisol	0.23
	Cortisone	<0.01
	Progesterone	2.65
	Testosterone	0.64
	Estradiol 17β	<0.01

Appendix J: Progesterone and Progestagen Profiles for Captive Female White Rhinos

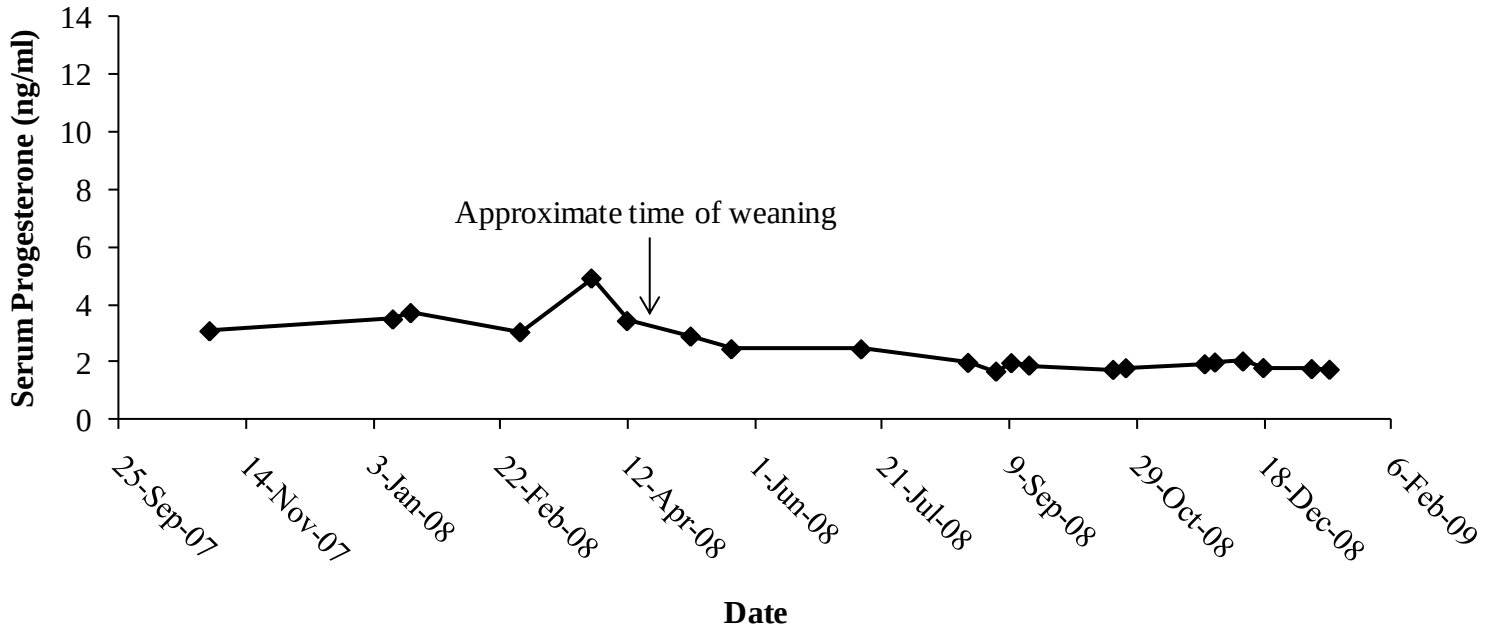

Fig. 10. Serum progesterone profile for adolescent Dakari who was weaned just before the birth of her mother's next calf. She did not commence cycling during the sample collection period.

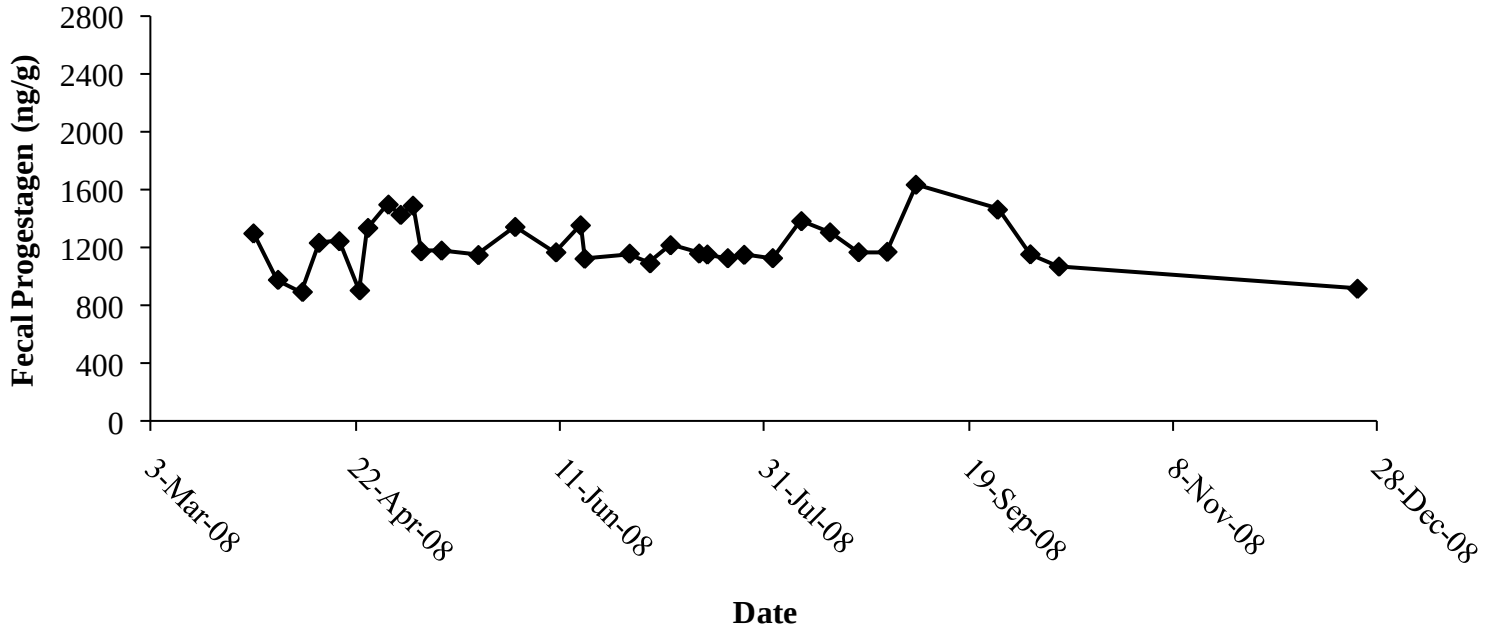

Fig. 11. Fecal progestagen profile for adolescent Evey during 2008. No luteal cycles were observed.

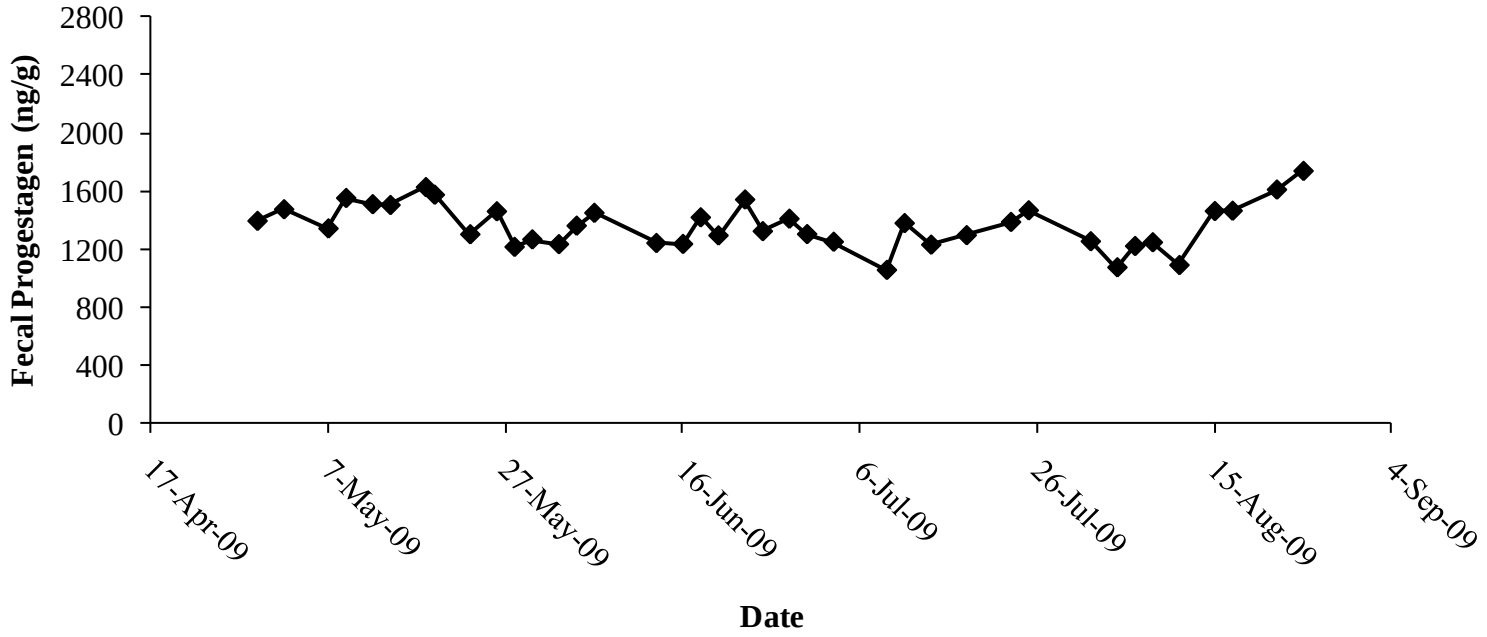

Fig. 12. Fecal progestagen profile for adolescent Evey during 2009. She might have started a luteal phase at the end of her profile, but this was not accompanied by any behavioral observations of estrus.

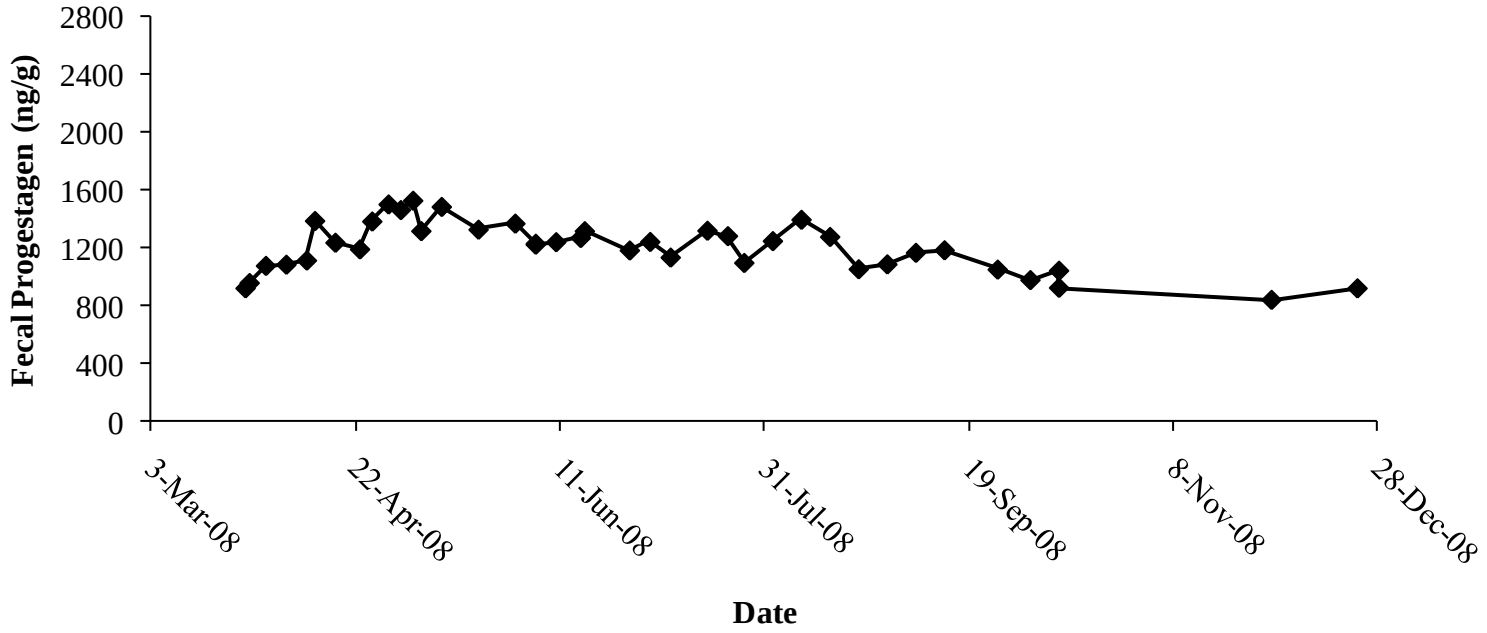

Fig. 13. Fecal progestagen profile for adolescent Sally during 2008. No luteal cycles were observed.

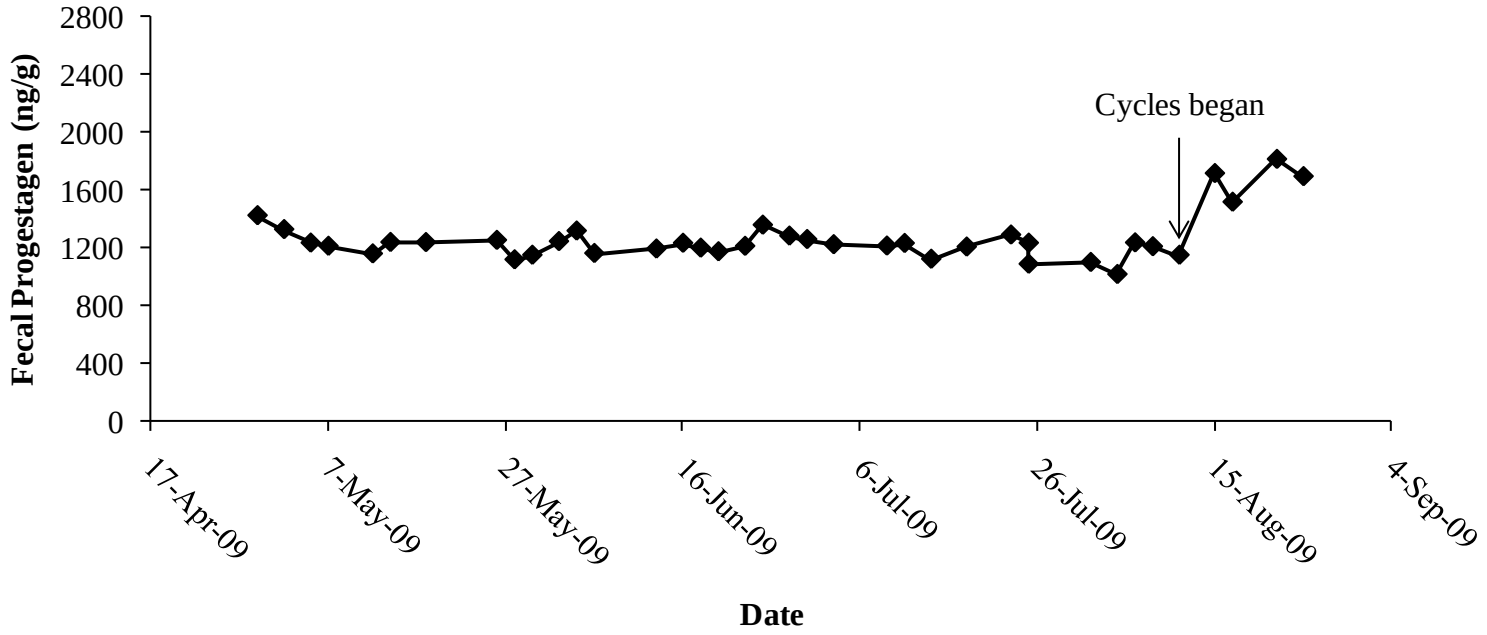

Fig. 14. Fecal progestagen profile for adolescent Sally during 2009. The beginning of her first luteal phase occurs after observations of excessive urine squirting and vaginal discharge on 11 August.

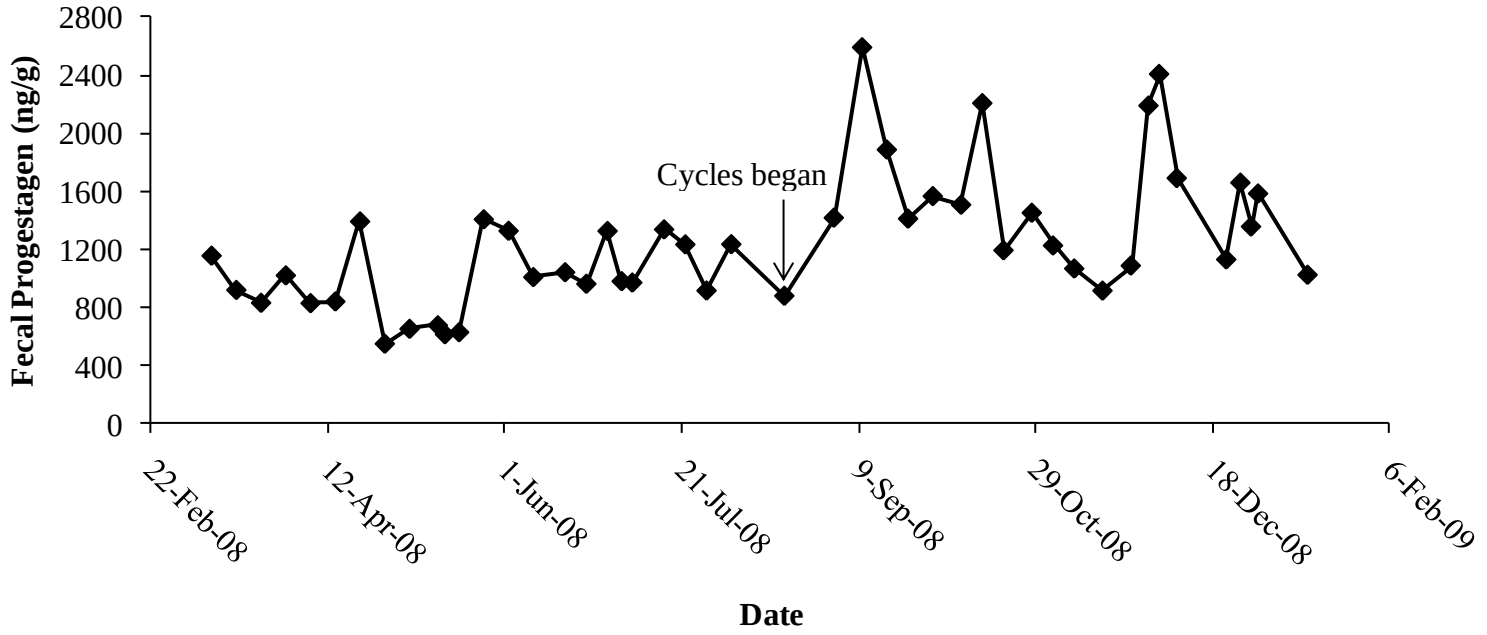

Fig. 15. Fecal progestagen profile for adolescent Kayla. She began cycling in 2008, the first cycle from 19 August to 17 November, and the second cycle from 17 November 2008 to 14 January 2009.

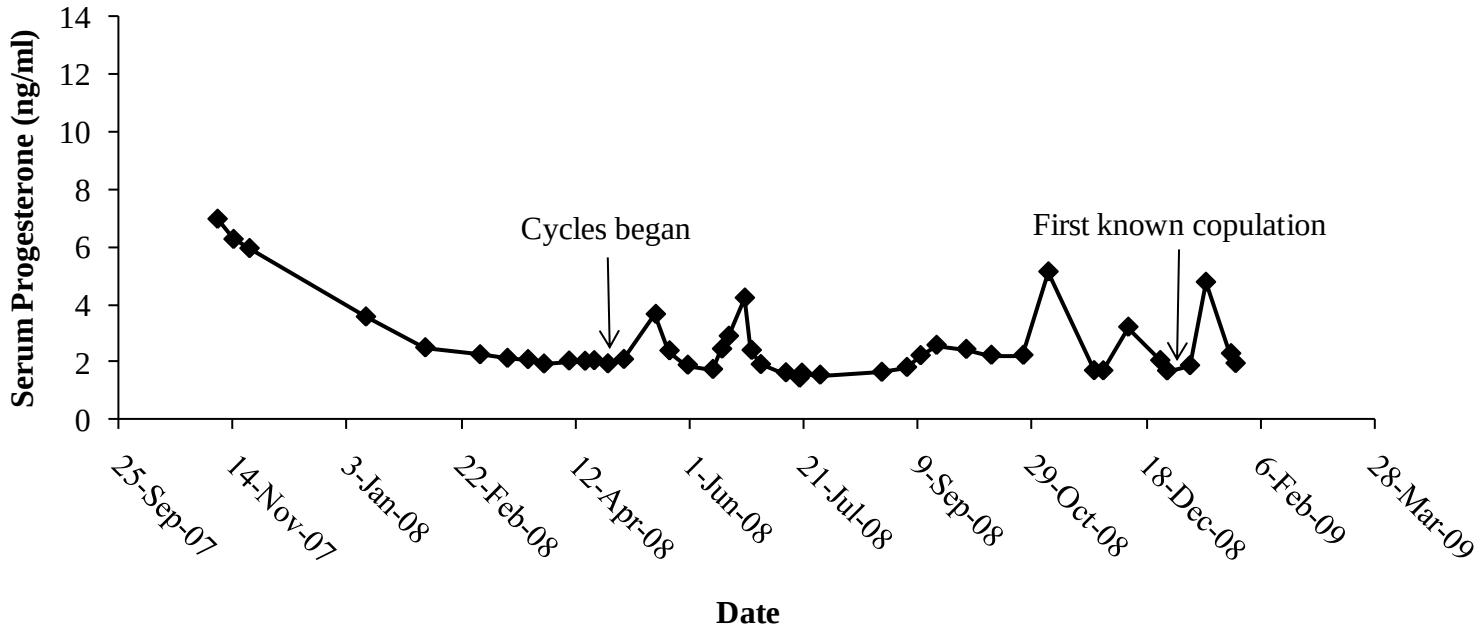

Fig. 16. Serum progesterone profile for adolescent Lucy at Busch Gardens. She had her first luteal cycles from 26 April to 11 June 2008, from 11 June to 13 July 2008, and 29 November 2008 to 6 January 2009. Keepers observed copulation on 3 January 2009.

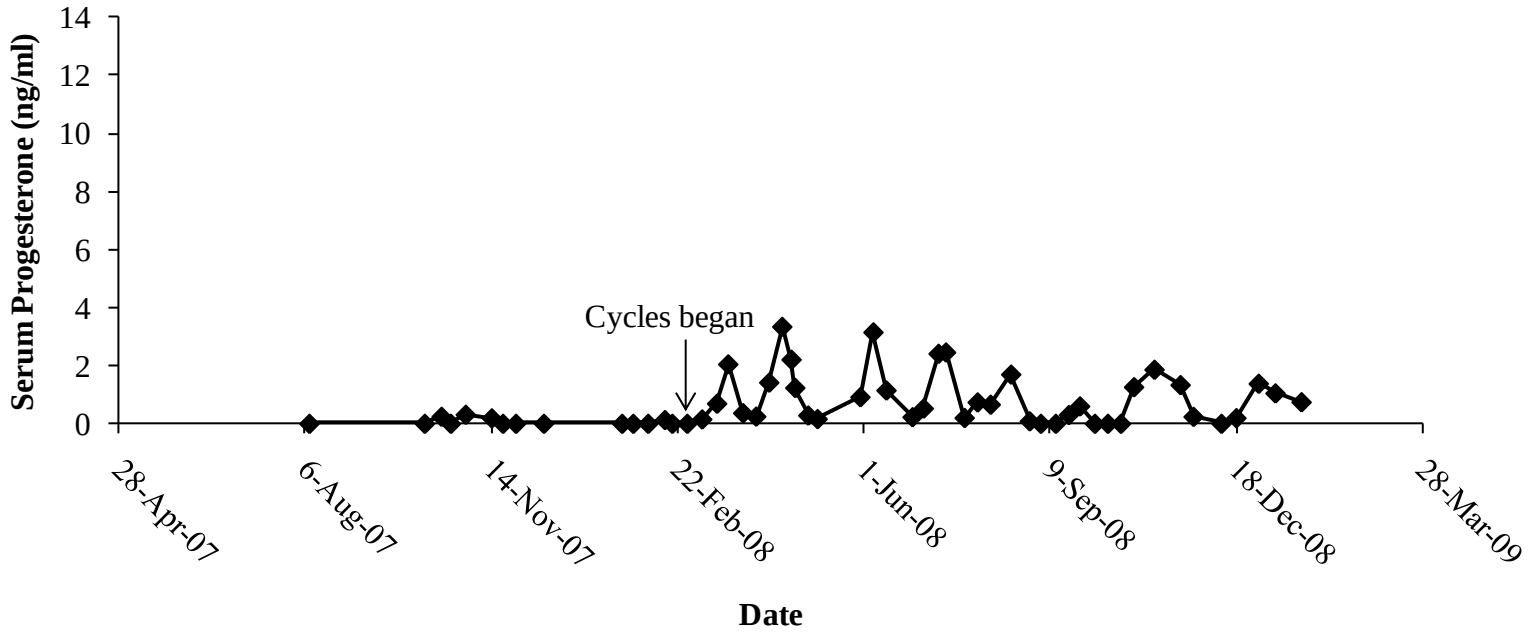

Fig. 17. Serum progesterone profile for adolescent Kelly. She began cycling in 2008, from 27 February to 4 April, from 4 April to 7 May, from 7 May to 27 June, from 27 June to 25 July, from 25 July to 12 September, and from 17 October to 10 December. Keepers observed mounting on 12 September and 13 December. Kelly became pregnant with her first calf early in 2009.

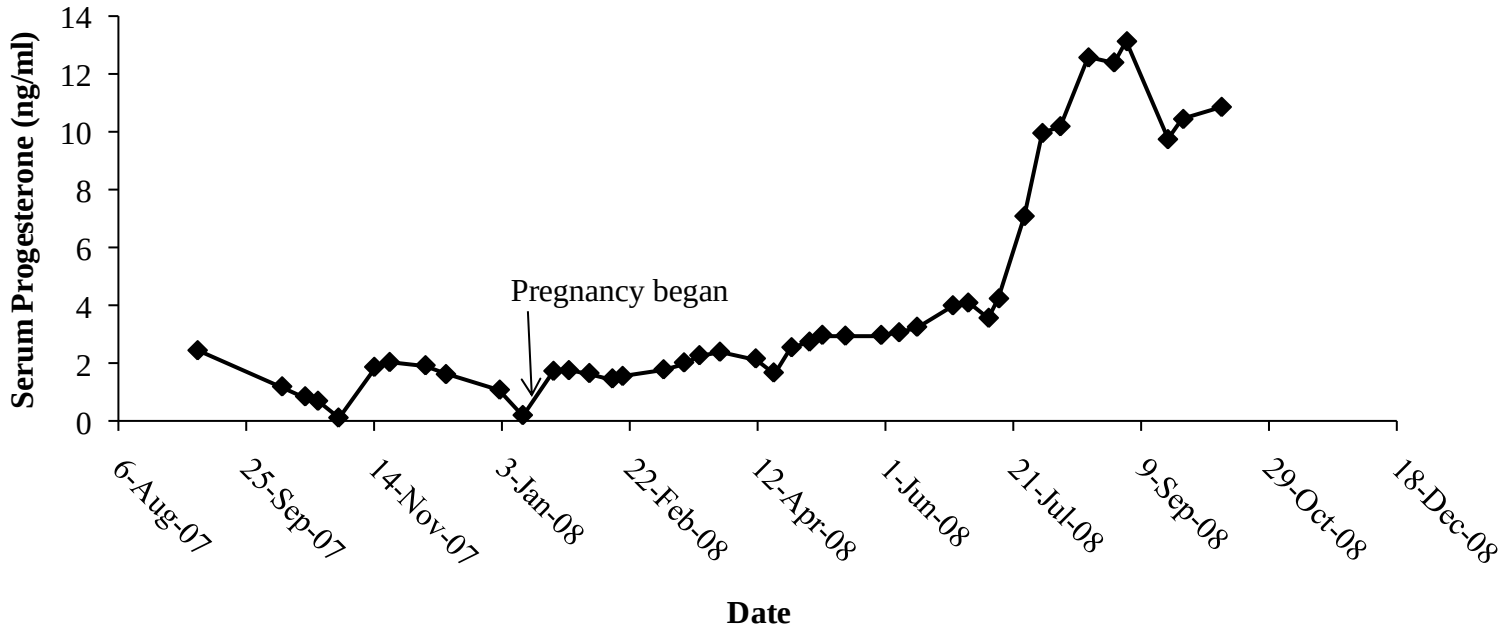

Fig. 18. Serum progesterone profile for captive-born, parous female Maggie at White Oak. She had a luteal cycle from 31 October 2007 to 11 January 2008. Some estrous behavior was observed on 13 and 15 January 2008, after which she became pregnant with her first calf.

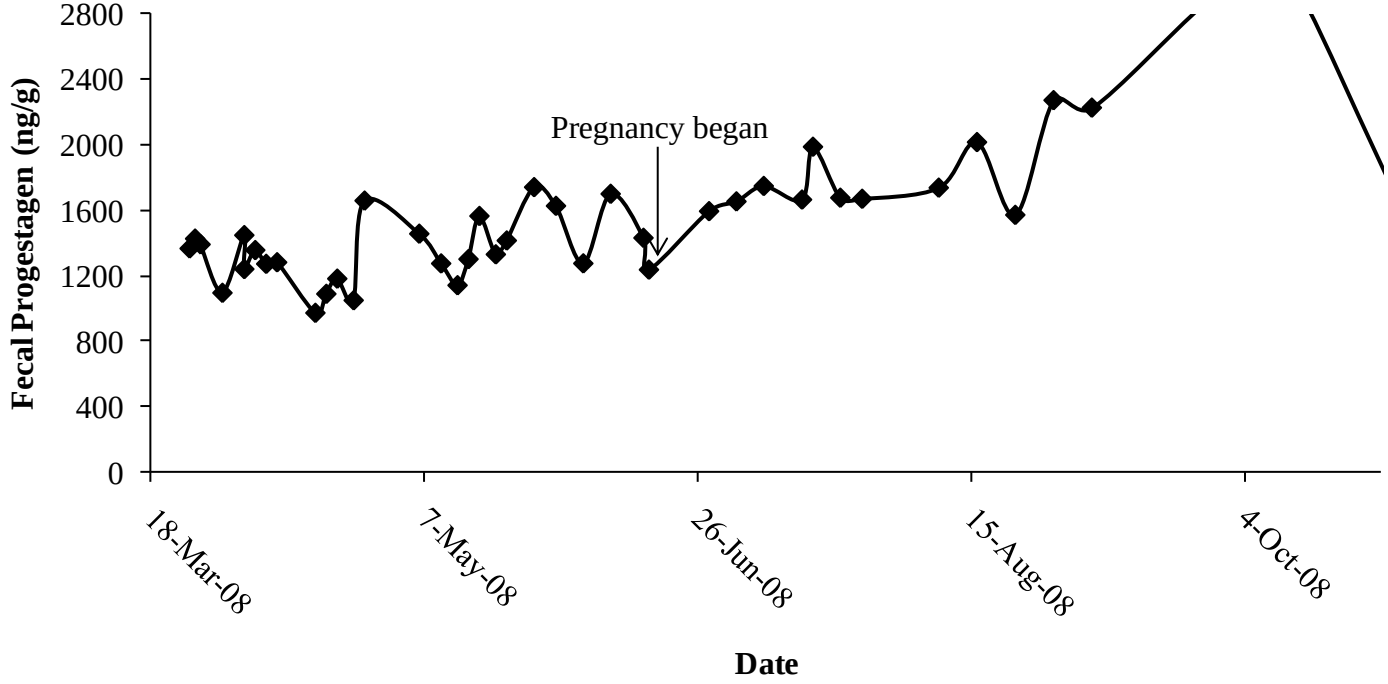

Fig. 19. Fecal progestagen profile for captive-born, parous female Zenzele. She had a luteal cycle from 13 May to 17 June 2008, which was preceded by mounting on 10 May and followed by this female's first pregnancy.

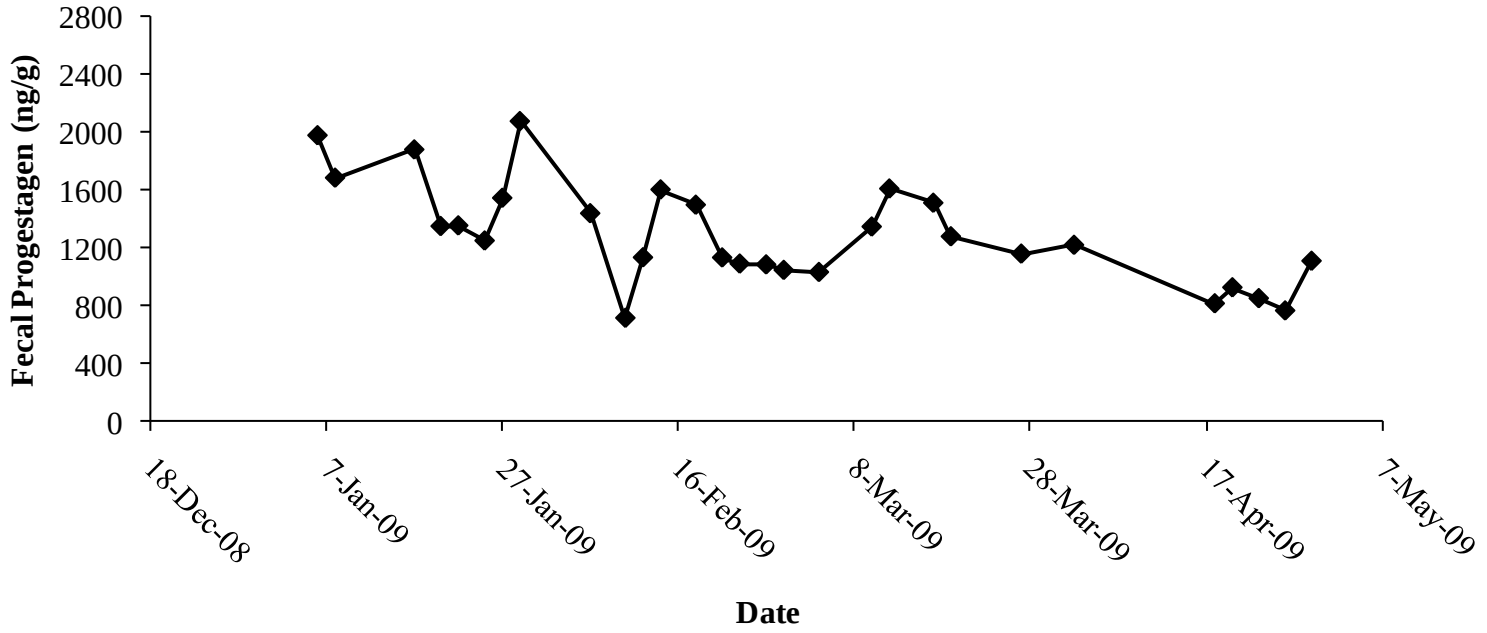

Fig. 20. Fecal progestagen profile for captive-born, parous female Bloom. Keepers observed copulation on 31 December 2008 before sample collection began, thus the first portion of the profile could be the luteal phase from a previous ovulation. A luteal cycle occurs here from 4 March to 20 April 2009.

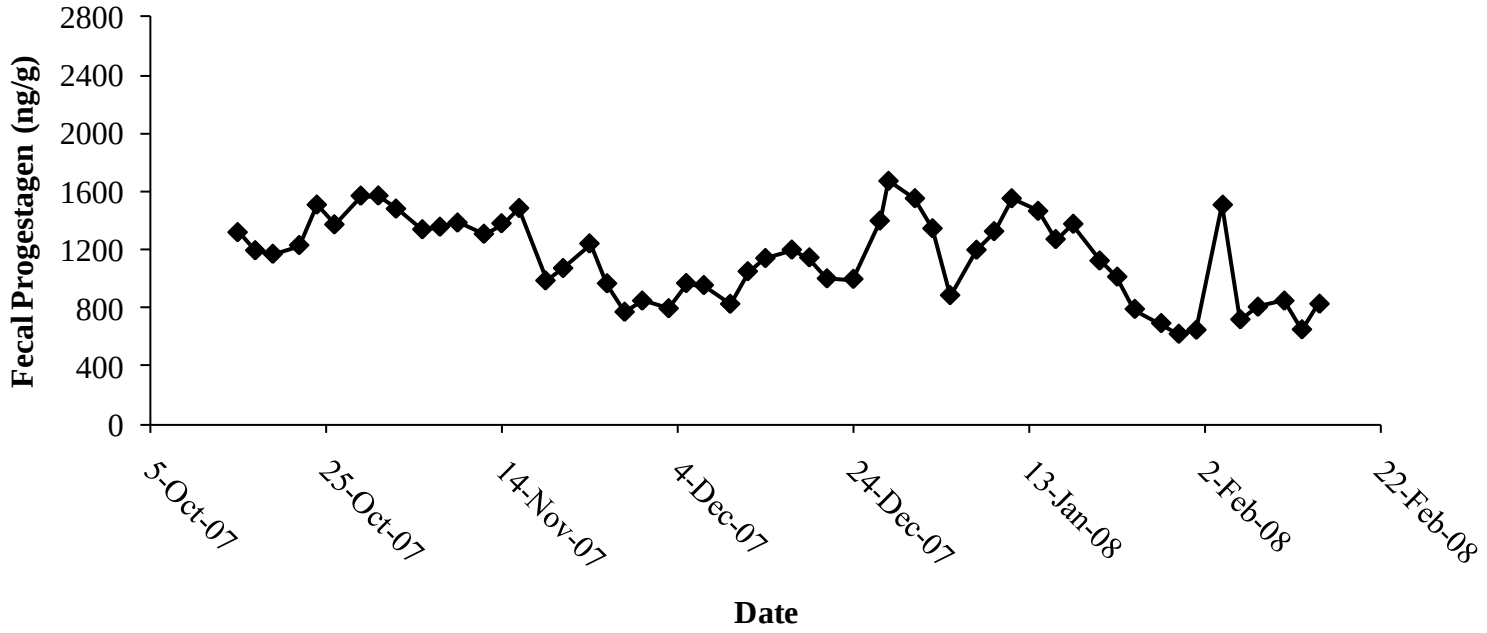

Fig. 21. Fecal progestagen profile for captive-born, parous female Gabby. She had a luteal cycle from 3 December 2007 to 28 January 2008.

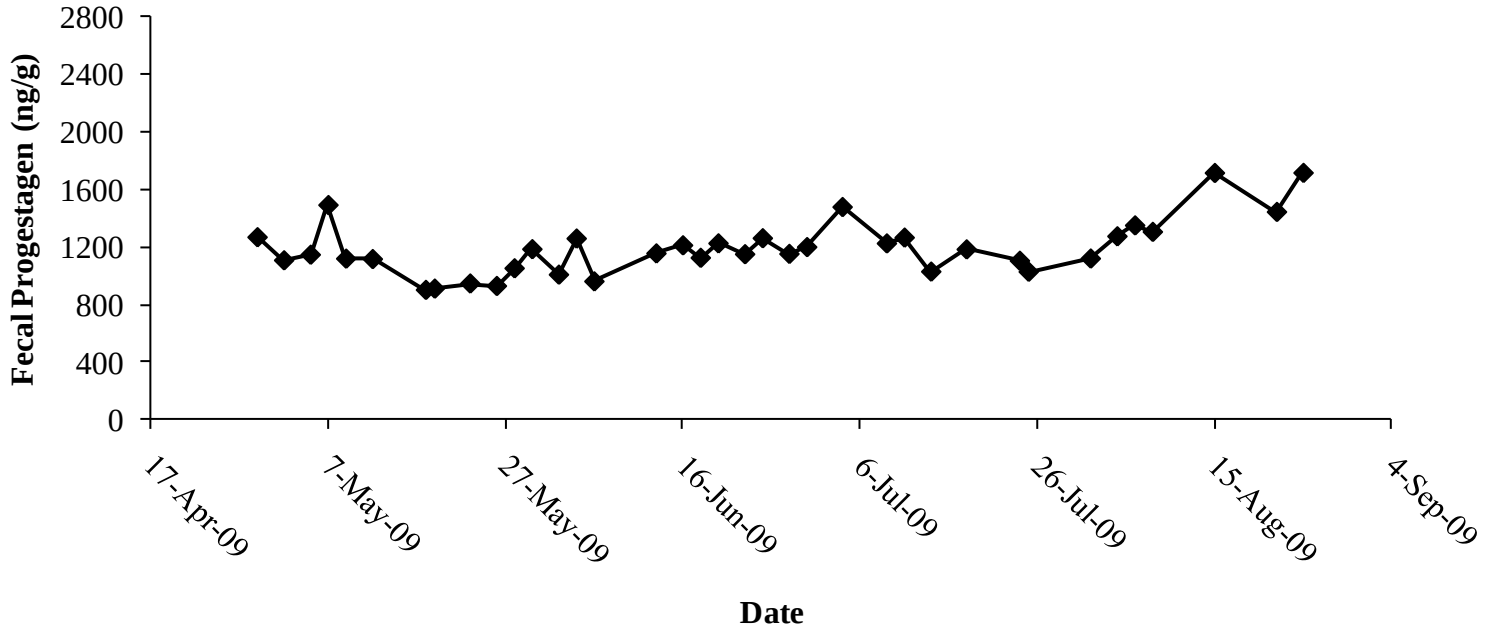

Fig. 22. Fecal progestagen profile for captive-born, parous female Julie. Keepers observed mounting on 21 June, after which a cycle was observed from 23 June to 25 July 2009. Keepers observed mounting again on 23 July. Rising luteal values following mounting suggest another ovulatory cycle.

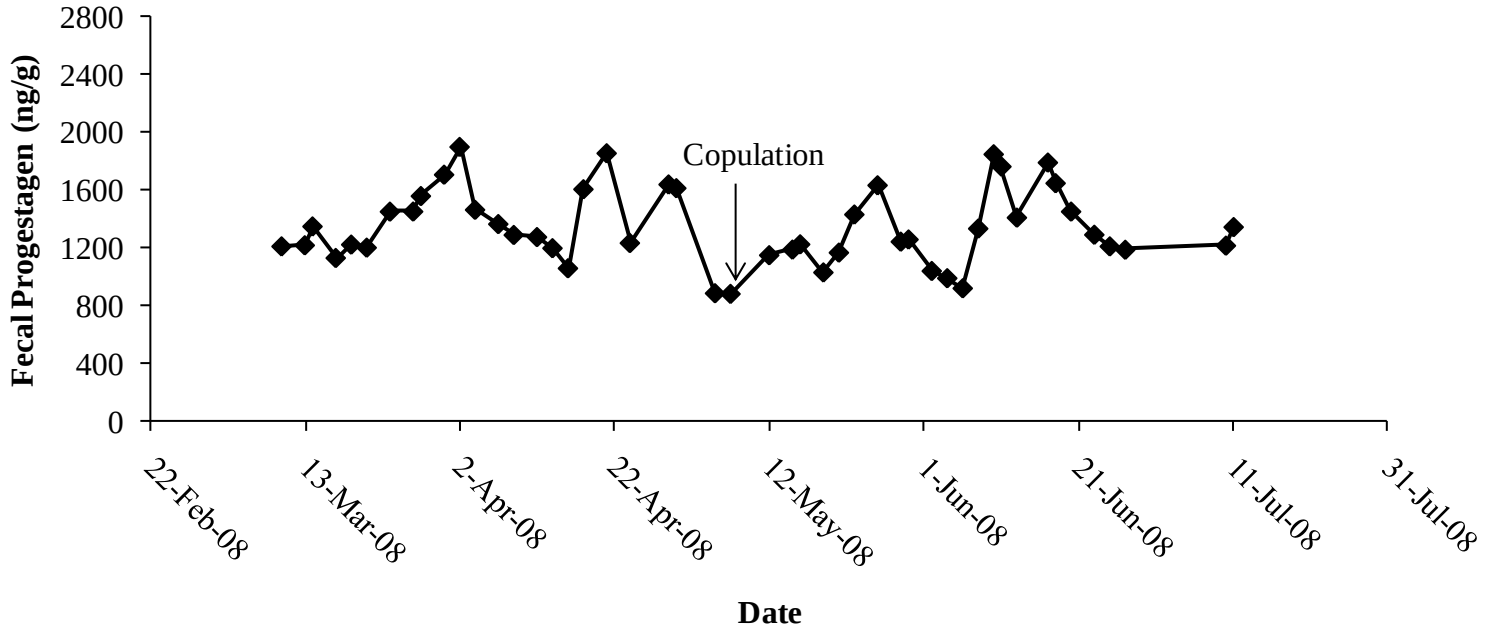

Fig. 23. Fecal progestagen profile for captive-born, parous female Kendi. Luteal cycles occur in 2008 from 21 March to 16 April, from 16 April to 7 May, and from 6 June to 25 June. Though keepers observed copulation on 8 May, luteal concentrations statistically were not sufficiently sustained to be confident that an ovulation occurred.

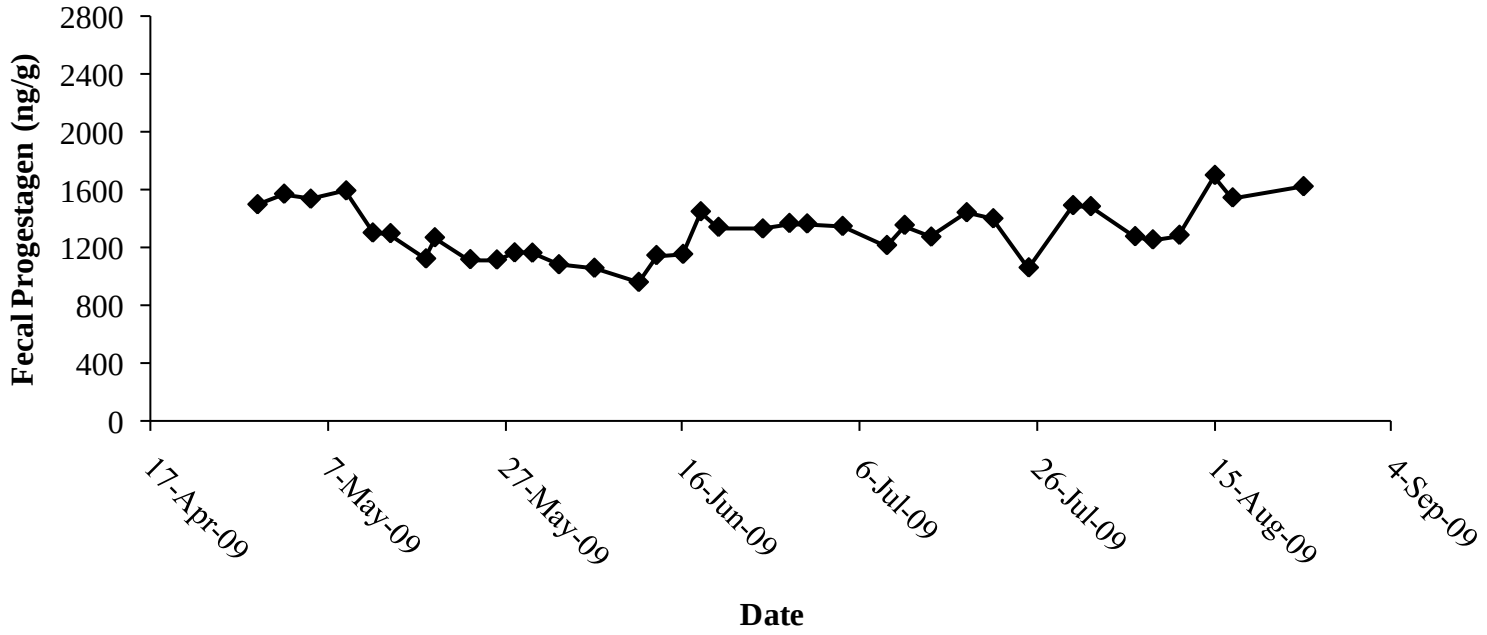

Fig. 24. Fecal progestagen profile for captive-born, parous female Maggie at the Wilds. She had a luteal cycle from 16 June to 8 August 2009.

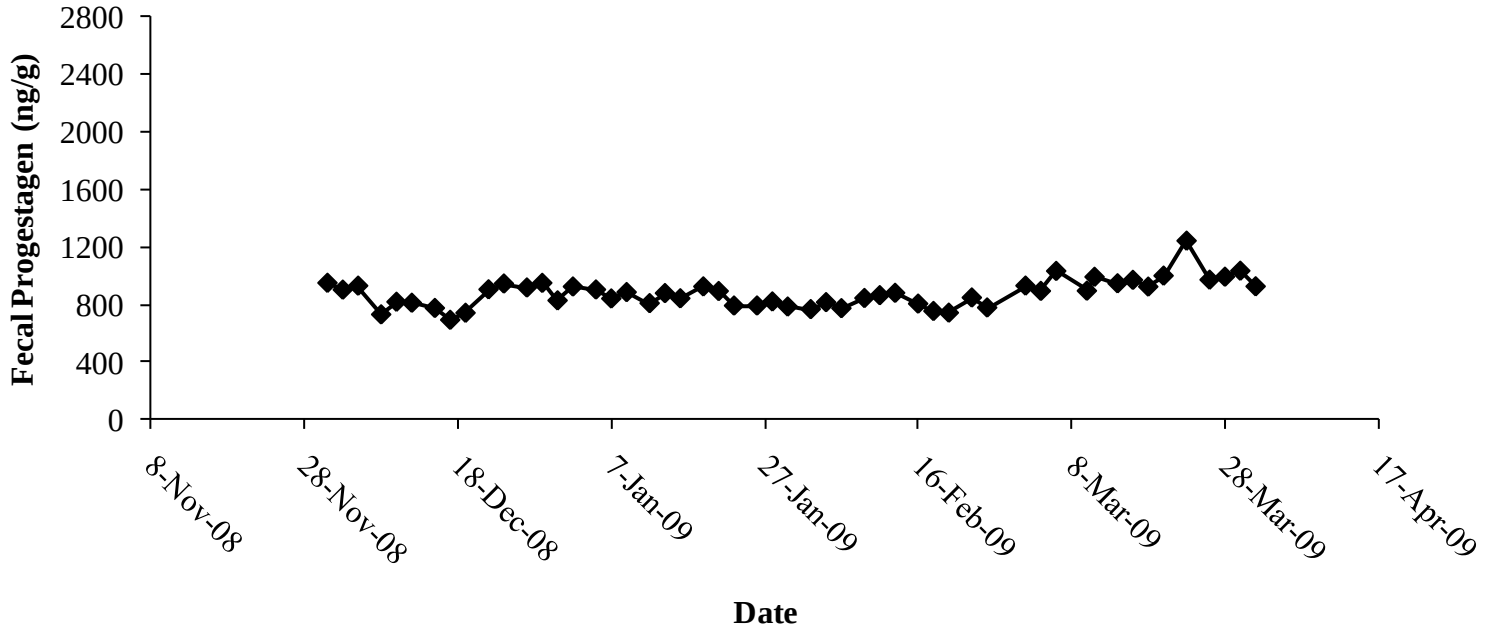

Fig. 25. Fecal progestagen profile for captive-born, nulliparous female Ajabu. She had a luteal cycle from 19 December 2008 to 26 January 2009. A second luteal cycle might have started at the end of the profile, which would be consistent with observations by keepers of possible estrous behaviors on 21 April 2009.

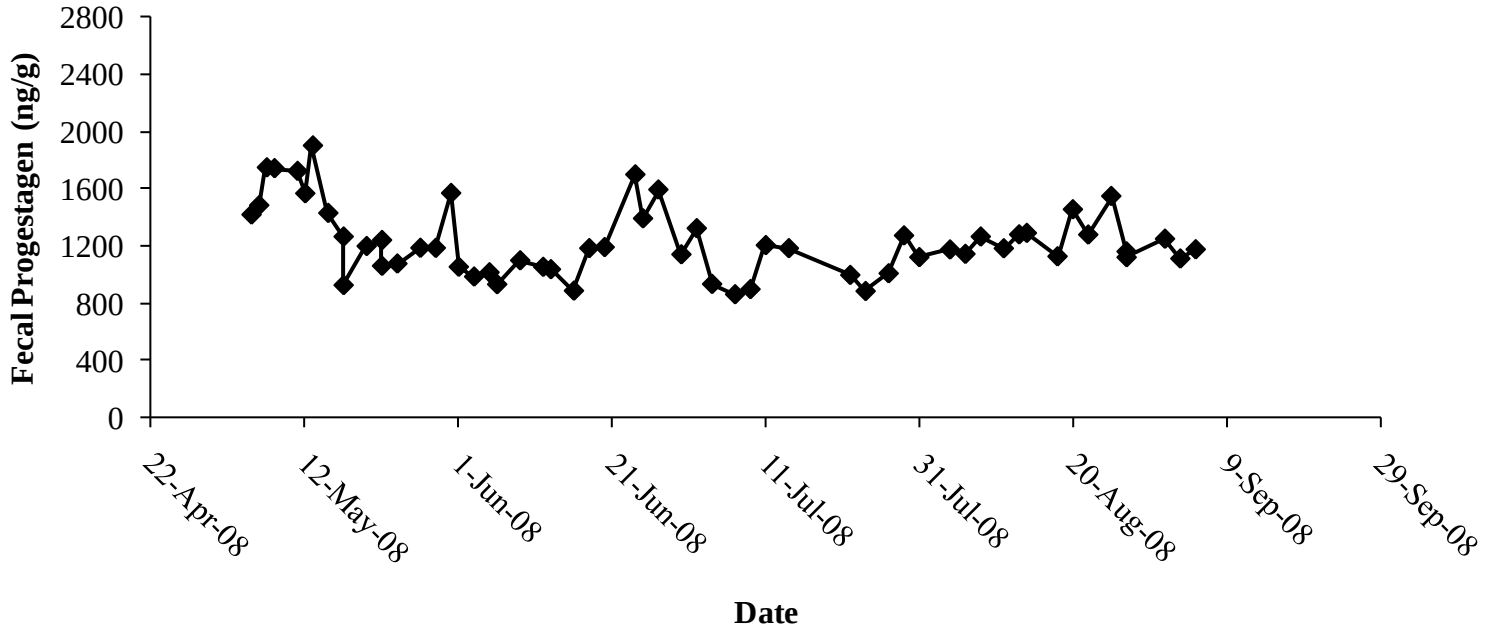

Fig. 26. Fecal progestagen profile for captive-born, nulliparous female Bonnie. Keepers observed copulation on 19 April 2008 before sample collection began, thus the first portion of the profile could be the luteal phase from a previous ovulation. A luteal cycle occurs from 6 August to 5 September 2008. Other values in June and July did not remain at luteal concentrations long enough to be considered part of an ovulatory cycle.

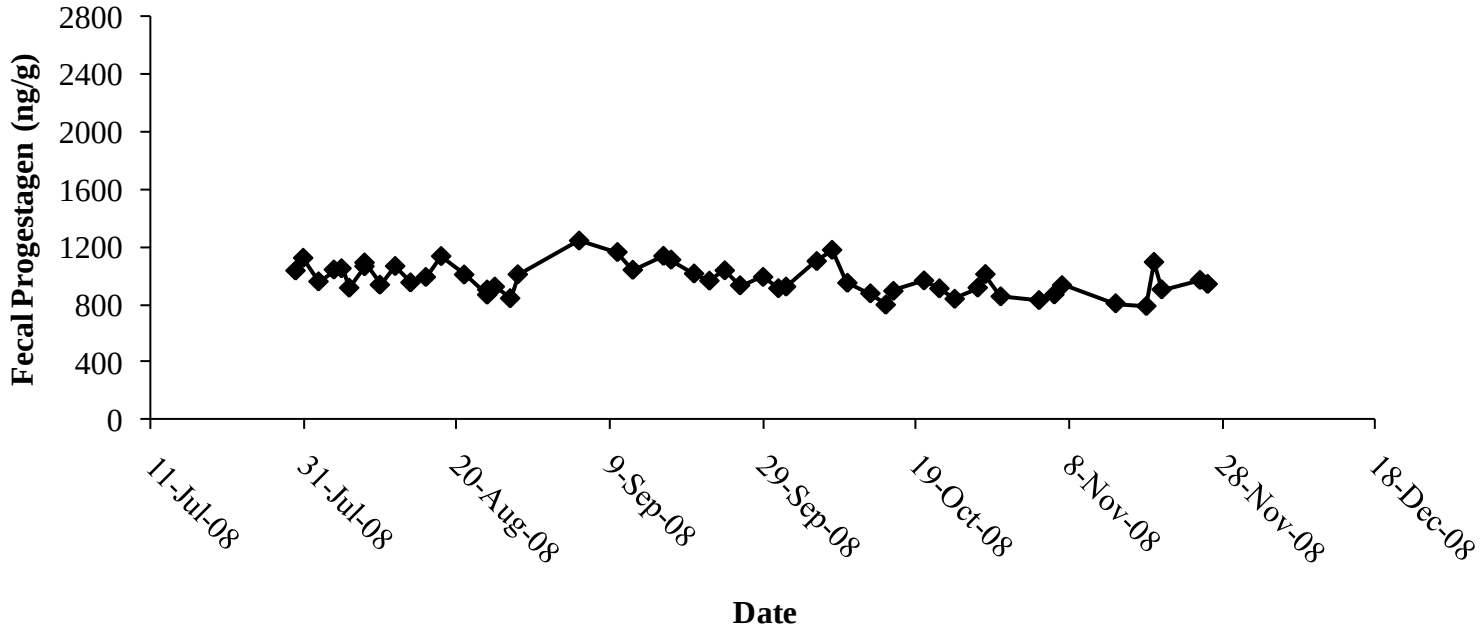

Fig. 27. Fecal progestagen profile for captive-born, nulliparous female Dumisha. She had a luteal cycle from 27 August to 2 October 2008.

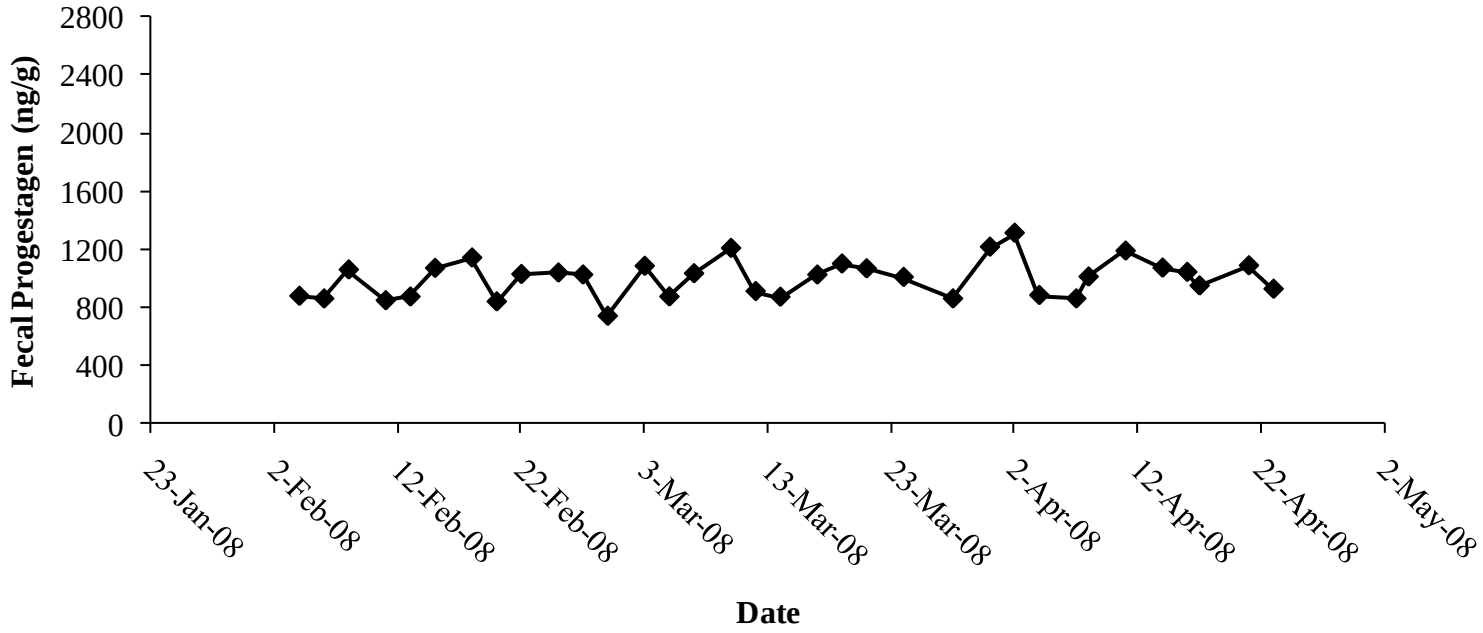

Fig. 28. Fecal progestagen profile for wild-caught, nulliparous female Emalah. She had a luteal cycle from 14 March to 7 April 2008.

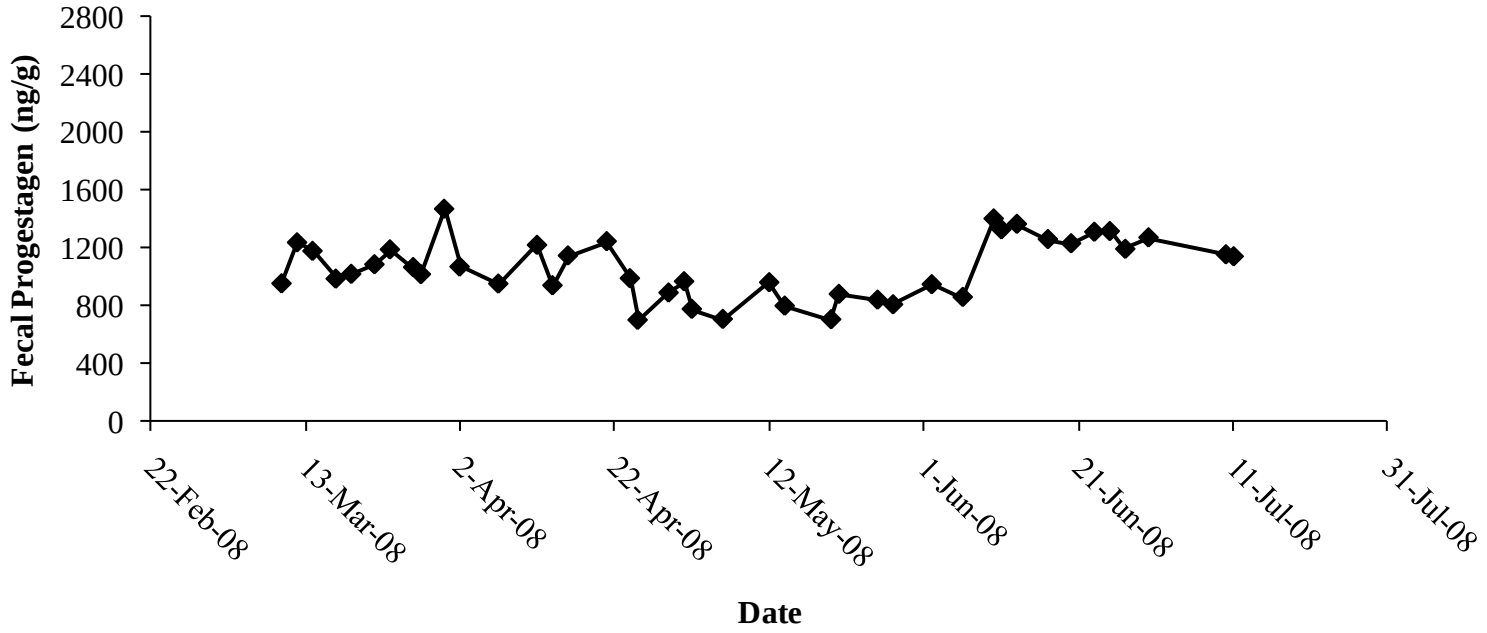

Fig. 29. Fecal progestagen profile for wild-caught, nulliparous female Helen. She had a luteal cycle from 19 March to 25 April 2008. A second luteal phase might have started at the end of the profile.

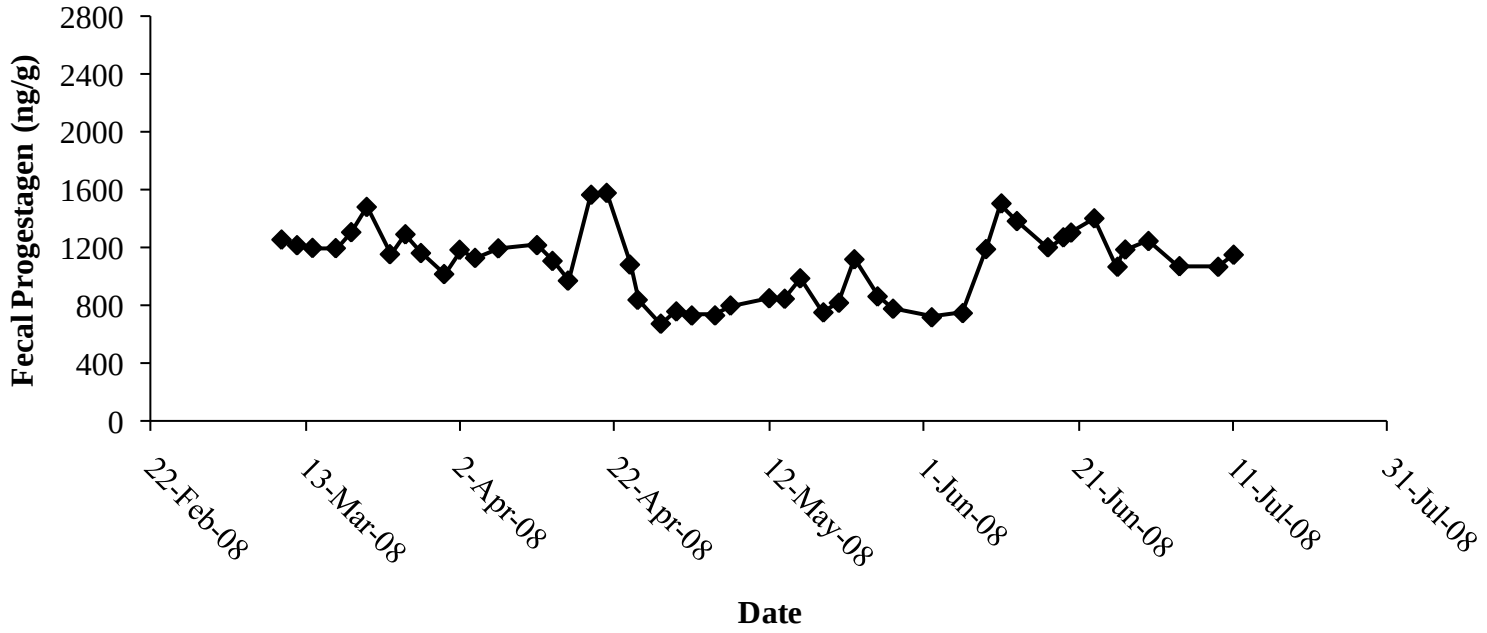

Fig. 30. Fecal progestagen profile for wild-caught, nulliparous female Jao. She had a luteal cycle from 9 June to 27 June 2008.

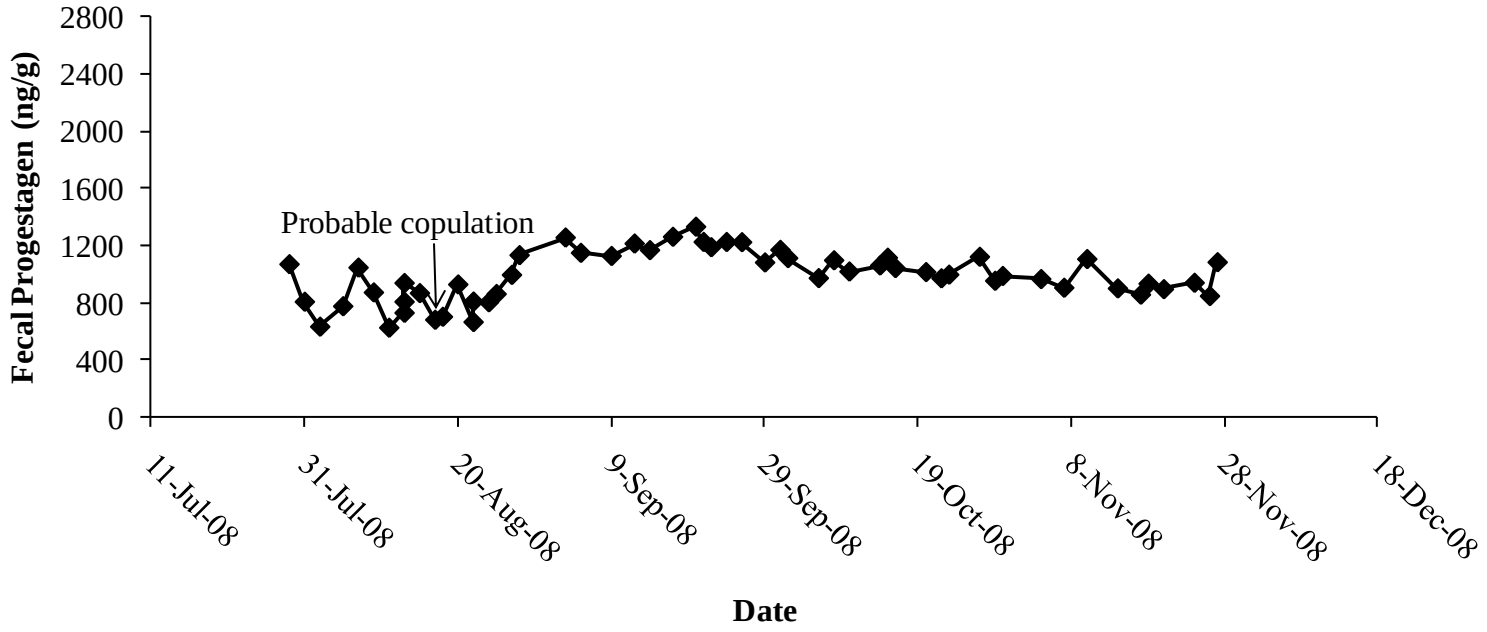

Fig. 31. Fecal progestagen profile for captive-born, nulliparous female Kiazi. Estrous behavior and mounting were observed on 16 and 17 August, and a cycle occurred from 17 August to 23 October 2008.

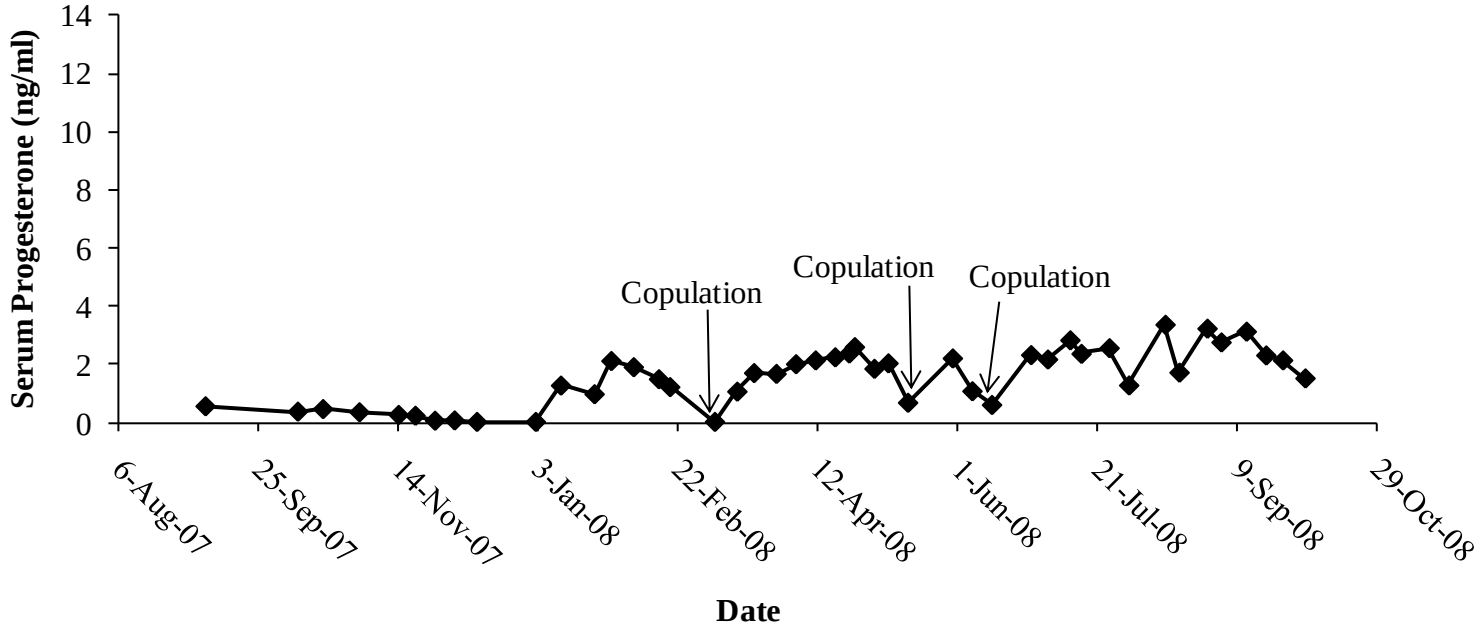

Fig. 32. Serum progesterone profile for captive-born, nulliparous female Lucy at White Oak. She had luteal cycles in 2008 from 2 January to 6 March, from 6 March to 14 May, from 14 May to 13 June, and from 13 June to 1 August. Interestingly, her cycles commence in 2008 after a novel male arrives on 18 November 2007 and is with the females all night for the first time on 30 December.

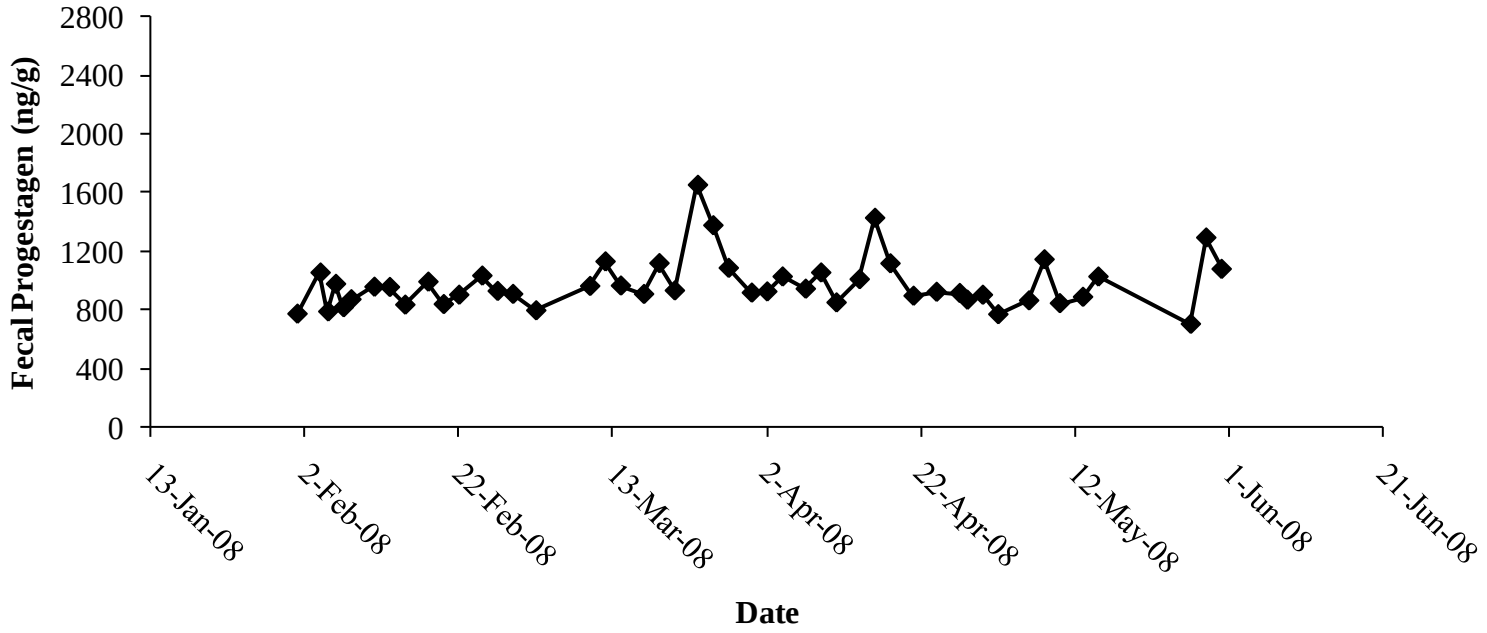

Fig. 33. Fecal progestagen profile for wild-caught, nulliparous female Mambo. She had luteal cycles from 3 March to 2 April and from 2 April to 24 April 2008.

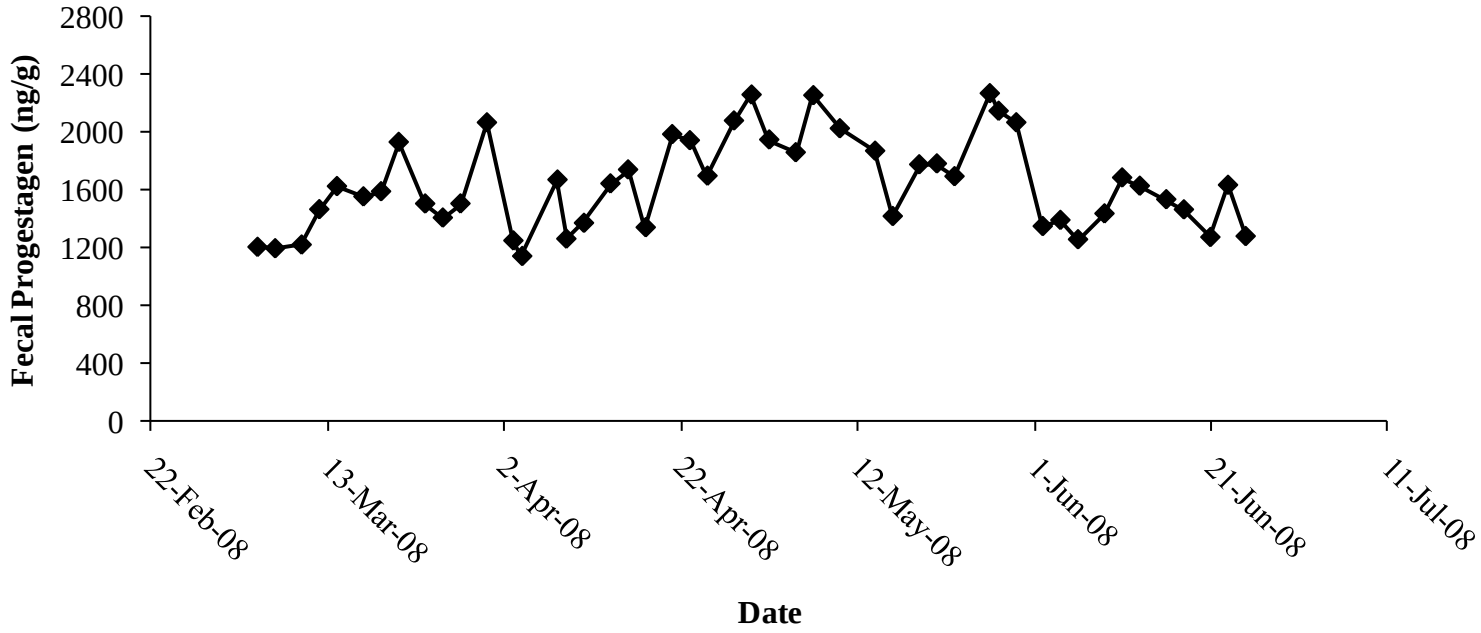

Fig. 34. Fecal progestagen profile for wild-caught, nulliparous female Marina. She had a luteal cycle from 10 March to 4 April 2008.

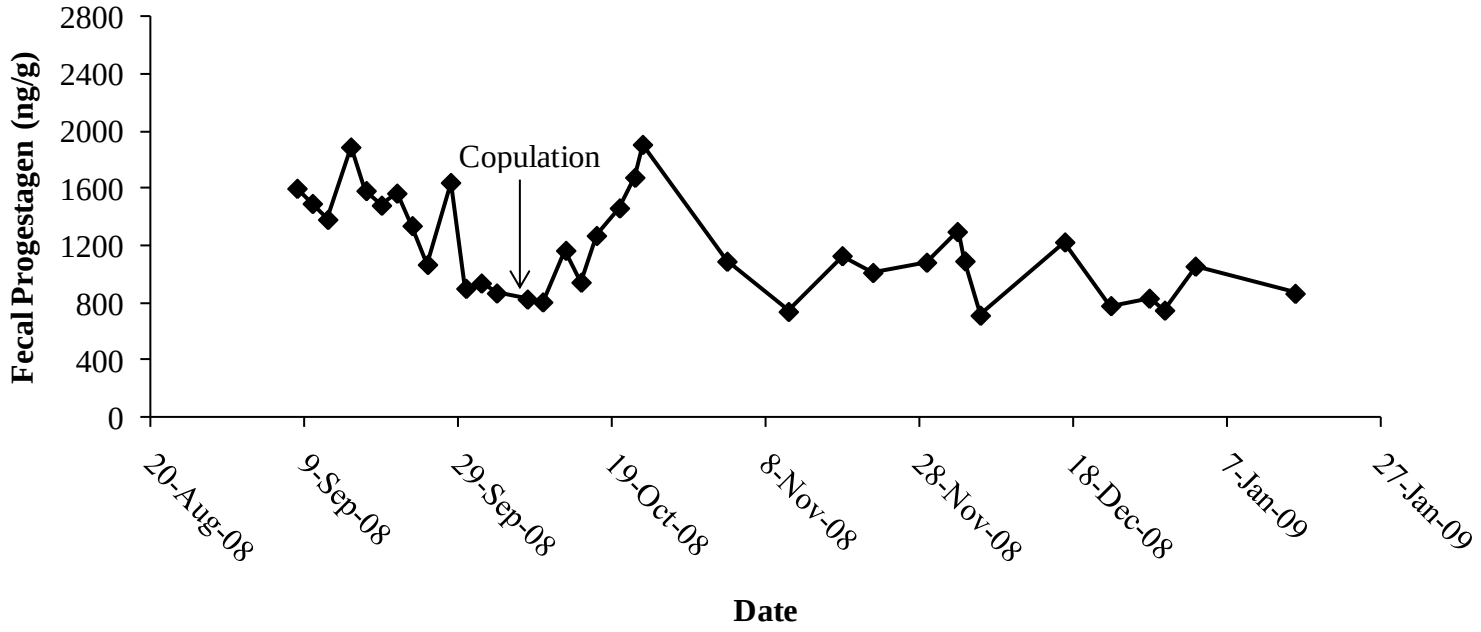

Fig. 35. Fecal progestagen profile for captive-born, nulliparous female Paddy. Keepers observed copulation on 7 October, and her profile indicates a luteal cycle from 10 October to 11 November 2008.

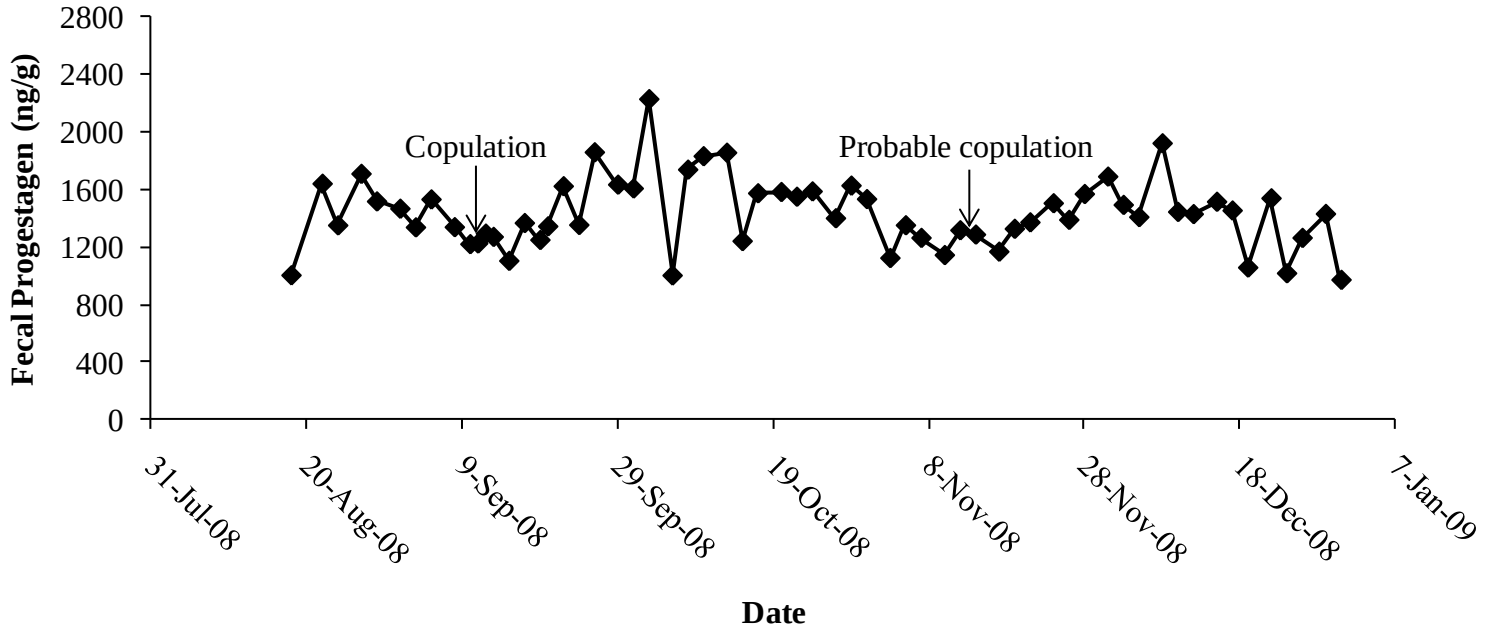

Fig. 36. Fecal progestagen profile for captive-born, nulliparous female Taryn. Copulation was observed on 11 September, and a luteal cycle occurred from 11 September to 13 November 2008. Estrous behavior and mounting were observed on 13 November, and a luteal cycle occurred from 13 November to 26 December 2008.

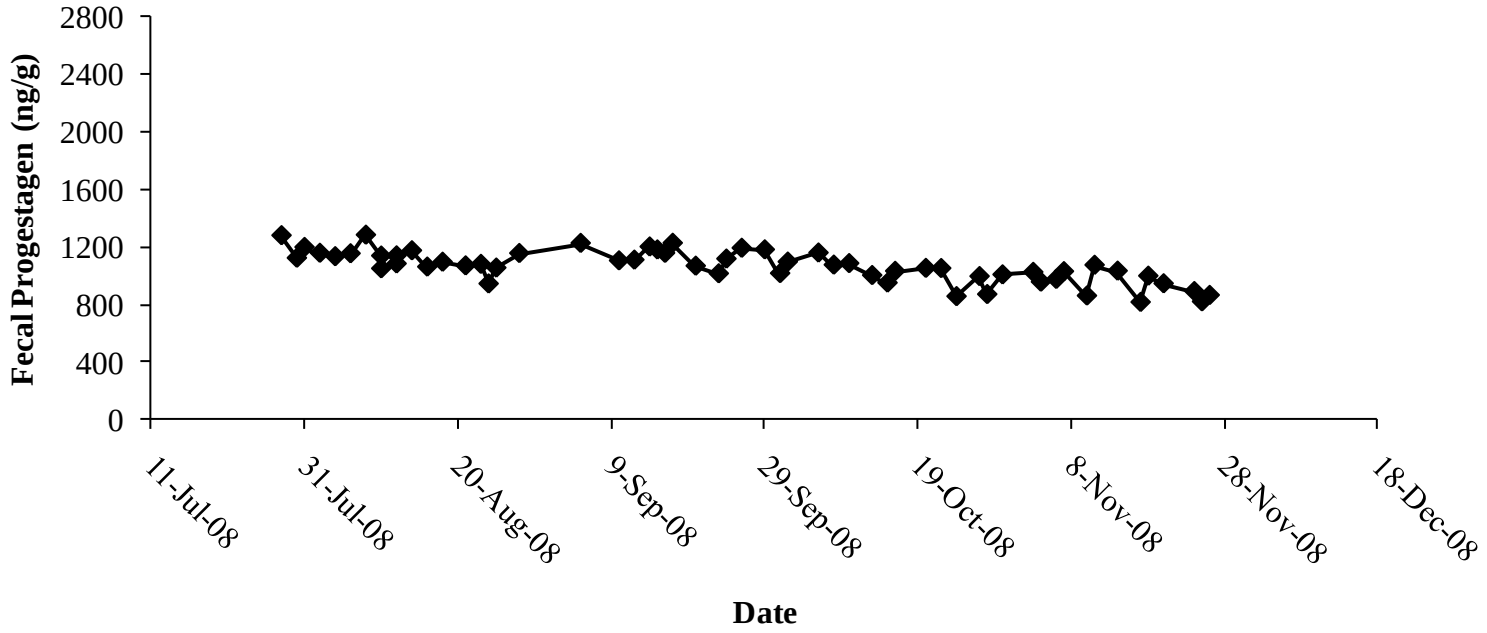

Fig. 37. Fecal progestagen profile for captive-born, parous female Holly. She was acyclic.

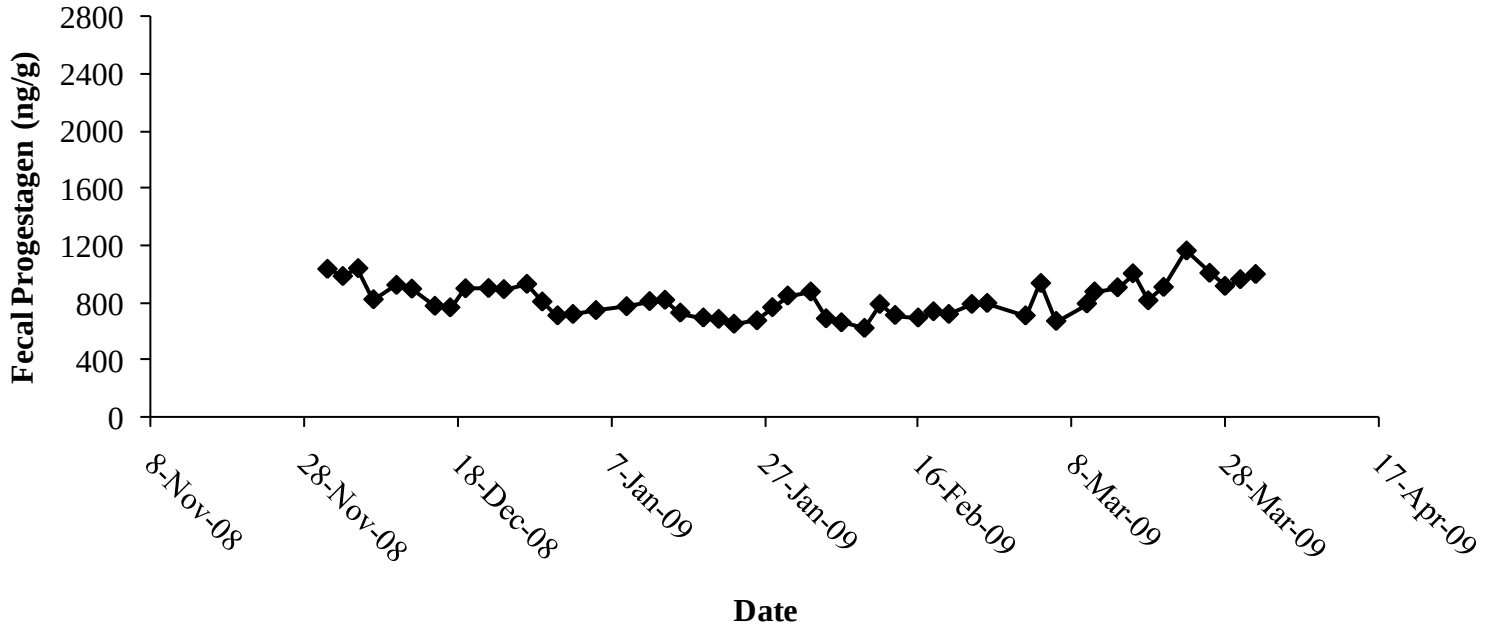

Fig. 38. Fecal progestagen profile for captive-born, parous female Laptop. She was acyclic, though a luteal cycle might have begun near the end of the profile.

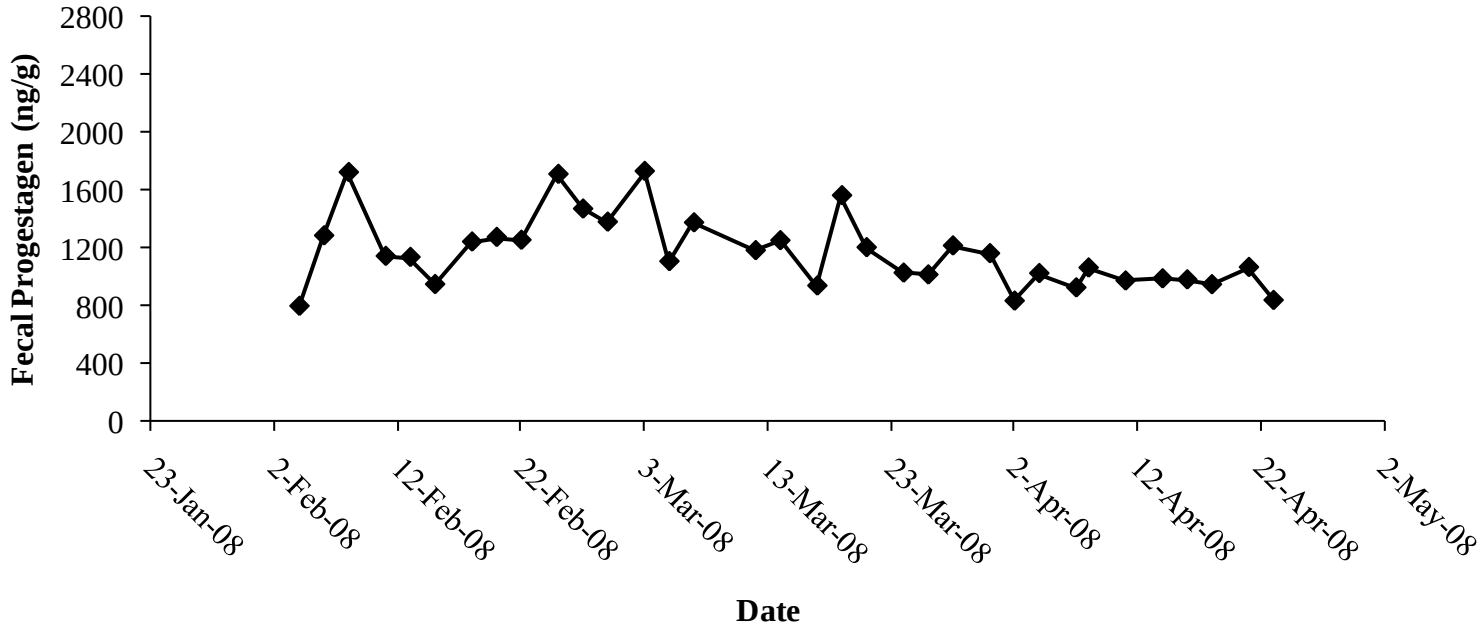

Fig. 39. Fecal progestagen profile for wild-caught, nulliparous female Bertha. She was acyclic.

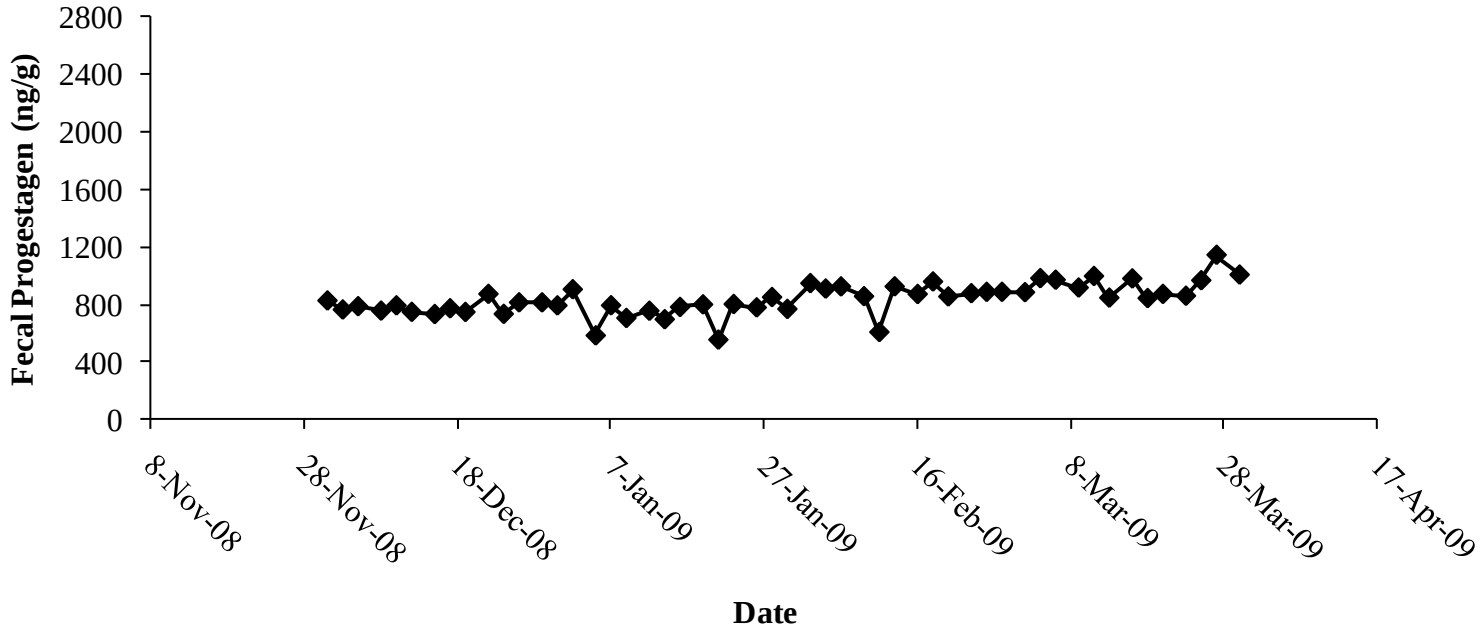

Fig. 40. Fecal progestagen profile for captive-born, nulliparous female Jeannie. She was acyclic.

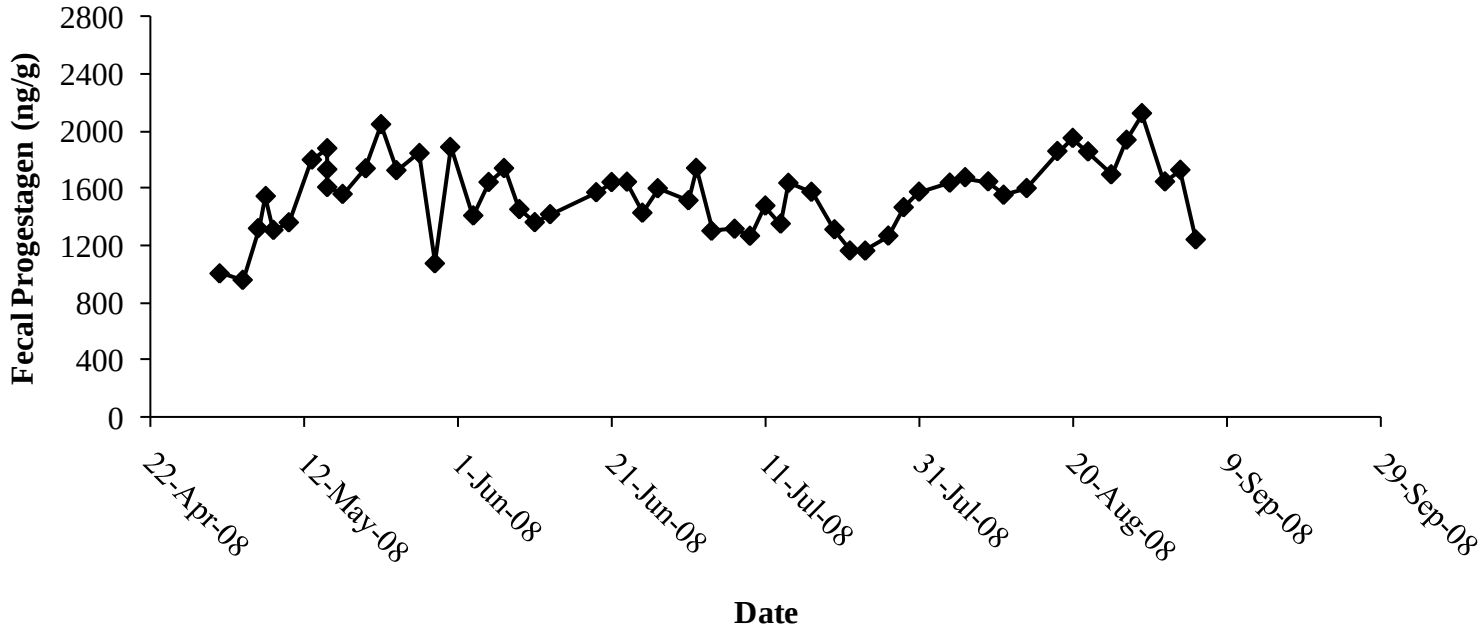

Fig. 41. Fecal progestagen profile for captive-born, nulliparous female Kiangazi. Keepers observed copulation on 28 April 2008 before sample collection began, but few of the values in May statistically were luteal concentrations. The same is true of values in the later portion of the profile. Therefore, this female was acyclic.

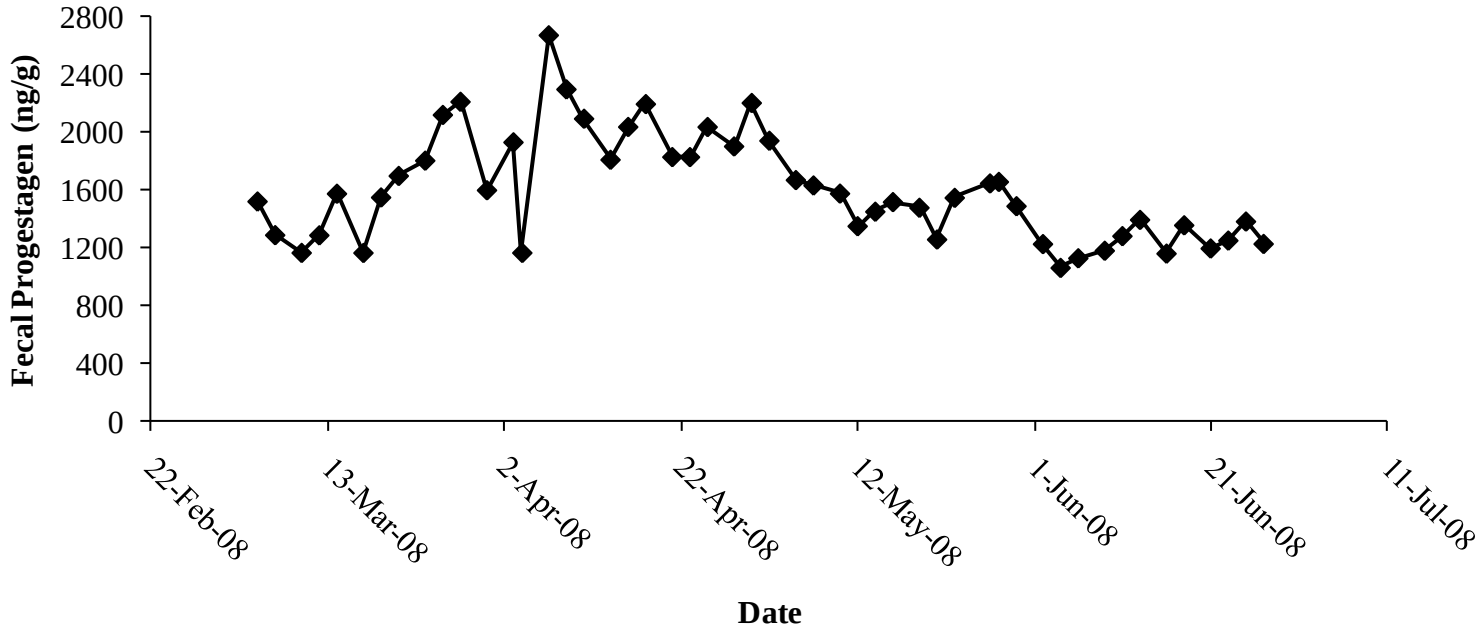

Fig. 42. Fecal progestagen profile for wild-caught, nulliparous female Mashile. She was acyclic.

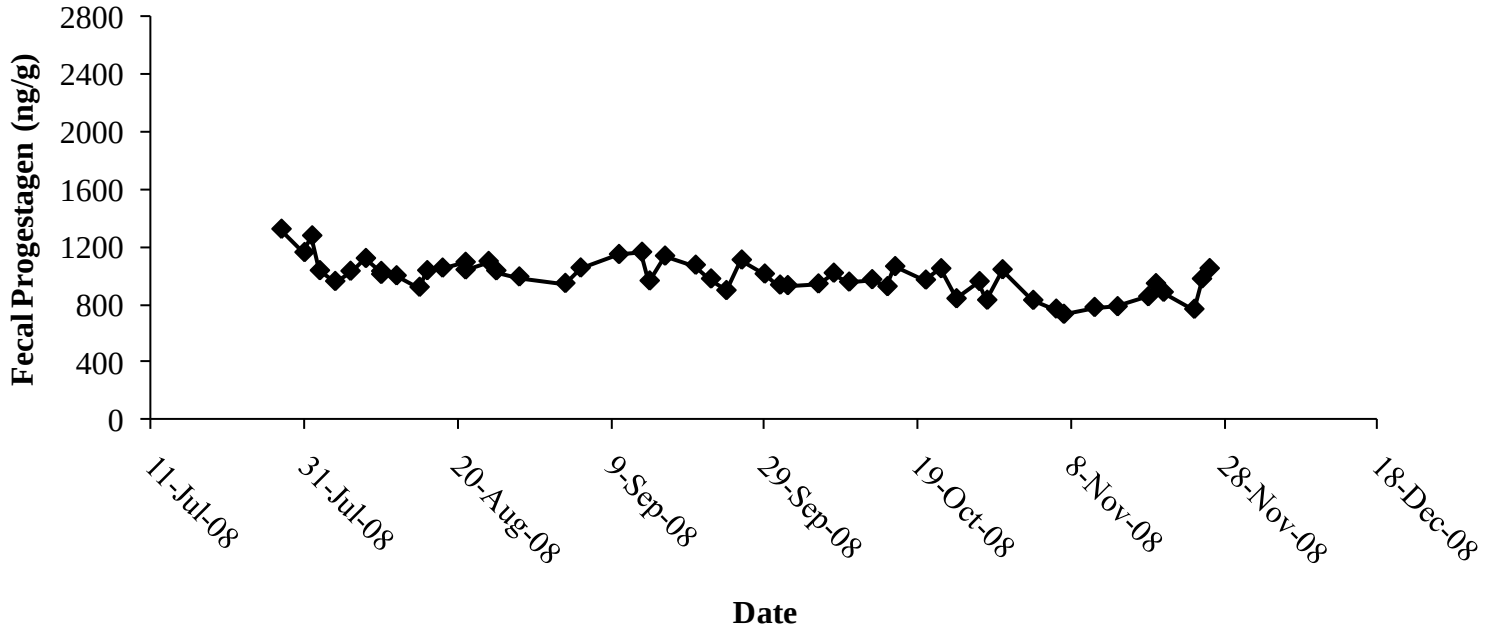

Fig. 43. Fecal progestagen profile for captive-born, nulliparous female Utamu. She was acyclic.

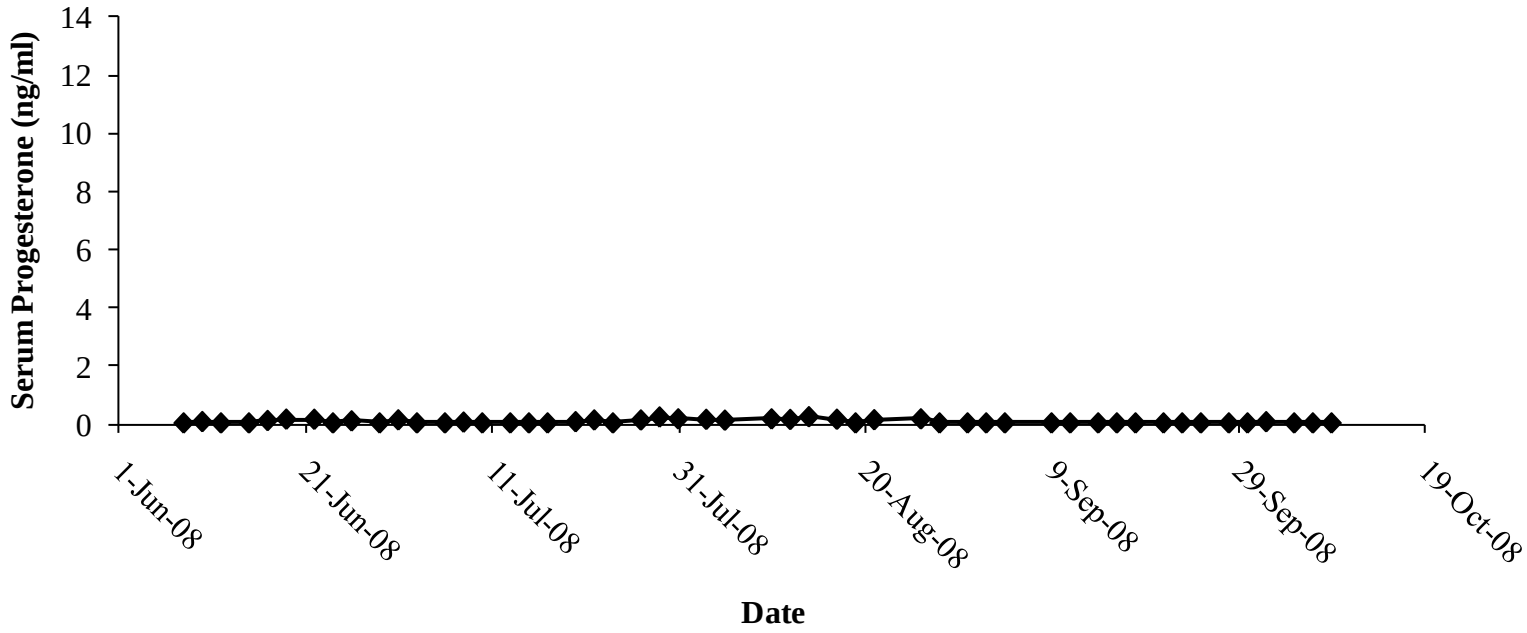

Fig. 44. Serum progesterone profile for captive-born, nulliparous female Yebonga. She was acyclic.

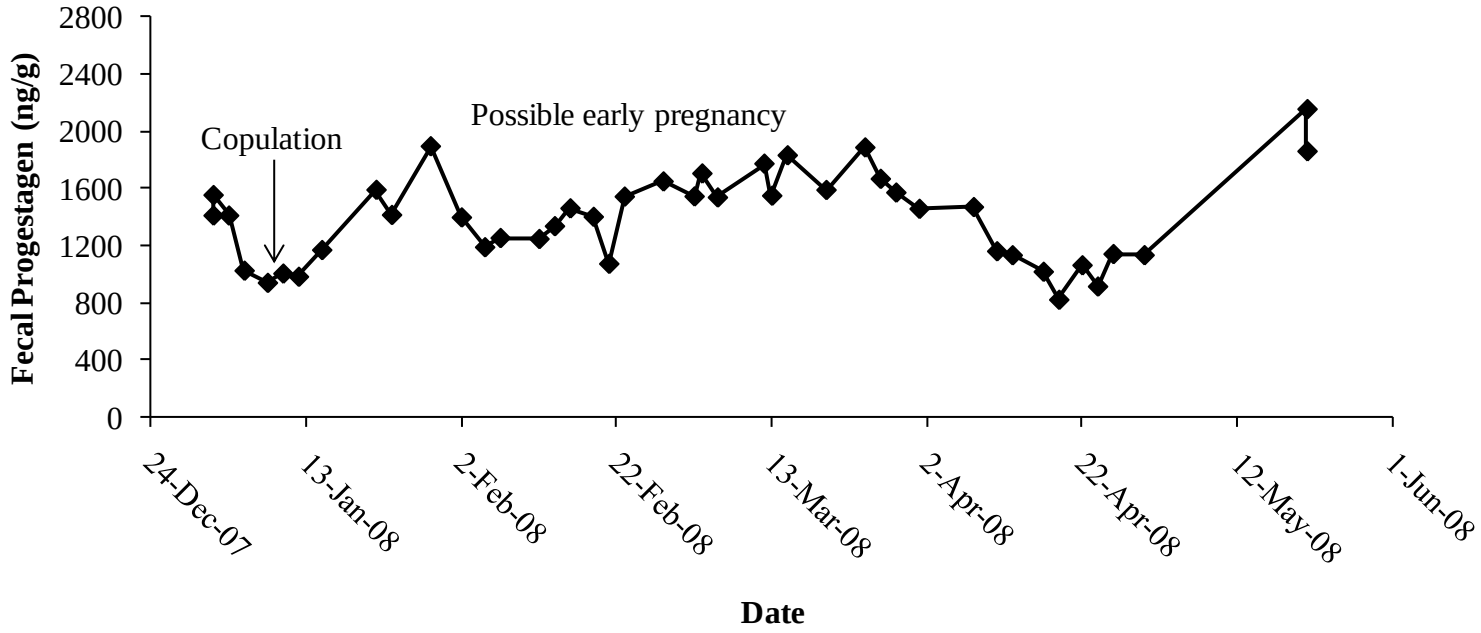

Fig. 45. Fecal progestagen profile for captive-born, parous female Lissa. She might have become pregnant after copulation on 9 January, but the pregnancy was lost after the last luteal concentration on 8 April 2008. Keepers observed copulation again on 8 July 2008.

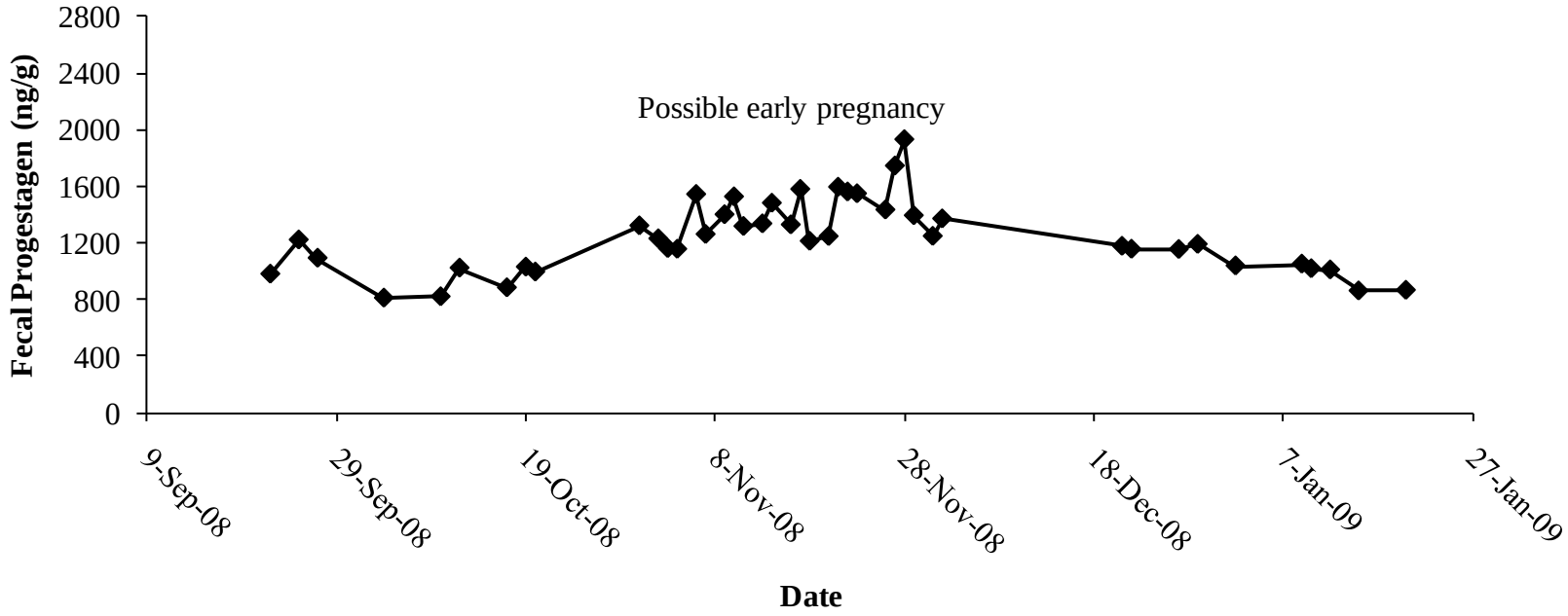

Fig. 46. Fecal progestagen profile for captive-born, parous female Yvonne. She might have become pregnant after 6 October, when keepers observed mounting, but the pregnancy was lost after the last luteal concentration on 29 December 2008. Estrous behaviors were not observed again until February 2009.

Appendix K: Corticosterone Profiles for Captive Female White Rhinos

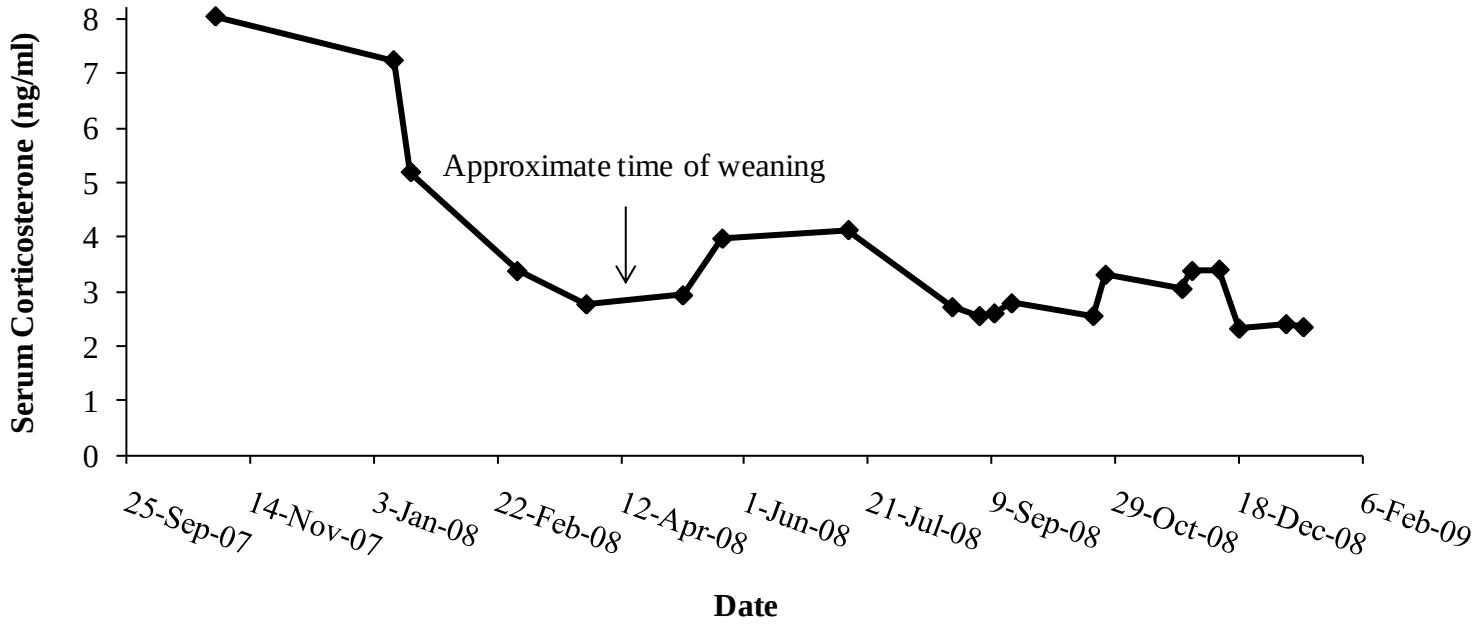

Fig. 47. Serum corticosterone profile for adolescent Dakari. Interestingly, concentrations were highly elevated at the beginning of the profile and actually decreased and subsequently leveled after she was weaned from her mother.

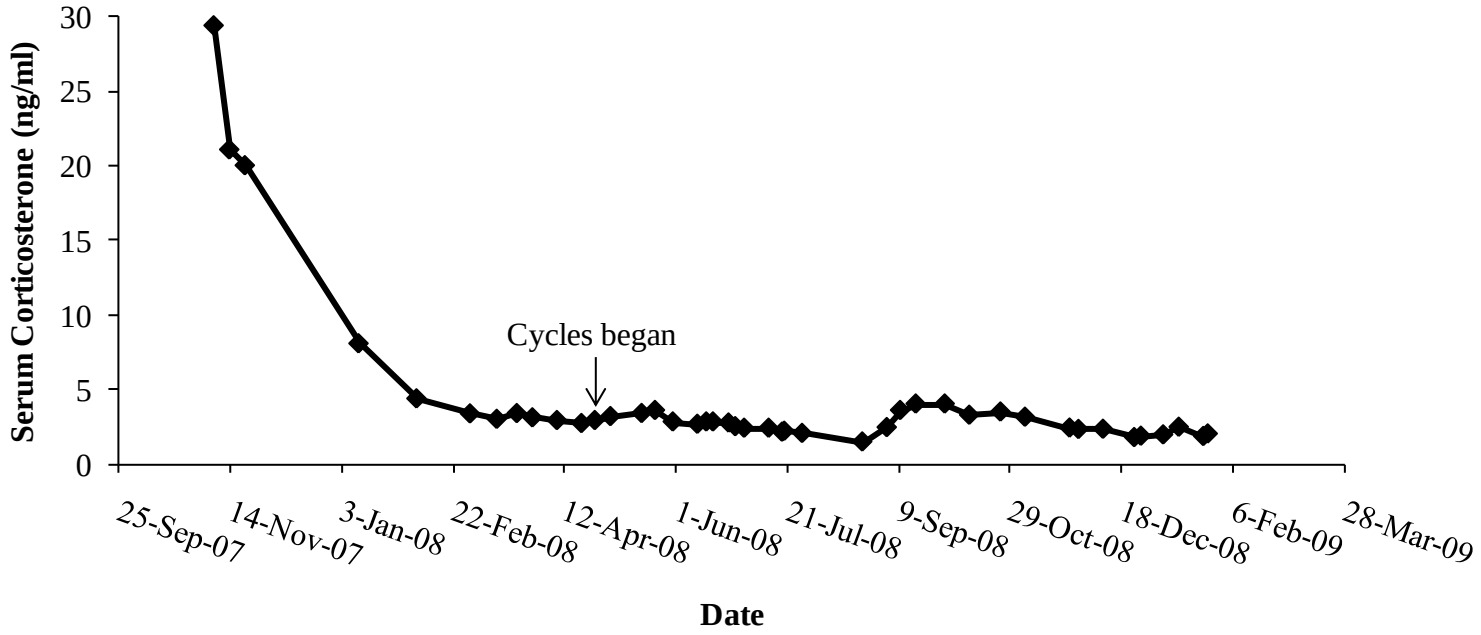

Fig. 48. Serum corticosterone profile for adolescent Lucy at Busch Gardens. Concentrations were extremely high at the beginning of the profile, but they gradually decreased and leveled.

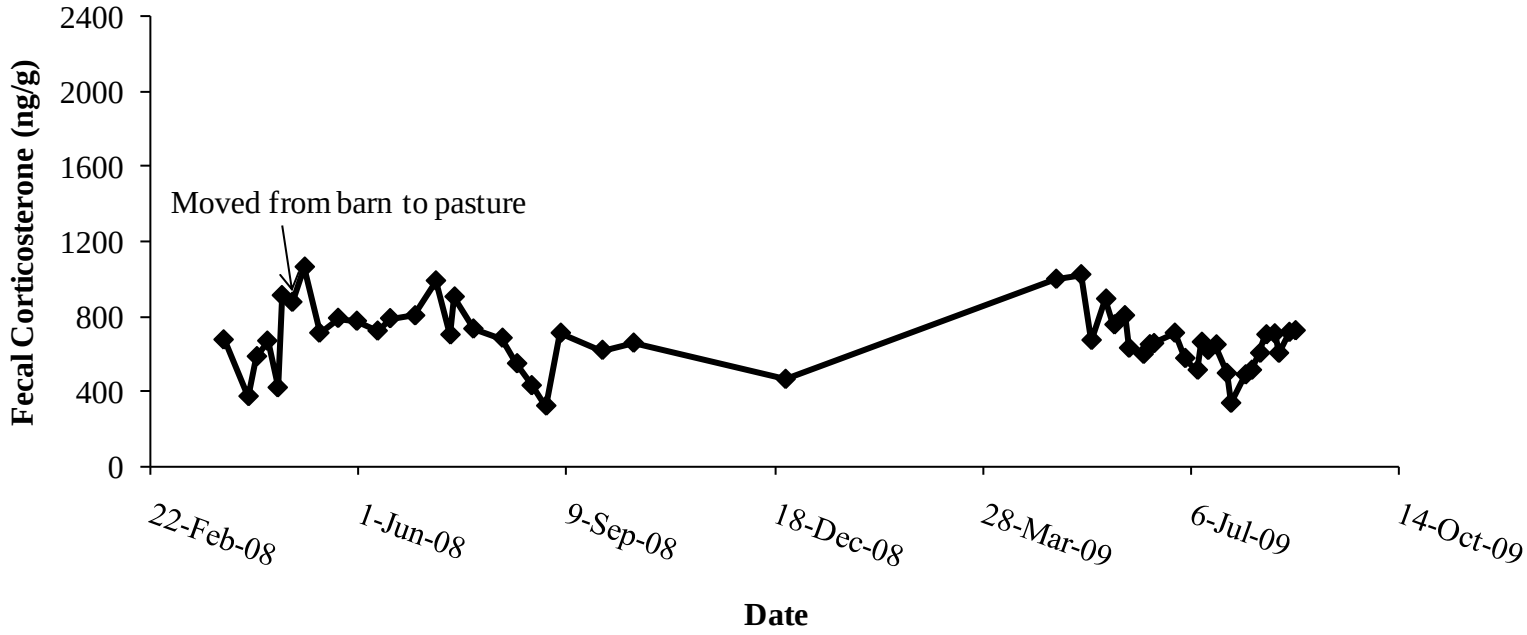

Fig. 49. Fecal corticosterone profile for adolescent Evey. She was housed inside initially and for the single value in December.

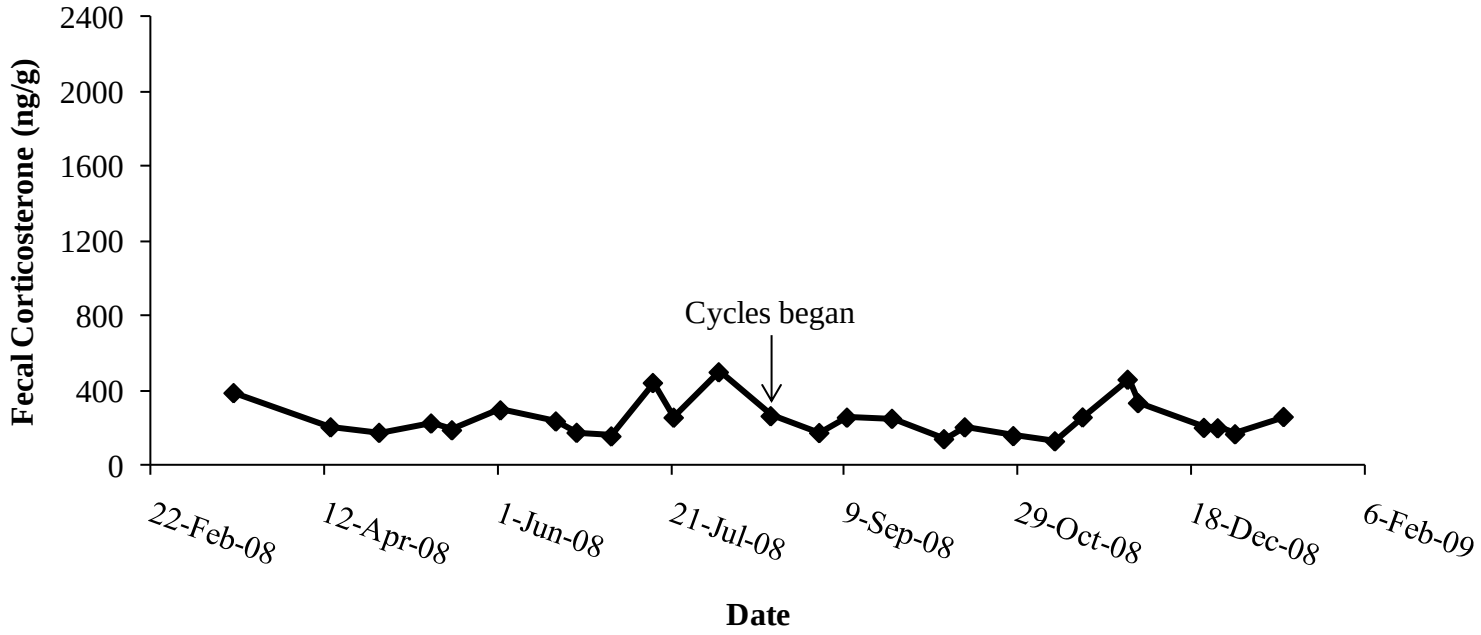

Fig. 50. Fecal corticosterone profile for adolescent Kayla.

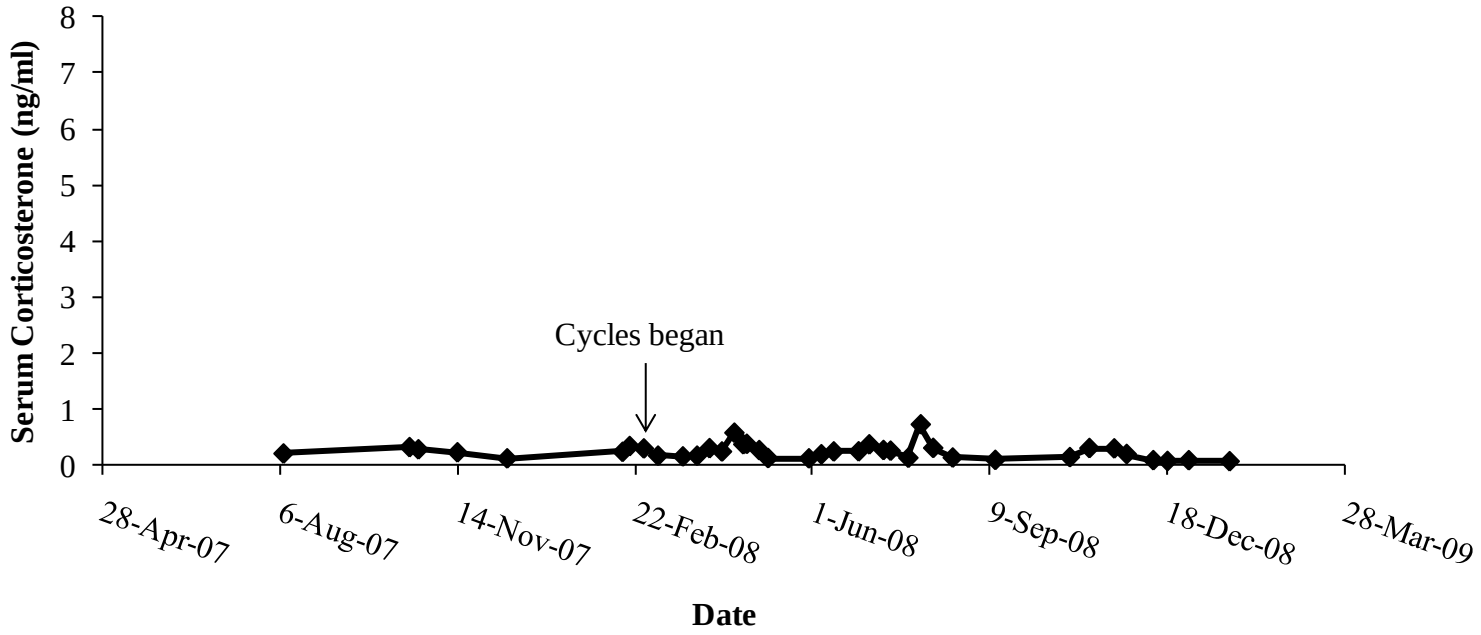

Fig. 51. Serum corticosterone profile for adolescent Kelly.

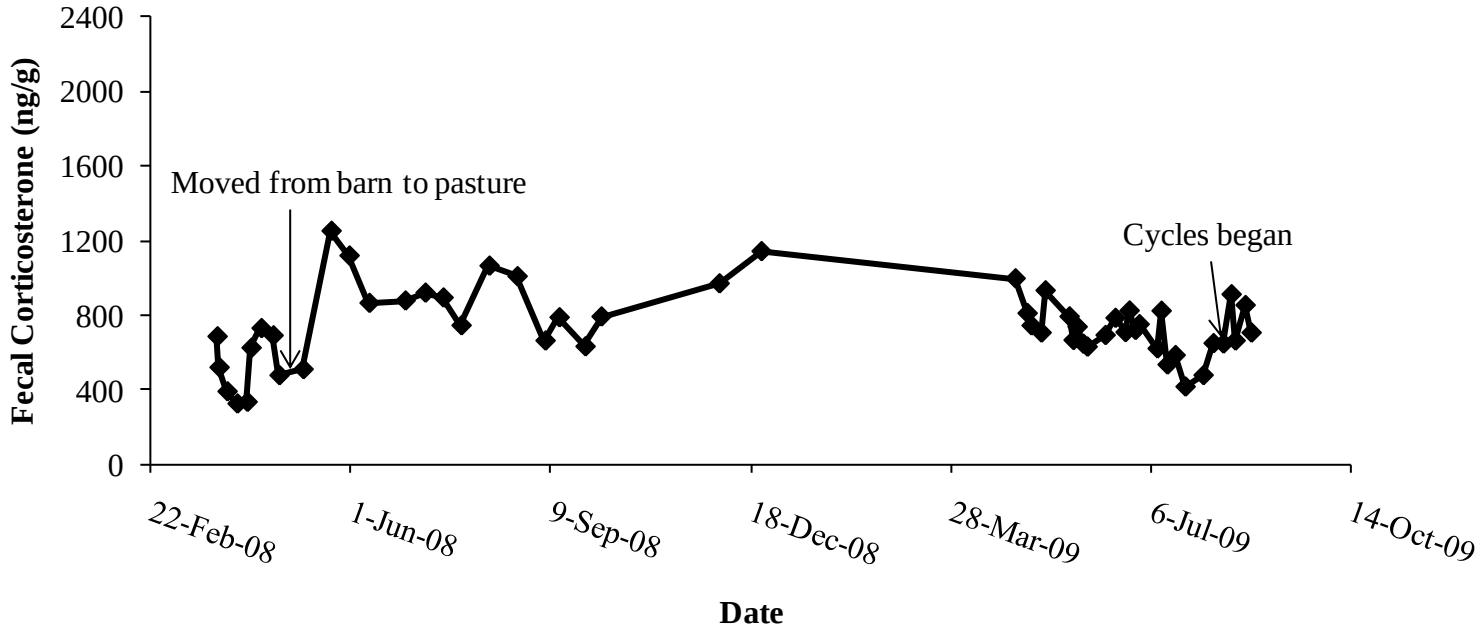

Fig. 52. Fecal corticosterone profile for adolescent Sally. She was housed inside initially and for the 2 values in December.

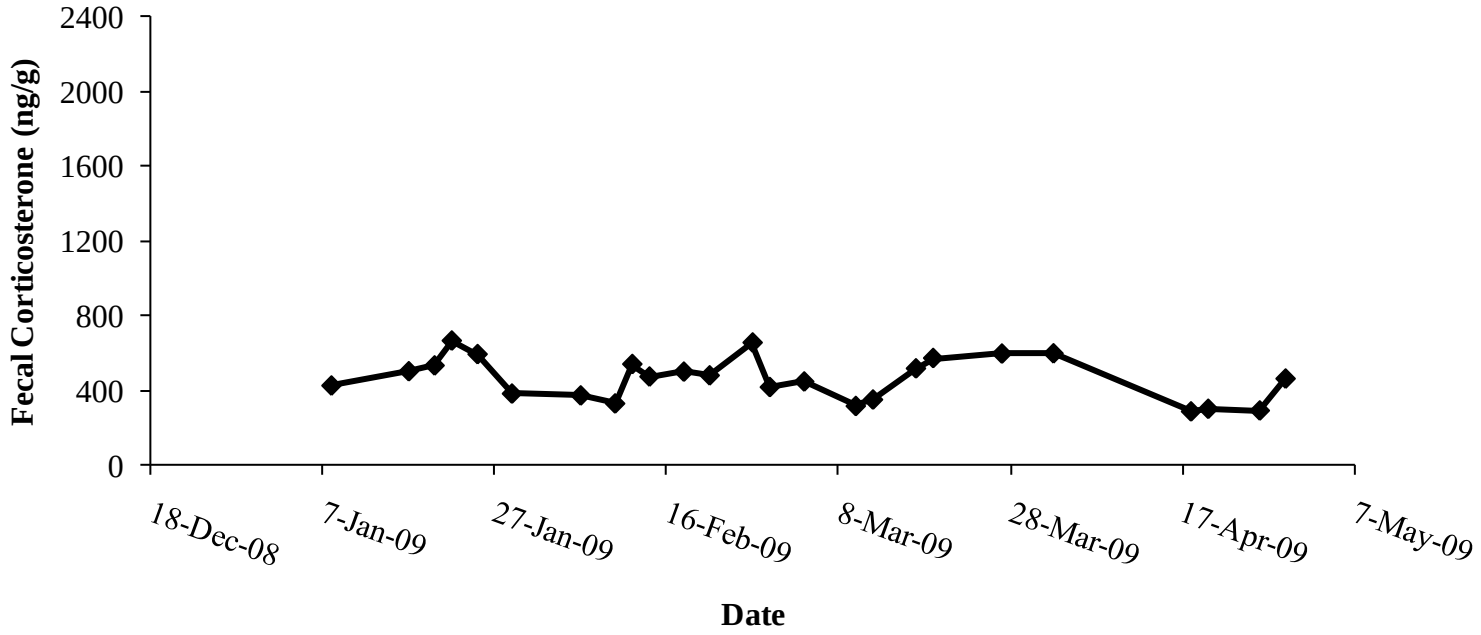

Fig. 53. Fecal corticosterone profile for captive-born, parous female Bloom.

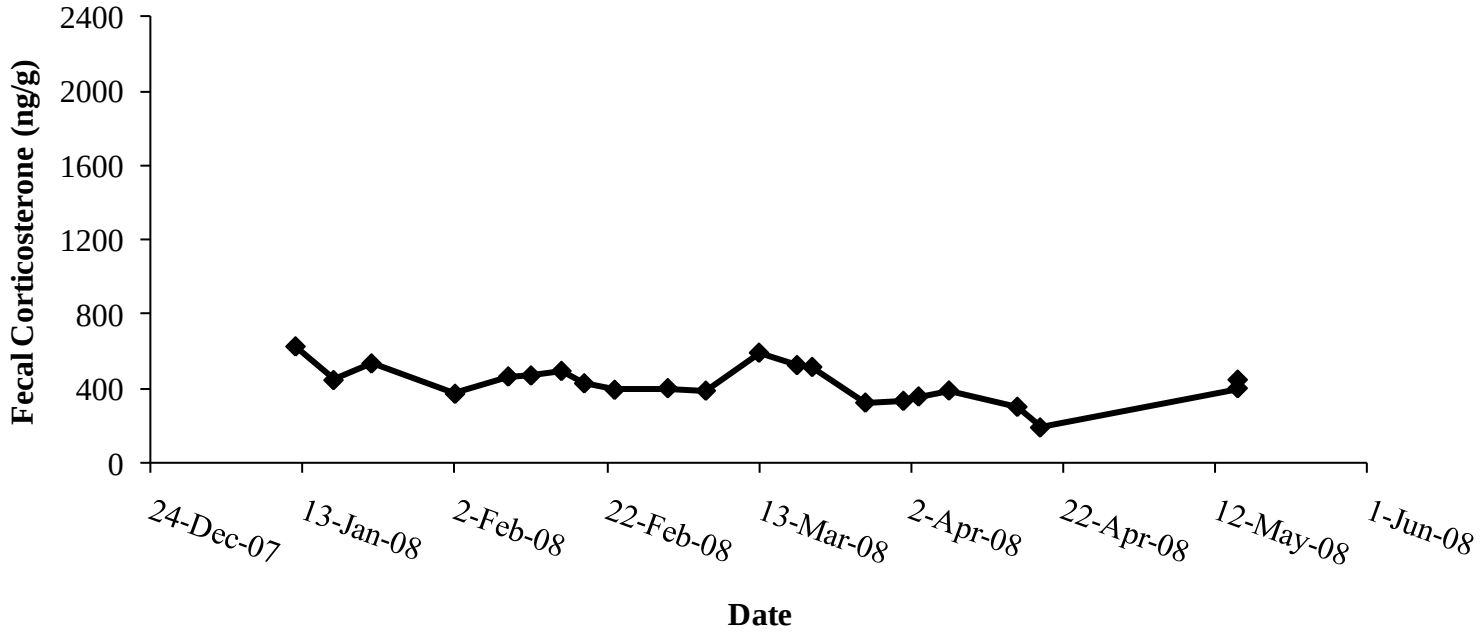

Fig. 54. Fecal corticosterone profile for captive-born, parous female Eliza. She was pregnant throughout this period.

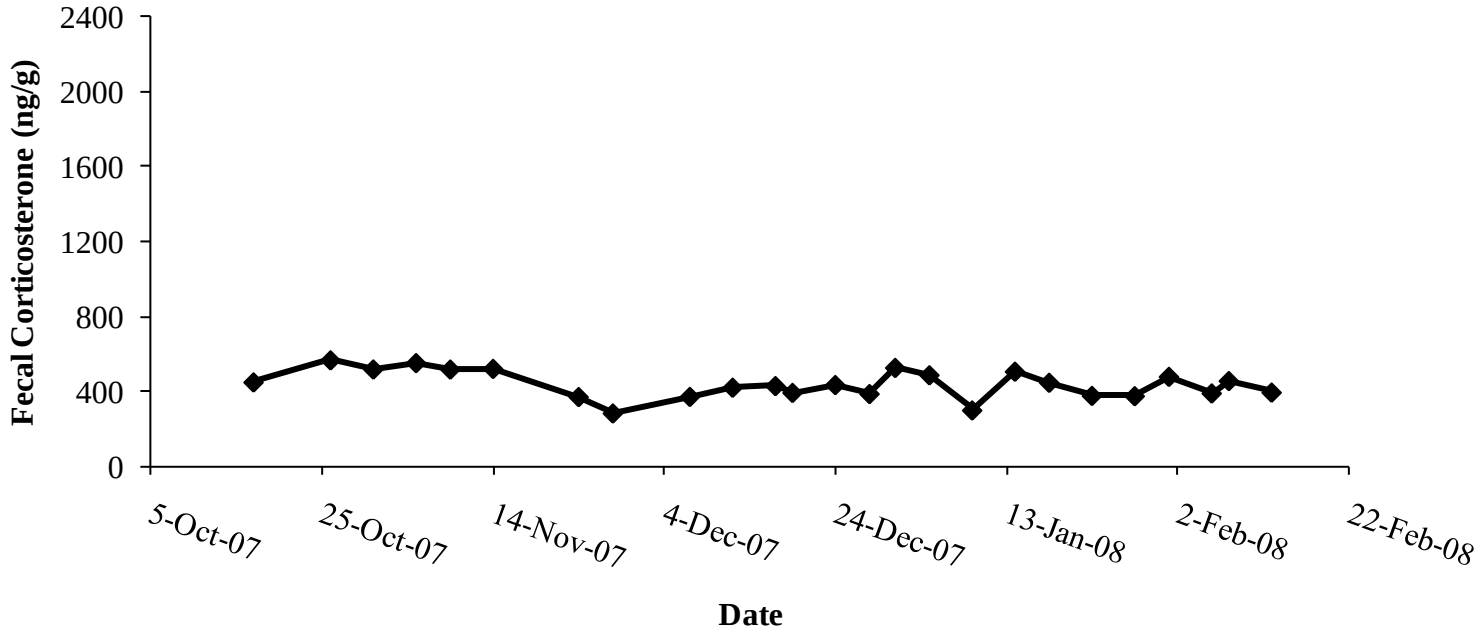

Fig. 55. Fecal corticosterone profile for captive-born, parous female Gabby.

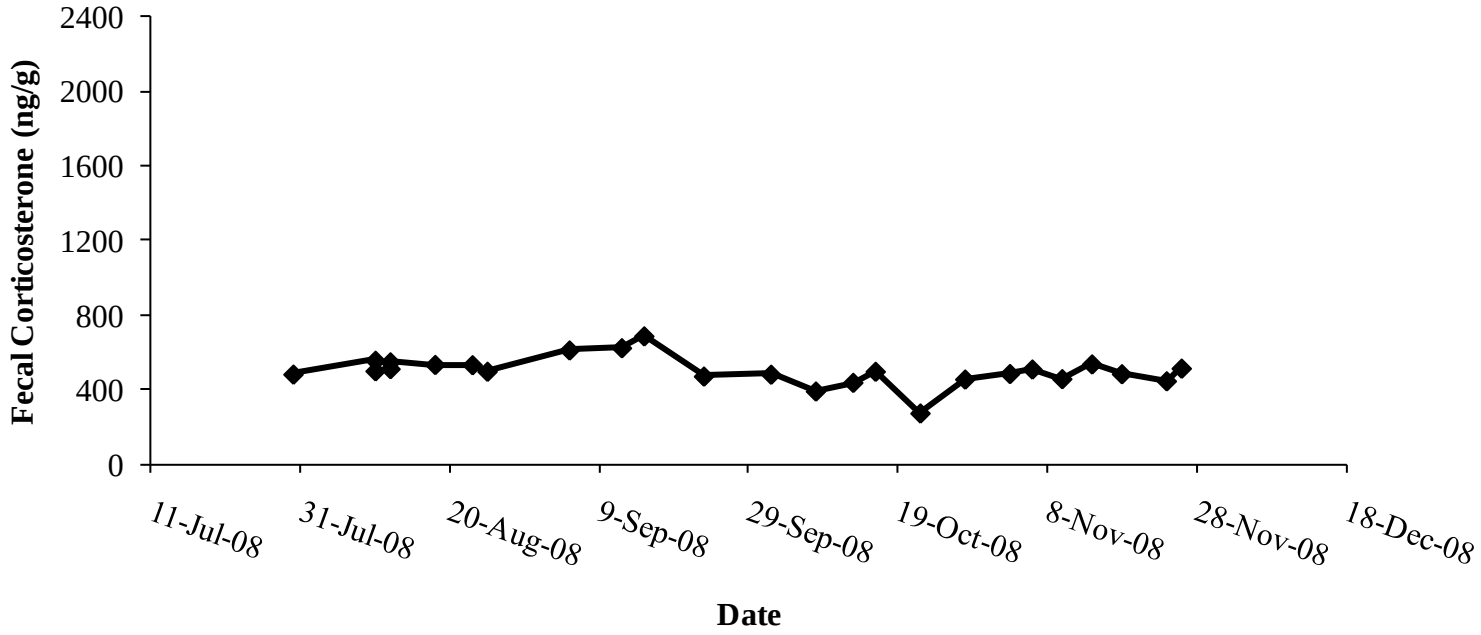

Fig. 56. Fecal corticosterone profile for captive-born, parous female Holly.

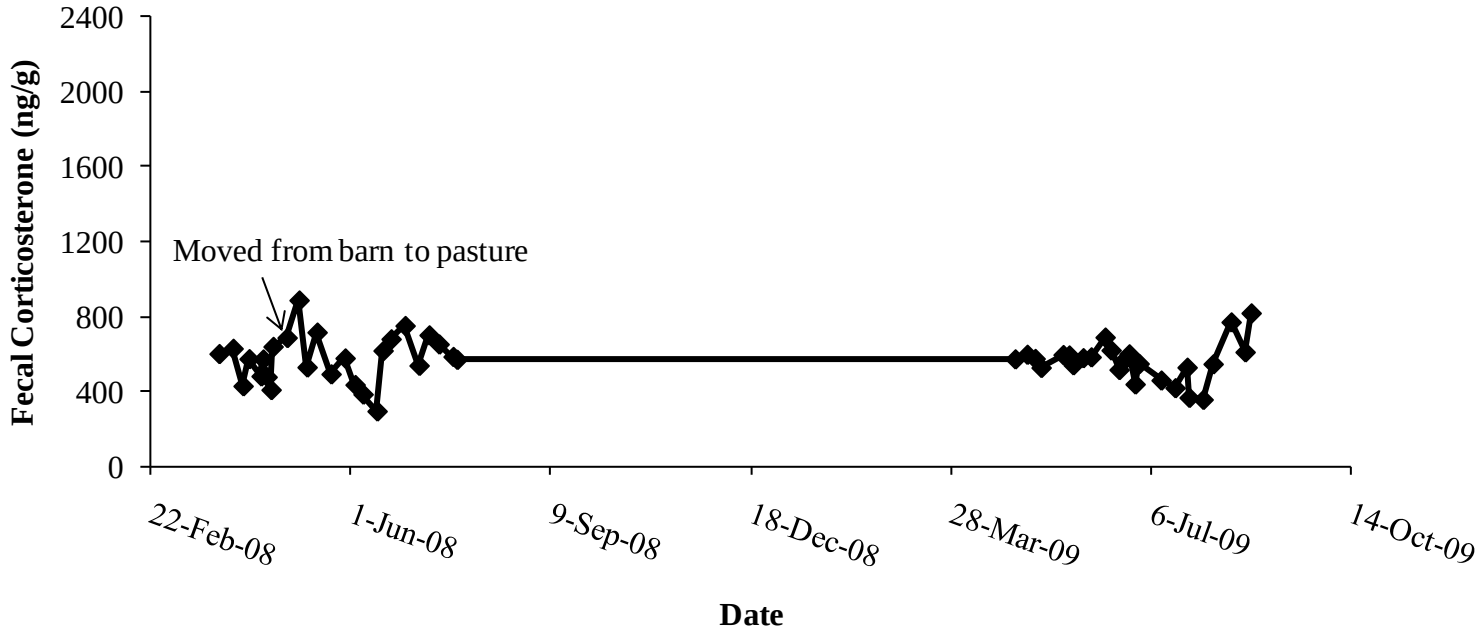

Fig. 57. Fecal corticosterone profile for captive-born, parous female Julie. She was pregnant in 2008 and lactating in 2009, and she was housed inside initially.

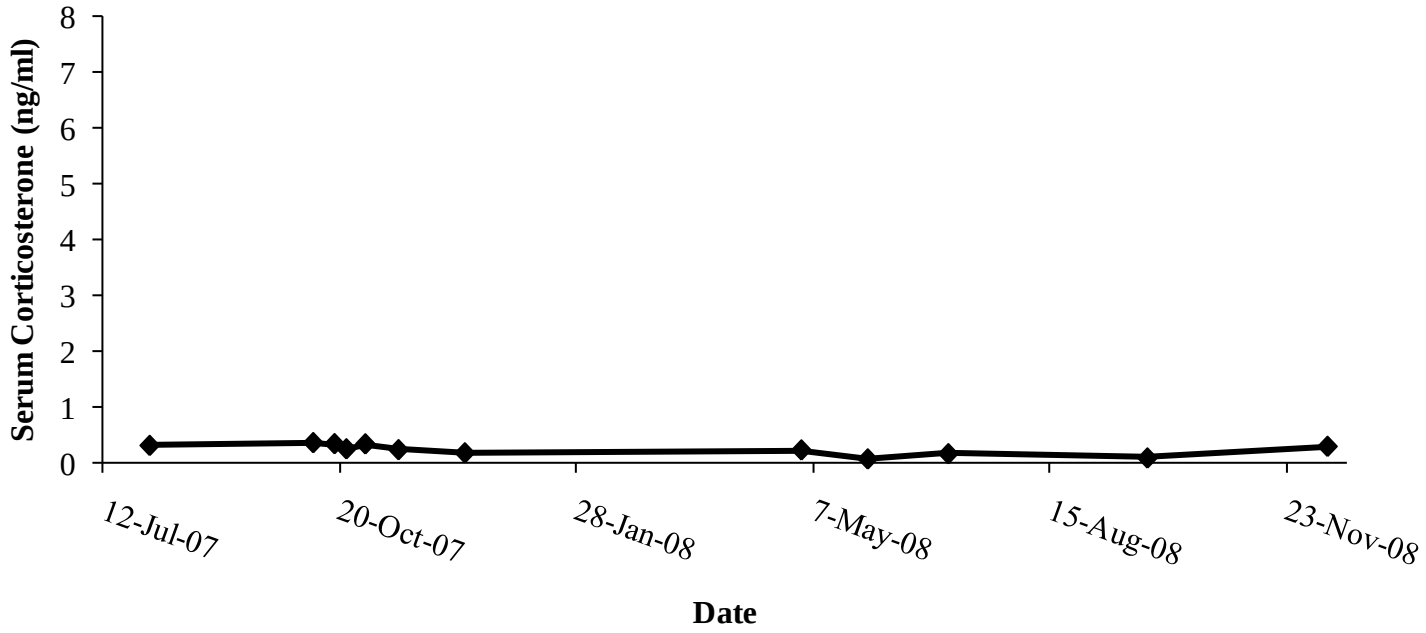

Fig. 58. Serum corticosterone profile for wild-caught, parous female Kathy.

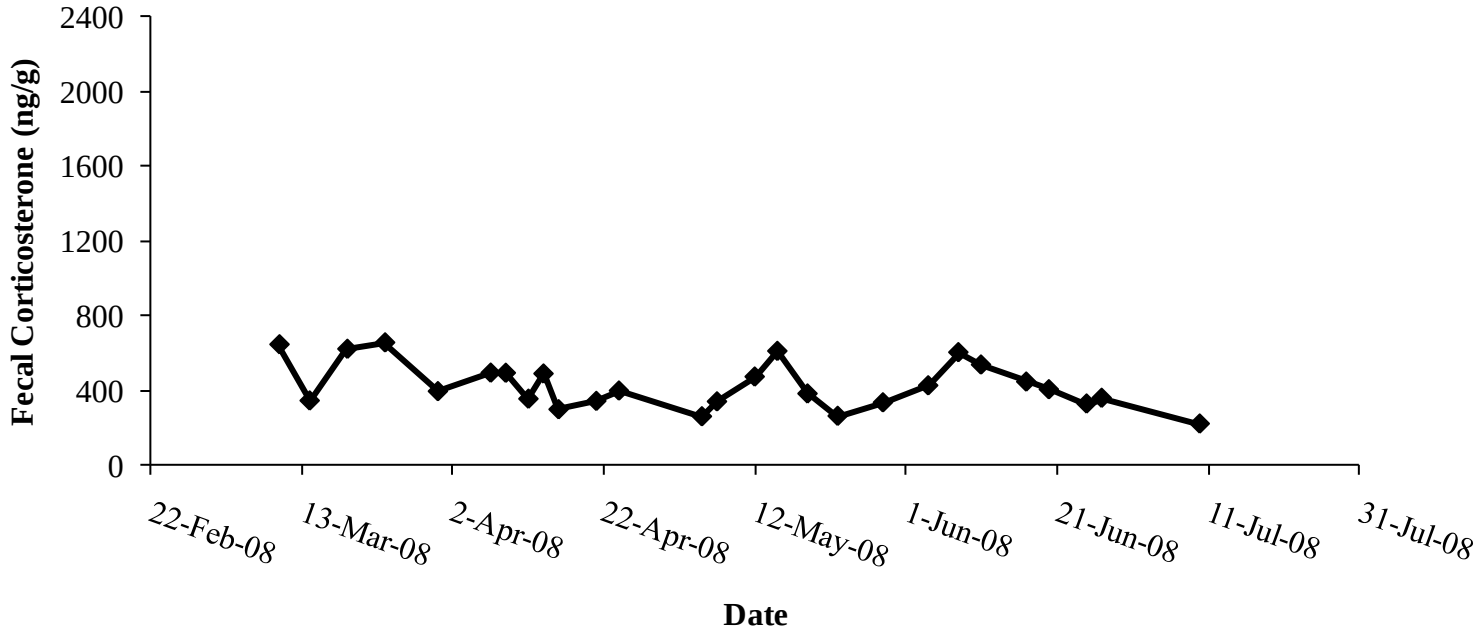

Fig. 59. Fecal corticosterone profile for captive-born, parous female Kendi.

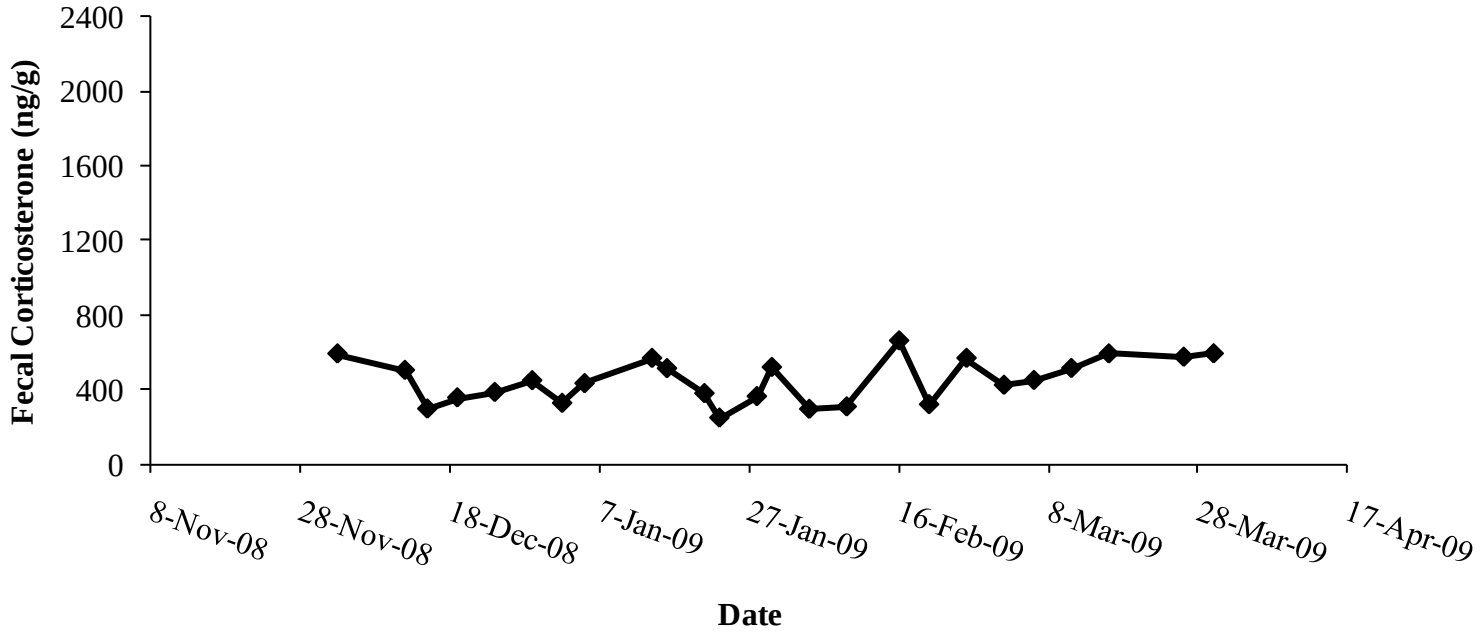

Fig. 60. Fecal corticosterone profile for captive-born, parous female Laptop. She was transferred to Birmingham from Fossil Rim in November 2008, just before sample collection began.

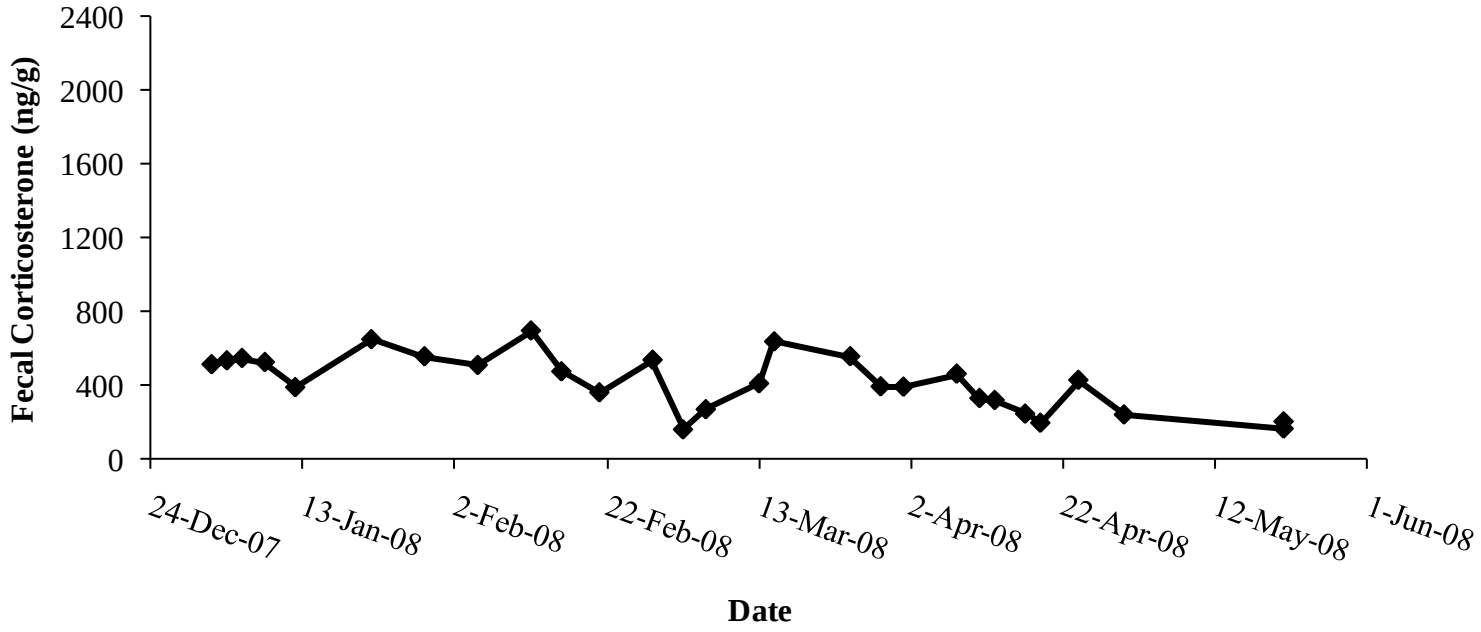

Fig. 61. Fecal corticosterone profile for captive-born, parous female Lissa.

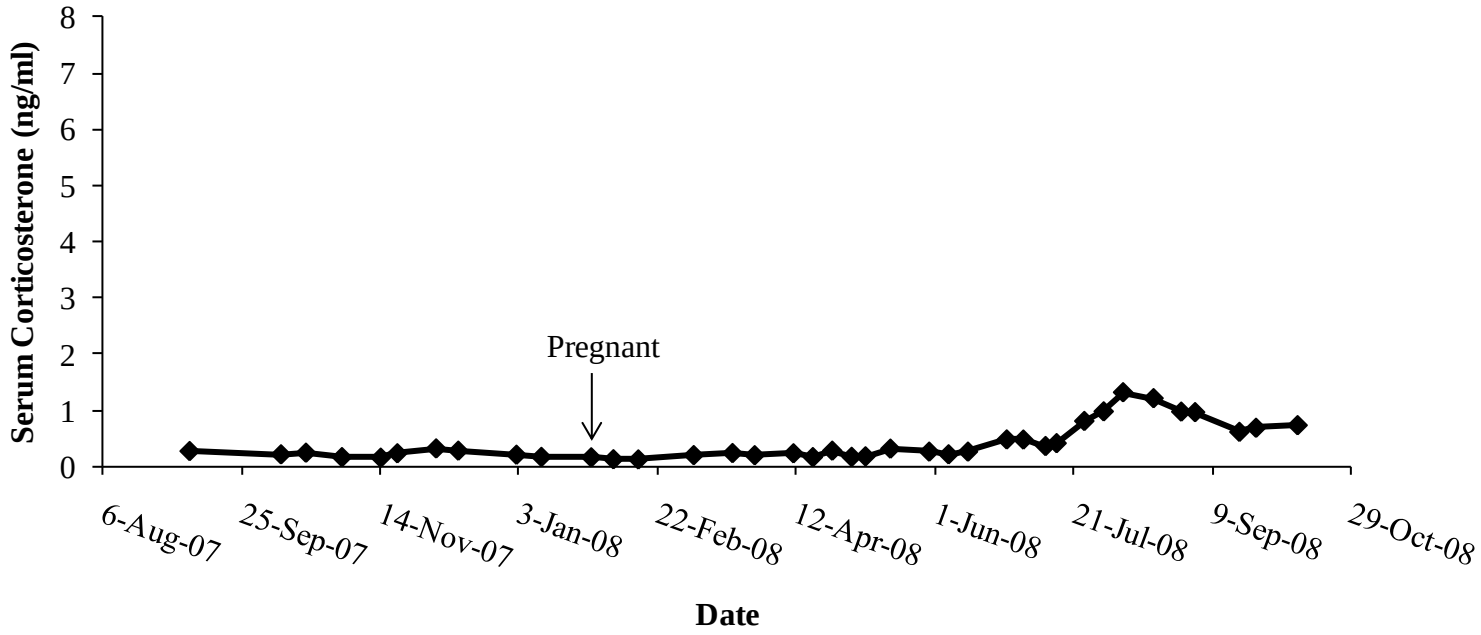

Fig. 62. Serum corticosterone profile for captive-born, parous female Maggie at White Oak.

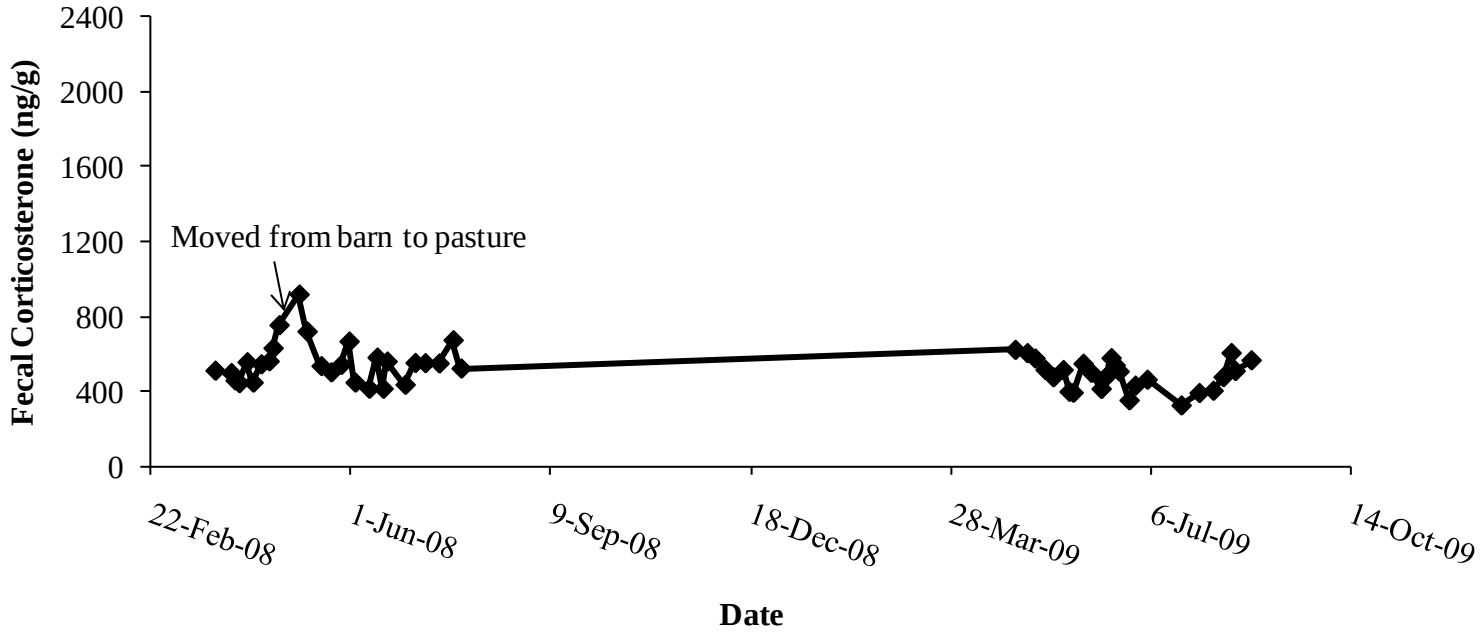

Fig. 63. Fecal corticosterone profile for captive-born, parous female Maggie at the Wilds. She was pregnant in 2008 and lactating in 2009, and she was housed inside initially.

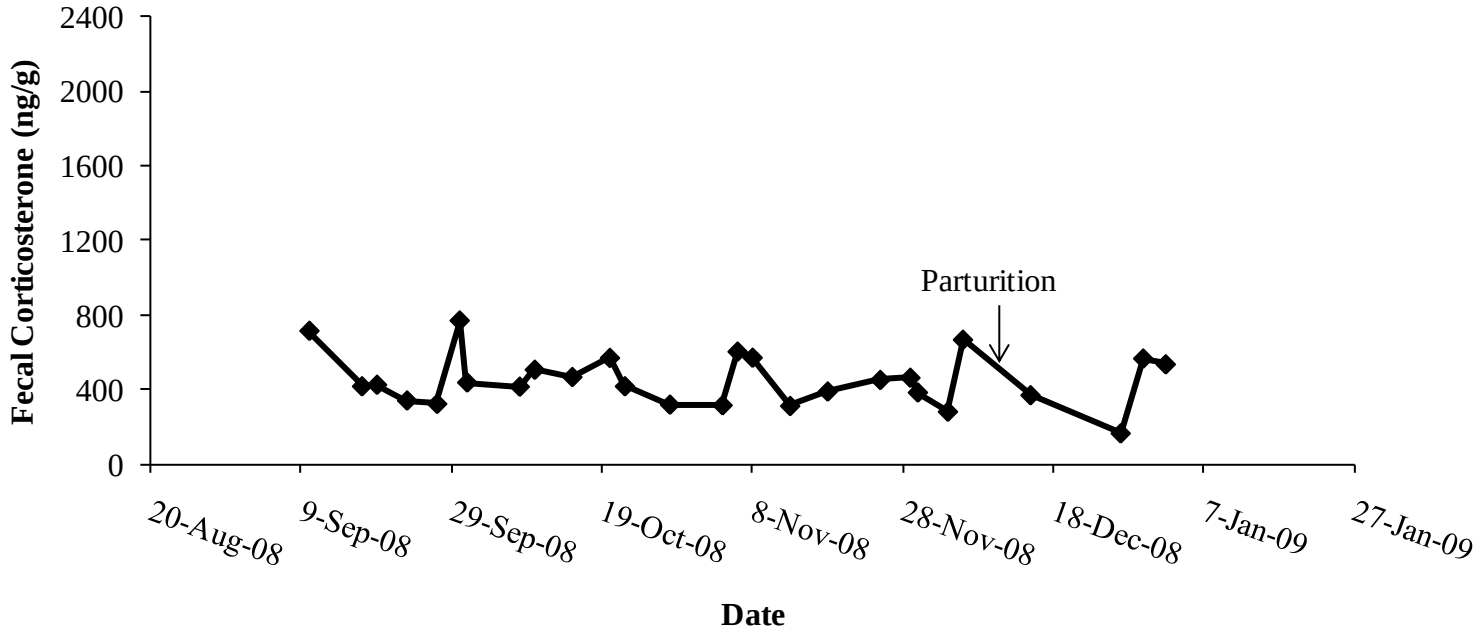

Fig. 64. Fecal corticosterone profile for captive-born, parous female Taraja.

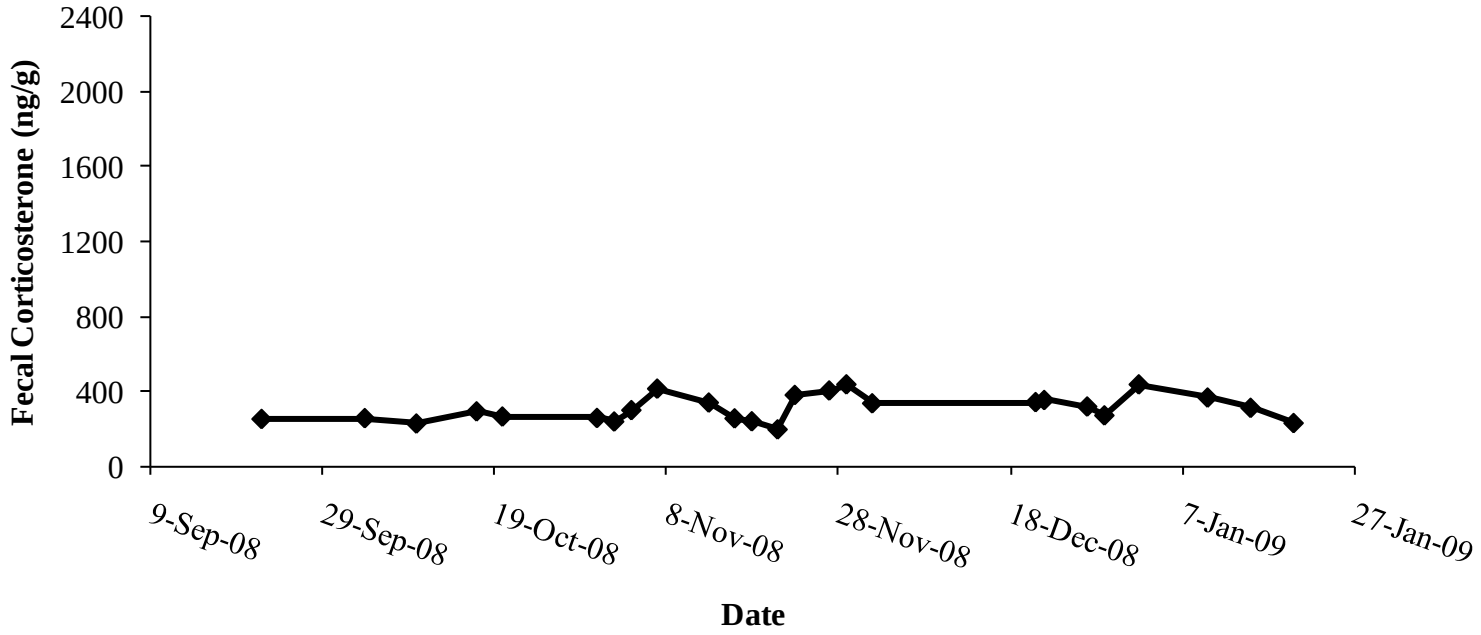

Fig. 65. Fecal corticosterone profile for captive-born, parous female Yvonne.

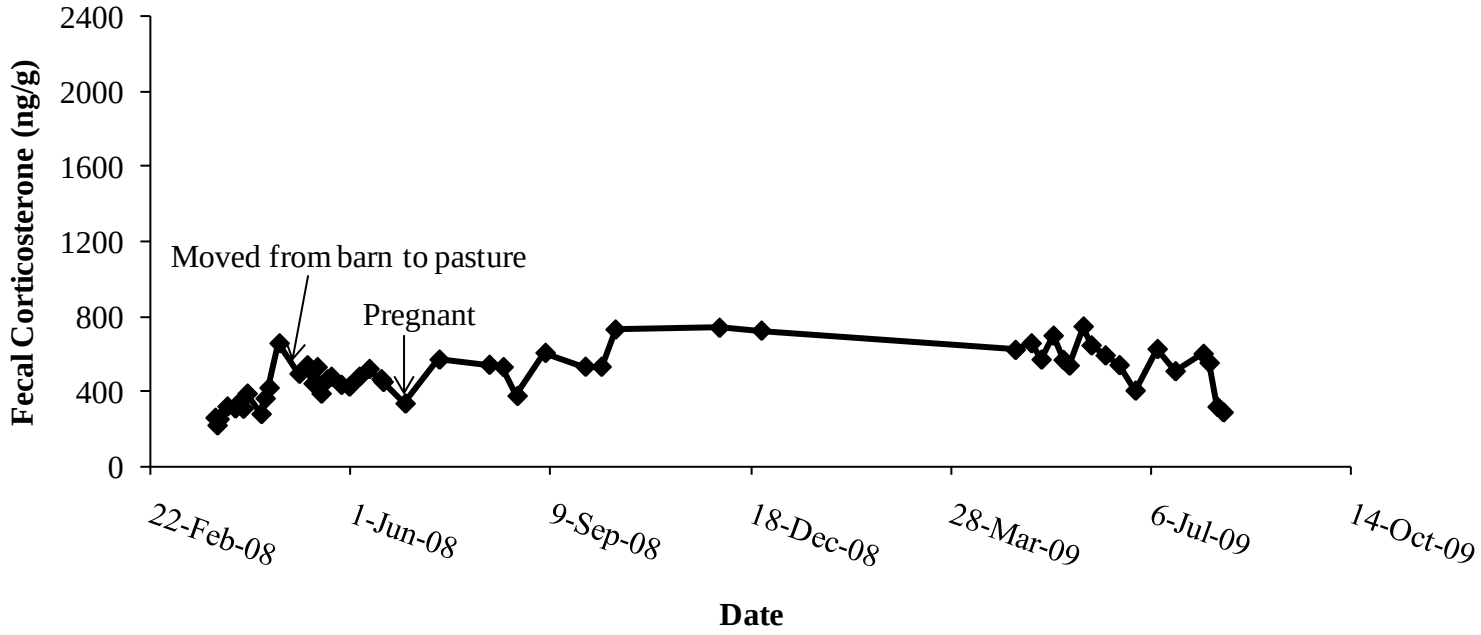

Fig. 66. Fecal corticosterone profile for captive-born, parous female Zenzele. She was housed inside initially and for the 2 values in December.

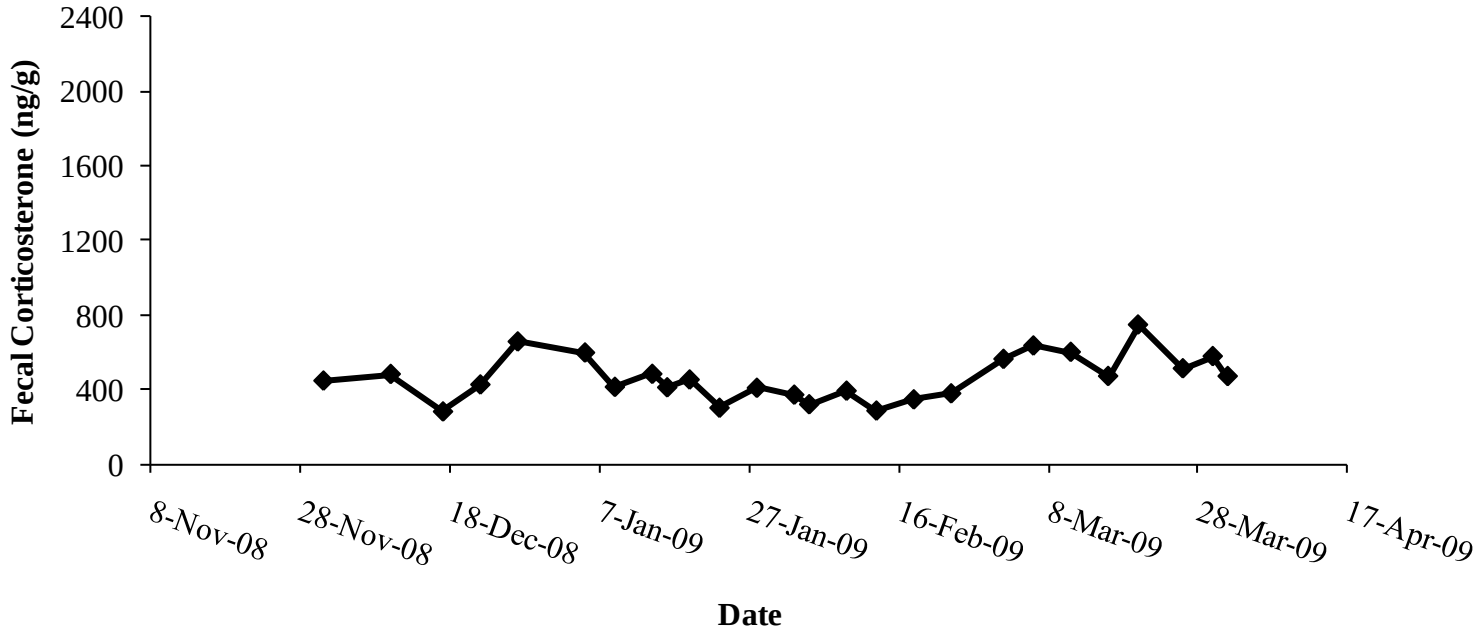

Fig. 67. Fecal corticosterone profile for captive-born, nulliparous female Ajabu. She was transferred to Birmingham from Fossil Rim in November 2008, just before sample collection began.

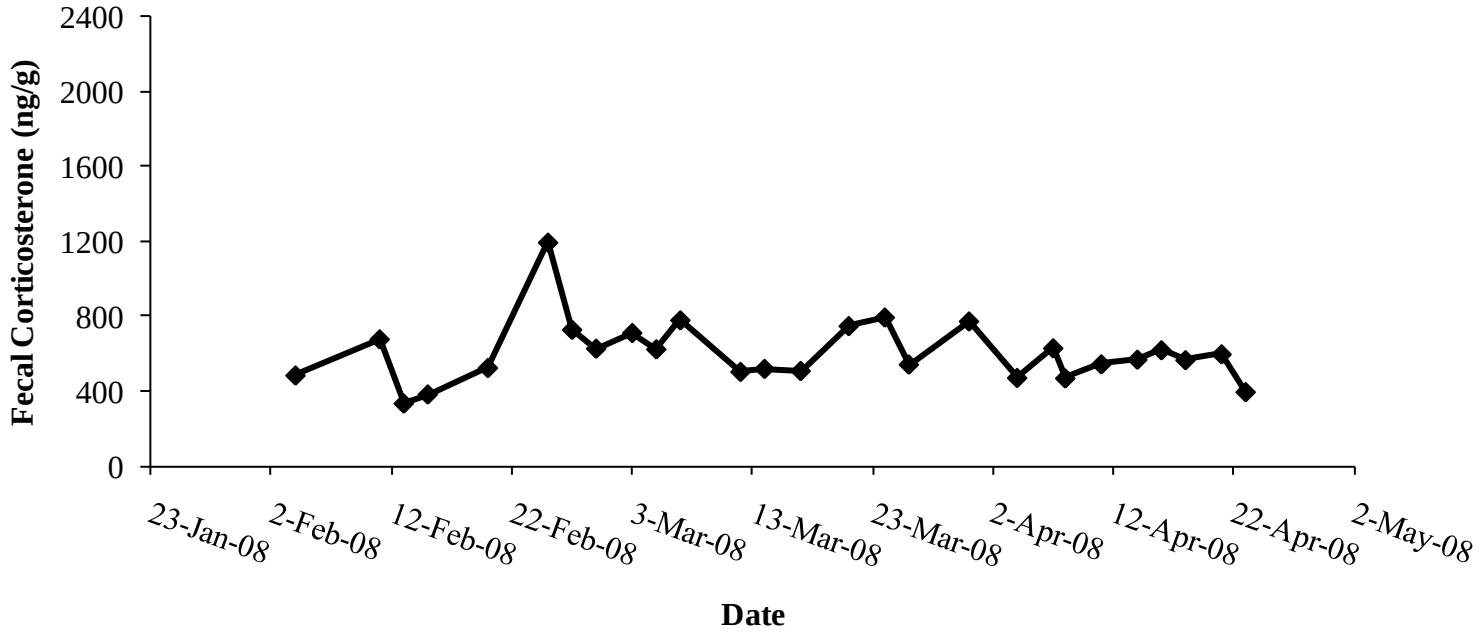

Fig. 68. Fecal corticosterone profile for wild-caught, nulliparous female Bertha.

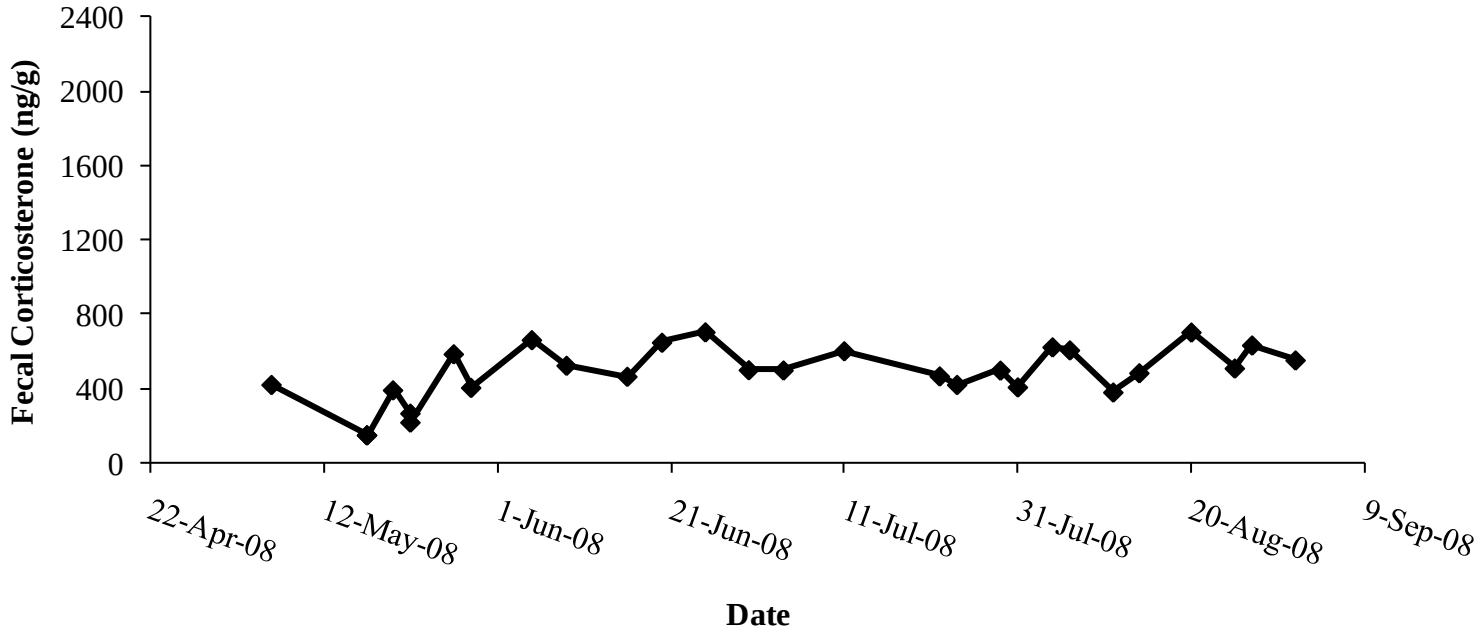

Fig. 69. Fecal corticosterone profile for captive-born, nulliparous female Bonnie.

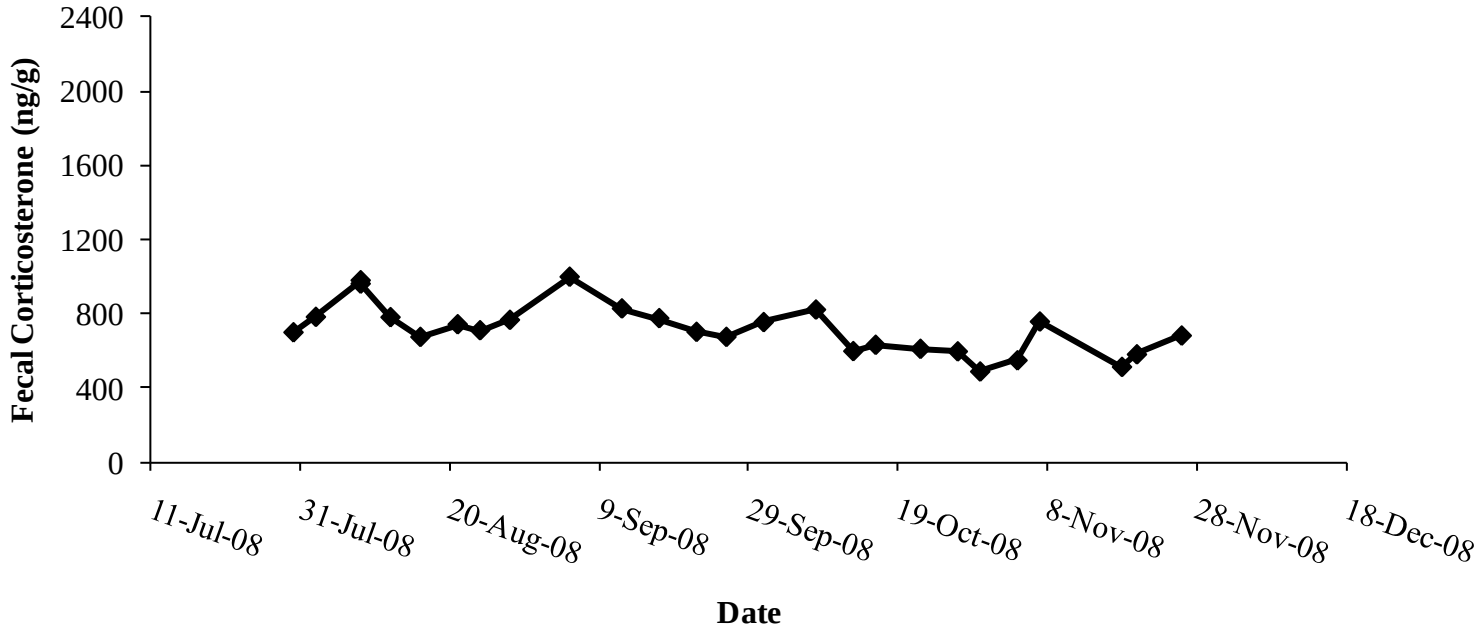

Fig. 70. Fecal corticosterone for captive-born, nulliparous female Dumisha.

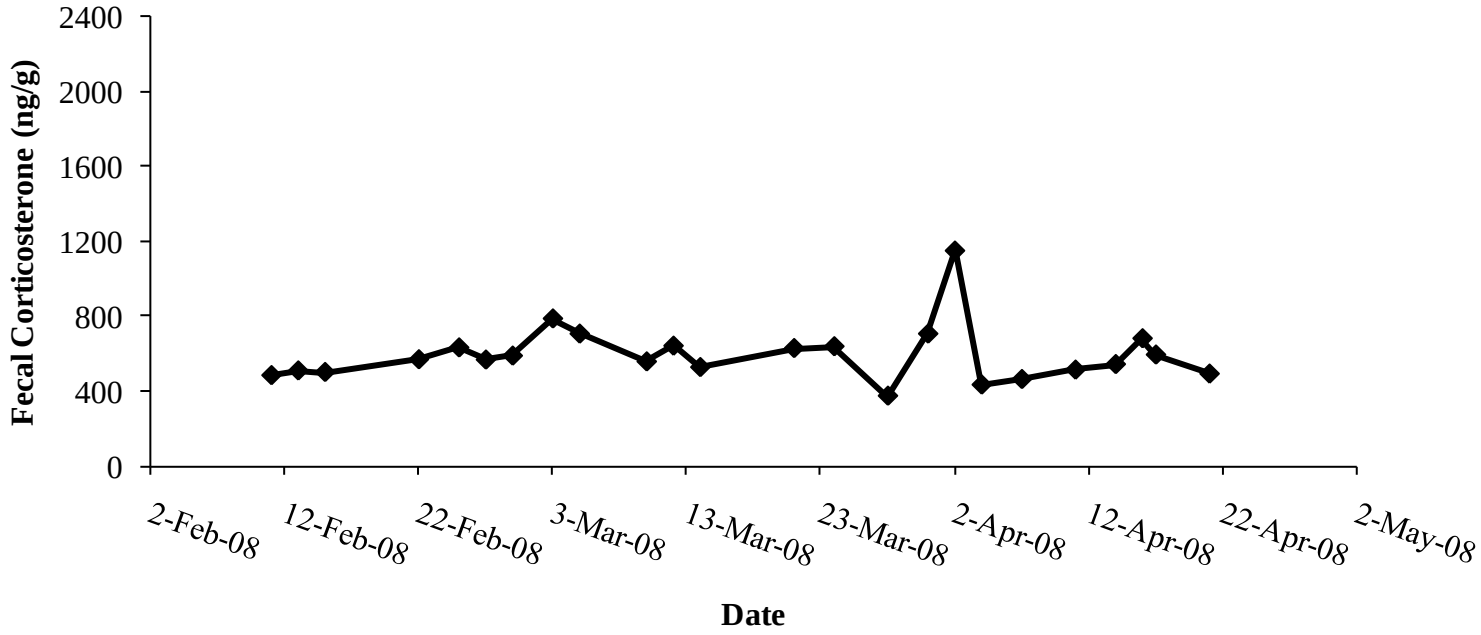

Fig. 71. Fecal corticosterone profile for wild-caught, nulliparous female Emalah.

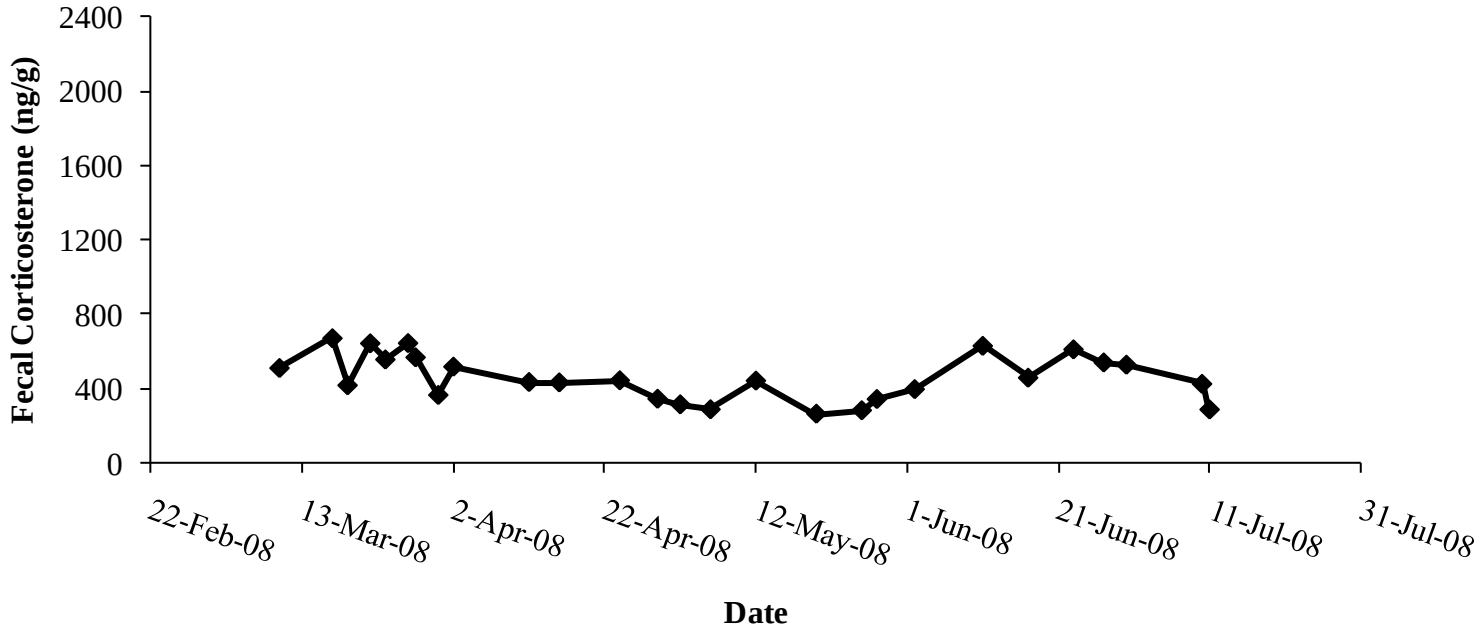

Fig. 72. Fecal corticosterone profile for wild-caught, nulliparous female Helen.

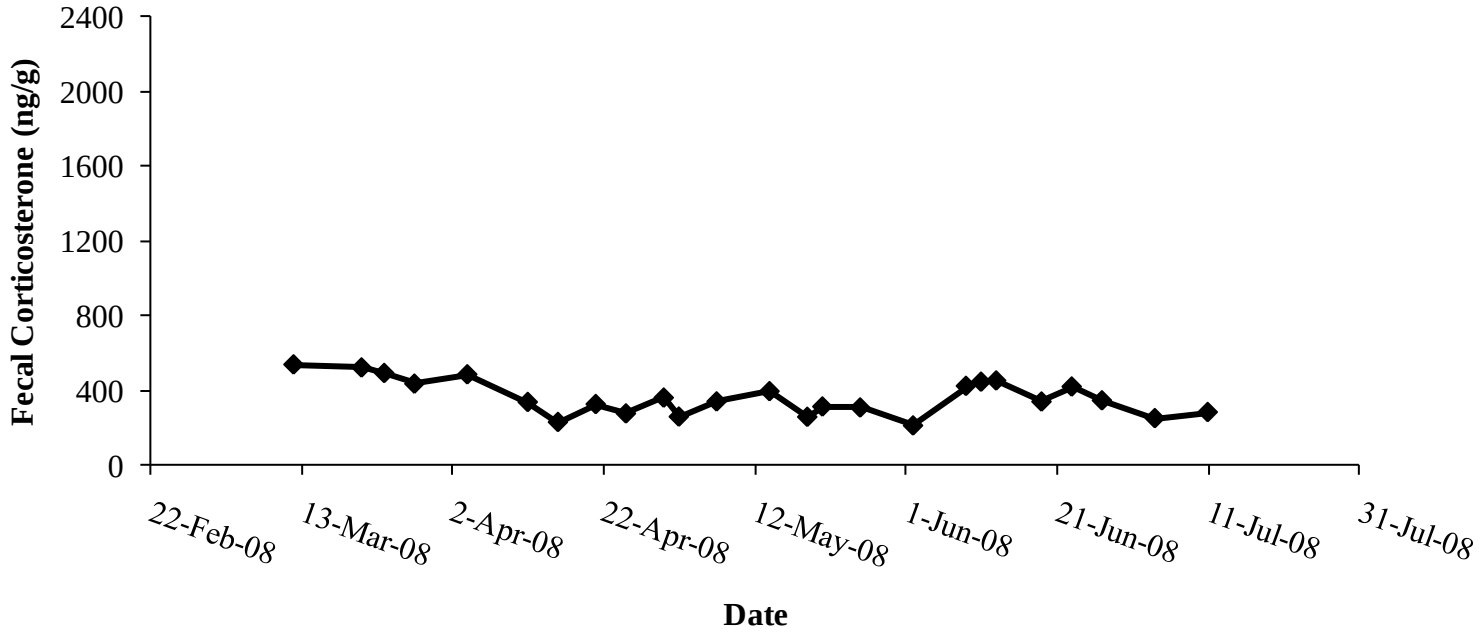

Fig. 73. Fecal corticosterone profile for wild-caught, nulliparous female Jao.

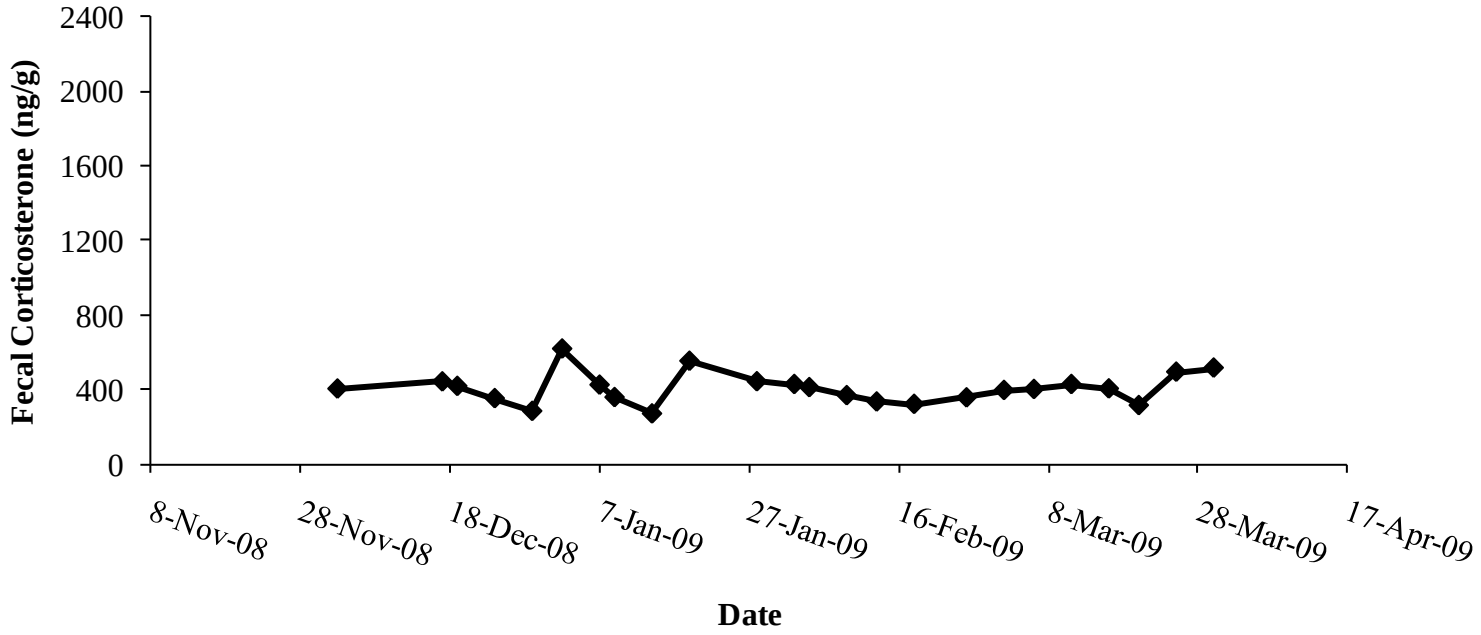

Fig. 74. Fecal corticosterone profile for captive-born, nulliparous female Jeannie.

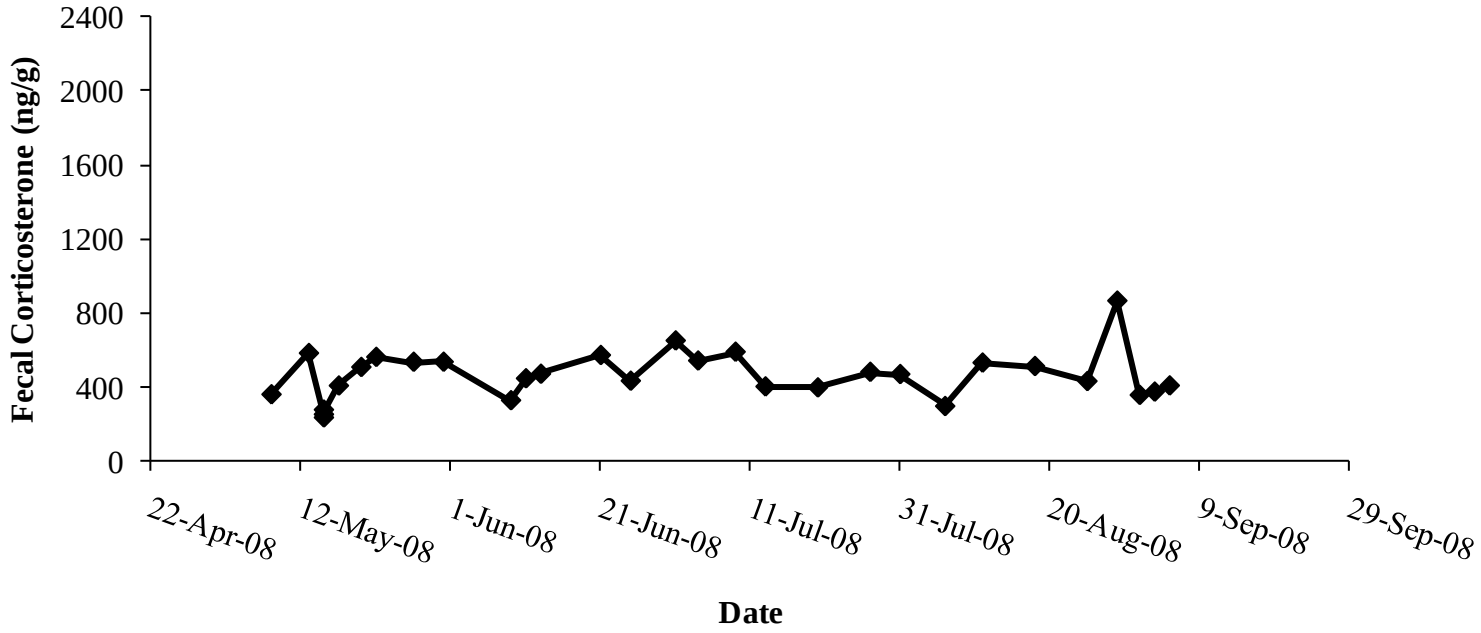

Fig. 75. Fecal corticosterone profile for captive-born, nulliparous female Kiangazi.

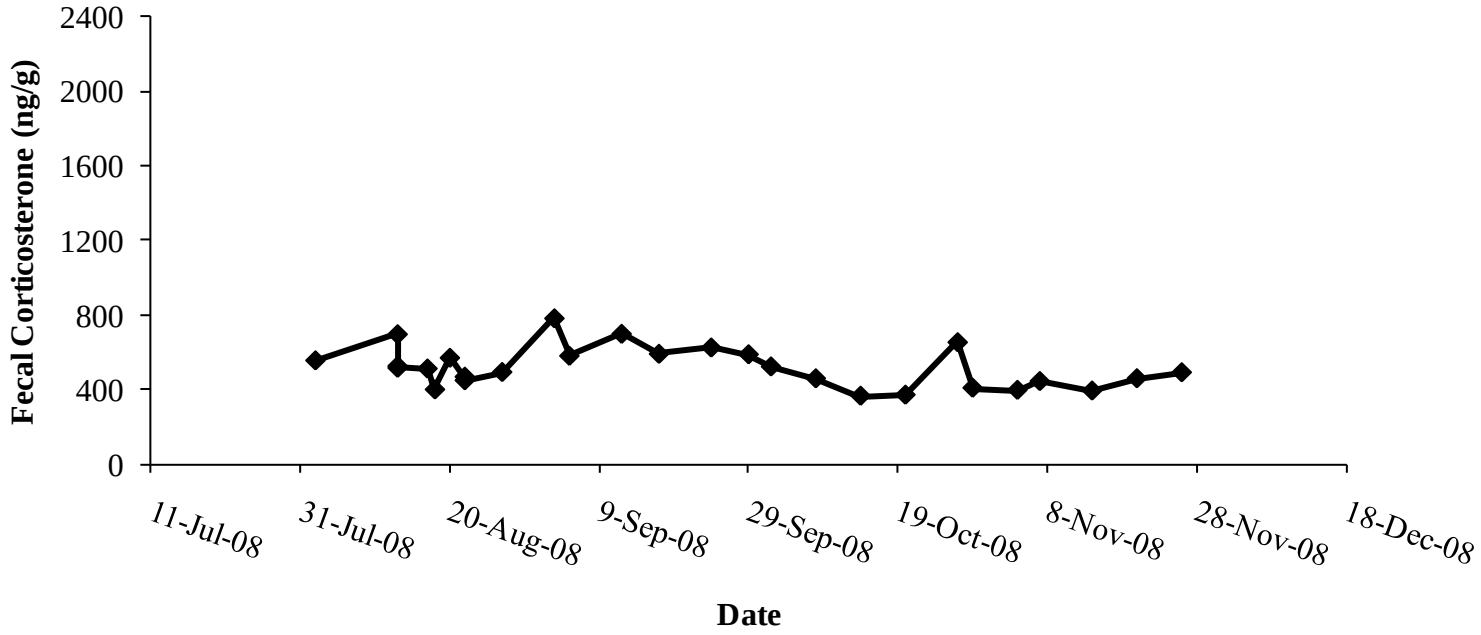

Fig. 76. Fecal corticosterone profile for captive-born, nulliparous female Kiazi.

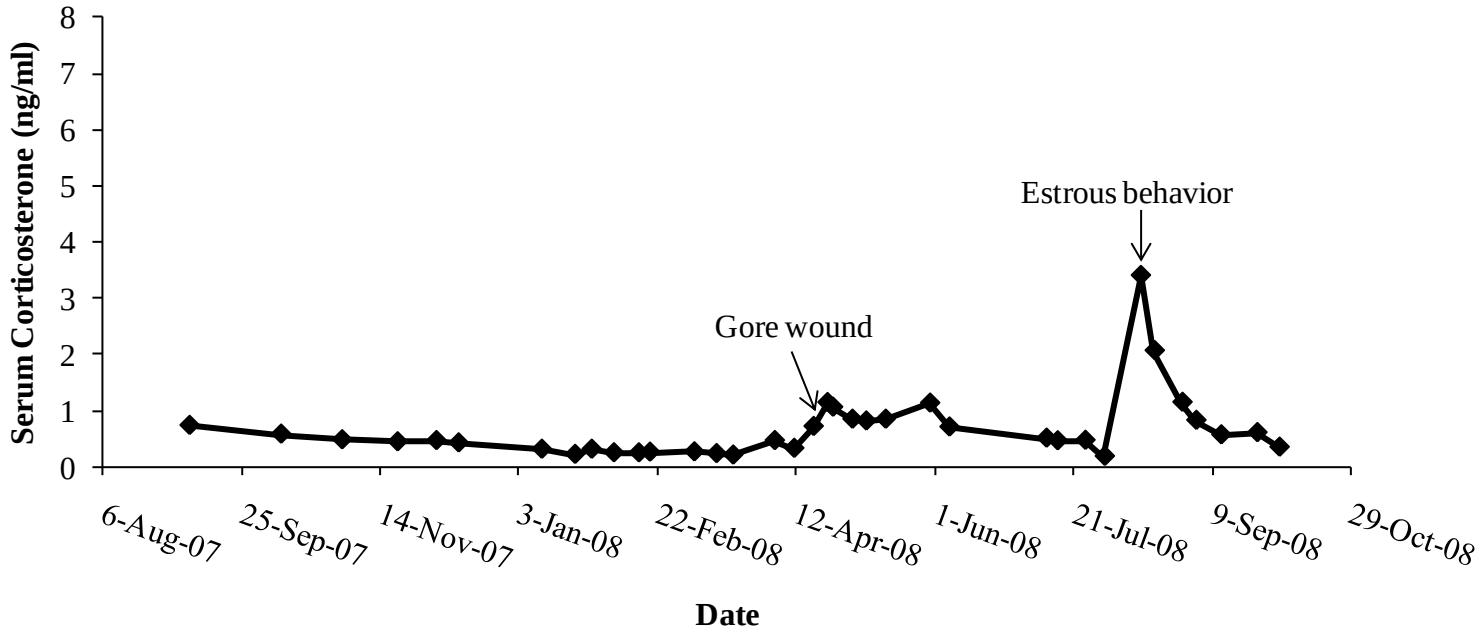

Fig. 77. Serum corticosterone profile for captive-born, nulliparous female Lucy at White Oak. Some elevated concentrations might be explained by a gore wound on 20 April 2008 and by estrous behavior on 14 and 15 August 2008 (full copulation was not observed), however copulation was not associated with similar elevations in corticosterone on other dates.

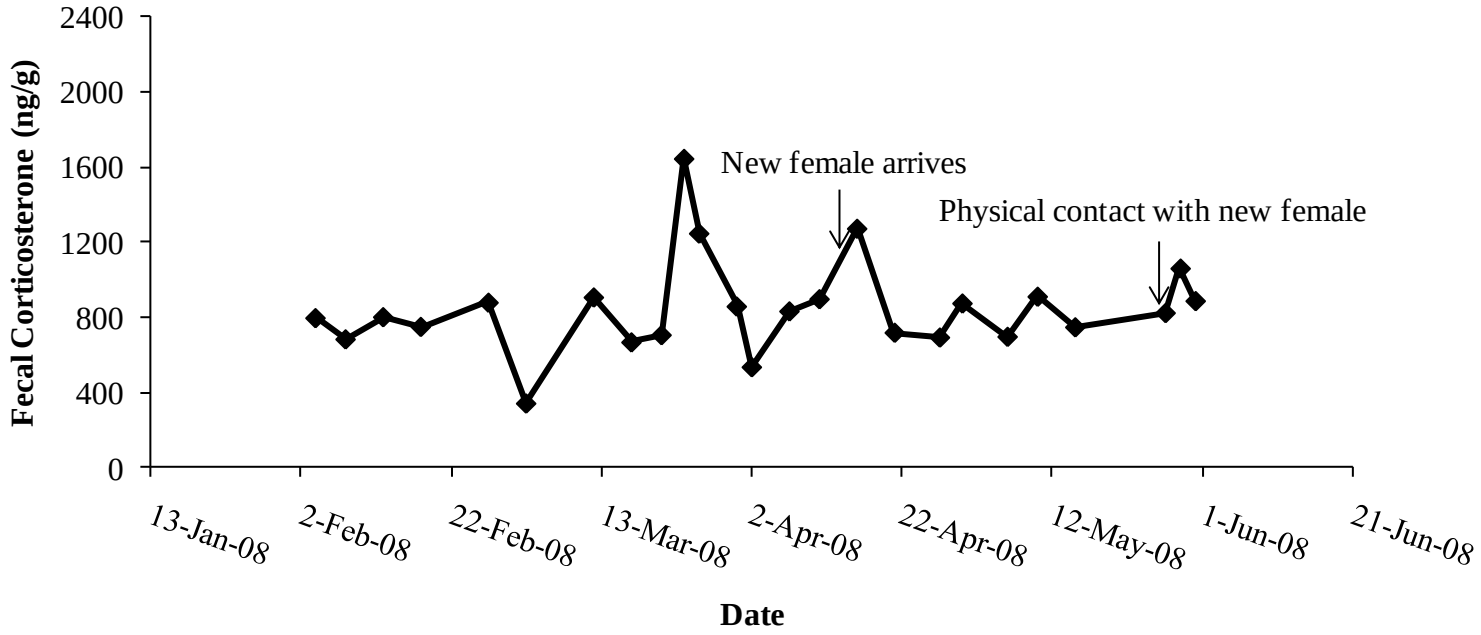

Fig. 78. Fecal corticosterone profile for wild-caught, nulliparous female Mambo. Some elevations in corticosterone concentrations might be explained by interactions with a newly-arrived female white rhino.

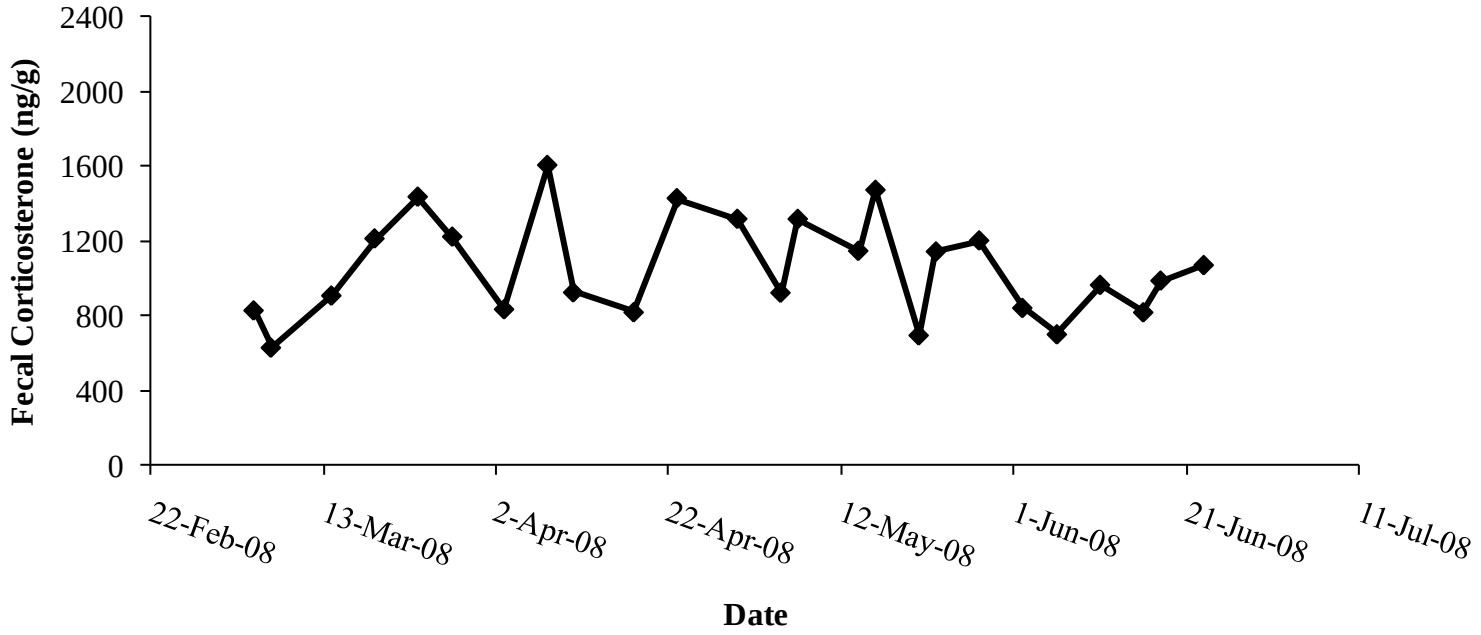

Fig. 79. Fecal corticosterone profile for wild-caught, nulliparous female Marina.

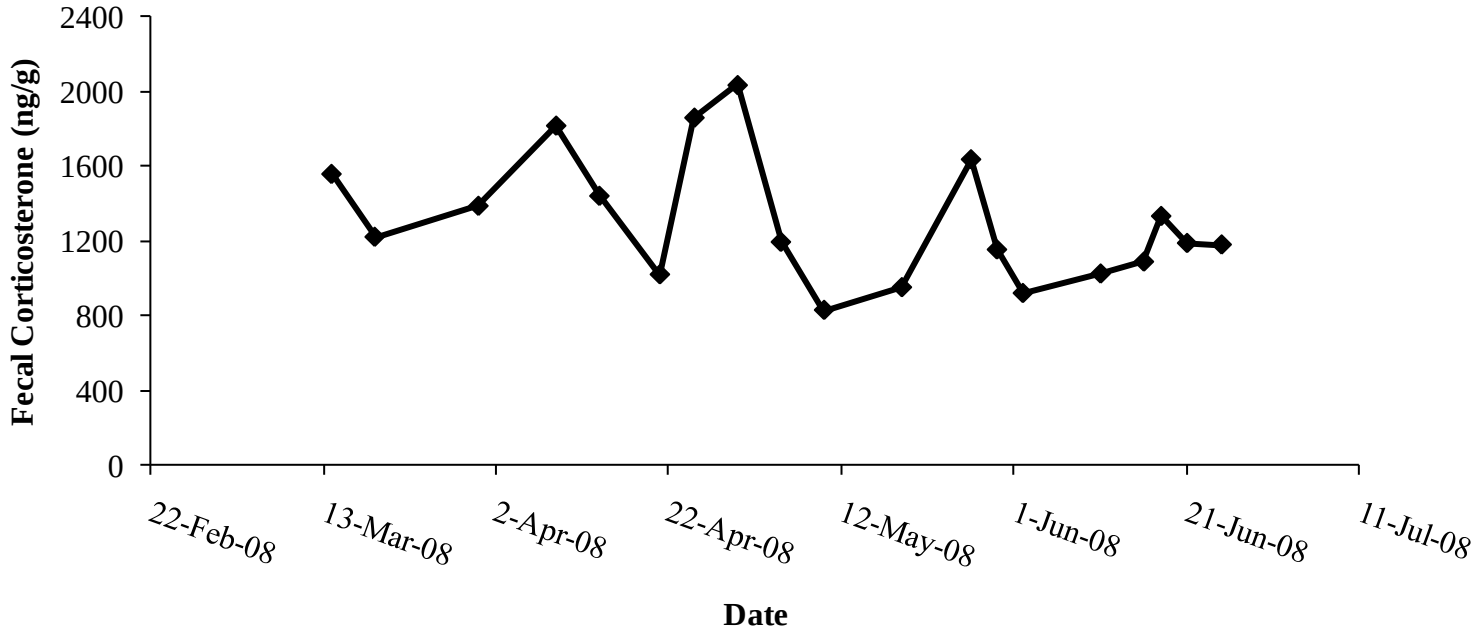

Fig. 80. Fecal corticosterone profile for wild-caught, nulliparous female Mashile.

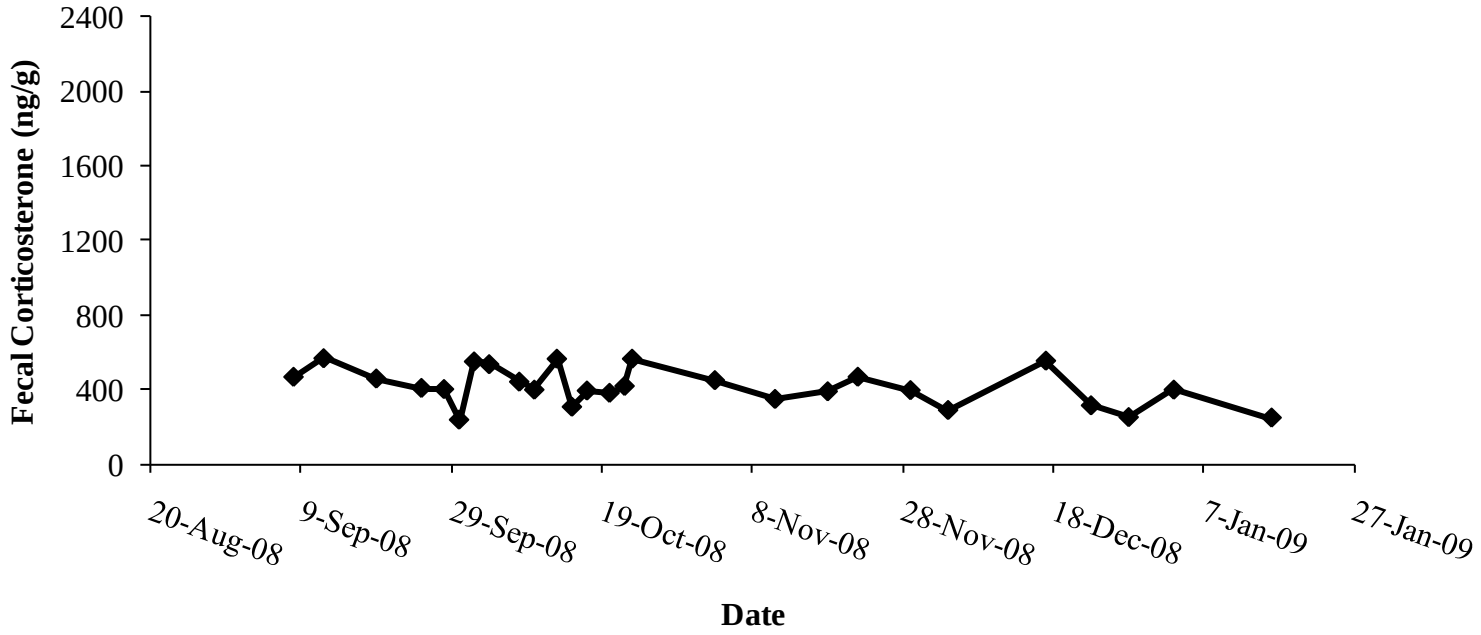

Fig. 81. Fecal corticosterone profile for captive-born, nulliparous female Paddy.

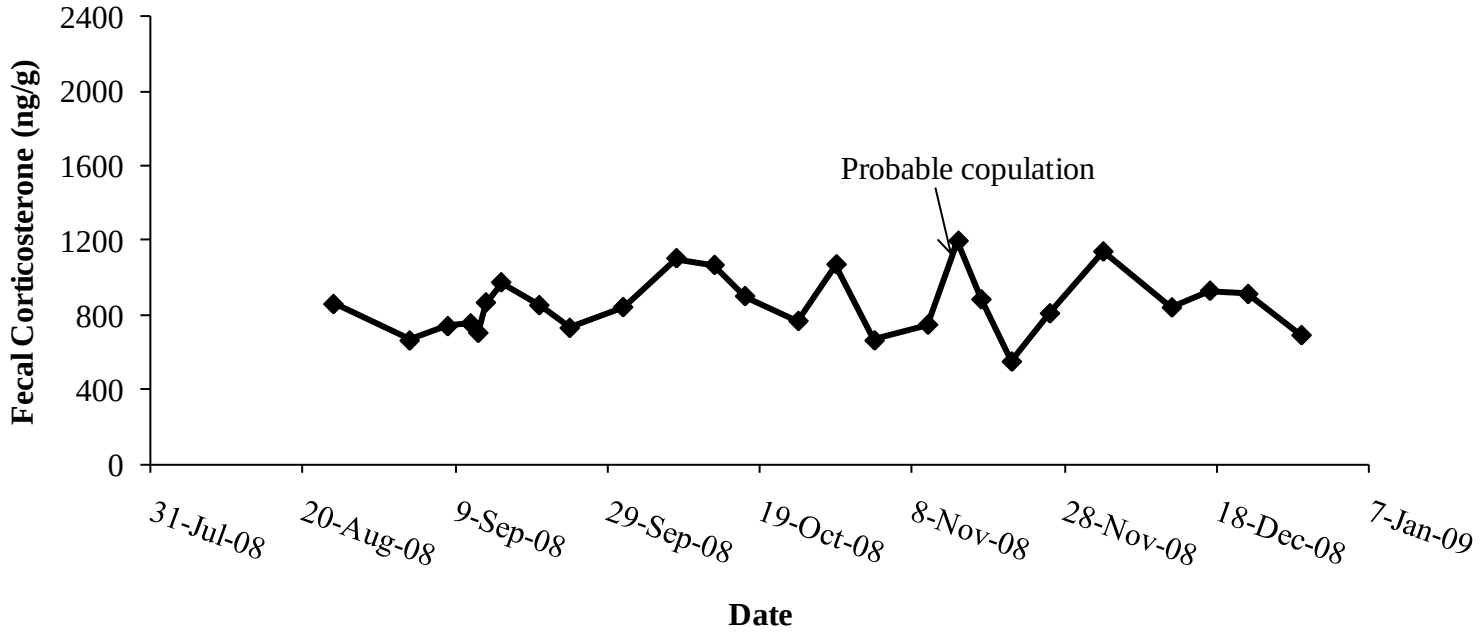

Fig. 82. Fecal corticosterone profile for captive-born, nulliparous female Taryn. Probable copulation on 13 November 2008 might explain elevated concentrations on 14 November, but concentrations were not elevated with copulation on 11 September.

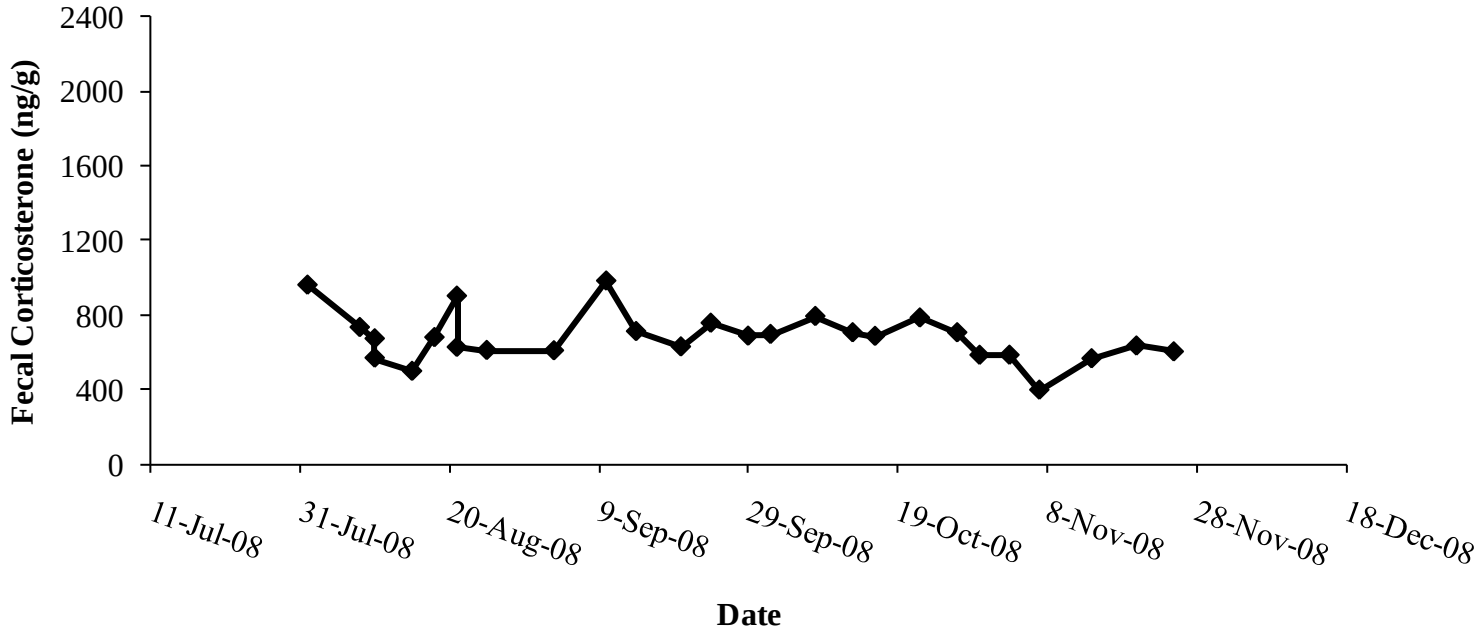

Fig. 83. Fecal corticosterone profile for captive-born, nulliparous female Utamu.

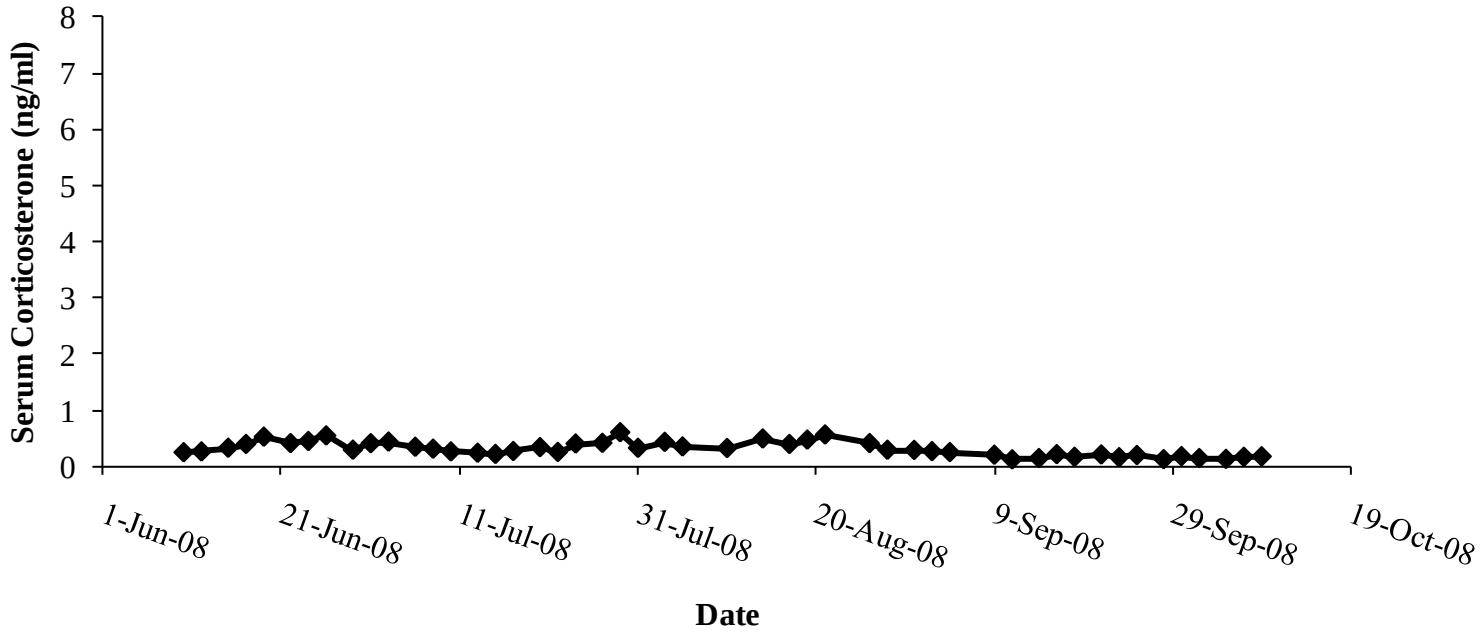

Fig. 84. Serum corticosterone profile for captive-born, nulliparous female Yebonga.